NANOREINFORCED AND CROSSLINKED HYDROCOLLOID FILMS TO PREVENT THE FORMATION OF TOXIC PRODUCTS DURING FOOD COOKING AND TO EXTEND FOOD SHELF-LIFE

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“To my family”

CONTENTS

RIASSUNTO

La cottura è uno dei metodi più antichi per consumare e conservare i cibi e migliorarne il sapore, l'aroma, la consistenza e la palabilità. Inoltre la cottura può inattivare eventuali patogeni, tossine, ed enzimi. Tuttavia, la cottura può procurare anche effetti indesiderati come la perdita di nutrienti e vitamine. Alcuni metodi di cottura possono produrre sostanze dannose e composti tossici quali i prodotti della reazione di Maillard come il furano, le amine aromatiche, l'acrilammide, l'acroleina, e gli isomeri trans degli acidi grassi. Tutti questi composti sono considerati carcinogenici, mutagenici, genotossici e teratogenici e sono, dunque, considerati un rischio per la salute umana. Nel corso del presente progetto, abbiamo voluto focalizzare l'attenzione sull'acrilammide (ACR), un composto altamente solubile in acqua, che ricercatori svedesi nel 2002 scoprirono formarsi in alcuni cibi quando venivano trattati a temperature di circa 120°C e in condizioni di bassi valori di umidità. La formazione di ACR, almeno in parte, è dovuta alla reazione di Maillard in cibi contenenti asparagine libere e zuccheri riducenti. Secondo il parere dell'EFSA (European Food Safety Authority), l'ACR si forma in diversi cibi cotti al forno o fritti, inclusi la patatine, il pane, i biscotti e nel caffè (si produce nei chicchi a seguito del processo di torrefazione). L'ACR è prodotta anche durante la combustione delle sigarette essendo presente nel fumo delle stesse.

Il rapporto EFSA 2015 riporta che ratti e topi esposti ad ACR mostrano segni di intossicazione, un'aumentata incidenza di deformazioni scheletriche, leggero aumento di peso, variazioni nell'istologia del sistema nervoso centrale con conseguenze neurocomportamentali.

Nel presente progetto sono stati studiati diversi cibi fritti (patatine, falafel e kobbah) con l'obiettivo di mitigare il contenuto in olio e ACR in modo da ottenere cibi più salutari. Soluzioni a base idrocolloidale contenenti pectina (PEC), o chitosano (CH) o farina di cicerchia (Grass Pea Flour, GPF) modificata mediante l'enzima transglutaminasi (TGase), e rinforzate con particelle di silica mesoporosa (MSN) o particelle di chitosano (CH-NP), sono state utilizzate per il raggiungimento dei nostri obiettivi. Le patatine, prima di essere fritte, sono state suddivise in gruppi e ogni gruppo rivestito da una delle seguenti soluzioni contenenti: GPF trattata e non con la TGase; CH; PEC. Quindi nei cibi cotti sono stati valutati la concentrazione di ACR, il contenuto in acqua e in olio. Inoltre, l'assunzione giornaliera (DI) e il margine di esposizione (MOE) sono stati calcolati per verificare eventuali variazioni dell'esposizione al rischio di assumere ACR come conseguenza dell'applicazione delle soluzioni protettive (coatings). I risultati hanno dimostrato che il più alto contenuto in ACR è stato osservato nel campione controllo, ovvero non rivestito dalla soluzione idrocolloidale, e pari a 2089 μg kg^{-1}. Il ricorso alle soluzioni idrocolloidali è un metodo efficace per ridurre la formazione di ACR, ridotta del 48% con soluzioni di PEC, del 38% con soluzioni di CH, del 37% con soluzioni contenenti GPF + TGase, e del 31% con soluzioni contenenti GPF non trattata con l'enzima. Infatti, il valore del MOE aumenta, diventando prossimo ai valori di sicurezza per evitare il rischio carcinogenico. Inoltre le soluzioni idrocolloidali sono state efficaci anche nel ridurre l'assorbimento di olio durante la cottura.

I falafel sono, in Medio Oriente, un cibo di strada. Essi sono fritti e di forma sferica. L'impasto è costituito da fave o ceci cotti e speziati. In questo progetto l'enzima

TGase (5 o 20U/gr di proteine di ceci) è stato aggiunto all'impasto del falafel per studiarne gli effetti. In seguito, i falafel sono stati divisi in due gruppi. I falafel del primo gruppo sono stati immersi in una soluzione idrocolloidale a base di PEC e quindi il contenuto in ACR, acqua e olio è stato determinato in tutti i falafel. Sono stati condotti anche esperimenti per determinare tecnologicamente i parametri (durezza, masticabilità, gommosità) tramite TPA (Texture Profile Analysis) e l'analisi sensoriale tramite assaggiatori appositamente addestrati in Palestina. Inoltre esperimenti di digestione *in vitro* sono stati condotti per verificare l'influenza dell'enzima sulla velocità di digestione. Il contenuto in ACR è risultato ridotto nei falafel al cui impasto è stato aggiunto l'enzima (10.8% in presenza di 5U TGase/gr e 34.4% in presenza di 20U TGase/gr). Nei falafel rivestiti con la soluzione di PEC, la riduzione in contenuto di ACR è risultata essere 59.3%, 65.3% and 84.5%, nei campioni allestiti in assenza di enzima e con 5U/g e 20U/g rispettivamente. Tuttavia, la presenza dell'enzima non influenza il contenuto in olio, mentre il rivestimento a base di PEC ne riduce l'assorbimento del 23.5%. Nessuna differenza riguardo i parametri studiati tramite TPA è stata riscontrata fra i controlli e i campioni rivestiti con PEC. Questi ultimi hanno incontrato i gusti degli assaggiatori, mentre i falafel preparati in presenza di TGase non sono risultati gradevoli. Gli studi di digestione *in vitro* hanno riportato che la presenza dell'enzima non influenza la digeribilità dei falafel.

I kobbah sono un cibo tipico dei paesi del Medio Oriente ma vengono consumati anche in altre nazioni come cibo etnico. I kobbah sono stati preparati secondo la ricetta tradizionale e quindi separati in gruppi e immersi in soluzioni differenti prima di essere fritti. Il primo gruppo di soluzioni idrocolloidali conteneva solo GPF (campioni denominati GPF), GPF trattata con TGase (campioni denominati GPF+TGase) o con nanoparticelle a base di mesoporosa (campioni denominati GPF+MSN) o con entrambi (campioni denominati GPF+TGase+MSN). Inoltre soluzioni colloidali a base di GPF sono state preparate ricorrendo anche ad altre nanoparticelle a base di chitosano (CH-NP) (campioni denominati GPF+CH-NP) in assenza e in presenza di TGase (campioni denominati GPF+CH-NP+TGase). Il secondo gruppo di soluzioni idrocolloidali era base di PEC nanorinforzate con MSN (campioni denominati PEC+MSN) o con CH-NP (campioni denominati PEC+CH-NP). Tutte le soluzioni idrocolloidali usate per rivestire i kobbah hanno provocato una riduzione del contenuto di ACR e di olio rispetto ai kobbah tradizionali. La percentuale di riduzione maggiore si è ottenuta nei kobbah rivestiti da PEC+MSN o PEC+CH-NP. Tuttavia anche nei campioni denominati GPF+MSN+TGase e GPF+CH-NP+TGase si è osservata una riduzione della concentrazione di ACR (rispettivamente del 41% e del 47.5%) rispetto ai campioni rivestiti di soluzioni GPF nanorinforzate allestite in assenza di enzima. I risultati indicano che il contenuto in acqua di tutti i kobbah rivestiti da soluzioni idrocolloidali risulta aumentato rispetto ai campioni controllo. La presenza di entrambi i tipi di nanoparticelle (MSN e CH-NP) è responsabile di questo effetto sia nelle soluzioni a base di GPF che di PEC.

Le soluzioni a base di PEC , preparate sia in assenza che in presenza di glicerolo, sono state usate per preparare bioplastiche reinforzate con MSN. Lo studio dell' influenza delle nanoparticelle sulle soluzioni filmanti ha dimostrato la riduzione delle dimensioni e nessun effetto sul valore del potenziale Zeta. Lo studio delle proprietà meccaniche ha dimostrato che le MSN provocano un modesto aumento della resistenza alla trazione mentre inducono un aumento significativo del modulo di Young. Tuttavia, il carico a rottura aumenta nei film PEC+MSN preparati in presenza

di glicerolo, mentre sia il modulo di Young che la resistenza alla trazione decrescono. Inoltre, le nanoparticelle riducono le proprietà dei film di PEC indipendentemente dalla presenza di glicerolo, mentre influenzano positivamente la stabilità termica e la forza di adesione. Le bioplastiche con migliori caratteristiche (0.6% di PEC con e senza 3% di MSN e contenenti il 30% di glicerolo sono state usate come involucri per proteggere campioni di fragole per allungare la shelf-life di questi frutti e conservarne alcune proprietà chimico fisiche. I risultati dimostrano che queste bioplastiche possono essere impiegate come materiali innovativi per proteggere la frutta.

SUMMARY

Food heating or cooking is one of the oldest method for food treatment that can be used to increase the food shelf-life and improve the palatability, aroma, taste, appearance and texture into the final product. Moreover, cooking provides safe food to the consumer by inactivation of pathogenic organisms/microorganisms, toxins, or enzymes. However, heating of foods might also have undesirable consequences, e.g., the loss of nutrients and vitamins. Thermal treatment of food also produces harmful substances and toxic compounds like Maillard reaction products, furan, heterocyclic amines, acrylamide, acrolein, and trans fatty acids. These compounds are considered as carcinogenic, mutagenic, genotoxic, and teratogenic and are a concern for human health.

During this project we focused on acrylamide (ACR), a compound highly soluble in water, about which heightened concerns regarding its exposure arose in 2002 when Swedish researchers discovered its formation in certain foods, when they were prepared at temperatures above 120ºC, and in the presence of low moisture. Its formation, at least in part, is due to the Maillard reaction in foods rich in free asparagines and reducing sugars. According to the European Food Safety Authority (EFSA), ACR forms in numerous baked and/or fried foods rich in free asparagine, including French fries, potato crisps, breads, biscuits, and coffee (roasted beans). ACR is also known to be present in cigarette smoke. The EFSA report in 2015 mentioned that rats and mice exposed to ACR have shown some signs of developmental toxicity, increased incidence of skeletal variations, slightly impaired body weight gain, histological changes in the central nervous system, and some neurobehavioral effects. Different fried foods (French fries, falafel and kobbah) were studied during this project to reduce ACR and oil content, with the aim of producing healthier fried foods. Hydrocolloid coatings were containing pectin (PEC), chitosan (CH), or grass pea flour (GPF) enzymatically crosslinked by means of transglutaminase (TGase) or reinforced with mesoporous silica nanoparticles (MSN) or chitosan nanoparticles (CH-NP), were used to achieve the objectives. In French fries, GPF treated or not by TGase, CH, and PEC hydrocolloid coating solutions were used to coat the potatoes before frying. ACR, water retention as well as oil content were evaluated. In addition, the Daily Intake (DI) and Margin of Exposure (MOE) were calculated to estimate variations in risk assessment by applying coating solutions before frying. Our results showed that the highest ACR content was detected in the control sample, reaching a value of 2089 µg kg^{-1}. Hydrocolloid coating solutions were demonstrated to be an effective way to reduce ACR formation, with the percentage of ACR reduction equal to 48% for PEC, 38% for CH, 37% for GPF + TGase, and 31% for GPF, respectively. The MOE value for coated French fries increased, resulting higher safety level to diminish carcinogenic risk. Moreover, the tailored coatings were effective in reducing oil uptake.

Falafel is traditional street food in Middle Eastern. It is a deep fried ball made of spiced fava beans and/or chickpeas. In this project TGase (5 or 20U/g of chickpea proteins) was added on falafel dough and the effect of the enzyme was investigated. Afterwards, the resulted falafel balls were either treated or not by dipping them into PEC coating solution. ACR, oil and water content were, hence, evaluated. Texture profile analyses (TPA) and sensory evaluation performed by trained Palestinian panellists were carried out. Moreover, *in vitro* gastric digestion experiments were also performed to study the effect of the enzyme and coatings on falafel digestibility. The ACR content was reduced by 10.8% and by 34.4% in the samples prepared with 5 and 20U TGase/g, respectively. In PEC-coated samples, the reduction of ACR was equal

to 59.3%, 65.3% and 84.5%, in falafel balls prepared either without TGase or containing 5U or 20U of the enzyme, respectively. However, TGase treatment did not affect oil content, while the PEC coating reduced oil uptake by 23.5%. No difference was observed between the control sample and the one dipped in PEC in regard to TPA while these properties changed in samples prepared with the enzyme. Panellists accepted falafel coated by PEC solution, ever better than the traditional ones, while a negative score was obtained by TGase-containing falafel. The digestion studies demonstrated that the falafel prepared in the presence of TGase was efficiently digested in the *in vitro* gastric environment.

Kobbah is a food typical in Middle Eastern countries but also used as an ethnic dish. It was prepared according to traditional recipe and then dipped using different solutions. The first set of dipping solutions was GPF-based and containing only GPF (GPF samples) reinforced by means of TGase (GPF+TGase samples) and/or by means of mesoporous nanoparticles (MSN) (GPF+MSN samples and GPF+MSN+TGase samples). Some GPF-containing samples were prepared also using, as nanoreinforcement, the (CH-NP), (GFP+CH-NP samples and GFP+CH-NP+TGase samples). The second set of dipping solutions was PEC-based reinforced by means of MSN (PEC+MSN samples) or CH-NP (PEC+CH-NP samples). All hydrocolloid-based materials, used to coat kobbah, were effective in provoking a significant reduction in ACR and oil content comparing to the controls. The highest reduction rates were obtained using both PEC+MSN and PEC+CH-NP samples. However, also GPF+MSN+TGase and GPF+CH-NP+TGase samples were responsible for ACR reduction (41.0% or 47.5% respectively) in comparison to the nanoreinforced GPF prepared in the absence of the enzyme. The results indicated that the water content was significantly increased after coating the kobbah by different coating solutions. Nanoreinforcement of the coating solutions by either MSN or CH-NP for both GPF-based and PEC-based coatings, showed the ability of increasing the water retention inside the kobbah. Water retention in these samples was significantly higher than retention exhibited from samples coated with GPF-based solution or PEC-based solutions.

PEC solutions, set up in the presence and in the absence of glycerol (GLY), were used to prepare bioplastics reinforced by MSN. Nanoparticles reduced significantly the particle size, and had no effect on the Zeta potential of PEC solutions. Mechanical characterization studies demonstrate that PEC containing MSN films exhibit a slightly increase in tensile strength and a significantly decrease in the Young's modulus in comparison to films made of only PEC. However, elongation at the break increased in the PEC-MSN films cast in the presence of GLY, while both Young's modulus and tensile strength were reduced. Moreover, nanoparticles were able to reduce the barrier properties of PEC films prepared with or without GLY, whereas positively affected the thermal stability of PEC films and the seal strength. The 0.6% PEC films reinforced or not with 3% nanoparticles in the presence of 30% GLY were used to wrap strawberries in order to extend the fruit shelf-life, over a period of eighty days, by improving their physicochemical properties, thus suggesting a possible use of these materials as novel bioplastics to protect fruit.

ABBREVIATIONS

ACR	acrylamide
AI	acceptance index
AOAC	association of official analytical chemists
ASTM	american society for testing and materials
$BMDL_{10}$	benchmark dose lower confidence limit
b.w.	body weight
CH	chitosan
CH-NP	chitosan nanoparticles
CTAB	cetyltrimethylammonium bromide
DI	daily Intake
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	differential scanning calorimetry
DTG	differential thermal gravimetry
EB	elongation at break
EFSA	European Food Safety Authority
EIC	extracted ion chromatogram
FFSs	film forming solutions
FT-IR	fourier-transform infrared
GLY	glycerol
GPF	grass pea flour
HPLC	high pressure liquid chromatography
HLB	hydrophilic-lipophilic-balanced
LOD	limit of detection
LOQ	limit of quantitation
MOE	margin of exposure
MSN	mesoporous silica nanoparticles
NPs	nanoparticles
PEC	pectin
RH	relative humidity
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	scanning electron microscopy
SPE	solid phase extraction
TA	titratable acidity
TEOS	tetraethylortosilicate
TGA	thermogravimetric analysis
TGase	transglutaminase
TPA	texture profile analysis
TPP	sodium tripolyphosphate
TS	tensile strength
UW	unwrapped
W	wrapped
WV	water vapor
YM	Young’s module

CHAPTER 1

INTRODUCTION

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1. INTRODUCTION

1.1 Edible films and enzymatic crosslinking by means of transglutaminase

The growing use of plastic materials in recent years has resulted in a global waste disposal problem. (Sabbah & Porta, 2017; Porta, 2019). Globally, production of plastics exceeds 300 million tons per year and it is likely that a similar quantity of plastics will be produced in the next eight years as it was produced during the 20th century (Thompson, 2017). Polymers cause serious damage to the environment since they remain undergirded for more than hundreds of years contaminating the wildlife in the ocean or land and also the food chain (Sadeghi & Mahsa, 2015). Most plastic-based materials are used in the food industry to protect food products and prolong shelf-life. In order to both minimize the environmental impact and satisfy the increasing demand for high quality foods, materials with faster degradation process are being researched and developed as substitutes to the traditional polymers (Imran et al., 2010; Fernando et al., 2015). A possible solution is the synthesis of bio-based and biodegradable/edible "bioplastics" (Falguera et al., 2011; Pathak et al., 2014). These biomaterials can reach satisfactory mechanical properties being able to prevent moisture loss and control gas exchanges (such as oxygen, carbon dioxide, and ethylene) which are involved in "respiration" processes of numerous coated food products. Some foods can be consumed fresh, but the current lifestyle has increased the demand for products that could be easily transported and stored in supermarkets and that still possess nutritional and sensorial values and other characteristics highly desired by consumers. These characteristics can be provided by recurring to the use of edible films and coatings, because of the advantages over synthetic films have received considerable attention in recent years.

Components used for the preparation of biodegradable/edible films can be classified into three categories: hydrocolloids (such as proteins or polysaccharides), lipids (such as fatty acids, acylglycerol, waxes), and composites (Song & Zheng, 2014). Polysaccharides used for edible films or coatings include cellulose, starch derivatives, PEC, seaweed extracts, exudates gums, microbial fermentation gums and CH. Moisture barrier properties of polysaccharide-based films are poor, and the addition of proteins can provide films with improved characteristics like acceptable barrier capacity to water vapor as well as mechanical resistance.

Barrier and mechanical properties of edible films can be reinforced recurring to reticulating agents. Reticulating agents can be chemical substances, i.e. glutaraldehyde, or organic molecules such as enzymes. Compared to chemical cross-linkers, enzymes offer numerous advantages. The total absence of toxicity is the most important. Among enzymes able to catalyze crosslinking, *Streptoverticillium mobaerense* transglutaminase has proved to be a powerful tool (Porta et al., 2011). Microbial transglutaminase (TGase) belongs to a family of enzymes (E.C. 2.3.2.13) (widely distributed in nature from microbes to animals and plants) capable of catalyzing iso-peptides bonds between endo-glutamine and endo-lysine residues belonging to proteins of different nature, giving arises to intra- and inter-molecular crosslinks (Fig. 1). Recently, TGase crosslinked protein-based edible films and coatings have met a great interesting among researcher, and many paper have been published using this enzyme as biotechnological tool. Recently, Sabbah et al. (2019a) have published an updated overview on the importance of TGase for the preparation of edible films and coating in the food sector. Moreover, TGase-mediated reticulation can affect functional properties of protein-based food products, as reported in the book chapter we

published in Enzymes in Food Technology with the aim of reporting the recent literature focused on the ability of plant and animal proteins to act as TGase-subtracts (See Appendix 6.5.1).

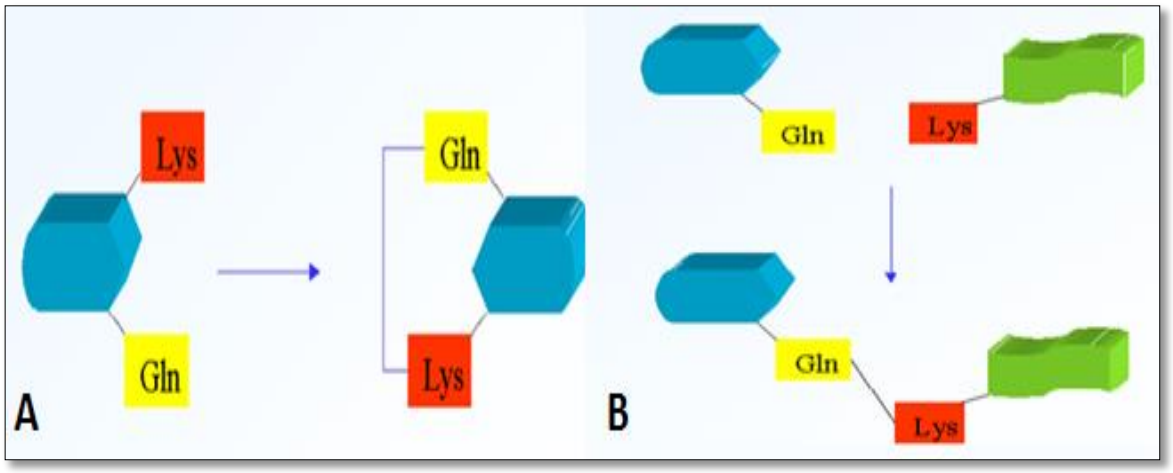

Fig. 1 TGase-mediated crosslinking can give rise to intra-molecular (A) and inter-molecular (B) isopeptidic bonds.

The use of the microbial isoform in food sector is successful because despite the others, is calcium independent, and acts in a wide pH and temperature ranges. Thus, TGase is widely used in the food industry as technological aid (according to Yokoyama et al., 2004), to improve texture of protein-based products, such as yoghurt, but also meat-based food such as sausages. Moreover it has been proved that TGase treatment also affects viscosity (Farnsworth et al., 2006; Jaros et al., 2006). Available in the market there are many TGase preparations from different producers, even though Ajinomoto is the most popular one. In the last decade many research papers have been published regarding the use of TGase to be used as biotechnological tool to reticulate the protein component of hydrocolloid edible films (Porta et al., 2016; Rossi Marquez et al., 2017; Giosafatto et al., 2018).

Moreover, Rossi Marquez et al. (2013) have demonstrated the effectiveness of whey protein/PEC-based coating obtained by means of TGase in improving a barrier to water, giving rise to fried donuts with low oil uptake and high water content, together with baked biscuits (named “Taralli” in Italian) that, when coated, exhibited a longer shelf-life. Thus, the present project was addressed to prepare hydrocolloid-based edible films or coatings, reinforced or not by nanoparticles into which the protein component can be reticulated or not by the mean of the TGase. In particular, we have meant to study the effectiveness of hydrocolloid-based coating in lowering the amount of toxic substances that are produced during Maillard reaction. We decided to coat some typical Palestinian foods (such as fried potato, falafel and kobbah) that are widely consumed at home and as street food in the Middle East.

1.2 Hydrocolloid materials for coating and wrapping

Hydrocolloids are effective coatings to enhance the quality of food products as reported in several reports (Porta et al., 2011; Coltelli et al., 2016). Despite their effectiveness in regulating the transmission of gases such as oxygen and carbon dioxide besides water vapor, an increasing interest in studying their role in ACR reduction in processed foods has been registered (Zeng et al., 2010; Suyatma et al.,

2015; Rannou et al., 2016). Polysaccharides and proteins are classified as hydrocollod compounds. In this project we have studied PEC and CH as polysaccharides, and grass pea flour as a source for proteins.

PEC is a plant cell wall structural polysaccharides composed mainly of galacturonic acid units with variations in composition, structure, and molecular weight (Lara-Espinoza et al., 2018). In general, PEC is used as food additive (E440), known as thickers or stabilizers, to prepare different food products like jelly, jam, marmalades and other products, due to their gelling properties (Padmaja & Bosco, 2014). PEC is also used in pharmaceuticals and cosmetics industry due to all these properties. Moreover, PEC applications are devoted to increase, since these biopolymers have great potential for future developments (Valdés et al., 2015). Coatings are one of the most important food preservation methods that are applied to protect highly perishable foods by creating a thin layer of edible materials onto surfaces of the products. For example, Yossef. 2014, found out that strawberry fruits dipped in PEC-based solutions retained physico-chemical properties comparable to the ones coated by soy proteins, gluten, or starch.

CH, is derived from chitin by deacetylation in an alkaline media (Abdou et al., 2008). Actually, CH is a copolymer consisting of β-(1–4)-2-acetamido-D-glucose and β-(1–4)-2-amino-D-glucose units with the latter usually exceeding 60%. CH is described in terms of degree of deacetylation and average molecular weight and its importance resides in its antimicrobial properties, in conjunction with its positive charge and film-forming properties. The potential of CH to act as a food preservative of natural origin has been widely reported on the basis of *in vitro* trials as well as through direct application on real complex matrix foods (Coma et al., 2003; Durango et al., 2006; Han et al., 2004; Park et al., 2004; Ribeiro et al., 2007). CH is also an excellent film forming material (Domard et al., 2001). CH films have a selective permeability to gasses (CO_2 and O_2) and good mechanical properties. However, the fact that CH films are highly permeable to water vapor limits their use being an important drawback since an effective control of moisture transfer is a desirable property for most foods, especially in moist environments.

Grass pea flour was used for its high content in proteins. Grass pea (*Lathyrus sativus* L.) belongs to the leguminous family and is a very popular crop in many Asian and African countries where it is cultivated either for stock feed or human consumption. It is characterized by a lot of advantageous biological peculiarities (resistance to pests) as well as agronomic features (resistance to drought, high grain-yielding capacity) (Campbell, 1997). We have recently demonstrated that grass pea flour (GPF) contains proteins able to act as TGase substrates (see Appendix 6.5.3), giving arise to novel bioplastics that were more resistant, more extensible, and less rigid than the ones prepared in the absence of the enzyme. Thus, in the present project GPF was chosen as protein component of our novel materials. In particular, we have set up different hydrocolloid-base solutions to be used either as edible coatings and wrapping bioplastics. Generally, coating application consists of applying a liquid or a powder ingredient onto a product, while wrapping is intended when the film represents a stand-alone protecting material. Hydrocolloid materials can be especially suitable for food coating or wrapping, as well as for separation of different food portions (Sabbah et al., 2019b). Recently, Sabbah et al. (2019b) used CH-based and bitter vetch protein-based films as wrapping of the Palestinian Nabulsi cheese, to improve the shelf-life of such dairy product widely consumed in the Middle East. Dipping is a common method for applying coatings on fruits and vegetables (Vargas, 2009). The coating solution must

exhibit some properties likes density, viscosity and surface tension (Cisneros-Zevallos & Krochta, 2003; Dhanapal et al., 2012).

1.3 Nanoparticles as reinforcement of edible films

Despite its biodegradability, renewable biopolymers have three main problems: performance, processing and cost (Petersen et al., 1999). In special, their poor mechanical and barrier properties are limiting their use, particularly in food packaging, thus these characteristics may be modified by adding some reinforcing compounds, generally on nanoscale dimension, forming composites/nanocomposites (Sadeghi & Mahsa, 2015). Nanotechnology refers to the use of particles that have dimension up to nanometers applied in several sectors, i.e. in diagnostic field to build up biosensors, to the food industry. In the latter, most attention is related to the use of nanotechnology food contact.

Among the NPs mostly studied in food packaging systems there are nanoclays and mesoporous silica nanoparticles (MSN) used as reinforcements, since both kinds are easily available, environmentally friendly, and low cost chemical substance. Nanoclays have found applications in many fields, including medicine (Ambre et al., 2010), pharmacy and cosmetics (Carretero & Pozo, 2010), besides than food packaging (Majeed et al., 2013; Shekarabi et al., 2014; Rostamzada et al., 2016). In particular, bentonite is absorbent aluminum phyllosilicate clay consisting mostly of montmorillonite (MMT). Bentonite is authorized as additive (E558) for plastic materials and articles in contact with foods (Regulation (EU) No 10/2011) with no specific restrictions. The bentonite is also an approved food additive included in the Directive 95/2/EC, and can be used as a carrier for colors with a maximum of 5% w/w in food (http://ec.europa.eu/food/safety_en). Few previous studies have dealt with the effect of MMT nanocomposite-based coatings to preserve fresh food, such as acerola cherries (Azeredo et al., 2012), melon (Danza et al., 2015), strawberries (Junqueira-Goncalves et al., 2017) and carrots (Costa et al., 2012).

SiO_{2-} based NPs of different composition are labelled as E551, E554, E556, or E559, and used for instance as an anti-caking agent. The amount ingested daily is estimated to be 1.8 mg/kg (around 126 mg/day for a 70 kg person) (Dekkers et al., 2011). MSN (Type MCM-41) are a kind of SiO_2–based NPs that are promising materials for application in numerous aspects of biomedical purposes. For example MSN-based delivery systems containing drugs and bioactive molecules can be suggested, since the advantages of mesoporous materials which are tunable in pore size, controlled in particle size and morphology, and dual-functional surface (external and internal) (Mathew et al., 2014; Rafi et al., 2016). Moerover, MSN appear promising for the application as selective media for the separations of large molecules such as proteins, which are important in the food and pharmaceutical industries (Bernardos & Kouřimská, 2013). Recently, Fernandez-Bats et al. (2018), prepared and characterized the active protein edible films nanostructured with MSN or with its amino-functionalized derivative, and they concluded that the film tensile strength and elongation at break significantly increased in the presence of both kinds of NPs. In this project we want to recur to NPs with the aim of obtaining hydrocolloid edible films that possess a higher capability of binding water. It is known from Cushen et al. (2012) that, when included in a polymer, nanosized fillers can effectively lengthening the path of diffusion through the polymer matrix (Fig. 2).

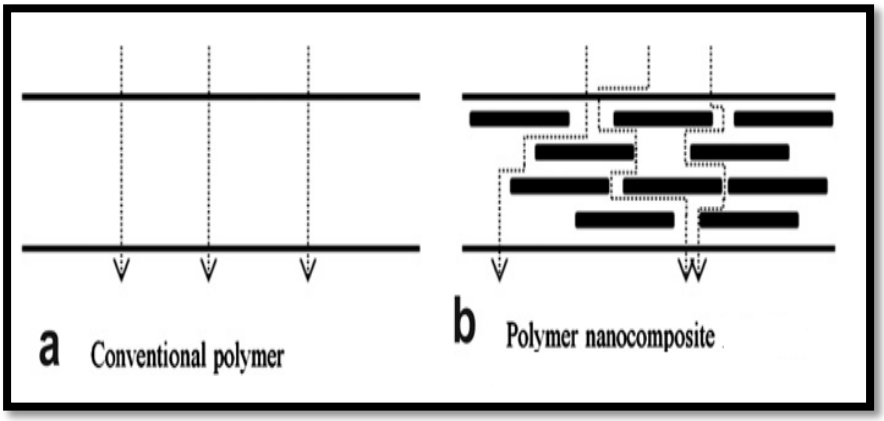

Fig. 2 Schematic illustration of formation of tortuous path created by the use of nanosized fillers in nanocomposites (b) compared to the conventional polymer (a).

Moreover, in this project, we want to test also CH-NP, since they can be easily prepared using sodium tripolyphosphate (TPP) which carries five negative charges per molecule. In this method, TPP solution is added to a CH solution dropwise, and the spontaneously formed particles are then spray dried (Gutiérrez, 2017).

CH and CH-NPs are natural material that are obtained from the marine by-products, with excellent physicochemical, antimicrobial, biodegradability and biological properties, which make them a superior environmentally friendly material and they possess bioactivity that do not harm humans and use as carriers for drug delivery (Malmiri et al., 2012; Divya & Jisha, 2018). Due to the all properties of CH-NPs now they are used in several applications such as tissue engineering, cancer therapy, antioxidant, drug delivery systems, water treatment, food packaging, antimicrobial agent and agriculture. Lorevice et al. (2016), obtained higher mechanical properties by adding CH-NPs to PEC films compared with control, allowing these novel materials to be an alternative to traditional food packaging production. Moreover, addition of small fractions of CH-NPs, enhanced mechanical and thermal stability of banana puree-based films (Martelli et al., 2013).

1.4 Acrylamide as a concern for public health

An undesirable compound that can be found in cooked foods is ACR (H_2C=CH-CO-NH_2), which is highly soluble in water. Heightened concerns about exposure to ACR arose in 2002 when it was discovered that it forms when certain foods are prepared at temperatures usually above 120 °C and low moisture (Mottram et al., 2002; Tareke et al., 2002). Its formation, at least in part, is due to a Maillard reaction between free asparagine and reducing sugars (Fig. 3), ACR forms in numerous baked or fried free asparagine rich foods, including French fries, potato crisps, breads, biscuits and coffee (roasted beans).

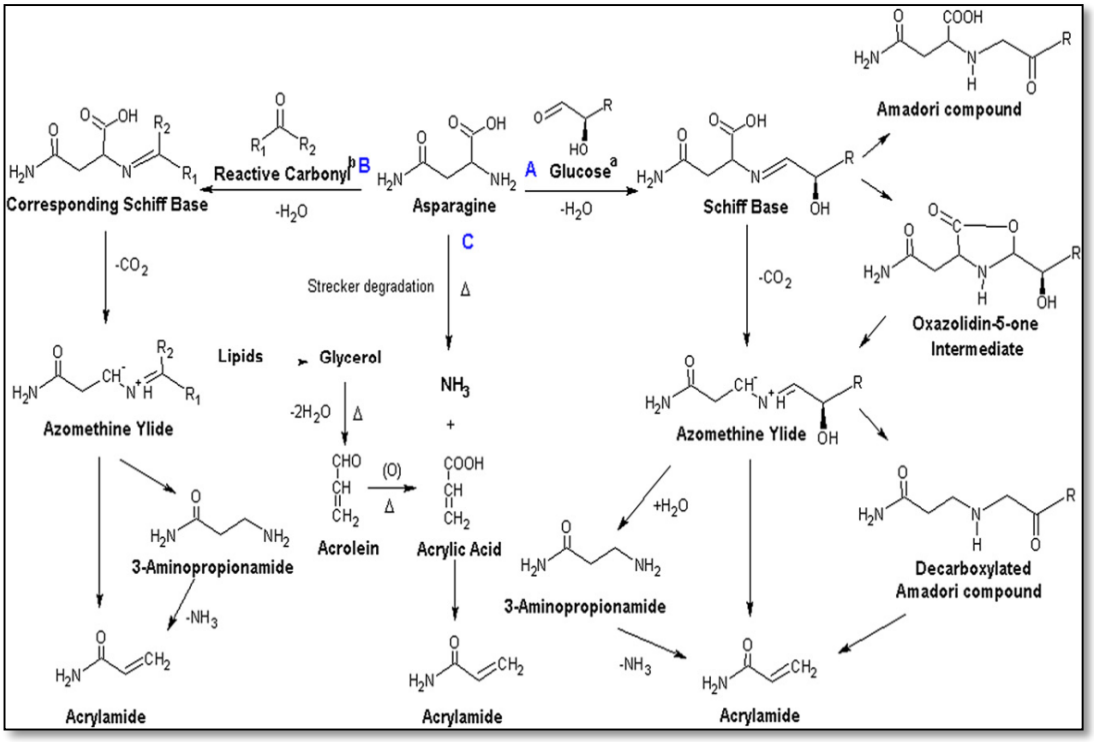

Fig. 3 Formation pathways of ACR from asparagine (Jin et al., 2013).

French fries can contain more than 2000 µg Kg^{-1} of ACR (Gökmen et al., 2006; Pedreschi et al., 2006). In 2015, it has been investigated the ACR content of 40 potato crisp brands from Spain market, that ranges from 108 to 2180 µg kg^{-1} (Mesías & Morales, 2015). ACR is also known to be present in cigarette smoke (EFSA, 2015). Rat- and mouse-based studies have shown some signs of developmental toxicity (increased incidence of skeletal variations, slightly impaired body weight gain, histological changes in the central nervous system, and neurobehavioral effects). However, ACR and its metabolite glycidamide (a reactive epoxide with the formula $C_3H_5NO_2$) are genotoxic and carcinogenic. Since any level of exposure to a genotoxic substance could potentially damage DNA and lead to cancer, EFSA scientists conclude that it is not possible to set a tolerable daily intake of ACR in food. Instead, EFSA's experts estimated the dose range within which ACR is likely to cause a small but measurable tumour incidence (called "neoplastic" effects) or other potential adverse effects (neurological, pre-and post-natal development and male reproduction). The lower limit of this range is called the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) and takes into account body weight (b.w.). For tumors, experts selected a $BMDL_{10}$ of 0.17mg/kg b.w./day. For other effects, neurological changes were seen as the most relevant with a $BMDL_{10}$ of 0.43 mg/kg b.w./day. By comparing the $BMDL_{10}$ to human dietary exposure to ACR, scientists can indicate a "level of health concern" known as the margin of exposure (MOE) which is a ratio between the dose at which a small but measurable adverse effect is first observed, and the level of exposure for a given population (EFSA, 2015).

MOE approach provides an indication of the level of health concern about a substance's presence in food without quantifying the risk. Use of the MOE can help in defining possible actions required to keep exposure to such substances as low as possible. EFSA Scientific Committee concluded that, for substances that are both genotoxic and carcinogenic, MOE of 10 000 or higher, based on a $BMDL_{10}$ from an animal study, and taking into account overall uncertainties in the interpretation, would

be of low concern from a public health point of view (EFSA, 2005; 2012). Since the calculated MOE values are all substantially lower than 10 000, the EFSA scientific panel on contaminants in the food chain (CONTAM Panel) concluded that the MOEs across surveys and age groups indicate a concern with respect to neoplastic effects. The MOEs for the cancer-related effects of ACR range from 425 for average adult consumers down to 50 for high consuming toddlers. These ranges indicate a concern for public health, especially for young people less than 18 years old (EFSA, 2015).

Thus, EFSA welcomes studies addressed to find solutions that could automatize processing of industrial foods to keep ACR content as lowest as possible. In the literature studies are present research that are devoted to minimizing ACR formation during cooking. Most of the studies regard fried potatoes, and pretreatments such as soaking in water or salt solution (Pedreschi et al., 2010), asparaginase treatment (Pedreschi et al., 2008), blanching (EL-Saied et al., 2008), microwave treatment (Erdogdu et al., 2007), addition of natural antioxidants (Ou et al., 2010), and the use of frying oil with high smoke points (Arribas-Lorenzo et al., 2009) have been examined as approaches to control ACR levels in the final product. Other studies have investigated the use of edible coatings to avoid excessive oil absorption and preserve product crispiness (Varela & Fiszman, 2011; Rossi Marquez et al., 2013). Moreover, Suyatma et al. (2015), have studied the synergistic effect of blanching and PEC coating of fried banana chips that resulted in great ACR reduction (up to 91.9 %).

Besides PEC other hydrocolloids, such as tragacanth, carboxy methyl cellulose, guar gum and xanthan gum, have been used to reduce oil uptake of various food products such as fried potato chips, vegetables (i.e. onions) and cereals (Albert & Mittal, 2002; Akdeniz et al., 2006; Garmakhany et al., 2008; Rossi Marquez et al., 2013). Interestingly, hydrocolloids could also reduce ACR formation in food model systems (rich in asparagine), wheat flower, and fried potato products (Zeng et al., 2010). Zeng et al. (2010) observed that ACR formation decreased in food models in the presence of 2% (w/w) PEC, alginic acid (> 50% reduction), and xanthan gum (20%), while for fried potato strips, they suggested that immersion time was a more important factor than hydrocolloid solution concentration. In fact, they showed inhibition of ACR formation by immersing potato strips in a 1% alginic acid solution for 5 hours was two times more effective than immersing in a 5% solution for 1 hour (60% versus 30% reduction).

1.5 Ideas developed in the present project

Palestinian authorities are not yet aware of the ACR concern in foods and, thus no studies are available about the level of this substance in traditional Palestinian food like fried products such as falafel, kobbah, and potato. Falafel is a fried ball principally made of spiced fava beans and/or chickpeas (Abdullah, 2015). (Fig. 4) Falafels were invented around 1000 years ago by the Egyptian Copts, who brought them to the rest of the Middle East. Originally falafel were made with fava beans. The dish migrated northwards to Palestine and Middle East where chickpeas were introduced instead. The chickpea was used as a food item in the East earlier than 4000 B.C. (Jodi, 2002).

Falafel dough is made of a mixture of soaked ground chickpeas, leafy vegetable, onions, spices and leavened by sodium bicarbonate, formed to patties just before deep-frying in vegetable oil until they are crust and thoroughly browned usually. Al-Dmoor et al. (2004), indicated that ACR in Jordanian fried falafel (cooked for 6-8 min at 160-180ºC) have very high values ranging from 2700 to 4200 µg kg^{-1}, moreover, the same study demonstrated that the excessive use of frying oil in food preparation

caused significant increases (~33%) in ACR content. Very little work has been done to decrease oil absorption in fried falafel balls and the only indications come from Abu-Alruz. (2015), which assessed that increasing falafel ball size provokes a reduction of oil uptake together with a decrease of frying time.

Fig. 4 Falafel balls, sandwich and frozen falafel (ready-to-eat after warming).

Kibbeh, kibbe, kobbah (also kubbeh, kubbah, kubbi) (pronunciation varies with region) is eastern dish made of a ground bulgur (wheat-based food) mixed with minced beef meat formed as balls stuffed with cooked ground meat, onion, nuts and spices (Fig. 5). They are usually cooked by deep frying at 160-180 ºC for 8-10 min in vegetable oil, thus they look gritty crust and thoroughly browned. They are home-made and consumed fresh or they are sold frozen in the super-markets and consumers can fry them at home. Al-Dmoor et al. (2004), also determined the ACR content in Jordanian fried kobbah found values that range from 2900 to 5300 μg kg^{-1}). Thus, both falafel and kobbah contain ACR in values that impose attention to protect the health of consumers.

Fig. 5 Kobbah and raw frozen kobbah.

Another objective reached in the present project was obtaining novel bioplastics nanoreinforced with NPs to be used as sustainable wrapping for strawberry, thus prolonging its shelf-life and physico-chemical characteristics. The strawberry (*Fragaria vesca*) is one of the most perishable fruits, and has very short postharvest time, because of its susceptibility to mechanical damage, physiological deterioration and possible attack of pathogens. Strawberry is classified as a healthy food, due to a high content of vitamin C, antioxidant activity, vitamin E and phenolic compounds which make *Fragaria vesca* important for human nutrition. To date, some studies have been devoted to extend the shelf-life of strawberry fruits by dipping fruits with edible coatings, and by packaging them using CH–poly(vinylalcohol) blend films (Liu et al., 2017). An objective of the present study was to investigate the effect of nanoreinforcement on both PEC films-forming solutions (FFSs) and cast film on strawberry physicochemical properties. Samples, prepared at two different concentrations of PEC, GLY and/or MSNs, were prepared with the aim of obtaining a suitable biomaterial useful for food packaging. We have here tested the efficiency of PEC-based films in protecting strawberries by extending their shelf-life at 4 °C.

CHAPTER 2

MATERIALS AND METHODS

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2. MATERIALS AND METHODS

2.1 Materials

Acrylamide (ACR) standard ≥ 99.8%, catalogue No. 23701 and methanol were obtained from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Acetonitrile HPLC (high pressure liquid chromatography) analytical grade, n-hexane, and formic acid were supplied from Carlo Erba reagents srl (Milan, Italy). Water purified by a Milli-Q-RO system (Millipore, Bedford, MA, USA) was used. Whereas Oasis HLB (Hydrophilic-Lipophilic-Balanced) 200 mg, 6 mL solid phase extraction (SPE) cartridges were from Waters (Milford, MA, USA). The syringe filters (0.45 µm, 0.22 µm PVDF (polyvinylidene difluoride) were from Alltech Associates (Deerfield, IL, USA).

Pectin (PEC) of Citrus peel low-methylated (7%) (Aglupectin USP (United States Pharmacopoeia)) was purchased from Silva Extracts srl (Gorle, Italy). Chitosan (CH mean molar mass of 3.7 -104 g/mol) was obtained from Professor R. Muzzarelli (University of Ancona, Ancona, Italy), with a degree of 9.0% N-acetylation. Glycerol (GLY) was purchased from the Merck Chemical Company (Darmstadt, Germany).

Tetraethylortosilicate (98%) (TEOS), cetyltrimethylammonium bromide (CTAB) were obtained from Sigma (Steinheim, Germany) that was used to synthesize the MSN, MSN- based solutions were prepared with distilled water.

2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Company (Pool, Dorset, UK), while ascorbic acid and sodium tripolyphosphate (TPP) were obtained from Merck Chemical Company (Darmstadt, Germany).

Grass pea seeds (GP), chickpeas and corn oil were purchased from a local market (Naples, Italy). Potatoes (cultivar Musica) were obtained from the Department of Agriculture, University of Naples “Federico II” (Naples, Italy) and stored at 4 ºC until use and strawberries variety "Sabrina" were purchased from local market Naples, Italy, strawberry experiments were carried out the day after their purchasing.

Transglutaminase was purchased from Prodotti Gianni (ITALY) which distributes the WM preparations sold by Ajinomoto, Japan.

Other chemicals and solvents used in this study were of analytical grade. Mater-Bi® (S 301)-based commercial material bags were from a local market, Naples, Italy.

2.2 Synthesis of mesoporous silica nanoparticles (MSN)

MSN (MCM-41) was synthesized by using (Chen et al., 2011; Fernandez-Bats et al., 2018) and, reported in the following scheme (Fig. 6).

2.3 Synthesis of chitosan nanoparticles (CH-NP) and their characterization

CH-NP was synthesized by using the ionic gelation method (Calvo et al., 1997; Hosseini et al., 2013; Chang et al., 2010; Lin et al., 2019). Reported in the following scheme (Fig. 7). The CH-NP then characterized by using Zetasizer Nano ZSP equipped with an automatic titration unit (MPT-2). Zeta potential and Z-average of CH-NP (1mg/mL) prepared at pH 2, than titrated automatically from pH2 to pH7 by using NaOH 0.1, 0.5 and 1N as titrant solutions.

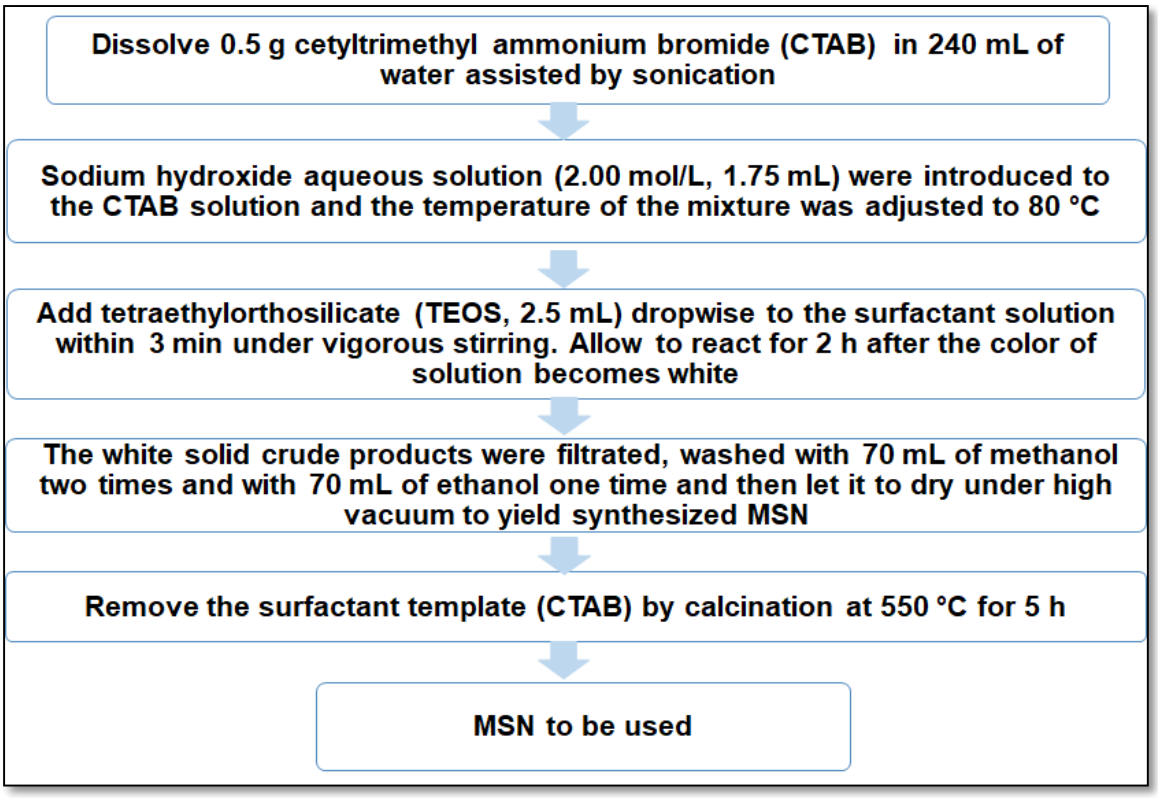

Fig. 6 Scheme for MSN synthesis.

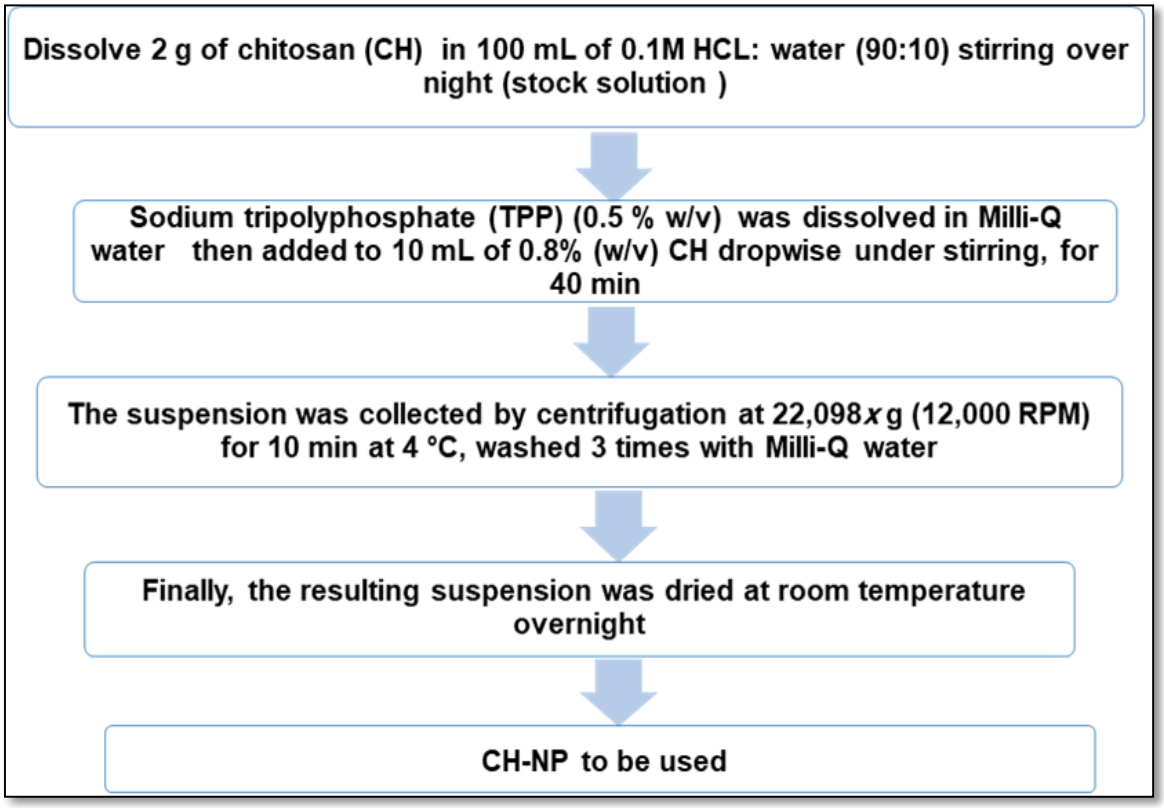

Fig.7 Scheme for CH-NP synthesis.

2.4 Hydrocolloid dipping solution preparation

To obtain GP flour (GPF), seeds were ground using a variable speed laboratory blender LB 20ES (Waring Commercial, Torrington, CT, USA), so that the GPF could pass through a 425-μm stainless steel sieve (Octagon Digital Endecotts Limited, London, UK), the flour samples were collected and stored in polyethylene bags at 4 °C until used for analysis. GPF-based solutions were prepared by dissolving 8.3 g of GPF (containing 24% w/w proteins) in 100 mL Milli-Q water. The solution was shacked for 1 h and its pH was adjusted to 9.0, followed by centrifugation at 12,096 x*g* for 10 min. After centrifugation, 60 mL of the supernatant were taken and the pH was adjusted to 7.0, and the final volume of 100 mL was reached with water after adding 16 μL of GLY (8% w/w with GPF proteins). The solution was then divided in two 50 mL falcon tubes and TGase (33U/g of GPF proteins) was added in only one tube; then both samples (GPF with and without TGase) were incubated for 2 h at 37 °C. The enzymatic reaction was stopped by adjusting the pH to 9.0. The ratio between the enzyme and its substrate in order to have TG-mediated crosslinking of GPF the proteins, was established in our laboratories (unpublished data). CH-based solutions (0.6% w/v) were prepared from a CH stock solution (2% w/v of hydrochloric acid 0.1 N stirred overnight) then diluted with water adjusting the pH to 4.0; finally the solution was stirred for 30 min at 25 °C. PEC-based solutions (1% w/v) were prepared according to Esposito et al. 2016 from a PEC stock solution (2% w/v), then diluted with water; the pH was adjusted to 7.5; finally the solution was stirred for 30 min at 25°C.

GPF-based solutions were prepared as described above, after centrifugation, 60 mL of the supernatant were taken and the pH was adjusted to 6.0, then added the NPs, either MSN or CH-NP (1% w/w GPF proteins) shaking for 30 min, and the final volume of 100 mL was reached with water after adding 16 μL of GLY (8% w/w GPF proteins). The solution was then divided in two 50 mL falcon tubes and TGase (33U/g of GPF proteins) was added in only one tube; then both samples in presence of NPs (GPF with and without TGase) were incubated for 2 h at 37 °C. PEC-based solutions (1% w/v) were prepared as descried above then added the NPs either MSN or CH-NP (1% w/w PEC) shaking for 30min the pH was adjusted to 6; finally the solution was stirred for 30 min at 25°C.

2.5 Food preparation and dipping process

2.5.1 Falafel preparation and dipping process

2.5.1.1 Falafel dough preparation

100 g of dough were made up of a mixture of water overnight soaked chickpeas (76 g), onion (10 g), parsley (3 g), falafel spices (0.3 g), salt (1.5 g), and sodium bicarbonate (0.2 g).The mixture was blended for 2 min with water. A special scoop (Inner diameter 4 cm, depth 1 cm) was used for preparing the falafel balls, each one with a diameter of 4 cm and weighing around 17 g. Then the balls were fried as described below.

2.5.1.2 Falafel dough treated with TGase

After prepared the falafel dough as described above, TGase (5 and 20U/g protein) was added to the dough and incubated at 37 °C for 2 h. The falafel balls were then formed and fried. The control falafel samples were obtained without TGase but treated under the same experimental conditions.

2.5.1.3 Dipping

The falafel balls, either treated or not with TGase, were frozen at −20 °C for 2 h, then dipped in 1% PEC, whereas the control was dipped in water. Each kind of dipped ball was frozen again for 30 min and then fried. In Table 1 the experimental design is described.

Table 1. Experimental design followed for performing all the analyses.

Falafel Type	TGase (U/g Protein)	Incubation 2 h at 37 °C	Dipping Solution
Traditional falafel	–	–	–
Incubated without TGase	0	√	–
Incubated with TGase (5U/g)	5	√	–
Incubated with TGase (20U/g)	20	√	–
Dipped	–	–	Water
Incubated without TGase	0	√	1% PEC
Incubated with TGase (5U/g)	5	√	1% PEC
Incubated with TGase (20U/g)	20	√	1% PEC

2.5.1.4 Falafel sensory evaluation

Sensory evaluation of falafel balls was carried out by 40 panellists who represented trained graduate students in the Department of Nutrition and Food Technology (An-Najah National University). Falafel belonged to three different groups: control (traditional falafel), falafel prepared adding TGase (5U/g) in the dough, traditional falafel coated by PEC 1% solution. Each group was stored at -20°C for 0, 30 and 60 days. The sensory test was performed to evaluate different attributes which were: appearance, taste, texture/mouthfeel, color and overall acceptability. The samples were evaluated using the preference test based on the five-point hedonic scale (1= Dislike a lot; 2= Dislike a little; 3= Neither like nor dislike; 4= Like a little; 5= Like a lot) (Singh-Ackbarali & Maharaj, 2014; Mansour, 2003). Acceptability Index (AI) of the falafel samples was calculated as AI (%) = ((Average score / highest score) x 100) (Granato et al., 2012). Fresh fried falafel balls were served on white plastic plates coded by a number. The sensory evaluation was performed in the laboratory under conditions of standard light and temperature of 25 °C.

2.5.2 Potato preparation and dipping process

Potatoes were cut into 1 cm x 1 cm x 6 cm sticks as described by Rossi Marquez et al. 2013, and treated as follows: 100 g of potatoes (18 sticks) were dipped for 30 s into either distilled water (sample used as “control”) or one of the following coating solutions: (1) grass pea flour (GPF); (2) TGase treated (GPF + TGase); (3) chitosan (CH); and (4) pectin (PEC). Then, each sample was allowed to drip for 2 min before frying. (Fig. 8)

2.5.3 Kobbah preparation and dipping process

Soaking of a ground bulgur flour into hot water (80°C) for 1 hour, then the wheat flour, oil, salt and spices were added and mixed with the soaked ground bulgur. The dough was store at the refrigerator for 1 hour. Stuffing: onions, salt and spices were mixed with minced beef meat then cooked with olive. The dough was shaped into balls stuffed with cooked ground meat, then treated as follows: 200 g of kobbah (5 pieces) were dipped for 30 s into either distilled water (sample used as “control”) or one of the following coating solutions: (1) grass pea flour (GPF); (2) GPF reinforced with MSN (GPF + MSN); (3) GPF reinforced with CH-NP(GPF + CH-NP); (4) TGase treated (GPF + TGase); (5) TGase treated reinforced with MSN (GPF + MSN +TGase); (6) TGase treated reinforced with CH-NP (GPF + CH-NP +TGase); (7) pectin (PEC); (8) PEC reinforced with MSN (PEC + MSN); and (9) PEC reinforced with CH-NP (PEC +CH-NP), as shown in Fig 8. Moreover, each sample was allowed to drip for 2min before frying.

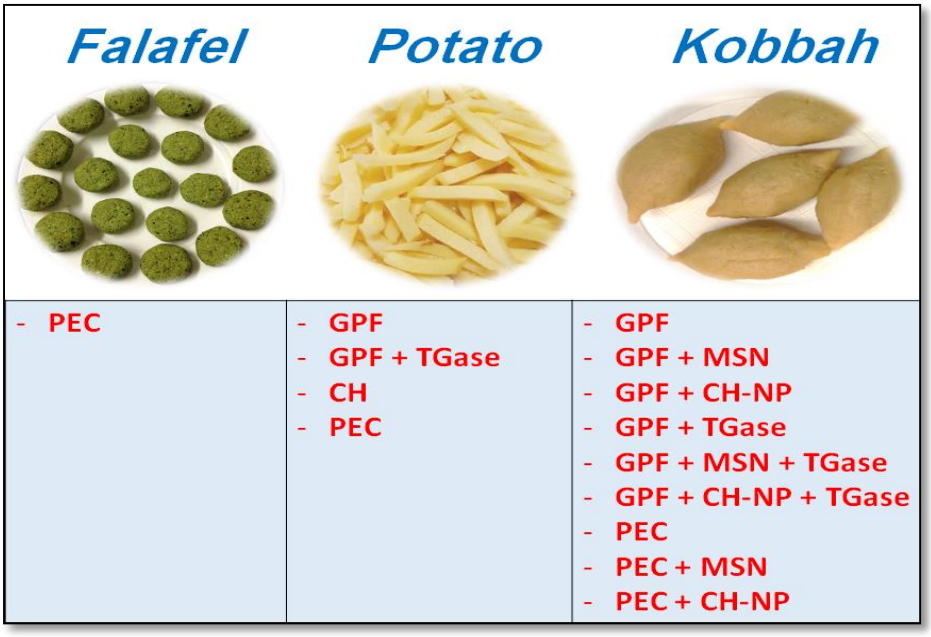

Fig. 8 Dipping solutions applied to the foods.

2.6 Frying process

Falafel balls, potato, and kobbah were fried at 180 ± 5 °C for 5 min, 170 ± 5 °C for 6 min, and 190 ± 5 °C for 4.5 min respectively, by using 1.5 - 2 L of corn oil was preheated, using a deep-fryer apparatus (Girmi, Viterbo, Italy) (Rossi Marquez et al., 2013) (Fig. 9). The oil was replaced with fresh oil for each group. Each fried group was flipping from side to side every 2 min. After frying, each sample was allowed to drain for 2 min to remove the excess oil.

Fig. 9 Frying tempterature and time for each kind of food.

2.7 Acrylamide detection

2.7.1 Acrylamide standard preparation

ACR standard stock solution (1.0 mg/mL) was prepared by dissolving 10.0 mg of the ACR standard in 10 mL of Milli-Q water by using a volumetric flask. From the stock solution, calibration standards at different concentrations (50,100, 250, 500, 1000, 2000, 3000, 4000, and 5000 µg/L), were prepared, respectively. All series of standard solutions were stored in glass dark bottles (light-resistant) at 4 °C until used.

2.7.2 Extraction of acrylamide

About 100, 160, 200 g of fried potato sticks, fried falafel balls, and fried Kobbeh respectively, accurately weighed after cooling, were immersed in n-hexane for 30 min to remove the oil from their surfaces (Zeng et al., 2010). The fried foods were then ground with a rotary mill Grindomix GM200, (Retsch GmbH, Haan, Germany) at a speed of 1300 rpm for 1 min. Each sample was allowed to dry by freeze-drying before being subjected to ACR extraction following the protocols reported by Wang et al. 2013, with some modifications: two different Falcon tubes were set up for each sample, one for detecting ACR formed in the sample itself (“basic” ACR), and the second one to carry out the “Recovery test”. In both tubes, 1.0 g (dry weight) of sample, accurately weighed, was put in both tubes and only in the second one was the ACR standard added. Fifty µL of Carrez reagent potassium salt and 50 µL of Carrez reagent zinc salts were added to each sample. In each tube, 10 mL of HPLC water were added. The samples were extracted in an incubated shaker for 30 min at 25 °C and 170 rpm, then followed by centrifugation at 7741x *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm syringe filter for the clean-up of the Oasis HLB SPE cartridges. The SPE cartridge was preventively conditioned with 2.0 mL of methanol followed by washing with 2 mL of water before loading 2.0 mL of the filtered supernatant, the first 0.5 mL was discarded and the remaining elute collected (~1.5 mL; exact volume was measured by weight and converted by means of density). All extracts were kept in dark glass vials at 4 °C before analysis. The clean sample extracts were further filtered

through 0.2 µm nylon syringe filters before HPLC-UV (ultra violet) analyzed for fried potato and kobbeh, while the falafel balls samples analyzed by TOF LC-MS. Each analysis was performed in triplicate.

2.7.3 Optimization of acrylamide analysis by HPLC

Improvement of the chromatographic conditions, and recovery. Acrylamide detection was performed by HPLC at 210 nm, by using the following columns: a) Alltima HP C18 stationary phase, 5µm particle diameter, 4.6 x 250 mm internal diameter and length (GRACE/Alltech, Maryland USA); b) Reprosil100 C18 stationary phase, 5µm particle diameter, 4.6 x 250 mm internal diameter and length (Dr. Maisch GmbH, Germany), c) Synergi™ Max-RP C18 stationary phase (Phenomenex, Torrance, CA USA), 4µm particle diameter, 4.6 x 250mm internal diameter and length),d) Phenomenex Synergi™ Hydro-RP 80 Å C18 stationary phase (Phenomenex, Torrance, CA USA), 4 µm particle diameter,4.6 x 250 mm internal diameter and length. All these columns allowed the separation of ACR by using to an isocratic and/or gradient program of elution. Three different mobile phases were tested changing the water/acetonitrile ratio: 90/10, 95/5, 97/3 v/v (all containing 0.1% v/v formic acid). In all cases mobile phase was used with the following flow rates: 0.50, 1.00 mL/min. The best performances were obtained using the Synergi 4 µm Hydro-RP 80 Å, column, 4.6 x 250 mm, with water/acetonitrile (97/3 v/v containing 0.1% v/v formic acid), according to the method already described by Michalak et al. (2013) with some modifications. In these conditions the best chromatographic separation was achieved with the elution of ACR at about 4.9 min. with at flow rate of 1.00 mL/min and UV detection at 210 nm.

Calibration curves were obtained by plotting the peak area of ACR versus concentration of ACR (range of concentration: 0.1-5 mg/mL). The equation obtained applying the linear regression was y=108.53x-1.6965, with a R^2 equal to 0.999; this equation was used to calculated the amount of ACR in all sample analyzed. The limit of detection (LOD) was 29.6 µg /L, and the limit of quantitation (LOQ) was 89.1 µg /L. (Fig. 10 and Fig. 11).

In addition, the recovery test was performed to assess the extraction efficiency for each sample; to this aim the ACR content before and after the addition of 500 µg/L of ACR standard was determined equation (1). Percentage recovery was determined according to the following formula.

$$\text{Recovery}(\%) = \frac{\text{ACR (detected after standard addition)} - \text{ACR (sample)}}{\text{ACR (standard added)}} \times 100 \qquad (1)$$

ACR determinations were repeated three times and results are reported as the means with standard deviations.

2.7.4 HPLC-UV analysis

HPLC-UV analysis was performed by using the RP-HPLC (RP: reverse phase) method on an Agilent 1100 series HPLC instrument equipped with an on-line degasser, a dual pump, and a diode array detector (Hewlett Packard, Wilmington, DE, USA). The column used was a Synergi™ 4 µm Hydro-RP 80 Å HPLC Column 250 x 4.6 mm (Michalak et al., 2011; 2013) (Phenomenex, Torrance, CA, USA).

The operating conditions were as follows: the wavelength detection was 210 nm, a gradient elution of 0.1% formic acid (v/v) in water: acetonitrile (97:3, v/v) was applied. Solvent A was water and Solvent B was acetonitrile, both solvents containing 0.10% (v/v) formic acid; flow rate, 1.0 mL/min. The gradient elution program was applied as follows: 97% A (3% B) for 10 min, increased to 20% A (80% B) from 10 to 12 min, and kept at 20% A (80% B) for 5.0 min, increased to 95% B (5% A) from 17 to 19 min, and kept at 95% B for 5 min, increased to 97% A (3% B) from 24 to 26 min, and kept for 4 min. The injection volume was 20 µL. The total chromatographic runtime was 30 min for each sample and the temperature was kept at 30 °C (GECKO 2000 “HPLC column heater”, Spectra Lab Scientific Inc., Markham, ON, Canada) to ensure optimal separation. In all samples (ACR standard and fried potato-derived), the ACR retention time was 4.9 min. The method presented a relative standard deviation lower than 5% with three repetitions; this result was in accordance with the data reported in literature.

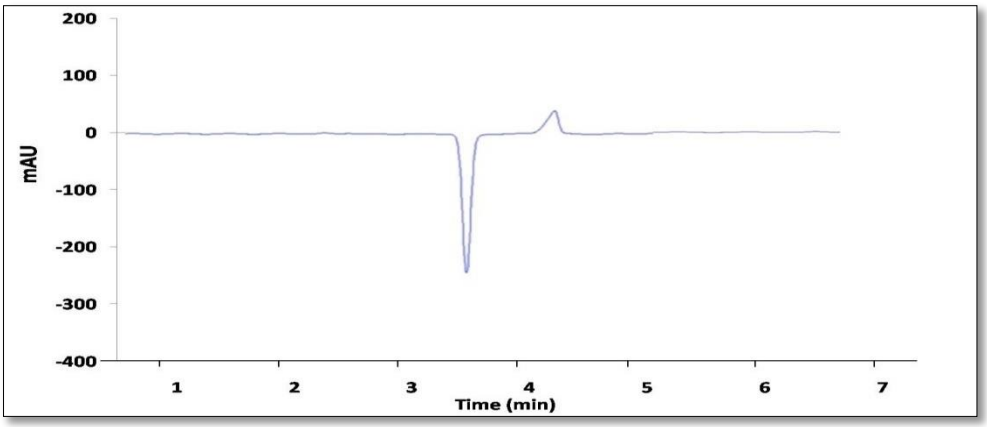

Fig. 10 HPLC chromatograms of water as blank obtained at 210 nm. The mobile phase was (97/3 v/v) water/acetonitrile containing 0.1% v/v formic acid at 1.00 mL/min.

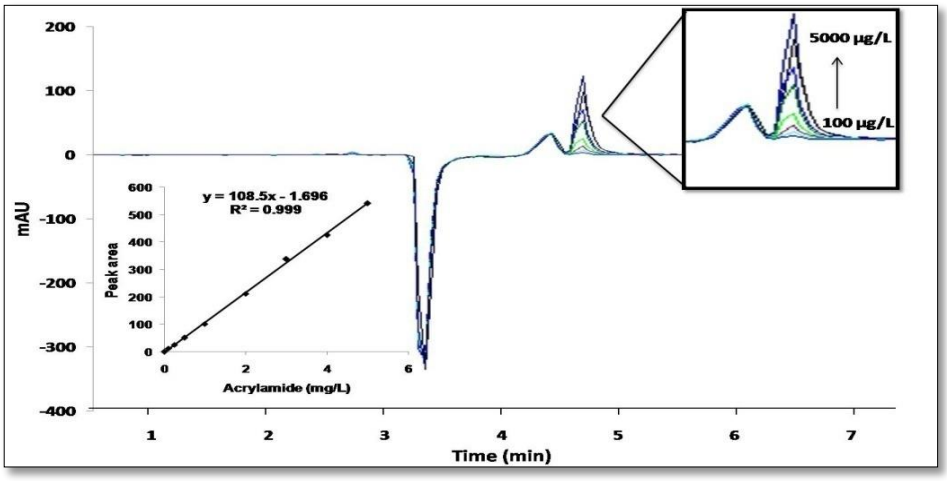

Fig.11 HPLC chromatograms of ACR standards obtained at 210 nm; ACR concentrations were 0.1, 0.25, 0.5, 1, 2, 3, 4 and 5 mg/L of ACR. The mobile phase was (97/3 v/v) water/acetonitrile containing 0.1% v/v formic acid at 1.00 mL/min.

2.7.5 LC-MS analysis

The determination of ACR concentration was performed using the Agilent 6230 TOF-LC/MS (Agilent Technologies, Santa Clara, US) coupled to a series HPLC system, a vacuum degasser, binary pumps, and a temperature-controlled column oven at 30 °C. The following MS parameters were used: positive ion mode, nebulizer pressure 35 psi, drying gas (N_2) 5 L/min and 325 °C, capillary voltage 3500 V, and fragmentor 175 V. The column used was a Synergi™ 4 µm Hydro-RP 80Å HPLC Column at 250 x 3 mm (Michalak et al., 2013) (from Phenomenex, Torrance, CA, USA). The operating conditions were as follows: the mobile phase was a gradient elution: mixture water/acetonitrile (97/3, v/v) containing 0.10% (v/v) formic acid Solvent A and Solvent B was acetonitrile containing 0.10% (v/v) formic acid. The program elution was applied as follows: 100% A (0% B) for 8 min, increased to 80% B (20% A) from 8 to 15 min, and kept at 80% B (20% A) for 10 min, increased to 100% A (0% B) from 25 to 30 min and kept at 100% A for 5 min, at a flow rate of 0.4 mL/min. The injection volume was 20 µL. The total chromatographic runtime was 35 min for each sample; ACR elutes at a retention time of 3 min, and then the peak identification was based on the Extracted Ion Chromatogram (EIC); by selecting the ion at m/z 72, calibration curves were obtained by plotting the peak area of ACR versus concentration of ACR (range of concentration: 0.05–10 mg/L). The equation was obtained by applying the linear regression of y = 102.21x − 10.706, with R^2 equal to 0.9991; this equation was used to calculate the amount of ACR in all analyzed samples.

2.8 Oil and water content analysis for fried foods

After frying and cooling, the oil content of each ground sample was determined in triplicate, and reported as a percentage on dry matter weight by n-hexane solvent extraction using the Soxhlet method (AOAC1990a) and the oil reduction due to coating was calculated as in the following equation.

$$\text{Oil reducing due to coating}(\%) = \frac{\text{oil content (control) –oil content (coated)}}{\text{oil content (control)}} \times 100 \quad (2)$$

Water content of fried potato samples was determined according to (AOAC 1990b). After frying the potato sticks that had been coated with a different hydrocolloids coating solutions and the control (coated with water), were dried in an oven at 105°C until constant weight was achieved. Water content, water loss during frying and water retention in the all samples were calculated as follows:

$$\text{water content}(\%) = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100 \quad (3)$$

$$\text{water loss during frying } (\%) = \frac{\text{(initial water –water after frying)}}{\text{initial water}} \times 100 \quad (4)$$

$$\text{water retention} = \left(\frac{\text{water contentof coated}}{\text{water content of control sample}}\right) - 1 \times 100 \quad (5)$$

2.9 Daily intake and margin of exposure of acrylamide risk assessment for French Fries

The DI and, consequently, the MOE were calculated taking into account the six following age groups (as stated from EFSA): Toddlers (1-3 years old); Children (3-10 years old); Adolescents (10-18 years old); Adults (18-65 years old); Elderly (65-75 years old); very elderly (more than 75 years old). DI (ng kg body weight^{-1}day^{-1}), was calculated according to the formula (Esposito et al., 2017):

$$\mathrm{DI} = \frac{\mathrm{C} \times \mathrm{Q}}{\mathrm{BW}} \quad (6)$$

Where C= Average concentration of acrylamide detected in each fried potato samples (ng g^{-1}). Q= Average daily consumption of fried potato samples of each age groups (g day^{-1}), BW= Body Weight of each age group.

The average daily consumption Q was taken from the EFSA report 2015, and BW values were taken by Leclercq et al. (2009), that considered BW of Italian consumers belonging to the described six age groups. The DI results were used to estimate the MOE, a parameter that indicates the level of health concern for toxic and/or carcinogenic molecules. Here we have calculated MOE for ACR, taking into account that it is classified both as neurotoxic and carcinogenic agent. To calculate MOE it is necessary to refer to the $BMDL_{10}$ = BenchMark Dose Lower confidence limit (mg kg BW^{-1}day^{-1}), which represents the minimum dose range of a substance that produces a clear, low level health risk, usually in the range of a 1-10% (EFSA, 2015). The $BMDL_{10}$ based on neurological changes (ACR as neurotoxic agent) is 0.43 mg kg BW^{-1}day^{-1}, while $BMDL_{10}$ considering ACR a carcinogenic agent is 0.17mg kg BW^{-1}day^{-1} from the EFSA report 2015.

The MOE assessment was calculated according the following equation (Esposito et al., 2017)

$$\mathrm{MOE} = \frac{\mathrm{BMDL_{10}}}{\mathrm{DI}} \quad (7)$$

2.10 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For the SDS-PAGE of falafel balls or fried kobbah , an aliquot of 250 µL of sample buffer (15 mM of Tris–HCl, pH 6.8, containing 0.5% (w/v) of SDS, 2.5% (v/v) of GLY, 200 mM of β-mercaptoethanol, and 0.003% (w/v) of bromophenol blue), was added to 25 mg of falafel dough and/or whole fried falafel (either untreated or treated with TGase) after grinding, also 25 mg of whole fried kobbah coated or not with GPF treated or not by means of TGase with or without MSN. Each aliquot was analyzed by 12% SDS-PAGE. The samples were heated at 100 °C for 5 min, and then centrifuged for 10 min at 13000 × g. Ten µL of each supernatant was analyzed by SDS-PAGE (12%). SDS-PAGE was performed as described by Laemmli 1970, at a constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250 (Bio-Rad, Segrate, Milan, Italy). Bio-Rad Precision Protein Standards were used as molecular weight markers.

2.11 *In vitro* digestion

Samples fried falafel balls either treated or not by TGase (20U/g protein), or fried kobbah coated or not with GPF treated or not by means of TGase with or without MSN were subjected to *in vitro* digestion (IVD) by using an adult model (Giosafatto et al., 2012; Bourlieu et al., 2014; Minekus et al., 2014), under gastric physiological conditions. For our analyses, 100 mg of each sample were incubated with 4 mL of Simulated Salivary Fluid (SSF, 150 mM of NaCl, 3 mM of urea, pH 6.9) containing 75 U of amylase enzyme/g protein for 5 min at 37 °C and 170 rpm. The amylase activity was blocked by adjusting the pH at 2.5. Afterwards, the samples were subjected to IVD as described by Giosafatto et al. 2012, with some modifications. Briefly, 100 µL of Simulated Gastric Fluid (SGF, 0.15 M of NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and added to 100 µL of oral phase and then incubated at 37 °C. Thereafter, 50 µL of pepsin (0.1 mg/mL dissolved in SGF) were added to start the digestion reaction. At intervals of 1, 2, 5, 10, 20, 40, and 60 min, 40 µL of the 0.5 M of ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease. The samples were then analyzed using the SDS-PAGE (12%) procedure described above.

2.12 Film preparation and characterization

2.12.1 Film forming solutions (FFSs) and film preparation

A stock solution of PEC (2.0 g) was dissolved in 100 mL of distilled water until the PEC solution was completely solubilized. Serial concentrations of PEC-based FFSs were prepared at pH 7.5 from 0.2-1%, containing MSN 3% w/w PEC in the absence and presence of GLY (30% w/w PEC). Then the 6 and 10 mg/mL PEC FFSs with different concentrations of MSN (1, 3 and 5 %w/w PEC), were prepared both in the absence and presence of different concentrations of GLY (10, 30 and 50% w/w PEC) in 50 mL of distilled water. CH-based film forming solutions (0.6% w/v) were prepared from a CH stock solution (2% w/v of hydrochloric acid 0.1 N, stirred overnight) at a pH 4.5, after that added MSNs or not (3% w/w of CH), were prepared in the absence or presence of GLY (30% w/w of CH), in 50 mL of distilled water. The same volumes (50 mL) of all the different FFSs, containing or not MSN and/or GLY, were cast onto 8 cm diameter polystyrene Petri dishes and finally the films were allowed to dry in an environmental chamber at 25°C and 45% RH for 48 h. The handle able dried films were peeled off intact from the casting surface after they were conditioned at 25°C and 53% RH for 2 h in a desiccators containing a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$.

2.12.2 Zeta potential and particle size measurements

Zeta potential and mean particle size hydrodynamic diameter (Z-average size) of the 1 mg/mL PEC FFSs prepared at pH 12.0, containing or not MSN 3%, GLY 30% or both (w/w PEC), were titrated automatically from pH 12.0 to pH 2.0, by measuring the dynamic light scattering by a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) using a He–Ne laser (wavelength of 633 nm) and a detector angle of 173°.

2.12.3 Film thickness and opacity

Film thickness was measured with an electronic digital micrometer (DC-516, sensitivity 0.001 mm) at different positions of each film sample. At least six measurements were taken on each film sample and the thickness mean values were considered in the different tests. The opacity test measurements, carried out six times for each sample, were performed, as described by Tonyali et al. 2018. Opacity was calculated as follows:

$$\text{Opacity } (mm^{-1}) = A_{600}/x \quad (8)$$

Where A_{600} was the absorbance at 600 nm and x was the film thickness (mm).

2.12.4 Film mechanical properties

The mechanical properties tensile strength (TS), elongation at break (EB) and Young's modulus (YM), were measured according to the ASTM D882-1997, by using a universal testing instrument model no. 5543A (Instron, Norwood, MA, USA). PEC films strips (1 cm wide and 5 cm long), obtained by using a sharp scissors, and were conditioned in an environmental chamber at 25°C and 53% RH for 2 h. Finally, 6 samples of each film type were tested, and the speed was 5 mm/min in tension mode.

2.12.5 Seal strength

The seal strength of each PEC films was tested by Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). PEC film samples were cut into strips of 5 x 2.5 cm, one strip was placed on the top of another. After being sealed, film samples were conditioned at 25°C and 50 ± 5% RH for 48 h (Farhan & Hani, 2017; Sabbah et al. 2019b; Tongnuanchan et al. 2016), and few drops of water were added to the seal area. Those two strips were sealed by heat sealer (MAGIC VAC® AXOLUTE Mod: P0608ED). The width of seal area was 0.3 cm^2. The sealing parameters were studied according to ASTM F 88-07a (2007) the seal strength (N/m) was calculated as following Eq. (9), (Farhan & Hani, 2017; Tongnuanchan et al., 2016):

$$\text{Seal strength (N/m)} = \text{Peak force}/\text{film width} \quad (9)$$

2.12.6 Film barrier properties

The gas permeability tests toward O_2 (ASTM D3985-05,2010), CO_2 (ASTM F2476-13, 2013), and water vapor (WV) permeability (ASTM F1249-13, 2013) of triplicate samples of each film were performed at 25°C under 50% RH, in aluminum masks having an area of 5 cm^2, by using MultiPerm apparatus (ExtraSolution s.r.l., Pisa, Italy).

2.12.7 Differential scanning calorimetry and thermogravimetric analysis

DSC, TGA and differential thermal gravimetry (DTG) analyses of the films were performed on a DSC Q-200 (TA Instruments). Samples (about 10 mg) were placed into platinum crucibles and then they were heated at 5°C/min, from room temperature to 400°C, under inert atmosphere (50 mL/min of N_2).

2.12.8 Morphology analysis

Film surfaces and cross-sections were analyzed by Scanning Electron Microscope (SEM). Films were cut using scissors, mounted on stub and sputter-coated with platinum-palladium (Denton Vacuum Desk V), before observation with Supra 40 ZEISS (EHT = 5.00 kV, in lens detector). Micrographs for sample surfaces were obtained at 25,000× magnifications, whereas cross-sections were obtained at both 25,000× and 50,000× magnifications.

2.12.9 Fourier transform infrared spectra

FT-IR measurements of the films were performed by a Bruker model ALPHA spectrometer, equipped with attenuated total reflectance (ATR) accessory. The measurements were obtained in 4000–400 cm^{-1} region at 4 cm^{-1} resolution for 24 scans.

2.12.10 Film moisture content

Moisture content of each film was measured gravimetrically according to Farhan & Hani 2017, and Singh et al. 2015 with some modifications. In particular, test specimens (5 cm x 5 cm) at different positions of each film type were uniformly cut and placed on glass Petri dishes. Film moisture content was determined by drying each specimen in an oven at 105 °C until a constant dry weight was obtained. Film moisture content was calculated as:

$$\text{Film moisture content (\%)} = ((W1-W2)/W1) \times 100 \quad (10)$$

Where W1 is the initial weight of the film and W2 is the film weight after drying at 105°C overnight. Each measure was carried out in triplicate.

2.12.11 Film moisture uptake

Moisture uptake of each film was measured gravimetrically in triplicate as described by Manrich et al. 2017. In particular, films were cut into 5-cm-sided squares, dried at 105 °C for 24 h, conditioned at 23 ± 2 °C into a desiccator, previously equilibrated at 50 % RH with a saturated $Mg(NO_3)_2$ solution for 24 h. The moisture uptake was calculated as:

$$\text{Film moisture uptake (\% dry matter)} = ((Ws-Wd)/Ws) \times 100 \quad (11)$$

Where Ws and Wd are the weight of swollen (24 h at 50% RH) and dried films, respectively. Each measure was carried out in triplicate.

2.13 Film applications to strawberry

2.13.1 Strawberry wrapping

The selected strawberries were of uniform size, color and without physical damages and fungal infections. They were randomly divided into four groups. Each group of eleven strawberries was wrapped (W) by different sealed films (10cm x 10cm) as following: 0.6% PEC+30% GLY film (W, PEC+GLY), 0.6% PEC+3% MSN+30%

GLY film (W,PEC+MSN+GLY), Mater-Bi® commercial material (W, Mater-Bi),and the control was unwrapped (UW). These samples were placed at 4°C, the quality of both wrapped and control samples were evaluated during storage at 0, 2, 4, 6, and 8 days.

2.13.2 Weight loss

The weight loss of the unwrapped and wrapped samples was calculated in triplicate at 0, 2, 4, 6 and 8 days of storage as follows Eq. (12) (Liu et al., 2017):

$$\text{Weight loss (\%)} = ((W_0-W_1)/W_0) \times 100 \quad (12)$$

Where W_0 and W_1 represent initial and final fruit weights, respectively.

2.13.3 Determination of pH and titratable acidity

Five fruits were taken from each group and then 5 g of fresh homogenate was suspended in 50 mL distilled water using a blender and then centrifuged at 5000 rpm for 10 min. Titratable acidity (TA as citric acid %, using 0.064 as conversion factor for citric acid), was calculated by titrating 5 mL of clear strawberry juice diluted in 50 mL of deionized water against 0.1 N NaOH solution (AOAC, 2000), pH of the samples was measured by a digital pH meter. All sample determinations were in triplicate during storage at 0, 2, 4, 6 and 8 days at 4°C.

2.13.4 Ascorbic acid content and DPPH radical scavenging activity

Ascorbic acid was determined using DPPH as Equivalent Antioxidant Capacity according to the procedure previously described by (Almeida et al., 2011; Brand-Williams et al., 1995; Giosafatto et al., 2014), with some modifications: the solution of DPPH (0.05 mg/mL) was diluted with methanol in order to obtain an absorbance of 1.516 ±0.04 at 517 nm. Homogenous strawberry fruit 100 µL or controls ascorbic acid were allowed to react with 900 µL of DPPH radical solution for 30 min in dark and the decrease in absorbance from the resulting solution was adjusted. The standard curve of 0 – 80 mg of ascorbic acid /100 mL was linear ($y=-0.017x+1.484$, $R^2=0.992$).
The same sample were used to study the DPPH radical scavenging activity assay as antioxidant activity %, based on the method previously described by (Giosafatto et al., 2014; Odriozola-Serrano et al., 2008).The absorbance was measured in triplicate as fresh weight at 517 nm for all samples. Results were expressed (antioxidant activity %) as follows Eq. (13), (Giosafatto et al., 2014):

$$\text{Antioxidant activity \%} = ((\text{Abs DPPH- Abs sample})/\text{Abs DPPH}) \times 100 \quad (13)$$

2.14 Texture profile analysis

The TPA were performed on strawberries and falafel. Unwrapped and wrapped strawberry samples were stored for 0, 4 and 8 days before studying TPA as described by (Pădureț et al., 2017; Rossi Marquez et al., 2017). Fried falafel were subjected to TPA according to Rossi Marquez et al. 2013. All the analyses were carried out using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA) equipped with a 2 kN load cell in compression mode with a cylindrical probe (55 mm in diameter) (Rossi Marquez et al., 2017). The test was

configured so that the TPA parameters: a) hardness (N), defined as maximum force of the first compression peak; b) chewiness (N. mm), defined as the total amount of work necessary to chew a sample until is ready for swallowing (Rossi Marquez et al., 2017); c) gumminess (N), calculated automatically by multiply the hardness with the ratio of the positive force areas under first and second compressions (cohesiveness). All TPA parameters were calculated by the software Bluehill by determining the load and displacement at predetermined points on the TPA curve. Pre- and post-test speeds were 2.0 mm/s, while test speed was 1.0 mm/s. The strawberries were centred and compressed to 60%, and 20mm of its original size of deformation, and average hardness, chewiness and gumminess values of at least six strawberries of each group were evaluated. Regarding falafel, at least eight balls per treatment were subjected to TPA experiments.

2.15 Statistical analysis

All the experiments were performed three times, and the data were analyzed by using the JMP version 10.0 software (SAS Institute, Cary, NC, USA). Statistical differences were considered to be significant at (p<0.05) using the 2-way ANOVA test, and the means were compared using the Tukey-Kramer HSD test differences were considered to be significant at p<0.05.

CHAPTER 3

RESULTS AND DISCUSSION

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3. RESULTS AND DISCUSSION

3.1 Acrylamide and oil uptake reduction in French chips by hydrocolloid-based coatings

One of the main challenges for the edible films and coatings for an industrial scale up was used the cost of the starting materials. Because of that, we used grass pea flour GPF as a protein cheap source for our novel coatings. GPF, also known as *Lathyrus sativus L*., is a very ancient legume already consumed in Mesopotamia 8000 years ago. Because of successful studies carried out using GPF as TGase substrate for making novel bioplastics (see Appendix 6.5.3), and for characterizing microstructure and physico-chemical properties when GPF was modified or not by means of the enzyme (see Appendix 6.5.5 and Appendix 6.5.2, respectively), coating solutions prepared to mitigate ACR formation in French chips were containing GPF, treated or not by TGase. French fries were also coated by CH-based solutions or PEC -based solutions, hydrocolloid coating solutions provokes in potato French fries a significant reduction in ACR formation, as well as in oil content, while an increase in water is observed. In addition, the DI and MOE were calculated to estimate variations in risk assessment by applying coating solutions before frying Sample used as “control” were dipped in water, then the potato sticks were fried for 6 min at 170°C. ACR analyses, based on fat-free dry matters, were carried out by RP-HPLC. Reduction in ACR formation, was equal to 48% for PEC, 38% for CH, 37% for GPF + TGase, and 31% for GPF, respectively. (Fig.12 Graphical abstract of potato paper). All results relative to this study have been published in the publication reported in the following page.

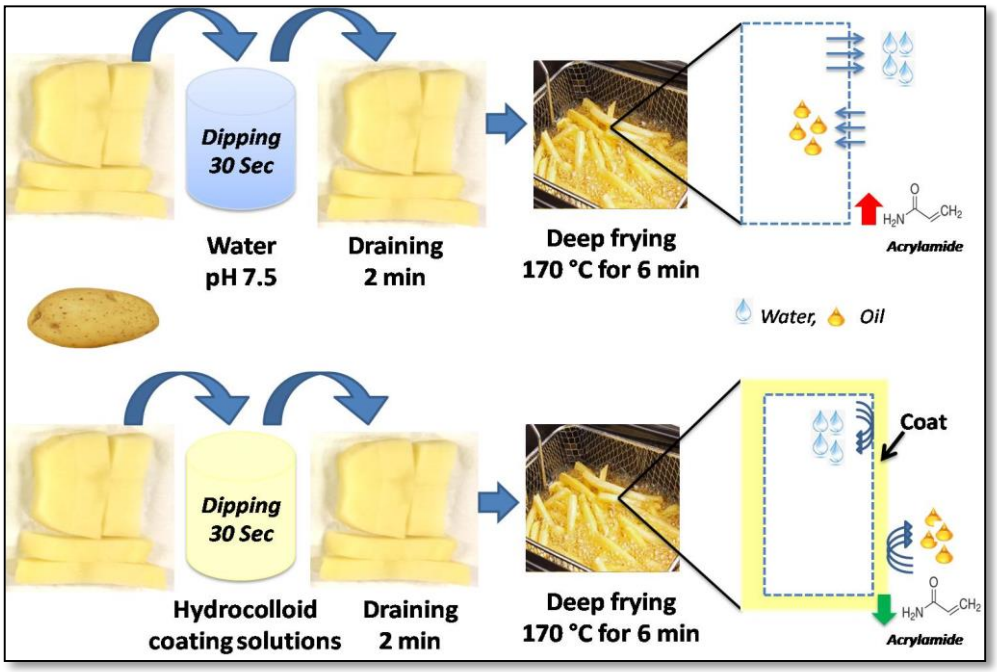

Fig.12 Proposed model of the effects of hydrocolloid-based coatings solution on the ACR content of French chips.

coatings

MDPI

Article

Hydrocolloid-Based Coatings are Effective at Reducing Acrylamide and Oil Content of French Fries

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Abstract: French fries are popular products worldwide. However, this product is a sufferable source of high acrylamide due to high temperature and low moisture. The main objective of this study was to evaluate the effect of grass pea flour (GPF), transglutaminase (TGase)-treated (GPF + TGase), chitosan (CH), and pectin (PEC) hydrocolloid coating solutions on the formation of acrylamide, water retention as well as on oil content. In addition, the Daily Intake (DI) and Margin of Exposure (MOE) were calculated to estimate variations in risk assessment by applying coating solutions before frying. Our results showed that the highest acrylamide content was detected in the control sample, reaching a value of 2089 μg kg^{-1}. Hydrocolloid coating solutions were demonstrated to be an effective way to reduce acrylamide formation, with the percentage of acrylamide reduction equal to 48% for PEC, >38% for CH, ≥37% for GPF + TGase, and >31% for GPF, respectively. We hypothesized that the coatings were able to increase the water retention and, thus reduce the Maillard reaction, which is responsible for acrylamide formation. In fact, the MOE value for coated French fries was increase, resulting in being closer to the safety level to avoid carcinogenic risk. Moreover, our coatings were effective in reducing oil uptake.

Keywords: French fries; acrylamide; hydrocolloid coating; transglutaminase; margin of exposure

1. Introduction

Acrylamide (H_2C=CH–CO–NH_2) is a compound that is highly soluble in water, about which heightened concerns regarding its exposure arose in 2002 when Swedish researchers discovered its formation when certain foods were prepared at temperatures above 120 °C and in the presence of low moisture [1,2]. Its formation, at least in part, is due to the Maillard reaction between free asparagines and reducing sugars. According to the European Food Safety Authority (EFSA), acrylamide forms in numerous baked and/or free asparagine rich fried foods including French fries, potato crisps, breads, biscuits, and coffee (roasted beans). Acrylamide is also known to be present in cigarette smoke. The EFSA report in 2015 mentioned that rats and mice exposed to acrylamide have shown some signs of developmental toxicity, increased incidence of skeletal variations, slightly impaired body weight gain, histological changes in the central nervous system, and some neurobehavioral effects. Acrylamide and its metabolite glycidamide (a reactive epoxide with the formula $C_3H_5NO_2$) are genotoxic and carcinogenic. Since any level of exposure to a genotoxic substance could potentially damage DNA and lead to cancer—as also stated by the International Agency for Research on Cancer (IARC, 1994) [3]—EFSA scientists conclude that acrylamide is a health concern [4].

The presence of acrylamide in food products has been studied in different countries and many food organizations have affirmed that deep-fried potato chips contain high amounts of acrylamide [5]. In addition, it was reported by Mestdagh et al. [6] that the ratio of fructose to glucose impacted both the color and acrylamide levels of fried potato strips, with higher fructose concentrations favoring acrylamide formation. Any factor, such as food formulation, pH, water content, temperature, and frying time can influence the Maillard reaction, which is also responsible for acrylamide formation [2]. French fries can contain more than 2000 μg kg^{-1} of acrylamide [7,8]. Recently, it has been found that the acrylamide content of 40 potato crisp brands from the Spanish market ranged from 108 to 2180 μg kg^{-1} [9]. Furthermore, the acrylamide level has recently been determined [10] in potato crisps, corn-based extruded snacks, and other savory snacks that are very popular in Italy together with the exposure risk assessment through the Margin of Exposure (MOE), showing that the acrylamide ranged from 21 to 3444 μg kg^{-1}, with the highest level in potato samples; the MOE assessment revealed that five out of six consumer groups showed exposure values associated with an augmented carcinogenic risk [10].

To prevent acrylamide formation during the frying process, some precautions have been reported [11]. The simplest precautions refer to the use of potato blanching either with water or acidic solutions, containing, for example, ascorbic acid or citric acid [12,13]. These strategies could also be applied together with a way of choosing specific potato cultivars described as containing lower amounts of both free asparagines and reducing sugars or controlling the storage conditions (potatoes stored at a temperature of 6 °C or higher have shown a lower amount of reducing sugars) or the recurring addition of salts in the soaking water [14]. Another more complex but expensive alternative process could be the recurring use of the asparaginase enzyme [15].

Some authors have proven the effectiveness of hydrocolloid solutions to control the acrylamide formation of different dried foods [16–18]. Hydrocolloids (proteins and polysaccharides) are hydrophilic polymers that can modify the functional properties of aqueous food systems such as thickening, gelling, and emulsifying properties [19,20]. Other studies have shown that the addition of some hydrocolloids to food preparations could enhance water retention [21–23], thus, becoming responsible for acrylamide reduction. In previous studies, the effect of enzyme transglutaminase (TGase, E.C. 2.3.2.13) on different hydrocolloid film properties was investigated [24–28]. This enzyme catalyzes the formation of isopeptide bonds among glutamine and lysine residues, thus providing a certain degree of reticulation in the hydrocolloid-based films [26]. Rossi Marquez et al. [29] demonstrated that a coating with whey protein/pectin film prepared in the presence of TGase resulted in a useful way to reduce oil content in fried potatoes. Moreover, a blanching pre-treatment and pectin coating were able to reduce the acrylamide formation in fried banana chips [16]. Additionally, alginic acid and PEC are promising inhibitors of acrylamide formation in fried potatoes [17]. Hydrocolloid-based coatings have also been used as effective protection to reduce oil uptake [29,30]. In fact, some studies have demonstrated that pectin [31,32], methylcellulose [32–34], whey protein, and egg white [35] were able to reduce the oil uptake in potato chips.

Hydrocolloids are effective coatings to enhance the quality of food products as reported in several reports [26,36]. Despite their effectiveness in regulating the transmission of gases such as oxygen and carbon dioxide besides water vapor, an increasing interest in studying their role in acrylamide reduction in processed foods has been registered [16,17,37].

The aim of the present study was to verify whether some hydrocolloid-based coatings, used previously in our laboratories [25,38] as effective film forming solutions, were also able to influence the formation of acrylamide in fried potatoes, the most widespread consumed fried food worldwide. In this study, we evaluated the effect of four different hydrocolloid coating solutions made of: (1) grass pea flour (GPF); (2) TGase-treated (GPF + TGase); (3) chitosan (CH); and (4) pectin (PEC) in reducing the formation of acrylamide during the frying process of potatoes, and to estimate the influence of the coating on the MOE in fried potatoes. Finally, the effect of coatings on reducing oil uptake was also investigated.

2. Materials and Methods

2.1. Materials

Acrylamide standard ≥ 99.8%, catalogue No. 23701 and methanol were obtained from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Acetonitrile HPLC (high pressure liquid chromatography) analytical grade, n-hexane, and formic acid were supplied from Carlo Erba reagents srl (Milan, Italy). Water purified by a Milli-Q-RO system (Millipore, Bedford, MA, USA) was used. Glycerol was purchased from the Merck Chemical Company (Darmstadt, Germany) whereas Oasis HLB (Hydrophilic-Lipophilic-Balanced) 200 mg, 6 mL solid phase extraction (SPE) cartridges were from Waters (Milford, MA, USA). The syringe filters (0.45 µm, 0.22 µm PVDF (polyvinylidene difluoride) were from Alltech Associates (Deerfield, IL, USA). Pectin (PEC) of *Citrus* peel low-methylated (7%) (Aglupectin USP (United States Pharmacopoeia)) was purchased from Silva Extracts srl (Gorle, Italy). Chitosan (CH) was obtained from Professor R. Muzzarelli (University of Ancona, Ancona, Italy), prepared as described by [39] with a degree of 9.0% *N*-acetylation. Grass pea seeds (GP) and corn oil were from a local market (Naples, Italy) and ACTIVA WM containing *Streptoverticillium* Ca^{2+} independent TGase was supplied by Ajinomoto Co. (Tokyo, Japan; Japan product No. AJ301402, lot No. 00.02.03).

2.2. Preparation of Coating Solutions

To obtain GP flour (GPF), seeds were ground using a variable speed laboratory blender LB 20ES (Waring Commercial, Torrington, CT, USA), so that the GPF could pass through a 425 µm stainless steel sieve (Octagon Digital Endecotts Limited, London, UK). GPF-based solutions were prepared by dissolving 8.3 g of GPF (containing 24% *w*/*w* proteins) in 100 mL Milli-Q water. The solution was shacked for 1 h and its pH was adjusted to 9.0, followed by centrifugation at 12,096× *g* for 10 min. After centrifugation, 60 mL of the supernatant were taken and the pH was adjusted to 7.0, and the final volume of 100 mL was reached with water after adding 16 µL of glycerol (8% *w*/*w* with GPF proteins). The solution was then divided in two 50 mL falcon tubes and TGase (33 U/g of GPF proteins) was added in only one tube; then both samples (GPF with and without TGase) were incubated for 2 h at 37 °C. The enzymatic reaction was stopped by adjusting the pH to 9.0. The ratio between the enzyme and its substrate in order to have TG-mediated crosslinking of GPF the proteins, was established in our laboratories (unpublished data). CH-based solutions (0.6% *w*/*v*) were prepared from a CH stock solution (2% *w*/*v* of hydrochloric acid 0.1 N stirred overnight) [25] then diluted with water adjusting the pH to 4.0; finally the solution was stirred for 30 min at 25 °C. PEC-based solutions (1% *w*/*v*) were prepared according to Esposito et al. [40] from a PEC stock solution (2% *w*/*v*), then diluted with water; the pH was adjusted to 7.5; finally the solution was stirred for 30 min at 25 °C. PEC and CH concentrations were chosen according to studies reported in the literature [38].

2.3. French Fry Preparation and Frying Process

Potatoes (cultivar Musica) were obtained from the Department of Agriculture, University of Naples "Federico II" (Naples, Italy) and stored at 4 °C until use. Before performing the experiments, potatoes were cut into 1 cm × 1 cm× 6 cm sticks as described by Rossi Marquez et al. [29] and treated as follows: 100 g of potatoes (18 sticks) were dipped for 30 s into either distilled water (sample used as "control") or one of the following coating solutions: (1) grass pea flour (GPF); (2) TGase-treated (GPF + TGase); (3) chitosan (CH); and (4) pectin (PEC). Then, each sample was allowed to drip for 2 min before frying. The frying conditions were the following: 1.5 L corn oil was preheated (using a controlled temperature deep-fryer apparatus (GIRMI, Viterbo, Italy) to the processing temperature (170 °C), then the potato sticks were fried for exactly 6 min [7]. The oil was replaced with fresh oil for each differently coated sample. Each potato stick was fried by flipping from side to side every 2 min. After frying, each sample was allowed drain for 2 min to remove the excess oil. The described procedure was repeated three times and the results were averaged.

2.4. Acrylamide Standard Preparation

A standard stock solution (1.0 mg/mL) was prepared by dissolving 10.0 mg of the acrylamide standard in 10 mL of Milli-Q water by using a volumetric flask. From the stock solution, calibration standards at different concentrations (100, 250, 500, 1000, 2000, 3000, 4000, and 5000 µg/L), were prepared, respectively. The limit of detection (LOD) was 29.6 µg/L, and the limit of quantitation (LOQ) was 89.1 µg/L. All series of standard solutions were stored in glass dark bottles (light-resistant) at 4 °C until used (Figures S1 and S2).

2.5. Extraction of Acrylamide from the Fried Potato Strips

About 100 g of fried potato sticks, accurately weighed after cooling, were immersed in n-hexane for 30 min to remove the oil from their surfaces [17]. The sticks were then ground with a rotary mill Grindomix GM200, (Retsch GmbH, Haan, Germany) at a speed of 1300 rpm for 1 min. Each sample was allowed to dry by freeze-drying before being subjected to acrylamide extraction following the protocols reported by Wang et al. [41], and Krishna et al. [42] with some modifications: two different Falcon tubes were set up for each sample, one for detecting acrylamide formed in the sample itself ("basic" acrylamide), and the second one to carry out the "Recovery test" (Table S1). In both tubes, 1.0 g (dry weight) of sample, accurately weighed, was put in both tubes and only in the second one was the 500 µg kg^{-1} of acrylamide standard added. In each tube, 10.0 mL of Milli-Q water were added. The samples were extracted in an incubated shaker for 30 min at 25 °C and 170 rpm, then followed by centrifugation at 7741 × *g* for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm syringe filter for the clean-up of the Oasis HLB SPE cartridges. The SPE cartridge was preventively conditioned with 2.0 mL of methanol followed by washing with 2 mL of water before loading 2.0 mL of the filtered supernatant, the first 0.5 mL was discarded and the remaining elute collected (≈1.5 mL; exact volume was measured by weight and converted by means of density). All extracts were kept in dark glass vials at 4 °C before analysis. The clean sample extracts were further filtered through 0.2 µm nylon syringe filters before HPLC-UV (ultra violet) analysis (Figure S3). Each analysis was performed in triplicate. The acrylamide recovery test was between 106% and 86% (Table S1) in accordance with the data reported in the literature.

2.6. HPLC-UV Analysis

HPLC-UV analysis was performed by using the RP-HPLC (RP: reverse phase) method on an Agilent 1100 series HPLC instrument equipped with an on-line degasser, a dual pump, and a diode array detector (Hewlett Packard, Wilmington, DE, USA). The column used was a Synergi™ 4 µm Hydro-RP 80 Å HPLC Column 250 × 4.6 mm [43,44] (Phenomenex, Torrance, CA, USA). The operating conditions were as follows: the wavelength detection was 210 nm, a gradient elution of 0.1% formic acid (*v*/*v*) in water: acetonitrile (97:3, *v*/*v*) was applied. Solvent A was water and Solvent B was acetonitrile, both solvents containing 0.10% (*v*/*v*) formic acid; flow rate, 1.0 mL/min. The gradient elution program was applied as follows: 97% A (3% B) for 10 min, increased to 20% A (80% B) from 10 to 12 min, and kept at 20% A (80% B) for 5.0 min, increased to 95% B (5% A) from 17 to 19 min, and kept at 95% B for 5 min, increased to 97% A (3% B) from 24 to 26 min, and kept for 4 min. The injection volume was 20 µL. The total chromatographic runtime was 30 min for each sample and the temperature was kept at 30 °C (GECKO 2000 "HPLC column heater", SpectraLab Scientific Inc., Markham, ON, Canada) to ensure optimal separation. In all samples (acrylamide standard and fried potato-derived), the acrylamide retention time was 4.9 min. The method presented a relative standard deviation lower than 5% with three repetitions; this result was in accordance with the data reported in literature.

2.7. Daily Intake (DI) and Margin of Exposure (MOE) of Acrylamide Risk Assessment

The daily intake (DI) and consequently, the Margin of Exposure (MOE) was calculated by taking into account the six following age groups (as stated from EFSA): Toddlers (1–3 years old); Children (3–10 years old); Adolescents (10–18 years old); Adults (18–65 years old); Elderly (65–75 years old); Very Elderly (more than 75 years old). DI (ng (kg body weight)$^{-1}$ day^{-1}) was calculated according to the equation [10]:

$$\mathrm{DI} = \frac{C \times Q}{\mathrm{BW}} \quad (1)$$

where C is the average concentration of acrylamide detected in each fried potato samples (ng g^{-1}), Q is the average daily consumption of fried potato samples of each age groups (g day^{-1}), and BW is the body weight of each age group (kg).

The average daily consumption Q was taken from the EFSA report [4], and the BW values were taken by Leclercq et al. [45] that considered the BW of Italian consumers belonging to the described six age groups. The DI results were used to estimate the MOE, a parameter that indicates the level of health concern for toxic and/or carcinogenic molecules. Here, we calculated the MOE for acrylamide, taking into account that it is classified as both a neurotoxic and carcinogenic agent. To calculate the MOE, it is necessary to refer to the BMDL_{10} = Benchmark Dose Lower confidence limit (mg (kg BW)$^{-1}$ day^{-1}), which represents the minimum dose range of a substance that produces a clear, low level health risk, usually in the range of 1%–10% [4]. The BMDL_{10} based on neurological changes (acrylamide as neurotoxic agent) is 0.43 mg (kg BW)$^{-1}$ day^{-1}, while the BMDL_{10} considering acrylamide as a carcinogenic agent is 0.17 mg (kg BW)$^{-1}$ day^{-1}, according to the EFSA report 2015 [4].

The MOE assessment was calculated according the following equation [10]:

$$\mathrm{MOE} = \frac{\mathrm{BMDL}_{10}}{\mathrm{DI}} \quad (2)$$

2.8. Oil Content

After frying and cooling, the oil content of each ground sample (3–5 g) was determined in triplicate and reported as a percentage on dry matter weight by n-hexane solvent extraction using the Soxhlet method [46], and the oil reduction due to coating was calculated as per the following equation.

$$\text{Oil reducing due to coating } (\%) = \frac{\text{oil content (control)} - \text{oil content (coated)}}{\text{oil content (control)}} \times 100 \quad (3)$$

2.9. Water Content Analysis

Water content of fried potato samples was determined according to AOAC (Association of Official Analytical Chemists) [47]. After frying, the potato sticks that had been coated with a different hydrocolloid coating solution and the control (coated with water) were dried in an oven at 105 °C until constant weight was achieved. Water content, water loss during frying and water retention in all of the samples were calculated as following:

$$\text{Water content } (\%) = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100 \quad (4)$$

$$\text{Water loss during frying } (\%) = \frac{(\text{initial water } - \text{water after frying})}{\text{initial water}} \times 100 \quad (5)$$

$$\text{Water retention } (\%) = \left(\frac{\text{water contant of coated}}{\text{water contant of control sample}} - 1\right) \times 100 \quad (6)$$

2.10. Statistical Analysis

All the experiments were carried out in triplicate, and the data were analyzed by using the JMP version 10.0 software (SAS Institute, Cary, NC, USA). Statistical differences were considered using the Tukey-Kramer HSD test to be significant at $p < 0.05$.

3. Results and Discussion

3.1. Effect of Hydrocolloid Coating Solutions on the Acrylamide Content

The aim of the present work was to evaluate the effectiveness of hydrocolloid-based coatings in reducing acrylamide formation in potato French fries. Acrylamide analyses, based on fat-free dry matters (FFDM), were carried out by RP-HPLC and reported in Figure 1.

As reported in the Materials & Methods (Section 2.3), potato chips were coated by dipping with four different hydrocolloid solutions. The first solution was made of GPF proteins, the second was made of the same proteins enzymatically modified by means of TGase, and the third was a solution made of 0.6% w/v CH, while the fourth one was made of 1% w/v PEC. A potato sample dipped in distilled water was used as the control.

The highest acrylamide content was detected in the control sample, reaching a value of 2089 ± 36 µg kg^{-1}. The results of Figure 1 show that the most effective coating solution was the one made of PEC, since the potato samples protected by this edible film, contained only 52% w/w of acrylamide when compared to the uncoated ones. Zeng et al. [17] tested PEC-based hydrocolloid solutions and demonstrated their effectiveness in reducing acrylamide in both the model systems and fried potatoes. Even though these authors obtained a 50% reduction in acrylamide content, they used a 2% pectin solution into which the samples were immersed for 5 h. These conditions are less sustainable and, thus, more expensive than the conditions we studied in the present paper. No comparison with previous results could be made with the GPF coating solutions since the present paper is the first study to have used this GPF and GPF + TGase as coatings. GPF was able to reduce the acrylamide content of 31% w/w, while in the presence of TGase, the acrylamide content was reduced to 37% w/w, thus suggesting that the presence of peptide bonds, catalyzed by the enzyme, increased the coating compactness and somehow influenced the acrylamide formation, probably due to the edible film possessing a more straightened protein network with reduced pores [24–28].

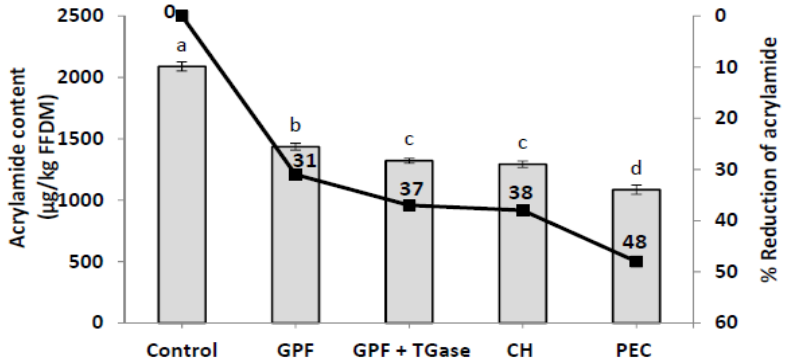

Figure 1. Effect of different hydrocolloid coatings on acrylamide content for French fries (y-axis on the left based on fat-free dry matters (FFDM)) and % reduction of acrylamide (y-axis on the right). Samples were coated with hydrocolloid-based coatings made of GPF, grass pea flour; GPF + TGase, grass pea flour treated by transglutaminase; PEC, pectin; CH, chitosan. "Uncoated" represents the control sample dipped in distilled water. Means with different letters (a–d) are significantly different (Tukey means comparison, $p < 0.05$). Data reported are the average values of three repetitions. Further experimental details are given in the text.

Our results also showed that the ability of reducing the acrylamide content of 38% (*w*/*w*) was exhibited by a CH coating solution, a reduction very similar to the one obtained using the coating made of GPF treated with TGase. While the latter reduction may be related to the pore dimension, the acrylamide reduction due to CH could be related to the high number of amino groups in this polysaccharide. In fact, Sansano et al. [18], while investigating the effect of CH on the reduction of acrylamide in fried batter, demonstrated that the polysaccharide influence was higher than the one performed by reducing sugars such as fructose added to the model system. Hence, the authors suggested that the CH amino groups might compete with free asparagine amino groups in binding the reducing sugars, which is the first step of the reactions that ends with acrylamide formation.

3.2. Effect of Coating to Acrylamide Risk Assessment

Risk assessment is usually evaluated by the MOE, which is calculated as the ratio between the dose at which a small but measurable adverse effect is first observed, and the daily level of intake that accounts for both the BW and different population groups. EFSA published in its report the MOE for acrylamide, which is classified as a substance that can be both genotoxic (neurotoxic) and carcinogenic. Acrylamide becomes a health concern for consumers that were divided in groups according to their ages, since the effect of exposure to a toxic/carcinogenic agent depends on the BW that is correlated, mostly, to age. In the present study, we verified that some hydrocolloid-based coatings were effective in reducing the acrylamide content and, thus, we also wanted to verify how the DI would change if the potato sticks were coated before being fried. Table 1 shows the DI calculated by Equation (1) in six different age groups. Indeed, we only excluded the Infants out of all seven groups that EFSA considered in its reports. In fact, Infants are classified as population members that are less than one year old and, thus, are not supposed to eat fried foods or potatoes. DI value relatives were calculated by multiplying the concentration of acrylamide detected in the samples by the average consumption (g day^{-1}) in each age group. The minimum, median, and maximum of the mean values were obtained by the EFSA through surveys across 20 European countries [4]. For the sake of brevity, Table 1 only reports the median values.

Table 1. Dietary intake of acrylamide consumption based on the median of the estimated consumption of fried potatoes treated with the coating solutions. Samples were coated with different hydrocolloid-based coatings made of GPF, grass pea flour; GPF + TGase, grass pea flour treated by transglutaminase; PEC, pectin; and CH, chitosan. "Uncoated" represents the control sample dipped in distilled water.

Fried Potato Samples	Age Groups	Acrylamide Intake (ng (kg body weight)$^{-1}$ day^{-1})
Control	Toddlers	1387
	Other children	1521
	Adolescents	1072
	Adults	719
	Elderly	536
	Very elderly	417
GPF	Toddlers	952
	Other children	1045
	Adolescents	737
	Adults	494
	Elderly	368
	Very elderly	287
GPF + TGase	Toddlers	877
	Other children	962
	Adolescents	678
	Adults	455
	Elderly	339
	Very elderly	264

Table 1. *Cont.*

Fried Potato Samples	Age Groups	Acrylamide Intake (ng (kg body weight)$^{-1}$ day^{-1})
CH	Toddlers	858
	Other children	941
	Adolescents	663
	Adults	445
	Elderly	332
	Very elderly	258
PEC	Toddlers	720
	Other children	790
	Adolescents	557
	Adults	374
	Elderly	279
	Very elderly	217

It was clear that the presence of the coating would allow all population groups to have a lower DI, so being more protected from the damage that acrylamide from fried potatoes could give them. The most effective was the PEC coating, since, taking into account the median DI value, we observed that all of them were roughly 50% less that the DI values exhibited in the control samples. We also verified that the GPF + TGase coating was as effective as the CH-based coatings even though they gave rise to a DI that was about 40% lower than the DI of the control. DI was useful to calculate the MOE according to Equation (2), and considered both the neurotoxic and carcinogenic risks of acrylamide. Thus, a $BMDL_{10}$ equal to 0.17 mg (kg BW)$^{-1}$ day^{-1} for carcinogenic risk (Figure 2, left panels) and a $BMDL_{10}$ equal to 0.43 mg (kg BW)$^{-1}$ day^{-1} for neurotoxic risk (Figure 2, right panels) were used to calculate the MOE in the six different age groups. Figure 2 also reports the MOE for the minimum (Figure 2A), medium (Figure 2B), and the maximum (Figure 2C) of acrylamide consumption levels as estimated from the EFSA throughout surveys across 20 European countries [4].

In each panel, the values of the MOE that were considered of no concern to the consumers according to EFSA regulation are reported: 10,000 and 125 are the minimum safety levels of the MOE for carcinogenic and neurotoxic risks, respectively. For the sake of clarity, it is worth reminding that, since MOE is inversely proportioned to the DI of acrylamide, a lower MOE level indicates a higher risk. Thus, as reported in Figure 2, for each age group, the MOE values were below the minimum safety level carcinogenic risk, even though it was clear that the use of hydrocolloid-based coatings increased the MOE values even though by only about 50%–60%. In any case, this result proved that recurring coatings could provide advantages to consumers, especially for the ones from 1 to 65 years old. In fact, this population consumes a higher amount of fried potato than the elderly or very old people. We want to underline that, as expected, the evaluation of the MOE indicated that the PEC-based hydrocolloid coating was the most effective among the others tested. Regarding the MOE values that assess safety for neurotoxic risk, we can see from the Figure 2, right panels, that a healthy concern due to a MOE value below the 125 safety level, was for the control samples of Toddlers and Other children groups, thus coating fried potatoes is effective in regard to maximum consumption levels (Figure 2C on the right). In fact, for minimum and median levels (Figure 2A,B on the right), acrylamide is not a health concern regarding neurotoxicity since the MOE values were much higher than the minimum safety level.

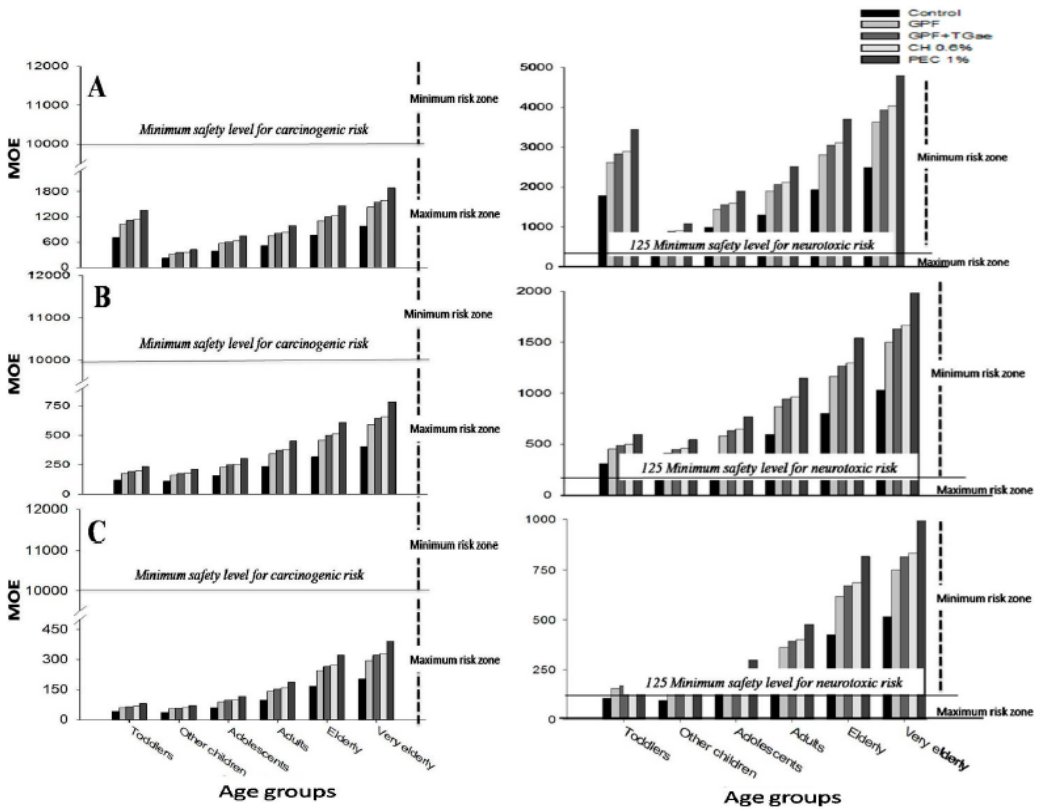

Figure 2. Margin of exposure (MOE) values for carcinogenicity (left panel) and neurotoxic (right panel) of acrylamide through the consumption of French fries that were both uncoated and coated with hydrocolloid coating solutions. Samples were coated with different hydrocolloid-based coatings made of GPF, grass pea flour; GPF + TGase, grass pea flour treated by transglutaminase; PEC, pectin; and CH, chitosan. "Uncoated" represents the control sample dipped in distilled water, across different consumer age groups: (**A**) minimum, (**B**) median, and (**C**) maximum of consumption levels estimated from the 2015 EFSA report. Further experimental details are given in the text.

3.3. Effect of Coating Materials on the Water and Oil Content

It is well known that during frying, the food water content decreases, while the oil replaces water. In a previous work, Rossi Marquez et al. [29] demonstrated the effectiveness of hydrocolloid-based coatings in reducing oil uptake in fried potato strips by using a coating made of PEC and whey proteins, modified by the means of TGase. In the present paper, the effectiveness of the four different hydrocolloid-based coatings in the oil uptake was also verified. As shown in Table 2, all four types of hydrocolloid-based coatings were effective in reducing oil content, even if the most significant reduction was obtained with the PEC-based coatings. Regarding water, as expected according to our hypothesis, the water content increased, reaching 56.1% (*w*/*w*) in PEC-coated samples, while the controls exhibited 41.9% (*w*/*w*) of water. During frying, the water evaporates through the dry crust and is substituted by oil. Oil uptake in fried foods can be considered a health concern since high consumption of fatty acids has been related to obesity and other health problems such as heart disease [48].

Table 2. Effect of hydrocolloid coatings on the quality of potato French fries. Samples were coated with different hydrocolloid-based coatings made of GPF, grass pea flour; GPF + TGase, grass pea flour treated by transglutaminase; CH, chitosan; and PEC, pectin. "Uncoated" represents the control sample dipped in distilled water *.

Coating Solutions	Oil Content (%)	Oil Reducing Due to Coating (%)	Water Content (%)	Water Loss During Frying (%)	Water Retention Due to Coating (%)
Control	20.1 ± 0.7^{a}	–	41.9 ± 1.1^{a}	36.9 ± 1.1^{a}	–
GPF	18.2 ± 0.4^{b}	9.3 ± 1.9^{a}	44.9 ± 1.1^{b}	33.9 ± 1.1^{b}	7.1 ± 0.7^{a}
GPF + TGase	$16.5 \pm 0.9^{b,c}$	15.9 ± 1.6^{b}	49.3 ± 0.7^{c}	29.5 ± 0.7^{c}	17.5 ± 1.7^{b}
CH	$15.7 \pm 0.5^{c,d}$	21.5 ± 2.7^{c}	51.3 ± 1.0^{c}	27.4 ± 1.3^{c}	22.3 ± 2.4^{c}
PEC	14.1 ± 0.4^{d}	29.4 ± 2.4^{d}	56.1 ± 0.6^{d}	22.7 ± 0.6^{d}	33.8 ± 1.4^{d}

*: Means with different letters ($^{a–d}$) are significantly different per column (Tukey means comparison, $p < 0.05$).

As shown in Table 2, all four hydrocolloid-based coatings used in this study were able to reduce the oil content when compared to the control, potato sticks coated with water. The most effective coating was PEC, which was able to reduce the oil content of the fried sample by about 29.4% (w/w) and retain 33.8% (w/w) of the water content of the sample. These results were in agreement with Hua et al. [32], since these authors indicated that the coating prepared with 1.0% (w/v) low-methoxyl sunflower head pectin and 0.05 mol/L $CaCl_2$ reduced the oil uptake by about 30%. Other authors [31,49] have reported that pectin leads to a lower oil uptake. In the present paper, the effectiveness of both protein-based hydrocolloid solutions (GPF and GPF + TGase) in reducing oil content uptake was also proven since the oil content was reduced by 9% and 15.9%, respectively. Thus, the TGase-mediated crosslinking was able to reduce the oil content twice when compared to the GPF sample. These results were better than the ones obtained by Aminlari et al. [35] where, using protein-based hydrocolloids such as whey and egg proteins, they only achieved 5% and 12% oil uptake reduction, respectively. The effectiveness of the hydrocolloid-based coatings in reducing the excessive oil uptake can be referred to their ability of reducing the heat transfer coefficient, as extensively discussed in the review by Kurek et al. [30].

4. Conclusions

In summary, it was demonstrated that a hydrocolloid-based coating was responsible for the reduction in acrylamide formation due to its capability to increase water retention. For the same reason, our hydrocolloid-based coatings were also effective in reducing oil uptake as they provided a reduction in the heat transfer coefficient during frying. It is worth noting that, of the four types of coatings studied, the PEC-based coating gave the best performances. We are confident that, due to their low cost and their colorless and tasteless properties, hydrocolloids could be hopefully adopted in the future as strategies for consumers, but also enterprises that produce commercial fried foods to maintain a lower acrylamide content.

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6412/8/4/147/s1, Figure S1: HPLC chromatograms of water as blank obtained at 210 nm. The mobile phase was (97/3 v/v) water/acetonitrile containing 0.1% v/v formic acid at 1.00 mL/min, Figure S2: HPLC chromatograms of acrylamide standards obtained at 210 nm; acrylamide concentrations were 0.1, 0.25, 0.5, 1, 2, 3, 4, and 5 mg/L of acrylamide. The mobile phase was (97/3 v/v) water/acetonitrile containing 0.1% v/v formic acid at 1.00 mL/min, Figure S3: HPLC chromatograms were: A (green line) 1000 µg/L acrylamide standard; B (violet line) acrylamide extracted from the French fry potato sample; and C (pink line) acrylamide from the French fry potato sample into which 500 µg/kg acrylamide standard was added and in the sample were eluted in the best chromatographic conditions by using mobile phasewater/acetonitrile (97/3 v/v) containing 0.1% v/v formic acid at 1.00 mL/min, and UV detection at 210 nm, Table S1: Recovery test for acrylamide in all samples (in each sample 500 µg/kg of standard were added) *.

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Author Contributions: Loredana Mariniello conceived and supervised the project. Loredana Mariniello, Asmaa Al-Asmar, and Daniele Naviglio designed the experiments. Asmaa Al-Asmar performed the experiments. Concetta Valeria L. Giosafatto and Daniele Naviglio analyzed the data. Asmaa Al-Asmar and Loredana Mariniello co-wrote the paper. All authors discussed the results and commented on the manuscript.

Conflicts of Interest: The authors declare that they do not have any conflict of interests.

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3.2 Acrylamide and oil uptake reduction in falafel by enzymatic treatment and/or pectin-based coatings

ACR represents an health concern in Europe and in other western countries, but this issue is highly underestimated elsewhere. In Palestine, in fact, most people are not aware of ACR production in foods, nor there are public agencies concerned about warning the population. In the Middle East one of the most popular street foods is the fried falafel balls, also consumed at home. The ACR is formed because of the Maillard reaction during cooking. In the present study, to verify whether or not both TGase and hydrocolloid-based coatings were effective in reducing ACR content, the enzyme was added to the falafel dough followed or not by dipping into PEC 1% coating solution. ACR, oil and water content of the fried falafel balls treated or not by TGase (5 or 20U/g of chickpea proteins) and coated or not with PEC-containing film forming solutions were evaluated. It was observed that the ACR content (detected by TOF LC/MS technique) was reduced, compared to control sample, by 10.8% and 34.4% in the

samples set up by adding 5 and 20U TGase/g respectively. In PEC-coated samples, ACR reduction was about by 59%, 65.3% and 84.5%, in falafel balls prepared either in the absence of TGase or containing 5U or 20U of the enzyme, respectively. Most probably TGase-mediated crosslinks among check pea proteins increase the water content inside the falafel balls, thus reducing the rate of Maillard reaction. However, TGase treatment does not affect oil content, while the PEC coating reduces the oil uptake about 23.5%. Finally, no difference was observed between the control sample and the one dipped in PEC regarding their texture properties hardness, chewiness and gumminess, while these properties were influenced in samples set up in the presence of the enzyme (Fig.13 Graphical abstract of the falafel paper). All results relative to this study have been published in the publication reported in the following page.

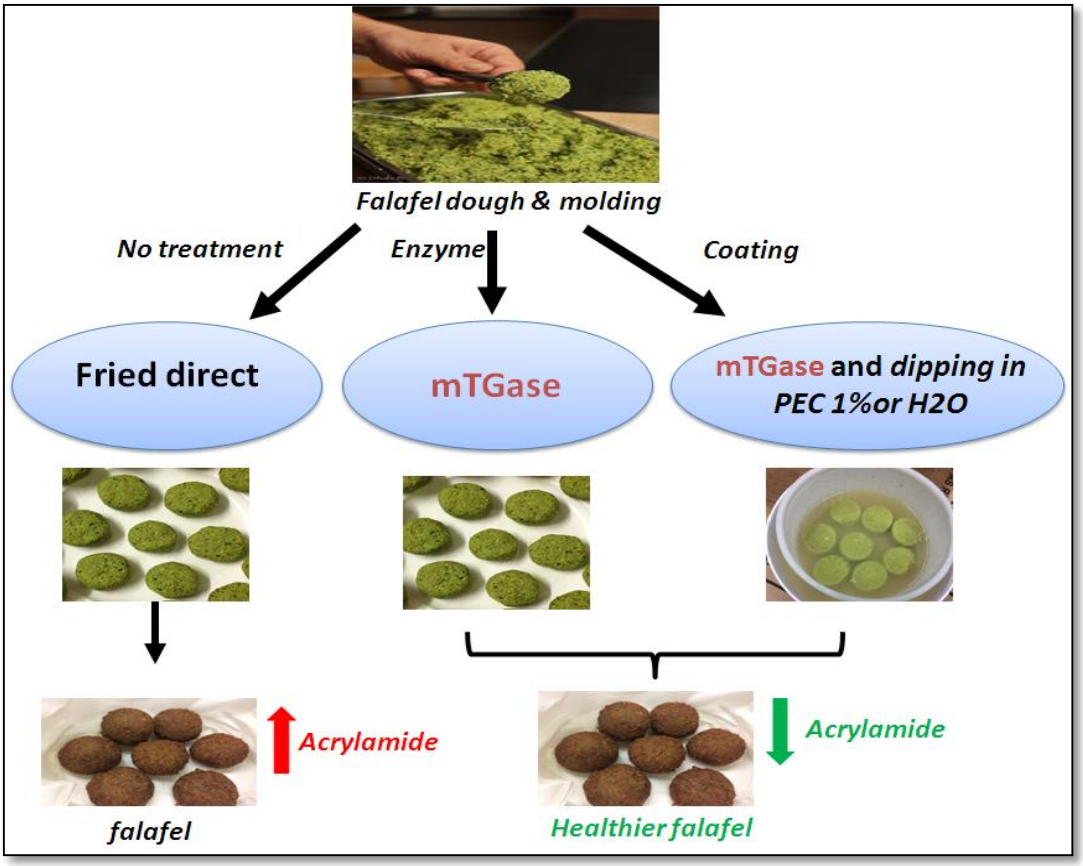

Fig.13 Proposed model of the effects of TGase enzyme and /or PEC-Coated on the ACR content of Falafel (Fried Middle Eastern Food).

coatings

MDPI

Article

The Effect of Transglutaminase to Improve the Quality of Either Traditional or Pectin-Coated Falafel (Fried Middle Eastern Food)

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Abstract: In this study, the effect of transglutaminase (TGase) (5 or 20 U/g of chickpea proteins) on falafel dough was investigated. The resulting falafel balls were either treated or not by dipping them into a pectin (PEC 1%) coating solution. Acrylamide (ACR), oil, and water content were then evaluated. Texture profile analyses and in vitro gastric digestion experiments were also carried out. The ACR content was reduced by 10.8% and by 34.4% in the samples prepared with 5 and 20 U TGase/g, respectively. In PEC-coated samples, the reduction of ACR was equal to 59.3%, 65.3%, and 84.5%, in falafel balls prepared either without TGase or containing 5 U or 20 U of the enzyme, respectively. However, TGase treatment did not affect oil content, while the PEC coating reduced oil uptake by 23.5%. No difference was observed in the texture properties between the control sample and the one dipped in PEC, while these properties changed in samples prepared with the enzyme. Finally, digestion studies, carried out under physiological conditions, demonstrated that the falafels prepared in the presence of TGase were efficiently digested in the gastric environment.

Keywords: falafel; acrylamide; transglutaminase; pectin; oil uptake

1. Introduction

Falafel is a traditional fast food or street food in the Middle East, also known as "ta'amiyya" in Egypt and Sudan; it is a deep fried ball made of spiced fava beans and/or chickpeas [1,2]. Nowadays, several countries, like Palestine, Jordan, Lebanon, Syria, and other Middle Eastern countries, use chickpeas to prepare this popular fast food that is eaten for both breakfast and dinner. Chickpeas have been used as a popular food in the East since 4000 BC [3]. Falafel dough is made of a mixture of soaked ground chickpeas, parsley, onions, spices and is leavened by sodium bicarbonate. The dough is shaped as balls just before deep-frying in vegetable oil until they become crusty and brown [2,4]. According to the United States Department of Agriculture (USDA) [5], homemade falafel contains 13.3% proteins, 17.8% total fat, 31.8% carbohydrate and is a rich source of different minerals like calcium, magnesium, phosphorus, potassium, sodium, and also vitamins, such as folate, vitamin C, and vitamin A.

The exposure of starch containing foods to temperatures above 120 °C in a low moisture environment provokes formation of acrylamide (ACR, $H_2C{=}CH{-}CO{-}NH_2$), which is highly soluble in water [6,7]. According to the European Food Safety Authority (EFSA), ACR is produced in numerous baked and fried foods, including French fries, potato crisps, breads, biscuits, and coffee (roasted beans). EFSA scientists conclude that ACR is a health concern [8]. Moreover, the pathway of

ACR formation probably involves Strecker degradation of amino acids, especially asparagine in the presence of dicarbonyl products from the Maillard reaction. Al-Dmoor et al. [4], indicated that ACR in Jordanian fried falafel (cooked for 6–8 min at 160–180 °C) is present in very high values ranging from 2700 to 4200 $\mu g \cdot kg^{-1}$, moreover, the same study demonstrated that the excessive use of frying oil in food preparation causes significant increases (~33%) in ACR content. Very little work has been done to decrease oil absorption in fried falafel balls. The only indications come from Abu-Alruz [2], who assessed that increasing falafel ball size provokes a reduction of oil uptake together with a decrease in frying time.

Transglutaminases (TGase, EC 2.3.2.13) are a widely distributed groups of enzymes that crosslink proteins through an acyl-transfer reaction resulting in an ε-(γ-glutamyl)lysine isopeptide bond. Recently, using TGase to improve the physiochemical properties of different food products, and also edible films and coatings, has increased in popularity, due to the ability of TGase to improve the crosslink network inside the food matrixes [9,10]. Due to the enzymatic cross-linking of milk proteins by TGase, yoghurt viscosity and yield stress were increased [11]. Moreover, many proteins were successfully found to be a substrate of TGase, such as egg proteins [12], fish proteins [13], soy proteins [14,15], bitter vetch proteins [16], and grass pea proteins [17].

Pectins (PECs) are plant cell wall structural polysaccharides composed mainly of galacturonic acid units with variations in composition, structure, and molecular weight [18]. In general, PECs are used as food additives (E440), known as thickers or stabilizers, to prepare different food products like jelly, jam, marmalades and other products, due to their gelling properties [19]. PEC is also used in pharmaceuticals and cosmetics industry due to all these properties. Moreover, PEC applications are devoted to increase, since these biopolymers have great potential for future developments [20]. Coatings are one of the most important food preservation methods that are applied to protect highly perishable foods by creating a thin layer of edible materials onto surfaces of the products. For example, Yossef. [21], found out that PEC coated strawberry fruits retained physico-chemical properties and a visual quality comparable to the ones coated by soy proteins, gluten, or starch.

Healthy food is becoming the most interesting objective for several industries and for a large portion of consumers, since many health problems are correlated to the consumption of food products. Recently, Al-Asmar et al. [22] concluded that the use of PEC as a dip coating material for French fries provokes a reduction of about 48% in ACR formation in comparison to uncoated samples. Moreover, Suyatma et al. [23] have studied the synergistic effect of blanching and PEC coating of fried banana chips, which resulted in high ACR reduction (up to 91.9%).

The objective of this study was to evaluate the effects of both TGase and PEC-based coating solutions on the ACR formation and quality of the fried falafel. Oil and water content, texture analysis profile, and in vitro gastric digestion were investigated.

2. Materials and Methods

2.1. Materials

ACR standard ≥99.8%, and methanol were supplied from the Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Acetonitrile HPLC analytical grade, *n*-hexane, and formic acid were obtained from Carlo Erba reagents S.r.l. (Cornaredo, Milan, Italy). Oasis HLB 200 mg, 6 mL solid phase extraction (SPE) cartridges were from Waters (Milford, MA, USA). Syringe filters (0.45 and 0.22 µm PVDF) were from Alltech Associates (Deerfield, Italy). PEC of a low-methylated citrus peel (7%) (Aglupectin USP) was purchased from Silva Extracts s.r.l. (Gorle, Bergamo, Italy), and Activa®WM *Streptoverticillium* TGase was supplied by Ajinomoto Co (Tokyo, Japan). All the other reagents were of analytical grade. Corn oil and chickpeas were purchased from a local super market.

2.2. Preparation of PEC Coating Solutions

PEC-based solutions (1% *w/v*) were prepared according to Al-Asmar et al. [22] and Esposito et al. [24], from a PEC stock solution (2% *w/v*), then diluted with water, and the pH was adjusted from 3.2 to 7.5 with NaOH 1 N, by means of a digital pH meter (Basic20, Crison, Barcellona, Spain). Finally, the solution was stirred for 30 min at 25 °C.

2.3. Falafel Preparation and Dipping Process

2.3.1. Falafel Dough Preparation

100 g of dough were made up of a mixture of soaked chickpeas (76 g), onion (10 g), parsley (3 g), falafel spices (0.3 g), salt (1.5 g), and sodium bicarbonate (0.2 g). The mixture was blended for 2 min with water. A special scoop (Inner diameter 4 cm, depth 1 cm) was used for preparing the falafel balls, each one with a diameter of 4 cm and weighing around 17 g. Then the balls were fried as described below.

2.3.2. Falafel Dough Treated with TGase

After prepared the falafel dough as described above, TGase (5 and 20 U/g protein) was added to the dough and incubated at 37 °C for 2 h. The falafel balls were then formed and fried. The control falafel samples were obtained without TGase but treated under the same experimental conditions.

2.3.3. PEC Dipping

The falafel balls, either treated or not with TGase, were frozen at −20 °C for 2 h, then dipped in 1% PEC, whereas the control was dipped in water. Each kind of dipped ball was frozen again for 30 min and then fried. In Table 1 the experimental design is described.

Table 1. Experimental design followed for performing all the analyses.

Falafel Type	TGase (U/g Protein)	Incubation 2 h at 37 °C	Dipping Solution
Traditional falafel	–	–	–
Incubated without TGase	0	√	–
Incubated with TGase (5 U/g)	5	√	–
Incubated with TGase (20 U/g)	20	√	–
Dipped	–	–	Water
Incubated without TGase	0	√	1% PEC
Incubated with TGase (5 U/g)	5	√	1% PEC
Incubated with TGase (20 U/g)	20	√	1% PEC

2.4. Frying Process

Falafel balls were fried at 180 ± 5 °C for 5 min, by using 2 L of corn oil, using a deep-fryer apparatus (Girmi, Viterbo, Italy) [22,25]. The oil was replaced with fresh oil for each group. Each fried group was flipping from side to side every 2 min. After frying, each sample was allowed to drain for 2 min to remove the excess oil [22].

2.5. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the SDS-PAGE of falafel balls, an aliquot of 250 µL of sample buffer (15 mM of Tris–HCl, pH 6.8, containing 0.5% (*w/v*) of SDS, 2.5% (*v/v*) of glycerol, 200 mM of β-mercaptoethanol, and 0.003% (*w/v*) of bromophenol blue), was added to 25 mg of falafel dough and/or whole fried falafel (either untreated or treated with TGase) after grinding. Each aliquot was analyzed by 12% SDS-PAGE. The samples were heated at 100 °C for 5 min, and then centrifuged for 10 min at 13000 × g. Ten µL of each supernatant was analyzed by SDS-PAGE (12%). SDS-PAGE was performed as described by Laemmli [26], at a constant

voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250 (Bio-Rad, Segrate, Milan, Italy). Bio-Rad Precision Protein Standards were used as molecular weight markers.

2.6. In Vitro Gastric Digestion

Fried falafel balls, either treated or not by TGase (20 U/g protein), were subjected to in vitro digestion (IVD) by using an adult model [12,27,28], under gastric physiological conditions. For our analyses, 100 mg of each sample were incubated with 4 mL of Simulated Salivary Fluid (SSF, 150 mM of NaCl, 3 mM of urea, pH 6.9) containing 75 U of amylase enzyme/g protein for 5 min at 37 °C and 170 rpm. The amylase activity was blocked by adjusting the pH at 2.5. Afterwards, the samples were subjected to gastric digestion as described by Giosafatto et al. [12] with some modifications. Briefly, 100 µL of Simulated Gastric Fluid (SGF, 0.15 M of NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and added to 100 µL of oral phase and then incubated at 37 °C. Thereafter, 50 µL of pepsin (0.1 mg/mL dissolved in SGF) were added to start the digestion reaction. At intervals of 1, 2, 5, 10, 20, 40, and 60 min, 40 µL of the 0.5 M of ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease. The samples were then analyzed using the SDS-PAGE (12%) procedure described above.

2.7. ACR Standard Preparation

The standard stock solution (1.0 mg/mL) was prepared as described by Al-Asmar et al. [22]. Then, it was diluted at different concentrations (50, 100, 250, 500, 1000, 2000, 3000, 4000, 5000, and 10,000 µg/L), respectively. All series of standard solutions were stored in glass dark bottles (light-resistant) at 4 °C until used.

2.8. Extraction of ACR from the Falafel Balls

ACR extraction from fried falafel balls was carried out according to Al-Asmar et al. [22], with some modifications. About 160 g of fried falafel balls, after cooling, were immersed in hexane for 30 min to remove the oil from their surfaces [29]. The falafel balls were then ground by using a rotary mill (Grindomix GM200, Retsch GmbH, Haan, Germany) at a speed of 1300 rpm for 1 min. Each sample was allowed to dry by freeze-drying before being subjected to ACR extraction following the protocols reported by Wang et al. [30] and Krishna et al. [31]. Two different falcon tubes were set up for each sample, one for detecting ACR formed in the sample itself, and the second one to carry out the recovery test. In both tubes, 1 g of the grinded sample was placed inside, but only in the second tube 100 µg/L of ACR standard were added. Fifty µL of Carrez reagent potassium salt and 50 µL of Carrez reagent zinc salts were added to each sample. In each tube, 10 mL of HPLC water were added. The samples were extracted in an incubated shaker for 30 min at 25 °C and 170 rpm, followed by centrifugation at 8000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.45 µm syringe filter for Oasis HLB SPE cartridges clean-up. The SPE cartridge was conditioned with 2 mL of methanol followed by 2 mL of HPLC water before loading 3 mL of filtered supernatant; the first 0.5 mL were discarded, and the remaining elute were collected (≈ 1.5 mL). The different extracts were all kept in dark glass vials at 4 °C before analysis. The clean sample extracts were further filtered through 0.2 µm nylon syringe filters and analyzed by TOF LC-MS. Each analysis was performed in triplicate.

2.9. LC-MS Analysis for ACR Content of Fried Falafel

The determination of ACR concentration was performed using the Agilent 6230 TOF-LC/MS (Agilent Technologies, Santa Clara, CA, USA) coupled to a series HPLC system, a vacuum degasser, binary pumps, and a temperature-controlled column oven at 30 °C. The following MS parameters were used: positive ion mode, nebulizer pressure 35 psi, drying gas (N_2) 5 L/min and 325 °C, capillary voltage 3500 V, and fragmentor 175 V.

The column used was a Synergi™ 4 µm Hydro-RP 80 Å HPLC Column at 250 mm × 3 mm [22,32] (from Phenomenex, Torrance, CA, USA). The operating conditions were as follows: the mobile phase

was a gradient elution: mixture water/acetonitrile (97/3, *v*/*v*) containing 0.10% (*v*/*v*) formic acid Solvent A and Solvent B was acetonitrile containing 0.10% (*v*/*v*) formic acid. The program elution was applied as follows: 100% A (0% B) for 8 min, increased to 80% B (20% A) from 8 to 15 min, and kept at 80% B (20% A) for 10 min, increased to 100% A (0% B) from 25 to 30 min and kept at 100% A for 5 min, at a flow rate of 0.4 mL/min. The injection volume was 20 µL. The total chromatographic runtime was 35 min for each sample; ACR elutes at a retention time of 3 min, and then the peak identification was based on the Extracted Ion Chromatogram (EIC); by selecting the ion at *m*/*z* 72, calibration curves were obtained by plotting the peak area of ACR versus concentration of ACR (range of concentration: 0.05–10 mg/L). The equation was obtained by applying the linear regression of $y = 102.21x - 10.706$, with R^2 equal to 0.9991; this equation was used to calculate the amount of ACR in all analyzed samples.

2.10. Oil Content

Each fried falafel ball was ground into pieces (3–5 g). The oil content was measured gravimetrically in triplicate by using the Soxhlet method [33] and reported as a percentage on dry matter weight.

2.11. Water Content Analysis

The water content of each fried falafel ball sample was measured gravimetrically in triplicate, according to Association of Official Analytical Chemists (AOAC) [34].

2.12. Texture Profile Analysis (TPA)

Texture profile analysis of the falafel balls for each sample was carried out as described by Rossi Marquez et al. [25], with some modifications. In particular, each falafel ball sample was analyzed using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA) equipped with a 2 kN load cell in compression mode with a cylindrical probe (55 mm in diameter). The instrumental TPA described by Bourne [35] was used. The test was configured so that the three TPA parameters, hardness, chewiness, and gumminess, were calculated at the time of the test by determining the load and displacement at predetermined points on the TPA curve. Pre- and post-test speeds were 2.0 mm/s, while the test speed was 1.0 mm/s. Samples, prepared as described above, were centered and compressed to 30% deformation. Hardness (N) was derived from the positive peak obtained at the first compression of the product or a maximum exhibited compression force. Chewiness (N·mm) was the mathematical product by the software Bluehill (Version 2.21) from the hardness, cohesiveness, and springiness [36]. Gumminess (N) was calculated by the software Bluehill automatically by multiply the hardness with the cohesiveness, which is a ratio of the positive force areas under the first and second compressions. All the TPA analyses were carried out with at least eight balls per treatment.

2.13. Statistical Analysis

All the experiments were performed three times, and the data were analyzed by using the JMP version 10.0 software (SAS Institute, Cary, NC, USA). Statistical differences were considered to be significant at ($p < 0.05$) using the 2-way ANOVA test.

3. Results and Discussion

3.1. Modification of the Protein Component of Falafel Balls by Means of TGase

Following the addition of different concentrations of TGase (0, 5, and 20 U TGase/g chickpea protein) to the falafel dough and incubation for 2 h at 37 °C, the samples (both the dough and the fried falafel) were analyzed by SDS-PAGE (12%). Figure 1 demonstrated that TGase (5 and 20 U/g protein) was able, under these experimental conditions, to modify chickpea proteins. The results indicated that chickpea proteins are an effective substrate of TGase. In fact, the disappearance of proteins indicates the presence of endo-glutamine and endo-lysine reactive residues. Moreover, by increasing

the concentration of the TGase (20 U/g protein), there was a concomitant increase of higher molecular mass polymers (Figure 1, Panels A and B).

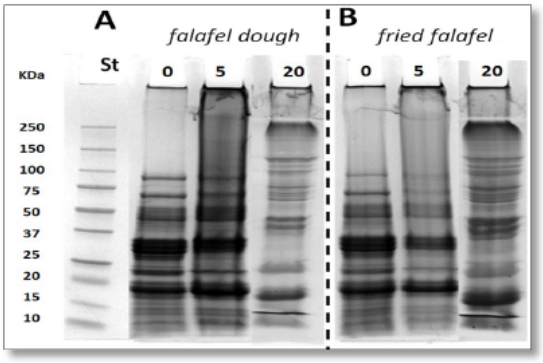

Figure 1. Panel A—SDS-PAGE of falafel dough without TGase (Lane 1), treated with 5 U/g of TGase (Lane 2) and treated with 20 U/g of TGase (Lane 3). Panel B—SDS-PAGE of fried falafel without TGase (Lane 1), treated with 5 U/g of TGase (Lane 2) and treated with 20 U/g of TGase (Lane 3). St, Molecular weight standards, Bio-Rad. Panel A: falafel dough; Panel B: fried falafel.

3.2. Effect of TGase and/or 1% PEC Coating Solution on the ACR Content of Falafel Balls

According to an EFSA report, ACR is a health concern. It is formed during frying, baking or roasting starchy rich food and also in food containing a high protein concentration and sulfhydryl groups [37,38]. Traditional fried falafel balls were analyzed for their ACR content, which was found to be equal to 7229 µg/kg. The falafel prepared by adding 5 or 20 U TGase/g protein, showed a reduction of ACR concentration equal to 10.8% and 34.4%, respectively (Figure 2, Panel A). This reduction could be explained in two different ways: (i) the ability of TGase to crosslink the lysine and glutamine provided a network that could retain free amino acids that were involved in the ACR formation; (ii) the water content of the falafel balls prepared in the presence of TGase was higher compared to the water content of falafel prepared in the absence of the enzyme (Figure 3). In fact, Al-Asmar et al. [22] have demonstrated that, in French fries, higher moisture content was effective in lowering ACR content.

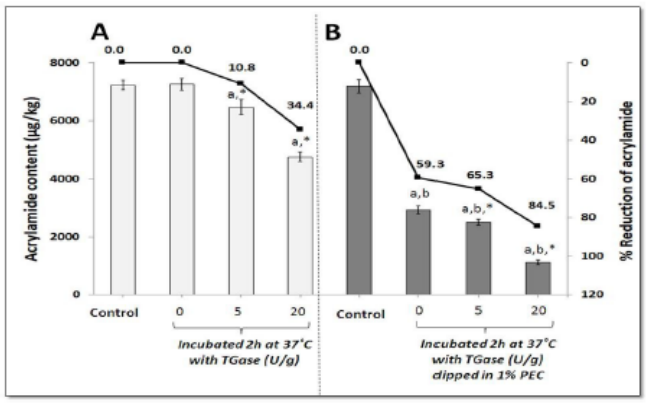

Figure 2. Effect of different concentrations of TGase on the acrylamide (ACR) content of fried falafel prepared without dipping (Panel A) or with dipping into 1% PEC-based coating solution (Panel B). The ACR content was determinated on fat-free dry matters and reported as percentage of the ACR reduction of fried falafel balls. The columns significantly different from those obtained by analyzing the control are indicated by "a", the columns indicated by "b" were significantly different from those obtained without dipping, whereas the columns indicated by "*" were significantly different from those prepared with different TGase concentrations (2-way ANOVA, $p < 0.05$). The control of Panel A represents ACR content in traditional fried falafel. The control of Panel B represents ACR content in fried falafel dipped in water.

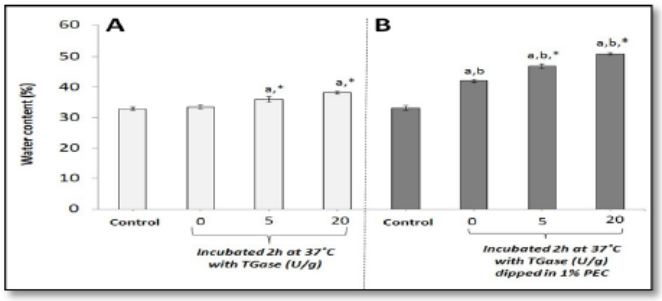

Figure 3. Effect of different concentrations of TGase on the water content of fried falafel balls prepared without dipping (Panel A) or with dipping into 1% PEC coating solution (Panel B). The columns significantly different from those obtained by analyzing the untreated are indicated by "a", the columns indicated by "b" were significantly different from those obtained without dipping, whereas the columns indicated by "*" were significantly different from those prepared with different TGase concentrations (2-way ANOVA, $p < 0.05$). The control of Panel A represents the water content in traditional fried falafel. The control of Panel B represents water content in fried falafel dipped in water.

To study the effect of coating, the ACR content was also determined in the falafel dipped in both water or 1% PEC-based solutions. The results, shown in Figure 2, Panel B, indicate that the ACR content was 40.7%, in comparison to the ACR content of falafel dipped in water or not dipped. As shown in both Panels A and B of Figure 2, falafel balls prepared by means of TGase followed by dipping into 1% PEC-based solution showed a significant reduction in ACR content in comparison to the uncoated falafel. The lowest amount of ACR (1118 µg/kg) was exhibited by falafel balls prepared by 20 U/g TGase and coated with 1% PEC-based solutions, where the percentage of the ACR reduction was equal to 84.5%. In addition, the recovery test was performed to assess the extraction efficiency for

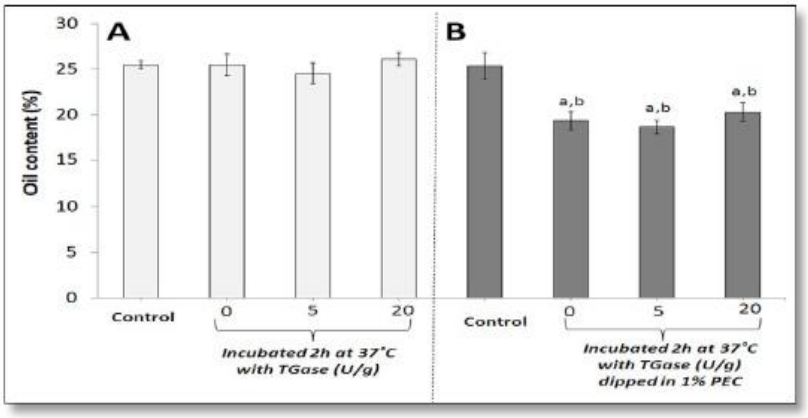

Figure 4. Effects of different concentrations of TGase without dipping (Panel A) or with dipping into a 1% PEC coating solution (Panel B), in oil content prepared based on the dry matters of the fried falafel balls. The columns significantly different from those obtained by analyzing the untreated ones are indicated by "a", whereas the columns indicated by "b" were significantly different from those obtained without dipping (2-way ANOVA, $p < 0.05$). The control of Panel A represents the oil content traditional fried falafel. The control of Panel B represents the oil content in fried falafel dipped in water.

The statistical analysis using 2-way ANOVA test indicated that both factors alone or together (dipping and TGase) significantly ($p < 0.05$) increased the water content of the falafel balls. On the other hand, dipping the falafel balls into a 1% PEC solution significantly reduced ($p < 0.05$) the oil content, while the TGase treatment, following or without the dipping process, did not significantly influence the oil content of falafel balls.

3.4. Effect of TGase and/or 1% PEC Coatings on Texture Profile Analysis (TPA)

To verify whether or not enzyme treatment and coating could influence the quality of the falafel balls, texture profile analysis (TPA) was performed. Thus, the falafel hardness, chewiness, and gumminess were assessed. As shown in Table 3, falafel balls prepared with TGase showed an increase of all three parameters. Further, TGase-treated falafel balls coated with 1% PEC-based solution showed significantly higher hardness, chewiness, and gumminess values compared to the sample incubated in the absence of the enzyme and dipped into the PEC-based solution. As matter of fact, the statistical analysis results, obtained by using a 2-way ANOVA test, indicated that the individual factors, or together (dipping and TGase), significantly increased ($p < 0.05$) the hardness, chewiness, and gumminess of falafel balls.

Table 3. Texture Profile Analysis (TPA) of falafel balls prepared in the presence or absence of different concentrations of TGase dipped or not into a 1% PEC solution prepared at pH 7.5.

Falafel Type	Hardness (N)	Chewiness (N·mm)	Gumminess (N)
Traditional falafel	56.41 ± 5.50	184.28 ± 3.10	23.20 ± 1.20
Incubated 2 h at 37 °C without TGase	52.22 ± 5.30	180.97 ± 2.80	22.15 ± 1.20
Incubated 2 h at 37 °C with TGase 5 U/g	70.88 ± 3.25 [a,*]	238.44 ± 2.70 [a,*]	Incubated 2 h at 37 °C with TGase 5 U/g
Incubated 2 h at 37 °C with TGase 20 U/g	96.57 ± 4.80 [a,*]	280.25 ± 15.10 [a,*]	49.64 ± 3.80 [a,*]
Dipped in water	52.18 ± 3.40	178.13 ± 4.10	21.42 ± 3.01
Incubated 2 h at 37 °C without TGase and dipped in 1% PEC	58.13 ± 4.90	183.23 ± 11.69	23.29 ± 4.50
Incubated 2 h at 37 °C with TGase (5 U/g) and dipped in 1% PEC	114.31 ± 8.20 [a,b,*]	453.18 ± 11.30 [a,b,*]	67.60 ± 4.50 [a,b,*]
Incubated 2 h at 37 °C with TGase (20 U/g)and dipped in 1% PEC	136.07 ± 12.28 [a,b,*]	518.50 ± 18.05 [a,b,*]	78.24 ± 2.01 [a,b,*]

The results were significantly different from those obtained by analyzing the untreated are indicated by "a", whereas the results indicated by "b" were significantly different from those obtained without dipping. The value indicated by "*" were significantly different from those prepared with different TGase concentrations (2-way ANOVA, $p < 0.05$).

3.5. Effect of TGase on the Digestibility of Falafel Balls

To test the effect of enzyme-treatment on digestibility of falafel, an IVD model was used. The experiments, carried out by using a protocol set up by the INFOGEST Cost Action [44], were performed on falafel balls prepared both in the presence and absence of 20 U TGase/g. At the end of the simulated gastric digestion, samples were analyzed by SDS-PAGE. As it is possible to see in Figure 5, the sample "C", incubated in the absence or presence of TGase (Figure 5, Lane "C", of both Panels A,B), contains proteins fully precipitated. Such samples were treated with only SGF without pepsin. This result is likely due to the gelation and aggregation of the food sample during the heat treatment [45]. On the other hand, as expected, when samples were treated with SGF containing pepsin, the protein component present in falafel was gradually digested. TGase slightly decreased the protein digestibility rate of the falafel balls, even though, at the end of digestion in both systems, they were completely hydrolyzed by the gastric enzyme pepsin (Figure 5). These results were also supported by the densitometry analysis (Figure 6) of the protein bands having a molecular mass between 35 and 25 kDa, (Figure 5, Panel A, samples prepared without TGase), and the high molecular mass polymers of 250 kDa (Figure 5, Panel B, falafel balls prepared by the means of TGase). As shown in Figure 6, the digestion rate is slower in the food incubated with the TGase enzyme, even though at the end of the digestion, the proteins analyzed were fully digested by pepsin in both untreated and TGase treated falafel.

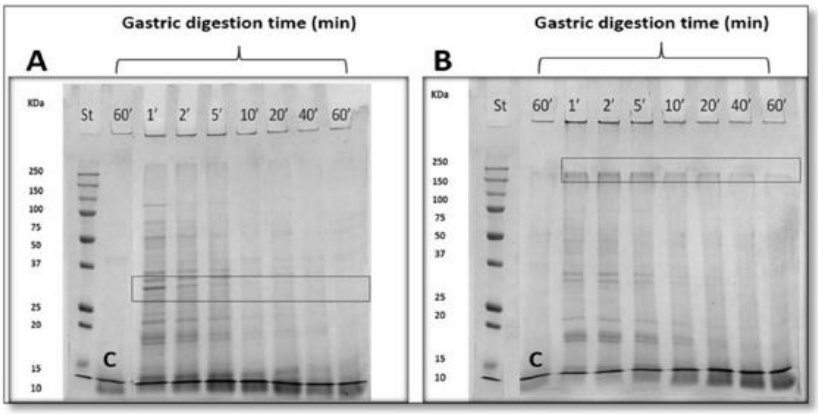

Figure 5. SDS-PAGE profile of falafel subjected to in vitro digestion (IVD) experiments. Panel A: traditional falafel prepared in the absence of TGase. Panel B: falafel prepared in the presence of TGase (20 U/g). The bands in the frame are those subjected to densitometry analysis. C is control sample incubated with simulated gastric solution not containing pepsin. St, Molecular weight standards, Bio-Rad.

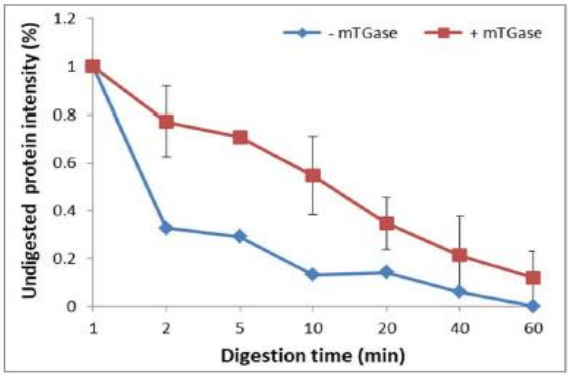

Figure 6. Densitometry analysis of the SDS-PAGE bands obtained after IVD shown in Figure 5. Both falafel types, traditional and (20 U/g) TGase-containing samples, were subjected to densitometry analysis.

4. Conclusions

In this paper, for the first time, falafel balls, a typical Middle Eastern food, were produced by using TGase enzyme in their dough. In addition, after their preparation, the balls were treated by dipping them in a PEC-based coating. The use of the enzyme provoked the reduction of the ACR in falafel balls, which was even more evident when TGase-prepared balls were coated by PEC, which was able to decrease the ACR concentration also present in the falafel prepared without TGase. However, TGase also had an effect on the texture profile parameters. On the other hand, the PEC coating protection reduced the oil content of this food product, either treated or not, by means of TGase. Moreover, protein gastric digestion, carried out under physiological conditions, showed that enzymatic treatment slightly decreased the digestion rate, although the proteins were fully digested at the end of the experiment in both unprocessed and TGase-processed systems.

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Conflicts of Interest: The authors declare no conflict of interest.

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3.3 The sensory properties of falafel balls

The sensory properties of falafel balls including appearance, taste, texture, color and overall acceptability are shown in Fig. 14. Falafel balls were prepared with and without TGase or coated by PEC then stored at -20ºC for different times (0, 30 and 60 days). The results have indicated that the lowest score for all properties was found to be for the enzymatically crosslinked falafel balls (5U TGase/g protein) at 0 day and 60 days of storage. However, the falafel balls enzymatically crosslinked by TGase and stored at -20ºC for 30 days have shown significantly higher scores in all sensory properties compared to the two storage times with the same preparations (Fig.14). Sensory results have shown that falafel balls coated by PEC and stored for 60 days at -20°C obtained significantly higher scores with respect to the control (traditional falafel kept at -20°C for 60 days).

The acceptance index (AI) of foods is taken into consideration when new ingredients are added or the formulation is optimized. It reflects the degree of liking or disliking for a food product, then is used to predict acceptability (Granato et al., 2012). The AI of samples varied from 87.2 to 29.4%. As shown in Fig. 15, the highest percentages were obtained in PEC-coated samples, showing a great commercial potential for such products. In particular falafel balls coated by PEC not stored at -20°C (fresh falafel) received the highest AI while falafel balls prepared with TGase and stored at -20°C for 60 days received the lowest AI.

In order to complete sensory evaluation analysis, the 40 panellists also evaluated the products according to the descriptive test, which is reported in Table 2. The taste of the products ranged from salty to fatty. The latter was characteristic of TGase-treated falafel not stored or stored for 30 days at -20°C. The 60 days stored TGase-containing samples had a taste that was classified as “burnt”. Texture results indicated that only falafel balls containing TGase were chewy, whereas the other products were crunchy as traditional falafel.

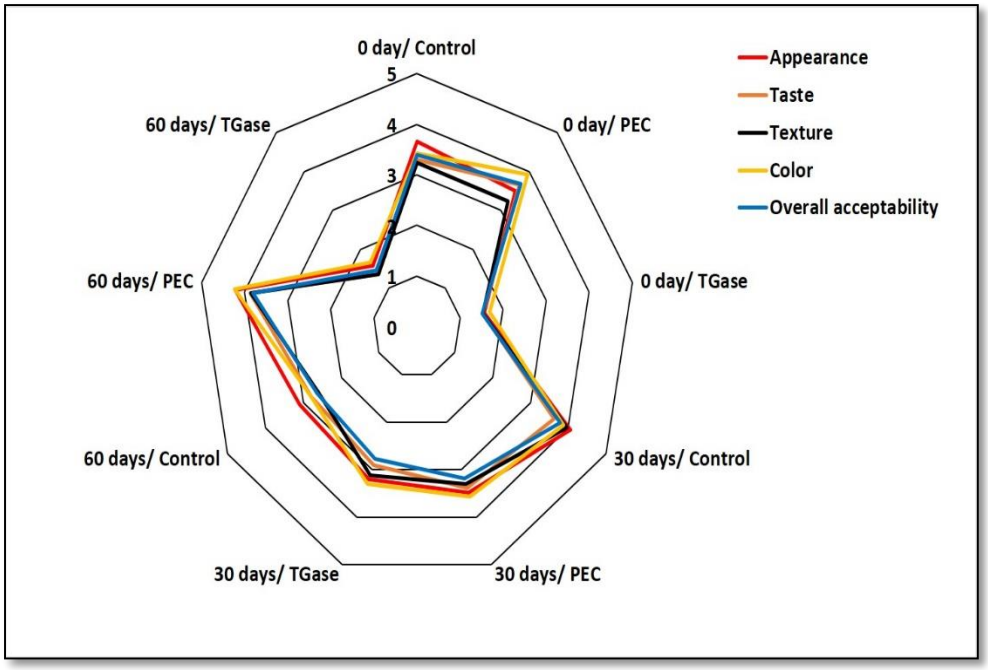

Fig. 14 Results of the sensory profile test of fried falafel prepared without (Control) or with 5U TGase/g protein (TGase) or coated by (PEC). The 5-point Hedonic rating test was used where: (1=Dislike a lot; 2= dislike a little; 3= neither like nor dislike; 4= Like a little and 5= Like a lot). All treatments were stored for different storage time 0, 30 and 60 days at -20ºC.

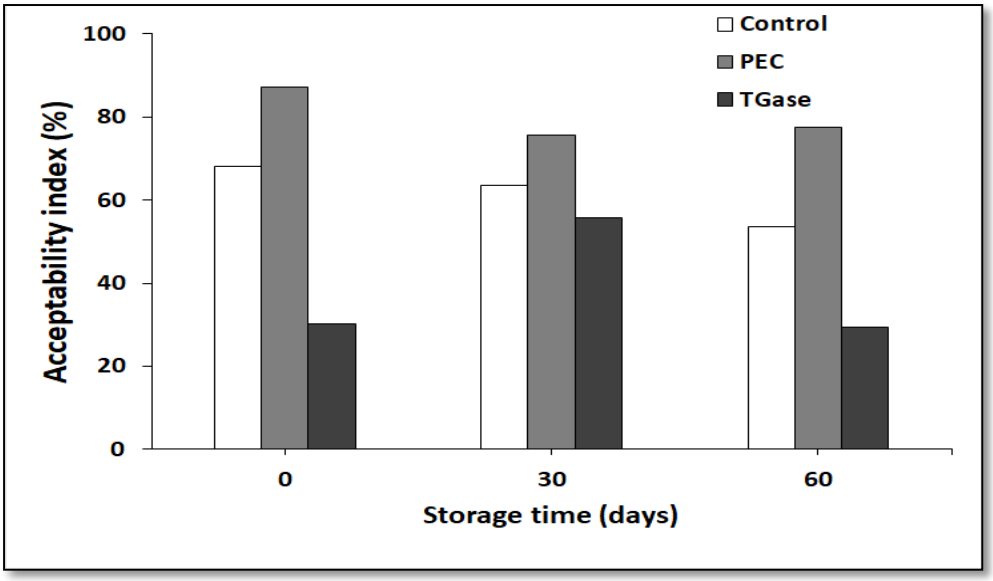

Fig. 15 Effect of PEC coating or TGase addition at different storage time (0, 30 and 60 days) on the acceptability index (AI) of respondents for the overall acceptability of fried falafel stored at -20ºC.

The color was variable from gold to brown. Moreover, the average overall acceptability results showed that only the falafel balls that coated with PEC were acceptable in all different storage times. On the other hand the panellists haven't accepted the falafel prepared with TGase due to fatty taste and chewy texture. Such results were also confirmed by the AI (Fig. 15) and hedonic scale test (Fig. 14).

In conclusion, storing falafel balls at -20^{o}C for 60 days were successfully achieved and the panellists have accepted the products. Moreover, coating the falafel balls with PEC was also successfully since higher AI was obtained and positive judgments on sensory properties were achieved. This means that fried PEC-coated falafel will be accepted also because they possess a lower ACR content and thus, can be classified as healthier food.

Table 2. Average descriptive test for sensory properties of fried falafel balls prepared in in the presence of TGase or coated by PEC then stored at different storage times (0, 30 and 60 days) at -20ºC. Control samples represent traditional falafel.

Storage time (Days)	Treatment	Taste	Texture	Color	Overall acceptability
0 day	**Control**	Salty	Crunchy	Gold	Slightly accepted
	PEC	Salty	Crunchy	Gold	Accepted
	TGase	Fatty	Chewy	Brown	Not accepted
30 days	**Control**	Salty	Crunchy	Brown	Slightly accepted
	PEC	Salty	Crunchy	Gold	Accepted
	TGase	Fatty	Chewy	Brown	Slightly accepted
60 days	**Control**	Fatty	Crunchy	Brown	Moderately accepted
	PEC	Salty	Crunchy	Gold	Accepted
	TGase	Burnt	Chewy	Brown	Not accepted

3.4 Effect of nanoreinforced and crosslinked hydrocolloid-based coatings on the content of acrylamide, water and oil in kobbah

3.4.1 Characterization of chitosan nanoparticles, mesoporous silica nanoparticles, and film forming solutions.

Zeta potential is an important value to indicate the stability of solutions. Moreover, Z-average shows the size of the particles. Fig. 16, show Zeta potential (panel A) and Z-average (panel B) of the CH-NP in the function of pH. The results indicate that Zeta potential of CH-NP was stable at +35 mV started from pH 2.0 to pH 6.0, and decreases to +20 mV when the pH is equal to 7.0. This finding is in accordance with other authors' results (Ali et al., 2011; Lorevice et al., 2016; Antoniou et al., 2015). Z-average of CH-NPs at pH 2.0 was around 99.5 d.nm and increased at higher pH to reach 800 d.nm at pH 7.0. Similar results were found also by Ali et al. (2011), who explain that at pH higher than 6.0 the protonated amino groups start to lose protons and the ionic bonds start decreasing. Thus, the rises the Z-average accompanied by sudden reduction in Zeta potential at pH 6.0 is due to the particle aggregation at this pH rather than the additional growth of individual particle size (Ali et al., 2010; 2011). In addition, we synthesized the MSN according to Fernandez-Bats et al. (2018), with the very similar Z-average size of MSN. The latter authors have characterized MSN

also by TEM observing an average size of 143 ± 26 nm. MSN were used to improve the physio-chemical of PEC and CH films the results reported in Giosafatto et al. 2019 (please see the Appendix section 6.5.4)

The coating solutions that used during this study were also characterized for their stability. Zeta potential and particle size results are reported in Table (3). The results showed that significantly increasing the stability after treated the GPF (-13.7 mV) with MSN (-16.8 mV) or TGase (-19.8 mV) also after enzymatically crosslinking the GPF nanoreinforced either with MSN (-18.4 mV) or CH-NPs (-18.2 mV) by means of TGase. However, no significant effect on Zeta potential were found by adding MSN or CH-NPs on FFSs stability. On the other hand, the Z-average of GPF solutions was 201.3 d.nm, but this value increased significantly when CH-NP were incorporated with or without TGase. No significant change on the Z-average of FFS after adding MSN on the GPF was observed and these results are in agreement with the ones published previously by (Fernandez-Bats et al., 2018). Adding TGase as crosslinker to the GPF together with MSN or CH-NP showed a significant increasing on the Z-average of FFSs. In addition, PEC FFS Z-average was (3198 d.nm) and it rises significantly to (3421 d.nm) after the addition of CH-NPs.

Table 3. Effect of 1% MSN or 1% CH-NPs on Zeta potential and Z-average on either GPF-based (in the absence or in the presence of TGase (33U/g protein)) or PEC-based film forming solutions at pH 6.

FFSs	Zeta potential (mV)	Z-average (d.nm)
GPF	-13.7 ± 0.6	201 ± 11
GPF + MSN	-16.8 ± 0.9[a]	191 ± 14
GPF + CH-NP	-14.1 ± 0.8[b]	386 ± 28[a,b]
GPF + TGase	- 19.8 ± 1.2[a,b]	241 ± 14[a,b]
GPF + MSN + TGase	-18.4 ± 0.5[a,]	333 ± 22[a,b,c]
GPF + CH-NP +TGase	-18.2 ± 0.9[a]	508 ± 19[a,b,c,d]
PEC	-33.7 ± 2.1	3198 ± 79
PEC + MSN	-31.8 ± 2.9	3110 ± 77
PEC + CH-NP	-32.4 ± 3.2	3421 ± 63*

The value significantly different from GPF FFSs are indicated by “a”, the value indicated by “b” were significantly different from GPF + MSN FFS, whereas the value indicated by “c” were significantly different from GPF + TGase FFS, the value indicated by “d” was significantly different from GPF + MSN +TGase FFSs, the value indicated by “*” was significantly different respect to the PEC and PEC+ MSN FFSs. Data reported are the average values of three repetitions using (2-way ANOVA, $p < 0.05$ for mean comparison). Further experimental details are given in the text.

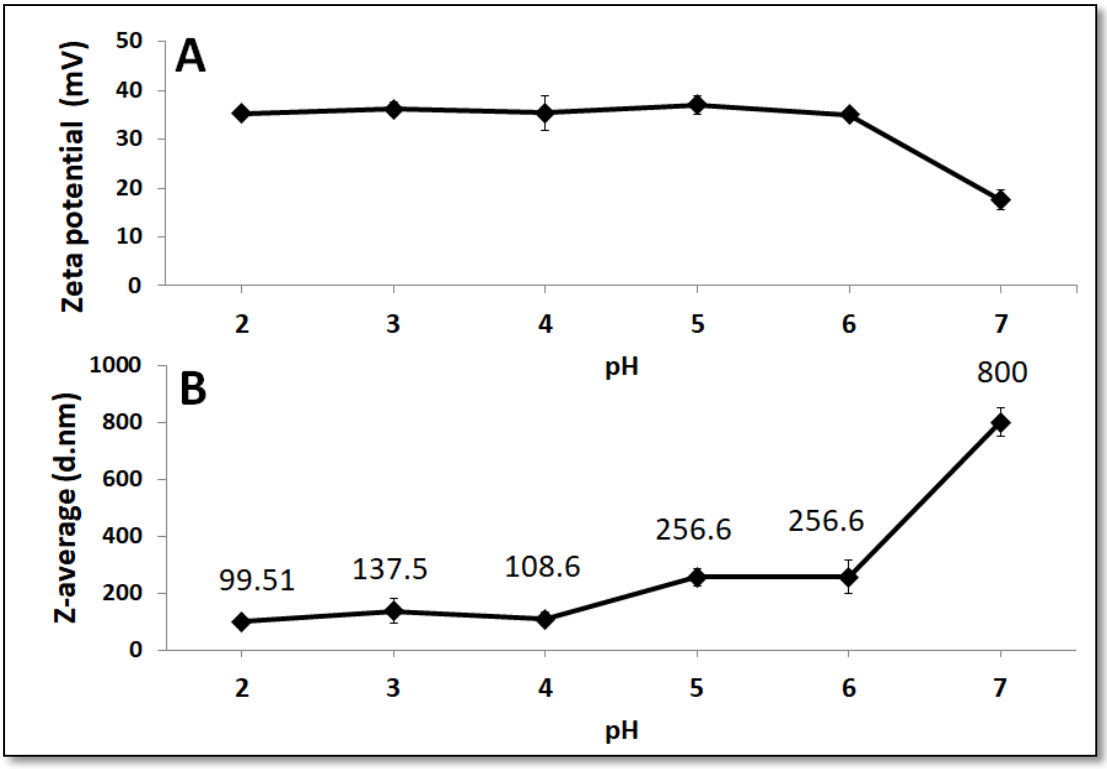

Fig. 16 Effect of different pHs on Zeta potential (Panel A) and Z-average (Panel B) of 1 mg/mL CH-NPs.

3.4.2 Effect of nanoreinforced and crosslinked hydrocolloid coating solutions on the acrylamide content

Kobbah is an ethnic food consumed dispersed among all the world not only in the Arabic region. The main purpose of this research was to study the effect of the different coating solutions to decrease the ACR content that is formed during frying. The ACR content was carried out by RP-HPLC and the results reported in Fig. 17. Two main different dipping solutions (GPF and PEC), reinforced in the presence or absence of two different nanoparticles (1% MSN and 1% CH-NP (w/w)) were used to coat the kobbah prior to frying. The GPF was enzymatically crosslinked by means of TGase in the presence or absence of NPs. The control sample was the kobbah dipped into distilled water. Fig. 17 shows that control exhibited the highest ACR content reaching a value of 3039.7 µg/kg. On the contrary, all used coating materials were able to significantly reduce ACR content. Kobbah dipped into GPF solution showed about 22.5% reduction in ACR content, while PEC-based coating solution reduced the ACR to 55.5%. The previous work studying potato French Fries showed that PEC alone reduced ACR formation about 48% (Al-Asmar et al., 2018). Coating solutions containing NPs (either MSN or CH-NP) in addition to GPF provoked slightly significant reduction of ACR comparing to the GPF-based coating sample not containing NPs. Higher significant reduction was observed when even MSN or CH-NP were mixed with PEC. The lowest ACR content was detected in the kobbah coated by PEC solutions containing CH-NP. In fact, in these samples the ACR content was 678 µg/kg with the 78.0% ACR reduction in comparison to the control. Recently, Mekawi et al. 2019 discovered that the addition of pomegranate peel NP extracts, to the sunflower oil during deep fat frying is responsible for ACR reduction to about (54%) in potato chips. The addition of the enzyme (33U TGase/g GPF protein) into nanoreinforced GPF (even

with MSN or CH-NP) reduced the ACR formation significantly (about 41.0% and 47.5% respectively) in respect to the nanoreinforced GPF prepared in the absence of TGase (Fig. 17). These results may indicate that a potential synergistic effect between NPs and TGase occurs which reduces the Maillard reaction. The ACR recovery test was between 93% and 108% (Table 4).

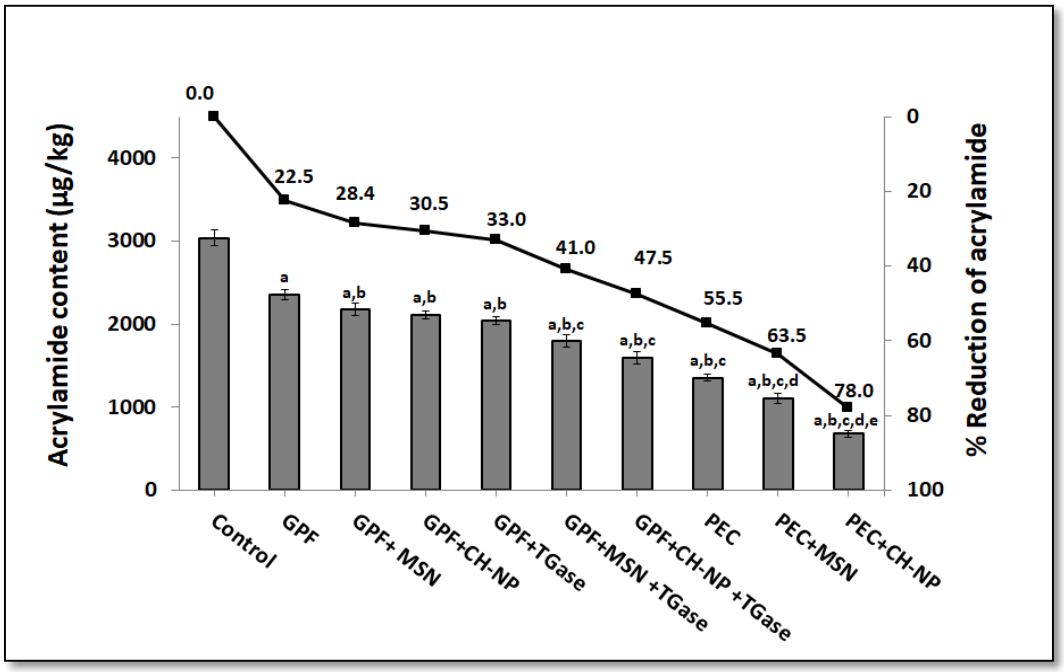

Fig. 17 Effect of different hydrocolloid coatings on ACR content for Kobbah (y-axis on the left based on fat-free dry matters (FFDM)) and % reduction of ACR (y-axis on the right). Samples were coated with hydrocolloid-based coatings made of GPF, GPF+MSN, GPF+CH-NP, GPF+TGase,GPF+MSN+TGase; GPF+CH-NP+TGase; PEC, PEC+MSN; and PEC+CH-NP. "Control" represents the kobbah sample dipped in distilled water. The columns significantly different from those obtained by analyzing the control are indicated by "a", the columns indicated by "b" were significantly different from kobbah coated only by GPF, whereas the columns indicated by "c" were significantly different from kobbah coated with GPF in the presence of nanoparticles or TGase alone, the columns indicated by "d" were significantly different from kobbah coated only by PEC and the column indicated by "e" was significantly different respect to the kobbah coated by PEC+MSN. Data reported are the average values of three repetitions using (2-way ANOVA, $p < 0.05$ for mean comparison). Further experimental details are given in the text.

Table 4. Recovery test for acrylamide in all Kobbah types (in each sample 150 µg\L of acrylamide standard were added).

Kobbah types	Acrylamide content in spiked sample (µg/Kg)	Recovery (%)
Control	3186 ± 61	98
Dipped in GPF	2511 ± 135[a]	103
Dipped in GPF + MSN	2329 ± 103[a,b]	101
Dipped in GPF + CH-NP	2255 ± 51[a,b]	96
Dipped in GPF +TGase	2186 ± 48[a,b]	98
Dipped in GPF + MSN + TGase	1934 ± 70[a,b,c]	93
Dipped in GPF +CH-NP +TGase	1744 ± 49[a,b,c]	99
Dipped in PEC	1495 ± 39[a,b,c]	95
Dipped in PEC + MSN	1250 ± 50[a,b,c,d]	95
Dipped in PEC + CH-NP	841 ± 37[a,b,c,d,e]	108

The value significantly different from those obtained by analyzing the control are indicated by "a", the value indicated by "b" were significantly different from kobbah coated only by GPF, whereas the value indicated by "c" were significantly different from kobbah coated with GPF in the presence of nanoparticles or TGase alone, the value indicated by "d" were significantly different from kobbah coated only by PEC and the value indicated by "e" was significantly different respect to the kobbah coated by PEC+MSN. Data reported are the average values of three repetitions using (2-way ANOVA, $p < 0.05$ for mean comparison). Further experimental details are given in the text.

3.4.3 Effect of nanoreinforced and crosslinked hydrocolloid coating solutions on the water and oil content

Water content of the kobbah (coated or not) was evaluated and the results reported in Fig. 18. The results indicate that the water content significantly increases in kobbah coated with any of the different hydrocolloid-based solutions used in this study. In fact, the lowest water content was found in the control sample (equal to 18%), while water content in coated kobbah by PEC-based solutions was (32.0%), significantly higher compared to the kobbah coated by GPF (21.0%). Nanoreinforcement by using either MSN or CH-NP in both GPF-based or PEC-based solutions, provokes the increasing in water content of the kobbah significantly higher in comparison to samples coated by solutions made of only GPF or PEC. Our findings are supported by Osheba et al. (2013) that concluded that CH-NP rise the moisture content of fish fingers up to 52.7%, while the uncoated samples exhibits 34.6% moisture. Regarding the use of TGase, our results prove that the enzyme action in both GPF-based and GPF+NP-based solutions show a significant higher water content respect to the kobbah coated without TGase. Similar results were obtained by Rossi Marquez et al. (2013), where the presence of TGase-mediated cross-links explains the reduction of the water evaporation during frying. Moreover, the results demonstrate that the water content of kobbah coated by GPF+CH-NP+TGase is significantly higher compared to the kobbah coated by only GPF+TGase and GPF+MSN+TGase (Fig. 18). Recently, Castelo Branco Melo et al. (2018), found out that CH-NP were responsible for delaying the ripening process of the grapes resulting in decreased weight loss, soluble solids and increased moisture retention inside that grapes.

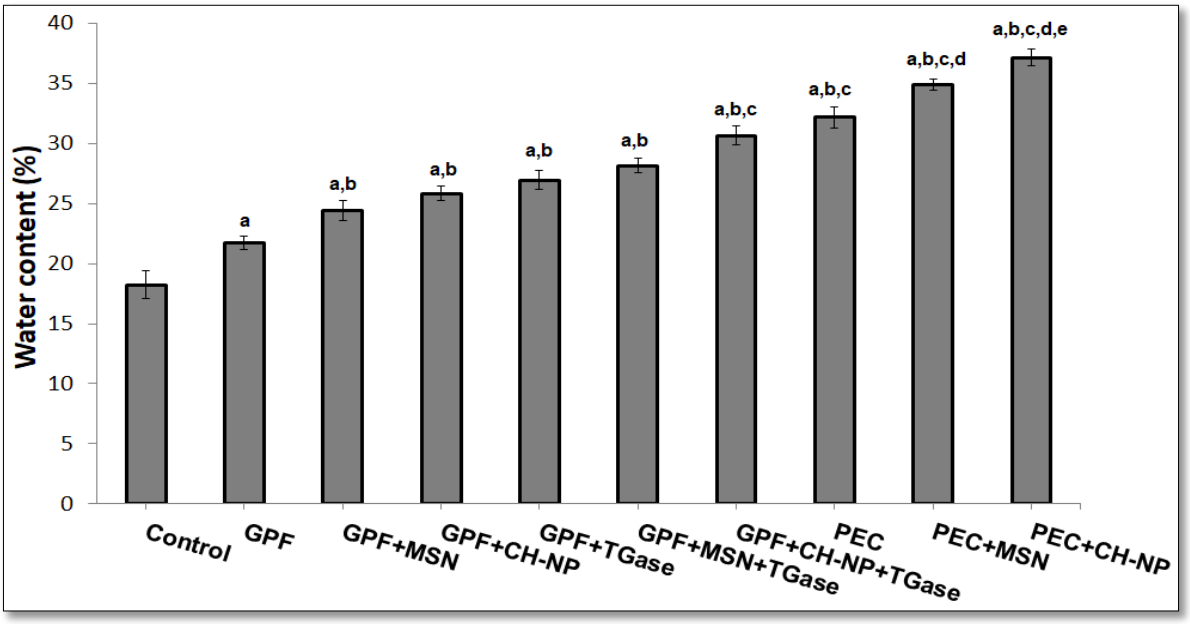

Fig. 18 Effect of different hydrocolloid coatings on water content for Kobbah. Samples were coated with hydrocolloid-based coatings made of GPF, GPF+MSN, GPF+CH-NP,GPF+TGase,; GPF+MSN+TGase; GPF+CH-NP+TGase; PEC, PEC+MSN; and PEC+CH-NP. "Control" represents the kobbah sample dipped in distilled water. The columns significantly different from those obtained by analyzing the control are indicated by "a", the columns indicated by "b" were significantly different from kobbah coated only by GPF, whereas the columns indicated by "c" were significantly different from kobbah coated with GPF in the presence of NP or TGase alone, the columns indicated by "d" were significantly different from kobbah coated only by PEC and the column indicated by "e" was significantly different respect to the kobbah coated by PEC+MSN. Data reported are the average values of three repetitions using (2-way ANOVA, $p < 0.05$ for mean comparison). Further experimental details are given in the text.

One of the main adversely health problems of our heath is the highest oil content of fried foods. Several studies concluded that coating the fried foods before frying by hydrocolloids materials reduced the oil uptake during frying (Rossi Marquez et al., 2013; Angor et al., 2013). Fig. 19 shows the oil content of kobbah just dipped into water (and used as control) or the ones coated with different solutions. Coating significantly reduces the oil content in comparison to the control, which shows the highest oil content (36.9%), whereas the lowest value was obtained in the fried kobbah coated by PEC+CH-NP (15.2%). No significant differences were detected between the GPF coated kobbah and the kobbah coated by GPF nanoreinforced with MSN or CH-NP. On the other hand, significantly difference in oil content of the fried kobbah were observed between PEC-coated samples and PEC+NP-coated samples. Enzymatically cross-linking of GPF, without and with NPs, demonstrated a significant oil uptake reduction in the coated fried kobbah compared to kobbah coated by GPF or in the presence of NPs (Fig. 19). PEC-based coating materials containing NPs (either MSN or CH-NP) induced a significant reduction in oil content of the coated kobbah (18.1% and 15.2% respectively) compared to kobbah coated with PEC (20.8%). Moreover, using CH-NPs for coating the fish fingers, Osheba et al., 2013 have demonstrated a

significant reduction on oil uptake which changed from 16.4 % in uncoated fish fingers to 4.5 % in coated ones.

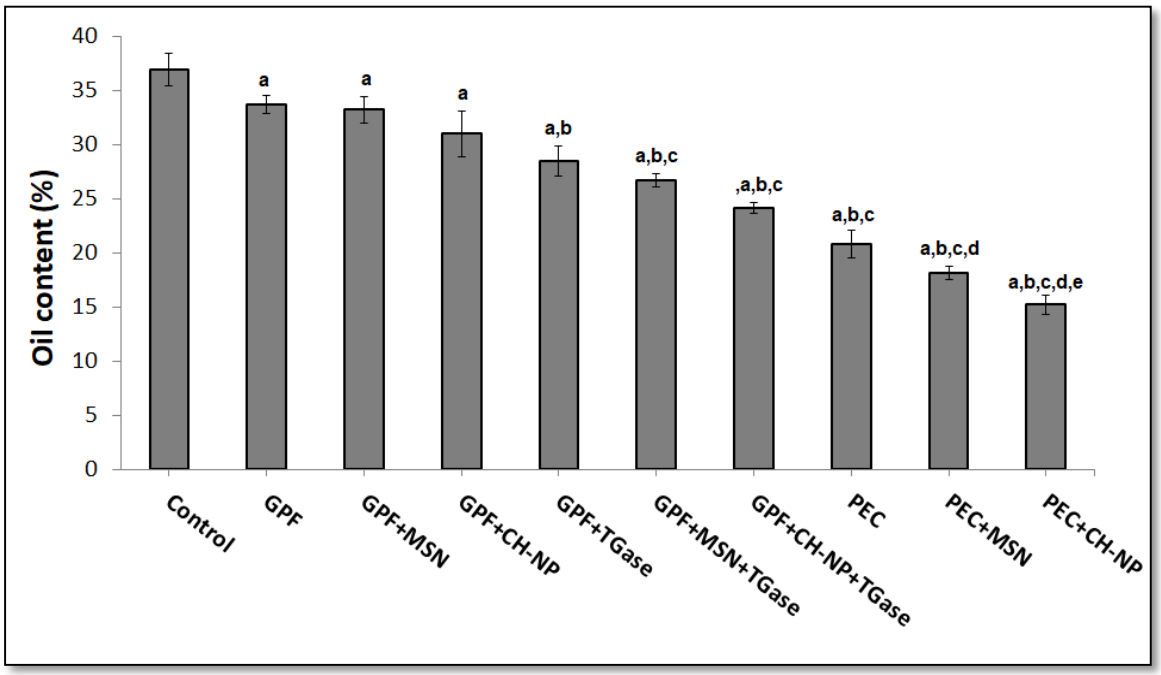

Fig. 19 Effect of different hydrocolloid coatings on oil content for Kobbah. Samples were coated with hydrocolloid-based coatings made of GPF; GPF+MSN, GPF+CH-NP ,GPF+TGase, GPF+MSN+TGase; GPF+CH-NP+TGase; PEC, PEC+MSN; and PEC+CH-NP. "Control" represents the kobbah sample dipped in distilled water. The columns significantly different from those obtained by analyzing the control are indicated by "a", the columns indicated by "b" were significantly different from kobbah coated only by GPF, whereas the columns indicated by "c" were significantly different from kobbah coated with GPF in the presence of nanoparticles or TGase alone, the columns indicated by "d" were significantly different from kobbah coated only by PEC and the column indicated by "e" was significantly different respect to the kobbah coated by PEC+MSN. Data reported are the average values of three repetitions using (2-way ANOVA, $p < 0.05$ for mean comparison). Further experimental details are given in the text.

In Fig. 20, all the samples analyzed for ACR, water and oil content are shown. It can be noted that kobbah with lighter color are the one that exhibit the highest ACR, water and oil reduction, namely kobbah coated by PEC+CH-NP. Colorimetric tests will be carried out in the future to quantify the color parameter.

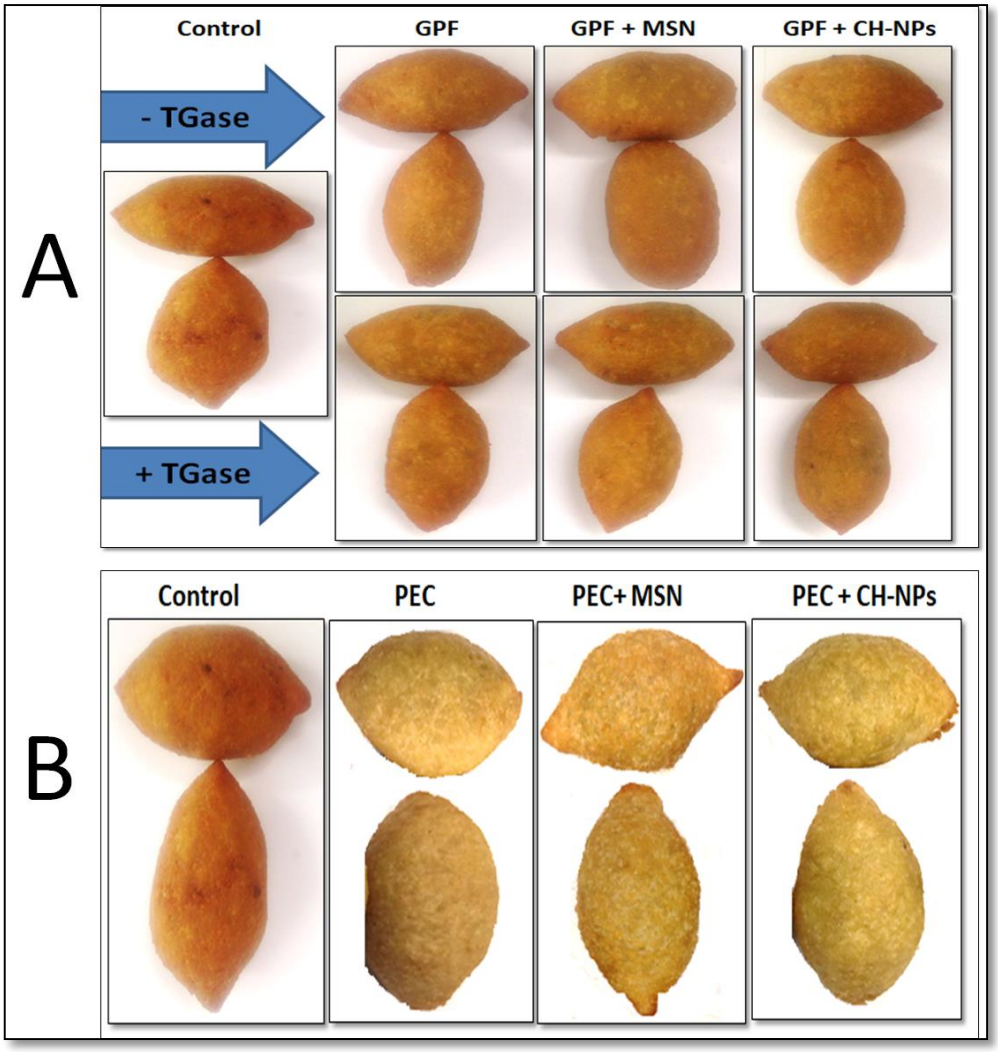

Fig. 20 Images of kobbah samples were coated with hydrocolloid-based coatings made of GPF,GPF+MSN, GPF+CH-NP, GPF+TGase; GPF+MSN+TGase and GPF+CH-NP+TGase (Panel A) ; PEC,PEC+MSN, and PEC+CH-NP (Panel B). "Control" represents the kobbah sample dipped in distilled water.

3.4.4 Effect of nanoreinforced and crosslinked hydrocolloid coating solutions on the digestibility of fried kobbah

To study the effectiveness of the enzyme in crosslinking the protein component (GPF) of hydrocolloid-based FFSs and how TGase effect could vary after the frying process of kobbah, samples were analyzed by SDS-PAGE. In particular, as described in the legend to Fig. 21, in Panel A different FFSs were analyzed, while Panel B shows kobbah coated with water (as control), or with other hydrocolloid-based FFSs. In order to verify whether the coating composition (GPF FFSs made of (Fig. 21): GPF, GPF+MSN, GPF+TGase, GPF+MSN+TGase) could affect digestibility of the fried food*, in vitro* digestion (IVD) experiments have been carried out by using a protocol set up by the INFOGEST Cost Action. (https://www.cost.eu/actions/FA1005/#tabs\T1\textbar Name:overview). According to INFOGEST protocol, IVD experiments were set up under physiological conditions, and samples were then analyzed by SDS-PAGE (12%) shown in Fig. 22.

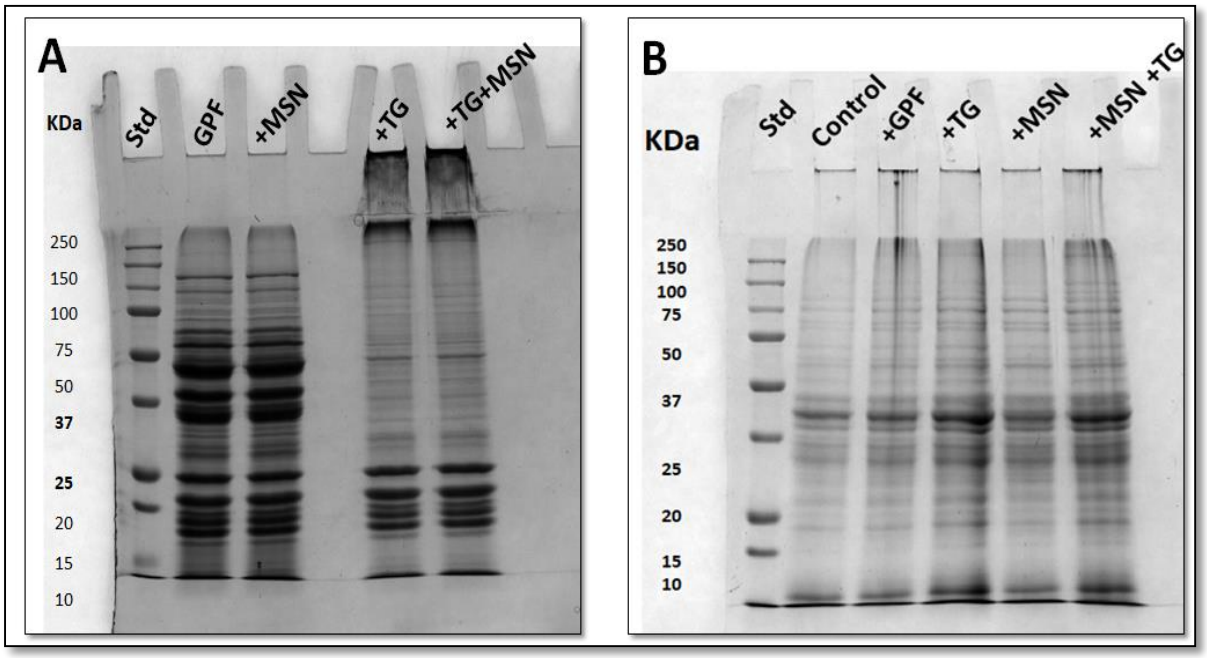

Fig. 21 Panel A - SDS-PAGE of GPF FFSs made of : GPF, GPF+MSN, GPF+TGase, GPF+MSN+TGase. Panel B - SDS-PAGE of fried kobbah dipped in : water (lane named “Control”), GPF, GPF+TGase, GPF+MSN, GPF+MSN+TGase . Std, Molecular mass standards, Bio-Rad.

Samples “C” represent the control since such samples were treated with SGF not containing pepsin. To study the digestibility rate two different kinds of bands were observed: 25 kDa band for samples containing GPF and GPF+MSN, and 250 kDa band for samples set up in the presence of the enzyme (GPF+TGase and GPF+MSN+TGase). By visual inspection of the SDS-PAGE patterns of Fig. 22, it is possible to assess that MSN do not effect digestibility (comparing Panel B to Panel A and Panel D to Panel C). However, looking at 250 kDa band present in TGase-treated samples (Fig. 22, Panels C and D) it is not possible to note significative differences among different samples following pepsin treatment. Thus, densitometry analysis was performed and the results reported in Fig. 24-Panel A. It is possible to note that a significant rate of digestibility of 250 kDa band present in TGase-treated FFS samples, was observed after 10 min pepsin incubation. Similar results were obtained studying digestibility of GPF-based bioplastics crosslinked by means of TGase. These studies were carried out during the development of the present project and published by (Giosafatto et al., 2018 see Appendix 6.5.3). Densitometry analysis results of 25 kDa (present in FFS samples not treated with the enzyme) confirmed what was observed by visual inspection, namely an higher digestibility rate already after 1 min pepsin incubation (Fig. 24, Panel A). Similar results were reported by Romano et al. 2018 (Appendix 6.5.2) by studies conducted in the progress of this thesis.

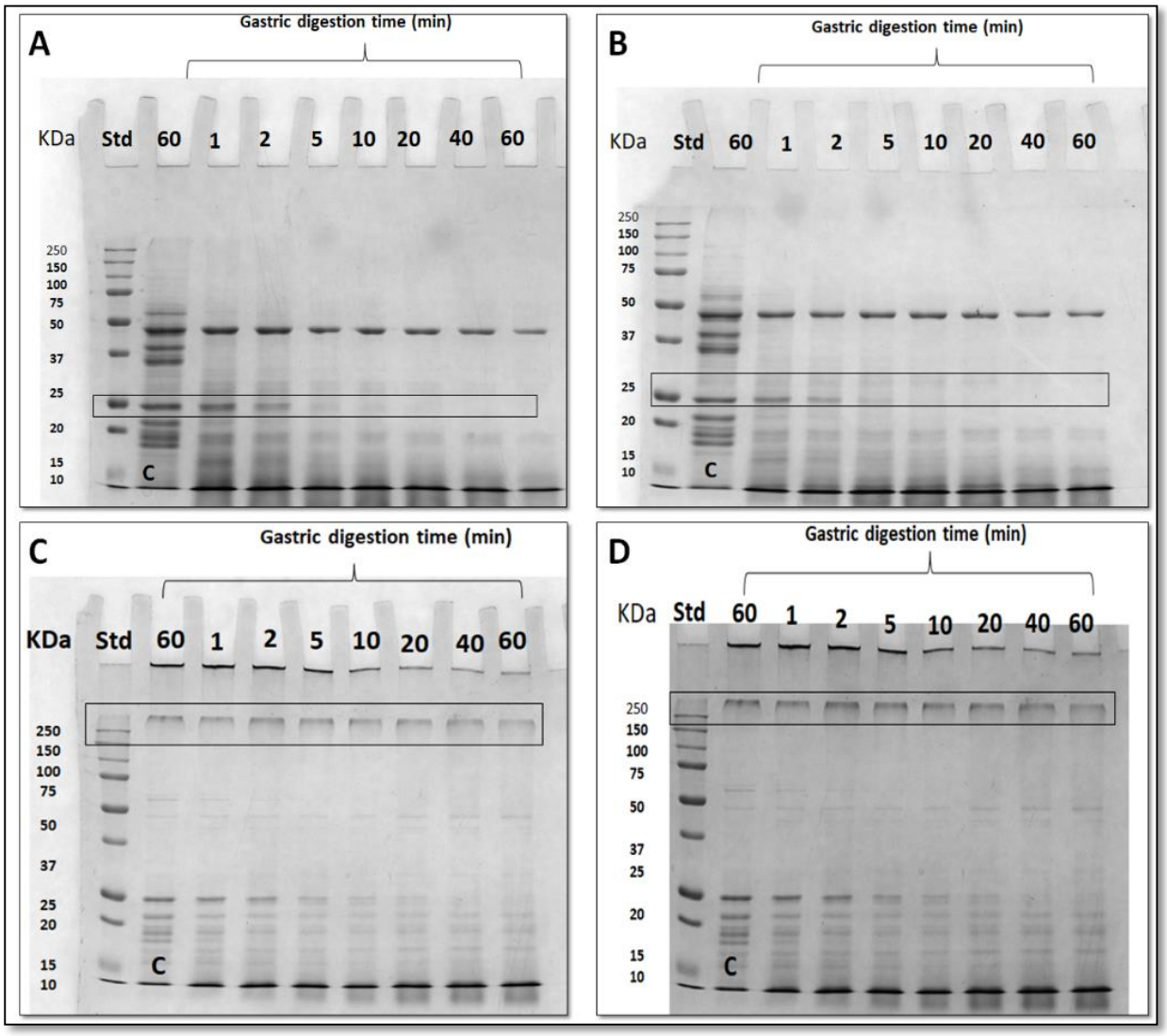

Fig. 22 SDS-PAGE profile of GPF FFSs subjected to *in vitro* digestion (IVD) experiments. Panel A: GPF; Panel B: GPF + MSN; Panel C: GPF+TGase (33U/g); Panel D: GPF+MSN+TGase (33U/g). The bands in the frame are those subjected to densitometry analysis. C is control sample incubated with SGF not containing pepsin. Std, Molecular mass standards, Bio-Rad.

IVD experiments were performed also using kobbah dipped in GPF or GPF containing MSN FFSs treated (Fig. 23). IVD treatment was effective on protein component of kobbah, mainly proteins present in kobbah ingredients (i.e. mostly bulgur flour, beef meat). The ~45 kDa band of samples not treated with TGase was subjected to densitometry analysis, while in enzyme–treated samples the 250 kDa was analysed. Densitometry analysis of those bands are observed in Fig. 24 Panel B.The digestion rate is slower in the food coated with protein crosslinked by means of TGase enzyme, even though at the end of the digestion, the proteins analyzed were fully digested by pepsin in all different coated kobbah (Fig. 24 Panel B).

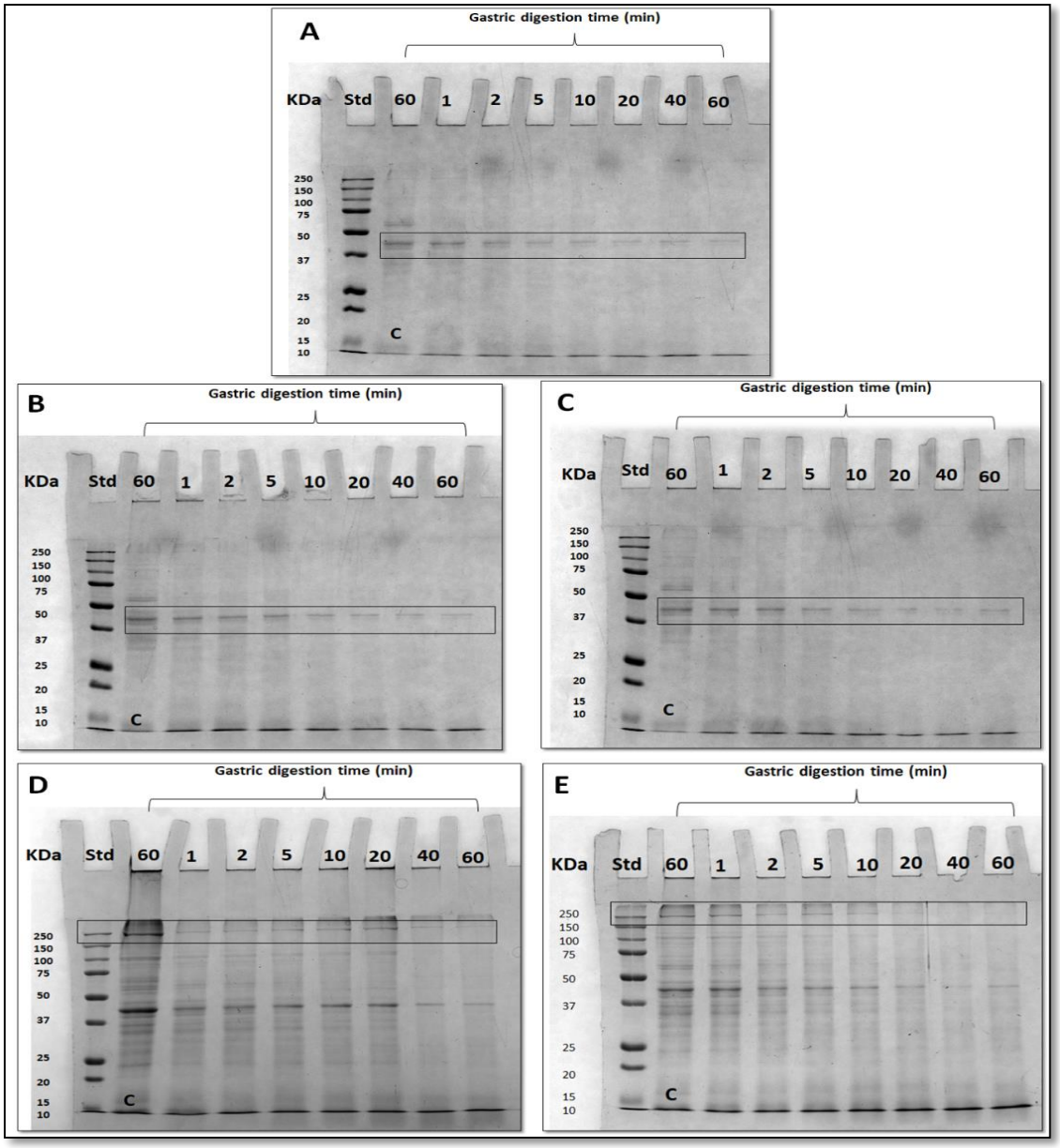

Fig. 23 SDS-PAGE profile of fried kobbah subjected to *in vitro* digestion (IVD) experiments. Panel A: kobbah dipped in water (control); Panel B: kobbah dipped in GPF; Panel C: kobbah dipped in GPF+MSN; Panel D: kobbah dipped in GPF+TGase (33U/g); Panel E: kobbah dipped in GPF+MSN+TGase (33U/g). The bands in the frame are those subjected to densitometry analysis. C is control sample incubated with SGF not containing pepsin. Std, Molecular mass standards, Bio-Rad**.**

3.4.5 Nanoparticles (NPs) safety issues

Until now the use of NPs in food is still limited, because of some potentially adverse health effects. The main concern regards NP dimension, no matter the chemical nature of them. In this thesis we have used MSN and CH-NP. Several studies have assessed the impact of MSN. Diverse and contradictory results have been observed in some cells or animals treated with MSN (Pérez-Esteve et al., 2015 ; Ruiz-Rico et al., 2017). However, the small size of NPs means that they may behave differently within the human body, compared to larger particles or bulk materials conventionally utilized as food ingredients (McClements & Xiao 2017).

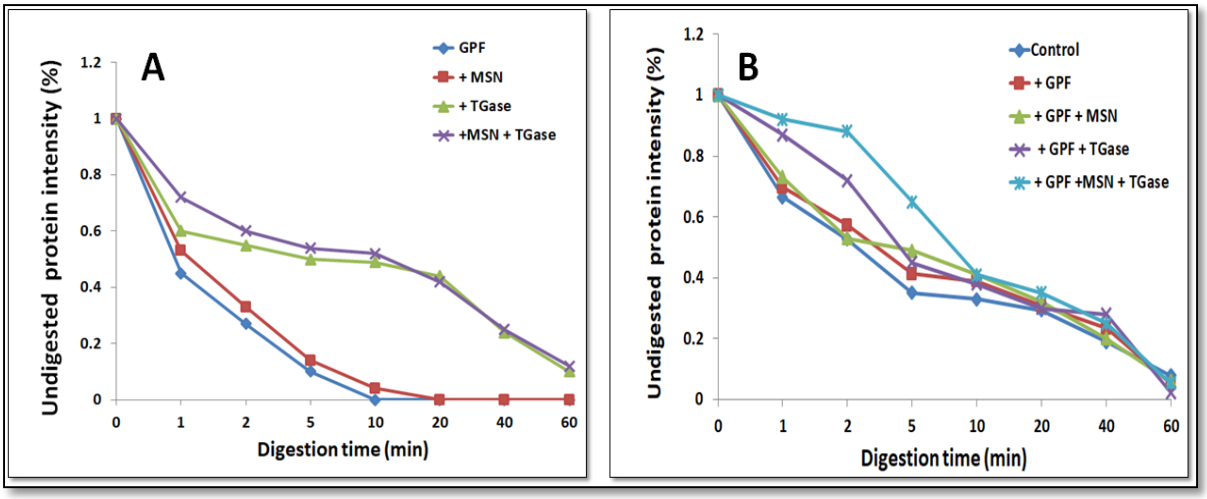

Fig. 24 Densitometry analysis of the protein framed bands in gels of Figures 22 and 23, obtained after IVD. Both GPF-based FFSs (Panel A) and fried kobbah coated with all GPF-based FFSs (Panel B) ,were subjected to densitometry analysis.

Cell culture and animal feeding studies suggest that high levels of SiO_2- based NPs with small size (9-26 nm) may cause adverse effects, such as cytotoxicity and generation of reactive oxygen species (Yang et al., 2017). In contrast McCarthy, (2012) observed that SiO_2- based NPs with the size of 150 nm and 500 nm do not perform toxic effects on Calu-3 cells. MSN have been successfully used to encapsulate the folic acid that was then added to fruit juices. Results indicated that folic acid MSN-encapsulation significantly improved its stability and contributed to control its release after fruit juice consumption by influencing this vitamin bioaccessibility (Ruiz-Rico et al., 2017). In our research group, nanoreinforcement of hydrocolloid materials with MSN in size (150-200 nm) were obtained and used to improve film properties (Fernandez-Bats et al., 2018) (see Appendix 6.5.4, Giosafatto et al., 2019). Thus, MSN were employed also in the present project, in the same time, CH-NP were prepared obtaining NPs with a size of 256 nm suitable to be used safely to reinforce or coatings.

3.5 Hydrocolloid-based films as bioplastics to protect Strawberry

The interest in biopolymers for manufacturing biodegradable/edible packaging materials is increasing due to the urgent demand of environmentally-friendly substitutes of conventional petroleum-based plastics. Among biopolymers, several hydrocolloids (polysaccharides and proteins), represent an abundant, inexpensive and renewable raw source. These natural molecules are proposed as film-forming agents, being able to establish intermolecular linkages by various interactions. Most of the times it is necessary to recur to plasticizers like GLY as an additive of such hydrocolloid films. In fact, in the absence of a plasticizer, many biomaterials are brittle and, as a consequence, very difficult to be manipulated. Recently, the use of different nanoparticles such as MSN has been proposed to reinforce biopolymer-based films because of their ability to reduce the permeability to CO_2, O_2 and water vapor. The aim of this work was to extend the shelf-life of strawberries by producing a packaging film made of Citrus PEC, plasticized by GLY and reinforced by MSN. Strawberry (*Fragaria vesca*), rich in vitamin C and antioxidant components, has short postharvest times and it is one of the most perishable fruits. PEC, a complex anionic polysaccharide mainly composed of homogalacturonan (1 → 4 linked α-D-galacturonic acid and its methyl

ester), has been used for many years as gelling/thickening agent, and also as stabilizer and emulsifier.

Strawberries were wrapped by 2 different films: 1. PEC+MSN+GLY containing films; 2. PEC+GLY containing films. The films were characterized according to seal strength and barrier properties toward CO_2, O_2 and water vapor. Moreover, strawberry shelf-life was evaluated by studying weight loss, pH, titratable acidity, ascorbic acid and antioxidant activity. The texture profile analysis of the fruit was aiso performed.

The results showed that MSN were able to positively affected the thermal stability of the film and reduce film barrier properties. In fact, in our study it has been reported that MSN-containing PEC-based films possess a more compact and homogeneous structure in comparison to the film prepared without MSN (Giosafatto et al., 2019 see Appendix 6.5.4), whereas seal strength was negatively influenced by the presence of both GLY and MSN. Strawberries were divided in 4 different groups: 1. wrapped with PEC+MSN+GLY films; 2. wrapped with PEC+GLY; 3. wrapped with the commercial bioplastic MaterBi®; 4. unwrapped. All samples were stored at 4 °C, and analyzed after 0, 4 and 8 days. Results indicated that both kinds of the PEC-based films were able to reduce fruit weight loss, and increase strawberry antioxidant activity, while pH, citric and ascorbic acid, and texture profile analysis were kept constant from 0 up to 8 days at 4°C in comparison with unwrapped samples and the ones wrapped with Mater-Bi®.

The effectiveness of MSN and GLY on improving the technological features of PEC films was demonstrated. It was observed a positive influence of films prepared in the presence of MSN on fruit weight loss and antioxidant activity. Hence it is possible to suggest PEC based films prepared in the presence of MSN and GLY as adequate candidates for extending the shelf-life of different fruits. All results relative to this study are summarized in Fig.25 as graphical abstract and have been published in the publication reported in the following pages.

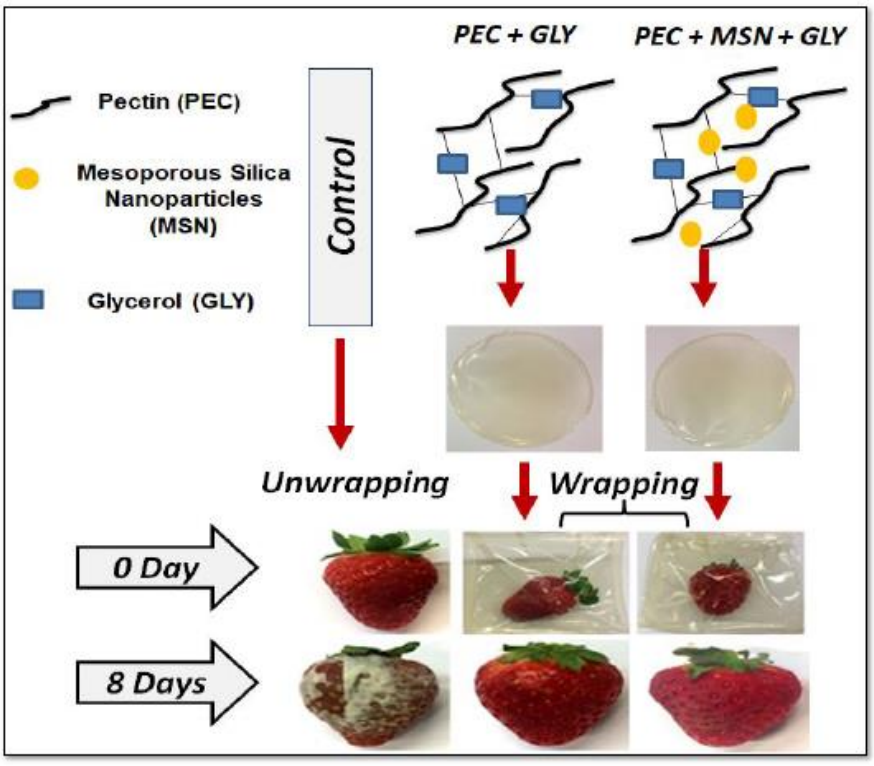

Fig. 25 Proposed model of PEC-based films as bioplastics to protect Strawberry.

nanomaterials

MDPI

Article

Effect of Mesoporous Silica Nanoparticles on The Physicochemical Properties of Pectin Packaging Material for Strawberry Wrapping

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Abstract: Citrus peel pectin was used to prepare films (cast with or without glycerol) containing mesoporous silica nanoparticles. Nanoparticles reduced significantly the particle size, and had no effect on the Zeta potential of pectin solutions. Mechanical characterization demonstrates that pectin+nanoparticles containing films slightly increased tensile strength and significantly decreased the Young's modulus in comparison to films made only of pectin. However, elongation at the break increased in the pectin+nanoparticles films cast in the presence of glycerol, while both Young's modulus and tensile strength were reduced. Moreover, nanoparticles were able to reduce the barrier properties of pectin films prepared with or without glycerol, whereas positively affected the thermal stability of pectin films and the seal strength. The 0.6% pectin films reinforced or not with 3% nanoparticles in the presence of 30% glycerol were used to wrap strawberries in order to extend the fruit's shelf-life, over a period of eighty days, by improving their physicochemical properties.

Keywords: pectin; mesoporous silica nanoparticles; wrapped strawberries; food packaging; biodegradable films

1. Introduction

Nowadays, people are more aware about the harmful effects of the presence in the environment of wastes derived from plastic materials [1]. Globally, production of plastics exceeds 300 million tons per year, and it is likely that a similar quantity of plastics will be produced in the next eight years as it was produced during the twentieth century [2]. A possible solution to reduce the consumption of traditional plastics is to produce innovative biomaterials with promising properties. Hydrocolloids are the main macromolecules naturally available to produce innovative products suitable in many sectors, i.e., food, agriculture, bioplastic and pharmaceutical industries [3]. Therefore, the development of potential hydrocolloid-based coatings and packaging biopolymers has increased during the last years.

However, some properties of these kinds of films are, so far, still poor, and further studies are necessary to find new additives to be added into biomaterials with improved both mechanical and barrier characteristics, both mechanical and barrier to gases and water vapor [4,5].

Pectin (PEC) is a complex anionic polysaccharide mainly composed of homogalacturonan (1 → 4 linked α-D-galacturonic acid and its methyl ester) [6]. PEC has been used in the food and beverage industries for many years. The principal applications of PEC are as a gelling agent, thickening agent, stabilizer and emulsifier [7,8]. Recently, Al-Asmar et al. [9] demonstrated that the PEC-based coating was the most effective among other hydrocolloid-based coatings (containing chitosan or proteins) in reducing acrylamide formation during the frying of French fries. However, as PEC-based films have some limitations regarding the mechanical properties, so many researchers have studied how to improve them by adding proteins [10–12], and/or new plasticizers [13], in order to improve flexibility and to reduce the rigidity and brittleness of the films.

The effect of active compounds inside the film generally depends on the physicochemical properties of the matrix structure, which in turn depends on its morphology at the nanoscale level [14]. For these reasons, many different studies are being devoted to film reinforcement by nanomaterials able to act also as nanocontainers for active compounds [14–18]. More recently, the use of nanoparticles in the development of nanocomposite materials is becoming to represent a new strategy also to improve the physical properties of several polysaccharide-based materials, and the food industry might benefit from it mainly through the production of innovative active and intelligent packaging [19]. Mesoporous silica nanoparticles (MSNs), one of the most important porous materials, have been recently widely used due to their unique features, such as high surface area, controllable pore structure, large pore volume, optically transparent properties, low toxicity, high chemical and thermal stability and a versatile chemical modifiable surface [20–23]. Recently, Fernandez-Bats et al. [14] prepared and characterized the active protein edible films nanostructured with MSNs or with their amino-functionalized derivative, and they concluded that the film tensile strength and elongation at break significantly increased in the presence of both kinds of nanoparticles. Moreover, Tyagi et al. [24], demonstrated that the incorporation of silica with polysaccharide has a significant reduction to the rate of water vapor transmission and water vapor permeability of the film. However, amorphous hybrids materials with the participation of pectin and silica derived from aqueous silicates and tetraethoxysilane (TEOS) were successfully prepared [25,26].

The possible health risk of the consumption of food containing nanoscale compounds transferred from the packaging is not yet fully studied. So far, few studies have reported that the safety of food-containing nanoparticles depends upon the particles' toxicity, size, morphology and the rates of migration and ingestion [27].

The strawberry (*Fragaria vesca*) is one of the most perishable fruits, and has very short postharvest time, because of its susceptibility to mechanical damage, physiological deterioration and possible attack of pathogens [28]. However, strawberry is classified as a healthy food, due to a high content of vitamin C, antioxidant activity, vitamin E and phenolic compounds which make *Fragaria vesca* important for human nutrition [29–31]. To date, some studies have been devoted to extend the shelf life of strawberry fruits, for example, by dipping fruits with edible coatings [32,33], by packaging using chitosan–poly(vinylalcohol) (PVA) blend films [34], and by using γ-irradiation [35].

The objective of this study was to investigate the effect of nanoreinforcement on both PEC films-forming solutions (FFSs) and cast film physiochemical properties. Samples, prepared at two different concentrations of PEC, glycerol (GLY) and/or MSNs, were prepared with the aim of obtaining a suitable biomaterial useful for food packaging. We have here tested the efficiency of PEC-based films in protecting strawberries with the aim of extending their shelf-life at 4 °C.

2. Materials and Methods

2.1. Materials

Citrus peel low-methylated (7%) PEC (Aglupectin USP) was obtained from Silva Extracts srl (Gorle, BG, Milan, Italy), while glycerol (GLY) (about 87%) was purchased from the Merck Chemical Company (Darmstadt, Germany). Tetraethylortosilicate (98%) (TEOS) and cetyltrimethylammonium bromide

(CTAB) were obtained from Sigma (Steinheim, Germany) that was used to synthesize the mesoporous silica nanoparticles (MSNs), as recently reported by Fernandez-Bats et al. [14]. MSN-based solutions were prepared with distilled water [14]. 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Company (Pool, Dorset, UK), while ascorbic acid was from Merck Chemical Company (Darmstadt, Germany). Other chemicals and solvents used in this study were of analytical grade. Mater-Bi® (S 301)-based commercial material bags were from a local market, Naples, Italy. The strawberry variety "Sabrina" was purchased from a local market in Naples, Italy, and strawberry experiments were carried out the day after their purchasing.

2.2. PEC Film Forming Solutions (FFSs) and Film Preparation

A stock solution of PEC (2.0 g) was dissolved in 100 mL of distilled water until the PEC solution was completely solubilized. Serial concentrations of PEC-based films-forming solutions (FFSs) were prepared at pH 7.5 from 0.2–1%, containing MSN 3% *w/w* PEC in the absence and presence of GLY (30% *w/w* PEC). Then the 6 and 10 mg/mL PEC FFSs with different concentrations of MSNs (1%, 3% and 5% *w/w* PEC), were prepared both in the absence and presence of different concentrations of GLY (10, 30 and 50% *w/w* PEC) in 50 mL of distilled water. The same volumes (50 mL) of all the different FFSs, containing or not MSNs and/or GLY, were cast onto 8 cm diameter polystyrene Petri dishes (6 and 10 mg PEC/cm^2, respectively) and finally the films were allowed to dry in an environmental chamber at 25 °C and 45% RH for 48 h. The handleable dried films were peeled off intact from the casting surface after they were conditioned at 25 °C and 53% RH for 2 h in a desiccator containing a saturated solution of $Mg(NO_3)_2 \cdot 6H_2O$.

2.3. Zeta Potential and Particle Size Measurements

Zeta potential and mean particle size hydrodynamic diameter (Z-average size) of the 1 mg/mL PEC FFSs prepared at pH 12.0, containing or not MSN 3%, GLY 30% or both (*w/w* PEC), were titrated automatically from pH 12.0 to pH 2.0, by measuring the dynamic light scattering by a Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) using a He–Ne laser (wavelength of 633 nm) and a detector angle of 173°.

2.4. Film Thickness

Film thickness was measured with an electronic digital micrometer (DC-516, sensitivity 0.001 mm) at different positions of each film sample. At least six measurements were taken on each film sample, and the thickness mean values were considered in the different tests.

2.5. Film Mechanical Properties

The mechanical properties tensile strength (TS), elongation at break (EB) and Young's modulus (YM), were measured according to ASTM D882 [36], by using a universal testing instrument model no. 5543A (Instron, Norwood, MA, USA). PEC films strips (1 cm wide and 5 cm long) were obtained by using a sharp scissors, and were conditioned in an environmental chamber at 25 °C and 53% RH for 2 h. Finally, six samples of each film type were tested, and the speed was 5 mm/min in tension mode.

2.6. Seal Strength

The seal strength of each of the PEC films was tested by the Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). PEC film samples were cut into strips of 5 × 2.5 cm, and one strip was placed on the top of another. After being sealed, film samples were conditioned at 25 °C and 50% ± 5% RH for 48 h [37–39], and few drops of water were added to the seal area. Those two strips were sealed by a heat sealer (MAGIC VAC® AXOLUTE Mod: P0608ED).

The width of the seal area was 0.3 cm^2. The sealing parameters were studied according to ASTM F 88-07a (2007) [40], the seal strength (N/m) was calculated as following Equation (1) [37,39]:

$$\text{Seal strength (N/m)} = \text{Peak force/film width} \quad (1)$$

2.7. Film Barrier Properties

The gas permeability tests toward O_2 [41], CO_2 [42], and water vapor (WV) permeability [43] of triplicate samples of each film were performed at 25 °C under 50% RH, in aluminum masks having an area of 5 cm^2, by using MultiPerm apparatus (ExtraSolution s.r.l., Pisa, Italy).

2.8. Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA)

Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) of the films were performed on a DSC Q-200 (TA Instruments, New Castle, United States). Samples (about 10 mg) were placed into platinum crucibles and then they were heated at 5 °C/min, from room temperature to 400 °C, under inert atmosphere (50 mL/min of N_2).

2.9. Strawberry Experiments

2.9.1. Strawberry Wrapping

The selected strawberries were of uniform size, color and without physical damages and fungal infections. They were randomly divided into four groups. Each group of eleven strawberries was wrapped (W) by different sealed films (10 cm × 10 cm) as following: 0.6% PEC + 30% GLY film (W, PEC+GLY), 0.6% PEC + 3% MSN + 30% GLY film (W, PEC+MSN+GLY), Mater-Bi® commercial material (W, Mater-Bi) and the control was unwrapped (UW). These samples were placed at 4 °C, the quality of both wrapped and control samples were evaluated during storage at 0, 2, 4, 6 and 8 days.

2.9.2. Weight Loss

The weight loss of the unwrapped and wrapped samples was calculated in triplicate at 0, 2, 4, 6 and 8 days of storage as following Equation (2) [34]:

$$\text{Weight loss (\%)} = ((W_0 - W_1)/W_0) \times 100 \quad (2)$$

where W_0 and W_1 represent initial and final fruit weights, respectively.

2.9.3. Determination of pH and Titratable Acidity

Five fruits were taken from each group and then 5 g of fresh homogenate was suspended in 50 mL distilled water using a blender and then centrifuged at 5000 rpm for 10 min. Titratable acidity (TA as citric acid %, using 0.064 as conversion factor for citric acid), was calculated by titrating 5 mL of clear strawberry juice diluted in 50 mL of deionized water against 0.1 N NaOH solution [44], and then the pH of the samples was measured by a digital pH meter. All sample determinations were in triplicate during storage at 0, 2, 4, 6 and 8 days at 4 °C.

2.9.4. Ascorbic Acid Content and DPPH Radical Scavenging Activity

Ascorbic acid was determined using DPPH as Equivalent Antioxidant Capacity according to the procedure previously described by Almeida et al.; Brand-Williams et al. and Giosafatto et al. [45–47], with some modifications: the solution of DPPH (0.05 mg/mL) was diluted with methanol in order to obtain an absorbance of 1.516 ± 0.04 at 517 nm. Homogenous strawberry fruit (100 µL) or controls ascorbic acid were allowed to react with (900 µL) of DPPH radical solution for 30 min in the dark, and the decrease in absorbance from the resulting solution was adjusted. The standard curve of 0–80 mg of ascorbic acid/100 mL was linear ($y = -0.017x + 1.484$, $R^2 = 0.992$).

The same sample were used to study the DPPH radical scavenging activity assay as antioxidant activity %, based on the method previously described by Giosafatto et al. and Odriozola-Serrano et al. [47,48]. The absorbance was measured in triplicate as fresh weight at 517 nm for all samples. Results were expressed (antioxidant activity %) as following Equation (3) [47]:

$$\text{Antioxidant activity \%} = ((\text{Abs DPPH} - \text{Abs sample})/\text{Abs DPPH}) \times 100 \quad (3)$$

2.9.5. Texture Profile Analysis (TPA)

The Texture Profile Analysis (TPA) of unwrapped and wrapped samples stored for 0, 4 and 8 days was performed as described by [49,50]. Texture analyses were carried out using an Instron universal testing instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA) equipped with a 2 kN load cell in compression mode with a cylindrical probe (55 mm in diameter) [50]. The test was configured so that the TPA parameters: (a) Hardness (N), defined as maximum force of the first compression peak; (b) chewiness (N.mm), defined as the total amount of work necessary to chew a sample until it is ready for swallowing [50]; (c) gumminess (N), calculated automatically by multiplying the hardness with the ratio of the positive force areas under first and second compressions (cohesiveness). All TPA parameters were calculated by the software Bluehill by determining the load and displacement at predetermined points on the TPA curve. Pre- and post-test speeds were 2.0 mm/s, while the test speed was 1.0 mm/s. The strawberries were centered and compressed to 60%, and 20 mm of its original size of deformation, and then the average hardness, chewiness and gumminess values of at least six strawberries of each group were evaluated.

2.10. Statistical Analysis

John's Macintosh Project (JMP) software 5.0 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. The data were subjected to analysis of variance (ANOVA), and the means were compared using the Tukey–Kramer honestly significant difference (HSD) test. Differences were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1. Zeta Potential and Particles Size of PEC and MSN-Based Solutions

In order to verify the effect of pH on the stability and particles size of PEC-based FFS and MSN-based solutions, automatically, titrations were performed starting from pH 12 to pH 2. Figure 1 (upper part of Panel A) shows that the Zeta potential of 0.1% PEC-based FFS is highly stable, being around −40 mV, starting form pH 12 to ≈ pH 4. These results are in good agreement with those previously obtained on the PEC-based films [13,51]. Moreover, the Z-average was ≈ 300 d.nm in the same pH range (Figure 1, lower part of Panel A). However, the 3% MSN-based solutions, rich in OH groups, show less stability compared to PEC-based FFS, since the Z-potential range was ≈ −28 mV to −20 mV from pH 12 to pH 5, respectively, reaching −10 mV at pH 2 (Figure 1, upper part of Panel A). These results were in agreement with those obtained by Fernandez-Bats et al. [14]. Regarding the Z-average, MSNs showed a smaller size of about 150 d.nm, at pH values ranging from 12 to 6 (Figure 1, lower part of panel A).

This value was very similar to the average size of MSN-based nanoparticles obtained by Fernandez-Bats et al. and Musso et al. [14,52]. The latter authors have characterized MSNs also by TEM, observing an average size of 143 ± 26 nm. At pH 3, the MSN particle size reached about 200 d.nm.

In general, PEC films present some limitations such as rigidity and brittleness [53]. In this work, GLY has been used as a plasticizer to improve PEC-based formulation properties by adding GLY or MSNs, or both. In particular, Figure 1, Panel B, reports the influence of pH on Zeta potential and Z-average size of 0.1% PEC FFSs containing 30% GLY, or 3% MSNs or both MSNs and GLY. The results show that, adding either GLY or MSNs does not change the PEC FFS stability from pH 12 to pH 6

(Figure 1, upper part of Panel B). Regarding the Z-average, Figure 1 (lower part of Panel B) shows that the presence of both MSNs and GLY into the PEC-based solution provokes a reduction of particle size from 600 to 400 nm at pH 2, reaching a diameter of around 200 nm at pH ≥ 3. To study the effect of different concentrations of each component, pH 7.5 was selected to cast the films in the further studies.

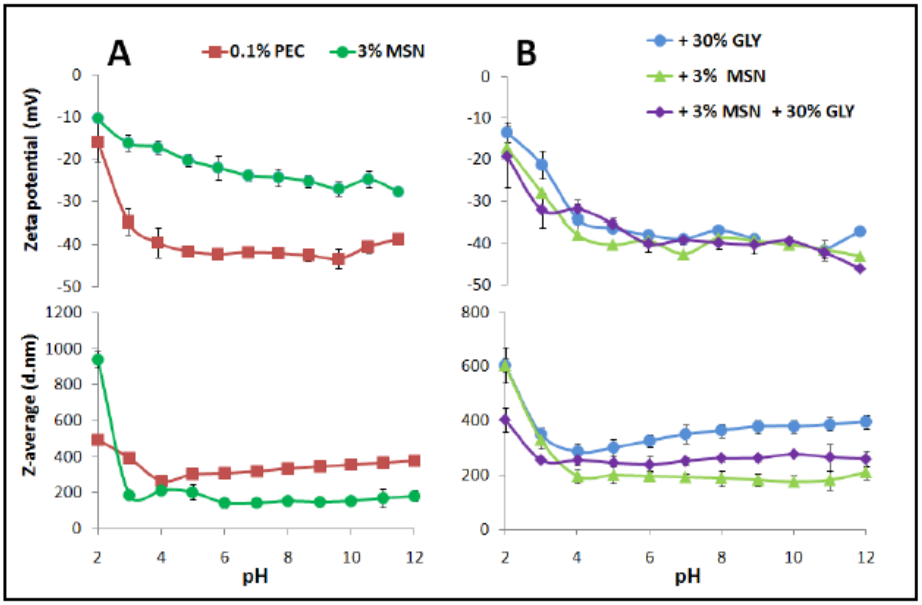

Figure 1. Effect of different pHs on the Zeta potential and Z-average size of 0.1% PEC-based and 3% MSN water solution (Panel **A**), and of 0.1% PEC-based FFS (prepared in the presence of 30% GLY (*w*/*w* with PEC) or 3% MSNs (*w*/*w* with PEC) or in the presence of both 3% MSNs and 30% GLY (Panel **B**).

3.2. Mechanical Properties

Figure 2 reports the film thickness and mechanical properties of PEC-based films prepared with different concentrations of PEC (0.2%, 0.4%, 0.6%, 0.8% and 1%) either in the presence or absence of 3% MSNs or 30% GLY and in the presence of both. The results were compared with the Viscofan NDX edible film that is a commercially available material prepared from collagen and mainly used in the meat industry [54].

The results indicate that film thickness increases depending on PEC concentration, becoming thicker, then also NDX at 0.4% and higher concentrations. MSNs do not affect PEC film thickness. Moreover, GLY influences thickness significantly when added alone or together with MSNs in films prepared at PEC concentrations equal or superior to 0.6%. PEC concentration seems do not affect EB that is very low in PEC films compared to NDX material. The presence of GLY and the concomitant presence of both GLY and MSNs influence EB significantly when 0.4% and higher PEC concentrations were used.

Giosafatto et al. [55], evaluated the effect of 3% MSNs or/and 30% GLY on the 0.6% PEC and 0.6% chitosan, and they demonstrated that MSNs and GLY significantly increased the plasticity of both kinds of films. In particular, the PEC-based films containing MSNs or GLY showed a higher value of EB, and a reduced YM, while a more marked effect on these parameters was obtained by adding both MSNs and GLY. At the same time, the concurrent presence of MSNs and GLY significantly reduced the tensile strength of the PEC-based films [55].

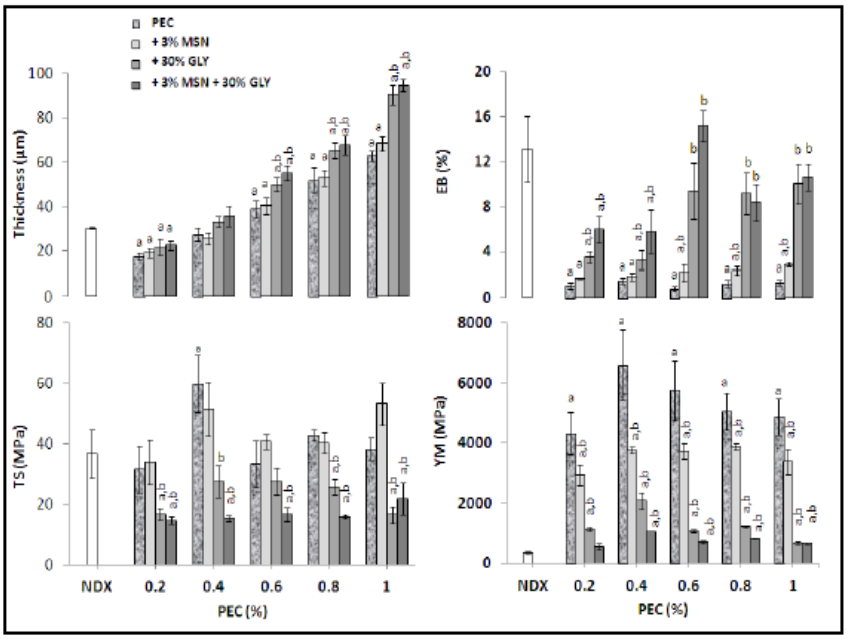

Figure 2. Effect of pectin (PEC) concentration on thickness and mechanical properties of films prepared at pH 7.5 in the presence or absence of mesoporous silica nanoparticles (MSNs) or glycerol (GLY) or in the presence of both. The values, significantly different from those obtained by analyzing the Viscofan NDX edible film (white bars) (Data from Porta et al. [54]), are indicated by "a", while the values indicated by "b" were significantly different from those obtained by using only PEC ($p < 0.05$). Further experimental details are given in the text.

It can be of interest to recall that Fernandez-Bats et al. [14] have demonstrated that protein-based films reinforced with MSNs showed higher TS and EB, reaching the highest values using 3% MSNs. Nesic et al. [56], studying highly methylated pectin biocomposite membranes reinforced by MSN type SBA-15, observed an increase of both TS and EB, but a reduction in YM.

For further studying the influence of both MSNs and GLY on film thickness and mechanical properties, 0.6% and 1% PEC-based films were chosen. Figure 3 shows that, in both kinds of films, thickness increases significantly by increasing the GLY concentrations, using 10%, 30%, or 50% of the plasticizer, respectively. No effect was observed on the 0.6% PEC film thickness by changing the MSN concentrations. Regarding 1% PEC films, the thickness was significantly reduced when the films were prepared in the presence of 10% GLY with both 1% and 3% MSNs. Thickness of films made in the presence of 30% GLY was affected only with 1% MSNs, while thickness of films made in the presence of 50% GLY was affected when 3% MSNs were added. Moreover, the presence of either 1% or 3% MSNs in FFS containing 50% GLY provokes an increased EB compared to the NDX material and to all other film kinds. However, 50% GLY-containing films showed lower TS and YM among all the studied films.

In summary, regarding the mechanical properties the GLY component is the one that affects TS (a measure of resistance), since it reduces this parameter, while it increases EB (a measure of extensibility) and decreases the YM (a measure of stiffness). On the contrary, MSNs influence positively the resistance and the extensibility, while they also contribute to obtain a more rigid material. Taking into account the film characterization results (thickness, mechanical and permeability properties), an efficient wrapping bioplastic could be obtained made of 0.6% PEC, 3% MSNs and 30% GLY.

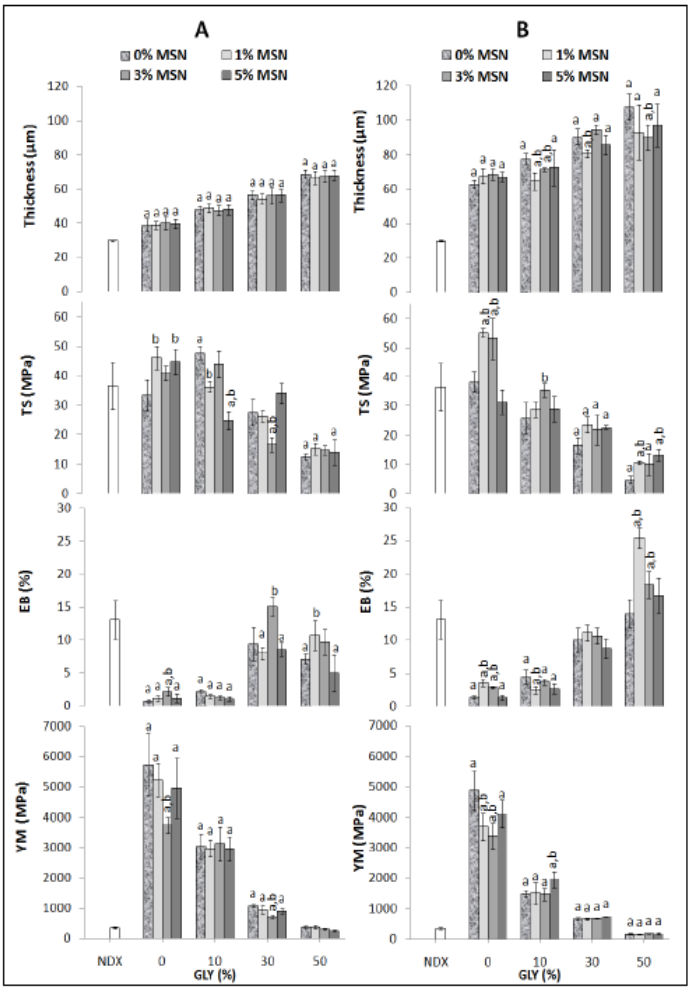

Figure 3. Effect of different concentrations of MSNs and GLY on thickness and mechanical properties of 0.6% PEC films (Panel **A**) and 1% PEC films (Panel **B**), both prepared at pH 7.5. The values significantly different from those obtained by analyzing Viscofan NDX (white bars) (Data from Porta et al. [54]), are indicated by "a", while the values indicated by "b" were significantly different from those obtained by analyzing films made of either only PEC or the one obtained with GLY ($p < 0.05$). Further experimental details are given in the text.

3.3. Heat Seal Strength

An important parameter for bioplastics, that have to be used for wrapping, is the heat seal strength. A higher heat seal strength value indicates that a higher strength is necessary to separate two parts of the same material that have been sealed to each other. Our findings indicated that the presence of either MSNs or GLY in PEC-based films increased the seal strength significantly. The 1% PEC-based films gave higher values compared to 0.6% PEC-films (Figure 4). However, the incorporation of both MSNs and GLY significantly reduced the seal strength in comparison to the values obtained with PEC+GLY.

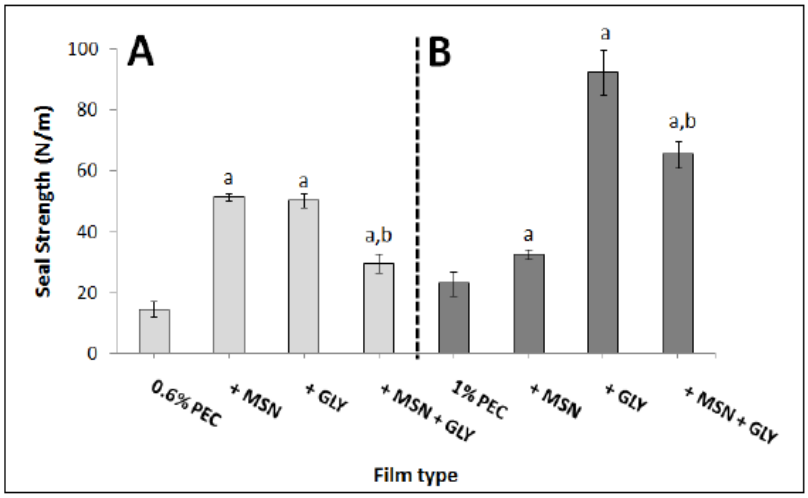

Figure 4. Seal strength of 0.6% PEC film prepared with or without 3% MSNs (*w*/*w* PEC), 30% GLY (*w*/*w* PEC) or in the presence of both (Panel **A**) and 1% PEC film prepared with or without 1% MSN (*w*/*w* PEC), 50% GLY (*w*/*w* PEC) or in the presence of both (Panel **B**). All the films were prepared at pH 7.5. The values significantly different from those obtained by PEC only (0.6% or 1%) are indicated by "a", while the values indicated by "b" were significantly different from those obtained by only adding GLY ($p < 0.05$).

In other words, MSNs counteract the sealability of the PEC+GLY-based films. Similar results were also reported by Voon et al. [18], since they found out that using both nanoparticle halloysite nanoclay and SiO_2 reduced the seal strength of bovine gelatin-based film plasticized with 20% GLY. However, increasing PEC (from 0.6% to 1%) and GLY (from 30% to 50%) concentrations improved the seal strength (Figure 4). This behavior was also observed by Farhan and Hani [37], that obtained an increase of the seal strength by increasing the plasticizer (GLY or sorbitol) concentrations in kappa-carrageenan films. These results could be likely due to higher heat transfer between the two sealed surfaces [37,57]. Generally, a higher value of seal strength is desirable when a higher capacity to resist the separation is required, whereas a lower value of seal strength might be advantageous in some applications where an easy peel opening is needed. For example, in the food field, consumers may prefer uncomplicated packaging to be open [18].

3.4. CO_2, O_2, and Water Vapor (WV) Permeability

Table 1 reported the effect of MSNs and/or GLY on gas (CO_2 and O_2) and the WV permeabilities of the PEC-based films. The permeability of 0.6% PEC film toward the CO_2 and O_2 were decreased significantly by adding 3% MSNs or 30% GLY or both.

However, adding 1% MSNs to 1% PEC-containing films does not decrease the CO_2 permeability, while it does reduce significantly the O_2 permeability. On the other hand, the only addition of 50% GLY to 1% PEC film increases significantly the permeability to both gases, while it decreases significantly when 1% of the MSNs were added to 1% PEC film prepared in the presence of 50% GLY.

Table 1. Gas and WV permeability of 0.6% and 1% PEC films prepared at pH 7.5 in the presence or in the absence of GLY and/or MSNs.

Film	Permeability (cm^3 mm m^{-2} day^{-1} kPa^{-1})		
	CO_2	O_2	WV
0.6% PEC	0.141 ± 0.009	0.151 ± 0.008	0.159 ± 0.048
+3% MSNs	0.110 ± 0.004 [a]	0.090 ± 0.014 [a]	0.083 ± 0.022 [a]
+30% GLY	0.105 ± 0.012 [a]	0.037 ± 0.007 [a]	0.110 ± 0.010
+3% MSNs + 30% GLY	0.076 ± 0.008 [a,b]	0.022 ± 0.001 [a,b]	0.026 ± 0.001 [a,b]
1% PEC	0.081 ± 0.031	0.013 ± 0.001	0.127 ± 0.016
+1% MSNs	0.076 ± 0.001	0.008 ± 0.002 [a]	0.091 ± 0.010 [a]
+50% GLY	0.415 ± 0.010 [a]	0.026 ± 0.012 [a]	0.122 ± 0.001
+1% MSNs + 50% GLY	0.286 ± 0.006 [a,b]	0.019 ± 0.003 [a]	0.085 ± 0.002 [a,b]
Chitosan (0.6%) *	15.81 ± 2.06	14.33 ± 0.29	0.05 ± 0.02
Viscofan (NDX) **	3.71 ± 0.16	0.03 ± 0.01	0.08 ± 0.01
Mater-Bi® (S-301) **	5.23 ± 0.01	0.74 ± 0.01	0.04 ± 0.01

The values significantly different from those obtained by PEC only (0.6% or 1%) are indicated by "[a]", while the values indicated by "[b]" were significantly different from those obtained by only adding GLY ($p < 0.05$), * Data from [58] ** Data from [54].

The strong effects of MSNs were markedly indicated by reducing significantly the WV permeability of both 0.6% and 1% PEC films obtained in the presence or in the absence of GLY. The gases and WV permeability values of MSN-reinforced 0.6% PEC film in the presence of GLY were lower compared to other polysaccharides like chitosan and commercial materials such as Viscofan NDX or Mater-Bi®. This result was obtained also by Fernandez-Bats et al. [14], that have studied protein films, concluding that the MSN addition gives rise to materials less permeable to gases and WV. The obtained results could be explained taking into account the structure of such films studied in a recent work [55]. In fact, in this study it has been reported that MSN-containing PEC-based films possess a more compact and homogeneous structure in comparison to the film prepared without any MSNs [55].

3.5. Thermal Properties

Figure 5 shows the TGA and differential thermal gravimetry (DTG) curves of 0.6% (Panel A) and 1% (Panel B) PEC films prepared with MSNs or GLY or with both. The weight loss curves of all samples exhibited a first weight loss appearing from room temperature to about 110 °C, representing evaporation of water molecules, present mainly as free water [59].

For all the films this weight loss is about 15%–17%. After this, a second and more significant mass fall appears, corresponding to the degradation of the organic films. In particular, for films containing only PEC, degradation started at about 200 °C for both concentrations of the FFS (0.6% and 1%), having a residue of 30% approximately at 400 °C. In contrast with the first step, the second process represents an exothermic transition, as it can be observed in Figure 6. This behavior agrees with previous works [60,61]. As it can be observed in both Panels of Figure 5, the presence of GLY in the films reduces the thermal stability, starting the degradation of the films (at around 170 °C), no matter the presence, or absence of MSNs. An additional sample weight loss was observed at the range of 260–400 °C in PEC film prepared with or without MSNs. In this case, due to the fact that a little difference percentage is observed, the weight loss could be related to the decomposition and pyrolytic de-polymerization of the PEC [62,63]. In films made of either 0.6% or 1.0% of PEC, the presence of MSNs results in a small increment of the residual mass, which can be justified according to the high stability of this inorganic nanomaterial [64].

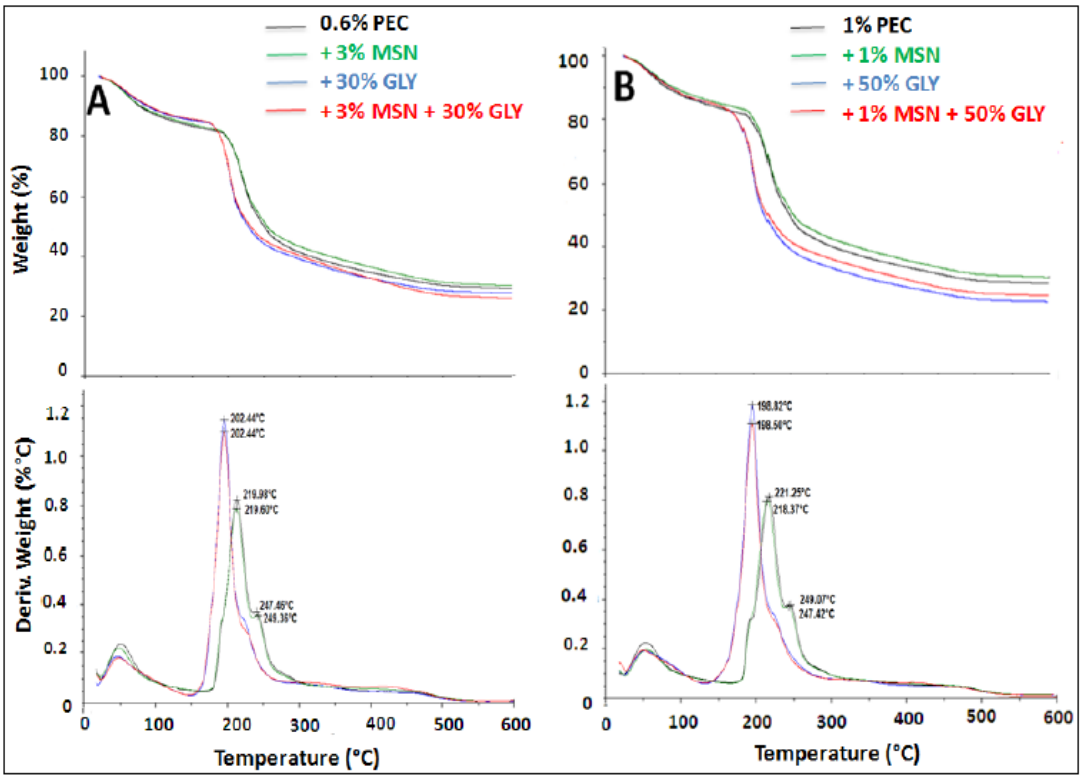

Figure 5. Thermogravimetric analysis (TGA) and differential thermal gravimetry (DTG) of 0.6% PEC film prepared at pH 7.5 with or without 30% (*w*/*w* PEC) GLY, 3% MSNs (*w*/*w* PEC) or with both (Panel **A**) and 1% PEC film prepared with or without 50% (*w*/*w* PEC) GLY, 1% MSNs (*w*/*w* PEC) or with both (Panel **B**).

Figure 6 and Table 2, show thermal properties of PEC-based films. Thermal properties depend mainly on both material chemical composition and state transition occurring during material processing, as well as on the interdependence of both these factors [65]. In Figure 6, it can be observed that, in both types of films (0.6% PEC, Panel A and 1% PEC, Panel B), heat flow alterations in thermal transition occurred between 65 and 100 °C. This behavior is mainly due to the water evaporation, which is an endothermic process. Similar results were obtained by Nisar et al. [66] that studied PEC-based materials as well.

Table 2. DSC thermal parameters, glass transition temperature (T*g*) and peak melting temperature (T*m*) of pectin films incorporated with GLY and MSNs, or with both.

Film	T*g* °C	T*m* °C
0.6% PEC	120.3	222.8
+3% MSNs	174.4	224.8
+30% GLY	84.9	205.7
+3% MSNs + 30% GLY	150.4	204.8
1% PEC	125.8	227.4
+1% MSNs	170.1	227.7
+50% GLY	91.4	202.0
+1% MSNs + 50% GLY	146.2	200.4

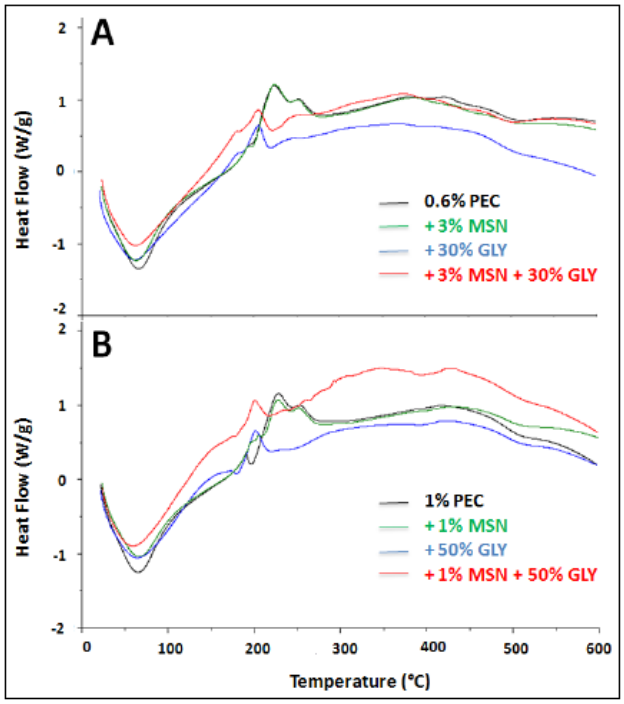

Figure 6. Differential Scanning Calorimetry (DSC) thermograms of 0.6% PEC film prepared with or without 30% GLY (*w*/*w* PEC), 3% MSNs (*w*/*w* PEC) or in the presence of both (Panel **A**) and of 1% PEC film prepared with or without 50% GLY (*w*/*w* PEC), 1% MSNs (*w*/*w* PEC) or in the presence of both (Panel **B**). All the films were prepared at pH 7.5.

In Table 2 there is the list of DSC thermal parameters such as glass transition temperature (T*g*) and melting temperature (T*m*). T*g* of the 0.6% and 1% PEC is 120.3 °C and 125.8 °C, respectively. A similar result was obtained by Seslija et al. [67], that reported a T*g* equal to 131.0 °C for 0.5% PEC solutions. We observed that the T*g* of the 0.6% PEC film decreased from 120.3 °C to 84.9 °C by adding 30% GLY, while was 91.4 °C when 50% GLY was added to 1% PEC. These were expected results because it is well known that GLY, acting as a plasticizer, reduces the T*g* and also the T*m* [68,69]. MSNs increased the film thermal stability of both kinds of films since the T*g* was 174.4 °C for 0.6% PEC films, and 170.1 °C for 1% PEC films. The addition of GLY gave rise to films with a T*g* equal to 150.0 °C and 146.0 °C, respectively, indicating the thermal protective effect of the plasticizer. Moreover, T*m* of 0.6% and 1% PEC films was at 222.8 °C and 227.4 °C, respectively, and it decreased in PEC+GLY samples, being 205.7 °C and 202.0 °C respectively. The presence of MSN in PEC+GLY+MSN films decreased to 204.8 °C and 200.4 °C, respectively. Our results are in accordance with the previous studies carried out by Nisar et al. [66], that found out that the T*m* of PEC film was 228.9 °C, a value very similar to the one obtained with 1% PEC-based films.

3.6. Quality of Wrapped Strawberries During Storage

Due to their characteristics (mechanical, sealing and permeability properties) 0.6% PEC+30% GLY films containing 3% MSNs (PEC+MSN+GLY) or not (PEC+GLY) were used to wrap strawberries. Experiments were set up using different groups of strawberries wrapped with different films, including Mater-Bi®, a commercial bioplastic. The results were compared to unwrapped strawberries (UW). The performed analyses were finalized to study the quality of strawberries from time 0 to time 8 days.

3.6.1. Weight Loss, pH and Titratable Acidity

Figure 7 shows the effect of wrapping on the weight loss, pH and acidity of wrapped fruits with films not containing silica (W, PEC+GLY) and containing silica (W, PEC+MSN+GLY), compared to the effect due to wrapping made with Mater-Bi® (W, Mater-Bi) as commercial material. Unwrapped strawberries (UW) were used as a control. The analyses were performed from 0 to 8 days. Up to two days, no significant differences were found among all the analyzed samples. However, for longer storage times, PEC+GLY and PEC+GLY+MSN films affected positively all the three studied parameters compared to both unwrapped and Mater-Bi® wrapped samples. Thus, as shown in Figure 7A, the weight loss was higher in the latter samples, while it was reduced when strawberries were wrapped with PEC+GLY films or with PEC+MSN+GLY films. The same trend was observed for pH (Figure 7B) that was close to the control values in samples wrapped with both PEC containing films. This is due to the low conversion rate of organic acids during respiration [37,70]. The content of citric acid (Figure 7C) was also higher in both PEC-containing films, even after eight days, compared to the unwrapped samples or the samples wrapped with Mater-Bi®. Generally, most fresh fruits are acidic, and the acid content usually decreases during ripening. This is due to the utilization of organic acids during respiration [70].

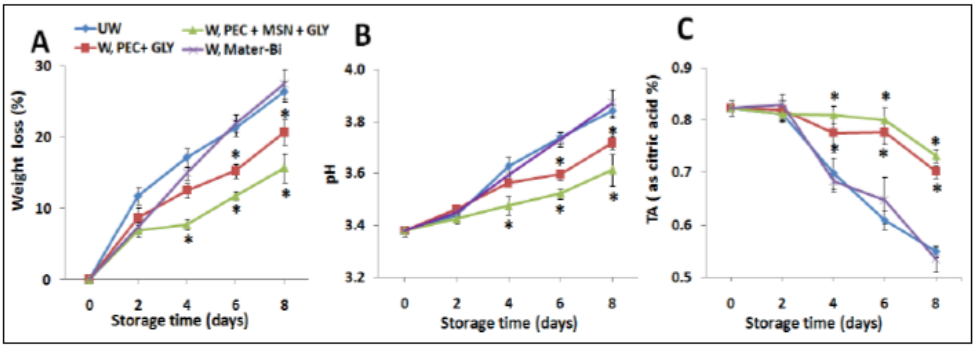

Figure 7. Effect of wrapping with pectin films on weight loss % (**A**), pH (**B**), and titratable acidity (**C**) of strawberries stored for several days (0–8 days). * indicates statistically significant differences at $p < 0.05$ compared to the unwrapping group (UW) used as a control, and to samples wrapped with Mater-Bi®.

3.6.2. Ascorbic Acid Content and Antioxidant Activity

Results shown in Figure 8 indicate a strong reduction of the ascorbic acid in the control UW strawberry and also strawberry wrapped by Mater-Bi® (W, Mater-Bi). Ascorbic acid drop is evident after two day storage. After this time only samples wrapped with both types of PEC-containing films kept high values of ascorbic acid that were only around 5% lower that the control.

Similar results were obtained by Liu et al. [34], that demonstrated that packaged strawberries with chitosan–poly(vinylalcohol) films reduced the oxidation of ascorbic acid compared to unpackaged ones. Regarding the antioxidant activity, strawberries wrapped by PEC+GLY with or without MSNs showed significantly higher values during the storage times compared to the UW and W, Mater-Bi and to time 0 (Figure 8B). A possible explanation could be related to the higher O_2 barrier provided by both PEC-based films compared to the barrier property of Mater-Bi (see Table 1). O_2 concentrations in wrapped samples contrast the evaporation of phenolic compounds that, as it is well known [35,71], are correlated to the antioxidant activity of the fruits.

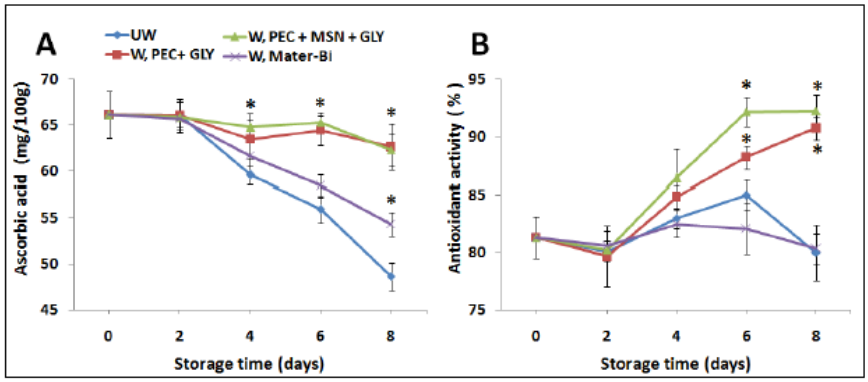

Figure 8. Effect of wrapping with pectin films on ascorbic acid content (**A**), and antioxidant activity (**B**) of strawberries stored for several days (0–8 days). * indicates statistically significant differences at $p < 0.05$ compared to the unwrapping group (UW) used as a control and to samples wrapped with Mater-Bi®.

3.6.3. Texture Profile Analysis (TPA)

The studied unwrapped and wrapped strawberries were analyzed also to assess their texture profile. Thus, Table 3 shows the TPA (hardness, chewiness and gumminess) of the fruit samples at different storage times (0, 4 and 8 days).

Table 3. Texture Profile Analysis (TPA) of strawberries wrapped or not with 0.6% PEC+30%GLY-based films prepared in the absence (W, PEC+GLY), and the presence of 3% MSNs (W, PEC+MSN+GLY). Unwrapped strawberries (UW) were used as control. A strawberry group was wrapped with Mater-Bi® (W, Mater-Bi). The analyses were performed at 0, 4 and 8 days.

Storage Time (Days)	Strawberry Sample	Hardness (N)	Chewiness (N.mm)	Gumminess (N)
0	UW	57.8 ± 8.9	92.5 ± 10.4	7.9 ± 2.8
4	UW	46.6 ± 5.4	89.2 ± 15.6	7.4 ± 1.9
	W, PEC+GLY	59.9 ± 6.4	81.5 ± 9.6	8.7 ± 2.7
	W, PEC+MSN+GLY	56.1 ± 3.1	74.2 ± 7.6	6.2 ± 1.0
	W, Mater-Bi	43.1 ± 1.3 [b]	76.7 ± 5.2	6.4 ± 0.4
8	UW	ND	ND	ND
	W, PEC+GLY	34.5 ± 3.8 [a,b]	50.4 ± 9.3 [a,b]	4.2 ± 0.8 [a,b]
	W, PEC+MSN+GLY	25.4 ± 7.3 [a,b]	41.1 ± 4.8 [a,b]	3.5 ± 0.4 [a,b]
	W, Mater-Bi	ND	ND	ND

The values significantly different from those obtained in UW in the same storage time are indicated by "[a]", while the values indicated by "[b]" were significantly different from those obtained by UW at 0 day ($p < 0.05$). ND: not detected.

The results show that, after four days' long storage, no significant differences were detected on the hardness, chewiness and gumminess between UW strawberries and both W, PEC+GLY and W, PEC+GLY+MSN wrapped fruits.

This means that wrapping is not necessary up to four days to have fresh strawberries. However, results after eight days clearly showed that, while both unwrapped and Mater-Bi®-wrapped samples appeared spoiled (Figure 9), the PEC-based wrapping films positively influence the fruit quality. It is interesting to note that W, Mater-Bi samples showed a hardness significantly lower that the UW strawberries, meaning that the fruits were more susceptible to spoiling. These results are consistent with the fact that Mater-Bi® provides a higher barrier to CO_2 (Table 1), a gas that influences ethylene production inside the wrapping film, thus promoting ripening [72].

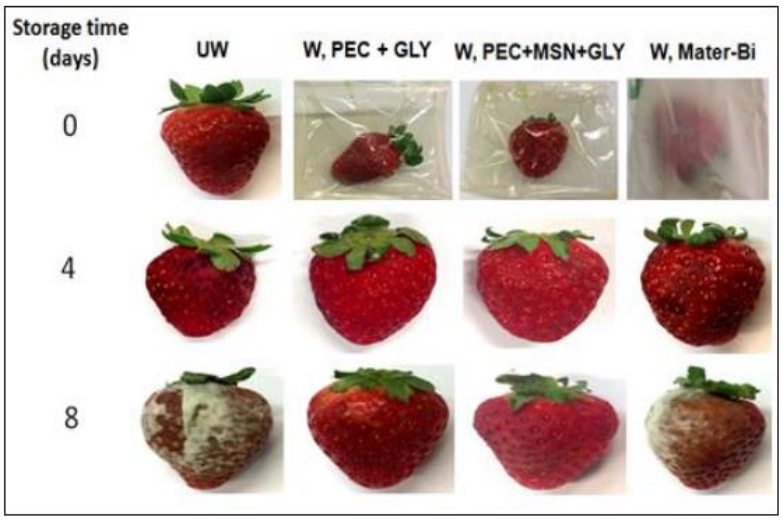

Figure 9. Images of unwrapped and wrapped groups of strawberries during storage time (days).

4. Conclusions

In this paper the effectiveness of MS nanoparticles and GLY on improving the technological features of PEC films was demonstrated. The GLY-enriched films either prepared in the absence or presence of MSNs exploited for strawberry wrapping and different fruit quality parameters were analyzed over a period of eight days.

The obtained results showed the positive influence of films prepared in the presence of MSNs on fruit weight loss and antioxidant activity, whereas pH, citric and ascorbic acid and texture, seem to be not significantly different from the unwrapped samples. Based on these studies it is possible to suggest PEC-based films prepared in the presence of MSN and GLY as adequate candidates for extending the shelf-life of different fruits.

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CHAPTER 4

CONCLUSIONS

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4. CONCLUSIONS

Reducing acrylamide content in French Fries, falafel and kobbah was successfully achieved by coating these products with both polysaccharides (pectin and chitosan) and proteins (grass pea flour) prepared in the presence or absence transglutaminase enzyme and mesoporous silica or chitosan nanoparticles. Our rational was confirmed by using hydrocolloid-based coatings with nanoreinforcement and enzymatically crosslinked capable to increase water retention and reducing the oil uptake, together with the ability of reducing significantly the ability to form acrylamide.

In this project, for the first time, falafel balls were produced by using transglutaminase enzyme in the dough. In addition, after the preparation, the balls were treated by dipping them in a pectin-based coating solution. Transglutaminase also had an effect on the texture profile parameters. On the other hand, the pectin coating protection reduced the oil content of this food product, either treated or not, by means of transglutaminase. Moreover, protein gastric digestion, carried out under physiological conditions, showed that enzymatic treatment slightly decreased the digestion rate, although the proteins were fully digested at the end of the experiment in both unprocessed and transglutaminase-processed systems. The sensory analysis indicated that the falafel balls stored for 60 days at -20ºC coated with pectin were accepted by the Palestinian panellists.

Hydrocolloid-based solutions were effective in reduction acrylamide, water and oil content in kobbah another Middle Eastern fast food. In this case, nanoreinforced coatings were used and the most effective was the pectin–based solution nanoreinforced with chitosan nanoparticles. In fact, fried kobbah coated by this solution showed the lowest acrylamide, water and oil content.

Pectin films containing mesoporous silica nanoparticles were also obtained and evaluated according the mechanical, permeability, thermal, FT-IR and morphology properties, the results showed that significant improvement of physiochemical properties of the obtained materials. The pectin films plasticized with glycerol, either prepared in the presence or absence of mesoporous silica nanoparticles, were exploited for strawberry wrapping and different quality parameters were analyzed over a period of eight days storage. The obtained results showed the positive influence on strawberry weight loss and antioxidant activity, whereas pH, citric and ascorbic acid, and texture seem to be not significantly different from the unwrapped samples.

According to this project results, it is reasonable to recur to hydrocolloid-based solutions to be used both as coatings to obtain healthier fried foods, or bioplastics to protect fruits and vegetable.

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6. APPENDIX LIST

Appendix 6.1

Experience in foreign laboratories

1. Stage in Department of Analytical Chemistry, Complutense University of Madrid (Spain)
Supervisor: Prof. Reynaldo Villalonga Santana
Topic: Physicochemical characterization of nanopaticales and pectin based film reinforced with mesoporous silica nanoparticles.
Date: April/2018- July/2018

REYNALDO VILLALONGA SANTANA, DNI 54523793Q
PROFESOR TITULAR DE UNIVERSIDAD
DEPARTAMENTO DE QUIMICA ANALITICA
FACULDAD DE CIENCIAS QUIMICAS
UNIVERSIDAD COMPLUTENSE DE MADRID

July 29th, 2018

TO WHOM IT MAY CONCERM

This is to certify that **Dr. Asmaa W. M Al-Asmar** from Università degli Studi di Napoli "Federico II", has been working in my research group at Complutense University of Madrid, Department of Analytical Chemistry from 28/4/2018 to 29/7/2018.

Please do not hesitate to contact me. I wish him all the best in her future endeavor.

FACULTAD DE CIENCIAS QUIMICAS
DEPARTAMENTO DE QUIMICA ANALITICA

Yours Sincerely
Prof. Reynaldo Villalonga
Department of Analytical Chemistry, Faculty of Chemistry
Complutense University of Madrid, 28040 – Madrid, Spain
Phone: +34 91 3944331; Fax: +34 91 3934329
E-mail: rvillalonga@quim.ucm.es

2. Stage in Department of Nutrition and Food Technology, in An-Najah National University.
Supervisor: Dr. Mohammed Altamimi
Topic: Sensory evaluation of falafel coated by edible films.
Date: September 2019- December/2019

An-Najah
National University
Faculty of Agriculture & Veterinary Medicine
Department of Nutrition and Food Technology

جامعة النجـــاح الوطنيـــة
كلية الزراعة والطب البيطري
قسم التغذية والتصنيع الغذائي

Nablus, 31st December 2019

TO WHOM IT MAY CONCERN

This is to certify that **Dr. Asmaa W.M Al-Asmar** from Università degli Studi di Napoli "Federico II", has been working in my research group at An-Najah National University, from September to December, 2019.

Please do not hesitate to content me. I wish her all the best in his future endeavor.

Dr. Mohammed Altamimi
An-Najah National University,
Faculty of Agriculture and Veterinary Medicine
Head of the Department
Nutrition and Food Technology
Email: m.altamimi@najah.edu phone: +972595591255

Dr. Mohammed Altamimi

جامعة النجاح الوطنية
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Faculty of Agriculture & Veterinary Medicine
Department of Nutrition and Food Technology

Appendix 6.2

Awards

1- Award of fellowship to attend FISV 2018

SOCIETÀ ITALIANA DI BIOCHIMICA E BIOLOGIA MOLECOLARE
Ente morale dal 1993

Rome, December 23rd, 2019

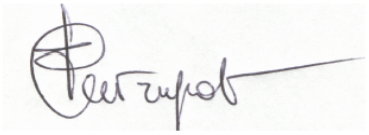
To whom it may concern

Ref. : Award of fellowship to attend FISV 2018 Meeting Dr. Asmaa W.M. AL-Asmar

I hereby declare that Dr. Asmaa W.M. AL-Asmar of the Department of Chemical Sciences, University of Naples Federico II (Italy) has been awarded a scholarship to attend the FISV (Federation of Italian life Sciences Societies) Meeting held in Rome (Italy) 18-21 September 2018.

Best regards

The SIB Secretary
Prof. Francesca Cutruzzoà



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Tesoriere:
Prof. Eugenio Monti
Dip.to di Medicina Molecolare e Traslazionale
Università degli Studi di Brescia
Viale Europa, 11 - 25123 Brescia

Appendix 6.3

Contributions to scientific meetings

1. Contribution: Oral Presentation

1.1 Workshop BIO/10 (Biochemistry Teachers and Researchers of the University of Campania) Napoli-Italy, Aula Magna del Complesso delle Biotecnologie,17th May 2019

BIOTEC.4

EDIBLE COATINGS AND FILMS TO REDUCE ACRYLAMIDE FORMATION IN FRIED FOODS AND TO EXTEND STRAWBERRY SHELF-LIFE

ASMAA AL-ASMAR [1,2] (asmaa.alasmar@unina.it; SIB), CONCETTA VALERIA L. GIOSAFATTO[1] (giosafat@unina.it; SIB), LOREDANA MARINIELLO[1] * (loredana.mariniello@unina.it; SIB)

[1] Department of Chemical Sciences, University of Naples "Federico II," 80126 Naples, Italy.
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*Corresponding author

According to European Food Safety Authority (EFSA), acrylamide is produced during cooking in numerous foods (e.g. french fries, breads). EFSA scientists concluded that acrylamide is a health concern. Higher temperature and low moisture content are very convenient conditions to produce acrylamide. Edible coating is a thin layer that covers the food surface that can be applied by dipping or spraying methods. Moreover, the edible coating or film can be consumed without causing any adversely health problem for consumers. Hydrocolloid materials are the most common materials used to prepare edible coatings and films. French fries are popular products worldwide with an acrylamide content that can reach 2089 µg/kg [1]. Recently, we ended up that pectin-based solutions are able to reduce acrylamide content in French fries of about 48%. Moreover, grass pea flour solutions treated with microbial transglutaminase also reduced acrylamide content of about 37%, while coating solutions containing grass pea flour not crosslinked by the enzyme could reduce acrylamide of about 31% [1]. In this work we checked the effectiveness of edible films in reducing acrylamide also in falafel, a traditionally fried and street food in Middle Eastern, made of grinded chickpeas with parsley and spices. Our studies have assessed that, after frying, the acrylamide content reached 7229 µg/kg. After adding transglutaminase (20 U/g chickpea proteins) to the falafel dough, the acrylamide content was reduced of about 34.4%, while the reduction was of about 84.5% when transglutaminase-containing falafels were dipped in the pectin solution [2]. We have also studied the effectiveness of mesoporous silica nanoparticles (MSN) in reinforcing pectin films and verified MSN ability to reduce the permeability to CO_2, O_2 and water vapor. Thus, cast pectin–based films containing MSN have been used to wrap strawberries. Results have indicated the ability of our films to increase strawberry shelf-life [3].

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2. Al-Asmar A., Giosafatto C.V. L., Panzella L., Mariniello L. Improving the health quality of fried falafel (Middle Eastern food) by using transglutaminase and/or pectin coating. Submitted to Coatings.
3. Al-Asmar A., Giosafatto C.V.L., Sabbah M., Sanchez A., Villalonga Santana R., Mariniello L. Effect of mesoporous silica nanoparticles on physicochemical properties of pectin packaging material for strawberry wrapping. Submitted to Nanomaterials.

Pectin-based film wrapping reinforced by mesoporous silica nanoparticles to extend the shelf life of strawberry

Asmaa Al-Asmar [1,2*], *Concetta Valeria L. Giosafatto*[1], *Banca Estela García Almendárez*[3] *and Loredana Mariniello*[1]

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[2]Analysis, Poison control and Calibration Center (APCC), An-Najah National University, Nablus, Palestine

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Abstract

The interest in biopolymers for manufacturing biodegradable/edible packaging materials is increasing. Among biopolymers, polysaccharides and proteins represent an abundant, inexpensive and renewable raw source. These molecules are proposed as film-forming agents, being able to establish intermolecular linkages by various interactions. Most of the times it is necessary to recur to plasticizers like glycerol (GLY) as an additive of such hydrocolloid films. Recently, the use of nanoparticles such as mesoporous silica nanoparticles (MSN), has been proposed to reinforce biopolymer-based films because of their ability to reduce the permeability to CO_2, O_2 and water vapor. The aim of this work was to extend the shelf life of strawberries by producing a packaging film made of *Citrus* pectin (PEC), plasticized by GLY and reinforced by MSN. Strawberry (*Fragaria vesca*), rich in vitamin C and antioxidant components, has short postharvest times. PEC, a complex anionic polysaccharide, mainly composed of homogalacturonan, has been used for many years as gelling/thickening agent, and also as stabilizer and emulsifier. Strawberries were wrapped by 2 different films: 1. PEC+MSN+GLY containing films; 2. PEC+GLY containing films. The films were characterized according to seal strength and gas barrier properties. Moreover, strawberry shelf life was evaluated by studying weight loss, pH, titratable acidity, ascorbic acid and antioxidant activity. The texture profile analysis of the fruit was as well performed. The results showed that MSN were able to reduce film barrier properties, whereas seal strength was negatively influenced by the presence of both GLY and MSN. Moreover, we demonstrated that both kinds of the PEC-based films were able to reduce fruit weight loss, and to increase strawberry antioxidant activity, while pH, citric and ascorbic acid, and texture profile analysis were kept constant from 0 up to 8 days at 4°C. PEC based films prepared in the presence of MSN and GLY can be considered as adequate candidates for extending the shelf life of different fruits.

Keywords: *Food packaging, pectin, mesoporous silica nanoparticles, shelf life.*

Acknowledgement *This work was supported by the Italian Ministry of Foreign Affairs and International Cooperation through the "Fourth Executive Program of Scientific and Technological Cooperation between Italy and the United States of Mexico", 2018-2020.*


SLiM 2019
SHELF LIFE INTERNATIONAL MEETING
9° Shelf Life International Meeting
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ISTITUTO PER I POLIMERI COMPOSITI E BIOMATERIALI
Consiglio Nazionale delle Ricerche
Institute of Polymers, Composites and Biomaterials
Dr. Giovanna G. Buonocore

1.3 60th Congress of the Italian Society of Biochemistry and Molecular Biology (SIB).Lecce-Italy, 18-0, September 2019.

SIB 2019

Sustainable hydrocolloid-based bioplastics for obtaining healthier foods

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[1]*DEPARTMENT OF* CHEMICAL SCIENCES, UNIVERSITY OF NAPLES "FEDERICO II", NAPLES, ITALY.
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Replacing petroleum-based plastics is of high interest for the worldwide community in many fields. Recurring to hydrocolloid-based bioplastics is sustainable , because they can be made of components that derive mostly from circular economy processes as wastes or byproducts. In the present work hydrocolloid-based bioplastics have been produced using polysaccharides (pectin, chitosan) or proteins (grass pea and check pea proteins) with and without the aim of transglutaminase (E.C. 2.3.2.13, TGase). This enzyme catalyzes the formation of isopeptide bonds between endo-glutamines and endo-lysines, thus acting as biotechnological tool to reticulate the protein components. In particular, our attention was first devoted to use hydrocolloid-based coatings to protect fried foods (fried potatoes and falafel, a typical Middle Eastern food) from the acrylamide formation during cooking. Fried potatoes were coated by polysaccharide solution (pectin or chitosan) or grass pea flour proteins either not treated or treated by the means of TGase. The latter solution was the more effective since 37% reduction of acrylamide was observed. Falafel, which are made of smashed check peas and spices, were prepared in the absence and in the presence of TGase and then dipped or not in a pectin-based solution. Check pea proteins were proved to act as TGase substrates and the enzyme-dependent reticulation, together with the pectin-based coating, was responsible for 65% reduction of the acrylamide content. Another part of this work has been devoted in preparing pectin-based wrapping material, reinforced with mesoporous silica nanoparticles, to protect the quality of strawberries. Results have proved the effectiveness of these novel bioplastics in extending the shelf life reducing their weight loss and preserving antioxidant activity of this perishable fruits.

2. Contribution: Web Conference

1st Coatings and Interfaces Web Conference. 15-29th March, 2019.

Certificate of Attendance

This certificate of attendance is given to

Asmaa W.M. AL-Asmar

for participating at the

1st Coatings and Interfaces Web Conference

15–29 March 2019

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3. Contribution: Poster Presentation

3.1 59th Congress of the Italian Society of Biochemistry and Molecular Biology (Caserta), 20-22. September, 2017

Hydrocolloid-based coatings to prevent acrylamide formation in fried foods

Asmaa Al-Asmar[1,2], Daniele Naviglio[1], C. Valeria L. Giosafatto[1], and Loredana Mariniello[1*]

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Acrylamide (Acry) is formed when certain foods are prepared at temperatures above 120°C and low moisture, especially carbohydrate-rich foods containing free asparagine and reducing sugars (such as french fries, potato chips, crisps, crispy bread, breakfast cereals, and biscuits). By comparing the data on the cancer-causing potential of Acry to dietary exposure, the EFSA's experts in 2015 concluded that Acry in food is a health concern for consumers. Even if people could escape to Acry exposition not eating such kind of foods, it is well known that fried and baked foods are preferred in many countries and cultures. Thus, it could be useful to find a way to reduce Acry food content to preserve human health. Recently, the use of hydrocolloid materials has been proposed to prevent the Acry formation in some foods, like banana and potato chips. Our group studies edible films and coatings made of hydrocolloids (proteins and/or polysaccharides) using transglutaminase (TG) to modify protein component to influence both mechanical and barrier properties. In fact, TG is able to produce inter- and/or intra-molecular ε-(γ-glutamyl)lysine isopeptide bonds covalently reticulating hydrocolloid edible films. The present study is aimed to assess whether or not edible films affect the formation of Acry in fried potatoes using coating solutions containing TG-modified proteins.

By the means of RP-HPLC, we measured the Acry content of fried samples. In particular, we demonstrate the effectiveness of coatings prepared in the presence of TG which are able to reduce the Acry content in fried potatoes up to 37% in comparison to uncoated samples, being more effective than coatings prepared in the absence of the enzyme. These results may be explained assuming that the protein reticulation produced by TG in the coated foods provides a higher barrier to water during food frying that is responsible for the Acry reduced formation.

Preparation and characterization of grass pea-based bioplastics prepared in the presence of transglutaminase

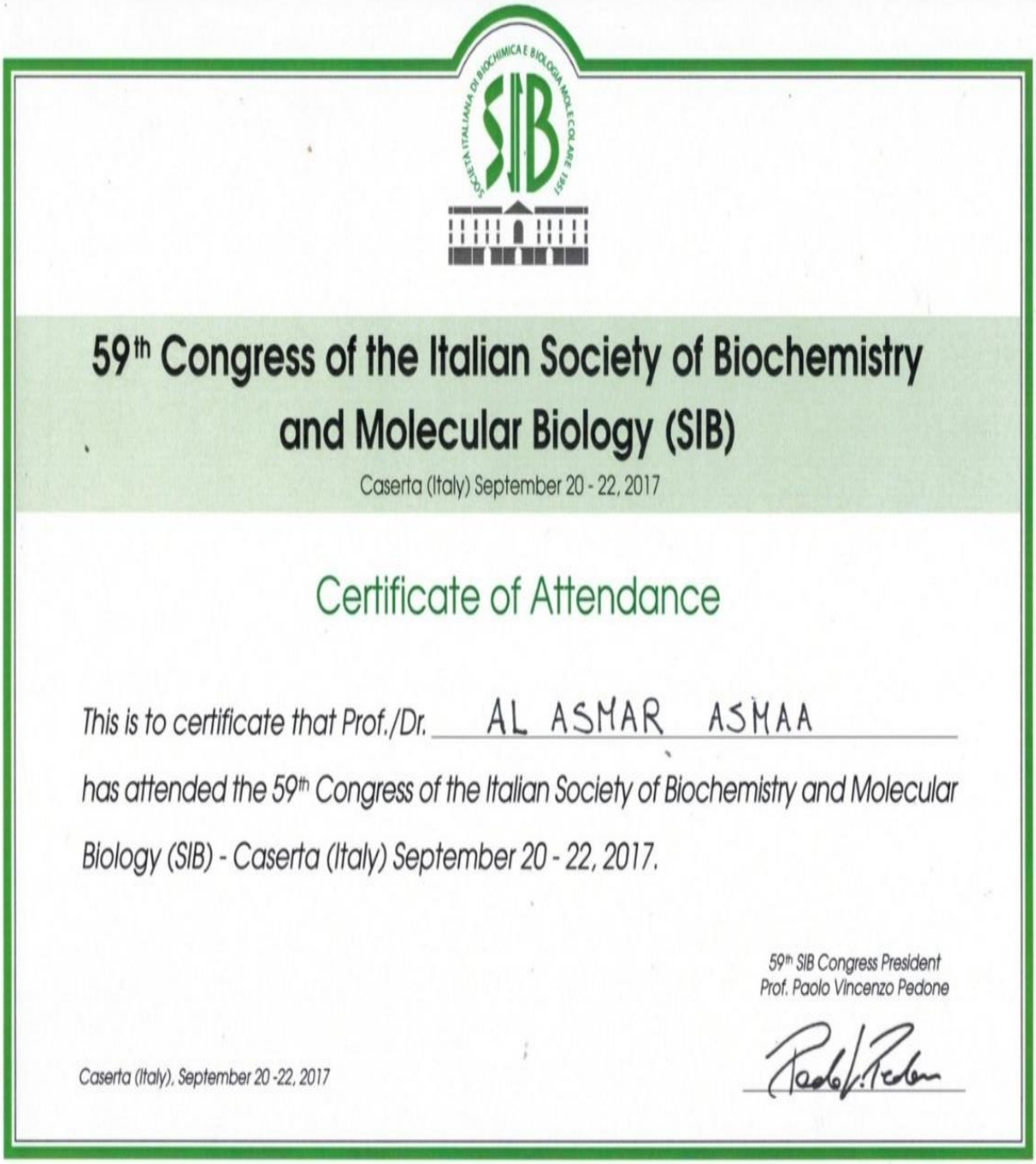
C. Valeria L. Giosafatto[1]*, Valentina Roviello[1], Asmaa Al-Asmar[1,2], Carlos Regalado-Gonzales[3], Antonio D'Angelo[1], and Loredana Mariniello[1]

[1]*Department of Chemical Sciences, University of Naples "Federico II", Naples, Italy.*
[2]*Analysis, Poison control and Calibration Center, An-Najah National University, Nablus, Palestine*
[3]*Departamento de Investigación y Posgrado en Alimentos, Facultad de Química, Universidad Autónoma de Querétaro, C.U., Cerro de la Campana s/n Querétaro, Mexico*
**Corresponding author: giosafat@unina.it*

The aim of this work was to prepare bioplastics from renewable and biodegradable molecules. Bioplastics are alternative to petroleum-based plastics, the latter extremely pollutant since their combustion contributes to the CO_2 enrichment in the atmosphere. In particular, we produced bioplastics by using as biopolymer source the grass pea (*Lathyrus sativus)* flour, the proteins of which were structurally modified by means of microbial transglutaminase (mTG), an enzyme able to catalyze isopeptide bonds between glutamines and lysines. mTG has been widely proposed for improving technological features of several protein-based edible films (1,2). We demonstrated that proteins from grass pea flour are endowed with glutamine and lysine residues able to act as effective acyl donor and acceptor substrates for mTG, as demonstrated by the formation of high molecular weight protein polymers following flour enzymatic treatment. After analyzing the film forming solutions by means of zeta-potential determination, the bioplastics, produced by casting, were characterized according to their mechanical, gas barrier and optical properties. The presence of mTG allowed to obtain films more mechanically resistant. On the other hand, the permeability and optical properties were not affected by the enzyme treatment. The visualization by Scanning Electron Microscopy (SEM) demonstrated that the enzyme-modified films possessed a more compact and homogeneous structure. In addition, digestion experiments under physiological conditions (1), performed in order to obtain information useful for applying these novel biomaterials as carriers in the pharmaceutical sector, indicated that the mTG-treated coatings might allow the delivery of bioactive molecules in the gastro-intestinal tract.

References

1. Giosafatto et al. Carbohydr. Polym. (2014) 106, 200-208.
2. Porta et al. Crit. Rev. Food Sci. Nutr. (2011) 51, 223-238.



59th Congress of the Italian Society of Biochemistry and Molecular Biology (SIB)

Caserta (Italy) September 20 - 22, 2017

Certificate of Attendance

This is to certificate that Prof./Dr. AL ASMAR ASMAA

has attended the 59th Congress of the Italian Society of Biochemistry and Molecular Biology (SIB) - Caserta (Italy) September 20 - 22, 2017.

59th SIB Congress President
Prof. Paolo Vincenzo Pedone

Caserta (Italy), September 20 -22, 2017

3.2 XV FISV Congress ("La Sapienza" University of Rome), 18-21 September. 2018.

P17.5 - Effect of mesoporous silica nanoparticles on the pectin film mechanical properties

Asmaa Al-Asmar[1,2], A. D'Angelo[1], M. Esposito[1], L. Mariniello[1]

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Nowadays, the increasing attention about the plastic pollution and environmental change open a research field devoted to replace traditional plastics by producing biomaterials with promising properties. Hydrocolloids are the main macromolecules naturally available to produce innovative products, i.e. edible films for the food industry. Citrus peel low-methylated pectin was used to prepare the films in the presence of mesoporous silica nanoparticles (MSNs) to contrast limited mechanical and barrier properties of the pectin film. In fact, due to the low toxicity, high surface area and ease of being obtained, MSNs have been used lately both in drug delivery and food packaging. The results reported in the present study show that MSNs reduced significantly the particles size of pectin aqueous solution while they do not affect zeta potential value. Moreover, MSNs slightly increase the tensile strength and decrease significantly the Young modulus. However, glycerol addition provokes elongation at break and film thickness increase, while reducing Young's modulus and tensile strength. Therefore, these films might be considered good eco-friendly candidates to replace traditional plastics.

Federazione Italiana Scienze della Vita

Rome, 21/09/2018

Certificato di Partecipazione

Con la presente si certifica che

Asmaa Sabah/Al-Asmar

ha partecipato al Congresso FISV 2018 (Roma, 18-21 settembre 2018).

Certificate of Attendance

This is to certify that

Asmaa Sabah/Al-Asmar

attended the FISV 2018 Congress (Rome, Italy, 18-21 September 2018).

In fede,

Giovanna Serino
Segretario–Tesoriere

Gennaro Ciliberto
Presidente

3.3 6th international conference of food digestion Granada 2019. Granada-Spain, 2-4 April 2019.

378/82. MICROBIAL TRANSGLUTAMINASE AS EFFECTIVE TOOL TO MODIFY FUNCTIONAL PROPERTIES AND IN VITRO DIGESTIBILITY OF GRASS PEA (LATHYRUS SATIVUS L.) FLOUR

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Introduction

Grass pea is a very popular crop in many Asian and African countries where it is grown either for stockfeed or human consumption. It is characterized by a lot of advantageous biological as well as agronomic features such as resistance to drought, high grain-yielding capacity and high protein content of its seeds. Thus, nowadays, this legume is rightly considered as one of the most promising sources of starch and proteins.

Objective

Our aim was to characterize grass pea flour following treatment with microbial transglutaminase (TG), an enzyme catalyzing intra and/or intermolecular isopeptide bonds between glutamines and lysines into proteins.

Methodology

The impact of the TG modification on microstructure by means of Scanning Electron Microscopy was explored. Protein digestibility was assessed by carring out in vitro digestion experiments following physiological conditions. Finally starch digestibility as well as expected glycemic index (eGI) of grass pea flour was evaluated by using an enzymatic assay kit and by taking into account the rapidly digestible starch and slowly digestible starch, important parameters used also for calculating the eGI.

Main findings

Results demonstrated that grass pea flour proteins act as effective substrate of TG. Microstructural results showed that the addition of TG produced a more compact structure likely due to TG-catalysed heteropolymers. Nutritional properties such as slowly digestible starch and expected glycemic index values followed the order: grass pea flour incubated in the absence of TG> grass pea flour incubated in the presence of TG > raw flour. The TG catalyzed heteropolymers were easily digested as demonstrated by in vitro oral and gastric digestion.

Conclusion

TG-modified grass pea flour can be considered as a new source of starch and proteins, as it possesses nutritional properties that make this legume an inexpensive food source suitable for for feeding a large spectrum of population.

Key words

Estimated glycemic index, food structure, grass pea flour. in vitro digestion, transglutaminase.

Appendix 6.4

1. Member of the Italian Society of Biochemistry (SIB)

SOCIETÀ ITALIANA DI BIOCHIMICA E BIOLOGIA MOLECOLARE
Ente morale dal 1993

To whom it may concern,
I certify that Asmaa W.M Sabah/Al-Asmar is a member of the Italian Society of Biochemistry (SIB) since 2018
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Appendix 6.5

Coauthor of published paper or book chapter during the PhD course

- **Chapter in Book**

6.5.1 Giosafatto C.V.L., **Al-Asmar A**., and Mariniello L. (2018). Transglutaminase protein substrates of food interest. In, Enzymes in Food Technology: improvement and innovation. Springer. pp. 293-317. DOI: 10.1007/978-981-13-1933-4-15.

Transglutaminase Protein Substrates of Food Interest

15

C. Valeria L. Giosafatto, A. Al-Asmar, and L. Mariniello

Abstract

Transglutaminases (TGase, EC 2.3.2.13) are a widely distributed group of enzymes that crosslink proteins through an acyl transfer reaction resulting in a ε-(γ-glutamyl)lysine isopeptide bond. The number of proteins acting as TGase substrate is restricted, since both protein's primary structure and conformation determine whether a glutamine or lysine residue can be reactive. The interest towards these enzymes is stimulated by their involvement in a growing number of human diseases such as celiac disease. Among the TGase isoforms, the microbial one (mTGase) has been object of special attention in the food sector, because of its calcium independency and its broad substrate specificity for the acyl donor. In fact, it was used to strengthen the texture of homogenized sausages made of pork, beef, or poultry meat, to increase the hardness of fishes, to improve the quality of different dairy products, as well as to enhance the elasticity of candies. In addition, the mTGase-catalysed modification of wheat flour proteins increases the elasticity and resilience of dough as well as the volume of bread. The purpose of this chapter is to present an overview of the literature focused on the ability of plant and animal proteins of food interest to act as mTGase substrates.

Keywords

Transglutaminase · Legumes · Fish · Food proteins · Eggs · Meat · Wheat · Digestibility

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15.1 Introduction

Since the last decade, protein enzymatic modification has gained great attention from the food industries, since the enzymes, possessing high substrate specificity, greatly reduce the risk of toxic product formation (Ozrenk 2006). Moreover, although chemical crosslinking reagents are easily available, their use is not advisable because the related formed compounds might be harmful and dangerous specially if applied in the food processing and tissue engineering sectors. As a matter of fact, the use of crosslinking enzymes is increasing, especially in the food field. Among these enzymes, transglutaminases (TGases, E.C. 2.3.2.13) have attracted a wide interest from both scientific and applied points of view. TGases, also named "protein glutamine γ-glutamyl transferases", are ubiquitous enzymes which catalyse post-translational modifications of proteins. They catalyse the crosslinking of proteins via acyl transfer reactions between the γ-carboxamide group of glutamine (Gln) residues and the ε-amino group of lysine (Lys) residues, leading to the formation of intermolecular and intramolecular isopeptide bonds. TGases can also be used in protein modification by the covalent bonding of the compounds containing primary amines. If the amine is bifunctional, the crosslinking of proteins can also occur via N, N′ (γ-glutamyl) polyamine bridges (Cortez et al. 2004). In the absence of co-substrates, the nucleophile may be represented by water, resulting in the deamidation of the glutaminyl residue (Fig. 15.1). The TGase family is made up of nine members (Table 15.1) that are represented by TGase2, also named tissue TGase (tTGase),

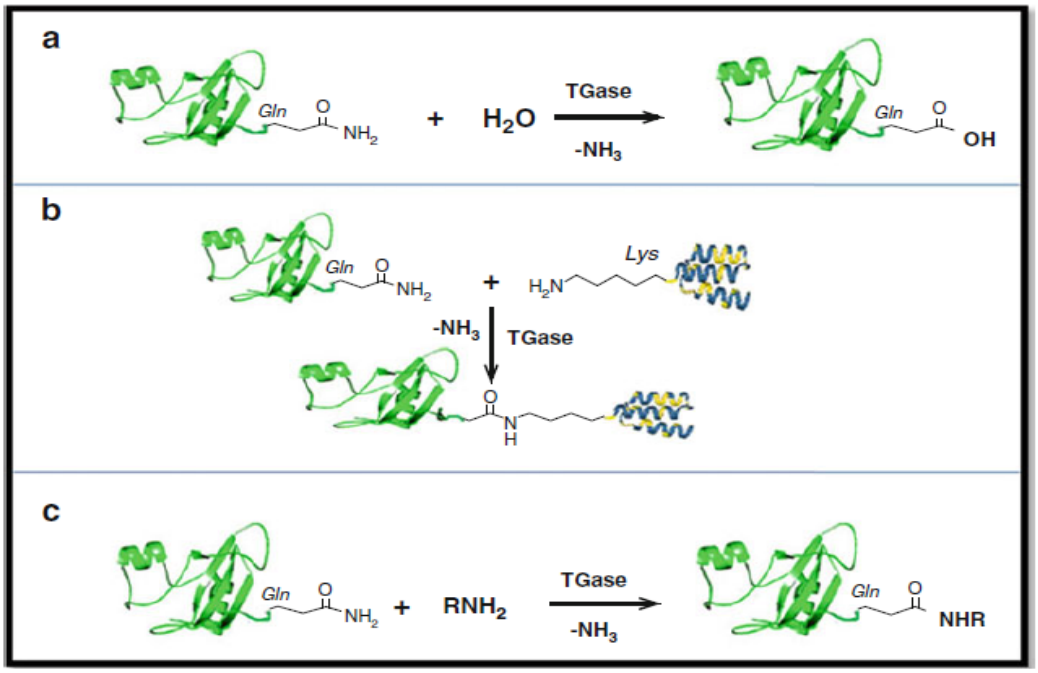

Fig. 15.1 TGase-catalysed reactions. (**a**) Deamidation, (**b**) crosslinking, (**c**) amine incorporation

cytosolic, type II, or liver TGase, TGase1, TGase3, and TGase5 isoforms, expressed mostly in epithelial tissue; TGase4, which is found in the prostate gland; factor XIII (FXIII), expressed in the blood; TGase6 expressed in the testis, lungs, and brain; and TGase7, mainly found in the testis and lungs. Another member of the TGase2 family is represented by band 4.2, an enzymatically inactive protein component of the erythrocyte membrane sharing homology with many TGases. An amino acid substitution (C > A) at its active site (Table 15.1) makes this enzyme to lose its activity. Band 4.2 is distributed on the erythrocyte membranes, bone marrow, foetal liver, and spleen, and it is crucial for maintaining erythrocyte skeletal network and shape as well as their mechanical properties (Table 15.1). Besides in mammalian tissues, over the last decades, TGases were found also in invertebrates, plants, fungi, and microorganisms. For example, in crustaceans TGases are implicated in the plasma clotting reaction as mammalian factor XIIIa. In the horseshoe crab, TGase and its clotting substrate proxin are both distributed in the amoebocytes, and, following its release into the plasma, TGase catalyses the crosslinking of the cell surface proxin to coagulin, whereas in crayfish, TGase located in haemocytes, released upon

Table 15.1 TGase enzymes and their biological function

TGase	Tissue expression	Biological function	References
Factor XIII	Dermal dendritic cells, placenta, plasma, synovial fluid	Blood clotting	Shi and Wang (2017)
		Vascular permeability	
		Optic nerve regeneration	
TGase 1	Keratinocytes, brain	Skin differentiation	Candi et al. (1998)
TGase 2	Ubiquitous	Apoptosis, cell adhesion, signal transduction, coeliac disease	Szondy et al. (2017)
			Facchiano et al. (2006)
TGase 3	Squamous epithelium brain	Cell envelope formation	Odii and Coussons (2014)
TGase 4	Prostate	Semen coagulation in rodents	Odii and Coussons (2014)
TGase 5	Nuclear matrix, cytoskeleton	Epidermal differentiation	Candi et al. (2002)
TGase 6	Testis, lung, brain	Neuropathy, 12 schizophrenia, 15 cerebral palsy	Guan et al. (2013)
			Gadoth et al. (2015)
TGase 7	Ubiquitous but predominantly in the testis and lung	Not known	Odii and Coussons (2014)
Band 4.2	Erythrocyte membranes, bone marrow, spleen	Erythrocyte skeletal network	Odii and Coussons (2014)

activation, is able to crosslink high-density lipoprotein in plasma. A similar clotting activity was also discovered in different shrimp species. A TGase enzyme was also found in *Saccharomyces cerevisiae* which plays important roles in the cell wall organization (Iranzo et al. 2002). Recently El-Hofi et al. (2014) have purified a TGase from rosemary (*Rosmarinus officinalis* L.) leaves at laboratory scale. The authors investigated the biochemical properties of the purified TGase in order to provide a suitable TGase to be applied in the food industry (El-Hofi et al. 2014). As far as TGases from microorganisms, the most characterized ones are those found in *Streptomyces* or *Bacilli*. They have been isolated from *Streptoverticillium* sp. and *Physarum polycephalum* and also detected in *Bacillus subtilis* spores. Moreover, as extracellular molecular form, they have been found in *Streptoverticillium cinnamoneum* subsp., *Streptoverticillium griseocarneum*, *Streptoverticillium ladakanum*, *Streptomyces netropsis*, and *Streptomyces lydicus*. Bacterial enzymes do not need cofactors such as Ca^{2+} or guanosine-5′-triphosphate and are active over a broad range of pH, buffers, and temperatures (Strop 2014). They typically have molecular masses of $\geq$38 kDa and possess high sequence homology with similar substrate specificities for both acyl-donor and alkyl-amine groups (Strop 2014). Moreover, more recently Steffen et al. (2017) discovered a microbial TGase from *Kutzneria albida* (KalbTG) which do not exhibit any cross-reactivity with known microbial TGase substrates. KalbTG, having a molecular mass of 26 kDa, was produced recombinant in *E. coli*. The production of KalbTG in *E. coli* in the presence of its natural inhibitor NH^{4+} enabled the high-throughput screening of substrate peptides by means of peptide array. The high activity and low molecular mass of KalbTG have a key advantage for mass production and enzymatic labelling purposes. Together, these properties make KalbTG a good candidate for a broad range of applications, such as the site-specific conjugation of biomolecules with various label molecules, e.g. production of therapeutic antibody-drug conjugates or chemiluminescent antibodies for in vitro diagnostic purposes (Steffen et al. 2017).

15.2 TGase from *Streptoverticillium mobaraense*

Special attention should be given to a microbial TGase, isolated for the first time in 1989 from a strain of *Streptomyces mobaraensis* (formerly classified as *Streptoverticillium mobaraense*) (Fig. 15.2). This isoform (mTGase) is widely used as a biological glue in many fields devoted to biomedicine, biotechnology, and food sector. The enzyme, easily purified from the culture medium of *S. mobaraense* (Kieliszek and Misiewicz 2014), is a single-chain protein with a low molecular weight of approximately 38 kDa and isoelectric point of 8.9 (Duran et al. 1998; Pasternack et al. 1998; Yokoyama et al. 2004). The active site of mTGase is constituted by the residue of cysteine, histidine, and aspartic acid or asparagine. mTGase possesses peculiar characteristics that make it different from the other isoforms. Contrary to TGases of animal origin, mTGase does not need calcium ions for activation. This feature is particularly desirable in the applicative sector, since the presence of calcium may let the precipitation of the substrate proteins.

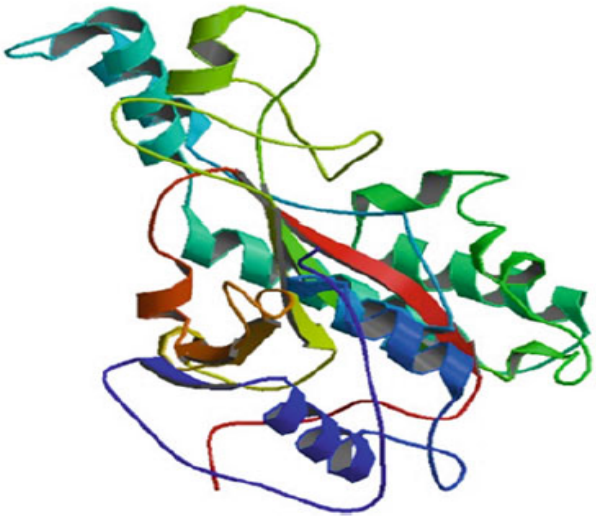

Fig. 15.2 Three-dimensional (3-D) structure of mTGase (PDB: 1 IU4)

mTGase activity is higher in the presence of Co^{2+}, Ba^{2+}, and K^{+} ions, whereas the inhibitors of the activity include Zn^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+} ions, which bind to the thiol group of cysteine found in the active centre (Kieliszek and Misiewicz 2014). In addition, the activity is exhibited at a broad range of temperatures and pHs with an optimal activity at approximately 40 °C and pH of 7–7.5, whereas the enzyme inactivation takes place after 5 min at a temperature above 75 °C. All these properties are important prerequisites for an application of an enzyme in the industrial sector, especially in the food field.

15.3 Food Proteins Acting as mTGase Substrate

The use of mTGase in the food sector is very diffused also because its specificity is very wide both towards acyl-donor and acyl-acceptor substrates. However, if a protein rich in Gln or Lys residues is tested as substrate, not all residues will be involved in TGase-mediated crosslinking. For example, using proteins acting as models such as granulocyte colony-stimulating factor, human growth hormone, and apomyoglobin has been demonstrated that, despite the fact that these three proteins have 17, 13, and 6 Gln residues, respectively, only one or two per protein were able to act as mTGase substrates (Rachel and Pelletier 2013). The authors have shown that such residues do not belong to a special amino acid sequence but to a region not organized in ordered secondary structures, demonstrating that flexibility is a feature of reactive Gln-containing sequences (Mero et al. 2009). Other studies conducted on collagen, α-lactalbumin, and fragment 205–316 of thermolysin (Stachel et al. 2010; Spolaore et al. 2012) have assessed that mTGase is less restrictive towards protein-bound K residues and that the ones located in disordered regions are even more reactive. No matter where or how many isopeptide bonds are formed, mTGase-

Fig. 15.3 Food source proteins able to act as mTGase substrates. In red foods of animal origin, in green foods of plant origin

mediated crosslinking is required in the food industry because it affects food structure (Macierzanka et al. 2011), thus influencing texture and firmness of the foods, as well as bioavailability of protein-derived products (amino acids, peptides). A list of food sources, the proteins of which act as mTGase substrates, is reported in Fig. 15.3, while an overview of the main effects of mTGase in the preparation of a variety of food products is summarized in Fig. 15.4.

15.3.1 Milk Proteins

Caseins represent about 80% of the bovine milk proteins and, together with calcium phosphate, constitute the so-called casein micelles. Caseins can be classified in four different types: α_{S1}-casein, α_{S2}-casein, β-casein, and k-casein. They are known as proteins poor in tertiary structure that do not have a well-defined secondary structure as well. These characteristics are responsible for their flexibility that makes them excellent substrates for TGase. Extensive studies have been carried out to assess their susceptibility towards the enzyme both using raw milk and individual caseins. In both systems the k-casein was found the most crosslinked substrate (Smiddy et al.

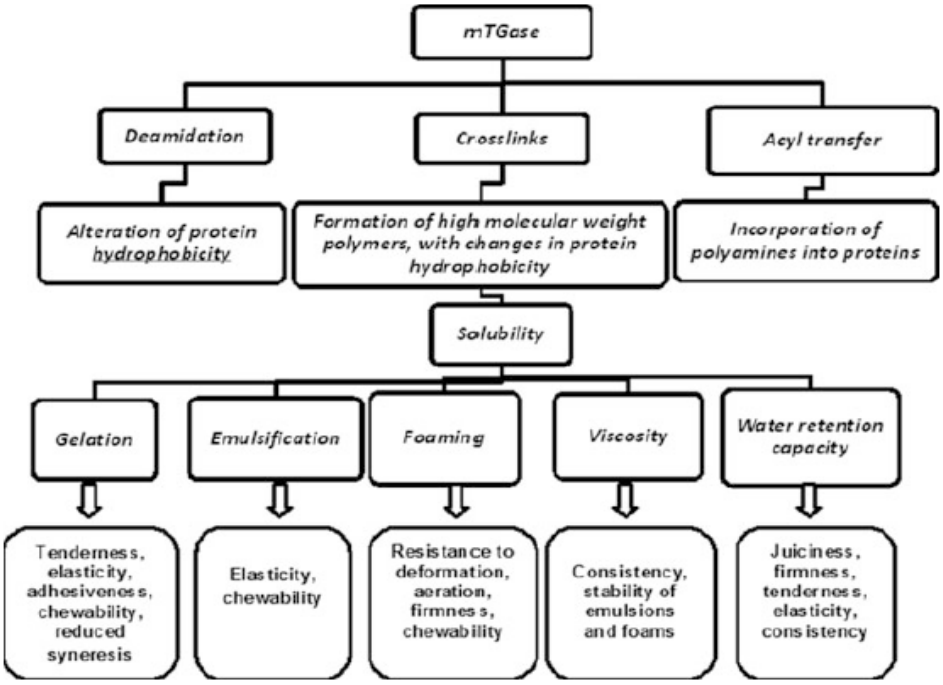

Fig. 15.4 mTGase-mediated reactions and main effects on the functional properties of food proteins. (Modified by Gaspar and de Góes-Favoni 2015)

2006). Even if the exact structure of casein micelles is still under debate (Bhat et al. 2016), the susceptibility of individual caseins can be related to their respective locations within micelles. Since it seems clear that k-casein is located on the surface, it is not surprising that it acts as the best substrate among the other caseins (Dalgleish 2011). Different susceptibility to mTGase is exhibited by whey proteins, in particular β-lactoglobulin and α-lactalbumin. In fact, due to their globular structure stabilized by disulphide bonds, they result to be poor substrates for mTGase in their native states. Enzymatic crosslinking can occur after denaturation promoted by heating, reducing agents, increasing pH, or recurring to high hydrostatic pressure. Denaturation has the effect of exposing internal residues, when the disulphide bonds have been reduced which became more prone to the action of the enzyme. Because of their ability to act as mTGase substrates, milk proteins are widely employed to prepare dairy products, such as cheeses, yoghurts, and other fermented products but also powders and caseinates. Romeih and Walker (2017) have recently published a list of patents where the mTGase has been used to prepare dairy products. The interest, both at research and applied levels, is due to the importance of dairy products at commercial level, since in the Western countries, there is a high demand of this kind of foods. Recurring to the use of mTGase results in higher cheese yield and improves texture and water-holding capacity of soft cheeses (Cozzolino et al. 2003). These properties are highly desired for achieving highest profit for cheese-manufacturing industries. On the contrary, many studies have assessed that the use of the enzyme does not provide better characteristics to hard cheeses because TG

addition prolongs coagulation time and delays ripening process. However, differences have been observed in respect to enzyme addition during the cheese production process. Another problem is represented by the persistence of the enzymatic activity, which may continue to produce protein crosslinks during cheese ripening and storage. In this respect an increased hardness of mTGase-containing cheese after storage at 4 °C has been reported (Özer et al. 2013). Because of this consequence, mTGase enzyme is generally inactivated before cheese making, the most commonly used method being the heat treatment at high temperatures (De Jong et al. 2003). Viscosity is one of the main attributes of several dairy products, like ice creams, being closely tied to their emulsification and gelation (Hong and Chin 2010). In experiments carried out with films made of α_s-casein and β-casein, modified by means of mTGase, it was observed that viscosity increased at the interface by up to 100-fold. This effect was more evident in β-casein films (Faergemand et al. 1999). As also reported by Rodriguez-Nogales (2005), this could be due to the fact that β-caseins, because of its flexible, open, and disordered structure, act as a more accessible substrate for mTGase. In ice creams, mTGase was shown to improve aeration and foam stability. The consistency, a rheological measurement of a fluid viscosity, is influenced by protein polymerization, and, since ice cream displays a pseudoplastic behaviour, fewer crosslinks are required to reach the desired consistency (Rossa et al. 2011, Gharibzahedi et al. 2017). mTGase has been also extensively used in yoghurt production, mainly to prevent syneresis since the enzyme has a positive impact on water-holding capacity of the milk gel (Motoki and Seguro 1998; Motoki and Kumazawa 2000; Kuraishi et al. 2001; Yokoyama et al. 2004; Bönisch et al. 2007; Abou-Soliman et al. 2017). Moreover, Pakseresht et al. (2017) demonstrated that addition of whey proteins to yoghurt, in the presence and in the absence of mTGase, influences firmness, since they obtained cohesively clotted end product of suitable viscosity and consistency without syneresis.

15.3.2 Egg Proteins

Eggs are considered as an affordable and popular source of high-quality proteins and are used extensively in the Western diet. In addition, the functional properties of egg proteins are useful in food preparation (e.g. leavening, emulsifying, and binding) (Bernhisel-Broadbent et al. 1994). It has been found that the proteins from both yolk and albumen of hen egg are able to act as substrate of mTGase (Di Pierro et al. 2007; Giosafatto et al. 2012; Porta et al. 2013; Marcet et al. 2017). In particular, proteins from the yolk have been modified by mTGase and then used to prepare edible films (Marcet et al. 2017). It has been found that when the egg yolk-delipidated proteins were treated with mTGase, the strength of the films was improved in comparison with films made with untreated proteins. Also egg white proteins treated with mTGase were exploited to prepare edible films. Peng et al. (2017) have revealed that the susceptibility of such proteins to mTGase-mediated crosslinking was enhanced by the addition of succinylated casein. Meanwhile, the films with mTGase were more homogeneous and smoother and possessed better water resistance and

thermal stability. Following enzyme treatment, the protein secondary structure changed; in fact in the content of α-helix, β-turn structures were increased, whereas β-sheet structure was decreased (Peng et al. 2017). The spatial conformation and degree of crystallinity of composite protein film were also affected by mTGase (Marcet et al. 2017). Egg white contains 24 different glycoproteins, as determined by crossed immunoelectrophoresis (Langeland 1982). Among them, ovalbumin, ovotransferrin, ovomucin, and lysozyme are the egg white proteins that can be used as functional components (Abeyrathne et al. 2013). In particular, lysozyme that is consisted of a single polypeptide chain with 129 amino acids (Cegielska-Radziejewska et al. 2008) with a molecular mass of 14.4 kDa and pI of 10.7 has been demonstrated to have Gln residues that act as mTGase substrates (Porta et al. 2013). On the other hand, ovalbumin is the most abundant protein present in egg white, predominantly contributing to its functional properties. It has a molecular mass of 45 kDa and pI of 5.4 and consists of 385 amino acid residues, of which nearly 50% are hydrophobic. Ma et al. (2015) reported the modification by means of mTGase of ovalbumin, the main protein of the egg white, and also of all the proteins present in toto in the albumen. They have used high hydrostatic pressure to enhance mTGase crosslinking, even though a substantial amount of monomeric proteins was left after mTGase treatment. The authors have shown that the digestibility of the mTGase-crosslinked proteins did not change significantly and that the enzyme-mediated modification keeps immunostimulatory and immunoreactivity properties of the proteins (Ma et al. 2015). On the other hand, Giosafatto et al. (2012) have successfully modified ovalbumin following heat treatment for 1 h at 80 °C. Analysis of simulated digestion under physiological conditions has demonstrated that the enzyme influences the digestion kinetic of the protein as it is described in the paragraph below. Furthermore, mTGase-treated ovalbumin gel conducted the formation of viscoelastic gel network which possessed higher modulus and lower phase angle values. These properties could suggest to use mTGase-modified ovalbumin as a potential ingredient to enhance viscoelasticity and gel strength. In 2013 Porta et al. have assessed that only the Gln residue (Gln115) occurring in egg white protein, ovomucoid, acts as effective acyl donor for mTGase. Although Gln115 is exposed on the surface of ovomucoid structure (Matsuda et al. 1985), the protein was able to act as substrate of mTG only after heating treatment, able to improve protein flexibility and surface hydrophobicity (Matsumura et al. 1996). Lim et al. (1998) have already demonstrated the importance of heating to promote TGase modification of total proteins extracted from the hen egg white.

15.3.3 Meat Proteins

One of the oldest industrial applications of mTGase in the food sector is the restructuring of meat products. Through the protein crosslink formation, the enzyme provides stability and improves meat mechanical properties, giving the treated product an appearance that is similar to natural muscle (Kuraishi et al. 2001; Barreiro and Seselovsky 2003; Ferreira et al. 2012). In studies using meat from different

origins (beef, poultry, pork), different results were achieved following enzyme-mediated gelation (Trespalacios and Pla 2007; Min and Green 2008; Benjakul et al. 2008; Herrero et al. 2008; Han et al. 2009; Chanarat and Benjakul 2013). This difference is probably due to the following factors: (i) muscle physiology and morphogenesis, (ii) Gln and Lys residues able to act as substrates for the enzyme, and (iii) the presence of possible enzyme inhibitors that may be present in different meats (Benjakul et al. 2008). It has been observed that myosin is modified by mTGase, while actin does not act as a substrate (Benjakul et al. 2008; Chanarat and Benjakul 2013). In their study on meat proteins, Herrero et al. (2008) found that mTGase-mediated crosslinking influenced intensely the structure of myosin heavy chain. In particular, the enzyme promoted reduction in α-helix structure, increased β-sheet, and allowed the formation of high molecular weight polymers. These structural modifications were responsible for stiffness, elasticity, cohesion, and adhesiveness, providing also compact and ordered protein gels. In particular, the rheological properties of pork batter weak gels were markedly improved by the combination of sodium tripolyphosphate and mTGase able to determine red, firm, and non-exudative gels (Lesiow et al. 2017). Furthermore, Hong and Xiong (2012) reported an increase of the solubility of pork myofibrillar proteins treated with mTGase at different pH values. Jira et al. (2017) have studied the influence of the enzyme on the sensory characteristics of a dry-cured formed ham. These authors demonstrated that rising mTGase content resulted in a modest increase in all sensory parameters.

15.3.4 Fish Proteins

mTGase has been widely used to improve textural features, such as elasticity and firmness, of low-quality surimi gel, minimizing food loss during thawing and cooking of frozen fish products (Kuraishi et al. 2001; Cardoso et al. 2010). However, enzyme addition to fish mince enhances gel network strength, and the performed experiments suggest that if setting process is initiated by adding the enzyme, a subsequent controlled heating markedly improves the fish textural characteristics (Saito et al. 2016). Huang et al. (2017) investigated the rheological properties and nanostructure of fish scales gelatin treated with pectin and mTGase. They observed that both have positive effect on the gelation and melting point, as well as on the viscosity and gel properties of the product. In addition, enzyme increasing concentrations result in lower gel strength and hardness due to a much rapid gel network formation (Karim and Bhat 2009). It is worthy to note that the fish restructuring technology allows to use numerous non-commercial fish species as well as to recycle commercial fish trimmings from filleting. Martelo-Vidal et al. (2016) investigated the possibility to obtain a reduced-salt restructured white tuna (*Thunnus alalunga*) and showed that mTGase allows to obtain a reduced-salt restructured fish product with an improved texture. Finally, recent studies of Yuan et al. (2017) demonstrated that mTGase-catalysed glycosylation induces shrimp tropomyosin structural changes and that these modifications result in the reduction

of IgG-/IgE-binding capacity. These findings indicated that the enzyme-mediated alterations in linear and conformational protein structure inactivate epitopes responsible for shrimp potential allergenicity. It deserves to be outlined that, compared with methodologies, such as heating, oxidation, and chemical browning, mTGase-catalysed glycosylation is able to induce site-specific modifications under mild conditions being more suitable in food process.

15.3.5 Wheat Proteins

Despite the mTGase innovative benefits of food production in the mentioned sectors, its industrial use to obtain derivatives of vegetal origin is not yet well exploited (de Góes-Favoni and Bueno, 2014). First Porta et al. (1990) reported the ability of several cereal proteins to act as TGase2 substrates. Among the various dietary proteins tested, they indicated wheat glutelins and gliadins, as well as purified α-gliadin, as the most effective acyl-donor substrates for the enzyme, opening the way to the hypothesis of a possible TGase involvement in etiopathogenesis of coeliac disease (Auricchio et al. 1990; Esposito et al. 2002, 2003). Marked effects of the enzyme on the emulsifying and foaming properties of soy proteins, as well as of chymotrypsin-hydrolysed gluten, were demonstrated (Babiker 2000; Agyare et al. 2009). It is likely that the deamidation of Gln to Glu may lead to an increased number of protein negative charges. The change in the protein hydrophobicity/hydrophilicity was suggested to reduce surface tension and to enhance binding ability to the water, giving the protein greater emulsifying capacity. Moreover, the addition of salt to the system was shown to increase the protein droplet hydrophobicity favouring emulsion coalescence. With regard to emulsion stability, Agyare et al. (2009) evaluated the action of mTGase on gluten, whereas Hong et al. (2012) studied pork myofibrillar proteins with reduced salt content. Both authors observed that the enzyme was able to produce crosslinked polypeptides which were adsorbed on the surface of the oil droplets. Such interaction promoted an electrostatic repulsion, preventing the approximation of the droplets and their flocculation, coalescence, and phase separation, thereby increasing the stability of the emulsion (Agyare et al. 2009; Hong et al. 2012). The ability of proteins to trap water is associated not only with the juiciness and tenderness of ground meat products but also with the desirable textural properties of breads and other gel-type products. Bread making is one of the most ancient food processing techniques, and numerous preparations of commercial enzymes are used in the baking industry in order to improve the quality of the different products. In fact, the biocatalysts are regarded as safe and considered as a valid alternative to the chemical additives (Steffolani et al. 2010). With regard to the water-holding capacity of emulsions and foams, Renzetti et al. (2008) reported that mTGase decreased the baking loss of buckwheat flour and whole rice bread, thus indicating an increase in the protein water-holding capacity. mTGase treatment was shown to have an impact on water-holding capacity also of both whole and shelled bean flour (Romano et al. 2016). Furthermore, Gerrard et al. (2000) observed a positive action of mTGase on yeasted croissant and puff pastry.

The effect of the enzyme was demonstrated to be strictly dependent on its amount and the quality of wheat flour utilized (Basman et al. 2002; Rosell et al. 2003), high levels of TGase resulting in low-volume bread due to high dough resistance, and CO_2 pressure not sufficient to achieve dough expansion. In addition, the isopeptide bonds produced by the enzyme form aggregates different from those derived by S-S bonds, and, as a consequence, the dough has less extensibility and high resistance (Steffolani et al. 2010). Finally, Scarnato et al. (2016) evaluated the effects of mTGase and sourdough on gluten-free flours. Their findings demonstrated that the enzyme is able to modify the gluten-free flour proteins improving their structural network. In addition, mTGase treatment of sourdough affected also the composition of volatile compounds and, as a consequence, the final organoleptic properties of the products. The application of the crosslinking enzyme in gluten-free systems was shown to modify also the viscoelastic properties of the dough, improving the quality and sensory properties of the resulting gluten-free breads (Gujral and Rosell 2004; Song and Shin 2007; Shin et al. 2010; Dlużewska et al. 2015). More recently the cooking qualities, as well as the eating and sensory properties of the whole-wheat noodle, have been the object of investigation following structural changes of the protein network by TGase (Choy et al. 2010; Bellido and Hatcher 2011; Niu et al. 2017).

15.3.6 Legume Proteins

Legumes are an inexpensive source of proteins, starch, dietary fibres, and minerals with a high nutritional profile and, after cereals, the next most important food source for humans (Butt and Batool 2010). Their proteins have been exploited by several authors (Mariniello et al. 2003; Mariniello et al. 2007a, b; Romano et al. 2016) as mTGase substrates with the aim to enhance both their biological features and physico-chemical properties. Hereinafter the major legumes, the proteins of which have been modified by mTGase, are described together with some of the applications that arise by this enzymatic treatment.

15.3.6.1 Soybean

Soybeans (*Glycine max*) are a great source of proteins and are used in various food products such as tofu, milk, cheese, flour, and edamame. Soybean consumption has been associated with the prevention of a few major health conditions, including breast cancer, prostate cancer, cardiovascular disease, menopausal symptoms, and osteoporosis (Moyad et al. 2001). The main soybean proteins are the glycinin, beta-conglycinin, soybean vacuolar protein, and Kunitz trypsin inhibitor. Such proteins, except the trypsin inhibitor, are known as effective substrates of mTGase (Mariniello et al. 2003) being endowed with Gln and Lys residues that act as both acyl donor and acyl acceptor of the enzyme. Glycinin is constituted of six subunits with molecular mass from 58 kDa to 62 kDa, while β-conglycinin possesses α, α1, and β subunits showing molecular weight ranging from 76 to 42 kDa. Mariniello et al. (2003) showed that all these subunits are effective substrates for *Streptoverticillium* TGase

concurring to the formation of high molecular weight species stabilized by isopeptide crosslinks. One of the most popular soybean-based foods is tofu that is widely consumed in Asian countries and vegetarian diets. Tofu has a great nutritional value because of its low-saturated fat and high-protein content (Xu et al. 2015). Moreover, as plant-derived product, tofu does not contain cholesterol; thus, its consumption has increased rapidly worldwide. However, texture is an essential sensory attribute in determining quality and consumer acceptability. Conventional tofu is made by coagulating heated soy milk with a coagulant, followed by moulding and pressing the curd to draw the whey. Various coagulants, such as $CaSO_4$, $MgCl_2$, glucono-delta-lactone (GDL), and mTGase, have been used to prepare tofu (Li et al. 2015). In particular, the use of mTGase leads to produce a *silken* tofu with a smoother and firmer structure (Yasir et al. 2007). In 2016 Hou et al. have used sugar beet pectin and soy protein to prepare the double-network (DN)-modified tofu by sequential laccase and mTGase treatments. The DN-modified tofu exhibited greater mechanical toughness than the corresponding single-network-modified tofu (Hou et al. 2016). Moreover, Gan et al. (2009) have demonstrated that soy protein-based noodles modified by mTGase had stronger texture and, above all, the lowest glycaemic index. The mTGase treatment of soybean proteins regards also the production of edible films. Mariniello et al. (2003) proposed the mTGase-modified proteins as protein component of hydrocolloid-based films, the carbohydrate component of which was represented by pectins from apples. It has been shown that the microbial enzyme was able to have materials with smooth and compact structure also at the cross-section level. Furthermore, the performed mechanical studies have demonstrated that the introduction of covalent isopeptide bonds into the protein framework considerably increased the strength of the films.

15.3.6.2 Pea

The proteins from the pea (*Pisum sativum*) legume also act as mTGase substrate. Djoullah et al. (2015) developed analytical tools capable to qualitatively follow the intramolecular crosslinking during the mTGase treatment applied to pea albumin fraction. In particular, they have used techniques for monitoring the intramolecular mTGase crosslinks of pea proteins, based on protein size determination. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of mTGase-treated low-concentration (0.01% w/w) pea albumin samples, compared to the untreated one (control), showed a higher electrophoretic migration of the major albumin fraction band (26 kDa), reflecting a decrease in protein size. This protein size decrease was confirmed, after DEAE column purification, by dynamic light scattering where the hydrodynamic radius of treated samples appears to be reduced compared to the control one. On the other hand, Lu et al. (2017) have covalently linked biogenic amines (BA) such as histamine (HIS) and tyramine (TYR) to the Q residues of alcalase-hydrolysed pea proteins (PPH). BAs also known as biogenically active amines are a group of low molecular weight organic bases that contain at least one primary amine group. Excessive amounts of BAs in food can cause health risks (Bulushi et al. 2009). By means of the HPLC, they demonstrated that the concentration of BAs in the mTGase-treated samples was

notably reduced. Moreover, the antioxidant activity of the protein extracts following the microbial enzyme treatment was greatly enhanced by reducing lipid peroxidation. These results confirm that TGase could be used to improve the antioxidant status of foods and in conjunction with Q-containing peptides as a tool to reduce the concentration of HIS or TYR in food. For instance, adding Q-rich hydrolysate from pea proteins or also from other food proteins (such as gluten) along with mTGase may have the possibility to decontaminate fermented beverages from BAs (Lu et al. 2017).

15.3.6.3 Common Beans

Common beans (*Phaseolus vulgaris* L.) are consumed worldwide, since they are rich in proteins, starch, unsaturated fatty acids, dietary fibre, and mainly soluble fibre, besides being an excellent source of some minerals (iron and zinc) and vitamins (Villavicencio et al. 2000; Kutos et al. 2003; Romano et al. 2015). Mariniello et al. (2007a) used mTGase as a biotechnological tool to modify phaseolin, the major storage protein found in the cotyledons of *Phaseolus vulgaris*. Phaseolin, structurally very similar to 11S globulins from soybean and other legumes, is a trimeric high-mannose glycosylated protein of about 150 kDa, containing almost identical monomers with a molecular mass ranging from 45 to 51 kDa and isoelectric points from 5.6 to 5.8 (Lawrence et al. 1994). The protein was shown to possess both Gln and Lys residues reactive for mTGase. Native and SDS-PAGE have shown that phaseolin is intra- and intermolecularly crosslinked by mTGase giving rise to different polymers as well as to modified forms of the protein with similar molecular weight but lower Stokes radius than the unmodified phaseolin. mTGase-modified phaseolin was more resistant to proteolytic hydrolysis than the unmodified counterpart, as demonstrated by in vitro trypsin and pepsin digestion experiments. Romano et al. (2016) evaluated the effect of microbial enzyme on the structural, physical, and thermal properties and in vitro digestion of undehulled (WB) and manually dehulled (SB) flour samples obtained from common beans. The scanning electron microscopy (SEM) observations of starch and proteins demonstrated that mTGase affected the structure of flour samples. In fact, in the presence of the enzyme, bean flour constituents are integrated in a more compact structure, because of the presence of mTGase-catalysed heteropolymers. WB and SB flour possessed a darker colour and higher water-holding capacity (WHC) than mTGase-treated samples. mTGase also influenced thermal parameters of flour samples, indicating an increase in resistance of the starch within the granules. In addition, the mTGase through the formation of isopeptide bonds controls also the gastric digestion of the bean flour. In fact, SB flour treated with mTGase was more easy to be hydrolysed by the gastric enzyme (Romano et al. 2016). Wu et al. (2016) provided theoretical basis for adding kidney bean protein as nonmeat protein into meat products. In fact, they investigated the effects of preheated bean protein isolate (KPI) on mTGase-modified myofibrillar protein isolate (MPI). SDS-PAGE displayed disappearance of original bands and the concomitant occurrence of new bands, showing that the microbial enzyme was able to catalyse the crosslinking between MPI and KPI mixtures. The functional properties of preheated KPI/MPI mixtures were significantly improved by mTGase

treatment. The gel hardness and viscosity of chicken meatball were shown to be greatly enhanced with both preheated KPI and the microbial enzyme addition (Wu et al. 2016).

15.3.6.4 Bitter Vetch

Bitter vetch (*Vicia ervilia*, BV) is an ancient grain legume of Mediterranean origins that is present all over the world. This annual *Vicia* genus exhibits positive characteristics, such as having high yields and being resistant to drought and insects. Moreover BV can be considered an inexpensive protein source for forage and seed yield because of its high nutritional value, capacity of nitrogen fixation, and ability to grow in poor soils (Arabestani et al. 2016a, b; Sadeghi et al. 2009). BV exhibits ~~Its~~ an amino acid profile very similar to the one of soybean. Both crops have been used as suitable inexpensive protein source to produce edible films for the food sector. Porta et al. (2015, 2016) have demonstrated that BV protein concentrates are able to be polymerized by mTGase being endowed with Gln and Lys residues that act as both acyl-donor and acyl-acceptor substrates of mTGase. The same authors successfully exploited the use of BV protein concentrate enzymatically modified as protein source to prepare edible coatings/bioplastics both in the absence and in the presence (Porta et al. 2015, 2016) of pectins as carbohydrate source. The presence of the microbial enzyme influenced both the microstructure and the technological features of the BV concentrate-based edible films. In fact, the enzyme gave rise to materials more compact and more homogeneous than the untreated ones. In addition, the novel mTGase-reinforced biomaterials showed improved mechanical and barrier properties towards the gases CO_2, O_2, and H_2O (Porta et al. 2015, 2016).

15.4 Physiological Functionalities Affected by mTGase-Mediated Crosslinking

The wide use of mTGase in the food sector has raised questions about the possible consequences of the formed isopeptide crosslinks on the physiological properties of the end product when consumed by humans such as digestibility, the bioavailability of the K residues incorporated, as well as allergenicity. There are a number of reports suggesting either positive or negative impacts of mTGase-mediated crosslinking on the physiological features of food proteins. Hereinafter we report the main study on the impact of the mTGase-mediated crosslinking on the physiological functionality of food-based proteins.

15.4.1 Digestibility

In relation to the absorption and bioavailability of Gln-Lys crosslinked dipeptides, 99% of these isopeptides are cleaved by the kidney enzyme γ-glutamylamine cyclotransferase into free Lys and 5-oxoproline that subsequently converted into glutamate by the ATP-dependent enzyme 5-oxo-prolinase (Jaros et al. 2006). Since

Lys is an essential amino acid, this pathway could help to satisfy nutritional requirements. On the other hand, the enzyme γ-glutamyl transferase, present mainly in intestinal brush-border membranes, kidneys, and blood (Meister et al. 1981), cleaves the Gln-Lys dipeptide into Glu and Lys (Seguro et al. 1995) without consuming ATP. Kuraishi et al. (1996), in order to evaluate the nutritional value of Lys in the Gln-Lys moiety, have demonstrated that rat fed with crosslinked caseins grew at same rate as those fed with untreated caseins, indicating that the Gln-Lys moiety contained in the crosslinked proteins is cleaved and the resulting Lys is incorporated into proteins (Kuraishi et al. 1996). Monogioudi et al. (2011) reported that mTGase crosslinking of the milk protein β-casein made the protein more resistant to the gastric enzyme digestion, when compared to the uncrosslinked one. Moreover, both gastric and duodenal digestions under simulated physiological conditions were performed to evaluate the effect of mTGase on the proteolysis also of ovalbumin (Giosafatto et al. 2012), the most abundant protein present in egg white. In particular, ovalbumin was modified via mTGase following heat denaturation because heating is able to determine the complete or the partial unfolding of many globular proteins and, consequently, to increase the extent of enzymatic crosslinking. The obtained results indicate that the introduction of isopeptide bonds confer an increased resistance of the protein to be digested in the gastric environment. In addition, the digestion experiments showed that a certain amount of mTGase-catalysed polymers persisted even through the duodenal environment, as demonstrated by the decrease in trypsin and chymotrypsin proteolysis. Further studies were carried out to evaluate the digestibility of legumes following mTGase treatment. The legumes are considered as poor man's meat, being good sources of slow release of both carbohydrates and proteins and are also good sources of minerals and vitamins. In particular, white beans (*Phaseolus vulgaris* L.) are widely grown and consumed in developing as well as developed countries, supplying significant amounts of proteins, starch, unsaturated fatty acids, and dietary fibre, together with iron, zinc, and several vitamins (Kutos et al. 2003). Romano et al. (2016) have shown that the digestion of bean flour was considerably reduced in mTGase-treated samples, likely because of a tighter and compact structure of the matrix obtained through the enzymatic crosslinking. In the same manner, the enzyme-catalysed crosslinking significantly decreased the in vitro digestibility of soy proteins (Tang et al. 2006), especially that determined by pepsin. In addition, Xing et al. (2016) have developed a novel bio-tofu by mixing soy and cow milk, fermented by *Lactobacillus helveticus* and *Lactobacillus plantarum*. They have shown that the enzymatic modification of soy and cow milk proteins led to firmer food matrices digested to a lower extent and with a lower chance to induce food allergy. In all instances, a tight and compact structure of food proteins determined by mTGase-catalysed crosslinking may explain the reduced observed digestion rate. Lastly Popović et al. (2013) demonstrated that the gelation, as well as the solubility properties of the mTGase-modified cucurbitin from pumpkin oil, was improved, thus showing that the functional properties of some proteins could be tailored by mTGase crosslinking to specific needs. It is worth to point out that cucurbitin possesses antihypertensive, antioxidant, anti-inflammatory, and hypocholesterolemic

properties that are all highly desirable for application as food ingredients. In 2017 Popović et al. also demonstrated that mTGase-modified cucurbitin was able to be hydrolysed by the main digestive enzymes, and the crosslinking by mTGase had no significant impact on the bioactive potential of the obtained hydrolysates. In conclusion, even though crosslinked proteins seem to be digested more slowly, the nutritional value and bioavailability of the deriving amino acid residues are similar to the ones contained in untreated proteins. Therefore, these findings suggest the possible development of novel functional foods possessing firmer structures able to be digested to a lower extent and to help in controlling energy intake.

15.4.2 Allergenicity

Porta et al. (2013) demonstrated the ability of the protein ovomucoid to act as mTGase substrate. Ovomucoid is a glycoprotein considered to be the most allergenic protein of the egg white (Urisu et al. 1997). Currently, heat and proteinase treatments are well-known strategies for reducing the allergenicity of food, but such methods are ineffective when applied to ovomucoid because of its heat resistance and ability to act as a trypsin inhibitor (Kato and Matsuda 1997). It has been proven that the Gln 115, the only Gln residue occurring in ovomucoid polypeptide chain, is an effective acyl donor of mTGase as demonstrated by monodansylcadaverine (a synthetic substrate used to identify proteins containing reactive Gln residues) incorporation into the protein. Upon enzymatic modification, the egg white protein reduced its capability of inhibiting the proteolytic activity of trypsin and was also less antigenic than the unmodified counterpart (Porta et al. 2013). In this context, Stanic et al. (2010) reported that polymerization of β-casein using mTGase caused a weak decrease in their IgE-binding potential. Li and Damodaran (2017) have produced, using mTGase, homologous and heterologous crosslinked polymers of whey protein isolate, soy protein isolate, and casein. The authors showed that the IgE reactivity of protein components in heterologous polymers was significantly lower than that in homologous polymers. By in vitro digestion experiments, Li and Damodaran (2017) assessed that both homologous and heterologous polymers were less digestible than the control, proteins not modified by the mean of the enzyme, but the peptides released during the time course of digestion were less IgE reactive, indicating that hypoallergenic protein products could be produced by mTGase-mediated heterologous polymerization of protein mixtures (Li and Damodaran 2017). On the other hand, it was demonstrated (Yang et al. 2017) that only appropriate heat denaturation prior to mTGase-mediated modification could decrease the immunoreactivity of the soy protein glycinin, whereas the digestibility of the protein decreased, and its IgG- and IgE-binding properties were increased because mTGase induced changes in its structure, including the primary structure and spatial structure. Thus, the authors (Yang et al. 2017) concluded that the combination of appropriate processes and mTGase modification may be an effective strategy to reduce the allergenic potential of glycinin. Yuan et al. (2017) reported the modification by means of mTGase of tropomyosin (TM), known as the major allergen of shrimp muscle. In particular TM

is a hydrophilic and heat-stable protein with a molecular weight of 36 kDa (Liu et al. 2010). The proteins contain Gln residues in the six epitopes. It has been demonstrated that these residues can be "glycated" by glucosamine (a natural amino sugar present in the crustacean chitosan) by means of mTGase (Yang et al. 2017). Western blotting and indirect ELISA with TM-specific polyclonal antibodies from rabbit and sera from patients allergic to shrimp demonstrated that antigenicity and potential allergenicity of TM decreased. Hence the authors ended up that mTGase-catalysed glycosylation has the potential to serve as a mild method for reducing the allergenicity of shrimp products (Yang et al. 2017).

15.5 Commercially Available mTGase Preparations

As a matter of the fact, there are different enzymatic preparations of mTGase on the market useful for the processing of different food products. All these preparations contain the enzyme obtained in a biosynthesis-dependent manner via *S. mobaraense*. The most popular one is Activa® that is the brand name of TGase preparations produced under the licence of Ajinomoto Co., Inc., Tokyo, Japan. The Activa® preparations contain stabilizers (such as maltodextrins, caseinates) helpful to increase the thermal stability of the enzyme (Cui et al. 2006) and also to protect it against degradation by extracellular proteolytic enzymes (Junqua et al. 1997). Under these conditions, at temperatures close to 0 °C, mTGase still maintains its total enzymatic activity (Yokoyama et al. 2004). Among Activa®, there are different types according to the specific food product application (http://www.ajinomoto.de/cms/front_content.php?idart=111). The most popular one is Activa® WM, a preparation of maltodextrins and mTGase, that standardizes sausage and cooked ham production. For dairy application Activa® YG, a mTGase preparation containing maltodextrin, lactose, and yeast extract, is specifically designed. In addition, this preparation used for yoghurt applications improves the texture by reducing non-fat solids, helps viscosity with perceived creaminess, and reduces syneresis. It can also increase the yield in cheese and the mouthfeel of frozen desserts. The patented combination of ingredients allows the enzyme preparation to have increased activity in milk systems. Activa® RM is an enzyme preparation that, in addition to maltodextrins, contains also sodium caseinate, and it is formulated for bonding or building texture in a diverse range of food materials such as red meat, poultry, and seafood pieces. It can be used as a pre-hydrated slurry or as a dry powder depending on the application. Activa® STG-MS is a mTGase-based formulation ideal for pasta and noodle applications. It is used to improve texture and deliver increased structure necessary for pasta that is processed under severe conditions such as retort or long cook time. It is also possible to use Activa® STG-MS to reduce the amount of egg whites needed in pasta formations in order to reduce costs. Specifically formulated to withstand the presence of oxygen, Activa® STG-MS can be blended with dry wheat flour and stored for an extended time before use. Activa® KS-TS-MH is designed specifically for use in surimi applications to enhance water retention and improve texture. It utilizes a special technology that allows a desirable accelerated reaction

time in a finished formulation. Activa® GS is a mixture of mTGase, maltodextrin, and gelatin, with the addition of polyphosphate salts and oil. It is an innovative mTGase preparation used for bonding relatively large pieces of meat, such as red meat cuts or salmon fillets. Finally, Activa® BF could be used in ham applications for textural and slicing improvement and non-ham marinade applications.

15.6 Legislations and Labelling for mTGase in Europe and Other Countries

Kuraishi et al. (2001) reported that mTGase is considered by the scientific community as a safe material for human intake. In May 2014, there was a report of "Labelling foodstuffs made with the enzyme transglutaminase" indicating that mTGase is a processing aid and is not an ingredient and under current law shall not be labelled in the list of ingredients. In fact, according to the requirements of Article 6.4 (c) of Directive 2000/13/EC and Article 20 (b) of Regulation (EU) N. 1169/2011 (Food Safety Authority of Ireland 2012, https://www.fsai.ie; Ajinomoto®, 2016, http://transglutaminase.com), food enzymes used as processing aids are not required to be declared in the list of ingredients of finished product. Of course in the case the enzymatic preparation which contains any allergenic sub-ingredients, the food operators are requested to label those ingredients clearly on the final foods. Moreover, mTGase is approved for food use in many other countries other than EU member states, including, but not limited to, the USA, Canada, Brazil, Japan, Korea, China, and Thailand. In the USA it is recognized by the Food and Drug Administration (FDA) as GRAS substance since 1998 (U.S. food and drug administration, 2016).

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- **Papers**

6.5.2 Romano A., Giosafatto C.V.L., **Al-Asmar A**., Aponte M., Masi P., Mariniello L. (2018). Grass pea (*Lathyrus sativus*) flour: microstructure, physico-chemical properties and in vitro digestion. European Food Research and Technology, 245: 191-198.

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ORIGINAL PAPER

Grass pea (*Lathyrus sativus*) flour: microstructure, physico-chemical properties and in vitro digestion

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Abstract
Flour from grass pea, a legume that is adapted to arid conditions containing high levels of proteins, was characterized according to microstructure, some functional properties and digestibility. Microstructural results showed that grass pea starch granules appeared surrounded by an integral matrix with heterogeneous sizes from 6 to 30 µm. Thermal properties displayed a single endothermic transition corresponding to starch gelatinization transition. The digestibility of starch was 79.6%, expressed as the ratio of non-resistant starch to the total amount of resistant and non-resistant starch. The flour was also relatively rich in phenolic substances possessing antioxidant properties as demonstrated by the 2-diphenyl-1-picrylhydrazyl radical method. Adult and elderly in vitro digestion demonstrated that proteins were easily digested. These findings suggest that this legume is suitable for feeding of a large spectrum of population, being endowed with attractive properties that make it potential enough as functional food.

Keywords Grass pea flour · In vitro elderly digestion · Microstructure · Novel foods

Introduction

Grass pea (*Lathyrus sativus*) is a very ancient legume already known in Mesopotamia 8000 years ago. It was introduced in Europe by Greek that gave it the name of "*lathyrus*", while the Romans named it "*cicercula*". For many centuries it has been cultivated, especially in Italy (where many different cultivars were developed), because of its agronomic characteristics. In fact, grass pea adapts to harsh and low-rainfall environments, being resistant to drought and low quality of soil. It is characterized by other advantageous biological and agronomic characteristics such as nitrogen fixation, resistance to insects and pests, high gran-yielding capacity. These features made this crop very popular in subsistence farming in certain developing countries. Nowadays, it is still used as food and feed, but the cultivation is limited at 1.50 million ha worldwide [1]. In fact, after the introduction of processed foods (such as legume in cans), grass pea use has been lowered since beans, lentils, peas, and chickpeas were more prone to be processed at lower costs. Meantime, it was discovered the molecular basis of the neurodegenerative disease named lathyrism occurring only when grass pea seeds are eaten as a large part of the diet and for a long time [2]. In fact, this legume is rich of a non-protein aminoacid β-N-oxalyl-L-α, β-diaminopropionic acid (β-ODAP). When grass pea is a part of a varied diet, β-ODAP is tolerated without any known adverse effect and only overconsumption of the seeds as a staple diet for at least 2–3 months might cause the neurodegenerative disease [3]. Moreover, agronomic studies have assessed that changing some cultivation conditions the content of β-ODAP can be reduced and, thus, this crop could be revaluated for its composition rich in proteins, starch, vitamins (group B vitamins and PP vitamin), fibers, calcium and phosphorous [3]. Nowadays, there is a particular interest in recovering the diffusion of this crop, also to defence biodiversity, the most important aim of Slow Food, an international non-profit

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organization. However, little information is still available on the structure, properties and practical applicability of flour from grass pea seeds. Developments in the industrial sector have increased interest in the identification of non-conventional protein sources with distinct technological and functional properties. Thus, it is necessary that these kinds of crops are studied to obtain structural parameters and information required to gain competitiveness in an international-scale industry. In this work, the flour from an Italian grass pea variety named Serra de' Conti, present in the market as a Slow Food Recommended Product [4], was characterized according to microstructure, and physico-chemical as well as functional properties. Moreover, starch and protein digestibility studies were performed. In particular, protein digestion was carried out under physiological conditions following the in vitro adult and elderly models. In vitro digestion models offer high throughput, robust and practically ethics-free evaluation of the digestive fate of ingested products [5]. Thus, the aim of the present work is to characterize grass pea flour to develop novel grass pea-based foods that might be of interest for special groups of consumers, i.e. vegetarians, vegans, athletes, but also aged population that is increasing in western countries. Hence, it is important to help food manufacturers, scientists and healthcare professionals to generate alimentary solutions that could meet also the needs of geriatrics. So far, few data are available on food protein digestion model that mirrors the physicochemical conditions of the elderly gastrointestinal system [6]. In fact, it is worthy to point out that the elderly population has marked physiological changes in both gastrointestinal secretions and composition of various digestive components, that are important aspects to functionally design foods with tailored functions [7, 8].

Materials and methods

Materials

Grass pea seeds (*Lathyrus sativus*) were purchased by La Bona Usanza (S.C.A.R.L.) as Slow Food Presidia [4]. Plants (Population C3, characterized for the ODAP content [7] were grown in the field in Serra de' Conti Municipality, Ancona Province (central Italy) in the summer 2016. ODAP mean content (g/100 g dry matter) determined by the colorimetric method varied for this population from 0.26 ± 0.01 to 0.53 ± 0.05 [7]. Chemicals for polyacrylamide gel electrophoresis (PAGE) and IPG-strips for two-dimensional (2D) PAGE were from Bio-Rad (Segrate, Milano, Italy). α-amylase (product A1031), pepsin from porcine gastric mucosa (product P6887) and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Chemicals were of analytical grade, unless specified.

Methods

Flour preparation

Grass pea seeds were ground using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the flour would pass through a 425-µm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK). The flour samples were collected and stored in polyethylene bags at 4 °C until used for analysis.

Scanning electron microscopy (SEM) analysis

The microstructure of grass pea flour was analyzed by a Scanning Electron Microscopy (SEM) (LEO EVO 40, Zeiss, Germany) as reported by Romano et al. [8] at a magnification of ×2000. The acceleration voltage was 20 kV and a magnification was of ×2000. Samples were mounted on aluminum stubs and sputter coated with a thin film of gold in an automated critical point drier (model SCD 050, Leica Vienna).

Physico-chemical and compositional parameters

The moisture content of flour was determined by the oven-dry method as reported by the AACC method (number 44–15.02) [9]. 2–3 g of sample was dried for 24 h at 105 °C. The results were expressed as percentage on the dry basis (%). The pH of flour was determined by means of a digital pH meter (MP220, Mettler, Toledo) according to the AACC method (number 02–52.01) [9].

The color of flour was determined as described in Romano et al. [10]. In particular, color was measured by means of a CR 300 tristimulus colorimeter (Minolta Chroma Meter, Milan, Italy) using CIELAB system with the parameters *L*, *a*, and *b* (chromatic coordinates): *L* (lightness $L = 100$ means white; $L = 0$ means black), *a* ($+a$ red; $-a$ green) and *b* ($+b$ yellow; $-b$ blue). The colorimeter was calibrated using a white standard plate ($L = 100/0$ white/black) at the beginning of each session.

The *L*, *a*, and *b* values were used for determination of Chroma using the following equation:

$$\text{Chroma} = \left(a^2 + b^2\right)1/2. \quad (1)$$

Each average value represents the mean of three independent measurements.

Water holding capacity

Water holding capacity (WHC) of the grass pea flour was determined according to the method of D'Appollonia [11] with some modifications as reported by Romano et al. [12].

Thermal properties

The thermal properties of the grass pea flour were analyzed by means of a differential scanning calorimeter (DSC Q200, TA Instruments, Milan, Italy) according to Romano et al. [13]. Samples (accurately 5–6 mg) with deionized water (moisture content 70%) were scanned from 30 to 100 °C at a rate of 10 C/min in a sealed aluminum pan. Thermal transitions of samples for gelatinization were characterized by onset temperature (To), peak temperature (Tp), conclusion temperature (Tc) and the enthalpy (ΔH). The enthalpy calculations were based on flour weight. Each average value represents the mean of three independent measurements.

Determination of resistant starch

The in vitro starch hydrolysis of flour was expressed as the ratio of non-resistant starch to the total amount of resistant and non-resistant starch. Measurement of resistant starch and non-resistant starch was determined using an enzymatic assay kit (Resistant Starch Assay Kit, Megazyme International Ireland) according to rate and extent of in vitro digestion by AACC method (number 32–40.01) [14]. All these results were expressed as percentage weight/weight on dry basis. All measurements were performed in triplicates.

Protein determination

Grass pea protein content was calculated by estimating nitrogen content (Kjeldhal method) [15].

2D-PAGE

Grass pea flour was dissolved in water at a concentration of 1 mg mL^{-1} and 200 µg of proteins, as determined by means of Kjeldhal method [15], was incubated with 125 µL of sample rehydration buffer (Bio-Rad). The second dimension (SDS-PAGE) was carried using acrylamide gels (5% stacking and 12% separating gels) and performed at a current of 200 V for 40 min. At the end of electrophoresis, the gels were stained with Coomassie.

Polyphenol content determination

The polyphenol extraction was carried out according to de Toledo et al. [16] with some modifications. 5 g of flour was mixed with 50 mL of methanol. The mixture was stirred for 24 h at 25 °C. The sample was centrifuged for 15 min at 14,000×g at 4 °C. The pellet was again resuspended in 20 mL of methanol, stirred for 1 h and re-centrifuged. The supernatants coming from the two centrifugations were collected and analyzed. The polyphenol quantification was determined following the procedure described by Kähkönen et al. [17]. Briefly, samples (200 µL) were introduced into test tubes with 1.0 mL of Folin–Ciocalteu's reagent and 0.8 mL of sodium carbonate (7.5%). The tubes were mixed and allowed to stand for 30 min. Absorption at 765 nm was measured (Perkin-Elmer λ25 UV–Vis spectrophotometer, Norwalk, CT). The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram of flour.

Grass pea flour radical scavenging capability using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method

The ability of grass pea flour to scavenge DPPH free radicals was assessed using the method described by Giosafatto et al. [18] with some modifications. Briefly, the flour (5 mg) was dissolved in 100 mL of methanol. After 1, 2, 3, and 24 h, 1 mL of solution was centrifuged (14,000×g for 15 min at 4 °C). Therefore, 100 µL of each supernatant solution was mixed with 900 µL of DPPH methanolic solution (0.05 mg/mL). After 30 min in darkness at RT (25 °C), the absorbance was recorded at 517 nm. The percentage of DPPH free radical quenching activity was determined using the equation reported in Giosafatto et al. [18]. Each sample was assayed at least five times.

In vitro protein digestion models

The simulation of human oral and gastric digestion was carried out following the adult [12, 19] and elderly models [7, 8]. For the adult model, to mimic oral phase digestion, 4 mL of grass pea flour (25 mg mL^{-1}) boiled for 15 min (to simulate the cooking process) was incubated for 2 min at 170 rpm and 37 °C with α-amylase dissolved (150 units mL^{-1}) in 4 mL of simulated salivary fluid (SSF) (0.15 M NaCl, 3 mM $CO(NH_2)_2$, pH 6.9). Therefore, 100 µL of oral samples, the pH of which was adjusted to 2.5 with 6 M HCl, was added to 100 µL of simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5) to start the gastric digestion reaction. The ratio of pepsin to test protein was 20:1 (w/w). After 1, 2, 5, 10, 20, 40, 60 min, 40 µL of 0.5 M ammonium bicarbonate (NH_4HCO_3) was added to each digested sample to stop the pepsin activity. The control was set up by incubating the sample for 60 min without the protease.

As far as the elderly digestion method, we took into account that the geriatric population has marked changes in various gastrointestinal secretions and the composition of various digestive components, starting from

concentrated saliva, through reduced pepsin levels in the stomach [8]. Therefore, the elderly digestion experiments differ from the "adult" ones in the following features:

1. The SSF: sample ratio is equal to 1:2, since the elderly population has a lower salivary secretion [8].
2. Human α-amylase concentration in the oral digestion mix was increased by a factor of 2 [7].
3. Pepsin concentration was decreased by roughly 25% in the gastric mix [8].
4. The stirrer rate is decreased both in the oral phase (65 rpm) and in gastric phase (85 rpm) [8].

At the end of the adult and elderly digestion experiments, 20 µL of each sample was analyzed by SDS-PAGE (4–20%).

SDS-PAGE

5 µL of sample buffer was added to aliquots of 20 µL of each proteolysed sample and analyzed by 4–20% SDS-PAGE, as described by Laemmli [20]. Electrophoresis was performed at 80 V for 2 h, and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

Image analysis

The SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) as described by Romano et al. [12]. Densitometry was performed by calculating the percentage of average intensity of 25-kDa protein normalized to control samples ("C"). Densitometry was carried out on three different sets of experiments.

Results and discussion

Microstructural analysis

Grass pea flour samples were subjected to scanning electron microscopy (SEM). As shown in Fig. 1, flour contains oval and ellipsoid starch granules, varying in shape from ovoid to ellipsoidal. The discernible globular or irregular particles, attached to or located between the starch granules, were the protein bodies or fragments of protein matrix disrupted during milling. Particles might also have included mineral and fiber components, as reported in different legumes by other authors [21].

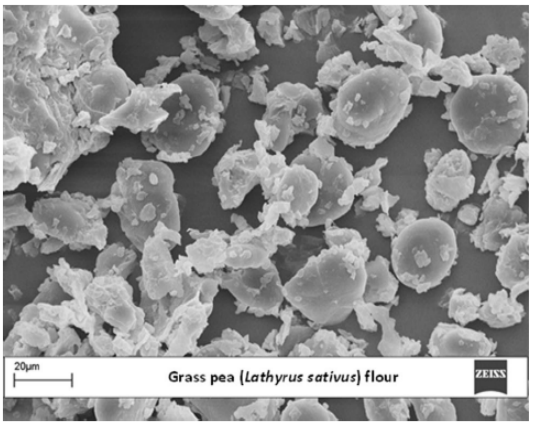

Fig. 1 Scanning electron micrograph of grass pea flour (2000 K)

Table 1 Properties of grass pea flour, expressed as means ± SD

Parameters	
Moisture content (%)	9.84 ± 0.06
pH	6.40 ± 0.01
WHC (%)	175.8 ± 6.8
Color parameters	
Lightness (*L*)	86.63 ± 0.39
Redness (*a*)	− 1.12 ± 0.08
Yellowness (*b*)	19.88 ± 0.78
Chroma	19.91 ± 0.78
Gelatinization parameters	
To (°C)	60.03 ± 0.05
Tp (°C)	68.29 ± 0.82
Tc (°C)	73.29 ± 0.41
Δ*H* (J/g)	1.50 ± 0.05

Physico-chemical, functional and thermal properties

As physico-chemical and functional properties, moisture content, pH, WHC and color parameters were investigated (Table 1). The moisture content of flour samples was of 9.8%. This value falls within the moisture range of 8.2–14.5%, as most flour samples used in the food industries (e.g. for bread making) [22]. The pH of flour was evaluated since pH is used generally to indicate the acidic or alkaline properties of liquid media. The pH was equal to 6.4, significantly higher than those reported for other legumes (e.g. beans), due to the fact that probably grass pea has lower content of tannins, phytic acid, and γ-aminobutyric acid.

WHC of grass pea flour samples was measured and equal to 176%. WHC of flour samples is important for certain product characteristics, such as the moistness of the

product, starch retrogradation, and the subsequent product staling [23]. WHC values for grass pea flour were similar to those given in the literature with respect to leguminous (e.g. beans) [8] and tubers (e.g. ahipa and taro) [24], while they were clearly greater than those given for the soft and hard wheat flours, which were in the range of 50–55 and 62%, respectively [25].

The color parameters (*L, a, b*) recorded for grass pea flour samples are shown in Table 1. The lightness (*L*) grass pea flour values was 86.6 and showed a similar pattern as was observed for ahipa, taro and whole white bean flour samples (*L** equal to 85.4, 81.2 and 86.8, respectively) [26]. The *a* (redness) and *b* (yellowness) values were − 1.1 and 19.9, the latter greater than the other reported leguminous flour samples [27]. The chroma that represents 'richness of color' (color intensity) was 19.9. A high value for lightness (*L*) and a low value for redness (*a*) are desired for the flour to meet the consumer preference. The whiteness of the flour is governed by two independent factors, lightness and yellowness. Lightness is influenced largely by the milling process, particle size and bran content and is correlated with flour ash content [28]. On the other hand, yellowness, principally, is due to the pigments (carotenoids). The seed coat color pattern and the type of cultivar represent an important component influencing the variability of phenolic profiles and levels. In particular, the color depends mostly on condensed tannins (i.e. proanthocyanidins), represented by flavan-3-ol-based biopolymers that possess antioxidant and antiradical activity [16].

Thermal analysis by means of DSC is a valuable tool for studying the impact of thermal processing on vegetable proteins and the phase transition of starch [10, 13, 29]. The thermal properties of samples: To—transition onset temperature, Tp—transition peak temperature, Tc—transition conclusion temperature and ΔH—transition enthalpy were studied by means of DSC and are summarized in Table 1. The To, Tp and Tc values of the grass pea flour were in according to those reported (To 58–69 °C, Tp 64–71 °C, Tc 74–82 °C) by Korus et al. [31]. The mean Δ*H* value of the grass pea flour (1.5 J/g) was instead of a lesser order of magnitude than those reported (12.4–15.3 J/g) for grass pea starches [30]. Lower Δ*H* values obtained for grass pea flour may be due to some degree of starch modification and protein denaturation, which occurred during processing of seed to flour as reported by Henshaw et al. [31] for cowpea flour.

Protein analysis

The grass pea proteins (200 µg) were analyzed by 2D-PAGE, after assessing that the flour has a protein content equal to 27%, in good agreement with data reported by Rosa et al. and Chattopadhyayet al [32, 33]. The proteins were resolved onto 7-cm IPG strips (pH 3–10). As it can be seen in Fig. 2,

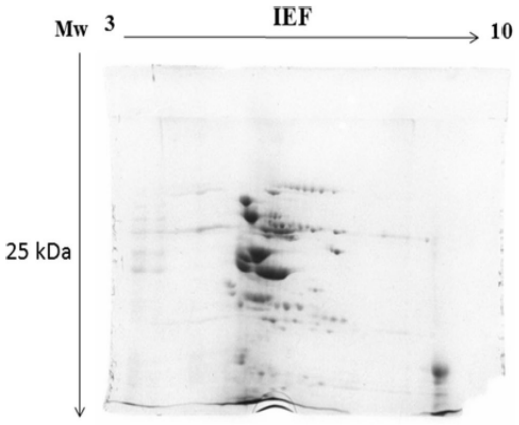

Fig. 2 2D-PAGE analysis of grass pea proteins. Experimental details are given in the text

most of the proteins possess a pI ranging between 4 and 8. These proteins are, namely globulins and albumins, the most abundant also in other legumes [33]. In fact, globulins, comprising > 60% of the seed legume proteins, are composed of many different polypeptides with a wide range of molecular masses. On the contrary, albumins, comprising 30% of the seed proteins, contain a 24-kDa polypeptide that represents more than half of the proteins present in seeds.

Total phenolic content and antioxidant activity

We have also studied the phenolic content and the resulting antioxidant activity of the grass pea flour. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers. In the present study, we have determined the total phenolic content of grass pea flour that yielded average value of 0.511 ± 0.02 mg/g, expressed as gallic acid equivalents (GAE; see Methods), evidencing a relative abundance of these molecules in the grass pea flour. The result was in agreement with those obtained by Desphande et al. [34], that investigated the total phenolic content in 100 lines of *Lathyrus sativus* germplasm. These authors found that the total phenolic content ranged from 0.039 to 0.8 mg/g when assayed by Folin–Ciocalteu method. Total phenolic content contributes to the overall antioxidant activities of plant foods; in fact, natural phenolics exert their beneficial health effects mainly through their antioxidant activity. Typical phenolics that possess antioxidant activity are mainly phenolic acid and flavonoids. Dietary antioxidants may have promising therapeutic potential in delaying the onset as well as in preventing the various life style and age-related diseases and related complications [35]. Measurement of antioxidant activities is one of the primary steps

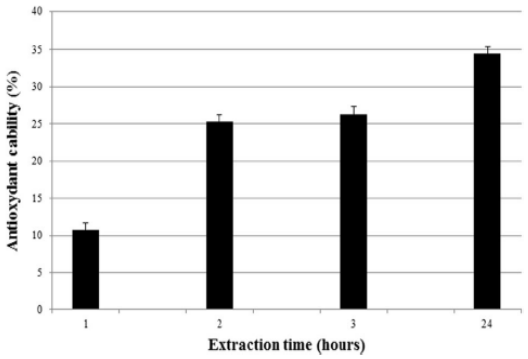

Fig. 3 Antioxidant capability of grass pea flour methanol extracts. The values are expressed as means ± SD. Experimental details are given in the text

towards development of a phytomedicinal profiling of a particular plant, since these compounds act as scavengers of singlet oxygen and free radicals. In this paper, extracts of grass pea flour were investigated for the radical scavenging capacity using DPPH assay that is characterized by a simple and rapid execution and good reproducibility. We have investigated the antioxidant activity after dissolving flour in methanol for 1 h, 2 h, 3 h and 24 h (Fig. 3). The DPPH radical scavenging activity of grass pea flour methanol extracts is given in Fig. 3. This activity was increased by increasing the incubation time with methanol, reaching, after 24 h, an activity equal to 34.4%. This value is comparable to that obtained by Tamburino et al. [36], that reported a value of antioxidant activity equal to 36.3% of a grass pea variety coming from a little town in South Italy.

Determination of resistant starch

The in vitro starch hydrolysis of grass pea samples was determined as the ratio of non-resistant starch (RS) to the total starch (equal to RS plus non-RS) [37]. The starch hydrolysis of raw flour was 79.6% ± 1.6 (mean ± SD), thus, lower than cereal (corn, wheat and rice) and bean (mung and broad) flour samples [38]. The obtained result appears promising for development of novel foods characterized by the slow release of glucose, that is to say low glycemic index and prevention of fasting hypoglycemia. Rapidly digestible starch is, in fact, associated with more rapid elevation of postprandial plasma glucose and insulin; it is generally the less desirable form of dietary starch [38].

Protein digestion under physiological conditions

In this work we have studied the digestibility of grass pea flour proteins following both the adult and elderly model to study the digestion of the flour sample proteins by the human gut. In fact, since the Western population is aging tremendously, it is important to collect more information on food characteristics to generate viable alimentary and pharmaceutical solutions that could help tackle the disorders of ageing. The digestion experimental conditions were set up under physiological conditions taking into account that the geriatric population has marked changes in various gastrointestinal secretions and the composition of various digestive components, such as a more concentrated saliva, reduced pepsin level in the stomach, altered intestinal secretions (i.e. bile and pancreatic secretions) and changes in the colon microbiome [39]. In Fig. 3, it is possible to compare the SDS-PAGE profiles of the grass pea proteins in an adult *versus* elderly model. The proteins seem to be fast digested both in the adult and elderly model, since most of proteins were promptly hydrolysed by the adult gastric environment already after 1 min incubation with pepsin (Fig. 4). Moreover, the proteins have a similar digestion rate (Fig. 4, Panel A) as shown also by densitometry analysis of the electrophoretic protein band having a molecular mass of 25 kDa (Fig. 4, Panel B) where no significant differences were found during the digestion process. Moreover, after 60 min incubation with pepsin, the proteins were completely broken down in both systems (Fig. 4). As a matter of fact, it was not necessary to perform the intestinal digestion.

These results are of a potential interesting interest, since to date no foods and/or oral formulations have been developed to the needs of the elderly. On the other hand, protein with fast digestion rate might be more beneficial than proteins with a slow one to improve postprandial protein gain and consequently to limit the body protein losses typical of elderly subjects.

It is worth to point out that the grass pea seeds, only when eaten continuously for long time, might cause lathyrism [2] due to the presence of β-ODAP, a neurotoxic aminoacid, the toxicity of which is also related to inhibition of mitochondrial complex I [40]. Nevertheless the disease affects generally less than 5% of population, also when the legume has been consumed as the sole dietary source [2, 3]. Moreover, Pratap Rudra et al. [40] have reported that the detoxification of β-ODAP by humans may explain the low incidence of neurolathyrism. Thus, nowadays, this legume might be rightly considered as one of the most promising sources of starch and proteins [3].

Conclusions

The results of this research provide interesting information on the microstructure and properties of the grass pea flour. The in vitro hydrolysis of grass pea starch has been proved to be lower than starch present in cereal and bean flour samples, which indicates that grass pea flour-containing foods

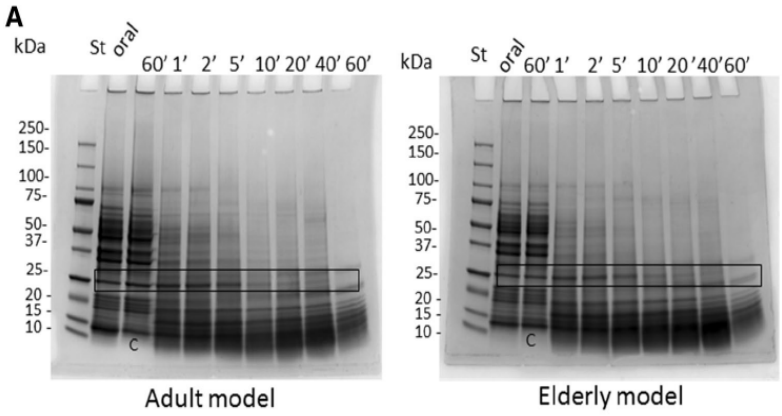

Fig. 4 Panel A-SDS-PAGE analysis of grass pea proteins subjected to oral and gastric in vitro digestion following the adult and elderly model. Lane "c" corresponds to the samples before the digestion. St, molecular weight standards, Bio-Rad. Panel B-Densitometry analysis of 25-kDa protein band (as indicated by the box) following the adult and elderly digestion. Further experimental details are given in the text

B

	Incubation time (min)	C	1	2	5	10	20	40	60
Adult model	Relative quantity (%)	100	92±0.1	78±0.02	63±0.13	37±0.14	24±0.01	25±0.07	0
Elderly model	Relative quantity (%)	100	92±0.01	89±0.01	68±0.10	45±0.01	35±0.02	34±0.01	0

could be of interest to control glycemia. On the other hand, the proteins of grass pea flour are easily digested in both adult and elderly models. All these results suggest that the aim of using grass pea flour to make novel foods for different groups of consumers could be pursued.

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Compliance with ethical standards

Conflict of interest None.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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Preparation and Characterization of Bioplastics from Grass Pea Flour Cast in the Presence of Microbial Transglutaminase

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Abstract: The aim of this work was to prepare bioplastics, from renewable and biodegradable molecules, to be used as edible films. In particular, grass pea (*Lathyrus sativus* L.) flour was used as biopolymer source, the proteins of which were structurally modified by means of microbial transglutaminase, an enzyme able to catalyze isopeptide bonds between glutamines and lysines. We analyzed, by means of Zeta-potential, the flour suspension with the aim to determine which pH is more stable for the production of film-forming solutions. The bioplastics were produced by casting and they were characterized according to several technological properties. Optical analysis demonstrated that films cast in the presence of the microbial enzyme are more transparent compared to the untreated ones. Moreover, the visualization by scanning electron microscopy demonstrated that the enzyme-modified films possessed a more compact and homogeneous structure. Furthermore, the presence of microbial transglutaminase allowed to obtain film more mechanically resistant. Finally, digestion experiments under physiological conditions performed in order to obtain information useful for applying these novel biomaterials as carriers in the industrial field, indicated that the enzyme-treated coatings might allow the delivery of bioactive molecules in the gastro-intestinal tract.

Keywords: grass pea; bioplastics; mechanical properties; transglutaminase; Zeta-potential

1. Introduction

Nowadays life without plastics seems to be unimaginable because of their important role in our society and applications in almost all the areas of daily life, from packaging to food, medical and communication technology to cars. The majority of these plastics are based on very unsustainable fossil resources, causing pollution that affects the entire environment. According to Geyer et al. [1], 8300 million metric tons (Mt) as of virgin plastics have been produced to date and in 2015, approximately 6300 Mt of plastic waste had been generated, around 9% of which had been recycled, 12% was incinerated, and 79% was accumulated in landfills or in the natural environment. In order to reduce pollution from plastics, during the last few decades, researchers have been developing different technologies to produce new kind of biobased plastics and bioplastics that are similar or better than the traditional ones [2–4]. According to European Bioplastic [5], bioplastics are a large family of different materials that are either biobased and/or biodegradable. Among bioplastics, it is worthwhile to talk

about edible films, that are important in the sector of food packaging and represent a potential new highly competitive market [6]. Edible films have received increasing attention mostly because of their advantages as components of food packaging over fossil-fuel materials [3,4]. An edible film is a preformed, thin layer, made of edible material, which can be placed either on or between food components, playing an important role on the conservation, distribution and marketing of foodstuff [7]. Some of its functions consist in protecting food products from mechanical damage, physical, chemical and microbiological activities [6,8,9]. The aim of this work was to prepare and characterize a new kind of hydrocolloid bioplastics, to be used as edible films, based on grass pea (*Lathyrus sativus* L.) flour, a legume from the family of *Fabaceae* [10,11]. Grass pea flour is very profitable because the legume is resistant to both abiotic (dryness, water stagnation and very poor and dry soils) and biotic (high capability to fix atmospheric nitrogen, high seeds and proteins yield) stresses [10]. The films were prepared by using grass pea flour either treated or not treated with microbial transglutaminase (mTGase, E.C. 2.3.2.13), an enzyme easily purified from the culture medium of *Streptoverticillium mobaraense* [12], able to catalyze the crosslinking of proteins via acyl transfer reactions between the γ-carboxamide group of glutamine residues and the ε-amino group of lysine residues, leading to the formation of inter-molecular and intra-molecular isopeptide bonds [13,14]. mTGase is Ca^{2+} independent, and it is active over a broad range of temperatures and pHs with an optimal activity at approximately 40 °C and pH of 7–7.5. These properties are important prerequisites for an application of an enzyme in the industrial sector. The film forming solutions prepared by using grass pea flour modified or not by mTGase have been characterized and the resulting bioplastics investigated according to their transparency, microstructure and mechanical properties. Moreover, digestibility studies carried out under physiological conditions were performed in order to apply such bioplastics in either food or pharmaceutical sector.

2. Materials and Methods

2.1. Materials

Grass pea seeds were bought in a local supermarket (Naples, Italy). Microbial transglutaminase (ACTIVA WM, Ajinomoto, Tokyo, Japan, specific activity 92 U/g) was purchased from Prodotti Gianni S.p.A. Milan, Italy. Glycerol, used as a plasticizer for the preparation of films, was purchased from Sigma (St. Louis, MO, USA). Acrylamide and Blue Brilliant Coomassie were purchased from Bio-Rad (Segrate, Milan, Italy). All other chemical reagents were purchased from the following companies: Amersham Pharmacia (Stockholm, Sweden), Merck (Rome, Italy), Roche (Grenzach-Wyhlen, Germany). The remaining chemicals and solvents used in this study were of analytical grade unless specified.

2.2. Grass Pea Flour Characterization

2.2.1. Protein Content

The amount of proteins was determined by measuring the nitrogen content of the material and multiplying that value by the factor 6.25 [15].

2.2.2. Zeta-Potential and Particle Size of Grass Pea Flour Suspension

The suspension was prepared dissolving the flour in distilled water at concentration of 1 mg mL^{-1}. In order to sediment the starch, the sample was kept overnight at 4 °C. After that, the sample was centrifuged at 10,000 rpm for 5 min at the temperature of 10 °C and the pellet was removed. Before the analysis, the supernatant was further filtrated with 0.45 micron filter and the pH was adjusted to 2 by using HCl 0.1 N. A titration as function of pH (from 2 to 12) was carried out to measure Zeta-potential and particle size of grass pea flour suspension by means of Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK). As titrants we have used 0.01, 0.1 and 1 N NaOH solutions, respectively. All results were analyzed by using the Zetasizer software (version 7.12).

2.3. Film Forming Solutions Preparation and Characterization

2.3.1. mTGase Preparation

The enzyme solution was prepared by dissolving the commercial preparation "Activa" (containing 1% of enzyme and 99% of maltodextrins, specific activity 92 U/g) in distilled water at a concentration of 20 U mL^{-1}. The mixture was stirred for 10 min to allow the solubilization of mTGase preparation.

2.3.2. Film Forming Solution (FFS) Preparation

Flour (41.5 g) was dissolved in 500 mL of distilled water (concentration of 83 mg mL^{-1}) and the stock solution was stirred for 1 h. Afterwards the pH was adjusted from 6.5 to 9 with NaOH 1 N. Then the solution was centrifuged at 10,000 rpm for 10 min at 4 °C and the pellet was removed. The pH of supernatant was adjusted to 7 by adding HCl 1 N and the solution was centrifuged under the same conditions (described above) in order to remove additional aggregates. FFSs without mTGase were prepared by mixing 30 mL withdrawn from solution and mixed with 200 µL (corresponding to 8% of glycerol in respect to protein content) of glycerol (100 mg mL^{-1} w/v) and 19.8 mL of distilled water. FFSs with mTGase were prepared as previously described and by adding 1 mL of mTGase (this amount corresponds to 33 U of enzyme/g of protein). Both FFSs, treated or not with mTGase, were incubated for 2 h at 37 °C. After incubation, the pH of FFSs was adjusted to 9. The final volume of each solution was 50 mL.

2.3.3. Zeta-Potential and Particle Average Size

Zeta-potential, average particle size, and polydispersity index of the FFSs, containing or not mTGase, were analyzed using the Zetasizer Nano-ZSP. Three independent Zeta-potential measurements at pH 9 were carried out on each sample of FFSs (1 mL) introduced in the measurement vessel. Temperature was set up at 25 °C, applied voltage was 200 mV and duration of each analysis was approximately of 10 min. The software calculated mean diameter of particles, determined at pH 9 by using dynamic light scattering, and the polydispersity index, representing the relative variance in the particle size distribution. The device uses a helium-neon laser of 4 mW output power operating at the fixed wavelength of 633 nm (wavelength of laser red emission). All the results were reported as mean ± standard deviation.

2.3.4. Viscosity

Standard Ostwald capillary viscometer was used for the experiments. The viscometer was thermostated to 30.0 ± 0.1 °C in a water bath. The flow time for water was approximately 83.3 ± 0.1 s. Flow times for the FFSs (untreated and treated with mTGase) were measured in duplicate using a stopwatch. Each FFS was diluted 1:2 starting from concentration of flour of 29.3 mg mL^{-1} to 1.83 mg mL^{-1}. Specific viscosity was obtained by using the following equation:

$$\text{Specific Viscosity} = (\text{FFS flow time} - \text{water flow time})/(\text{water flow time}) \quad (1)$$

2.4. Film Preparation and Characterization

2.4.1. Film Casting

FFSs, prepared as described above, were poured in Petri's dishes and placed in a climatic chamber at 25 °C and 45% of R.H. for 48–72 h.

2.4.2. Thickness

Thickness was obtained using a micrometer (Metrocontrol Srl, Casoria, Naples, Italy, mod. H062 with the precision of ± 2 µm). The results were obtained measuring thickness in four random points, then the average and the standard deviation were calculated.

2.4.3. Opacity

The opacity of each samples was investigated reproducing the method used by Shevkani et al. [16]. This method is based on the measurement of absorbance at 600 nm (spectrophotomer UV/Vis SmartSpec 3000 Bio-Rad, Segrate, Milan, Italy) divided by the thickness (mm). All the samples (our bioplastics and commercial material used for references) were cut into pieces of 1 cm × 3 cm and they were let adhere perfectly to the wall of the cuvette.

2.4.4. Scanning Electron Microscopy (SEM)

SEM analysis of both surface and cross-section of grass pea flour-based films was carried out by using field emission scanning electron microscope (Nova NanoSem 450-FEI-Thermo Fisher, Scientific, Waltham, MA, USA). Briefly, the samples were placed on an aluminum stub by using a graphite adhesive tape. A thin coat of gold and palladium was sputtered at a current of 20 mA for 90 s. The sputter-coated samples were then introduced into the specimen chamber and the images were acquired at an accelerating voltage of 3 kV, (4.4–5.2) mm working distance, through the Everhart Thornley Detector (ETD, 450-FEI-Thermo Fisher, Scientific, Waltham, MA, USA). Two different samples of each type of films were subjected to SEM and four micrographs of each sample were taken. Micrographs of surfaces and cross-sections were obtained taking parts at 2600× magnification of the samples.

2.4.5. Mechanical Properties

Film tensile strength, elongation at break and Young's modulus were determined by using an Instron Universal Testing Instrument (model no. 5543A, Instron Engineering Corp., Norwood, MA, USA). Film sample strips (1 cm wide and 5 cm long), obtained by using a sharp razor blade, were equilibrated for 2 h at 50% RH and 25 °C in an environmental chamber, and four samples of each film type were tested. Tensile properties were measured according to the ASTM D882-97 [17]. The initial grip separation was 40 mm, and the crosshead speed was 5 mm min^{-1} in tension mode. The acquisition and elaboration of the data were made by the using the software BlueHill 2.21.

2.4.6. In Vitro Film Digestion

The films prepared in the absence and in the presence of mTGase were subjected to a three-stage in vitro digestion by using adult model [18–20], under simulated oral, gastric and duodenal physiological conditions. For our analyses, 5 mg of each type of films were incubated in 600 µL of Simulated Salivary Fluid (SSF, 150 mM of NaCl, 3 mM of urea, pH 6.9) for 5 min at 170 rpm. Afterwards the samples were subjected to gastric and duodenal digestion as described by Giosafatto et al. [18] with some modifications. Briefly, aliquots (100 µL) of Simulated Gastric Fluid (SGF, 0.15 M of NaCl, pH 2.5) were placed in 1.5 mL microcentrifuge tubes and incubated at 37 °C. 75 µL of films dissolved in SSF, the pH of which was adjusted to 2.5 with HCl 6 M, were added together with pepsin (1:20 *w*/*w* respect to grass pea protein content) to each of the SGF vials to start the digestion reaction. At intervals of 1, 2, 5, 10, 20, 40, 60 min, 40 µL of 0.5 M of ammonium bicarbonate (NH_4HCO_3) were added to each vial to stop the pepsin reaction. The control was set up by incubating the sample for 60 min without the protease. Duodenal digestions were performed using, as the starting material, the gastric digests after 60 min, adjusted to pH 6.5 with 0.5 M Bis-Tris HCl pH 6.5. Bile salts (sodium taurocholate and sodium glycodeoxycholate) dissolved in Simulated Duodenal Fluid (SDF, 0.15 M of NaCl at pH 6.5) were added to a final concentration of 4 mM. After equilibrating at 37 °C for 10 min, trypsin, chymotrypsin (the ratio of trypsin and chymotrypsin with test proteins was 1:400 (*w*/*w*) and 1:100 (*w*/*w*), respectively) were added to the duodenal mix. Aliquots were removed over the 120 min digestion time course and proteolysis was stopped by addition of a two-fold excess of soybean Bowmann-Birk trypsin-chymotrypsin inhibitor above that calculated to inhibit trypsin and chymotrypsinin of the digestion mix. The control was carried out by incubating the sample without

the proteases for 120 min. The samples were then analyzed using the SDS-PAGE (12%) procedure described below.

2.4.7. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For SDS-PAGE of FFSs, an aliquot of 5 µL of sample buffer (15 mM of Tris–HCl, pH 6.8, containing 0.5% (*w*/*v*) of SDS, 2.5% (*v*/*v*) of glycerol, 200 mM of β-mercaptoethanol, and 0.003% (*w*/*v*) of bromophenol blue) were added to aliquots of 20 µL of FFS (either untreated or mTGase treated) and analyzed by 12% SDS-PAGE. The SDS-PAGE of cast films was carried out by dissolving 20 mg of each film in 250 µL of sample buffer. The samples were treated at 100 °C for 5 min, and then centrifuged for 10 min at 13000 ×g. Three µL of each supernatant were analyzed by SDS-PAGE (12%). For the analysis of film digestion carried out under physiological conditions, 5 µL of sample buffer were added to 20 µL of each protolyzed film sample and analyzed by 12% SDS-PAGE.

In all cases SDS-PAGE was performed as described by Laemmli [21], at constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250 (Bio-Rad, Segrate, Milan, Italy). Bio-Rad Precision Protein Standards were used as molecular weight markers.

2.4.8. Densitometry Analysis

Densitometry analysis was carried out by means of Image Lab software (version 5.2.1) from Bio-Rad Laboratories. Each SDS-PAGE image was analyzed by detecting all the lanes and protein bands. Protein bands, possessing a relative molecular mass (*Mr*) of 50 kDa were used to determine the band intensity of film digested in the absence of mTGase respect to the control carried out without proteases. Protein bands >250 kDa were used to determine the band intensity of film digested in the presence of the microbial enzyme with respect to control incubated without proteolytic enzymes.

2.5. Statistical Analysis

All data were analyzed by means of JMP software 5.0 (SAS Institute, Cary, NC, USA), used for all statistical analyses. The data were subjected to analysis of variance, and the means were compared using the Tukey-Kramer HSD test. Differences were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1. Stability of Grass Pea Flour Suspension and FFSs

In order to evaluate the pH stability of grass pea flour dissolved in water at a concentration of 1 mg mL^{-1}, a titration as function of pH was carried out to measure Zeta-potential. The charge of particles depends on the solvent used [22]. Zeta-potential is a function of the surface charge of the particle, of adsorbed layer at the interface, and of the nature and composition of the surrounding suspension medium. Generally, Zeta-potential values higher than ±25 mV indicate that the solution is quite stable [22]. The data reported in Figure 1 show a moderate stability of grass pea flour suspension, in fact, the potential changes from +27 to –25 mV by varying the pH from 2 to 12. At pH 4, the suspension became unstable (0.01 ± 0.53 mV) since this pH is close to isoelectric point of grass pea proteins (globulins and albumins), which are in the range of 4–6, as also demonstrated by Romano et al. by performing two-dimensional gel electrophoresis [23]. Also, the dimension of particles was quite stable (data not shown) during the titration, being the main particle size diameter equal to roughly 200 nm of diameter for all the pHs analyzed (data not shown).

FFSs were prepared, both in the presence and the absence of mTGase, at pH 9, since, as reported in Figure 1, we have an acceptable stability at this pH (Zeta-potential = −25 mV). After the preparation, 1 mL of each solution was analyzed at Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) to confirm the stability.

In Table 1 results about average size, polydispersity index and Zeta-potential of FFSs are reported. The solutions possess a similar Zeta-potential, regardless the presence of mTGase. The average size

seems to be slightly reduced in the FFS prepared in the presence of the enzyme as already reported by Porta et al. [8]. It is important to note that polydispersity index is around 0.5 indicating that the size of particles is quite uniform in both the systems.

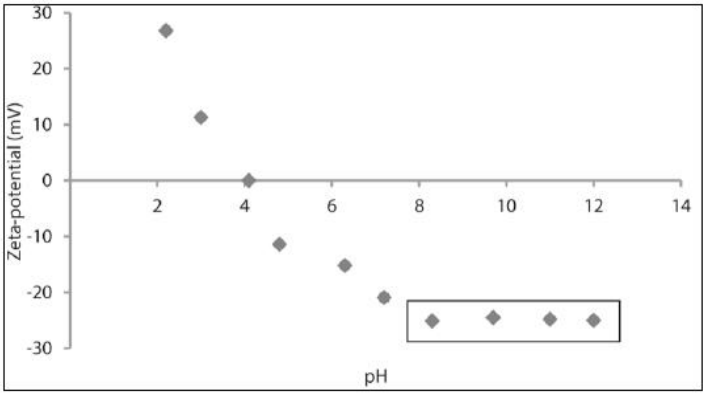

Figure 1. Zeta-potential of grass pea flour suspension as function of pH. Values in the frame represent the Zeta-potential range of stability.

Table 1. Average size, polydispersity index and Zeta-potential of FFSs treated or not by mTGase.

Sample pH 9	Average Size (d/nm)	Polydispersity Index	Zeta-Potential (mV)
FFS	139.40 ± 1.06 [a]	0.53 ± 0.01 [a]	−27.10 ± 1.90 [a]
FFS + mTGase	127.30 ± 2.50 [a]	0.57 ± 0.02 [a]	−28.00 ± 1.63 [a]

Values are mean ± standard deviation; Means followed by the same letters are not significant different (Tukey-Kramer test, $p < 0.05$).

3.2. Modification of Grass Pea Flour Proteins by Means of mTGase

Both FFSs and cast films were analyzed by means of SDS-PAGE (12%). The Figure 2 demonstrated that mTGase was able, under these experimental conditions, to modify grass pea proteins. In fact, from the gel (Figure 2) it is possible to note the formation of *Mr* polymers and the concomitant disappearance of lower *Mr* protein bands in the sample treated with mTGase both in FFSs (Figure 2A) and the solubilized films (Figure 2B), indicating that the mTGase-catalyzed reaction occurs also in the casting system. This result was also supported by viscosity analysis that demonstrated that FFS treated with mTGase has a higher viscosity than the one untreated (Supplementary Materials). An increase of viscosity is due to mTGase activity that, by forming intra and intermolecular ε-N-(γ-glutamyl)-lysine crosslinks between proteins, reinforces the network. These results are in good agreement with those obtained by Nio et al. [24], and Temiz et al. [25] that studied the gelation of casein and soybean globulins by mTGase, demonstrating that the enzyme treatment increases the viscosity of solution.

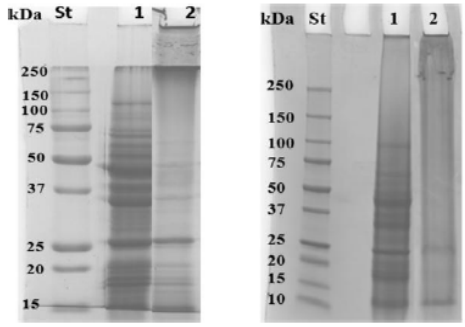

Figure 2. Panel A-SDS-PAGE of untreated (lane 1) and mTGase-treated (lane 2) FFSs. Panel B-SDS-PAGE of solubilized films cast in the absence (lane 1) and presence (lane 2) of mTGase. St, Molecular weight standards, Bio-Rad. (**A**) FFSs; (**B**) FILMS.

3.3. Opacity

As shown in Table 2, grass pea-based films, cast in the absence of mTGase, possess an opacity value of 7.74 ± 0.26 A_{600nm}/mm that is similar to the ones obtained by Shevkani et al. [16] which studied hydrocolloid edible films made up of proteins from bean (*Phaseulus vulgaris*) and pea (*Pisum sativum*). mTGase-treated films have a opacity value (4.04 ± 0.06 A_{600nm}/mm) that is statistically lower ($p < 0.05$) than the ones exhibited by grass pea-based films. The opacity was also determined in traditional commercial plastics such as cellulose triacetate (CTA) and polypropylen (PP5). As expected CTA, glossy plastic sheets used for projecting, appeared very transparent (0.53 ± 0.08 A_{600nm}/mm), whereas PP5, normally used for bakery products, macroscopically opaque, showed an opacity value equal to 32.02 ± 3.35 A_{600nm}/mm.

Table 2. Opacity of grass pea flour film cast with and without mTGase, compared to commercial plastics.

Film Features	Thickness (mm)	Opacity (mm^{-1})
Grass Pea-Based Films	0.084 ± 0.005 ^{b}	7.74 ± 0.26 ^{b}
Grass Pea-Based Films + mTGase	0.12 ± 0.02 ^{a}	4.04 ± 0.06 ^{c}
Kidney Bean-Based Films *	0.064 ± 0.002	8.9 ± 0.3
Field Pea-Based Film *	0.064 ± 0.002	7.3 ± 0.3
CTA	0.131 ± 0.001 ^{a}	0.54 ± 0.09 ^{d}
PP5	0.054 ± 0.003 ^{c}	32.02 ± 3.35 ^{a}

Values are mean ± standard deviation; Means followed by the same letters are not statistically different (Tukey-Kramer test, $p < 0.05$); * Data from Shevkani et al. [16]; CTA, cellulose triacetate; PP5, polypropylene.

3.4. Scanning Electron Microscopy (SEM)

The film both cast in the presence and absence of mTGase macroscopically appear quite handleable and flexible with a homogeneous structure. Figure 3 shows the SEM images of untreated and mTGase-treated bioplastics. As it is possible to see from Figure 3A, the surface of film cast in the absence of mTGase has a very heterogeneous structure with a high grade of roughness and deep cracks. On the other hand, film surface of films treated with mTGase appears smoother and homogeneous. This observation can be better appreciated in the cross sections of the films, shown in Figure 3B, where the untreated film is highly wrinkled, appearing not compact; instead in the presence of mTGase the film sections appear more homogeneous and uniform, with less cracks. These results reflect those obtained by Giosafatto et al. [3] and Mariniello et al. [26] that state that mTGase treatment confers a smoother and compact structure in pectin and phaseolin-based films.

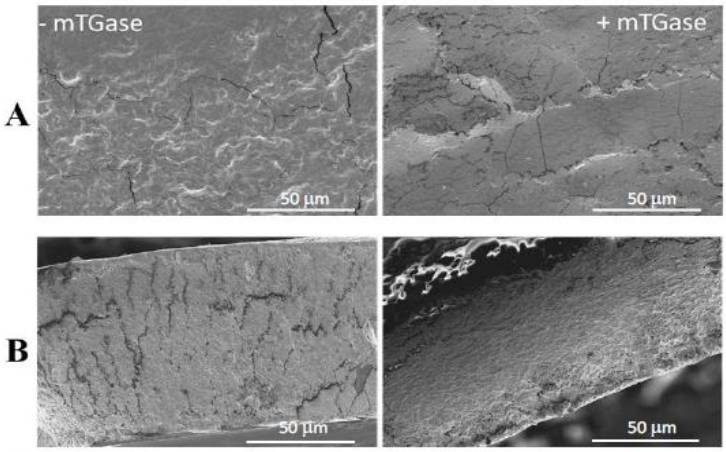

Figure 3. SEM micrographs of surface (**A**) and cross-sections (**B**) at 2600× magnification of grass pea flour-based films prepared in the absence and the presence of mTGase.

3.5. Oral, Gastric and Duodenal in Vitro Digestion of Grass Pea Flour-Based Edible Films

Gastric and duodenal digestion experiments were performed under physiological conditions in order to study the possible digestion of the films by the human gut [3,18]. As it is possible to note from SDS-PAGE (12%) shown in Figure 4A unmodified proteins are more susceptible to be digested in the gastric environment than the mTGase-crosslinked ones (Figure 4B). In fact, low *Mr* proteins occurred only following the pepsin hydrolysis of untreated grass pea proteins; on the other hand, the mTGase-catalyzed polymers seemed quite resistant and stable even after 60 min of incubation with pepsin (Figure 4B). In fact, densitometry analysis showed (lower part of Figure 4B) that mTGase-modified forms start being digested only after 20 min incubation with pepsin, and about 76% of these polymers were still present following 60 min incubation in comparison to the band intensity of control (lower part of Figure 4B), whereas the undigested proteins represented only the 36% in the samples that were not subjected to mTGase-mediated modification (lower part of Figure 4A).

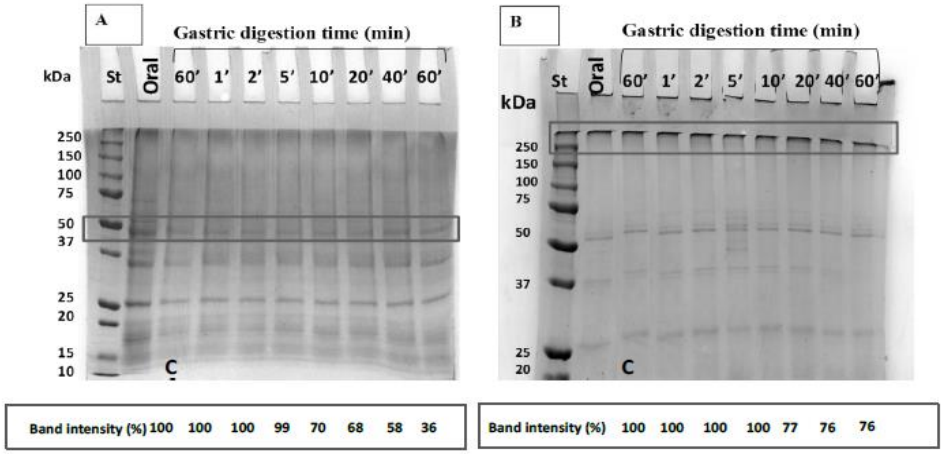

Figure 4. (**A**) Oral and gastric in vitro digestion and densitometry analysis of 50 kDa protein bands of grass pea film cast without mTGase; (**B**) Oral and gastric in vitro digestion and densitometry analysis of protein bands of >250 kDa of grass pea film cast in the presence of mTGase (33 U/g). C is control sample incubated without pepsin. St, Molecular weight standards, Bio-Rad.

The samples obtained after 60 min of pepsin digestion were further processed by recurring to trypsin and chymotrypsin, with the aim of mimicking duodenal digestion (Figure 5). We found that both unmodified (Figure 5A) and mTGase-modified (Figure 5B) were more difficult to be digested, even though, once again, the samples incubated in the absence of the crosslinking enzyme appeared more prone to be hydrolyzed by the intestinal enzymes. mTGase-derived polymers are gradually digested and after 120 min incubation (lower part of Figure 5B) with trypsin and chymotrypsin, 61% of unbroken polymers are still detectable. On the contrary, densitometry analysis of residual intact 50 kDa protein present in the unmodified grass pea flour indicated that 41% of protein was observed still intact following 120 min digestion with trypsin and chymotrypsin (lower part of Figure 5A). These results clearly indicate that the TGase-mediated intra- and inter-molecular crosslinks confer resistance to gastric and duodenal digestion as demonstrated by other proteins when modified by mTGase [18,27]. These characteristics make such materials usable as scaffolds for the incorporation of active molecules to be delivered in the intestinal tract.

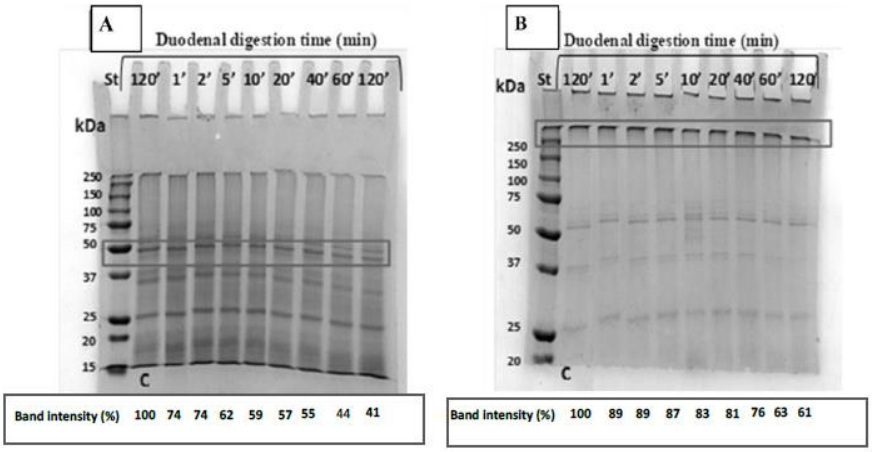

Figure 5. (**A**) Duodenal in vitro digestion and densitometry analysis of 50 kDa protein bands of grass pea film cast without mTGase; (**B**) Duodenal in vitro digestion and densitometry analysis of protein bands of >250 kDa) of grass pea film cast in the presence of mTGase (33 U/g). SDS-PAGE 12%. Molecular weight standard, Bio-Rad. C is control sample incubated without chymotrypsin and trypsin. St, Molecular weight standards, Bio-Rad.

3.6. Mechanical Properties

Tensile strength (TS), Elongation to break (EB) and Young's Modulus (YM) are shown in Table 3. As it possible to see, TS of grass pea flour-based film mTGase-untreated is lower than the one treated with mTGase. These results are in agreement with data reported by our research group [6]. The mTGase induces an increasing of TS because of the occurrence of the mTGase-catalyzed isopeptide bonds within film matrix [28–31]. Also, EB is higher for grass pea flour-based film treated with mTGase than the one performed by untreated sample. It has been reported that deamidated gluten films crosslinked by mTGase showed a gaining of EB likely due to the formation of covalent linkages by mTGase which confers more flexibility [31]. These results are also in agreement with the ones obtained by Mariniello et al. [32], and Tang et al. [33], who suggest that there is a development of a more compact and more elastic film structure after the mTGase treatment. YM data show that the films cast in the absence of mTGase are more rigid than the ones cast with mTGase, the latter possessing lower values of YM. The results reflect those reported from Porta et al. [6], that studied bitter vetch protein concentrate (BVPC) films treated or not with mTGase and affirmed that a treatment with the microbial enzyme induces an increase of resistance and a reduction of stiffness (Table 3). Moreover, from Table 3 it is possible to compare mechanical properties of grass pea flour based-films with those

performed by Viscofan® and Mater Bi® [34] plastics, already available on the market and based on natural molecules. In particular, Viscofan® is obtained from collagen, cellulose and fiber-reinforced cellulose [35], whereas Mater Bi® is made up of corn starch mixed with some vegetal oils [36] in order to improve the technological features. Viscofan® has a higher value of TS and YM (Table 3) than our bioplastics prepared both in the presence and the absence of mTGase, demonstrating that this bioplastic is more mechanically resistant but more rigid than our bioplastics.

On the other hand, EB (Table 3) performed by Viscofan® is lower than that one performed by grass pea flour based-film, indicating that the latter is more extensible than the commercial bioplastic. As far as Mater Bi® is concerned, it is possible to note again that the grass pea flour-based bioplastics are less resistant, less stiff and less extensible then the starch-based one (Table 3).

Table 3. Mechanical properties of films cast in the presence and the absence of mTGase compared to commercial plastics.

Film Type	TS (MPa) Resistance	EB (%) Extensibility	YM (MPa) Stiffness
Films	0.70 ± 0.03^{b}	32.2 ± 4.4^{b}	26.2 ± 0.7^{a}
Films + mTGase	1.04 ± 0.10^{a}	59.1 ± 6.1^{a}	17.1 ± 2.8^{b}
* BVPC	1.59 ± 0.18	32.08 ± 2.52	78.14 ± 3.04
* BVPC + mTGase	2.14 ± 0.47	21.04 ± 1.29	65.13 ± 2.10
** Viscofan NDX®	36.6 ± 8.1	13.1 ± 2.9	356 ± 29
** Mater Bi (S-301)®	18.4 ± 2.7	317.9 ± 35.9	75.2 ± 2.7

Values are mean ± standard deviation; Means followed by the same letters are not significant different (Tukey-Kramer test, $p < 0.05$); * Data from Porta et al. [6]; ** Data from Porta et al. [34].

4. Conclusions

It has been demonstrated that grass pea flour suspension treated or not with mTGase in the presence of a very low amount (8%) of glycerol, used as plasticizer, is able to produce edible films. Zeta-potential and polydispersity index of the resulting FFSs do not seem to be affected by treatment with mTGase, while average protein agglomerate size appears to be slightly affected by enzyme treatment, resulting on a reduction of particle size. Optical analyses show that grass pea flour-based films are quite transparent in the presence of mTGase, the film opacity being 7 times greater than that performed by the transparent CTA and 8 times lower than the opaque PP5. Morphology studies demonstrated that mTGase confers a smoother and uniform structure as evident from the SEM micrographs of both film surface and cross-section. Digestibility analysis carried out under physiological conditions demonstrated that the grass pea flour proteins were more easily broken down by both gastric and duodenal proteolytic enzymes when the bioplastics were prepared in the absence of mTGase, whereas, the enzyme was able to produce high molecular weight polymers that resulted very resistant to the hydrolysis. Finally, mechanical analyses showed that the bioplastics prepared in the presence of mTGase were more resistant, more extensible and less rigid that the ones prepared in the absence of the enzyme. Further studies will be devoted to assess barrier properties toward O_2, CO_2 and water vapor permeability

Supplementary Materials: The following are available online at http://www.mdpi.com/2079-6412/8/12/435/s1, Figure S1: Specific viscosity of grass pea flour FFSs prepared in the absence and the presence of mTGase.

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Effect of Mesoporous Silica Nanoparticles on Glycerol-Plasticized Anionic and Cationic Polysaccharide Edible Films

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Abstract: This study describes the production of reinforced polysaccharide (PS)-based films, by adding mesoporous silica nanoparticles (MSNs), to either pectin (PEC) or chitosan (CH) film forming solutions, either containing glycerol (GLY) as a plasticizer, or not. Film characterization demonstrated that MSNs and GLY were able to significantly increase the plasticity of both PS-based biomaterials and that the interactions between PSs and nanoparticles were mainly due to hydrogen bonds. Moreover, MSN-containing films were less transparent, compared to controls prepared with either PEC or CH, in the absence of GLY, while all films containing MSNs, but obtained with the plasticizer, were as transparent as the films prepared with PEC or CH alone. MSN addition did not influence the thickness of the PEC-based films, but increased that of CH-based ones, prepared both in the absence and presence of GLY. MSN-containing PEC-based films possessed a more compact and homogeneous morphology, with respect to both control films, prepared, with or without GLY, and to the CH-based films, containing MSNs, the structure of which showed numerous agglomerations. Finally, moisture content and uptake were reduced, in all films prepared in the presence of MSNs. The suggested addition of MSNs might have given rise to novel biomaterials for food or pharmaceutical applications.

Keywords: chitosan; pectin; silica nanoparticles; edible films; glycerol

1. Introduction

In the last decades, there has been a worldwide demand for replacing the highly pollutant oil-based plastics, by renewable and biodegradable materials. In fact, there has been an increased interest in the development and application of biodegradable/edible films and coatings in the food industry, as a consequence of the increasing consumer demand for safe and stable functional foods, with a low environmental impact [1]. Ideal candidates for reducing the huge amount of conventional plastics are the "bioplastics, manufactured exclusively with natural polymers, which are able to be fully

recycled into the environment, in a short time, following their utilization as packaging materials" [2–4]. Renewable resources for producing bioplastics are represented by several polysaccharides (PSs) that are able to constitute the matrices for edible films and coatings [5,6]. Nevertheless, films based on natural polymers are widely known to exhibit poor mechanical resistance, as well as limited barrier and thermal properties. Consequently, these biomaterials had so far restricted commercial applicability in the food packaging sector. More recently, much attention has been given to the production of biopolymer composites, where at least one of their components has nanometric dimensions (1–100 nm) [7]. Such nanoreinforcement is due to the nanoparticles of different chemical natures that are added to a variety of biopolymers, with the aim of obtaining biomaterials with enhanced mechanical and other chemico-physical properties. A uniform nanoparticles dispersion, within a polymer matrix, leads to a very large matrix/filler interfacial area, which changes the molecular mobility, the relaxation behavior, and the resulting thermal, and tensile properties of the material. In this context, special consideration was given to the mesoporous silica nanoparticles (MSNs), due to their unique features, such as the high surface area, controllable pore structure, large pore volume, optically transparent properties, low toxicity, high chemical and thermal stability, and versatile chemically modifiable surface [8]. These nanoparticles act as material reinforcements because they allow an intercalation/exfoliation of silicate layers in the polymer matrix, and therefore, their use is turning into a promising option, to improve the technological features of biopolymer-based films. Hence, the objective of the present work was the reinforcement of either pectin (PEC) or chitosan (CH)-based films, by means of MSNs. PEC is an anionic PS, occurring in fruits and numerous vegetables which represents the major structural component of cell walls [9]. It is currently used in fruit jellies, pharmaceuticals, and cosmetics, for its thickening and emulsifying properties, and ability to solidify to a gel [10]. Conversely, CH is a cationic biopolymer being derived from the deacetylation of chitin [11], the second most abundant polysaccharide, existing in nature, mainly found in the exoskeleton of crustaceans, and in fungal cell walls. CH is frequently used in designing drug delivery systems, because of its biodegradability, non-toxicity, antibacterial, and hemostatic characteristics. Despite the individual advantages, both PSs give rise to films, with some drawbacks, due to their brittleness and poor mechanical and moisture barrier properties. Hence, PEC-MSN and CH-MSN biocomposites, plasticized with glycerol (GLY) or not, have been prepared and partially characterized to find possible applications of the novel biomaterials, both in food and pharmaceutical sectors.

2. Materials and Methods

2.1. Materials

Citrus peel low-methylated (7%) PEC (Aglupectin USP) was purchased from Silva Extracts s.r.l. (Gorle, BG, Italy). CH (mean molar mass of 3.7×104 g/mol) with a degree of 9.0% N-acetylation, was purchased from Professor R.A.A. Muzzarelli (University of Ancona, Italy) [12]. GLY was obtained from the Merck Chemical Company (Darmstadt, Germany). MSNs were prepared and characterized, as recently reported by Fernandez-Bats et al. [8]. All other chemicals and solvents were of analytical grade.

2.2. Film Preparation

PEC-based film forming solutions (0.6% w/v) were prepared from a PEC stock solution (2% w/v of water), at pH 7.5. CH-based film forming solutions (0.6% w/v) were prepared from a CH stock solution (2% w/v of hydrochloric acid 0.1 N, stirred overnight) at a pH 4.5. All film forming solutions, with added MSNs or not (3% w/w of PEC or CH), were prepared in the absence or presence of GLY (30% w/w of PEC or CH), in 50 mL of distilled water and characterized, as previously described [12,13]. The film forming solutions were then cast onto 8 cm diameter polycarbonate Petri dishes and allowed to dry in an environmental chamber, at 25 °C and 45% Relative Humidity (RH) for 48 h. The dried films were peeled, intact, from the casting surface and tested, as described below.

2.3. Film Thickness and Opacity

The thickness was performed for each film, in six different points, with a digital micrometer (DC-516, sensitivity 0.001 mm). The opacity test measurements, carried out six times for each sample, were performed, as described by Tonyali et al. [14]. Opacity was calculated as follows:

$$\text{Opacity } (\text{mm}^{-1}) = \text{A600/x} \quad (1)$$

where A_{600} was the absorbance at 600 nm and x was the film thickness (mm).

2.4. Film Mechanical Properties

Mechanical properties of films, namely tensile strength, elongation at break, and Young's modulus, were obtained by using a universal testing instrument Model No. 5543A (Instron, Norwood, MA, USA). The obtained films were cut into strips (10 mm × 50 mm), by using a sharp razor blade. Each strip was conditioned in an environmental chamber, at 25 °C and 50% RH, for 2 h. Finally, six strips of each film type were tested. The mechanical properties were measured, according to the ASTM D882-97 [15].

2.5. Morphology Analysis (SEM)

Film surfaces and cross-sections were analyzed by Scanning Electron Microscope (SEM). Films were cut using scissors, mounted onto a stub, and sputter-coated with platinum–palladium (Denton Vacuum Desk V), before being observed by the Supra 40 ZEISS (EHT = 5.00 kV, in lens detector). Micrographs for sample surfaces were obtained at 25,000× magnifications, whereas cross-sections were obtained at both 25,000× and 50,000× magnifications.

2.6. Fourier Transform Infrared (FT-IR) Spectra

FT-IR measurements of the films were performed by a Bruker model ALPHA spectrometer (Bruker model ALPHA spectrometer, Leipzig 04318, Germany) equipped with attenuated total reflectance (ATR) accessory. The measurements were obtained in 4,000–400 cm^{-1} region, at 4 cm^{-1} resolution for 24 scans.

2.7. Film Moisture Content

Moisture content of each film was measured gravimetrically, according to Farhan and Hani [16], and Singh et al. [17], with some modifications. In particular, test specimens (5 cm × 5 cm) at different positions of each film type were uniformly cut and placed on glass Petri dishes. Film moisture content was determined by drying each specimen in an oven, at 105 °C, until a constant dry weight was obtained. Film moisture content was calculated as:

$$\text{Film moisture content } (\%) = (W1 - W2)/W1 \times 100, \quad (2)$$

where $W1$ is the initial weight of the film and $W2$ is the film weight, after drying at 105 °C, overnight. Each measure was carried out in triplicates.

2.8. Film Moisture Uptake

Moisture uptake of each film was measured, gravimetrically, in triplicates, as described by Manrich et al. [18]. In particular, films were cut into 5-cm-sided squares, dried at 105 °C for 24 h, conditioned at 23 ± 2 °C, into a desiccator, previously equilibrated at 50% RH, with a saturated $Mg(NO_3)_2$ solution for 24 h. The moisture uptake was calculated as:

$$\text{Film moisture uptake (\% dry matter)} = (Ws - Wd)/Ws \times 100, \quad (3)$$

where *W*s and *W*d are the weight of the swollen (24 h at 50% RH) and the dried films, respectively. Each measure was carried out in triplicates.

2.9. Statistical Analysis

All data were analyzed by the JMP software (SAS Institute, Cary, NC, USA, version 5.0). The data were subjected to analysis of variance, and the means were compared, using the Tukey–Kramer HSD "honestly significant difference" test. Differences were considered to be significant at $p < 0.05$.

3. Results and Discussion

3.1. Film Thickness and Opacity

Thickness is a useful parameter, as it serves as a basis for determining several functional properties of the films. As for CH-based films, the data reported in Table 1 indicates that the thickness value significantly increased when MSNs were presented into the film matrix, whereas the addition of MSNs did not lead to a statistically significant change of PEC-based film thickness. This result could be explained with the increase of viscosity of nanoparticle-containing CH-based film-forming solution [7], which is able to affect the spreadability and thickness of the derived materials [19]. Conversely, the addition of GLY to the film-forming solutions was observed to increase the thickness of the CH-based, as well as of that of the PEC-based films. In addition, in the presence of both GLY and MSNs, CH film thickness resulted in almost three-times greater than that measured with films made by CH alone. Since the appearance of the coated foods plays an important role in consumer acceptability, the opacity of the biomaterials was evaluated, by measuring the light transmission through the films, at a wavelength of 600 nm. Table 1 clearly indicates that the opacity values of both PEC- and CH-based films, markedly increased, when MSNs were present into the film matrices and GLY was absent. Nevertheless, it is worthy to note that the concomitant presence of GLY had a remarkable effect in decreasing film opacity, since the MSNs-containing both CH- and the PEC-based films appeared to be transparent, as the control films, thus, indicating that the GLY allowed to obtain a more homogenous nanoparticle dispersion inside the film matrix, as it was confirmed by SEM experiments (see below).

3.2. Film Mechanical Properties

We have previously observed that the mechanical properties of protein-based films were significantly improved in the presence of MSNs, reaching the maximum values, at a concentration of 3% of nanoparticles [8]. In addition, 30% GLY concentration was found to satisfactorily plasticize the PS-based films [12,13]. Therefore, we selected such a formulation of film-forming solutions to compare the tensile strength, elongation at break, and the Young's modulus of the nanoparticle-containing films, to those of the control films. Table 1 shows that both MSN and GLY were able to significantly increase the plasticity of, both, the PEC- and the CH-based biomaterials. As for the PEC-based films, MSN, and GLY alone, increased the film elongation at break, and reduced Young's modulus, even though a more marked effect on these parameters was obtained by adding both MSN and GLY. At the same time, the concurrent presence of nanoparticles and plasticizer significantly reduced the tensile strength of the PEC-based films. Similar results were obtained by analyzing the CH-based biomaterial, even though, in this case, GLY alone exhibited the most plasticizing effect. Conversely, previously reported data on the MSN effects on the mechanical properties of protein-based films, showed a concomitant increase of both tensile strength and elongation at break [8], thus, indicating different interactions of the same nanoparticles grafted into the matrices of different biopolymer nature.

Table 1. Effect of mesoporous silica nanoparticles (MSNs) on film thickness, transparency, and mechanical properties of the pectin (PEC) and the chitosan (CH) films plasticized with glycerol (GLY), or not.

Film	Thickness (μm)	Opacity (mm^{-1})	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)
PEC-based					
Control	37.5 ± 1.3	1.91 ± 0.16	33.4 ± 7.8	0.8 ± 0.2	5750.2 ± 612.2
+ MSN	40.1 ± 1.2	3.63 ± 0.72 *	40.8 ± 2.5	2.2 ± 0.7	3732.5 ± 264.0 *
+ GLY	56.8 ± 2.5 *	2.05 ± 0.28	27.7 ± 4.4	9.4 ± 2.5 *	1067.6 ± 49.3 *
+ MSN + GLY	58.2 ± 2.1 *	1.88 ± 0.29	16.6 ± 2.4 *	15.2 ± 1.4 *	712.3 ± 52.4 *
CH-based					
Control	30.1 ± 3.1	2.20 ± 0.28	56.2 ± 4.2	7.7 ± 1.8	3438.1 ± 501.3
+ MSN	51.3 ± 1.2 *	6.62 ± 1.88 *	42.4 ± 8.3	13.0 ± 5.6	2088.8 ± 137.3 *
+ GLY	61.2 ± 2.3 *	1.44 ± 0.89	12.5 ± 2.3 *	82.5 ± 2.0 *	130.5 ± 52.2 *
+ MSN + GLY	81.7 ± 3.7 *	1.87 ± 0.35	28.1 ± 2.2 *	54.9 ± 4.2 *	427.3 ± 64.2 *

* Values significantly different at $p < 0.05$ from those obtained without MSN and GLY.

3.3. Film Morphology

Figure 1 shows the images of the different films and their surfaces and cross-sections obtained from the SEM analysis. The control, PEC-based films, prepared in the absence of GLY and MSNs, appeared to be more compact and homogenous, in both, the surface (Figure 1,A1) and the cross-section (Figure 1, inset A2), than the control CH films (Figure 1, insets C1 and C2). The presence of MSNs conferred to the surface of the PEC-based films, prepared either in the presence or absence of GLY, an even more compact and homogeneous structure, compared to the control films, probably due to a widespread inclusion of MSNs, into the PEC network responsible for the major smoothness of the matrices obtained (Figure 1, inset A3 and B3). This hypothesis seemed to be confirmed from the film cross-section SEM images, at 25,000× magnification, which shows a more compact and homogeneous structure of the MSN-containing film matrices, exhibiting more evident continuous zones, in comparison with the control sample (Figure 1, inset A4 and B4), as well as from the zoomed SEM micrographs (obtained at 50,000× magnification), showing the pervasive inclusion of nanoparticles in the PEC film matrix (Figure 1, inset A5 and B5). Numerous aggregations were observed, by contrast, in the cross-sections of the CH-based films prepared in the presence of MSNs, without (Figure 1, inset C4 and C5) or with (Figure 1, inset D4 and D5) GLYs. In fact, these films exhibited many protuberances or fractured morphology, probably due to the MSNs interactions with the CH chains. These results were similar to those reported by Liu et al. [20], who found that the ultrastructure of the starch-based materials was much rougher and exhibited numerous aggregations, due to the asymmetrical MSN dispersion in the film matrix.

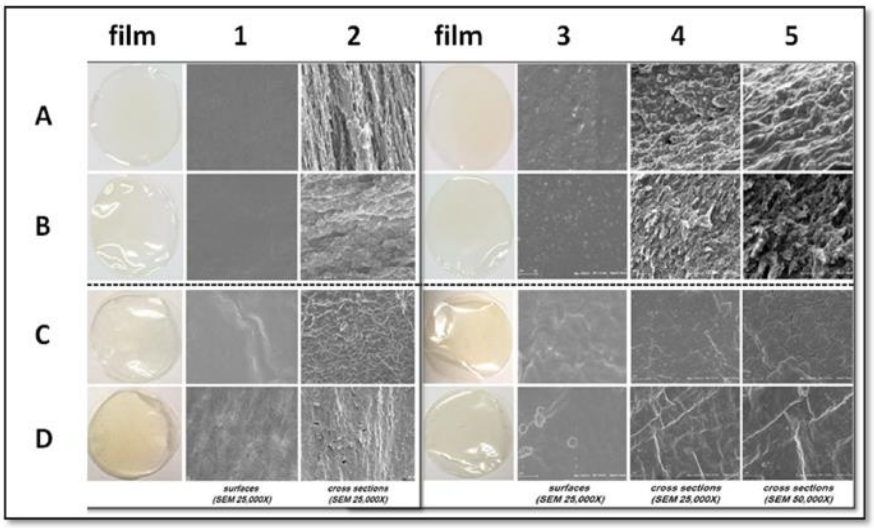

Figure 1. SEM morphology of the PEC (**A**,**B**) and the CH (**C**,**D**) films, prepared in the absence (**A1**,**A2**,**C1**,**C2**) or presence of either GLY (**B1**,**B2**,**D1**,**D2**), MSN (**A3–5**,**C3–5**), or both GLY and MSN (**B3–5**,**D3–5**).

3.4. Film FT-IR Analyses

To elucidate the molecular interactions of both PEC and CH with MSNs, all films were also analyzed by the ATR-FT-IR (Figure 2). The bands detected in the region below 1,000 cm^{-1} arose from inter-atomic vibrations, the bands around 832 and 400 cm^{-1}, being characteristic of the silanol groups (Si–OH), and being related to the Si-O stretching and deformation frequencies [21,22]. The spectra of all PEC-based films (Figure 2, panel A) results were quite similar, representing bands originating from the vibrations of the functional groups of the compounds existing in their matrices, and showing only some differences in the relative intensities and positions. Nesic et al. [23] reported that the interactions between the PEC and the silica, occur between the hydroxyl groups of the PS and the oxygen containing groups of MSNs. The absorption bands in the range between 3,400 and 3,000 cm^{-1} and 1,410 cm^{-1} were typical of hydroxyl groups of bound water, as well as of Si–OH linkage [24], whereas the peaks at 1,751 and 1,628 cm^{-1}, corresponded to the C=O stretching of esterified carboxylic groups (–COOCH3) and free carboxylic groups (–COOH), respectively. The stretching, asymmetric, symmetric, and deformation vibrations of the siloxane network, could be identified with the bands around 1,177 cm^{-1} [25]. All these interactions were more marked in the CH film (Figure 2, panel B) spectra, showing a broad band at 3,267 cm^{-1}, apparently related to the hydroxyl and amino groups occurring on the polysaccharide chains [26]. Finally, the band at 2,881 cm^{-1} was assigned to the C–H stretching vibrations, the band near 1,461 cm^{-1} owed to the stretching frequency of the carbonyl amidic groups present within the CH chains, with some degree of acetylation, whereas the band in the range of 1,250–1,030 cm^{-1} was related to the asymmetric stretching vibration of the Si–O–Si group. In conclusion, all the film FT-IR data showed evident interactions of the PS matrix, with both GLY and MSNs, by hydrogen bonds, and these interactions became increasingly pronounced in the CH–based films.

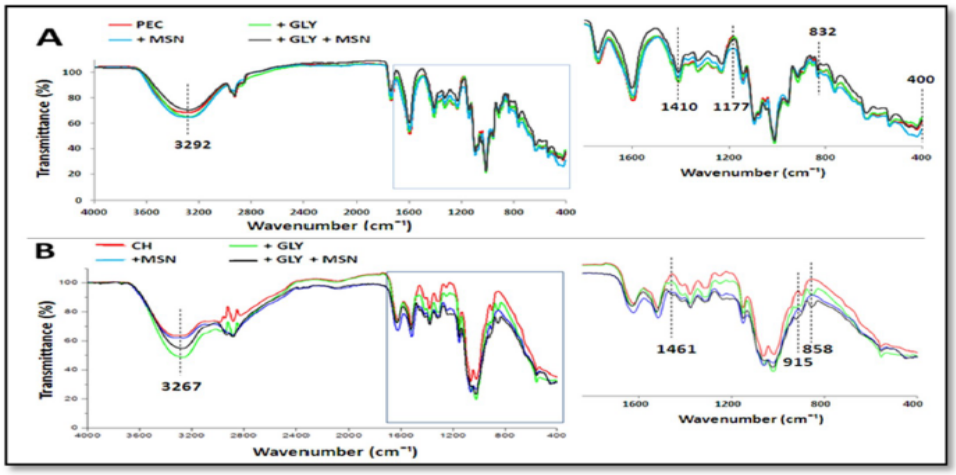

Figure 2. FT-IR spectra of PEC (**A**) and CH (**B**) films prepared in the presence or absence of GLY or MSN.

3.5. Film Moisture Content and Uptake

The prepared films were further characterized for their water moisture content and uptake, as these characteristics were extremely important for potential food packaging applications, especially, when water activity is high or when the film must be in contact with water, and acts as a food protective barrier [27]. In fact, higher moisture content might considerably limit edible film use as a packaging material. Figure 3 shows the positive influence of the MSNs on the film moisture content and uptake, indicating that the addition of MSNs to film-forming solutions significantly reduced both parameters, by using, both, PEC and CH as a film matrix, in the absence or presence of GLY, thus, suggesting that MSNs enhanced the moisture resistance of the prepared biomaterials. This phenomenon is likely due to the modified matrix structure by the hydrogen bond interaction between the two types of PSs and the nanoparticles. These results can be related to the interaction of nanoparticles, with both biopolymers, due to the hydrogen bonds, as described above. Consequently, the resulted dens network reduced the diffusion of water molecules into the matrix [28]. In fact, the equilibrium moisture uptake value depended on the film hydrophilic feature, as well as on its morphology. Therefore, MSNs probably produced a tortuous pathway and, consequently, a reduced water uptake. These findings were in agreement with the data previously reported for montmorillonite-containing films, made either with starch/carboxymethyl cellulose [29], or with methyl cellulose [30].

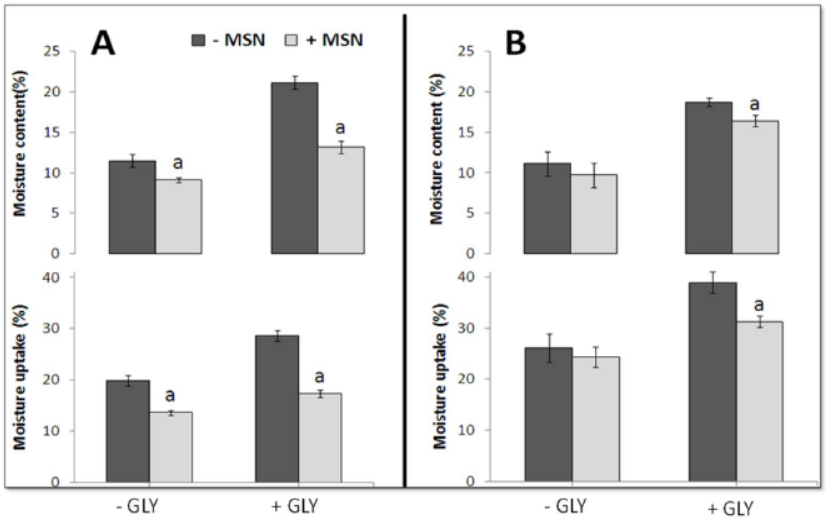

Figure 3. Effect of MSN on moisture-content and uptake of PEC (**A**) and CH (**B**) films, prepared with or without GLY. "a" values significantly different at $p < 0.05$, from those obtained without MSN.

4. Conclusions

In the present study, PEC- and CH-based films, containing MSNs, were prepared by casting to obtain innovative biomaterials. FT-IR showed a strong interaction between PSs and nanoparticles, which influenced the behavior and the stability of both kinds of films. Morphology studies demonstrated an evident nanoparticle dispersion into the PEC matrix, as well as the absence of any nanoparticle agglomeration observed, conversely, in the CH-based films. MSNs were shown to decrease the moisture content and uptake of the prepared films, as a consequence of their interaction with both PEC and CH, by hydrogen bonds, and the formation of the resulting dens network which is able to reduce the diffusion of water molecules inside the biomaterial. In addition, MSN and GLY significantly increased the plasticity of both PEC- and CH-based biomaterials, also influencing their thickness and transparency. Therefore, MSNs are suggested as effective additives of PS-based films, for food industrial applications, when coatings or wrappings with reduced hydrophilic properties are required.

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ORIGINAL PAPER

Structure and in vitro digestibility of grass pea (*Lathyrus sativus* L.) flour following transglutaminase treatment

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Abstract
The impact of transglutaminase (TG) modification on microstructure and in vitro protein and starch digestibility of grass pea flour was investigated. Results demonstrated that grass pea flour proteins act as effective substrate of TG. Microstructural results showed that the addition of TG produced a more compact structure likely due to TG-catalyzed heteropolymers. Nutritional properties such as slowly digestible starch and expected glycemic index values followed the order: grass pea flour incubated in the absence of TG>grass pea flour incubated in the presence of TG>raw flour. The TG-catalyzed heteropolymers were easily digested as demonstrated by in vitro oral and gastric digestion carried out under physiological conditions. Therefore, TG-modified grass pea flour can be considered as a new source of starch and proteins suitable for feeding a large spectrum of population.

Keywords Grass pea flour · In vitro digestion · Food structure · Transglutaminase · Estimated glycemic index

Introduction

Grass pea (*Lathyrus sativus* L.) is a very popular crop in many Asian and African countries where it is grown either for stockfeed or human consumption [1]. It is characterized by a lot of advantageous biological as well as agronomic features such as resistance to drought, high grain-yielding capacity and high protein content of its seeds [2]. There is a great potential for the expansion in the utilization of grass pea in dry areas and zones which are becoming more drought prone as a result of climate change [3]. Grass pea belongs to the leguminous family and is high in protein (28.70 g/100 g) and lysine contents [3]. However, the grass pea seeds, only when eaten as a large part of the diet for long time, can cause lathyrism [4] due to the presence of a non-protein aminoacid β-*N*-oxalyl-L-α, β-diaminopropionic acid (β-ODAP). When grass pea is a part of a varied diet, β-ODAP is tolerated without any known adverse effect. Thus, nowadays, this legume is rightly considered as one of the most promising sources of starch and proteins [5].The use of pulses is bound to increase in the future, and especially in combination with cereal raw materials they may find new applications, meeting both sensory and nutritional needs of consumers worldwide. However, it is necessary that different kinds of crops are studied to obtain structural parameters and information required to gain competitiveness in an international-scale industry. Pulses have recently gained interest as protein sources because of their high-quality protein (about 20–40%) [6], nutrient density [7] and as suitable ingredients in gluten-free foods. It has long been established that pulses are low glycemic index [8, 9] and there is growing evidence that eating pulse foods regularly reduces serum cholesterol [10]. Additional health benefits of pulses have been revealed through recent research [11].

As a follow-up to a former study about properties and in vitro digestion of raw Grass pea (*Lathyrus sativus*) flour [12, 13], one of the aims of the present research was to study the proteins of grass pea flour as microbial transglutaminase (TG) substrate. TG catalyzes intra- and/or intermolecular isopeptide bonds between the γ-carboxamide group of

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glutamine (acyl donor) and ε-amino group of lysine residues (acyl acceptor) isopeptide bonds between glutamines and lysines into proteins [14–17]. Hence, the impact of the TG modification on microstructure, in vitro protein and starch digestibility as well as on the expected glycemic index (GI) of grass pea flour was explored.

Materials and methods

Seed materials

Grass pea seeds (*Lathyrus sativus*) were purchased by "La Bona Usanza (S.C.A.R.L.) as Slow Food Presidia [18]. Plants (Population C3, characterized for the ODAP content [19] were grown in the field in Serra de' Conti Municipality, Ancona Province (central Italy) in the summer 2016.

Reagents

TG, Activa TI (specific activity 92 U/g), Ajinomoto, Japan, was provided by Prodotti Gianni Reagents. Gels for SDS-PAGE were from Bio-Rad (Segrate, Milano, Italy). α-amylase (product A1031), pepsin from porcine gastric mucosa (product P6887) and all other reagents were purchased from Sigma Chemical Company (Pool, Dorset, UK). Chemicals were of analytical grade, unless specified.

Flour preparation

Grass pea flour was prepared according to Romano et al. [13] and Al-Asmar et al. [20] by grounding the grass pea seeds using a variable speed laboratory blender (LB20ES, Waring Commercial, Torrington, Connecticut, USA), so that the grass pea flour (raw) would pass through a 425-µm stainless steel sieve (Octagon Digital Endecotts Limited, Lombard Road, London, UK).

Some flour samples were boiled in water for 15 min to simulate the cooking process. Moreover, cooked samples were treated for 2 h at 37 °C in the absence (GP) and presence of TG (20 U/gr of substrate) (GP/TG) as described in the next paragraph. All grass pea flour samples (raw, GP and GP/TG) were collected and stored in polyethylene bags at 4 °C until used for analysis.

TG-mediated modification of grass pea flour

The enzymatic modification of raw flour by means of TG was carried out by following the procedure described in Mariniello et al. [14] and Porta et al. [21] with some modifications. It is worth to note that the samples were treated for 15 min at 100 °C to allow protein denaturation and in the same time to simulate the cooking process. Briefly, 100 µg of protein flour were incubated in Tris–HCl 80 mM pH 7.5 with increasing (5, 10, 20 U/g) amounts of TG for 2 h at 37 °C in a final volume of 100 µL. The same experiment was carried out also on unheated flour with the aim of comparing the extent of TG-catalyzed reaction following the denaturation process.

Microstructural analysis

All flour samples (raw, GP and GP/TG) were dried at the critical point and coated with gold particles in an automated critical point dryer (model SCD 050, Leica Vienna). Microstructure of samples was examined by means of Scanning Electron Microscopy (SEM) (LEO EVO 40, Zeiss, Germany) as reported by Romano et al. [13] at a magnification of ×2000.

In vitro starch digestibility and expected glycemic index

Measurement of resistant starch (RS) and non-resistant starch (solubilised, Non-RS) were determined using an enzymatic assay kit (Resistant Starch Assay Kit, Megazyme International, Ireland) by AACC [22]. Starch hydrolysis is expressed as the ratio of Non-RS to the sum of RS and Non-RS starch [23]. All these results were expressed as percentage weight/weight on dry basis.

Rapidly digestible starch (RDS) and slowly digestible starch (SDS) were measured after incubation for 30 min and a further 120 min, respectively [24].

The digestion kinetics were described by means of a non-linear model in the following equation found by Goñi et al. [25]:

$$C = C_{\infty}(1 - e^{-kt}), \quad (1)$$

where C is the hydrolysis degree at each time, C∞ the maximum hydrolysis extent and k is the kinetic constant. The hydrolysis index (HI) was calculated as the relation (as percentage) between the area under the hydrolysis curve (AUC, 0–180 min) of each sample and the AUC of white bread as reference food. Previous research has shown HI to be a good predictor of glycemic response [25]. Last, expected glycemic index (eGI) was calculated using the equation proposed by Goñi et al. [25]:

$$eGI = 39.71 + 0.549HI. \quad (2)$$

Protein determination

Grass pea protein content was calculated by estimating nitrogen content [26].

In vitro protein digestion models

The simulation of human oral and gastric digestion of both unmodified and TG-modified grass pea flour was carried out following the procedure described in Giosafatto et al. [27].

At the end of the digestion experiment, 20 µL of each sample was analyzed by SDS-PAGE (4–20%).

SDS-PAGE

5 µL of sample buffer was added to aliquots of 20 µL of each sample and analyzed by 4–20% SDS-PAGE, as described by Laemmli [28]. Electrophoresis was performed at constant voltage (80 V for 2–3 h), and the proteins were stained with Coomassie Brilliant Blue R250. Bio-Rad Precision Protein Standards were used as molecular weight markers.

Image analysis

The SDS-PAGE gel images were acquired using Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1) following the procedure described in Giosafatto et al. [27].

Statistical analysis

All experimental results are reported as means and standard deviation of at least three independent experiments. One-way ANOVA with Duncan's multiple comparison test at the 95% confidence level ($p \leq 0.05$) were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) on all experimental data.

Results and discussion

TG-mediated modification of grass pea flour

To study the effect of protein structure on the biological properties of grass pea proteins, TG-mediated crosslinking assays were performed. It is worth to note that TG has been successfully exploited by our research group to modify several proteins of food interest. In particular, recently proteins from other legumes were subjected to TG treatment as reported in Romano et al. [17], who have modified, by means of the microbial enzyme, proteins from beans (*Phaseolus vulgaris*). On the other hand, Porta et al. [21, 29] have used TG to treat the proteins from bitter vetch (*Vicia ervilia)*, another legume used mainly for animal feeding, with the aim to prepare biopolymer materials with improved technological properties. In this study, we have performed some experiments that successfully demonstrated that also the proteins from grass pea, as from other legumes [14, 30, 31] act as effective substrate for TG. In particular, 100 µg of protein flour was incubated with increasing amount of Activa (5, 10 and 20 U/g of proteins, respectively) for 2 h at 37 °C. At the end of incubation, the TG assay was stopped by boiling the samples for 2 min. The extent of polymerization was evaluated by SDS-PAGE. It is worth to point out that the same assay was carried out on both denatured and undenatured proteins to verify the effect of protein denaturation on the extent of crosslinking. To this purpose, the flour was boiled for 15 min and, in this way, besides promoting the denaturation, we also mimicked the cooking process. As it is possible to see from Fig. 1, TG is able to catalyze the formation of high MW polymers with the concomitant decrease of the grass pea characteristic protein bands even using the lowest amount of TG (5 U/g). However, this effect is much more evident on the heat-treated samples. In fact, for obtaining the almost complete protein modification, 10 U/g of enzyme represents a sufficient amount for the denatured samples, whereas for the undenatured ones the same result is obtained only using 20 U/g of TG (Fig. 1). Nevertheless, the low MW bands, either from undenatured or denatured samples, seem quite resistant to act as TG substrate (Fig. 1). Under these experimental conditions, the grass pea proteins appear an effective substrate of TG and also more efficient than other legume proteins. Porta et al. [21] analyzing the effect of TG on proteins (undenatured) isolated from *Vicia ervilia* seeds have found that the lowest amount of enzyme able to lead to exhaustive polymerization is equal to 20 U/g; on the other hand phaseolin, main storage protein of the

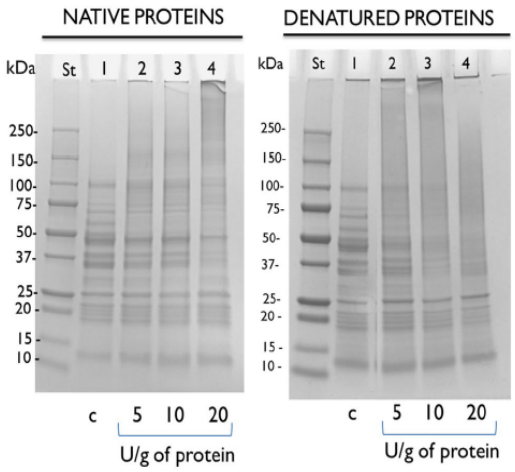

Fig. 1 TG-catalyzed reaction of grass pea flour proteins. Both native and undenatured flour proteins (100 µg) were incubated in the absence (lane 1, C) and presence of increasing amounts (lane 2–4) of Activa for 2 h at 37 °C. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

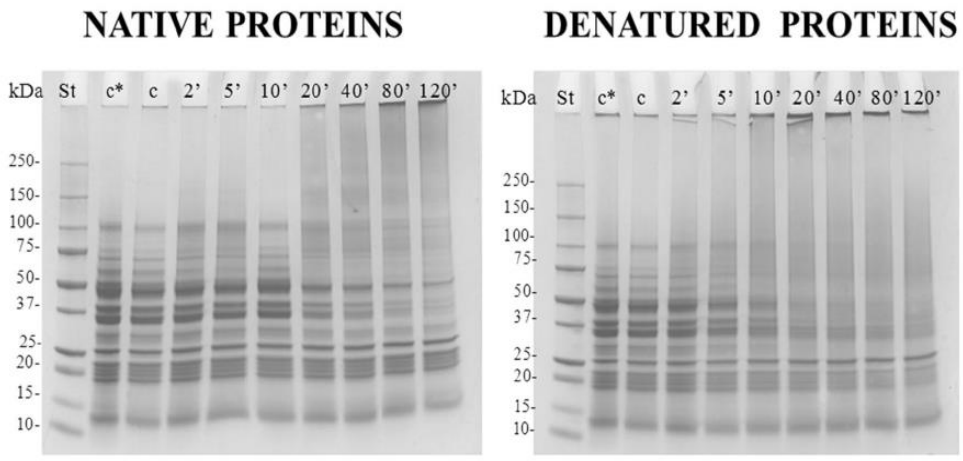

Fig. 2 TG-catalyzed reaction of grass pea flour proteins. Both native and undenatured flour proteins (100 µg) were incubated in the presence of 5U/g of Activa for different times at 37 °C. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). c* and c are the controls representing the protein samples treated without Activa, not incubated and incubated for 2 h, respectively. St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

seeds of *Phaseolus vulgaris*, can be modified after 2-h incubation using 15 U/g of microbial enzyme [14].

To prove that the polymerization is enzyme dependant, a time-course assay was also performed (Fig. 2). As it is possible to note from Fig. 2, the enzyme catalyzes the formation of intermolecular crosslinks among protein molecules more rapidly when the proteins are denatured. The 50-kDa protein band is still present at the end of incubation (120 min) when the proteins are not heat treated, while in the case of thermic denaturation already after 80 min such a band disappears together with other proteins possessing a MW ≥ 24 kDa. In addition, the formation of polymers that have a MW ≥ 250 kDa and of polymers unable to enter the gel is already evident after 10 min if the proteins are denatured. In the case of undenatured samples, the high MW polymers appear only over 20-min incubation.

Microstructure characteristics of flour

To study the microstructural changes arising as a result of the TG treatment, the microstructure of grass pea flour samples was investigated by means of SEM. Figure 3 shows representative SEM micrographs of grass pea flour samples: GP (Fig. 3a) and GP/TG (Fig. 3b).

GP samples (Fig. 3a) possess starch granules that appear swelled compared to the ones present in GP raw flour studied by Romano et al. [13] which contained oval and ellipsoid starch granules with heterogeneous sizes. The swelled aspect of starch granules in incubated GP samples is due to the partial hydration of their amorphous regions (partially gelatinized). On the top surface of starch granules, strands of protein bodies were also observed.

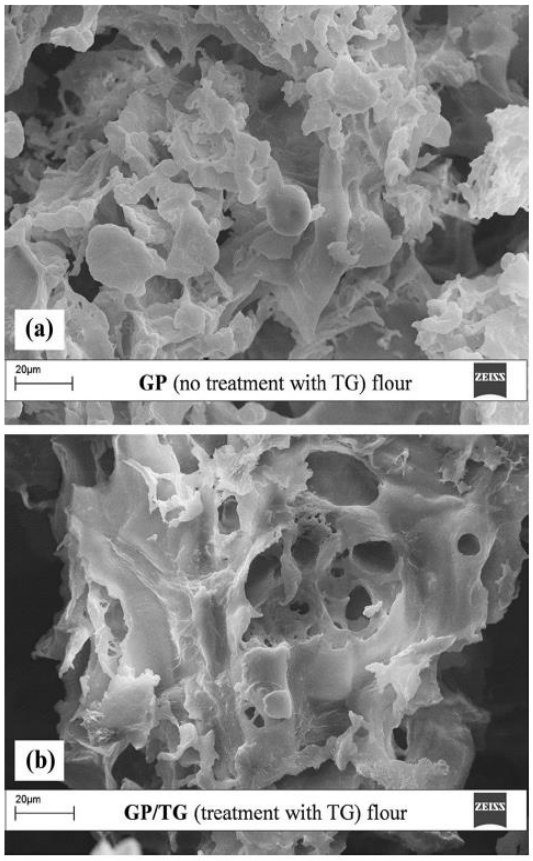

Fig. 3 Scanning electron micrographs (2000 K) of grass pea flour incubated for 2 h at 37 °C in the absence of TG (GP, panel a), and in the presence of TG (GP/TG, panel b)

As expected, GP/TG samples possessed a distinctly different structure (Fig. 3b) from raw flour [13] and GP samples (Fig. 3a). GP/TG samples showed in fact a more compact and homogeneous structure due, most probably, to the formation TG-mediated crosslinking that reinforces the protein–protein interactions. Similar microstructural observations were reported previously by Romano et al. [17] when TG was added to bean flour and by Bonet et al. [32] that studied the glucose oxidase effect on wheat flour dough at molecular level.

In vitro starch digestibility and expected glycemic index

The results regarding starch digestibility (in vitro) is depicted in Fig. 4. In vitro starch digestion was investigated by measuring the released glucose content during starch digestion and the hydrolysis curves of samples were compared with those performed by white bread used as control. All of the samples investigated showed a starch digestibility lower than white bread. The raw samples showed the lowest digestibility. The hydrolysis curves of GP flour showed a significantly ($p < 0.05$) increase upon 120 min with a more starch hydrolysis and digestibility than GP/TG. This increase could be due to the presence of swelled starch granules observed in Fig. 3a. The hydrolysis kinetics of GP/TG flour showed a similar hydrolysis trend, although the percentage of digested starch is lower at the plateau. This result could be explained because of the presence of TG-mediated protein network that could give rise to insoluble complexes also affecting in vitro digestibility of proteins [17].

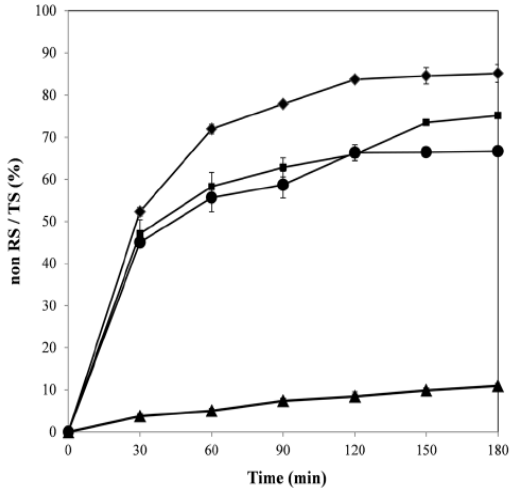

Fig. 4 Total starch hydrolysis rate of white bread (filled diamond) and grass pea flour: not incubated (filled triangle), incubated for 2 h at 37 °C in the absence of TG (filled square) and in the presence of TG (filled circle)

Starchy food can be classified according to their digestibility. Rapidly digestible starch (RDS) and slowly digestible starch (SDS) of different starches are shown in Table 1. RDS is rapidly and completely digested in the small intestine and is associated with more rapid elevation of postprandial plasma glucose, whereas SDS is more slowly digested in the small intestine and is generally the most desirable form of dietary starch [23, 33]. The RDS content was in the range of 1.5–18.7%, while the SDS contents ranged from 3.3 ± 0.4% of raw flour to 26.1 ± 0.6% of GP. In particular, the GP/TG samples showed significantly ($p < 0.05$) lower RDS, SDS (Table 1) in comparison with GP samples. Certain indigestible polymers and some associated non-fibrous compounds may, in fact, reduce the rate of starch digestion in vitro and in vivo, resulting in low metabolic responses [34].

The expected glycemic index (eGI) for different samples is shown in Table 1; eGI for the flour samples was in order GP>GP/TG>raw. The eGI results differed significantly ($p < 0.05$) varying between 45.03 of raw flour and 85.58% of grass pea flour without TG (Table 1). The eGI was correlated with the parameters of the starch fractions, including RDS and RS. In particular, RDS is found to be a positive and main contributing factor to the eGI. Higher percentages of RDS in starch are usually related to a higher degree of eGI [35, 36], while the RS content had an inverse relationship with eGI [24]. eGI influences the nutritional quality of foods and the benefits of a low GI food in reducing insulin demand, improving satiety, improving blood glucose control with diabetic people, reducing blood lipid level and increasing colonic fermentation which are well documented [37]. The protein network formed reduces the rate of grass pea flour starch digestion, being the glycemic index of grass pea flour modified by TG lower than the non-treated one (GP).

Considering the in vitro digestibility results of TG samples, the latter might be a potential ingredient in the formulation of products for diabetics and weight management, and could lead to the formulation of novel foods characterized by the slow release of glucose, that is to say low glycemic index and prevention of fasting hypoglycemia.

Table 1 Effect of TG on starch nutritional fractions (RDS, rapidly digestible starch, and SDS, slowly digestible starch) and expected glycemic index (eGI) of the analyzed samples. Each value is expressed as mean ± S.D

Grass pea flour	RDS (%)	SDS (%)	eGI (%)
Raw	1.46 ± 0.21^{a}	3.29 ± 0.42^{a}	45.03 ± 0.18^{a}
GP	18.65 ± 1.32^{c}	26.10 ± 0.61^{c}	85.58 ± 0.37^{c}
GP/TG	16.67 ± 0.09^{b}	24.55 ± 0.70^{b}	82.97 ± 0.53^{b}

[a–c]Means within the same column with different letters are significantly different ($p < 0.05$; Duncan test)

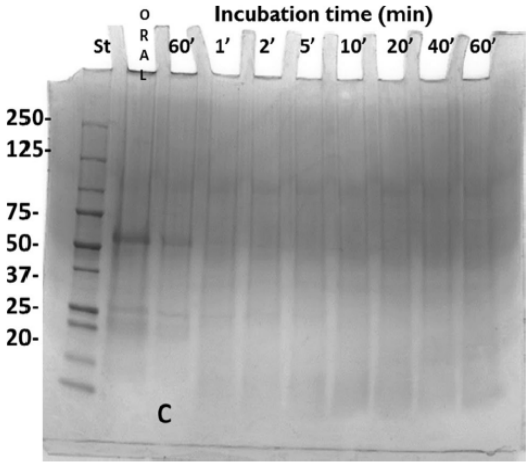

Fig. 5 Digestibility of grass pea flour proteins. TG-treated samples were subjected to oral and gastric digestion following the conditions found in the adult human. At the end of incubation, the samples were analyzed by SDS-PAGE (4–20%). Lane "c" corresponds to the samples before the digestion. St, Bio-Rad Precision Protein Standards were used as molecular weight markers. Further details are described in the text

Protein digestibility

In this work, we have also studied the digestibility of grass pea flour proteins following TG treatment, to study the effect of food structure on the human gut. To this aim, the TG (Activa, 20 U/g)-modified sample was subjected to in vitro oral and gastric digestion carried out under physiological conditions as described in Giosafatto et al. [27] and the products are analyzed by SDS-PAGE. The results reported in Fig. 5 demonstrated that TG did not influence the digestibility of grass pea flour proteins. In fact, the crosslinked polymers (with a MW ≥ 250 kDa) as the unmodified proteins [13] were still easily and gradually digested upon incubation with pepsin. In fact, densitometry analysis shows that only about 20% of TG-modified forms were present after 20-min gastric digestion in comparison with control (Fig. 5). At the end of incubation with pepsin, only 13% of high MW polymers are still detectable. These results are very interesting since some previous papers showed that food processing influences the protein digestion [38, 39] and in particular, TG-mediated crosslinked protein forms appear very stable and more resistant to the hydrolysis catalyzed by digestive enzymes. In fact, Giosafatto et al. [13] demonstrated that ovalbumin polymers obtained by means of TG persisted even through duodenal digestion suggesting that the TG-induced crosslinking of the egg protein affects the rate of digestion. Similar results were also observed by Tang et al. [40] and Monogioudi et al. [41] that found the covalent crosslinking of soy as well as β-casein decreased the in vitro digestibility especially that observed for pepsin digestion. Nevertheless, cucurbitin protein from pumpkin oil cake crosslinked by TG was still prone to be digested by gastrointestinal enzymes and the obtained hydrolysates still maintained their bioactive potential [42]. Based on our results, it is possible to assess that TG was able to modify grass pea flour proteins providing a novel flour ingredient which might be used to obtain food highly digestible and with a low glycemic index. Nowadays, the demand of "easy to use" foods is increasing in western countries; thus the results reported in the present paper could represent a basic study to develop sustainable novel foods with desired characteristics for different groups of consumers, such as athletes, diabetics or common people that do care about a healthy diet.

Conclusions

Grass pea flour proteins either or not heat treated act as effective TG substrate, even though the heat processing markedly improves the capability of these proteins to be modified by the microbial enzyme. The TG-crosslinked proteins were easily digested in vitro and possess nutritional properties that make grass pea an inexpensive legume suitable for feeding a large spectrum of population.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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Appendix 6.6

- Schools, workshops,courses, and seminars followed during PhD course.

A. Schools

1. Summer School “In Silico Methods for Food Safety”, 13-15, June, 2017- in Parma (EFSA)

B. *Workshops*

1. Workshop on NanoBiomedicine in Naples: The next future of Theranostices. Napoli-Italy. 22, March 2019.
2. Sustainable polymers for a circular economy. Napoli-Italy. 24, June 2019. Aula Magna, Complex of San Giovanni, Federico II University. 5th Blue Sky Conference

C. Courses

1. Eugenio Notomista. “Protein structure visualization, analysis and modeling”, 5,9,16/5/2017.
2. Angela Arciello. “Biotechnological applications of bioactive peptides and peptidomimetics” ,18-19/5/2017.
3. Valeria Giosafatto. “Enzymes in food biotechnology”, 25-26/5/2017.
4. Moh Mousori “Communicating and disseminating your research work”, 5-7/6/2017.
5. Valeria Giosafatto .“Bioplastics”, 21,27/6/2017.
6. Cinzia Pezzella. “Microbial cell factories in industrial biotechnology”, 22-23/6/2017.
7. Piero Pucci. “Advanced mass spectrometry”, 26-30/6/2017
8. Daniele Naviglio. “The techniques of solid-liquid extraction used in the preparation of the sample for chemical analysis and production of extracts for industrial uses”, 10-13/7/2017.
9. Raffaele Velotta. “Introduction to data analysis”, 25-28/9/2017.
10. Bedini Emilianno. “Glycochemistry”, 4-7/12/2017.
11. Valeria Giosafatto. “Bioplastics”, 5-6/10/2018.
12. Valeria Giosafatto. “Enzymes as additives or processing aids”, 8-9/10/2018.
13. Rachelle Isticato. “Enzymatic and microbial applications in biotransformations and nanotechnology”, 10-11/10/2018.
14. Viola Calabrò. “Genome editing”,15-16/10/2018
15. Daria Monti. “First steps in writing and publishing a scientific manuscript”, 11/6/2019
16. Fabio Borbone. “Microscopic methodologies for Life and Materials Sciences”, 11,27/6/2019

D. Seminars

1. Caserta S. ""How “smart" a cell can be? Do transport phenomena play a role in dynamic evolution of cell systems?”, 8/2/2017.
2. Ricci F. “DNA-based nanodevices for diagnostic and drug-delivery applications”, 15/2/2017
3. Sacha Lucchini. “From the farm to the gut: mechanisms of bacterial adaptation”, 22/2/2017.
4. Andrea Strazzulli. “Discovery novel hyperthermophilic carbohydrate active enzymes for plantbiomass degradation: a metagenomic approach”, 8/3/2017.
5. Giovanni Libralato. “Hygiene research and implications of emerging compounds: personal care products, pharmaceuticals and engineered nanomaterials”, 14/3/2017.
6. Luis I. Díez. “Oxy-combustion of coal/biomass in the framework of CO2 capture and storage (bio-CCS)”, 6/4/2017
7. Pietro Lupetti. “Ultrastructural studies on intraflagellar transport trains”, 28/4/2019
8. Giuseppe Nicotra. “Challenges beyond the nanoscale, and the new Sub –A S/TEM microscope at CNR”, 28/4/2017.
9. Angharad M. R. Gatehouse. “The Role of RNA interference in crop protection”, 11/5/2017.
10. Thierry Tron. “On the use of surface functionalized enzymes.”, 11/5/2017.
11. Aurel Radulescu. “Elucidation of the morphology of hydrocarbon polymer electrolyte membranes by small-angle neutron scattering technique”, 15/6/2017
12. Philip Jessop. “CO2-Switchable Surfaces and Coatings”, 23/5/2017
13. Gabriel Luna-Barcenas “Nanocomposites for biomedical & environmental applications”, 5/7/2017.
14. Mohammad Altamini. “Anti adhesive effect of oligosaccharide micro flora using cell culture model”, 7/7/2017.
15. W.A.L. van Otterlo. “Synthetic “fun and games” with compounds inspired by bioactive natural products” , 5/12/2017
16. Henk Haasgmann. “Immunomosulatory antimicrobial peptides: biology and applications”, 15/02/2018.
17. Gianfranco Pacchioni. “Supported Metal Particles in Catalysis: Role of Metal-Oxide Interface”, 15/2/2018
18. N J Clayden. “A life of magical spinning (Solid state NMR spectroscopy in chemistry)”, 20/2/2018
19. Philippe Zinck. “Chain shuttling polymerization - a powerful tool for the design of multiblock polymers”,20/2/2018
20. N J Clayden. “Muons useful in chemistry”,22/2/2018
21. Charlotte Blom. “Enzyme discovery at novozymes”, 13/3/2018

22. Michael M.Cox. “On the Nature of Science”, 13/4/2018
23. Sabine Flitch. “The golden age of glycoscience-new tools in synthesis and analysis”, 24/9/2018.
24. Carmen Galan. “Novel synthetic glycol-tools for biology research”, 26/9/2018.
25. Arianna Mazzoli. “Search of new therapeutic targets of obesity and obesity-related diseases”, 21/11/2018
26. Alfonso De Simone. “Catching the invisible molecular interactions behind Parkinson’s Disease”, 8/1/2019
27. Marco Moracci. “IBISBA 1.0: Industrial Biotechnology Innovation and Synthetic Biology Accelerator”,26/3/2019
28. Anna Maria Masdeu Bulto.” Utilization of CO_2 from solvent to reactant”, 9/4/2019
29. Bruno Iafelice. “Innovation and entrepreneurship: The silicon valley mindset for scientists”,12/4/2019
30. Antonietta Demaio. “Innovation and entrepreneurship: Legal issues for entrepreneurship”, 12/4/2019
31. Michale condry. “Innovation and entrepreneurship: Smart medical system to predict cancer and other gastro intestinal disorders”, 12/4/2019

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