IMPROVEMENT OF LIPASE STABILITY IN THE PRESENCE OF COMMERCIAL TRIGLYCERIDES

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BRIEF SUMMARY

Lipases (EC 3.1.1.3) constitute the most important group of biocatalysts for biotechnological applications. They are serine hydrolases and catalyse both the hydrolysis and the synthesis of esters from glycerol and long chain fatty acids. Their use in the oleochemical industry for the modification of fats and oils is particularly attractive. Unfortunately, lipases showed instability in the presence of commercial preparations of triglycerides, the products of triglycerides peroxidation being the main cause for inactivation. Aldehydes in particularly seem to affect the lipase stability to a great extent. The interaction with the lipase involves specific residues of the protein such as lysine, cysteine and histidine, with the formation of covalent bonds.

This PhD thesis was characterized by two aspects. First, the effect of soybean oil that had undergone an oxidation process by means of either several day incubation in air, or use in a frying process lasting several hours, was analyzed on the commercial lipase Novozym® 435. Additionally, stabilization via chemical modification of this lipase was investigated. Secondly, the possibility of generating a stable lipase by protein engineering, was analyzed. The prolipase from *Rhizopus oryzae* (proROL) was successfully produced in *E. coli* Origami strain, and genetically modified in order to substitute those residues responsible for the interaction with the products of lipid oxidation. High quality libraries of mutants carrying single mutations of all the histidine residues and of some lysine residues were generated, and a high-throughput screening assay was developed. in order to rapidly select those mutants having better stability than the wild type proROL. The library screening led to some interesting hits. The best variants found were H201S and H201A, with an increase of the lipase stability of almost 50%. Recombination of some stable mutants was also considered in order to increase the stabilization effect.

Work on the Novozym® 435 lipase and analysis in semi-micro scale of the variants' stability were carried out at the Department of Chemical Engineering, University of Napoli Federico II (Naples, Italy)

Experimental work on ROL and proROL expression, mutagenesis and libraries screening, was carried out at the Department of Biotechnology and Enzyme Catalysis of the Ernst-Moritz-Arndt University of Greifswald (Germany).

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Thanks in general to those who were so close to me during this time.

RIASSUNTO ESTESO

Miglioramento della stabilità delle lipasi in presenza di trigliceridi commerciali

Introduzione

Le lipasi appartengono alla classe degli enzimi lipolitici e sono in grado di idrolizzare lipidi complessi. I substrati naturali delle lipasi sono i trigliceridi, che vengono idrolizzati formando acidi grassi, glicerina, mono- e di- gliceridi. Tuttavia, le lipasi sono anche attive su una vasta gamma di composti derivati dai trigliceridi (acidi grassi, esteri)

L'impiego di lipasi in mezzi non acquosi ha aperto la strada a nuove trasformazioni di interesse industriale, consentendo la sintesi di numerosi prodotti ad alto valore aggiunto destinati in particolare ad industrie alimentari, cosmetiche, farmaceutiche e mediche. In molte di queste produzioni di interesse industriale, i trattamenti enzimatici rappresentano una valida alternativa rispetto ai processi tradizionali condotti con catalizzatori inorganici, vista la loro maggiore specificità e la possibilità di utilizzare temperature e pressioni operative sensibilmente più basse (Casey et al., 1992; Akoh, 1995; Gandhi, 1997).

Particolarmente interessante dal punto di vista commerciale è la produzione di trigliceridi ad alto contenuto di residui di acidi ω-3 insaturi, al fine di modificare la composizione degli acidi grassi contenuti in oli e grassi di origine vegetale o animale per conferire loro alto valore dietetico. Negli ultimi anni, inoltre, si sta considerando la possibilità di riciclare oli e grassi di scarto. Gli oli residui dei processi industriali di frittura e dei processi di lavorazione delle carni animali, che generalmente vengono inceneriti o inviati in discarica, possono essere trasformati in esteri alchilici di acidi grassi, ed impiegati come biodiesel o come lubrificanti per applicazioni speciali (Villeneuve et al., 2000; Fukuda et al., 2001; Lee et al., 2002).

Tuttavia, nelle condizioni operative di interesse industriale, la scarsa stabilità nel tempo dell'attività catalitica delle lipasi ne limita pesantemente l'applicabilità. I prodotti dell'autossidazione dei trigliceridi sono i maggiori responsabili di questi fenomeni di disattivazione. Schematicamente, l'autossidazione dei trigliceridi ha luogo in due stadi: produzione di idroperossidi, che rappresentano i prodotti primari della ossidazione e successiva, ulteriore ossidazione degli idroperossidi in composti carbonilici. I prodotti di ossidazione primaria e secondaria interagiscono con alcuni gruppi funzionali delle lipasi, decurtandone la reattività. In particolare, aldeidi come malondialdeide (MDA), 4-idrossinonenale (Stirton et al.) e acroleina, generate dall'ossidazione di acidi grassi presenti in trigliceridi commerciali, interagiscono con specifici residui (lisina, cisteina e istidina) coinvolgendo rispettivamente i gruppi ε-amminici, i gruppi sulfidrilici e l'azoto dell'imidazolo. L'interazione è di tipo covalente e dà luogo generalmente ad addotti di Michael e a fenomeni di polimerizzazione tra le proteine (Agerbo et al., 1992; Szweda et al., 1992; Bolgar et al., 1996; Dean, 1997; Brunet, 1999). Questi processi sono stati ampiamente studiati in ambiente acquoso, in particolare nell' organismo umano. Al contrario, i meccanismi di disattivazione in ambienti virtualmente anidri, quali quelli necessari per le applicazioni precedentemente citate, non sono ancora ben chiari e richiedono ulteriori approfondimenti.

Esperimenti condotti in precedenza presso il Dipartimento di Ingegneria Chimica dell'Università Federico II di Napoli sulla lipasi commerciale immobilizzata Novozyme 435 in solventi organici, hanno evidenziato, l'esistenza di interazioni proteina-prodotti di ossidazione analoghe al caso di ambienti acquosi. Infatti, pretrattamenti della lipasi con il p-idrossimercurbenzoato (pHMB), hanno dato luogo a perdite di attività meno marcate, dimostrando il coinvolgimento nel processo dei gruppi sulfirilici (Pirozzi, 2003).

Partendo da questi risultati, il mio lavoro di Tesi si è sviluppato in due distinte direzioni.

Da una parte, ho condotto uno studio ulteriore sulla lipasi Novozyme 435 in solventi organici, caratterizzandone la disattivazione in presenza di oli ossidati ed di oli sottoposti a processi di frittura e considerando anche la possibilità di stabilizzare la proteina attraverso il blocco dei residui amminoacidi particolarmente suscettibili all'interazione con le aldeidi.

Dall'altra, ho tentato di conseguire una maggiore stabilità dell'enzima attraverso interventi di ingegneria proteica, mediante la sostituzione dei residui coinvolti nel processo di disattivazione.

Il lavoro di ricerca è stato condotto in parte presso il Dipartimento di Ingegneria Chimica dell'Università Federico II di Napoli ed in parte presso il Dipartimento di Biotecnologia e Catalisi Enzimatica dell'università Ernst-Moritz-Arndt di Greifswald, Germania.

.

RISULTATI ED OBIETTIVI CONSEGUITI

Disattivazione delle lipasi in ambienti anidri

I primi esperimenti sull'effetto disattivante di oli ossidati sono stati condotti sull'enzima commerciale Novozyme 435 (lipasi B da *Candida antartica,* immobilizzata su resina a scambio ionico). Quando questa lipasi è stata sottoposta ad incubazione con oli a differente grado di ossidazione, si è osservata una caduta della attività residua dipendente dal grado di ossidazione dell'olio. Una disattivazione più marcata è stata osservata nel caso di incubazione in reattori in continuo (Figura 1).

Successivamente, sono stati svolti alcuni esperimenti di stabilizzazione del Novozyme 435, attraverso l'impiego di Piridoxal 5-fosfato e di N-etilmaleimmide, che sono in grado di complessarsi rispettivamente con i gruppi ε-amminici della lisina e con il gruppo tiolico della cisteina. I risultati conseguiti sono promettenti:

1) il trattamento non ha comportato variazioni significative dell'attività catalitica;
2) incubando con olio ossidato sia l'enzima trattato, sia quello non trattato, dopo una disattivazione iniziale, si osserva una stabilizzazione nel solo caso di enzima trattato.

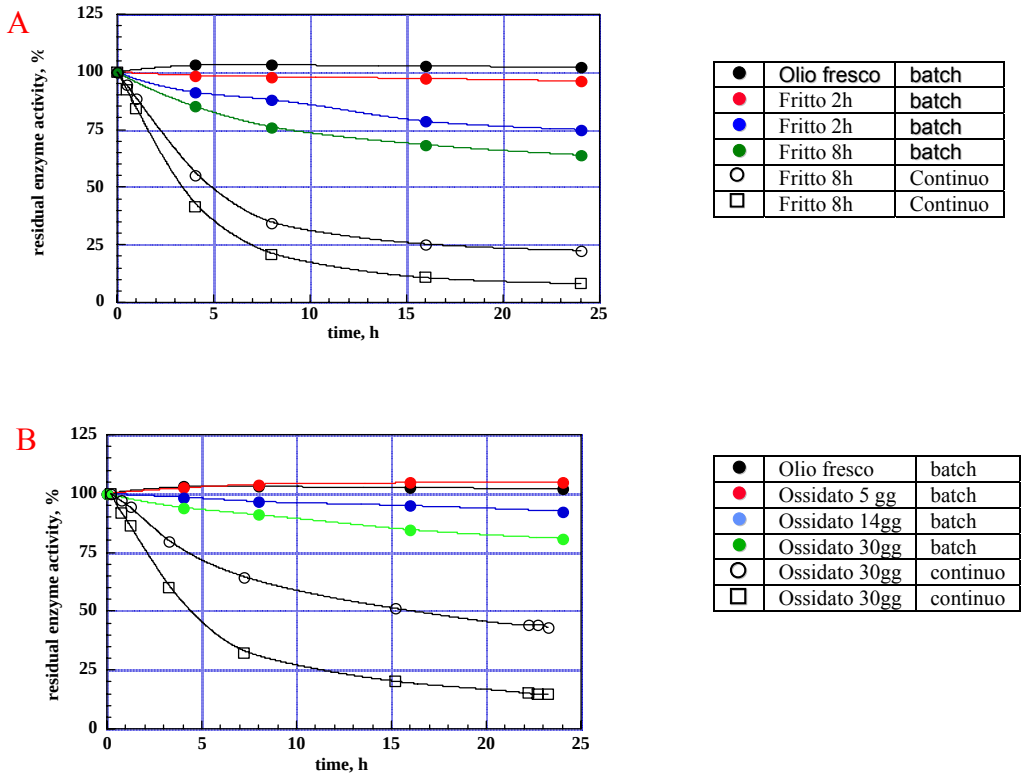


Figura 1: A) effetto della incubazone del Novozyme 435 con olio di soia fritto; B) effetto della incubazione del Novozyme 435 con olio di soia ossidato

Stabilizzazione delle lipasi attraverso mutazioni genetiche

Gli esperimenti riguardanti questa seconda parte del lavoro di Tesi, sono stati effettuati sulla lipasi proveniente dal fungo *Rhizopus oryzae*.
Il lavoro si è svolto attraverso una successione di obiettivi sviluppati in modo coordinato.

- Produzione soddisfacente della proteina di interesse, in termini di resa e di attività enzimatica.
- Localizzazione dei residui di lisina istidina e cisteina, mutazione di tali residui e generazione di genoteche di mutanti.
- Messa a punto di un efficiente metodo per caratterizzare quantitativamente la stabilità delle genoteche prodotte, attraverso il quale selezionare i mutanti con stabilità migliore rispetto alla lipasi wild type.

Produzione
Inizialmente, i geni codificanti direttamente per la prolipasi (proROL) e per la lipasi matura (ROL), clonati nel vettore pET11(+) sono stati espressi nel ceppo BL21 di *Escherichia coli*. Come risultato si è ottenuta la produzione di una proteina insolubile (corpi di inclusione). Si è cercato di ottenere una maggiore produzione di proteina in forma solubile, esprimendo il gene sia attraverso il ceppo di *E. coli* Origami, che agevola la formazione di ponti disolfuro, sia

mediante il ceppo di *E. coli* BL21 Rosetta, utilizzato per l'espressione in *E.coli* di geni codificanti per codoni rari. I risultati migliori sono stati ottenuti con il ceppo Origami, che ha consentito di ottenere sia la proROL, sia la ROL in forma solubile e attiva.
In parallelo, al fine di semplificare il trattamento dei corpi di inclusione, la proROL e la ROL sono state nuovamente clonate in vettori contenenti una coda di istidina nella posizione C-terminale (pET 22-b) e nella posizione N-terminale (pET 28-b). Una volta denaturata la proteina ricombinante con urea, la purificazione dei pellet è stata resa possibile attraverso l'interazione tra la coda di istidina e una resina contenente cobalto quale chelante. Il refolding è poi avvenuto in presenza di cisteina. Anche in questo caso è stato possibile ottenere una proteina attiva. Nonostante la semplificazione indotta da questa metodica sul trattamento dei corpi di inclusione, negli esperimenti successivi si è preferito utilizzare il ceppo Origami per l'espressione della proteina di interesse. Dal momento che la ROL è altamente tossica per le cellule di *E.coli,* la sua produzione è molto bassa, e quindi l'attenzione è stata focalizzata solo sulla proROL, meno tossica e quindi prodotta in quantità notevolmente maggiori.
L'espressione della proROL in Origami è stata poi ottimizzata. È stato studiato l'effetto della temperatura di crescita e della densità di cellule al momento dell'induzione sulla produzione e sull'attività. Inoltre, per semplificare la purificazione, si è clonato il gene della proROL in un vettore contenente la sequenza codificante per una coda di sei istidine e se ne è valutata l'influenza della posizione (N-terminale o C-terminale) sull'attività enzimatica. A tal proposito, sono stati considerati i vettori PET-22b, PET-28b(+) ed un vettore PET-11d(+) modificato, a scopo di confronto. In quest'ultimo vettore è stata inserita la sequenza codificante per la coda di istidina nella posizione N-terminale. Dagli esperimenti è emerso è che la coda di istidina nella posizione N-terminale ha un effetto negativo sull'attività enzimatica della proteina, mentre la coda in posizione C-terminale è ininfluente. Una possibile spiegazione di questo risultato è da ricercare nella possibile interazione della sequenza di istidine in posizione N-terminale con la sequenza pro della proteina, che partecipa al processo di folding e, dunque, influenza la sua attività.
Le condizioni migliori di produzione si sono verificate con il vettore PET-22b, espresso a 20°C con OD_{600} di induzione di 0.5. In queste condizioni, l'attività specifica misurata è stata di 116 U/mg (Di Lorenzo et al., 2005).

Mutazione
Attraverso lo studio della struttura tridimensionale della proROL è stato possibile localizzare i residui di istidina, lisina e cisteina, presenti nella proteina. Dall'analisi, è risultato che le 6 istidine sono tutte vicine al sito attivo, con la sola eccezione di quella in posizione 134, mentre le lisine sono distribuite sulla superficie della proteina. Le cisteine sono coinvolte nei tre ponti di solfuro che stabilizzano la struttura della proteina. Si è deciso, secondo un criterio puramente cautelativo, di indirizzare la mutazione solo su alcuni residui di lisina e su tutti i residui di istidina, trascurando le cisteine, indispensabili per una corretta formazione della struttura secondaria.

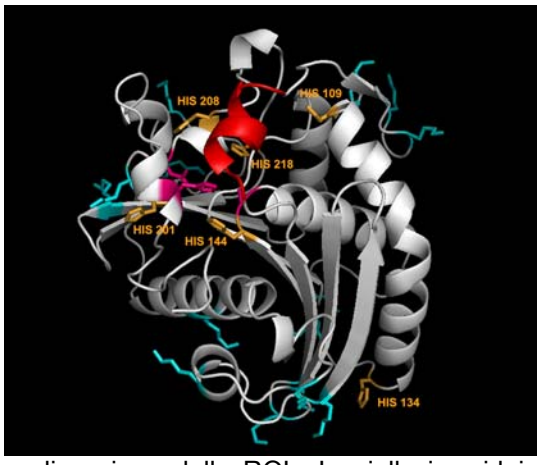


Figura 2: Visualizzazione della ROL. In giallo i residui di istidina in azzurro i residui di lisina, in rosso il lid, in fucsia il sito attivo

La mutazione effettuata è del tipo *site-directed* per saturazione, ovvero la sostituzione di uno specifico residuo amminoacido viene saturata con tutte le possibili combinazioni di triplette (mutazione del tipo NNN). L'adozione di questo tipo di mutazione discende dal fatto che, se da un lato è nota la posizione di un residuo "indiziato", dall'altro poco si sa su quale specifico sostituto potrebbe migliorare le caratteristiche di stabilità della proROL. Al fine di utilizzare le condizioni di espressione già ottimizzate per la proteina wild type, le genoteche di mutanti sono state prodotte nel ceppo Origami. Purtroppo, data la scarsa concentrazione di plasmide ottenuta dopo il processo di mutazione, non è stato possibile utilizzare direttamente questo ceppo, ma Il DNA palsmidico mutato è stato inserito in prima battuta nel ceppo di *E. coli* DH5α. Successivamente, nel caso dei residui di istidina, i cloni in DH5α sono stati inoculati in LB e lasciati crescere per qualche ora prima di estrarre il DNA ed effettuare la trasformazione in Origami. Inoltre, data la vicinanza delle istidine con il sito attivo, per la produzione delle genoteche sono stati considerati solo mutanti attivi, selezionati attraverso un test di attività condotto su piastre solide di agar. Questa selezione iniziale ha consentito di individuare, già in questo stadio, due posizioni particolarmente critiche per la reattività dell'enzima: His144 e His218. Infatti per ciascuna di queste due posizioni, la frazione di colonie attive per piastra è risultata inferiore al 10%, mentre la percentuale più alta di colonie attive si è verificata nel caso della istidina 134, che è la più lontana dal sito attivo.
Per quanto riguarda i mutanti con mutazione relativa ai residui di lisina, l'estrazione del DNA dai cloni in DH5α e la successiva trasformazione in Origami, sono state effettuate direttamente in piastre multipozzetto, dal momento che, in questo caso, la diluizione delle genoteche non poteva essere ovviata da un'iniziale scrematura dei cloni non attivi, visto che i residui di lisina sono distribuiti sulla superficie della proteina e, quindi, sono ininfluenti in termini di attività.
Create le genoteche, il passo successivo è stato quello di adattare l'espressione dei mutanti all'uso di piastre multipozzetto (MTP), tenendo conto dei risultati ottenuti dalla ottimizzazione della produzione in beuta della proteina wild type. A tal proposito, è stata indagata sia la possibilità di espressione in MTP normali (vol. tot 300μl), sia in piastre deep well (vol. tot 2000μl), col risultato che in piastre normali la produzione di proteina è ovviamente più

scarsa, ma è associata ad una maggiore semplicità operativa.

Analisi dei mutanti

Per quanto riguarda il test di stabilità, l'effetto dei prodotti dell'ossidazione dei trigliceridi è stato limitato solo ai prodotti secondari più disattivanti, ed in particolare alle aldeidi. In questa fase esplorativa, non sono stati considerati i prodotti tipici della perossidazione dei trigliceridi, quali MDA e acroleina, a causa della loro scarsa stabilità operativa, né 4-HNE, di costi troppo elevati. Invece, per l'analisi delle genoteche in MTP si è utilizzata un'aldeide diversa, l'ottanale, di costo contenuto e di volatilità relativamente bassa. Questa scelta parte dall'assunto che i meccanismi di interazione della lipasi con quest'aldeide siano gli stessi, viste le analogie delle cinetiche di disattivazione della proteina wild type incubata, rispettivamente con 4-HNE e con ottanale. Nei test di stabilità dei mutanti in MTP, si è utilizzata una concentrazione di aldeide tale da determinare una vita media della proteina wild type di circa 4 ore, associata ad un processo di disattivazione con cinetica del primo ordine. Il confronto tra i mutanti con la proteina wild type è riconducibile al confronto tra le costanti cinetiche. Il seguente schema riassume il procedimento seguito per l'espressione dei mutanti ed il test di stabilità. Ogni piastra contiene la proteina wild type (controllo positivo) ed un bianco (Vedi figura 3).

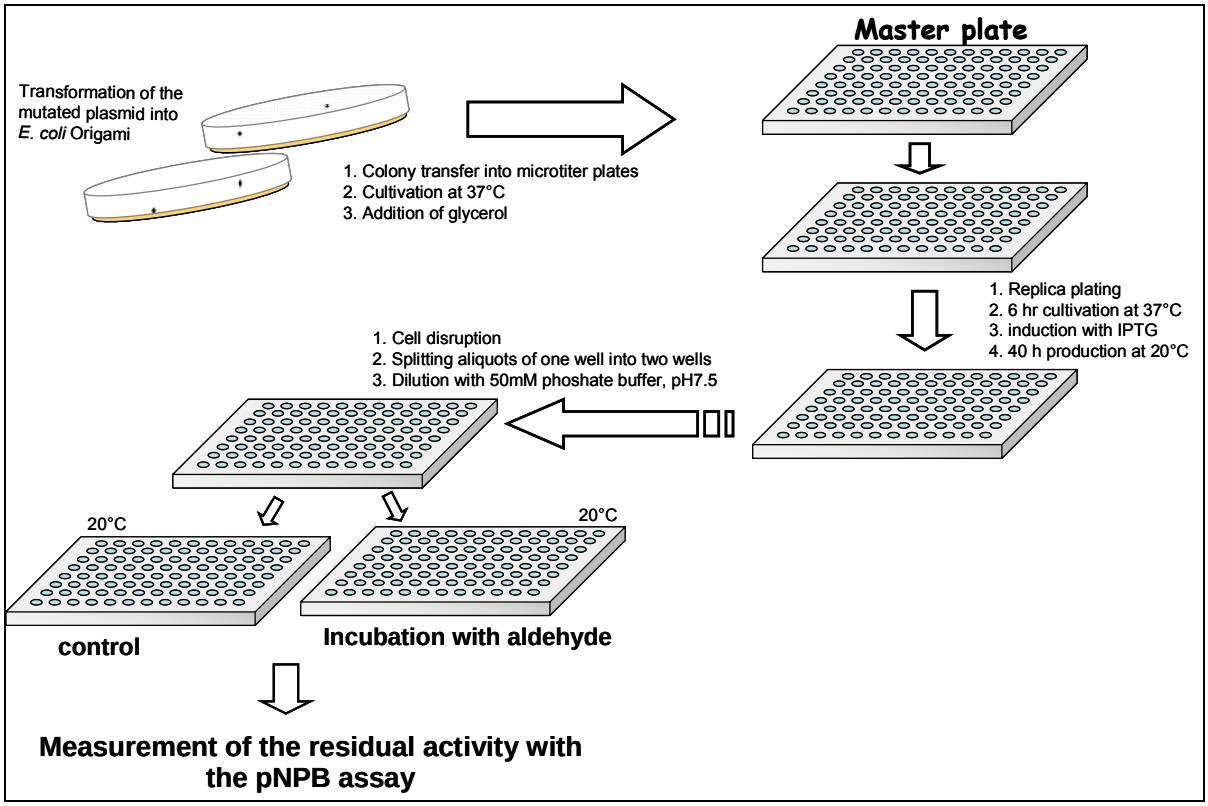


Figura 3 : Schema rappresentativo del processo di produzione dei mutanti e dello screening effettuato

Per quanto si sia ottimizzato il processo in MTP, non si è riusciti ad eliminare una qualche instabilità sperimentale imputabile ai ridotti volumi di lavoro. Dopo una prima selezione in MTP, i mutanti caratterizzati da migliore stabilità sono stati prodotti ed analizzati in condizioni sperimentali più facilmente controllabili

(volumi maggiori), al fine di confermarne le proprietà. Dall'analisi delle genoteche sono emersi alcuni risultati interessanti. Per quanto riguarda le genoteche relative alla sostituzione dei residui di istidina, i risultati migliori si sono ottenuti nel caso dei mutanti His201Ser e His201Ala per i quali si è osservato un aumento del 50% della vita media della proteina (Figura 4). Per quanto riguarda invece le genoteche relative ai residui di lisina, si sono osservati minori incrementi della stabilità della proteina, dell'ordine del 10%. Questi risultati confermano che la stabilità delle lipasi può essere migliorata agendo sui residui coinvolti nel processo di disattivazione, ma mettono anche in risalto che una singola mutazione non è sufficiente a ridurre di molto l'interazione con il disattivante. Sviluppi futuri di questo lavoro prevedono la ricombinazione dei cloni più stabili ed aventi mutazione in posizione diverse, al fine di ottenere mutanti con migliori caratteristiche.

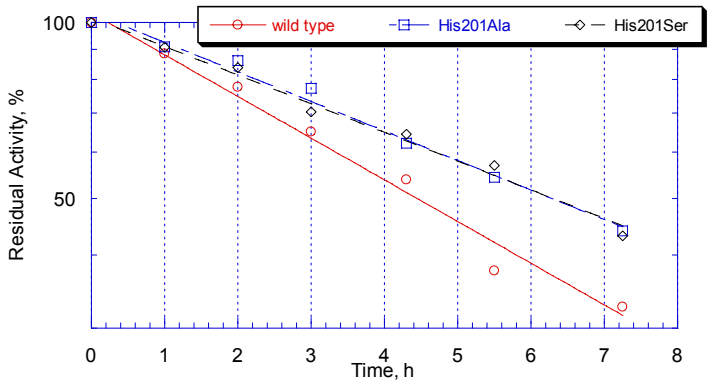


Figura 4 : Confronto tra le cinetiche di disattivazione della proROL wild type (K_d=0.16) e dei mutanti H201A (K_d=0.11) e H201S (K_d=0.11), per effetto dell'incubazione con ottanale, 50mM.

1 INTRODUCTION

1.1 INTRODUCTION TO LIPASES

Lipases (triacyglycerol ester hydrolases, E.C. 3.1.1.3) promote the hydrolysis of acylglycerides and in general of a wide range of low and high molecular weight fatty acid esters, thiol esters, amides, polyol/polyacid esters, etc. Furthermore, in suitable environmental conditions, lipases are capable of catalyzing the reverse reaction of synthesis just as efficiently.
These enzymes are distributed among higher animals, plants and microorganisms, in which the play a key role in the biological turnover of lipids.
Acting as digestive enzymes, lipases facilitate the transfer of lipids from one organism to another and they are also responsible for the deposition and the mobilization of fat that is used as an energy reservoir within the organism. They are also involved in the metabolism of intracellular lipids and, therefore, in the functioning of biological membranes.
The biochemical and physiological properties of lipases have been extensively characterized. Lately, their industrial applications have been investigated, as well (Gandhi, 1997; Kazlauskas et al., 1998; Schmid et al., 1998a; Villeneuve et al., 2000).

1.1.1 STRUCTURAL CHARACTERISTICS OF LIPASES

Lipases are ubiquitous, since they are produced by virtually every living organism. Despite differences in size, sequence homology, substrates, activators, and other properties, most of them adopt a similar core topology, known as the α/β hydrolase fold (Ollis et al., 1992; Gandhi, 1997; Bornscheuer et al., 2002). The interior topology of α/β hydrolase fold proteins is composed largely of parallel β-sheets (at least five in lipases), separated by stretches of α-helix, and forming, overall, a superhelically twisted-pleated sheet. Helical-peptide sections packed on both faces of this sheet form much of the outer surface of the protein (Cygler et al., 1997; Villeneuve et al., 2000).
Despite widely varying degrees of sequence homology between the members of this family, one sequence is exceptionally highly conserved: the pentapeptide Gly-X-Ser-X-Gly (Ueda et al., 2002). The conservation of this serine, and the loss of catalytic activity upon its modification or replacement, argue that this amino acid is crucial to catalysis (Brady et al., 1990). In addition to the catalytic serine, the active centres of all lipases consist of a histidine residue and a negatively charged amino acid (Asp or Glu) the three residues forming a catalytic triad whose topology is highly conserved among the lipases.
The catalytic triad of lipase sits in a hydrophobic cavity in the enzyme. In many cases, as for the *Candida rugosa* lipases (Grochulski et al., 1994), the active site is at the mouth of a deep hydrophobic cavity. The hydrocarbon chain of the substrate fatty acid enters the cavity, leading with its terminal methyl end and ultimately aligning the scissile ester bond with the catalytic residues.

Generally, lipases exhibit low activity against water-soluble substrates, and much higher activity against micelles formed when a substrate exceeds its solubility limit (critical micellar concentration, or cmc). This “interfacial activation” results from a conformational change in the enzyme at the surface of the insoluble substrate. In the inactive state, the substrate-binding region and the active site are covered by a peptide loop of the enzyme, termed “lid”. In this state, the active site is not accessible from the bulk solvent. The lid is able to pivot away from its location over the active site, exposing the substrate-binding and catalytic site, increasing the hydrophobicity of the surface in the region surrounding the substrate-binding cleft, and thereby facilitating interaction between the enzyme and the hydrophobic substrates (Cygler et al., 1997; Shiraga et al., 2002). During the catalytic event an oxyanion hole, critical for the stabilization of the tetrahedral intermediates is formed. Therefore the interaction of lipases with insoluble substrates does not conform to Michaelis-Menten kinetics, but involves a four-step mechanism (Derewenda et al., 1994) (Figure 1.1):

1. Nucleophilic attack of the serine oxygen on the substrate, yielding the formation of a tetrahedral intermediate stabilized by histidine and aspartate
2. Release of the alcohol with the formation of an acyl-enzyme complex
3. Nucleophilic attack, forming another tetrahedral intermediate (water acts as the nucleophile in hydrolysis, whereas an alcohol plays the same role in trans-esterification)
4. Cleavage of acyl-enzyme bond and release of the product (an acid in hydrolysis or an ester in esterification)

Lipases can be classified on the basis of their specificities towards triglycerides: some lipases are non-specific, i.e. they hydrolyze the fatty acids in all three positions of the triglyceride. *sn*1,3-regiospecific lipases can only hydrolyze ester bonds of primary alcohols, so that their application to triglycerides yields *sn*2-monoglycerides.

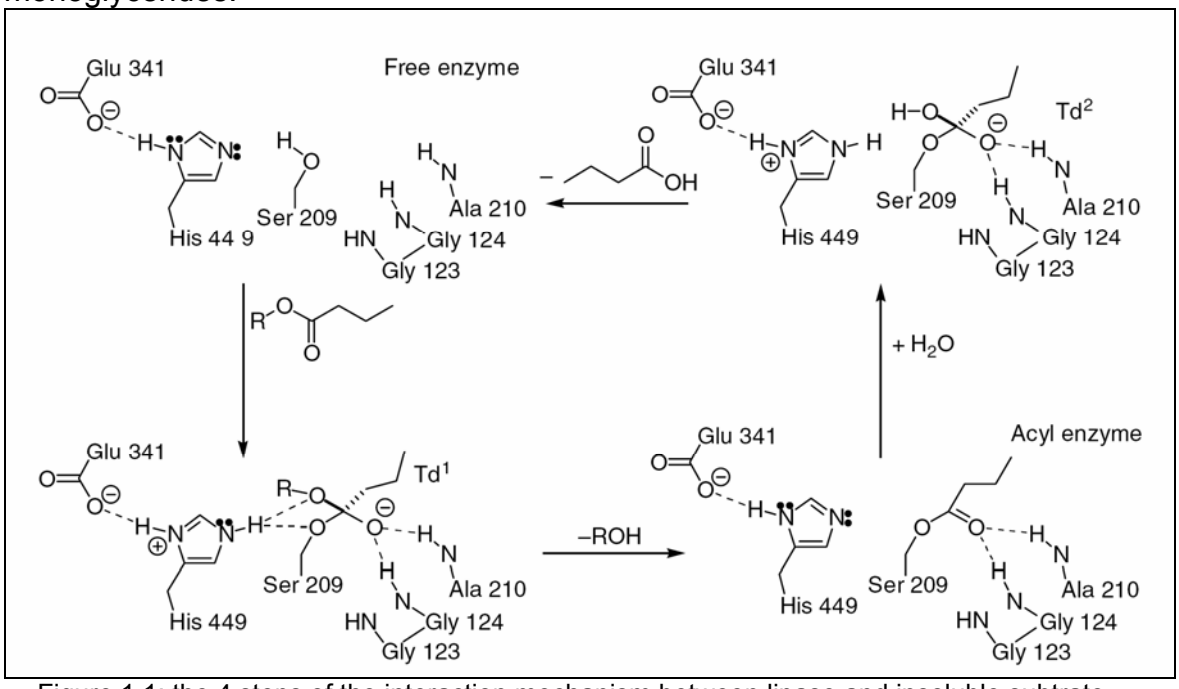


Figure 1.1: the 4 steps of the interaction mechanism between lipase and insoluble subtrate (Bornscheuer et al., 2005)

1.1.2 *Rhizopus* LIPASES

Approximately twenty years ago, the fungus *Rhizopus delemar*, along with other members of the *Rhizopus* genus like *R. niveus* and *R. javanicus*, was reclassified and renamed *Rhizopus oryzae*. All these members contain a gene for an extracellular lipase, whose amino acid sequences are identical. Some minor differences in the properties of those enzymes are probably due to proteolytic cleavage of the same prolipase (Schipper, 1984).
Being fungal enzymes, they are usually glycosylated, but recombinant lipases obtained through heterologous expression in *E. coli* show that the glycosylation is not necessary for lipase activity, both pH optimum (pH 8 for mature lipase) and temperature optimum (30°C for mature lipase) being the same for native and recombinant lipase.
Among the broad number of lipases, enzymes from the genus *Rhizopus* are in particular attractive as potential catalysts in lipid modification processes. They are, in fact, active only against esters of primary alcohols: in the hydrolysis and synthesis of glycerides they are positionally selective acting only at the *sn*1- and *sn*3-location, thus they could change the physical properties of a lipid quite markedly (Holzwarth et al., 1997; Beer et al., 1998; Bornscheuer et al., 2000; Bornscheuer et al., 2003).
Analysis of the DNA sequence indicated that the *Rhizopus oryzae* lipase is initially synthesized as a pre-pro-enzyme, consisting not only of the 269 amino acids of the mature enzyme, but also of a 97 amino acid propeptide fused to its amino terminus and a 26 amino acid-long export signal peptide at the amino terminus of the propeptide (Haas et al., 1999).
The function of the pre-sequence can easily be deduced: it is a signal sequence for membrane translocation. The pro-sequence, on the other hand, seems to have various functions. It contains one Cys-residue, which seems to be vital for the correct formation of the three disulfide bridges formed by the six Cys-residues in the mature lipase. Incorrect or lacking formation of the disulfide bridges leads to a misfolded, inactive enzyme, which is why heterologous expression in *E. coli* so far has only yielded enzyme that was fully inactive or scarcely active, and that had to be refolded using a cumbersome and time-consuming renaturation procedure. The fact that the prolipase, in the absence of substrate, has a higher thermostability than the mature lipase also suggests its role in facilitating the folding process. Therefore, the role of the pro-sequence could be correlated to the one played by the chaperone-like proteins, termed “modular proteins, foldases or activator proteins”, for the prokaryotic lipases, such as *Pseudomonas* lipases. These chaperones in fact assist the correct folding of the protein in the periplasm, and are encoded by complete different genes, located immediately downstream of the lipase structural genes. However, the pro-sequence has been shown not to be required for *in vitro* folding of the mature lipase.(Beer et al., 1996; Shiraga et al., 2002)
Another function of the pro-sequence seems to be the modulation of lipase activity, comparable to the function of zymogens (Takahashi et al., 2001). The mature lipase has phospholipase activity which makes it extremely toxic to the cells (even the *E. coli* cells expressing very low amounts of active mature lipase were lysed). This resulted in surprisingly low transformation frequencies, elevated plasmid instabilities and reduced host viabilities, so it became obvious

that tightly regulated expression plasmids had to be used. pET11d is an example of such an expression vector. The cloned gene is placed behind a bacteriophage T7 promoter recognized only by a T7 RNA polymerase. This polymerase is synthesized by the bacterial host under the control of a *lac* promoter, the cloned gene is also additionally controlled by the *lac* repressor/operator system (Ueda et al., 2002).
As long as the pro-sequence is attached to the lipase, the bacteria continue growing after induction, even with prolipase activities 100 fold higher than those of the lethal mature lipase activities. The lipase activity does not appear to be repressed through the pro-sequence as would be the case for a typical zymogen. Some substrate specificities appear to be altered, though, as the mature lipase could be purified via oleic acid affinity chromatography, while the recombinant prolipase did not bind to this column (Takahashi et al., 2001)

1.2 LIPASES IN THE INDUSTRY

1.2.1 LIPASE CATALYZED REACTIONS

The biological function of lipases is to catalyze the hydrolysis of ester especially long chain triacylglycerols, and glycderol. In anhydrous organic solvents, biphasic systems and in micellar solution the lipase can catalyze the reverse reaction as well (Villeneuve et al., 2000).
The two main categories in which lipase catalyzed reactions may be classified are as follows:

Hydrolysis $RCOOR' + H_2O \rightleftharpoons RCOOH + R'OH$

Synthesis

Reactions under this category can be further separated in:

Esterification	$RCOOH + R'OH \rightleftharpoons RCOOR' + H_2O$
Interesterification	$RCOOR' + R''COOR^* \rightleftharpoons RCOOR^* + R''COOR'$
Alcoholysis	$RCOOR' + R''OH \rightleftharpoons RCOOR'' + R'OH$
Acidolysis	$RCOOR' + R''OOH \rightleftharpoons R''COOR' + RCOOH$

The last three reactions are often schematically grouped together and referred as transesterification reactions.

1.2.2 APPLICATIONS OF LIPASES

The wide range of reactions catalyzed by lipases leads to a very high number of industrial applications according to the kind of transformation considered (see Table 1.1) (Bornscheuer et al., 2003). Examples regarding the reaction of hydrolysis include the employment of lipases in the detergents industry. Lipases are in fact, important components in many detergent formulations for both fabric cleaning and dish washing. Fatty acids and glycerol, generated by lipid hydrolysis, are largely used in soap production. In the leather manufacture, the ability of lipases to break down lipids is exploited to remove residues of fats and protein debris during the processing of hides and skins. Lipases are also utilized in activated sludge and other aerobic waste processes, where a thin layer of fat must be removed from the surface of aerated tanks to permit oxygen transport. In the food industry, lipases can be used in flavour development for diary products, such as butter and margarine, soybean milk preparation, smoked carp processing, vegetable fermentation, production of fat-free meat.
As already mentioned, lipases are also able to catalyze the reverse reaction of synthesis, thus increasing their application range. With esterification reactions, water and ester are produced, while the transesterification processes, such as alcoholysis, acidolysis and interesterification, give rise to alcohol, acid or ester instead of water. Low-molecular weight esters, such as geranyl acetate, isoamyl butyrate and benzyl propionate are mainly used for their flavour and aroma qualities. Polyesters such as polymers of dimethyl terephthalate, vinyl acetate, butyl acrylate, etc. are used in plastics, coatings adhesives, and laminates. Lipases can be used in the synthesis of monoglycerides, sugar esters and fatty acyl amino-esters, which find use as food biosurfactants to stabilize emulsions, such as salad dressings (Nagao et al., 1989). Currently, an important group of lipid products with immense potential for lipase action are synthetic fat substitutes which are in great demand because they can substitute for various high-caloric-fats and oils. The production of mono-,di-, and tri-glycerides of octanoic and decanoic acids leads to dyes and perfume bases in cosmetics, toiletries, and pharmaceuticals. Moreover, the interesterification of fats is attractive for the production of synthetic triglycerides with desirable characteristics, such as the manufacture of a cocoa butter-equivalent from palm oil (Gandhi, 1997; Ghamgui et al., 2004).

Table 1.1: examples of industrial applications of lipases (Villeneuve et al., 2000)

Field of industry	Application	Product
Hydrolysis		
Food (diary)	Hydrolysis of milk fat	Flavouring agent for diary products
Chemical (oil processing)	Hydrolysis of oils and fats	Fatty acids, diglycerides, and monoglycerides Reagent for lipid analysis
Chemical (detergent)	Removal of oil strains, spots and lipids	Detergent for laundry and household uses
Medical	Blood triglyceride assay	Diagnostic kits

Field of industry	Application	Product
Esterification		
Chemical (fine chemical)	Synthesis of esters	Chiral intermediates
		Esters, emulsifiers
Food (chemical and pharmaceutical)	Transesterification of natural oils	Oils or fats (e.g., cocoa butter equivalent)

1.2.3 WHY LIPASES?

Basically all the lipase applications mentioned above could be performed with traditional methods involving chemical catalysts. Fatty acids are normally produced by splitting oils and fats with water in a countercurrent process run at high temperature and pressure. As a practical example, the esterification of fatty acids with glycerol to produce medium-chain triglycerides with the traditional chemical reaction, requires a temperature range of 200-250 °C and pressures of 50 Bar. The chemical reaction is also tedious, non-selective and wastes large amounts of energy. The product obtained has to be further purified by alkaline washings, steam refining, molecular distillation, ultrafiltration, activated carbon treatment, etc. Another example is represented by the hydrolysis of vegetable oils, such as olive oil to produce fatty acids and glycerol. The traditional process requires pressures of 3000-5000 kN/m^2 and temperature of 250°C. The process is clearly energy consuming and because of the high temperatures, gives rise to a variety of undesiderable side reactions, such as polymerization of highly unsaturated fatty acids and production of ketones and hydrocarbons. As a consequence, the resultant fatty acids need to be redistilled to remove by-products (Gandhi, 1997).
On the other hand, the enzymatic catalysis requires ambient pressure and low temperature (40-60°C), allowing much lower energy costs. The reaction vessels need not be corrosion and pressure resistant, since no mineral acids are used as catalysts and working pressure is atmospheric. Moreover, because of the high lipase specificity, regioselectivity and enantioselectivity, few or no side-products are generated. The overall cost is then reduced as the amount of downstream processing required is lower. Furthermore, the lower temperatures ensure minimal thermal degradation and allow the production of fatty acids from unstable oils that contain conjugated or highly unsaturated fatty acids (Kaewthong et al., 2005).

1.2.4 LIPASES IN LOW WATER MEDIA

The reverse synthetic reactions described above need to be performed in low water media, since the substrates required have greater solubility in organic

solvents and the reaction equilibria need to be shifted in the desired direction (in the synthesis reaction water is a product).
Traditionally, enzymology regarded enzymes in their natural environment, i. e. water. Only in the middle 80s it was discovered that enzymes could be utilized in media with a very low water amount (up to 0.01%) as well. In organic solvents not only the enzyme activity is conserved but also some of its properties are improved. In this condition in fact, the protein looses its natural conformational mobility, showing a kind of rigidity which determines a general higher stability and, in particular, an enhanced thermostability, up to temperatures as high as 100°C. The choice of the solvent influences not only the lipase characteristics, but also its reactivity, substrate specificity, regiospecificity and enantioselectivity. Therefore, by means of medium engineering it is possible to optimize the reaction conditions according to specific requirements. The use of organic media leads also to the possibility of recovery and reusability of the lipase without immobilization (Klibanov, 1989; Gupta, 1992).

1.2.5 TWO INTERESTING APPLICATIONS IN THE OLEOCHEMICAL INDUSTRY

1.2.5.1 Structured triglycerides

The oleochemical industry is based on the modification of natural triglycerides coming from plants and animal to obtain products with a wide range of applications. The nutritional value of triglycerides and their physicochemical properties are determined by the fatty acid composition and also by the positional distribution of the acyl groups bonded to the glycerol. The structure of an oil or a fat can be modified by mixing up different types of triglycerides with a chemical modification of the fatty acids (hydrogenation), or with a "rearrangement" of the fatty acids on the fat (interesterification)
Because of their excellent stereo- and regioselectivity, lipases are ideal catalysts for the modification of lipids, whereas these transformation are exceedingly difficult to carry out by chemical synthesis, since a traditional catalyst cannot discriminate among the *sn*1,2,3-positions of a triglyceride. With a *sn*1,3-regiospecific lipase, a specific fatty acid can be introduced in the 2 external positions of a triglyceride, keeping the one in the centre, with a process called transesterification (Casey et al., 1992).
With enzymatic technology it is then possible to produce new fats according to specific requirements. For example it is possible to improve the quality of a fat such as the palm oil which is normally characterized by a low nutritional value but is easily available in high amount. Special triglycerides of the ABA-type containing medium-chain fatty acids (e.g. C8) in the *sn*-1,3 positions and long-chain unsaturated fatty acid (e.g. C16-C22) in the *sn*-2 position are an effective energy source for patients with malabsorption, e.g., pancreatic insufficiency. Polyunsaturated fatty acids (PUFAs) like eicosapentaenoic acid (EPA, $C_{20:5}$) and docosohexaenoic acid (DHA, $C_{22:6}$) have been reported to have several advantages compared to conventional fatty acids, such as reduction of blood

pressure and plasma triglyceride levels, and control of overactive immune functions. DHA is recognized as being important for brain and eye development in infants (Schmid et al., 1998b). Though crude triglycerides can be directly added to commercial products, a growing industrial interest is devoted to the EPA/DHA-enriched triglicerides (Schmid et al., 1999; Harwood et al., 2006). Fish oils are the best-exploited natural source of ω-3 fatty acids and are often used as starting material to produce fats and oils with high nutritional value.

1.2.5.2 Biodiesel

Alternative fuels for diesel engines are becoming increasly important due to diminishing petroleum reserves and the environmental consequences of exhaust gases from petroleum-fuelled engines. Biodiesel (fatty acid monoalkyl esters), has attracted considerable attention during the past decade as renewable, biodegradable, and non-toxic fuel (Varese et al., 1996). It is produced from various vegetable oils and has viscosity close to those of diesel. Biodiesel is derived from triglycerides by transesterification with alcohol (Figure 1.2) and many processes have been developed for its production, among them the utilization of alkali-catalysts gives high yield of products in short reaction times.

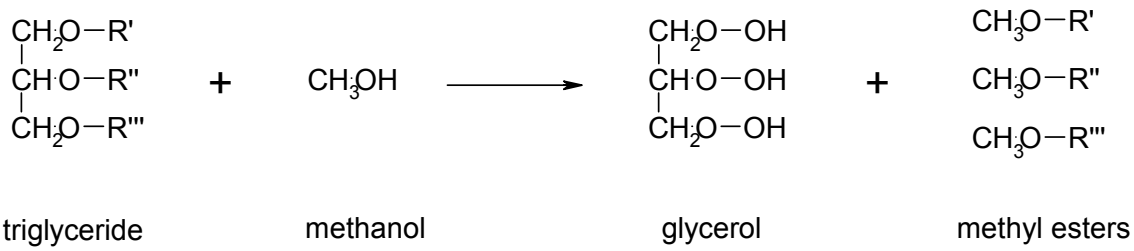


Figure 1.2: biodiesel production from methanol

This process, however, has several drawbacks such as the difficulty of removal of glycerol and the need to separate the catalyst and of wastewater treatments. Several steps, such as the evaporation of methanol, removal of saponified products, neutralization, concentration etc., are needed to recover glycerol as by-product. Recently, enzymatic transesterification using lipases has become more attractive for biodiesel production,since the glycerol produced as a side product can easily be recovered and the purification of fatty methyl esters is simple to accomplish. Because biodiesel fuel has a higher price than petroleum fuel, the use of lower-cost feedstocks would be advantageous. Therefore, the possibility of utilized used fried oils as source of biodiesel is particularly attractive, thus helping also to reduce the cost of wastewater treatment in sewerage systems and generally assisting in the recycling resource. Lard, a co-product of the meat-packing industry, has been also considered as a source for biodiesel (Fukuda et al., 2001; Hsu et al., 2001; Matsumoto et al., 2001; Lee et al., 2002).

1.3 LIPASE DEACTIVATION

1.3.1 LIPASE STABILITY

A serious bottleneck in lipase utilization in non aqueous media is the poor operational stability of this enzyme in the presence of commercial triglycerides. It has been shown that lipases are likely to be deactivated by impurities contained in raw substrates. The products generated by the oxidation of triglycerides strongly affect enzyme stability (Pirozzi, 2003). During storage and use, vegetable oils can easily be subjected to conditions that promote oxidation of their components. The fatty acid composition of a fatty material is an important factor influencing oxidation: the higher the concentration of unsaturated components, the lower the stability against oxidation (Knothe et al., 2003). Fish oil is an example of a very unstable oil, thus making the utilization of this substrate difficult (Ke et al., 1975; Horiuchi et al., 1998). Moreover, many countries import increasing amounts of their supplies from very far areas. As a consequence, oxidation becomes a major problem in the long storage of oils and fats. The possibility of using spent fried oils as substrate for enzymatic biodiesel production is seriously limited by the high concentration of oxidation products formed at the high temperature reached during the frying process.

1.3.2 OXIDATION OF TRIGLYCERIDES

Hydrolysis and oxidation reactions lead to the deterioration of fats and oils, decreasing their quality. Nevertheless while the hydrolysis is quite simple to minimize by acting on the water present in the system, a reduction of the oxidation is more difficult to achieve since the storage and transportation processes would be otherwise very expensive.
The oxidation processes in the open air involve mainly the unsaturated components of fats (Subramanian et al., 2000). The oxidation velocity of fatty acids increases with the unsaturation number. In Table1.2 the relative autoxidation velocities of oleic, linoleic and linolenic acid are compared.
Basically, the relative oxidation velocity of a fat or oil can be evaluated by multiplying the decimal fraction of a single unsaturated acid in it contained by its relative velocity of reaction and afterward by adding the results obtained.
Table 1.3 reports the oxidation velocity values of the main fats and oils measured with this method.

Table 1.2 : Relative oxidation velocities of some of the most common fatty acids in commercial triglycerides (Stirton et al., 1945)

Fatty acids	Velocity of oxidation in presence of O_2
Oleic 18:1 (9*Z*)	1
Linoleic 18:2 (9*Z*,12*Z*)	10
Linolenic 18:3 (6*Z*, 9*Z*, 12*Z*)	25

Table 1.3: Relative velocity of oxidation in the presence of O2 of the main commercial oils and fats

Oils and fats	Relative velocity of oxidation
Soybean	7.0
Sunflower seeds	6.8
Wheel	6.2
Rape seeds	5.5
Cotton seeds	5.4
Peanuts	3.7
Lard	1.7
Olives	1.5
Palm	1.3
Tallow	0.86
Almonds	0.27
Coconut	0.24

The main fatty acids contained in the most common oils and fats are listed in Table 1.4

Table1.4: Principal fatty acids in the triglycerides of commercial oils and fats (Gunstone et al., 1965).

	Fatty acids (double bonds)			
Chain lenght	Saturated	Monounsaturated	Diunsaturated	Triunsaturated
C-4	Butyric			
C-6	Caproic			
C-8	Caprilyc			
C-10	Capric			
C-12	Lauric			
C-14	Mirystic	Mirystoleic		
C-15	Pentadecanoic			
C-16	Palmitic	Palmitoleic		
C-17	Margaric	Margaroleic		
C-18	Stearic	Oleic	Linoleic	Linolenic

	Fatty acids (double bonds)		
C-20	Arachidic	Gadoleic	Eicosadienoic
C-22	Behenic	Erucic	
C-24	Lignoceric		

The percentages of fatty acids in commercial oils are reported in Table 1.5: linoleic and oleic acids results to be in much higher concentration.

Table 1.5: Fatty-acid percentage in some commercial oils (Gunstone et al., 1965)

Fatty acids		**% Soybean oil**	**Rape oil**	**Sunflower oil**	**Sesame oil**	**Palm oil**	**Coconut oil**	**Olive oil**	**Cotton seeds**
Palmitic	C-16:0	7-10							
Stearic	C-18:0	3-5	1-2	3-6	3-8	2-6	1-3	1-3	1-4
Oleic	C-18:1*n*-9	15-33	60-67	14-35	35-46	38-52	5-8	64-86	13-44
Linoleic	C-18:2*n*-6	43-56	19-22	50-75	35-48	5-11	1-3	4-15	33-58
Linolenic	C-18:3*n*-3	0-2	8-9	0,1	trace	0-1	---	0-1	0-2
Arachidonic	C-20:0	0-3	0-1	0-1	0-1	---	---	trace	0-1

The “autoxidation” or “peroxidation” of unsaturated fatty acids is an extremely complex process characterized by two steps (Velasco et al., 2004). The first step, described by the scheme below, regards hydroperoxide formation via a radical mechanism. This process requires an initiator, as shown in Figure 12, concerning the case of the linoleic acid, one of the most common polyunsaturated acids in the oil triglycerides. It begins with the generation of a pentadienylic radical via the extraction of an bisallylic hydrogen atom which reacts with the atmospheric oxygen to produce a cis-trans peroxidic radical (Figure 1.3). The subsequent destiny of this radical depends on several factors, such as the structural characteristics of the unsaturated fatty acid (number and position of the double bonds, etc.) and the chemical environment.
The peroxi- trans-cis radicals formed in this step can lead to the abstraction of a hydrogen atom from another fatty acid molecule (Figure 1 3) or undergo a preliminary isomerisation to give trans-trans type hydroperoxides.

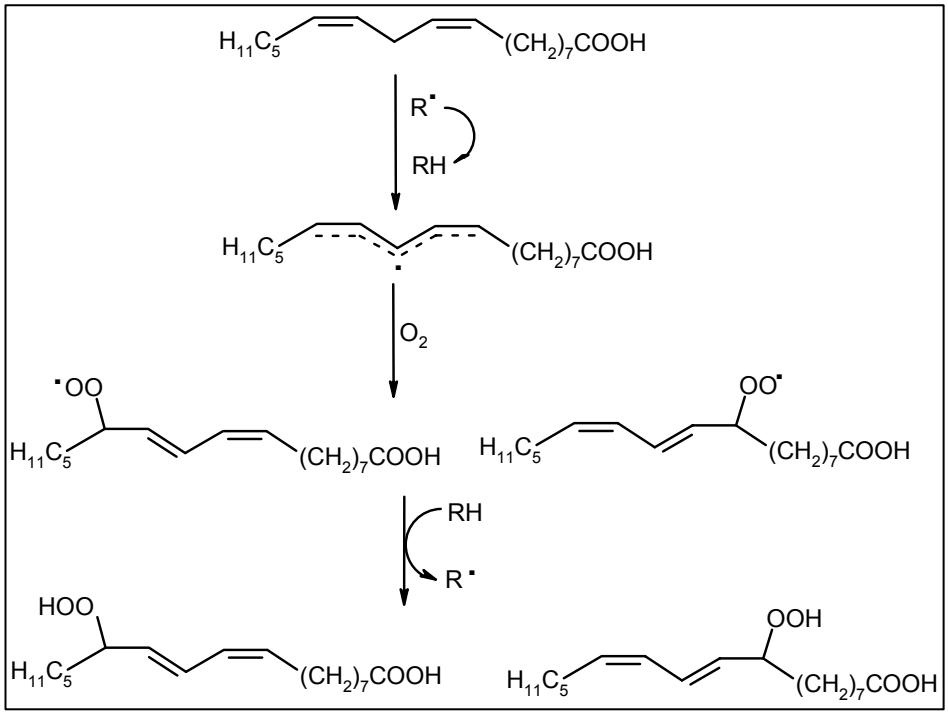


Figure 1.2: Initial steps of the lipid peroxidation process with formation of hydroperoxides with regards to the linoleic acid

The second step of the peroxidation step involves further oxidation processes of the hydroperoxides (13-hydroperoxi-9, 11-octadecadienoic acid, 13 HPODE and 9-hydroperoxi-10,12-octadecadienoic acid, 9-HPODE in the case of linoleic acid) with the formation of epoxidic hydroperoxi- or carbonyl intermediates, leading at the end to the fragmentation of the alkyl chain of the starting fatty acid (Figure 1.4). These processes are strictly influenced by the reaction conditions, for example the presence of metal salts, particularly iron, and they may lead initially to the generation from the intermediate hydroperoxide to an alcoxyl radical that can follow several reactions such as i) hydrogen extraction from a donor, like the starting product or some intermediates, to produce an alcohol; ii) attachment to the double bond of another fatty acid molecule to generate a dimer, iii) formation of an aldehyde by homolytic fission of the α C-C bond (Figure 1.4) (Nawar, 1985)

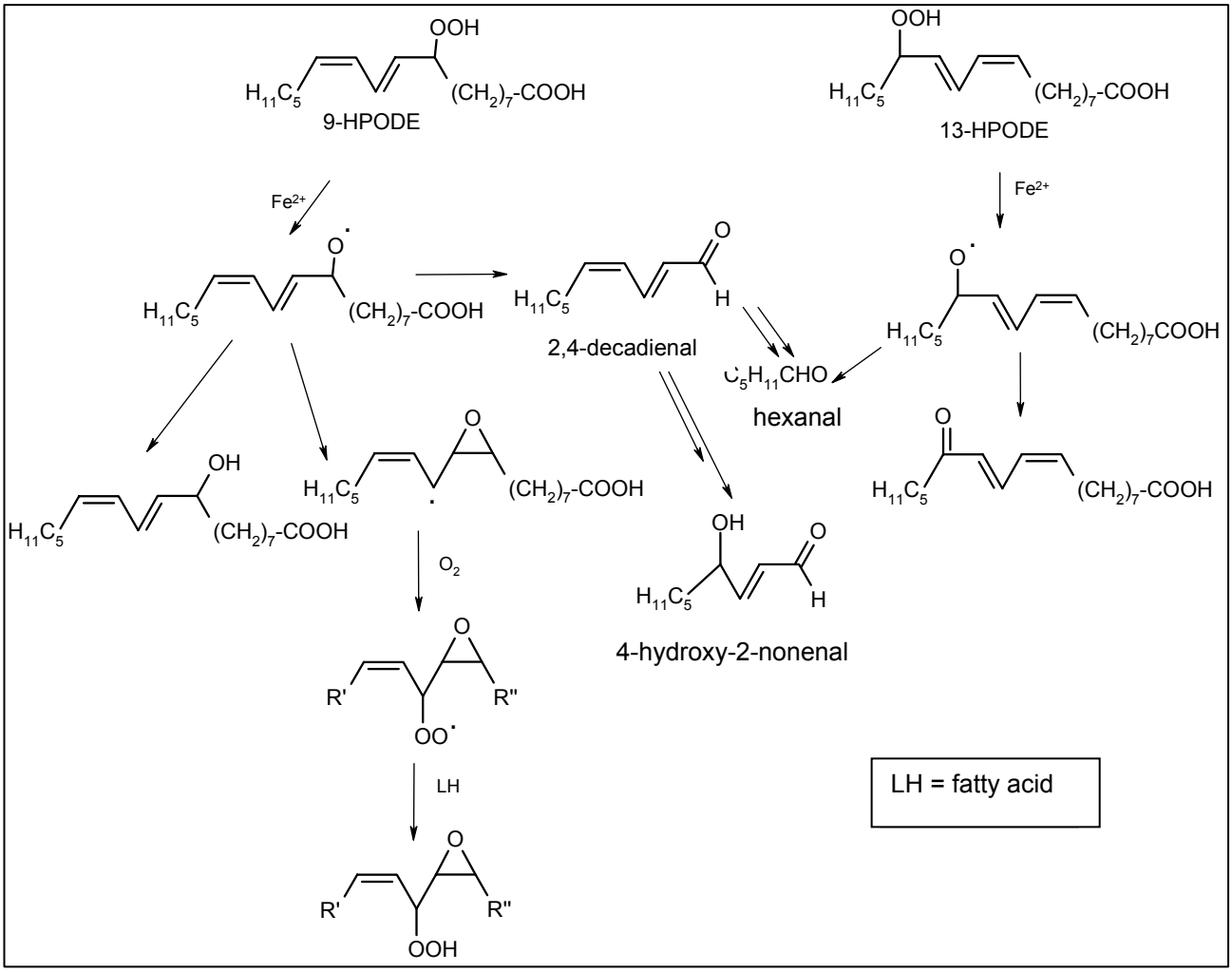


Figure 1 3 hydroperoxides tranformations generated by the acid linoleic peroxidation

In the case of fatty acids containing three or more bonds, such as linolenic and arachidonic acid, the peroxyl radicals not only are converted in hydroperoxides (for example hydroperoxi-5,8,11,13 eicosatetraenoic acid, 15-HPETE in the case of arachidonic acid) from which epoxide and dienoic species could be produced, but they can also form cyclic epoxides and enoperoxides by intermolecular reaction. The latter can undergo fission with the break of the C-C bonds and production of aldehyde products among which 4-hydroxynonenal and malonaldehyde (MDA) (Figures 1.5 and 1.6) (Dahle et al., 1962).

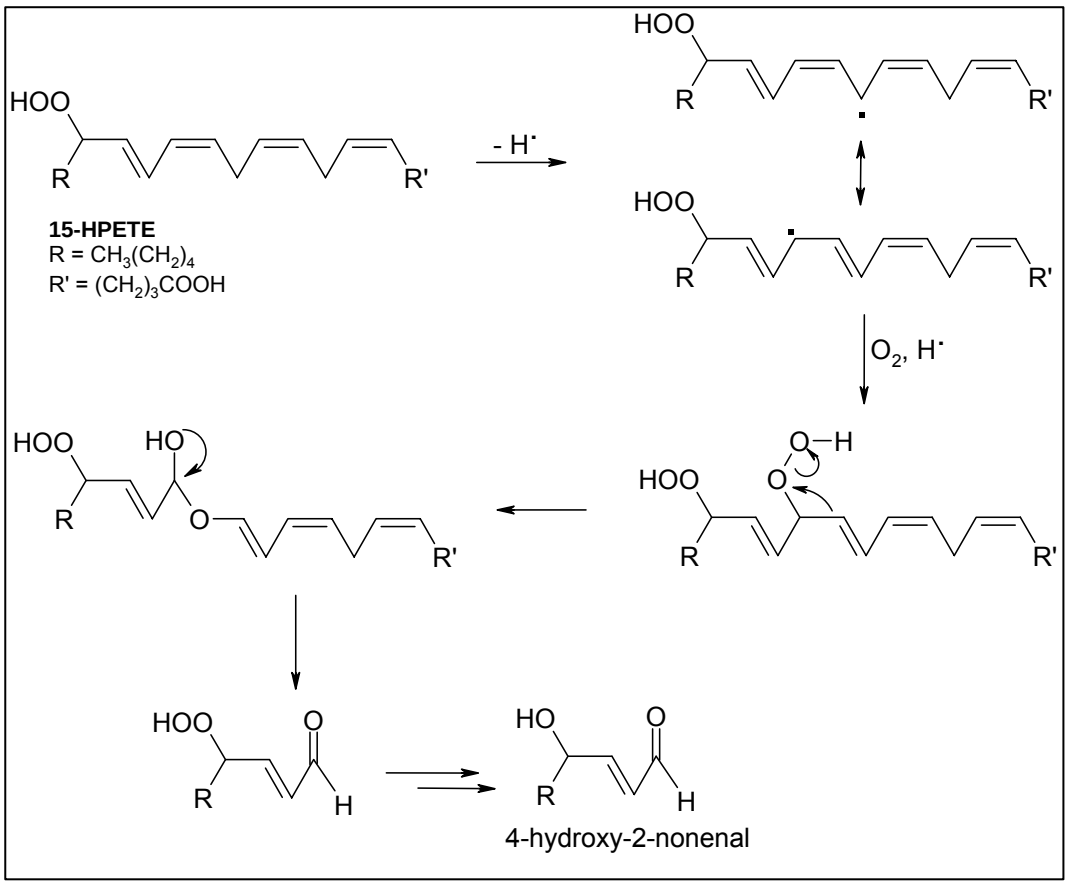


Figure 1.4**:** Transformation of the intermediate peroxides formed by oxidation of fatty acid with three or more bonds, with regards of the arachidonic acid.

By considering how complex these mechanisms are it is clear that the definition of the peroxidation processes that may take place in oils and fats of commercial interests during their storing and manipulation is extremely difficult because of the instability of the reaction intermediates and of the final products, because of the complex effects due to impurities that can allow or inhibit the oxidation, and because of the acceleration of the oxidation process caused by the exposition to light.

Temperature reduction does not lead to variations of the auto-oxidation velocity because the activation energy is relatively low.

An evaluation of the rancidity grade of fats and unsaturated oils is performed by ,measuring the peroxides content which are the initial products of the auto-oxidation process. The peroxides number of a fat (P.V. *Peroxide Value*) is a measurement of its amount of reactive oxygen in mmol of peroxide. During the initial step the oxidation velocity is quite low. The reaction velocity increases rapidly after the formation of a critical quantity of hydroperoxides. The starting step of a relatively low oxidation is called induction period. If the oxidation process is free to follow the further step, the peroxidation value (PV) reaches a maximum and then decays as soon as the peroxides start to break down or polymerize at a velocity higher than the formation one (Esterbauer, 1991; Esterbauer et al., 1993; Velasco et al., 2004)

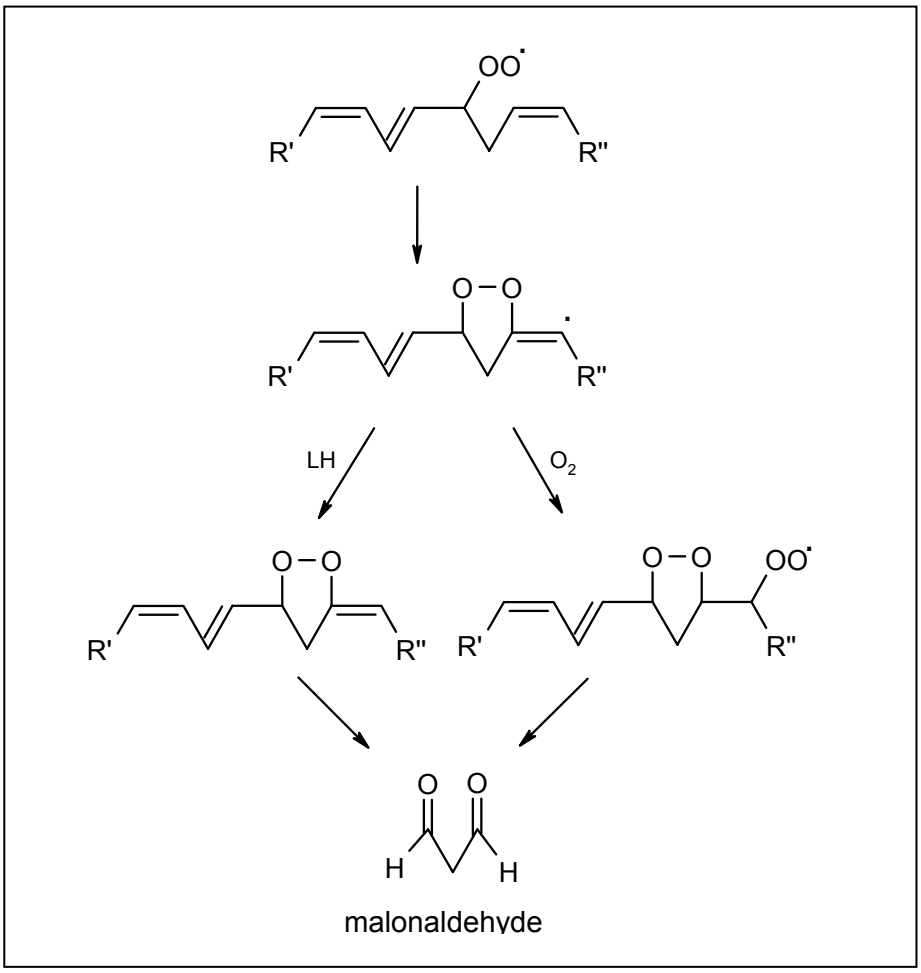


Figure 1.5: MDA formation from peroxiradicals generated by oxidation of fatty acids with three or more double bonds, in the case of arachidonic acid

1.3.3 INTERACTION BETWEEN ENZYMES AND OXIDATION PRODUCTS IN AQUEOUS SYSTEMS

The available information about the mechanism of interaction between triglyceride oxidation products and proteins are based on studies concerning mainly enzymes in animal cells, with the aim of investigating the effects of the membrane lipid oxidation.
The oxidation mechanisms of membrane lipids are analogous to those concerning the triglycerides, described in the previous section. In this case, the oxidation is started by the radicals generated both from the electron release due to reactions caused by metal ions (Fenton reactions), than from the lipid and sugar auto-oxidation. In particular the most common free radicals in the biological systems are the super oxide radical ($O_2^{-\cdot}$) in equilibrium with its protonated form, and the hydroperoxide radical ($HO_2^{\cdot}$). The oxidation of cellular membrane lipids causes several damages, among which loss of the cellular membrane structure and generation of many intermediate and final products virtually able to interact with the biomolecules, causing the deactivation of some

enzymes. These phenomena originate several cellular disfunction diseases like hepatic disfunctions, modifications in cholesterol metabolism, cancer and atherosclerosis (Chio et al., 1969; Agerbo et al., 1992).
The secondary oxidation products showed to be more reactive. Among them, the hydrophilic ones are more harmful since they quickly permeate the cell membrane and interact with biomolecules such as proteins, amino acids and nucleic acids, modifying them irreversibly. In particular it has been found that α,β-unsaturated aldehydes are potent enzymes inhibitors. The mono-unsaturated compounds are much more toxic than the bis-unsaturated aldehyde, despite the larger degree of conjugation in the latter. This behaviour may be due to sterical hindrance, as the dienal molecule possesses a rather rigid planar part. Moreover, among the aldehydes, the ones characterized by the presence of an oxo-group conjugated to the C=C bond (2-alkenals) showed higher reactivity (Agerbo et al., 1992). The reactivity increases with the increasing chain length. Malonaldehyde (MDA), 4-hydroxy-2-nonenal (Stirton et al.) and acrolein are principal products of lipid peroxidation reaction, whose inhibitory effect *in vivo* and *in vitro* has been thoroughly studied.
It resulted that the interaction with enzymes involves specific residues of the protein such as cysteine, lysine and histidine, typically via covalent modifications involving an attack by nucleophilic amino acids on the unsaturated β-carbon, e.g. the sulphydril group of cysteine, imidazole group of histidine, and the ε-amino group of lysine(Kaga et al., 1994; Weber et al., 1995; Bolgar et al., 1996).
In the case of acrolein it has been reported that the nucleophilic addition of the lysine groups at the double bond (C-3) forms a secondary amine derivative, with retention of the aldehyde group. This intermediate further reacts with another acrolein molecule via Michael addition and generates an imine derivative. After aldol condensation followed by dehydration, the reaction is completed to generate a novel acrolein-lysine adduct, N^{ε}-(3-formyl-3,4-dehydropiperidino)lysine (FDP- lysine) derivative. Even in the presence of a large excess of lysine molecules, the secondary amine derivative is scarcely detected and, instead, FDP-lysine is exclusively formed (Chio et al., 1969). Acrolein can also react via Michael addition mechanism with either or both nitrogens of the imidazole ring of histidine to generate $N^{\pi(\tau)}$-formylethylhistidine (Uchida et al., 1998; Kaminskas et al., 2004).
Buko et al., in analysing the inactivation of the cytocrome P-450 in rat liver to cytocrome P-420 caused by MDA, concluded that MDA modifies the surface layer of the microsomal membrane, interacting with surface SH-groups and forming protein cross-linkages. This led to neutralization of the charges located on the membrane surface and to changes to the cytochrome P-450 conformation, which improved a penetration of other MDA molecules to more hydrophobic areas of the hemoprotein . A cross-linking action among proteins, causing their polymerization, has been reported also for acrolein and 4-HNE (Uchida et al., 1998). The protein activity decay of the polymers is related to alteration of functional groups, directly affecting the enzymatic activity, but of course also to a restriction for substrate diffusion. Some studies report that the interaction of aldehydes with the lysine residues can also involve the formation of Schiff base type aldehyde-lysine groups.

1.3.4 LIPASE DEACTIVATION IN ORGANIC MEDIA

Analysis of the mechanism of interaction between oxidation products and lipases in non-aqueous media is a necessary prerequisite for setting up efficient stabilization methods to improve the lifespan of lipases in the presence of commercial triglycerides. Unfortunately, the mechanism of enzyme deactivation in low water media is less known compared to the case of aqueous systems. We will refer particularly to the results obtained from previous experiments, carried out on the immobilized lipase from *Candida cylindracea*, commercially known as Novozym® 435. The inactivating influence of specific compounds in organic solvent, using soybean oil as solvent, has been characterized. To distinguish between the effect of primary and secondary oxidation products, the Novozym® 435 was incubated with soybean containing 13-hydroperoxi-9,11.octadecanoic acid (13-HPODE), obtained from oxidation of linoleic acid and in parallel with the oxidation product mixture obtained from the further oxidation of 13-HPODE in presence of Fe. The result was that the time-dependent inactivation effect was much higher in the case of secondary oxidation products, in particular when the single effect of several secondary products was analyzed, and aldehydes in particular were shown to affect the enzyme stability more.
Moreover, when the Novozym® 435 sulfhydril groups were blocked by pre-treatment with p-hydroxymercuribenzoate (pHMB) the activity loss in the presence of MDA and 4-HNE was reduced, thus suggesting that aldehydes interaction with sulfidryl groups may cause enzyme inactivation. However, lipase activity recovery was not complete even at higher concentration of pHMB, suggesting the involvement of other amino acids such as lysine and histidine in the deactivation process. The involvement of SH-bonds of the protein has been also confirmed by the reduction of the enzyme deactivation when the substrate was pretreated albumin.
It could be concluded then that in organic solvent the inactivation mechanism may be the same than in water systems (Pirozzi, 2003).

1.4 STRATEGIES TO IMPROVE LIPASE STABILITY

1.4.1 MODIFICATION OF LIPASES

1.4.1.1 PHYSICAL MODIFICATION

The physical modification that is currently most applied to lipases is immobilization (Montero et al., 1993; Cao, 2005). This process usually involves attaching the enzyme onto an insoluble support. The use of such a modified lipase is very advantageous since, besides a generally improved operational and temperature stability, it can be easily recovered at the end of the reaction and continuously recycled.

The nature of the support is obviously very important for the efficiency of the immobilization. It has been shown that large pores (> 100nm) are required for the efficiency (activity/loading) to become independent of pore diameter, below this value, the enzyme activity was highly dependent on pore diameter, as a result of limitation in the rate of diffusion of substrate. Generally, hydrophobic materials are better supports: the amount of enzyme adsorbed is in fact usually larger and higher enzyme activities are obtained. On the contrary, hydrophilic supports tend to compete with enzyme for the available water in the reaction. When the lipase and the support are fully hydrated, the hydrophilic supports lead to a higher water concentration in the environment of the enzyme favoring hydrolytic reactions The most used supports are ion exchange resins, celite and biopolymers such as Accurel EP100 (Lee et al., 1998). The immobilization of a lipase can preserve it from deactivation by chemicals produced during the enzyme-catalyzed reaction. Kaga showed that the immobilization of *Candida rugosa* lipase could preserve it from deactivation by aldehydes produced in (trans)esterification reactions, by protecting the lysine residues and avoiding the Schiff-base formation in a Maillard-type reaction (Kaga et al., 1994).
The enzyme encapsulation onto organic host materials, using sol-gel processing, demonstrate to provide stabilization towards external regents (Dunn et al., 1998).
Actually lipase immobilization, either with physical methods or with chemical methods (discussed in Section 1.4.1.2) finds application in oil and fat bioconversion and many publications deal with immobilized lipase-catalyzed structured triglyceride synthesis.

1.4.1.2 CHEMICAL MODIFICATION

Chemical modification of lipases using various types of modifiers can improve their native properties and endow them with useful new functions. The procedure involves either chemical modification of amino acid residues, covalently coupling the enzyme to a water-insoluble material, lipase attachment to a matrix by covalent bonds, or formation of a cross-linked lipase matrix using various bi-functional reagents.
Examples of chemical modification of amino acid residues is represented by the modification of lysine residue with pyridoxal-5'-phosphate (PLP) and of disulfide bonds with dithiothreitol (DTT), which were effective on the esterification activity without affecting the hydrolysis activity (Kawase et al., 1989). Some authors, considering the mechanism of reductive alkylation of the lipase amino groups *via* the Schiff base formation with an aldehyde, performed a chemical modification of lipases with *n*-butyraldehyde, isobutyraldehyde and acetone, thus obtaining about a 50% increase in the V_{max} value.
With the covalent coupling method, the lipase is covalently bound to a water insoluble material via reaction between functional groups of the protein and reactive groups on the activated material. Examples include PEG-modified lipase where PEG is a polymer which has the advantage of having an amphipatic nature; fatty acid-modified lipase, prepared by reacting amino

groups in the enzyme using a water-soluble acylating reagent, such as dimethylsulfoniophenyl (DSP) ester; amidinated lipase obtained with imidoester hydrochlorides of different hydrophobicity and detergent-lipase using a synthetic detergent, such as didodecyl glucosyl glutamate. These methods demonstrated to increase the catalytic efficiency in esterification reactions, the thermostability and solvent-stability.(Tsuzuki et al., 1991; Murakami et al., 1993)
The attachment of lipases to a matrix by covalent bonds is not as common as the physical adsorption, but presents the advantages of avoiding the desorption phenomenon. As a consequence this kind of immobilization is very solid and gives an high stability to the protein to extreme conditions (pH range, temperature). The covalent attachment has to be directed to those amino acids not involved in the catalytic machinery, but this may be difficult to achieve and commonly part of the enzyme initial activity is lost. Typically prior to the covalent attachment the support must be activated by the incorporation of a chemical group capable of reacting with the side groups of protein. One of the most common compounds is glutaraldehyde, which easily polymerizes to give polymers containing ω unsaturated aldehyde functions which can react with amino groups of the support. Other activating agents are carbodiimides, which are used to create a link between carboxylic functions of the support and amino group of the protein. Since many of the covalent protein interactions with the support involve particular protein amino acids groups, such as lysine groups, the lipase stability against attacks from triglycerides oxidation products increases. However, when the *Rhizopus oryzae* lipase was covalently attached to alumina by treatment with γ–aminopropyltriethoxysilane in acetone, followed by reaction with glutaraldehyde, only 23% of its original activity was retained.
Another process for the protein stabilization is the formation of a cross-linked lipase matrix using various bi-functional reagents. The procedure includes a preliminary step involving immobilization on an ion exchange resin followed by treatment with a buffered solution of glutaraldehyde to form a Schiff base as a product of reaction with amine residues. The resulting mixture is then treated with hydrogen sulphide solution to reduce the excess Schiff base and glutaraldehyde (Villeneuve et al., 2000).
In recent years, CLEAs, prepared by chemically cross-linking protein aggregates, have attracted increasing attention, due to their simplicity, broad applicability, high stability and high volume activity (Cao, 2005).

1.4.1.3 PROTEIN ENGINEERING

Tailor-made biocatalysts can be created from wild-type enzymes by protein engineering using computer-aided molecular modelling and site-directed mutagenesis, or by directed evolution techniques (Bornscheuer et al., 2001). As opposed to more traditional forms of mutagenesis (e.g. chemically induced), these changes in the genetic material can be localized to precise sites or regions within the protein. Rational design usually requires both the availability of the structure of the enzyme and knowledge about the relationship between sequence, structure and mechanism/function. However, by using molecular

modelling, it has been possible to predict how to increase the selectivity, activity and the stability, even if there were no structural data available and the structure of a homologous enzyme was used as a model (Kazlauskas, 2000). Useful strategies such as reinforcement of a promiscuous reaction, change of enzyme mechanism, substrate specificity, cofactor specificity, enantioselectivity and stability, as well as the elucidation of enzyme mechanisms have been reported(De Sanctis et al., 1999; Cedrone et al., 2000) Rational protein design has successfully been used for stabilization of lipases towards thermoinactivation, protease attacks and oxidation (Eijsink et al., 2005). Anyway, this is a restrictive method of enzyme improvement because it relies on a preconceived decision about what part(s) of the enzyme determine the property of interest. As an alternative to rational design, directed evolution can be employed. It involves either random mutagenesis of the gene encoding the target protein, or recombination of gene fragments. Libraries created in this way are then usually assayed using high-throughput technologies to identify improved variants. Both random approaches can be repeated by using sequential cycles of mutagenesis and screening to create successive generations of improved variants. A combination of both methods is also attractive, for example using saturation mutagenesis on a structure-based determined specific point of the enzyme, which is mutated with all the possible combination of triplets (NNN type mutagenesis).

1.4.2 SUBSTRATE TREATMENT

Another interesting approach is the possibility of reducing the lipase deactivation by pre-treating the substrate (i.e. oil or fat) to remove the inhibitory components, such as secondary oxidation products of triglycerides. Traditional oil refinement processes are neutralization, decolorization and deodorization. All of them could reduce the concentration of the triglycerides oxidation products but do not have however a real specific effect on them and therefore do not guarantee the elimination of those compounds which are very reactive like MDA, 4-HNE and acrolein. Moreover these methods are relatively expensive if considered in large scale (see Table1.6), also because a loss of oil in each process cannot be avoided.

Table 1.6: indicative prices of the most traditional processes of oil refinement (Data supplied by Casa Olearia di Monopoli, Bari)

Purification step	Cost (Cent Euro/lt of oil)
Neutralization	6
Decolouration	4
Deodorization	25
Total	12.5

It should be also considered that the characteristics of an edible oil are not necessary those needed to reduce enzyme deactivation effect. one could remove only some components, without considering those that do not affect enzyme stability but only influence the oil nutritional characteristics such as taste. For example MDA and 4-HNE are not enough volatile to be removed

during the deodorization, neither enough polar to be removed with a simple water extraction.
Alternative methods include membrane processing and filtration with active filters as diatomaceous earth, magnesium and calcium silicates, zeolites and alumina (Subramanian et al., 2000). Oils could be treated with specific adsorbents which compete with lipase in the interaction with aldehydes. Previous experiments on oil pre-treatment with albumin led to satisfactory results but the process cost rules out any possible industrial application. It is then fundamental to develop methods to regenerate and recycle the albumin, in order to reduce the overall process cost.

2 OBJECTIVES

This PhD thesis has the aim of improving the stability of lipases in the presence of commercial triglycerides for industrial purposes, by acting on particular residues of the protein, such as lysine, cysteine, histidine, that are reported to be involved in the deactivation process.
Preliminary studies for a tentative stabilization of the commercial lipase Novozym® 435 with a site-blocking reagent methodology that involves these residues, will serve as basis for a subsequent part of this Thesis, which deals mainly with the lipase from the fungus *Rhizopus oryzae*. In particular, the possibility of improving the stability of this lipase by substituting the target residues with an appropriate mutagenesis strategy, will be investigated.

3 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 LIPASE ORIGIN

Lipase B from *Candida antarctica*, immobilized on anion exchange resin beads, (Novozym® 435), was a gift from Novozymes (Bagsvaerd, Denmark)

The lipase utilized for the genetic engineering comes from the fungus *Rhizopus oryzae*. The genes coding directly for the pro- and mature form of this lipase, cloned in pET11-d(+) and expressed in *E. coli* BL21 (Figure 3.1), were kindly provided by M. Haas (United States Department of Agriculture, ERRC, ARS, Wyndmoor, Pennsylvania).

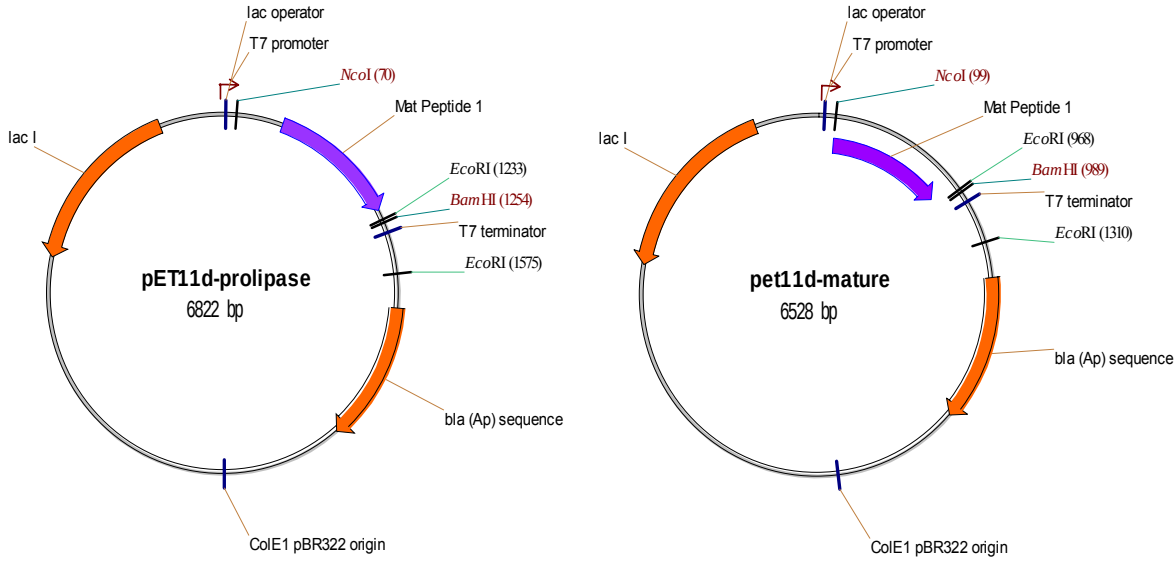


Figure 3.1: pET11 constructs of the prolipase and mature lipase from *Rhizopus oryzae*. The vector pET11d carries an ampicillin resistance and an IPTG inducible promoter system.

3.1.2 BACTERIAL STRAINS, PLASMID AND GROWTH CONDITIONS

E. coli strain DH5α [*sup*E44 Δ*lac*U169(ϕ80*lac*ZΔM15)*hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1] was used as host for genetic manipulation of plasmids. *E. coli* BL21(DE3) [F^- *omp*T *hsd*S_B($r_B^-m_B$) *gal dcm*(DE3)], Rosetta(DE3)[F^- *omp*T *hsd*S_B($r_B^-m_B$) *gal dcm*(DE3) pRARE^2 (Cm^R)] and Origami(DE3) [Δ*ara-leu*7697 Δ*lac*X74 Δ*pho*APv*u*II *pho*R *ara*D139 *ahp*C *gal*E *gal*K *rps*L (Sm^R)$^4F^1$[*lac*$^+$(*lac*I^q) *pro*] *gor*522::Tn10 (Tc^R) *trx*B::kan (DE3)] strains were used for the overexpression of proteins. Plasmids pET-11d, pET-15b, pET-28b(+) and pET-

22b(+) (Novagen) were used for cloning and for expression. The *E. coli* strains were grown in Luria-Bertani (LB) medium containing 100mg/l ampicillin, 30mg/l Kanamycin, 10mg/ml tetracycline, 34mg/ml chloramphenicol, as required. Transformation of *E. coli* was performed as described previously.

3.1.3 CHEMICAL REAGENTS

The stability screening assay were performed with the following pure aldehydes (99% purity, purchased by Sigma): Hexanal, heptanal, octanal, trans-2-nonenal, malonaldehyde bis-(diethylacetal) and acrolein.

4-Hydroxynonenal was provided by Cayman Chemical, as a 10mg/ml solution in ethanol.

3.2 METHODS

3.2.1 MOLECULAR BIOLOGY METHODS

3.2.1.1 SUBCLONING INTO pET VECTORS

To produce N-terminus His-tagged coding genes the pro- and mature lipase genes were amplified by PCR by using respectively the primers 1F and 2F to substitute the *Nco*I site with a *Nde*I site and the T7 terminator primer (see table 3.1 for primer sequences). For the construction of C-terminus His-tagged coding genes the amplification by PCR were also performed with the primers 1F and 2F but the reverse primer in both cases was the 3R primer in order to substitute the stop codon into an *Eco*RI site.

Table 3.1: Primers used for the PCR -subcloning fo the ROL-coding genes. The introduced restriction sites are shown underlined.

NAME	SEQUENCE
1F	5’ AAGGAGATATCATATGGTTCCTGT 3’
2F	5’ GAGATATCATATGGATGGTGGTA 3’
3R	3’ ACAAACTTCTTAAGAACTGCACAA 5’

For every PCR reaction , the following reagents were combined in a sterile PCR tube:

	Volume (µl)
sterile H_2O	35
10x Taq buffer	5
$MgCl_2$	5
dNTP mix	1
primer forward	1
primer reverse	1
Taq polymerase	1
template	1

These were incubated in the thermocycler using the following temperature program:

Number of cycles	**segment**	**t (min)**	**T (ºC)**
1	1	5	96
29	1	1	94
	2	1	43
	3	1*kbp	72
1	1	10	72

*time calculated as 1min for each 1kbp

The PCR products were purified and digested with *Eco*RI and *Nde*I and ligated into plasmid vectors pET-28b(+) and pET-22b(+), previously digested with the same endonucleases (Figure 3.2 and 3.4).
pET-15b was considered as an ampicillin selectable vector for the expression of the protein in *E. coli* Origami (Figure 3.3). The pET-28b(+) constructs carrying the pro- and mature lipase genes were digested with *Nde*I and *Xho*I, and the DNA fragments corresponding to the lipase and prolipase were purified and ligated into the empty pET-15b vector digested with the same enzymes.
To evaluate the influence of the His-tag in the expression, the prolipase gene preceded by a His-tag sequence was cloned into pET-11d(+). For this purpose the pET-15b vector containing the prolipase gene was digested with *Nco*I and *Bam*HI, purified and introduced, by a ligation reaction, into the pET-11d(+) vector digested with the same restriction enzymes in the same conditions (Figure 3.5).
To visualize DNA fragments, the DNA preparations were mixed with 1/6 10x loading buffer and separated on a 0.8% (w/v) agarose gel containing 0.01% (w/v) ethidium bromide.

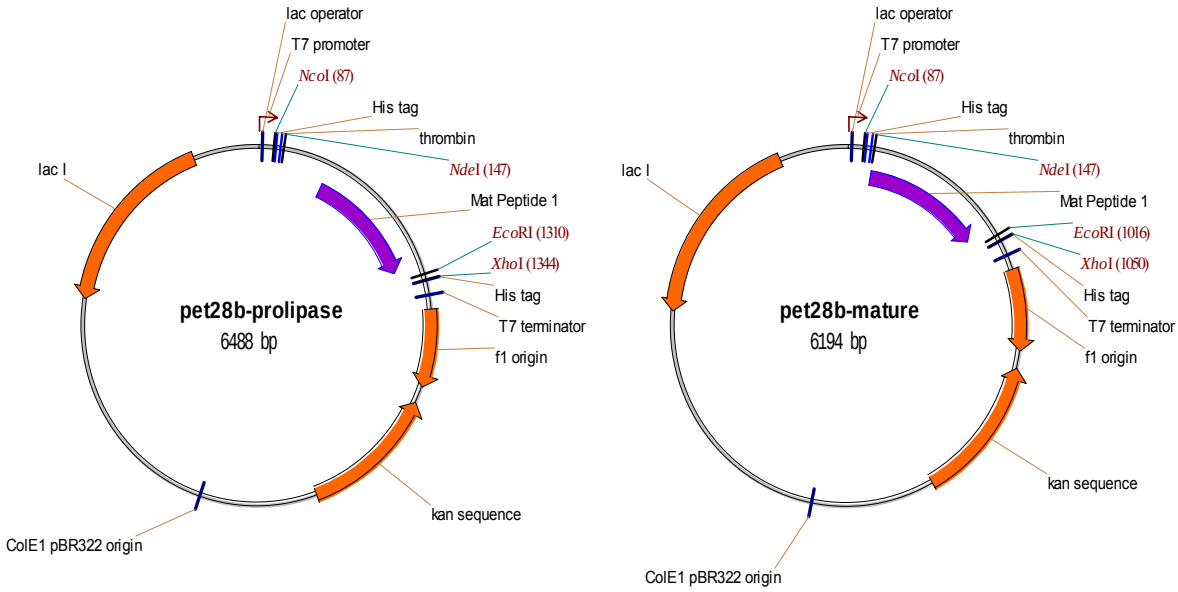


Figure 3.2: pET28b constructs. This vector contains a kanamycin resistance gene, an IPTG inducible promoter system and a N-terminal His tag. It is not suitable for expression in *E. coli* Origami which already carries a kanamycin resistance and it was only used to purify the His-tagged enzyme in insoluble form.

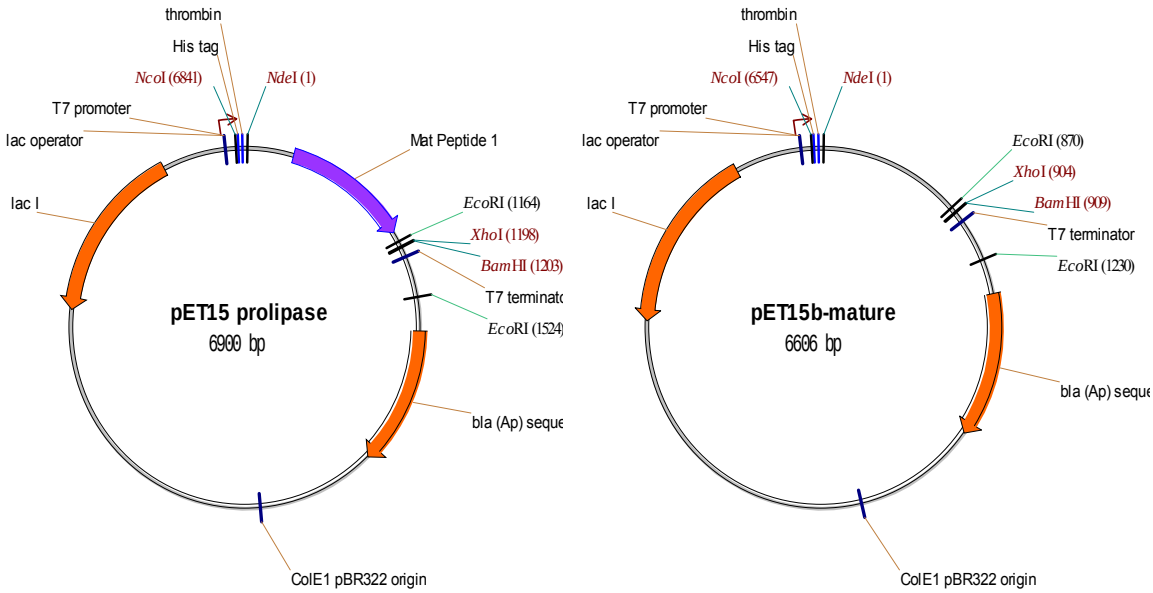


Figure 3.3: pET15 constructs. The vector pET15 carries an ampicillin resistance, an IPTG inducible promoter system, and an N-terminal His-tag.

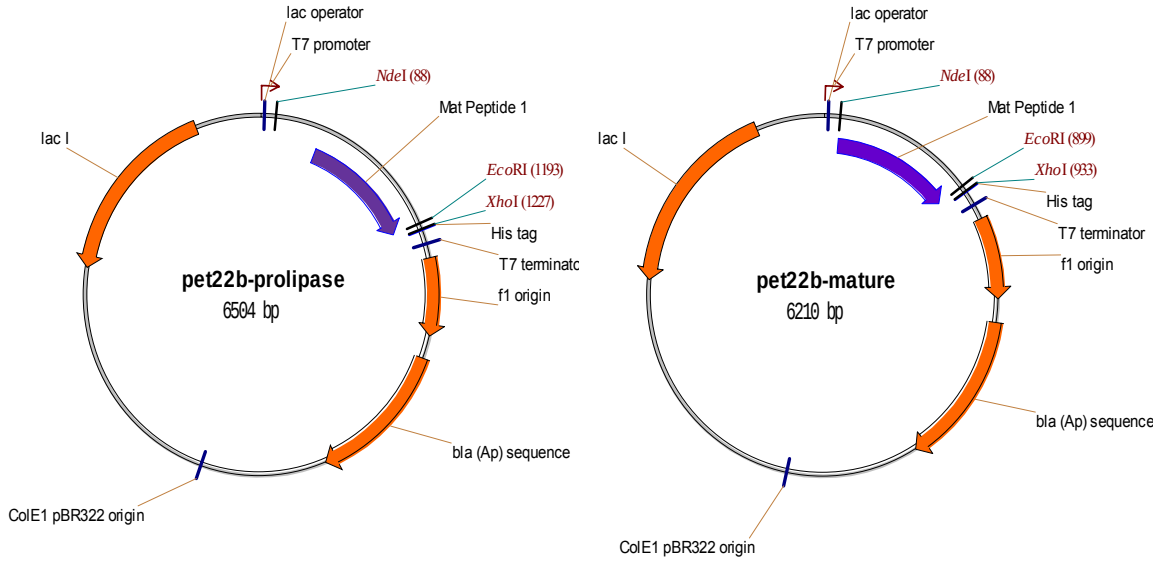


Figure 3.4: pET22 constructs. The vector pET22 carries an ampicillin resistance, an IPTG inducible promoter system, and a C-terminal His-tag.

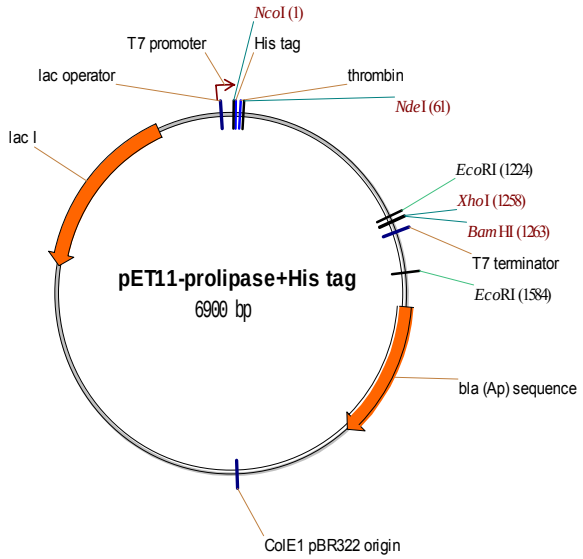


Figure 3.5*:* N-terminus His-tagged prolipase-coding gene cloned in plasmid pET11-d

3.2.1.2 PREPARATION OF COMPETENT CELLS

Escherichia coli cells were made competent chemically using the rubidium chloride method. A 100 ml culture of the target strain in SOB medium (Table 3.2) was grown until a cell density equivalent to OD_{600}=0.4. The culture was then chilled to 4 ºC, centrifuged (20min, 800 xg) and the pellet resuspended in 16ml filter sterilized Tfb1 buffer, incubated on ice for 15 min and centrifuged again (20min, 800g). The resulting pellet was then resuspended in 4 ml of filter sterilized buffer Tfb2, incubated on ice for 15 min, aliquoted (50μl) in cold

Eppendorf tubes and stored at -80°C. Tables 3.3 and 3.4 reports the Tfb1 and Tfb2 buffer compositions.

Table 3.2: SOB medium, quantities referred to 1lt. Before autoclaving, the pH is adjusted to 7. Sterile filtered MgCl2 is added after autoclaved.

Trypton	20g
Yeast extract	5g
NaCl	0.5g
KCL	0.4mM
MgCl2	10mM

Table 3.3: composition of the Tbf1 buffer

Tbf1	
RbCl	100mM
MnCl2	50mM
Potassium acetate	30mM
CaCl2	10mM
Glycerol	15% final
Water	To final volume

Table 3.4: composition of the Tbf2 buffer

Tbf2	
0.2M MOPS	10mM final
RbCl	10mM
Glycerol	15%
Water	To final volume

3.2.1.3 PLASMID ISOLATION

The overnight culture of bacteria carrying the desired plasmid was centrifuged (13000 rpm, 10 min, 4°C) and the pellet treated according to the protocol in the QIA Prep Miniprep kit (QIAGEN, Hilden, Germany). The purified plasmid DNA was eluted in 50 µl of elution buffer as indicated by the manufacturer's instructions.

3.2.1.4 PLASMID PRECIPITATION AND VERIFICATION

The plasmid preparation from the Miniprep kit, was diluted to 180 μl with ultrapure water, and precipitated by adding 20 μl 10M ammonium acetate and 500 μl isopropanol, mixing thoroughly and freezing for 1 h at -80°C. The sample was then centrifuged at 800 xg and 4°C for 30 minutes, the supernatant carefully discarded, and the pellet washed with 70% (v/v) ethanol at -20°C, centrifuged again for 15 minutes at 800 xg. The supernatant was again discarded carefully and the pellet air-dried.
DNA was sequenced by automatic methods (MWG Biotech, Martinsried, Germany), using the T7 forward and T7 terminator as sequencing primers.

3.2.2 LIPASE PRODUCTION IN FLASKS, PELLET TREATMENTS AND STORAGE

3.2.2.1 OVEREXPRESSION OF ROL AND PROROL, SEPARATION OF THE SOLUBLE AND INSOLUBLE FRACTION

The *E. coli* strains harbouring the pET recombinant plasmids were grown in 100 ml LB medium supplemented with the required antibiotics, at a temperature optimized according to the specified requirements, indicated in the results section. IPTG (isopropyl-β-D-thiogalactopyranoside) was used as inducer to a final concentration of 0.1 mM. At different time intervals, aliquots (equivalent to 5 ml at OD_{600}=1) were centrifuged 10 min at 800 xg to harvest the cells. The cells were then resuspended in 300μl 50 mM phosphate buffer, pH 7.5, and disrupted by sonication (1 min, 50% pulse). The soluble fraction and the particulate material were separated by centrifugation as shown in Figure 3.6, and 10μl from these preparations were analyzed by SDS-PAGE on 12.5% polyacrylamide gels and stained with Coomassie blue, as described by Laemmli (Laemmli, 1970). The soluble fraction was also subjected to activity measurement by using the pNPB assay (see Section 3.2.5). The protein concentration of the samples was determined according to Bradford (Bradford, 1976).

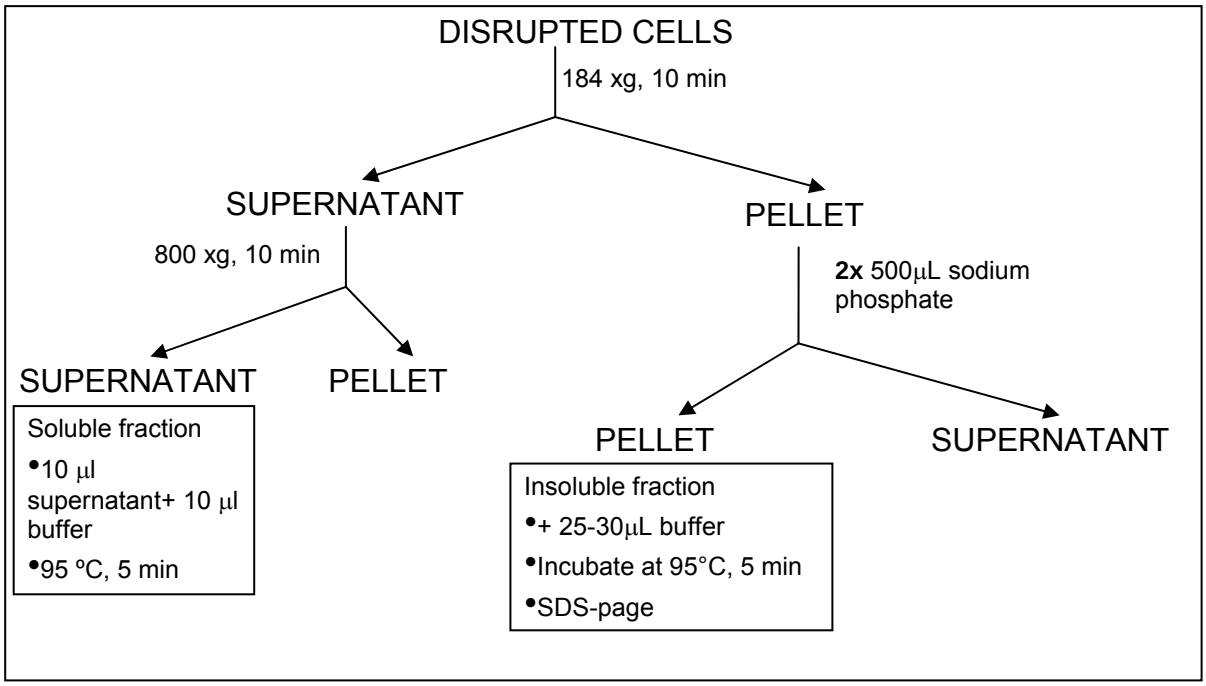


Figure 3.6: Protocol to separate the soluble and insoluble fractions of crude cell extracts

3.2.2.2 PROTEIN STORAGE

The pellet obtained from all the cultivation was resuspended in 3ml of 50mM phosphate buffer, pH 7.5 and sonicated for 10 minutes. The lysate was then centrifuged for 10 minutes at 800 xg and the supernatant poured in a Petri dish. After freezing for 1 hour at -80°C, the supernatant was lyophilized overnight and stored at -20°C.

In addition to the lyophilisation the *E. coli* Origami cells were also stored in glycerol. An overday culture of 3 ml in LB Medium with the corresponding antibiotics was inoculated with 30 µl of an overnight culture and incubated at 37°C with high shaking for about 8 hours. Then, 60%(v/v) sterile glycerol were added to the overday culture to a final concentration of 20% (v/v). The glycerol stocks were then stored at -80°C.

3.2.2.3 PROTEIN PURIFICATION

The cells obtained from a 100ml culture of *E. coli* BL21 harbouring the pET-28b pro- and mature lipase constructs, were resuspended in 15ml of 50mM phosphate buffer, pH 7.5 , sonicated (10 min; 50% pulse; 50% power) and centrifuged at 800 xg. The insoluble pellet was resuspended in 15ml of Triton x-100 0.1%, incubated at 37°C for 10min and centrifuged at 16000 xg. Pellets were then washed with 20ml of 50mM phosphate buffer, pH 7.5. The inclusion

bodies were resuspended in 2ml of sodium phosphate buffer pH 7, containing 8M urea and incubated at 37°C for 20 min. The total sample volume was then added to 2ml of the cobalt based Talon cellThru IMAC resin (BD Biosciences, Palo Alto, CA, U.S.A) and lightly shaken for 20min. The mixture was then centrifuged at 700 xg for 5min and washed with 2ml of sodium phosphate buffer, pH 7, containing 8M urea and 5mM imidazol. Elution was then carried out with 3 ml of sodium phosphate buffer, pH 7, containing 8M urea and 150mM imidazol. Figure 3.7 shows the mechanism of the His-tagged protein interaction with the resin.

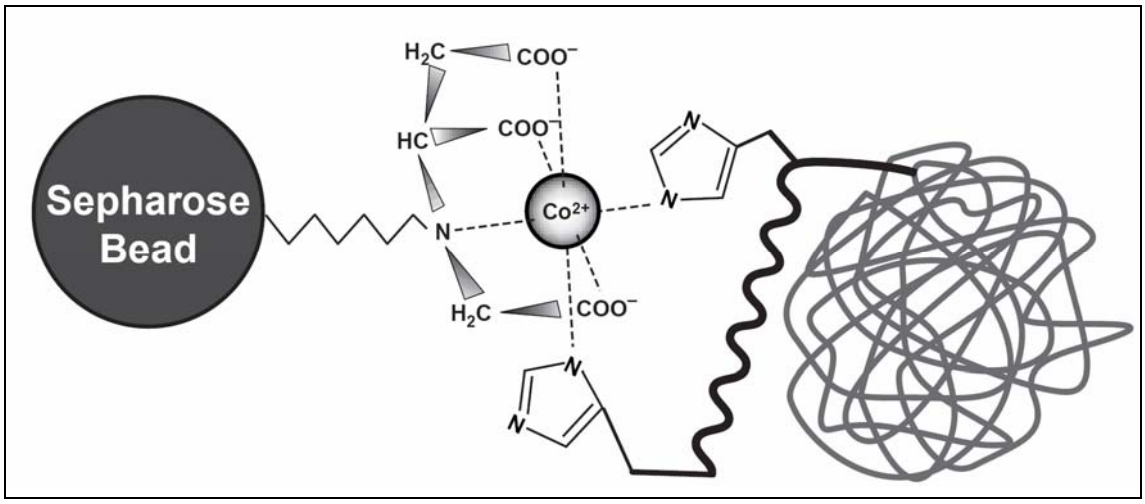


Figure 3.7: Mechanism of interaction between the protein His tag and the TALON resin

3.2.2.4 REFOLDING OF THE PURIFIED PROTEIN

The supernatant from the washing steps and eluted fractions was analysed by electrophoresis. The first and the second eluted fraction were centrifuged using a filtering device (Centricon Plus-20, 30.000NMWL, Millipore, Milford, MA, U.S.A.) for 10 minutes at 4000 xg , cystine (dissolved in 0.6 M NaOH) was added to a final concentration of 15 mM. The pH of the solution was maintained at 8.5 to 9 for 10 min, afterwards the solution was added to 20 volumes of cold 50 mM sodium phosphate, pH 7.5 mM EDTA, 5mM cysteine. This solution was incubated for 48 h at 4°C to allow refolding of the denaturated protein. The solution was then filtered with a filtering device (Centricon Plus-20, 30.000NMWL) and the concentrated solution was diluted with 500 µl of 50mM phosphate buffer, pH 7.5. The activity of the refolded protein was measured with the pNPB assay (see below).

3.2.3 MUTAGENESIS METHODS

3.2.3.1 SATURATION MUTAGENESIS

The site directed mutation, by saturation mutagenesis, of specific residues of the target protein was performed by following the procedure of the Quikchange™ protocol. The principles of the Quikchange™ are resumed in Figure 3.8. Basically the mutant strand synthesis is performed as follows: thermal cycling is performed to denature DNA template, mutagenic primers containing the desired mutation anneal, primers are extended and incorporated with *Pfu* DNA polymerase. Finally, the digestion of the parental methylated and hemimethylated DNA with *DpnI* and the mutated molecules are transformed into competent cells for nick repair.

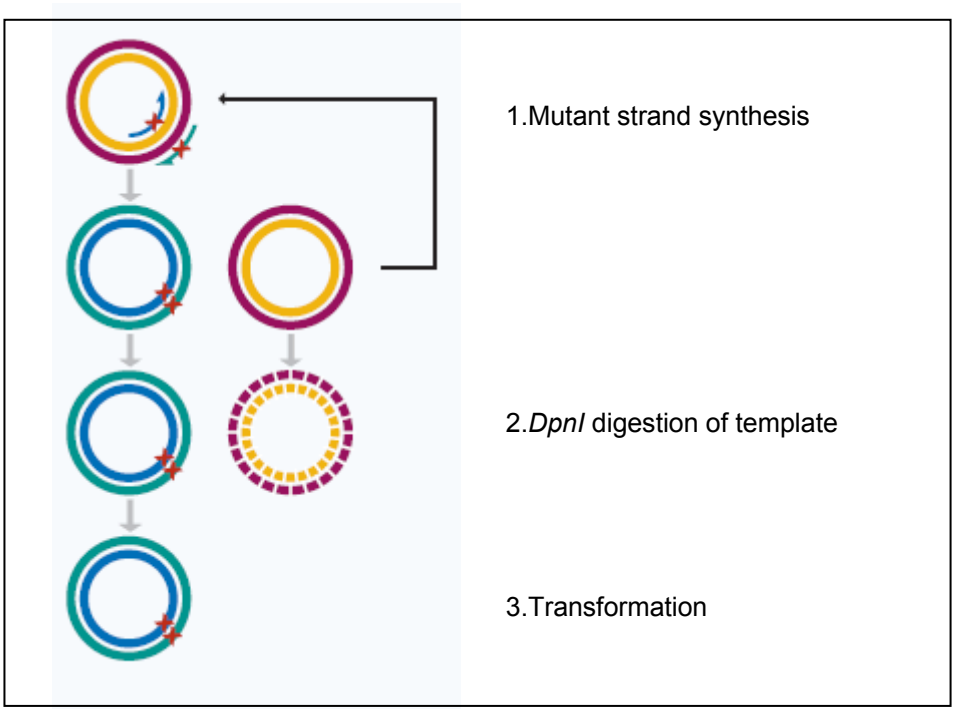


Figure 3.8: scheme of the Quikchange™ protocol

The primers designed to introduce a desired mutation in the protein are listed in Tables 3.5 and 3.6.

Table 3.5: primers utilized for the mutations of the histidine residues

NAME	SEQUENCE
His201 FW	CCACCGGTATCCCTTTCCAACGTACCGTTNNNAAGAGAGATATCGTTCC
His201 RV	GGAACGATATCTCTCTTNNNAACGGTACGTTGGAAAGGGATACCGGTGG
His144 Fw	CTTATAAGGTCATCGTTACCGGTNNNTCACTCGGTGGTGCACAAGC
His144 RV	GCTTGTGCACCACCGAGTGANNNACCGGTAACGATGACCTTATAAG
His218 FW	CCTCAATCCTTCGGATTCCTTNNNCCCGGTGTTGAATCTTGG
His218 RV	CCAAGATTCAACACCGGGNNNAAGGAATCCGAAGGATTGAGG
His134 FW	CTGTCGTCCAAGAACAATTGACCGCCNNNCCTACTTATAAGGTCATCG
His134 RV	CGATGACCTTATAAGTAGGNNNGGCGGTCAATTGTTCTTGGACGACAG
His109 FW	CTGTCAAGGGCGCCAAAGTTNNNGCTGGTTTCCTTTCCTCTTATG
His109 RV	CATAAGAGGAAAGGAAACCAGCNNNAACTTTGGCGCCCTTGACAG
His208 FW	CCGTTCACAAGAGAGATATCGTTCCTNNNGTTCCTCCTCAATCCTTCG
His208 RV	CGAAGGATTGAGGAGGAACNNNAGGAACGATATCTCTCTTGTGAACGG

Table 3.6: primers utilized for the mutation of the lysine residues

NAME	SEQUENCE
Lys5 FW	CAGCGCCTCTGATGGTGGTNNNGTTGTTGCTGCTACTACTGC
Lys5 RV	GCAGTAGTAGCAGCAACAACNNNACCACCATCAGAGGCGCTG
Lys37 FW	GTCGTTCTGTTGTCCCTGGTAACNNNTGGGATTGTGTCCAATGTC
Lys37 RV	GACATTGGACACAATCCCANNNGTTACCAGGGACAACAGAACGAC
Lys45 FW	GGGATTGTGTCCAATGTCAANNNTGGGTTCCTGATGGCAAG
Lys45 RV	CTTGCCATCAGGAACCCANNNTTGACATTGGACACAATCCC
Lys101 FW	CGTCTTCAACTTTTCTGACTACNNNCCTGTCAAGGGCGCCAAAGTTC
Lys101 RV	GAACTTTGGCGCCCTTGACAGGNNNGTAGTCAGAAAAGTTGAAGACG
Lys104 FW	CTTTTCTGACTACAAGCCTGTCNNNGGCGCCAAAGTTCATGCTG
Lys104 RV	CAGCATGAACTTTGGCGCCNNNGACAGGCTTGTAGTCAGAAAAG
Lys168 FW	ACGTGAACCAAGATTGTCTCCCNNNAATTTGAGCATCTTCACTGTCG
Lys168 RV	ACGTGAACCAAGATTGTCTCCCNNNAATTTGAGCATCTTCACTGTCG
Lys 202 FW	CCCTTTCCAACGTACCGTTCACNNNAGAGATATCGTTCCTCACGTTC
Lys202 RV	GAACGTGAGGAACGATATCTCTNNNGTGAACGGTACGTTGGAAAGGG

3.2.3.2 RECOMBINATION

Recombination of stable mutants by site directed mutagenesis, was performed, according to the Quikchange™ protocol. The primers utilized for the mutation are listed in Table 3.7

Table 3.7 :primers for double recombinants

NAME	SEQUENCE
H201A FW	CCACCGGTATCCCTTTCCAACGTACCGTTGCTAAGAGAGATATCGTTCC
H201A RV	GGAACGATATCTCTCTTAGCAACGGTACGTTGGAAAGGGATACCGGTGG
H201S FW	CCACCGGTATCCCTTTCCAACGTACCGTTTCGAAGAGAGATATCGTTCC
H201S RV	GGAACGATATCTCTCTTCGAAACGGTACGTTGGAAAGGGATACCGGTGG

3.2.4 HIGH-THROUGHPUT PRODUCTION OF PROROL MUTANT LIBRARIES

3.2.4.1 GENERATION OF MUTANT LIBRARIES

After mutation, DH5α was utilized as cloning strain. In the case of the mutants carrying a single mutation of the histidine residues, only after growing the mutants (200 colonies) in 1ml of LB at 37°C for 4h the plasmid purification and the transformation in *E. coli* Origami could follow. The libraries were produced by considering only the active mutants, previously selected with an activity staining test performed in solid agar plates.
In the case of the lysine residue a high-throughput plasmid isolation and transformation was performed.

3.2.4.2 PLASMID ISOLATION AND TRANSFORMATION IN MTP

The purification in MTP of the mutated plasmid DNA was performed with the DirectPrep 96 kit from Qiagen (Figure 3.9). In the basic high-throughput transformation procedure, the previously purified plasmid DNA was added to PCR tubes containing 25μl of *E. coli* Origami competent cells. The tubes were chilled and then subjected to heat shock, performed with a thermocycler. One hundred microliter of SOC medium was then added to the cells and 4 hours incubation at 37°C followed. The transformants were then selected by plating on solid medium containing the appropriate antibiotic.

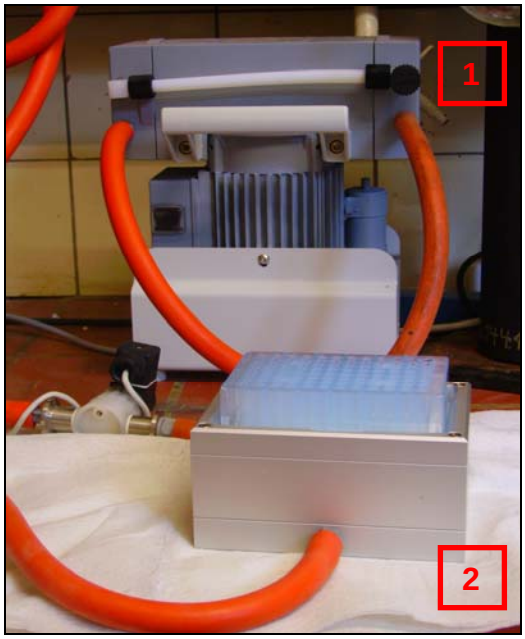


Figure 3.9: equipment for the high-throughput plasmid purification. 1) vacuum pump; 2) MTP for plasmid purification from Qiagen.

3.2.4.3 EXPRESSION IN MTP

Two hundred microliter of LB containing the necessary antibiotics were pipetted in each well of a microtiter plate (MTP). Each transformant was picked with a sterile toothpick and used to inoculate each well. The plates were grown for 24h at 37°C, and afterwards 100µl sterile 60% glycerol (v/v) were added, the plates mixed shortly and stored at -80°C as master plates (see figure 3.10).
With the 96-spike replicator, new MTP containing 200µl LB with antibiotics were inoculated and grown for 24h. From these plates, new plates containing 100µl LB with antibiotics were inoculated with 10µl, and incubated at 37°C for 6h. IPTG was added to each well for a final volume of 0.1mM, and the MTP were incubated for approximately 20h and centrifuged at 213 xg and 4°C for 30min. The supernatant was discarded and the plates were stored at -20°C

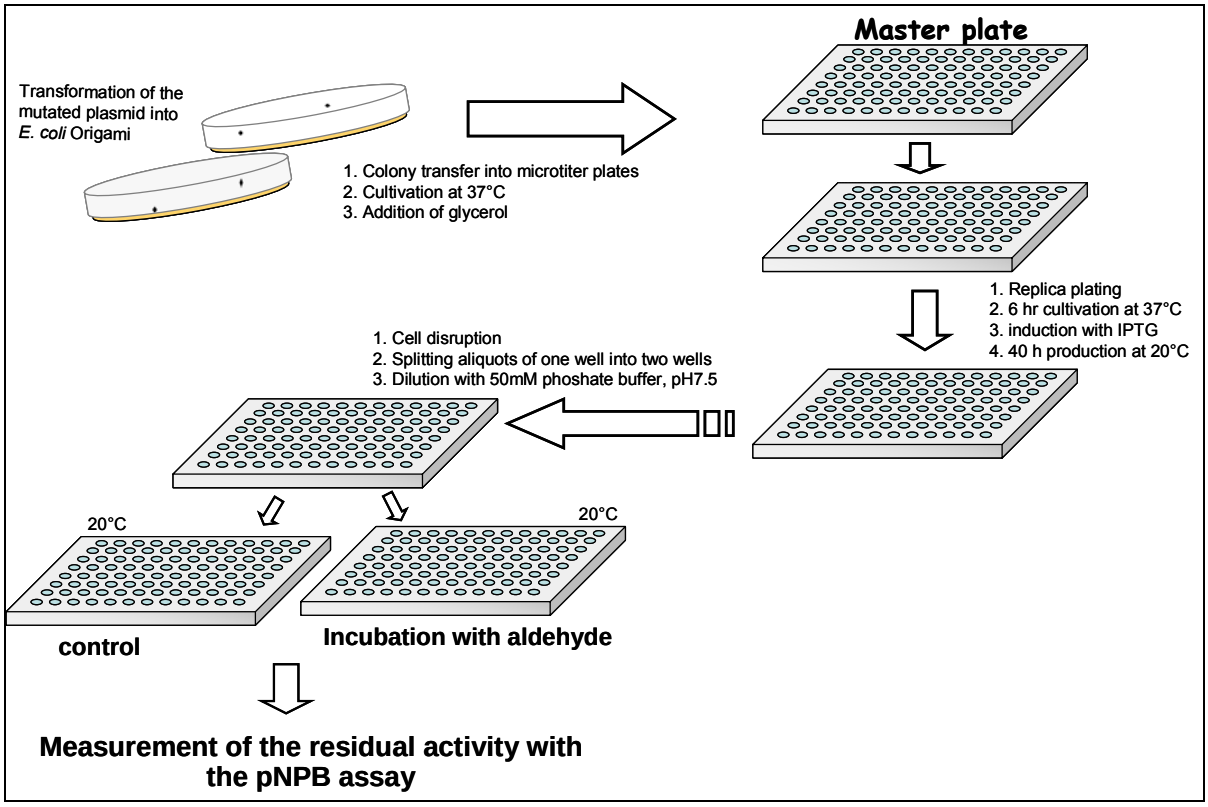


Figure 3.10: scheme of high-throughput expression and screening of mutants

3.2.4.4 CELL DISRUPTION

In the case of expression in MTP the cells disruption was carried out *via* an enzymatic lysis. The culture was centrifuged (213 xg, 30min) and after

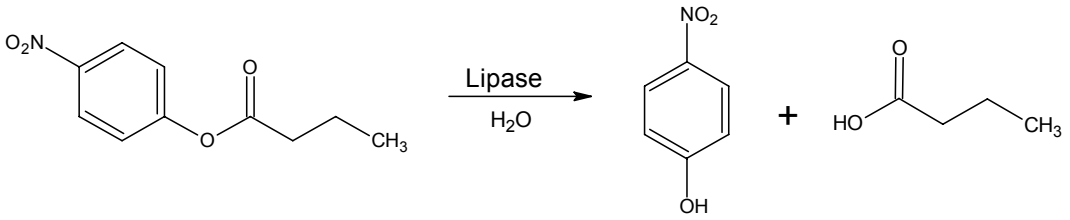
supernatant elimination, the pellets were resuspended with 50mM sodium phosphate buffer, 300mM NaCl pH 8, containing 0.1% DNAse, 1mg/ml lysozyme. The plates were incubated for 30 min at 4°C, frozen for 1h at -80°C, thawed for 30 min at 37°C, centrifuged for 30min at 213 xg.

3.2.5 ACTIVITY ASSAYS

3.2.5.1 pNPB ASSAY

3.2.5.1.1 Semi-micro scale

The activity was measured *in vitro* by monitoring the amount of *p*-nitrophenol released upon hydrolysis of a 1 mM solution of *p*-nitrophenyl butyrate (pNPB) in 50 mM phosphate buffer, pH 7.5 at room temperature with a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corp. Kyoto, Japan) (Figure 3.11). Aliquots (100µl) of the cell fraction assayed were added to 900 µl of the reaction mixture and the increase in absorbance at 410 nm was measured for 1 minute. One unit of hydrolase activity was defined as the amount of enzyme releasing 1 µmol of 100 *p*-nitrophenol (pNP) per min at room temperature. The activity was calculated with the following formula $\Delta E = \varepsilon_{mol} \cdot C \cdot d$ with ε being the molar absorption coefficient, ΔE being the measured absorption difference, *d* being the path length of the cuvette (here: 1 cm) and *C* being the molar concentration of the chromogenic substrate. The extinction coefficient ε was determined with a calibration curve obtained by measuring the absorption at 410 nm of pNP solutions in a concentration range of 0.2-1 mM.



p-Nitrophenylbutyrate p-Nitrophenol

Figure 3.11: Enzymatic hydrolysis of *p*-Nitrophenylbutyrate

3.2.5.1.2 MTP scale

In a MTP 20 µl of supernatant were combined with 160 µl of phosphate buffer. The MTP was then placed in a FLUOstar Optima fluorimeter (BMG Labtechnologies, Offenburg, Germany) and 20 µl of *p*NPB were added using the fluorimeter pump just before starting the measurement. The absorption at

410 nm was measured within 10 cycles. The activity was then calculated as described above in the case of the assay in cuvettes.

3.2.5.2 ACTIVITY STAINING TEST IN AGAR PLATES

Plates containing clones of *E. coli* Origami harbouring the mutagenic plasmid were replicated with a sterile cloth onto a plate containing the appropriate antibiotics and 0.1 mM IPTG as protein inducer. The replicated plates were incubated at 20°C. After two days these plates were covered with 10ml of soft agar (0.5% w/v) containing 160μl of Fast blue (89mg/ml in DMSO) and 80μl of α-naphthyl acetate (40mg/ml in DMF). Active clones (coloured in red) were selected for the master plates.

3.2.6 TRANSESTERIFICATION REACTION BY GC

3.2.6.1 BATCH REACTOR

In residual activity measurements after incubation with deactivants, the Novozym® 435 was washed twice with ethanol and twice with hexane, in order to remove inactivating compounds. Transesterification of 100mM geraniol and 100mM ethyl caproate in petroleum ether, was carried out as sample reaction. 1ml of substrate solution was incubated for 10 minutes at 37°C with 10mg of enzyme. Afterwards, 1μl of solution was introduced in the column (RTX 5, 30m, 0.25 inner diameter) of a Shimadzu Gas Chromatograph (GC) to measure the amount of geranyl caproate (t_r=15.5min) produced. Nonadecane (t_r=17.7) was used as standard.

3.2.6.2 CONTINUOUS REACTOR

In the case of continuous reactors the output flow collected in 10ml sealed tubes to minimize air contact, was analyzed at defined time intervals with the GC to check the amount of product obtained. The tubes were also weighted to evaluate the weight output flow and thus. By knowing its density, the volumetric output flow in the reactor was thus determined. The sample reaction was the same as in the case of the batch reactor and nonadecane was used as standard.

3.2.7 STABILITY ASSAYS

3.2.7.1 STABILITY ASSAY IN SEMI-MICRO SCALE

For the screening of the proROL stability, the effect of the triglyceride oxidation products has been simplified by considering only aldehydes. After cell disruption the lysate was incubated, in a concentration depending on the protein amount, with aldehyde in phosphate buffer (50mM, pH7.5) for a total volume of 1ml. A control, i.e. enzyme in buffer without aldehyde, was also considered to evaluate the natural protein deactivation. The aldehyde concentration was optimized each time in order to observe a linear time-dependent activity decay, during an incubation time of approximately 8 hours. To avoid thermal inactivation effects, the incubation temperature was maintained to 20°C. The residual activity was measured at defined time intervals with the pNPB assay.

3.2.7.2 HIGH-THROUGHPUT STABILITY SCREENING ASSAY

After cell disruption in MTP the lysate was incubated in MTP with the aldehyde in phosphate buffer (50mM, pH7.5) for a total volume of 200 μl. The aldehyde concentration was optimized each time in order to observe a linear time-dependent activity decay, and to reach the half-life of the wild type protein during an incubation time of approximately 4 hours. A control MTP, containing only variants in buffer without aldehyde, was also considered. In each plate a positive control (i.e. the wild type proROL) and a blank consisting of buffer without enzyme were also introduced. At defined time intervals the residual activity was measured with the pNPB assay and the mutants showing an higher stability (i.e. higher half life) with respect to the wild type protein were selected. A 40% of experimental error in the evaluation of the wild type residual activity was considered for the mutant selection. The selected mutants were sequenced to confirm the mutation, expression in flask was carried out as described above and the stability tested again in semi-micro scale as previously described.

3.2.7.3 DEACTIVATION OF NOVOZYM® 435 IN BATCH REACTORS

The oxidation of raw soybean oil was performed in air at 37°C. Afterwards, 10 mg of Novozym® 435 were incubated with 1ml of oxidised oil at 37°C, with light shaking. At a defined period of incubation, the enzyme was recovered and its residual activity measured as described in the activity assay section. In the same conditions the incubation of Novozym® 435 with soybean oil, fried for a defined time, was also performed. The incubation with oxidized and fried soybean oil was also performed for the Novozym® 435 pretreated with pyridoxal-5'-phosphate (PLP) and with dithiothereitol (DTT). The pre-treatment

involved the incubation of 100mg of Novozym® 435 with 10ml of a 20mM solution in phosphate buffer (50mM, pH7.8) of the stabilizer for 2h at room temperature with light shaking. Before the incubation of the enzyme with oil the solvent was removed and the enzyme dehydrated.

3.2.7.4 DEACTIVATION OF NOVOZYM® 435 IN CONTINUOUS REACTORS

All the reactor components were made either of glass or Teflon to allow the utilization of organic solvents. The solution containing the substrates and eventually the additives was kept in a sealed glass container. The solution was fed to the reactor by a peristaltic pump (Miniplus 3, Gilson) through a Teflon tube ID 1/16''. The reactor was immersed in a thermostatic bath (W6, Grant). Samples were collected in sealed tubes at the reactor outlet, to avoid solvent evaporation, and subjected to activity assay, as described above.
The reactor scheme is shown in Figure 3.12B. The overall system scheme of is reported in Figure 3.12A.
Before starting the test, the catalyst was dehydrated, to control the water amount in the system and to reduce the electrostatic interactions among catalyst particles, during enzyme loading in the reactor. Prior to feeding the reactor, substrate solutions were equilibrated for 24h, in order to reach the predetermined value of water thermodynamic activity. The enzyme was loaded rapidly, to minimize its contact with the external environment. Novozym® 435 was weighed in a large spout Pasteur pipette, which was then introduced in the Teflon tube. Enzyme loading in the reactor was performed by introducing a second Pasteur pipette with a smaller spout inside the first one. The use of the second pipette was fundamental to avoid interactions among catalyst particles and among the latter and the glass walls. Upon loading the enzyme in the reactor, the system was kept at room temperature for the time needed to let 10ml of substrate flow, in order to allow system stabilization and to pack the catalyst particles on top of the porous septum. The reactor was then immersed into the thermostatic bath and the test was started.

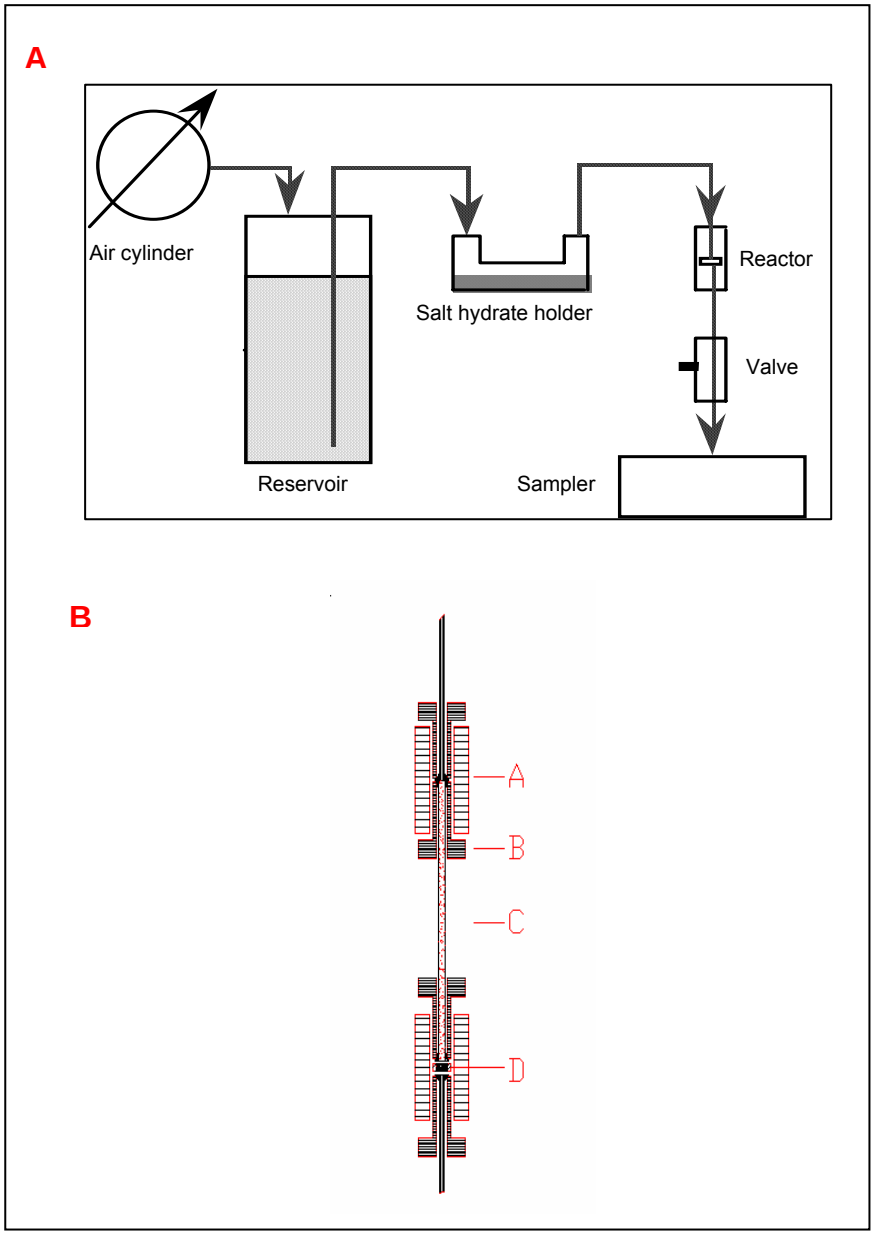


Figure 3.12: A) Scheme of the system equipment. B) Reactor scheme During the test, the enzyme was contained in a 1/8" Teflon tube (C), to allow its loading. A polyethylene frit (D) blocked the enzyme, leading to the formation of a fixed bed. Two tapped connection (B) made the dead volume negligible. The solvent was carried through 1/16" tube to avoid back mixing of the components in the solution. A union (A) allowed the contact between the 1/16" tube with the 1/8" tube at the top of the reactor.

4 RESULTS AND DISCUSSION

4.1 NOVOZYM® 435 DEACTIVATION

Previous experiments on the commercial lipase Novozym® 435, carried out at the Chemical Engineering Department of the University of Napoli, Federico II, led to the conclusion that the mechanism of lipase inactivation in nonaqueous media is the same as that holding in aqueous system. These experiments were performed by considering raw soybean oil in which a defined oxidation product was introduced, in order to analyse the effect of each individual compound on enzyme stability (Pirozzi, 2003).
In this Thesis, the overall oxidation products generated by the oil oxidation was analyzed. Soybean oil that had undergone a natural oxidation process by means of either several day incubation in air, or use in a frying process lasting several hours, was then considered. It was thus possible to evaluate enzyme deactivation occurring in working conditions, typical of oleochemical industry. The enzyme was incubated with the oxidized oil, according to the procedures described in the methods section, within both a batch reactor and a continuous flow one. Obviously, the use of a batch reactor is easier, but a continuous reactor allows to monitor some deactivation mechanisms not easily controlled in discontinuous systems, where concentration levels of enzyme-deactivating impurities and of potentially stabilizing feed components vary markedly in the course of reaction time.
The dimensions of the continuous reactor, described in the methods section, are extremely small, thus enabling let perform long tests with limited amounts of reagents.
Figure 4.1B compares the results of the Novozym® 435 incubation with oxidized oil, in a batch reactor with those produced in a continuous one. Enzyme deactivation increases with the increasing storage time of the oil, being almost negligible for a 5 day storage time . In the continuous reactor the deactivation is much higher, (figure 4.1B refers to incubation with an oil stored for 30 days), and increases with the feed flow. For a 6ml/h flow, the half life of the enzyme is approximately of 4 hours. Similar results, were obtained with oil fried for 2-8 hours. Almost the same results were in fact observed in the case of a 6ml/h flow continuous reactor for the Novozym® 435 incubated with soybean oil fried for 8 hours.

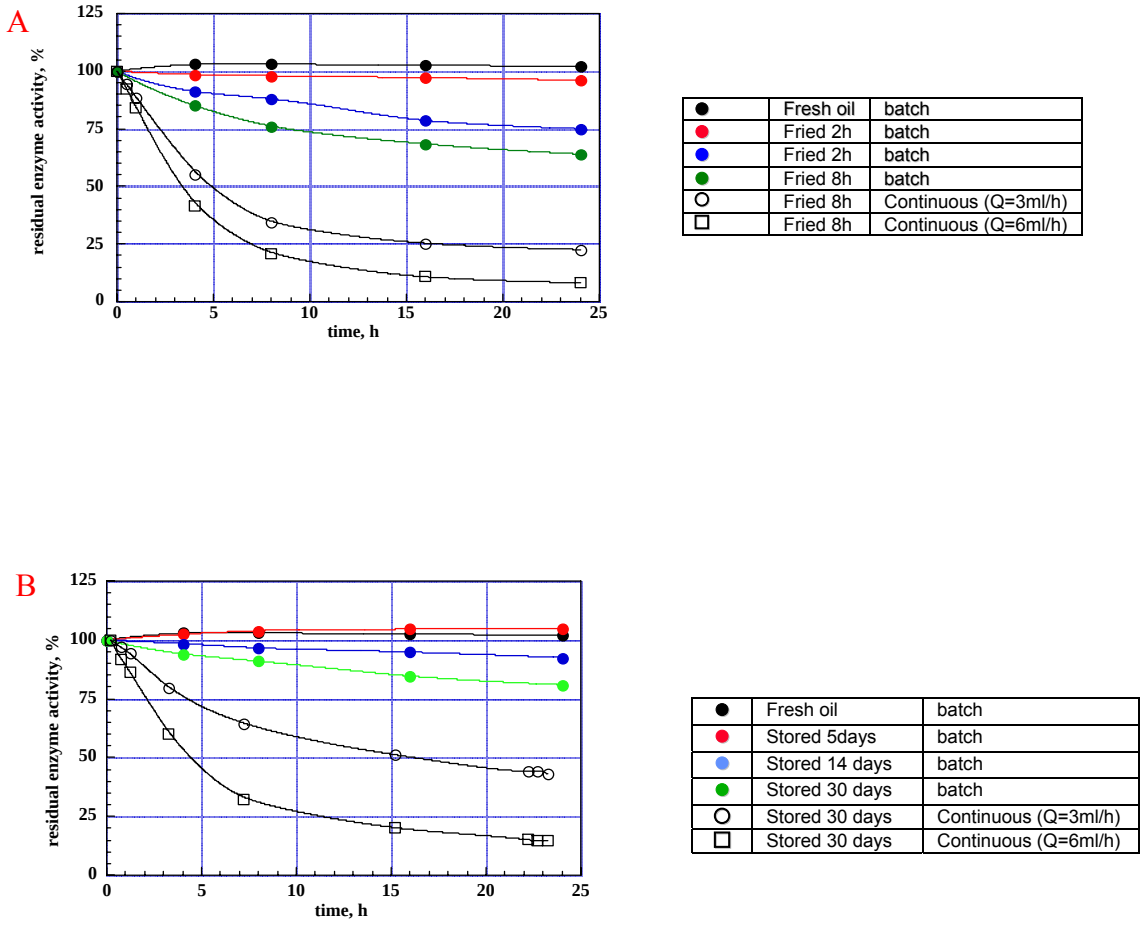


Figure 4.1: effect of the incubation of the Novozym® 435 with raw soybean oil, oxidized by storing on air or frying, A) Incubation with fried soybean oil B) Incubation with soybean oil oxidized by storing in the open air for several days.

4.1.1 STABILIZATION WITH PLP AND DTT

It is already known that pyridoxal-5'-phosphate (PNP) and dithiothreitol (DTT) interact with specific lipase residues, such as lysine groups and cysteine groups, respectively. An improvement of the enzymatic activity of the lipase from *Candida cylindracea* had been previously reached by the chemical modification with PNP and DTT. These modifications had the aim of improving the activity by changing some amino acid residues which could be proton donors or proton acceptors, with the assumption that protons participate in the reaction catalyzed by lipases (Kawase et al., 1989). Since these modifications were directed to those residues involved in the deactivation process, we investigated the Novozyme 345 stability after a pre-treatment with those chemical compound. As a blank it was considered not only the no treated

enzyme, but also the Novozym® 435 incubated only with buffer. This was done in order to take into account the tendency of the resin, on which this lipase is immobilized, to trap water because of its ionic nature. As shown in the Figure 4.2, even if the enzyme was dehydrated before the stability assay, this water interfered on the enzymatic activity, since the activity decay of the enzyme treated with buffer, was higher than the one observed for the Novozym® 435 not treated at all. The picture also shows that there is a two steps time-dependent activity decay. The first deactivation step is equal both for the treated enzyme than for the not treated one. On the contrary in the second step a stabilization of the treated enzyme was observed. With a prolonged time, referred to normal industrial working time, a saturation effect occurred in the case of the PNP-Novozyme and the DTT-Novozyme. The latter had a stability slightly higher.
This experiment demonstrated that an improvement of the lipase stability could be performed by properly acting on the residues susceptible of being involved in the interaction with the triglyceride oxidation products.
Essentially, these results are interesting because gave the basis for the next part of my Thesis, which concerned the possibility of improving the lipase stability by genetically modifying this time, the residues involved in the interaction, thus generating a more stable protein.

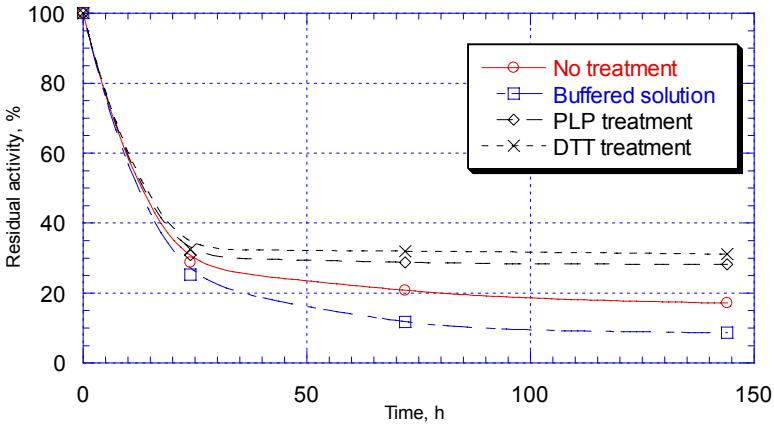


Figure 4.2: Effect of the incubation of the Novozyme 435 with pyridoxal-5'-phosphate and dithiothreitol

4.2 EXPRESSION OF ROL AND PROROL

As already mentioned in the Objectives section, the second part of this Thesis analyzes a lipase from the fungus *Rhizopus oryzae*.
It is clear that an efficient expression of the target protein is a fundamental and necessary step before trying to improve its characteristics for industrial purposes. The production of an active mature *Rhizopus* lipase has been performed in *Pichia pastoris* (Minning et al., 1998; Minning et al., 2001; Resina et al., 2004) and in *Saccharomyces cerevisiae*.(Swenson et al., 1994; Takahashi et al., 1998; Shiraga et al., 2002) Unlike these yeasts, *E. coli* in principle allows for an easier expression system and a higher yield of enzyme. It is a frequently used host for the production of recombinant proteins since, among the many hosts available for heterologous protein production, it remains one of the most attractive. *E. coli* is in fact, characterized by a high ability to grow rapidly and at high density on inexpensive substrates, its genetics are well-known and the number of its cloning tools available is high (Baneyx, 1999). Despite this, there is no *a priori* guarantee that every gene can be expressed efficiently and functionally in this host. Factors influencing the expression level include unique and subtle structural features of the gene sequence, the stability and efficiency of mRNA, correct and efficient protein folding, codon usage, degradation of the recombinant protein by ATP-dependent proteases and toxicity of the protein (Schumann et al., 2004).
Since *E. coli* lacks the necessary proteases to process fungal maturation signals, the *Rhizopus oryzae* lipase cDNA were previously expressed in *E. coli,* for both the unprocessed lipase precursor and the mature product, in unsoluble form. Moreover, as the *Rhizopus oryzae* lipase is highly toxic for the host, even at non-inducing conditions, probably due to its activity on phospholipids, the mature lipase and prolipase were expressed using tightly regulated systems employing *E. coli* BL21(DE3) as a host for expression, and the pET-11d plasmid as vector (Joerger et al., 1993).
Therefore, it was not surprising that the expression of the pET-11d(+)proROL and pET-11d(+)ROL constructs in *E. coli* BL21(DE3) led to an insoluble and inactive product. As shown in Figure 4.3 the protein is present only in the insoluble fraction (inclusion bodies), while no bands are visible in the soluble one (Figure 4.3B).

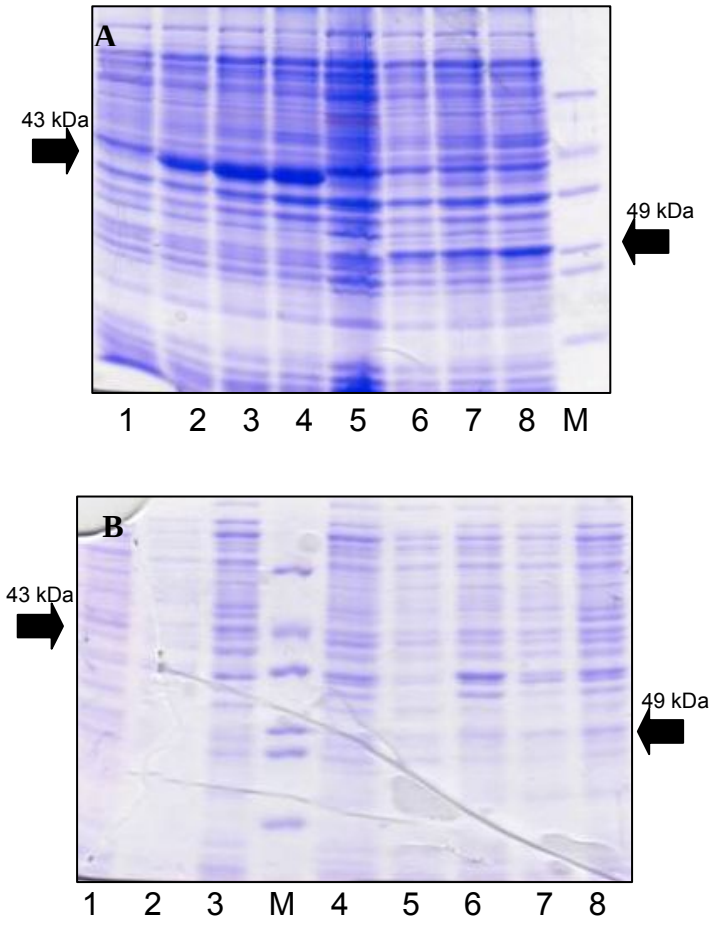


Figure 4.3: Expression of *E. coli* BL21pET-11d(+) proROL and ROL. The temperature of expression was 37°C and the target protein was induced for OD_{600} = 0.5. Panel A. Lane 1: prolipase before induction, pellet; lane 2: prolipase after 1h of induction, pellet; lane 3: prolipase after 3h of induction , pellet; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 4: prolipase after 4h of induction , pellet; lane 5: mature lipase before induction, pellet; lane 6: prolipase after 1h of induction, pellet; lane 7: prolipase after 3h of induction, pellet; lane 8: prolipase after 5h of induction, pellet.
Panel B. Lane 1: prolipase before induction, supernatant; lane 2: prolipase after 1h of induction, supernatant; lane 3: prolipase after 3h of induction , supernatant; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 4: prolipase after 4h of induction, supernatant; lane 5: mature lipase before induction, supernatant; lane 6: mature lipase after 1 h of induction, supernatant; lane 7: mature lipase after 3h of induction, supernatant; lane 8: mature lipase after 5h of induction, supernatant

In this Thesis, strategies have been set up, in order not only to simplify the pellet treatment but also to directly allow the production of a soluble recombinant lipase.

4.2.1 TREATMENT OF THE INCLUSION BODIES

A time-consuming purification and refolding process of the unfolded protein, involving the utilization of two different chromatographic columns, has been reported (Joerger et al., 1993).
To simplify the protein purification, the genes coding for the proROL and the ROL have been recloned into vectors containing a His-tag-coding sequences, the purification of the His-tagged protein is in fact easy to perform *via* an immobilized metal-ion affinity chromatography on tailor-made supports. and if necessary, a one-step, tailor-made, purification procedure can be designed, by carefully engineering the metal-chelate supports (Beer et al., 1998; Armisén et al., 1999; Mateo et al., 2001; Hidalgo et al., 2004).
In particular the vector pET-28b(+) was utilized. *E. coli* BL21, BL21 pLysS and BL21 pLysE strains were considered for the expression of the proROL and ROL pET28 constructs, in order to investigate the one which could give the highest protein amount. The best expression was obtained with *E. coli* BL21 strain (Figure 4.4).
As shown in Figure 4.4, the overexpression of the pET-28b(+)proROL in *E. coli* BL21 led to a much higher protein amount than in the case of the ROL. This was mainly due to the high toxicity of the ROL which in the case of the proROL is modulated by the pro-sequence (Beer et al., 1996). The pellets of both proROL and ROL were purified and refolded as described in the Methods section. The purification of the proROL from the inclusion bodies was easily achieved by immobilized metal-ion affinity chromatography (IMAC) chromatography (Figure 4.5). On the contrary, in the case of ROL the IMAC purification was unsuccessful, since the amount of protein produced was much lower and most of the protein was lost after the adsorption step on the resin (data not shown).
The purified proROL was refolded in the presence of cysteine, leading to an active enzyme preparation (0.6 U/ml). The specific activity could not be evaluated, because the protein concentration was lower than the sensitivity of the method utilized, due to the high protein dilution from the refolding step. Moreover, the storage of the refolded enzyme was difficult to perform. Unfortunately the refolded proROL was inactive either after storage at –20 ºC, at 4 ºC or when lyophilized. Previously low thermostability was reported for the refolded prolipase (Haas et al., 1999).

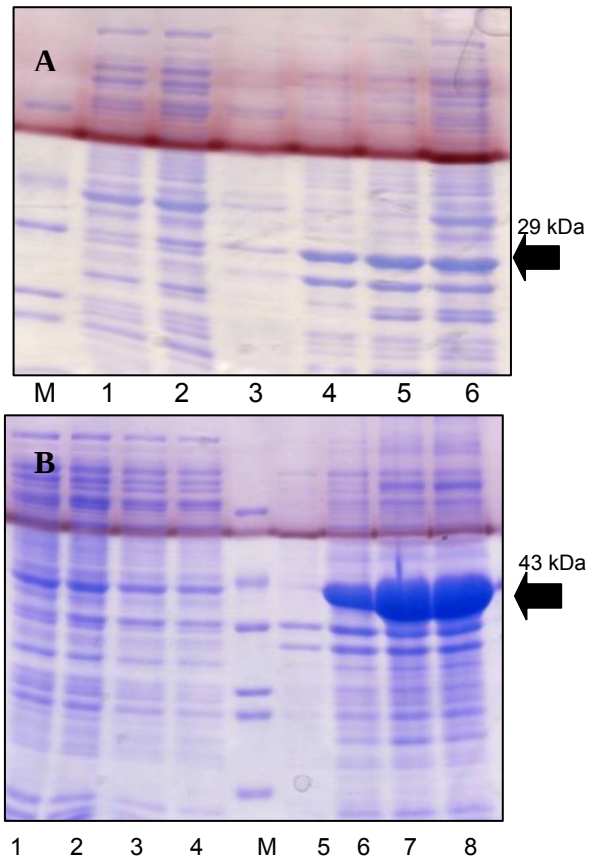


Figure 4.4: Expression of *E. coli* BL21pET-28b(+) proROL and ROL. The temperature of expression was 37°C and the target protein was induced for OD_{600} = 0.5. Panel A. M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 1: ROL before induction, supernatant; lane 2: ROL after 1h of induction, supernatant; lane 3: ROL after 5h of induction, supernatant; lane 4: ROL before induction, pellet; lane 5: ROL after 1h of induction pellet; lane 6:ROL after 5h of induction, pellet.
Panel B. Lane 1: proROL before induction, supernatant; lane 2: proROL after 1h of induction, supernatant; lane 3: proROL after 3h of induction, supernatant; lane 4: proROL after 5h of induction, supernatant; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 5: proROL before induction, pellet; lane 6: proROL after 1 h of induction, pellet; lane 7: proROL after 3h of induction, pellet; lane 8: proROL after 5h of induction, pellet.

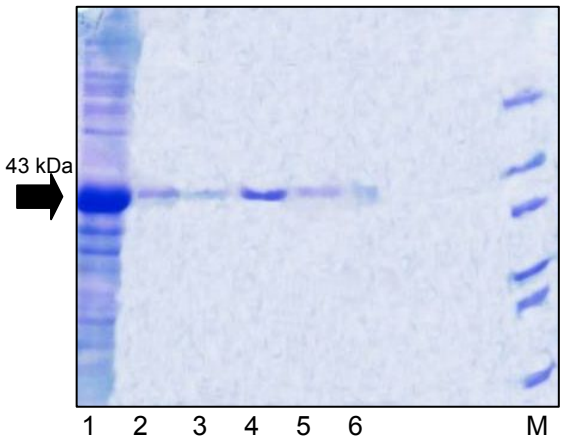


Figure 4.5 *:* purification of the pellets from the expression of the pET-28b proROL in BL21. Lane1: pellet solution;2,3 washings; 4,5 and 6 elution; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa).

4.2.2 IMPROVING THE EXPRESSION OF A SOLUBLE PROTEIN

Although the proROL accumulated in inclusion bodies within *E. coli* BL21 was successfully purified, and the refolding procedure led to an active enzyme, the product did not have the desired stability and the procedure was time consuming. Therefore, strategies to directly enhance the production of a soluble protein were also investigated.
In the literature, reports are found of a correctly folded lipase production was by translocation of the product to the periplasm, or even to the extracellular space, as in the case of the expression of a *Burkholderia* ABC transporter in the host, in order to secrete the *Burkholderia cepacia* lipase produced in *E. coli* (Ahn et al., 1999).
A problem in heterologous expression is the different codon usage in eukaryotes and prokaryotes that may cause ineffective protein expression (Mitraki et al., 1989). Adequate tools to overcome such codon library problems have been developed, such as strains that carry a plasmid encoding additional tRNAs for rare codons, like *E. coli* Rosetta (Novagen, Madison, U.S.A). In many cases the proper formation of specific disulfide bonds of a protein involves post-translational modifications. When extracellular proteins are expressed in conventional *E. coli* strains, missing disulfide bonds or incorrectly bonded cysteine residues often cause formation of insoluble aggregates, as these strains lack such post-translational modification mechanisms.
To overcome this problem, *E. coli* strains with enhanced disulfide bond formation capacity, such as Origami (Novagen, Madison, U.S.A.) may be used. This strain carries a thioredoxin reductase (*trx*B$^-$) and glutathione reductase (*gor*522$^-$) double mutation that stimulates proper folding in the host strain with a more oxidizing cytoplasmatic environment. Recently, successful heterologous expressions that would not been possible otherwise have been achieving using *E. coli* Origami as the host (Lehmann et al., 2003).
Expression of the ROL and proROL in *E. coli* Rosetta did not lead to good results, the protein being expressed mainly as insoluble aggregates (data not shown). On the contrary, the use of *E. coli* Origami as expression strain resulted in soluble protein with accurately formed disulfide bonds and lipolytic activity was observed (Figure 4.7).
In fact the *Rhizopus oryzae* lipase contains six cysteine residues, which form three disulfide bridges (Figure 4.6). These bonds constitute a conserved characteristic of the homologous family of lipases from filamentous fungi, involved not only in the correct protein folding but also contributing to thermostability in some of the family members, especially the long disulfide bond between Cys29 and Cys268 (Derewenda et al., 1994).

Figure 4.6: Visualization of the three disulphyde bridges in the *Rhizopus* lipase. In red the lid, in pink the active site, in yellow the disulfide bonds

Figure 4.7 shows the results of the expression of ROL and proROL in *E. coli* Origami. The figure 4.7B also shows that in the case of the ROL the protein concentration at the beginning increase, with a maximum after 3 hours of induction, but afterwards the protein concentration decays probably mainly due to its toxicity.

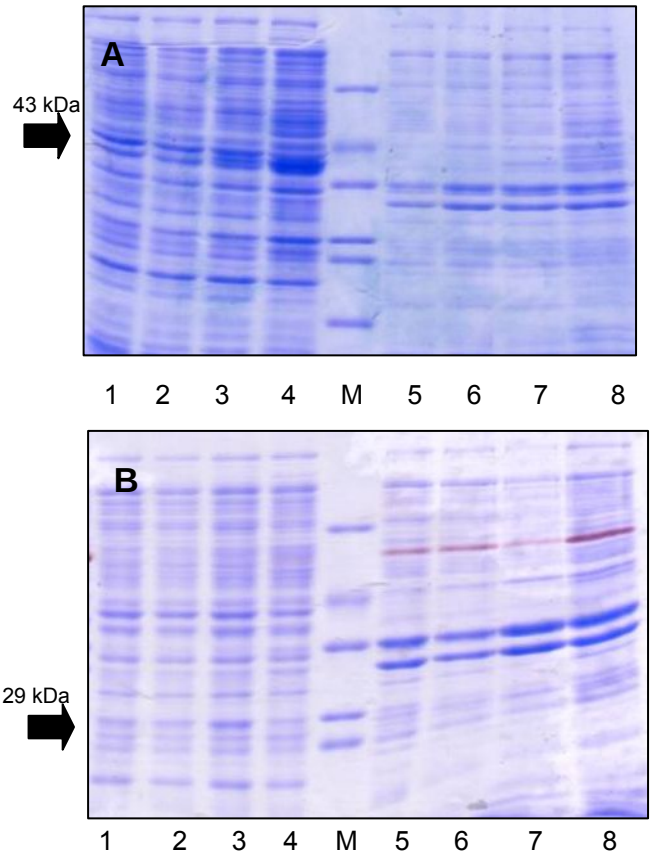


Figure 4.7: A: expression at 25°C of *E. coli* Origami harbouring the pET11-d Prolipase. Lane 1: prolipase before induction, supernatant; lane 2: prolipase after 1h of induction , supernatant; lane 3: prolipase after 3h of induction , supernatant; lane 4: prolipase after 24h of induction , supernatant; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 5: prolipase before induction, pellet; lane 6: prolipase after 1h of induction, pellet; lane 7: prolipase after 3h of induction, pellet; lane 8: prolipase after 24h of induction, pellet.
B. expression at 25°C of *E. coli* Origami harbouring the pET11-d mature lipase. Lane 1: lipase before induction, supernatant; lane 2: lipase after 1h of induction, supernatant; lane 3: lipase after 3h of induction , supernatant; lane 4: lipase after 24h of induction , supernatant; M: molecular weight marker (66, 45, 36, 29, 24, 20.1 kDa); lane 5: lipase before induction, pellet; lane 6: lipase after 1h of induction, pellet; lane 7: lipase after 3h of induction, pellet; lane 8: lipase after 24h of induction, pellet.

4.2.3 INFLUENCE OF SONICATION TIME ON THE SPECIFIC ACTIVITY OF THE PROLIPASE

Cell disruption by sonication must be carried out effectively, prior to protein production under optimal conditions and attempts to improve its activity. Sonication time can in fact influence the specific activity of enzymes in two ways. On one hand, a prolonged sonication time can enhance the activity because more cells are disrupted and thus more lipase is released into the medium. On the other hand, despite refrigeration during sonication, sample temperature can increase, thus yielding inactivation of proteins with low

thermostability. To study the effect of the sonication time on the specific activity, several samples were taken 24 hours after induction, and sonicated for different times. The results are shown in Table 4.1.

Table 4.1: Specific activities of pET11-d(+) proROL after different times of sonication

Sonication time [s]	Protein content [mg/ml]	Specific activity [U/mg]
20	0,9	40,7
30	1,1	35,9
50	1,9	26,8
60	2,1	24,6
2x 20	1	21

This table shows quite clearly that the sonication time has a dramatic effect on the specific activity. Two factors account for the loss of specific activity after long sonication times: the protein content rises with increased sonication time, but more lipase is deactivated due to the heat that is transmitted.

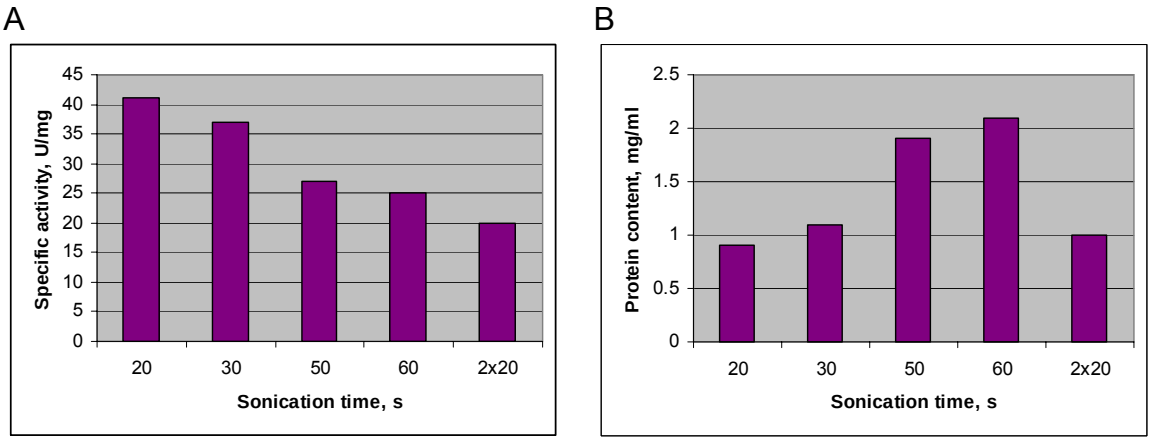


Figure 4.8: Influence of sonication time on (A) specific activity and (B) on protein content

The first factor, i.e. increased protein release, is easily confirmed reviewing the protein content determined according to Bradford (Figure 4.8). The latter is confirmed through the values obtained for the last result listed in Table 4.1. These samples were sonicated for 20 seconds, then centrifuged for 5 minutes at 800 rpm and the supernatant sonicated again for 20 seconds. The second sonication should not disrupt more cells and thus add more protein to the sample, as the cell pellet was removed before repeated sonication. The loss of half of the specific activity compared to that achieved with only one 20 seconds sonication round, must be attributed mainly to sample heating.Two parallel samples were prepared for each sonication condition. Although they followed, significant differences, due to.

Although they both followed the same general trend, significant differences emerged, as wel, due to irreproducibility of the experimental conditions. Indeed, the position of the sonication rod within the eppendorf tube and the overall amount of ice and water surrounding the sample were both relevant.

4.2.4 OPTIMIZING THE EXPRESSION IN *E. coli* ORIGAMI

The amount of soluble protein produced and its activity was improved by decreasing the rate of protein synthesis with a lower temperature of expression and induction at a higher OD_{600}

Much of this work was conducted using the prolipase gene rather than the lipase gene. The prolipase has a specific activity comparable to the mature lipase but it is less toxic and consequently the amount of prolipase produced is much higher. The role of the prosequence might be double. First, it facilitates the folding process via a single Cys residue, which acts as an intramolecular thiol-disulfide reagent (Beer et al., 1996). This intramolecular chaperone-like function can also occur when the prosequence is expressed in *trans* (Takahashi et al., 2001). Secondly, similar to other preproenzymes, the prosequence modulates the enzyme activity of the mature lipase so that it can be synthesized without damaging the host, in this case as a result of a decreased affinity of the prolipase for phospholipids in comparison to the mature lipase. This modulation also causes the prolipase and the mature lipase to have different affinities for their substrates (Takahashi et al., 1999), although whether this fact is caused by an interaction between the prosequence and the peptide “lid” that sits atop the active site in an inactive form of the mature lipase is not confirmed since a resolved crystal structure of the prolipase is not available. The prolipase is also much more thermostable than the prolipase, retaining full activity after exposure to temperatures as high as 70°C, while the mature lipase has been reported to deactivate at temperatures higher than 40°C. The fact that the prolipase is active implies that the part of the expression product corresponding to the mature peptide is already correctly folded.

Other post-translational modifications, such as glycosylation have not been reported to be crucial for functional expression of ROL in *Pichia pastoris*, although the protein contains four potential N-glycosylation sites (Minning et al., 1998).

The expression of *E. coli* Origami pET-11d-prolipase was optimum at a growth temperature of 20°C with induction at OD_{600} =1 (Table 4.2). After 20 h of cultivation under these conditions the lipolytic activity of the supernatant was 110.7 U/mg. Although the functional expression of *Rhizopus* sp. lipase has been already performed in *Pichia pastoris* (Minning et al., 1998; Minning et al., 2001; Resina et al., 2004) and *Saccharomyces cerevisiae* (Takahashi et al., 1998; Shiraga et al., 2002), we hesitate to compare the data as different activity assays with different substrates and conditions have been used. In an effort to simplify the future purification of the protein, the *Rhizopus delemar* prolipase gene was cloned into pET-15b(+) and pET-22b(+), vectors which lead to a product with a His-tag respectively in the N-terminal and in the C-terminal position. The expression was performed under the conditions optimized for the pET-11d. The presence of a N-terminal His-tag negatively influenced the activity of the recombinant prolipase: when the vector pET-15(+) was utilized, the activity was 37% of that obtained with the pET11d construct (Table 4.3). This result was confirmed when a His-tag coding sequence was introduced in the N-terminus of the prolipase gene in the vector pET-11d. In this case the activity decreased much more: the N-terminal his-tagged protein exhibited only 17% of the activity obtained from a product without His-tag. On the contrary the His-tag

in the C-terminal position did not influence the prolipase activity, but in this case the optimal expression conditions were 20 ºC and induction at OD_{600} of 0.5. The effect of the position of the His-tag on the production and solubility remains largely an empirical factor. There are examples in the literature that describe little or no effect, others report a significant improvement when N-tagged products are expressed, and yet some others report best results when a C-terminal His-tag is used (Busso et al., 2003; Woestenenk et al., 2004). It can be concluded that the effect is protein- and system-specific. In our case, the effect of the His-tag was related to the activity instead of the solubility of the recombinant product. The fact that the C-terminal position of the His-tag does not have an effect on activity, while the N-terminal markedly does, may be related to the role of the N-terminal pro-sequence as an intramolecular chaperone, to assist the folding of the mature peptide. Although no structural data on the prolipase are available, it seems logical that the environment of the prosequence should be kept as unmodified as possible, since it has been previously reported to influence the formation of disulfide bonds (Beer et al., 1998).

Table 4.2**.** Influence of growth temperature and of the OD_{600} value of induction on the yield of the prolipase from *Rhizopus oryzae* produced in *E. coli* Origami (pET 11-d construct).

Growth temperature	OD_{600} at induction	Protein conc. [mg/ml]	Specific activity [U/mg]
20°C	0.5	0.7	60.4
20°C	1	1.5	110.7
25°C	0.5	0.9	47.3
25°C	1	0.7	42.4

Table 4.3. Influence of the vector and the OD_{600} value at induction time on the activity of the prolipase from *Rhizopus oryzae* expressed in *E. coli* Origami at 20 ºC.

Vector	His-tag position	OD_{600} at induction	Protein conc. [mg/ml]	Specific activity [U/mg]
pET11	none	0.5	0.7	60.4
pET11	none	1	1.5	110.7
pET15	N-terminal	0.5	1.6	32.5
pET15	N-terminal	1	0.9	40.8
pET11+ His-tag	N-terminal	0.5	1.6	20.7
pET11+ His-tag	N-terminal	1	1.7	18.8
pET22	C-terminal	0.5	0.7	116
pET22	C-terminal	1	0.7	70.7

4.3 MUTAGENESIS

The increasing interest in applying enzymes in industrial processes has spurred the search for biocatalysis with new or improved properties. Evolutionary design approaches have enjoyed considerable success in recent years. Numerous enzymes have been improved by directing their evolution in the laboratory, which usually involves iterations of random mutagenesis or recombination followed by screening or selection (Farinas et al., 2001).

4.3.1 VISUALIZATION OF TARGET RESIDUES

Rational protein design requires both the availability of the structure of the enzyme and knowledge about the relationship between sequence, structure and mechanism. Since publication of the first high-resolution 3-D lipase model in 1990 (Brady et al., 1990), many additional structures have been published (Cygler et al., 1997). Rapid progress in solving protein structures by NMR spectroscopy instead of by X-ray diffraction of crystals and the enormously increasing number of sequenced stored in public data bases have significantly eased access to data and structure (Bornscheuer et al., 2001). The structure and function of several *Rhizopus* lipases have been deeply investigated and are nowadays deeply known (Bornscheuer et al., 2000).
The *Rhizopus oryzae* lipase 3-D coordinate file in the Brookhaven protein data bank (PDB) is 1TIC. Unfortunately, this file containes only the α-carbon skeleton, (2.6 Å). However, the position of the side chains was needed in order to determine accessibility of residues. Therefore a similarity search for a structure was performed. Lipase from *Rhizopus niveus* (RNL) was the closest model found (PDB= 1LGY). It differs from ROL only by two conservative mutations, H134N, I254L (Bornscheuer et al., 2000), and particularly, in the case of proROL, by Met1(Joerger et al., 1993). From the RNL structure, by inserting these mutation the ROL structure was obtained (Figure 4.9). The structural characteristics of the ROL are the follows:

	Signal
Preprolipase	aa 1-25
Prolipase	aa 26-86
Mature lipase	aa 87-393
Lid	aa 208-215

The active site is characterized by the triad: S268, D327, H380. As already mentioned, the protein is characterized by three sulfhydril bonds: C152-C391; C163-C166; C358-C367.

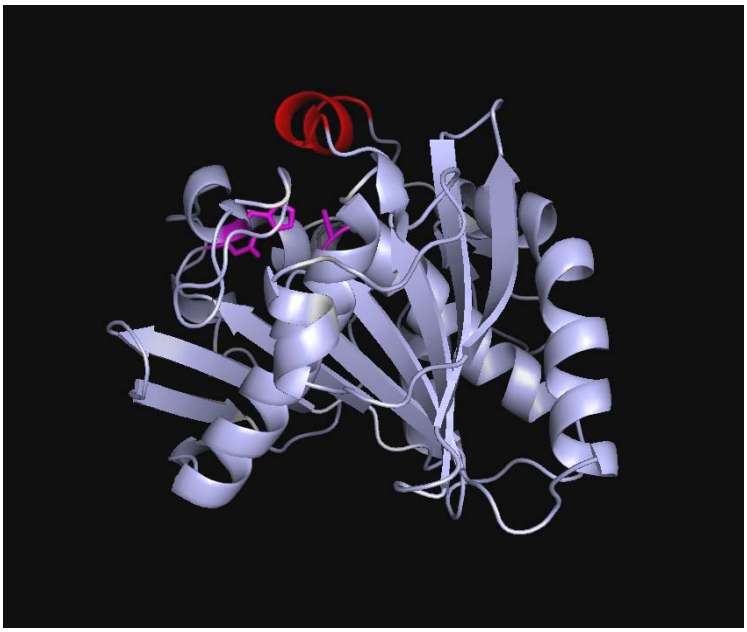


Figure 4.9: visualization of the 3D structure of the *Rhizopus oryzae* lipase, in red the lid, in pink the active site. The model was created from the Rhizopus niveus lipase.

By analyzing the ROL 3-D structure, the residues involved in the interaction with the products of the triglyceride oxidation were located. It resulted that there are six cysteine groups and are all involved in the three sulfhydryl bonds of the protein; the lysine residues are fifteen and are all distributed on the protein surface, some of them are involved in H-bonds; the histidine residues are seven and are all but one, His134, very close to the active site (Figure 4.10).

Figure 4.10: Visualization of the target residues on the ROL. In red the lid, in pink the active site, in orange the histidine groups, in light blue the lysine groups, in green the cisteine groups bounded with SH-bonds.

4.3.2 MUTATION STRATEGIES

Since the cysteine groups are all involved in sulfhydryl groups, it was decided not to change these residues. Indeed it is very likely that the single change of one cysteine causes undesirable changes on the protein secondary structure, leading to an unfolded and thus inactive, protein. It has been already mentioned that the use of *E. coli* Origami as expression strain led to a soluble protein because it helps forming sulfhydryl bonds in the cytoplasm. On the other hand, the protein deactivation may be determined by a change in its structure caused by the disruption of a SH-bond from the inactivant which may compete in the interaction with one cysteine. An interesting option could be then to change both cysteine residues involved in a disulfide bond. However, this option was not considered in this Thesis, and we preferred to concentrate on the histidine and the lysine groups diffusion into the active site. In particular, since the histidine groups are all (with the exception of His134) very close to the active site, we expected that the change in these residues could affect the protein activity, by changing the binding site environment. Alternatively, it could be possible to obtain a more stable protein, since a reason for the protein deactivation might be that the interaction between the inactivant and the histidine does not allow substrate diffusion into the active site. With regard to the lysine groups, we decided to direct the mutagenesis only to lysine groups not involved in H-bonds, or involved in up to 2 H-bonds, as summarised in Table 4.4.

Table 4.4: Number of H-bonds in which the lysine residues substituted with the site-directed mutagenesis are involved

RESIDUE	H-BONDS
Lys168	none
Lys101	none
Lys104	none
Lys37	1
Lys202	1
Lys5	2

The mutations were performed as described in the Methods section. Basically, the technique utilized was a combination of rational design and random mutagenesis at defined position, in the form of focused libraries, e.g. the effect of each of the 20 amino acids were tested at selected position in the enzyme. This concept of spatially pre-determined saturation mutagenesis has been already successfully employed with the aim of enhancing substrate selectivity or influencing enanthioselectivity (Reetz et al., 2005; Reetz et al., 2006).

4.3.3 LIBRARY GENERATION

For each position to be mutated, to be sure that all the possible cases (i.e. one position is saturated with all the 19 remaining amino acids), are present in the library, a 10–fold oversampling was considered, and thus a number of 200 clones should be screened. It is clear that the quality of the genomic libraries generated for each mutated position has to be high, theoretically that means that each clone of the libraries should be different. The lower the library quality (multiple copies of every different individual), the higher the number of clones to be screened to consider all possible cases. Another aspect is that the strain utilized for the expression of proROL variants should be obviously *E. coli* Origami, in order to be sure that the prolipase is expressed as soluble protein in the cytoplasm, and the protein expression conditions based on those previously optimized for the expression of the wild type protein in flask, to ensure high protein yield and activity.
Unfortunately, when the *E. coli* Origami strain was used for the direct transformation of the mutated DNA, no transformants were obtained, not even when electrocompetent Origami cells were used. A reason could be the very low competence of the Origami strain. On the other hand, when *E. coli* DH5α was used as cloning strain, enough transformants could be obtained per mutation. Different strategies were then considered, in order to generate the genomic library in the Origami strain, as represented in Figure 4.11.
In Protocol I, 200 colonies obtained after the transformation of the mutated proROL construct in DH5α competent cells were pooled and resuspended in LB containing the necessary antibiotic and incubated overnight at 37°C. Plasmid preparations were then isolated and transformed into *E. coli* Origami strain. In this way, high numbers of transformants could be obtained after transformation in *E. coli* Origami but the libraries were critically diluted. That means that the quality of the libraries generated was very low and a number of mutants much higher than 200 should be screened to cover all possible mutations in one position, leading to an extremely time-consuming screening procedure. It is evident that this protocol had to be discarded. Its optimization is represented by Protocol II. In this case, in order to reduce the library dilution, the 200 colonies were pooled and resuspended only in 1ml of LB with antibiotic and incubated at 37°C for a time that was optimized to obtain the minimum amount of plasmid DNA necessary to obtain enough colonies per plate, after transformation in *E. coli* Origami. The experiments showed that four hours was the minimum incubation time needed.
Finally, according to Protocol III, the single colonies obtained with the transformation in *E. coli* DH5α, were grown in MTP and subsequently, a high-throughput DNA purification was performed. The transformation in *E. coli* Origami of each single mutant followed. It is evident that in this case no library dilution occurred and the quality may be regarded as very high: for each position only two MTPs had to be screened. The bottleneck of this method is unfortunately represented by the high cost of the kit required for the DNA purification in MTP.

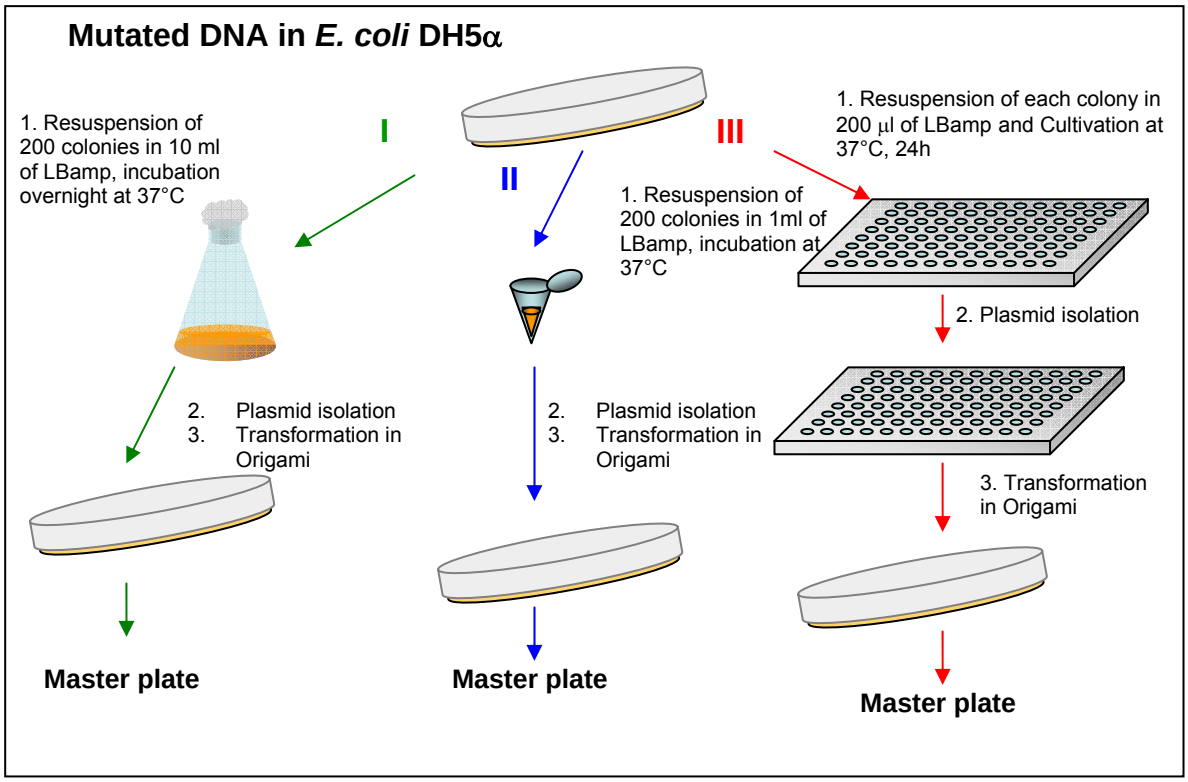


Figure 4.11: Protocols for libraries generation in *E. coli* Origami

For library creation we decided to perform Protocol II in the case of the histidine residues, and Protocol III in the case of the lysine residues and the histidine residue far from the active site (H134).

In the case of the histidine residues close to the active site, the excessive number of variants to be screened could be reduced with a previous activity staining test carried out in solid agar plates, since it was very likely that their mutation could negatively affect protein activity. In this way then, the library size could be reduced by including only variants that showed activity.
Some authors already refer to the production of focused libraries, in order to reduce the number of variants to be screened, by choosing from a prior analysis of the protein structure, the site of mutation most likely to improve the target enzyme's properties (Reetz et al., 2005; Reetz et al., 2006). Furthermore, mutations in sites close to the active site of the enzyme have been reported to cause greater effects on the property of interest (Kazlauskas, 2005; Morley et al., 2005).
In Figure 4.12 some results from the overlay agar activity staining, concerning the mutation of the histidine in the position 109, are shown. Many clones on plates 1 and 2 had no activity. The proROL wild type was screened as positive control. A negative control was also performed by screening the empty pET22b vector transformed in *E. coli* Origami.

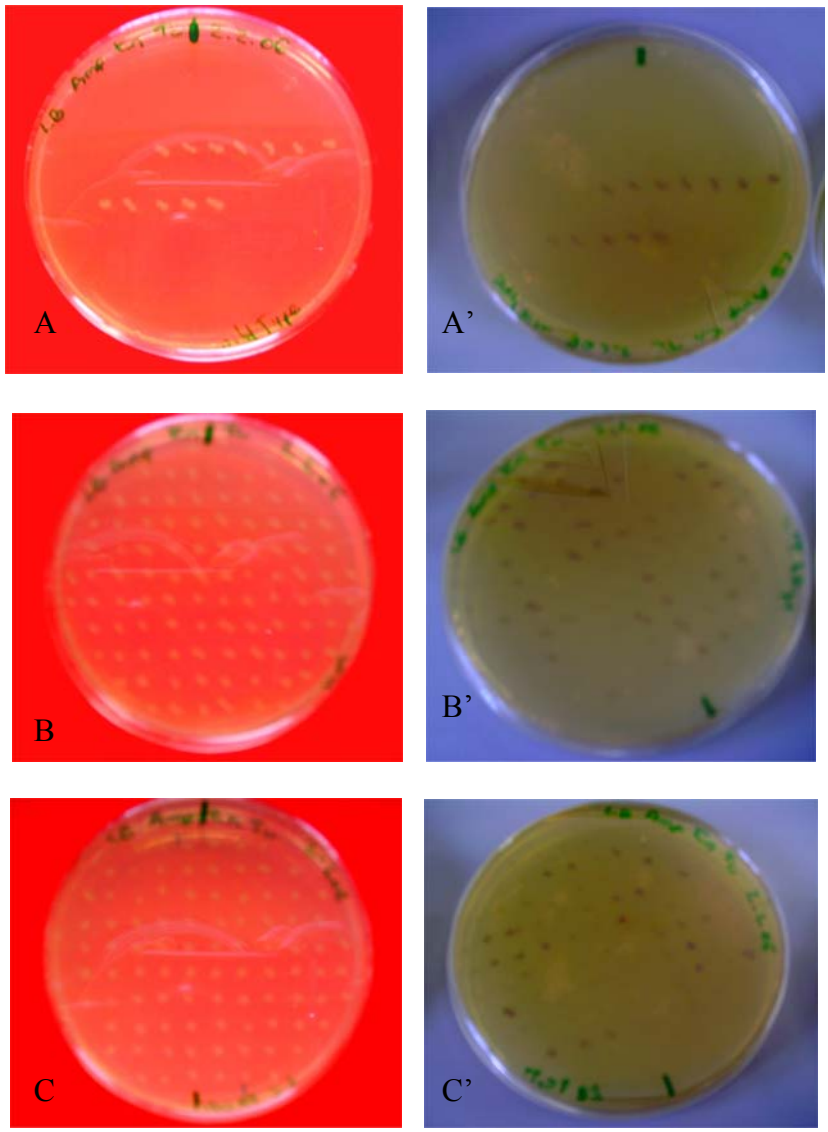


Figure 4.12. Activity staining tests. A,A' wild type; B,B' clones carrying a mutation of the histidine in position 109, plate 1; C, C' clones carrying a mutation of the histidine in position 109, plate 2.

The results of the activity-based filtering of the libraries provided an approximate notion about the influence of the position mutated on the lipase activity. The percentage of the clones per plate that retained activity for each histidine residue mutated were compared (Figure 4.13). In the case of Histidine 134, the highest percentage of active clones was observed, while, according to the results obtained, positions 218 and 144 (Figure 4.14) have shown to be essential for the enzymatic activity, the number of active clones per plate being in both cases less than 10%.

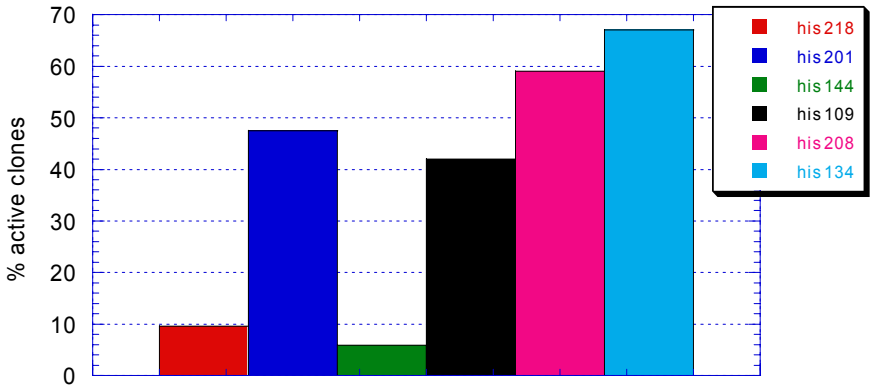


Figure 4.13: Percentage of active clones revealed by the activity staining test. The percentages have been calculated on an average of 10 plates per position mutated. In the case of His134 the percentage was referred to the two MTPs obtained from the protocol III

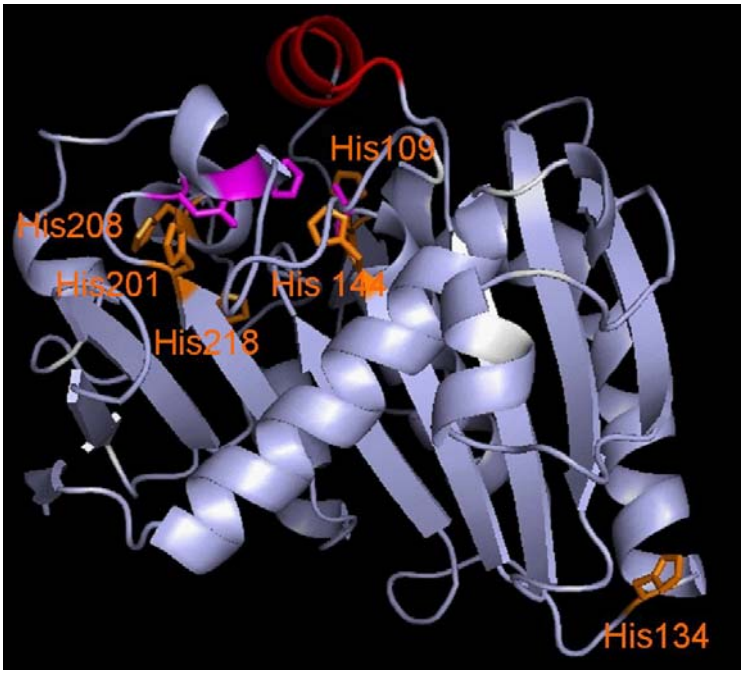


Figure 4.14: Visualization of the ROL histidine residues

Some of the active and inactive clones from the His218 and His144 plates, were sequenced in order to have an idea of the residues that preserve the activity or inactivate the proROL when the histidine of the wild type protein is replaced (Table 4.5). Many of the clones picked were represented, as expected, by the wild type protein, but some different mutants were also found. It would be interesting to analyze the reason why some particular histidine substitutions make the proROL inactive.

Table 4.5: Sequencing results of some His218 and His144 active and not active clones

His218	
Active	Val Ser
Not active	Met Phe Cys
His144	
Active	Asn Pro
Not Active	Ile Arg

In conclusion, Table 4.6 summarises the protocol utilized for the generation of mutant libraries and their number. In case of the high-throughput purification, two MTPs of mutants were created for each position mutated, while in the other cases, the library was generated by picking the active clones resulting from the activity staining test of 10 agar plates containing an average number of clones varying from 200 to 300.

Table 4.6: Library of single mutants generated, methods utilized and total number of plates created for each position mutated

Library	**Method**	**Num of 96-well library plates**
His109	Activity staining test	3
His134	High-throughput plasmid purification	2
His144	Activity staining test	1
His201	Activity staining test	7
His208	Activity staining test	3
His218	Activity staining test	1
Lys101	High-throughput plasmid purification	2
Lys104	High-throughput plasmid purification	2
Lys37	High-throughput plasmid purification	2
Lys202	High-throughput plasmid purification	2
Lys5	High-throughput plasmid purification	2

It can be concluded that owing to the prior activity staining performed, a high number of clones was rapidly screened, with the results that the libraries were characterized by very few MTPs, also when, for the library generation the

Protocol II was followed. The only exception was represented by position 201, for which it was not possible to reduce the number of variants to be analyzed and 7 MTPs had to be screened.

4.3.4 OPTIMIZATION OF THE EXPRESSION AND CELL DISRUPTION IN MTP

The expression in MTP of the wild-type proROL was derived from the conditions previously optimized in the case of the expression in flasks, leading unfortunately, to a very low activity, of approximately 3mU/ml. proROL expression in deep well MTPs was considered, with the aim of achieving a higher enzymatic activity. In this way, the expression volume could be increased by a factor of seven, compared to standard 96-well MTPs. Accordingly, the cell lysis protocol had to be optimized for deep-well plates, with particular attention to the amount of the lysis buffer to be utilized for the pellet resuspension after centrifugation, and the concentration of lysozyme and DNAse contained in the buffer.
The buffer volume should be sufficient to resuspend the cell pellet, but should be kept to a minimum, in order to avoid enzyme dilution. DNAse and lysozyme concentrations should provide an efficient cell disruption and a decrease of the lysate viscosity. By increasing the expression time of in the normal MTPs, up to 16h, a much higher activity could be reached. With these modifications, the activity obtained after cell disruption amounted to 50mU/ml. On the contrary, the expression in deep well MTPs did not increase the enzymatic activity as expected, and the activity reached was only 12 mU/ml. The expression time should have been increased to 16 hours in this case as well, thus obtaining a much higher activity, but we preferred to utilize standard 96-well plates for easier manipulation. The lysate viscosity in the case of deep well was in fact still higher than the normal MTP, thus making pipetting difficult. Moreover, the amount of total protein per well was excessive, causing protein precipitation in the wells of the incubation plate, which interfered with the kinetic assay.
The optimized conditions found for the deep and normal MTPs are compared in Table 4.7, together with the resulting activities.

Table 4.7. Conditions for the cell disruption assay in the case of normal MTP and of deep well MTP. The activity value in the case of normal MTP, corresponds to an increased incubation time of 16 hours

MTP size	Volume of the expression [μl]	Volume of lysis buffer [μl]	Lysozyme [mg/ml]	DNAse [%]	Activity [mU/ml]
Deep well	1400	450	5	0.5	12
Normal	200	150	1	0.1	50

4.4 SCREENING

4.4.1 DEACTIVATION OF THE WILD TYPE PROROL IN SEMI-MICRO SCALE

The deactivating effect of the triglycerides auto-oxidation products on the proROL, , was investigated by considering only aldehydes, as typical secondary oxidation products. Obviously, the effect of oxidized oils on lipases is much more complex since several oxidation products are produced at concentration levels, that depend on oil or fat (Knothe et al., 2003). Previous experiments have shown, however, that aldehydes decrease lipase stability to the highest extent among al carbonyl compounds produced in the oxidation of oils and fats (Pirozzi, 2003). Moreover, the interaction between aldehydes from oils and fats peroxidation and proteins has been studied in-depth (Agerbo et al., 1992; Kaga et al., 1994; Uchida et al., 1998; Buko et al., 1999; Wu et al., 2001; Pirozzi, 2003).
The proROL stability screening was performed as described in the materials and methods section. Even if we are mainly interested in nonaqueous environments, and transesterification reactions, in the experiments concerning the proROL, an aqueous system was considered and the reaction investigated in the screening was the hydrolysis of the *p*-nitrophenyl butyrate (pNPB). An efficient assay for lipase activity characterization based on the fluorescence of methylumbelliferone has been reported (Nùria Prim, 2003). In our specific case, however, it was unsuccessful perhaps because of the interaction taking place between the substrate and the aldehyde.
The main reason for choosing an aqueous system were the simplicity and throughput required for library screening. It is obviously much more difficult and extremely time-consuming to perform a purification of the mutants after cell disruption in MTPs in order to use an organic solvent and consider a transesterification reaction.
At this stage, the main objective is to carry out a fast selection of mutants with better stability than the wild type protein. Subsequently, this improved stability has to be confirmed in organic media and the effect of mutation on the (trans)esterification reactivity of the protein, must be analyzed.
Before performing a high-throughput screening of the mutants, the methods had to be previously developed in 1.5ml tubes, for the wild type proROL expressed in flask, in a total volume of 1000μl.
It was crucial to identify conditions under which:

- deactivation was high enough to be measured
- deactivation was due to the aldehyde and not to other factors
- the control (i.e. enzyme without deactivant) was stable, i.e. minimum deactivation during the assay
- deactivation was slow enough to allow measuring various points along the curve, enabling the determination of a first order deactivation kinetic

In order to prevent microbial growth, so that deactivation was not due to bacteria growing on the proteins in solution, NaN_3 was added to the protein solution. Moreover, the incubation temperature had to be low enough to avoid thermal inactivation. To ascertain that the aldehyde is the main deactivation factor, it is of paramount importance that the control without inactivating agent is stable, throughout the whole experiment.
The dilution factor of the enzyme solution after the cells disruption plays a critical role on enzyme stability during the assay. When highly diluted enzyme solutions were used, the control underwent deactivation
Recently, most lipases have been shown to have a natural trend to form bimolecular aggregates, by adsorption of open lipases on open lipases *via* the large hydrophobic pocket formed around the active centre. These aggregates have entirely different catalytic properties when compared to the individual lipase molecule and are more stable than the enzyme in monomeric form. With increasing enzyme dilution, enzyme stability decreases (Wilson et al., 2005). The relation between the residual activity of the enzyme and the dilution factor has been analysed in order to choose a factor which leads to a stable control in the stability assay. Figure 4.15 shows that only 10% of the enzyme activity is lost after 8 hours of incubation, when dilution factors of 50 and 100 are used, whereas over the same time-span the deactivation is approx.80% with a factor of 400.

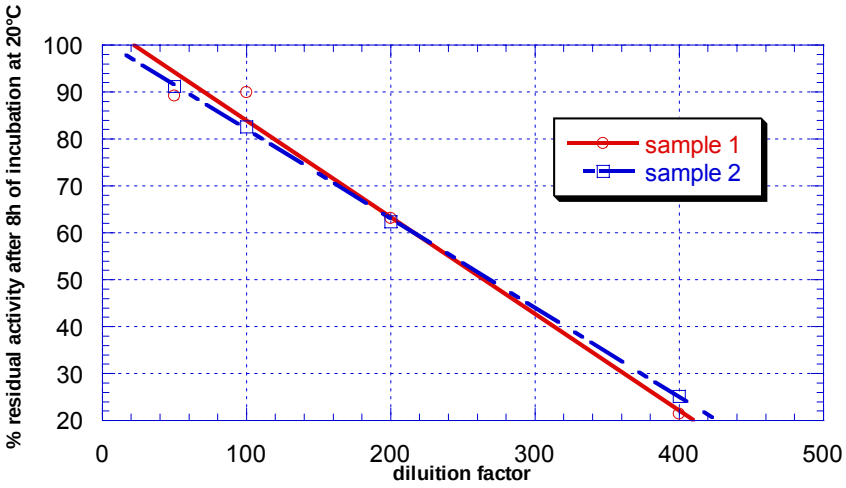


Figure 4.15. Relation between the residual activity of the proROL after 8h of incubation at 20°C and the dilution factor utilized for the enzyme solution after the cells disruption.

The other important factor was the concentration of inactivating agent in the deactivation process. If the concentration of aldehyde were too high, the deactivation would proceed too fast and it would be difficult to take enough measurements, also in this case it would have been difficult to assure the same initial activity value of the control and the protein incubated with aldehyde at the time zero, necessary to be able to compare the stability of both samples. On the other hand a too low aldehyde concentration might have determined the deactivation not to be high enough to be measured, or other factors would have

outweighed the aldehyde effects, thus causing only little differences between the control and enzyme with aldehyde.
At the beginning of this Thesis work, we investigated the effect of proROL incubation with some of the most reactive aldehydes generated by lipid peroxidation, i.e. malonaldehyde (MDA), 4-hydroxy-nonenal (4-HNE) and acrolein.
Figure 4.16 shows the deactivation kinetics in the case of the wild type proROL incubated with (MDA). The latter was obtained from the acid hydrolysis of 1,1,3,3-tetramethoxypropan as described by Kwon (Kwon et al., 1963). Case A, refers to an incubation temperature of 37°C, case B to a temperature of 20°C. It is evident that 1M and 0.5M concentrations of MDA are too high, thus leading to a too fast deactivation effect. Moreover, the initial activity was not the same in each case. Table 4.8 indicates a 46% enzyme deactivation occurring immediately after MDA addition to a final concentration of 1M. Obviously, temperature further increases the deactivation rate (Table 4.8).

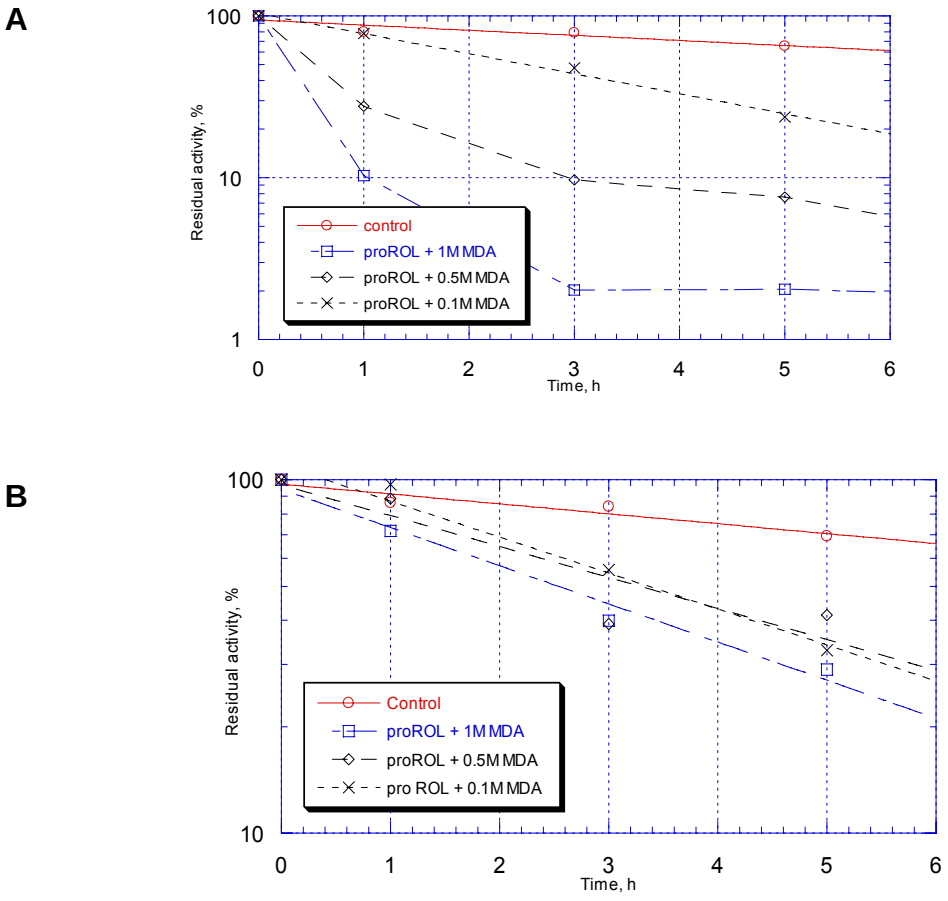


Figure 4.16:Effect of the incubation of the proROL with MDA. Dilution, in phosphate buffer (50mM, pH7.5), of the enzyme solution obtained after cells disruption : 1:100. Concentrations refer to the final concentration of the MDA solution obtained from the hydrolysis. A) Incubation performed at 37°C; B) incubation performed at 20°C

Table 4.8: Analysis of the influence of incubation temperature on the enzyme half life and of the MDA concentration on the initial enzyme deactivation with respect of the control

MDA solution concentration [M]	Activity at t=0, [U/ml]	% deactivation	Half life at 20°C .[h]	Half life at 37°C.[h]
none	80	/	8.8	7.6
0.1M	79	1	3.4	2.3
0.5M	73.4	8	3.3	0.7
1M	42.9	46	2.5	0.5

Figure 4.16 also shows that at 20°C, over the time-span considered, the activity decay data can be interpolated with exponential curves for all MDA concentrations investigated. This suggests that, under these conditions, first order kinetics in active enzyme concentration is followed. On the contrary, at 37°C the inactivation curves are more complex and can be interpreted with first order kinetics only for the control and of a 0.1M MDA concentration.
According to these results, the incubation temperature was fixed to 20°C.for the following experiments. It was also decided not to utilize MDA as deactivator for the stability assay, as it was difficult to define exactly its concentration, since its conversion efficiency from the acid deprotection reaction required, is not know. In fact the concentration values indicated above, refer to the concentration of the aldehyde solution after the hydrolysis reaction and are evidently too high for being the aldehyde concentration in a 100% conversion case. The experiments results were also difficultly reproducible, confirming on one hand, that the aldehyde might be produced with different efficiency each time and on the other that this aldehyde is very unstable. The high MDA instability has been already reported (Kwon et al., 1963). Moreover, the MDA solution showed a red-orange colour, evidencing a polymerization of the aldehyde molecules, the colour changing with the time and influencing the activity assay. Additionally, since the assay time is not very long, having a maximum of 6 hours, NaN_3 was not added to the solution, as no risk of microbial growth could occur.
The proROL was also incubated with acrolein, and its deactivation at three different aldehyde concentrations analyzed (Figure 4.17). It is possible to observe that the acrolein concentration considered were much lower than those of MDA, demonstrating once again, that the concentration values indicated above for the MDA solution can not be referred to a 100% efficient MDA conversion. An acrolein concentration of 50mM is highly deactivating and thus the deactivation does not follow first order kinetics.

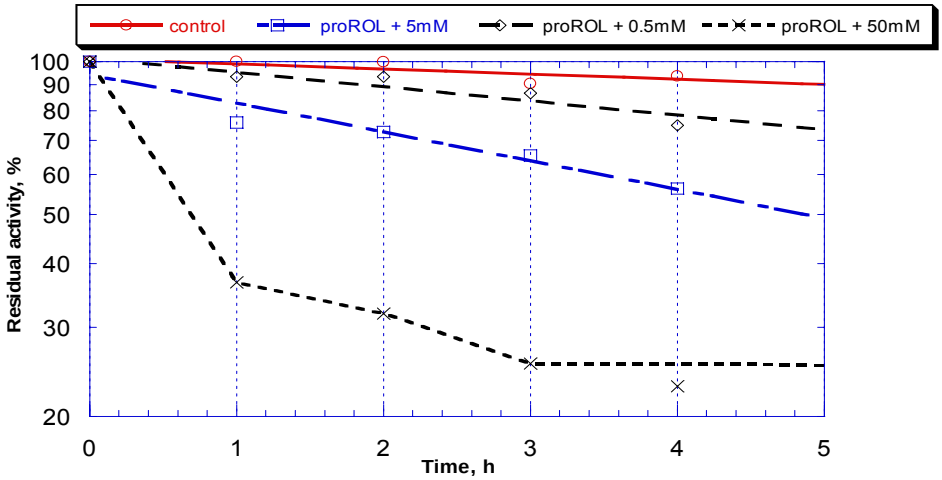


Figure 4.17: Effect of the incubation of the proROL with acrolein. Dilution, in phosphate buffer (50mM, pH7.5), of the enzyme solution obtained after cells disruption : 1:100. Incubation performed at 20°C

Some irreproducibility resulted from the high volatility of acrolein that determined random variations in the actual aldehyde amounts introduced in the 1.5ml incubation tubes. Furthermore, because of the instability of this aldehyde storage problems arose.
Another product of the lipid peroxidation investigated was 4-HNE. Figure 4.18 shows the effect of the proROL incubation with several 4-HNE concentrations.
Here the aldehyde concentrations are again much lower than the case of MDA, a 2mM concentration of 4-HNE resulted to be already highly deactivating, the half life of the enzyme being of approximatly. 3 hours. Figure 4.18 also shows that the proROL deactivation during 4 hours follow an exponential.

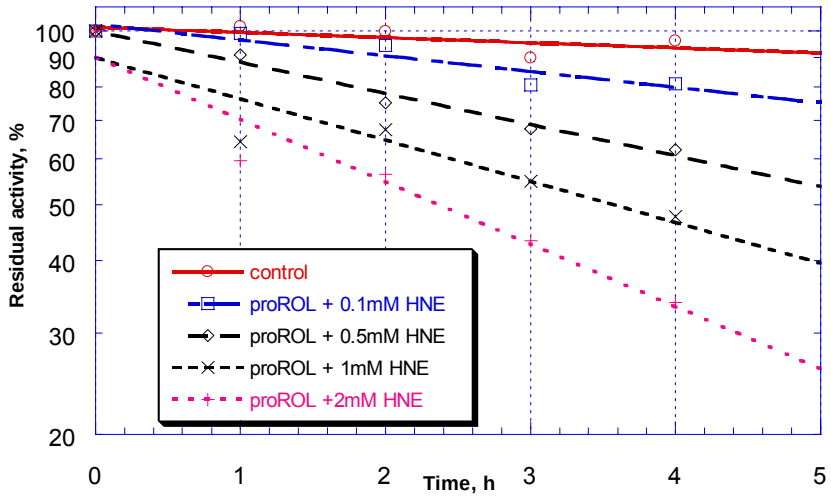


Figure 4.18. Incubation of the proROL wild type with 4-HNE at different concentration. Initial enzyme activity 35 mU/ml, dilution of the enzyme solution after cell distruption 1:200, incubation temperature 20°C

4-HNE is not as volatile as MDA or acrolein, it does not undergo polymerization and thus no colour changes interfere with the assay. As a consequence of its stability, the experiments were this time much more reproducible. A serious drawback is the high cost of HNE for the screening of all the mutant libraries., A less expensive aldehyde is thus mandatory, at least for the initial selection of the mutants.
Several other aldehydes were considered and their effect on the proROL analysed and compared with 4-HNE. We assumed that the mechanism of the interaction between aldehyde and lipase was the same as in the case of 4-HNE. The aldehydes had different hydrocarbon chain lengths. For each of them, the deactivating effect was lower than that of 4-HNE, so higher concentration had to be adopted. Figure 4.19 shows results regarding the incubation of the proROL wild type with 50mM solutions of some of the aldehydes investigated. Basically the inactivating effect of the aldehyde increases with chain length. A particular case is represented by the trans-2-nonenal as the activity loss of the proROL incubated with 1mM, 10mM and 50mM solutions of this aldehyde, after a defined time, was the same (data not shown). The increasing deactivation effect with the increasing aldehyde chain length has been already reported in human body, in the case of aldehydic products of lipid peroxidation (Buko et al., 1999). Thus, the deactivation mechanisms holding in our case appear to be the same.

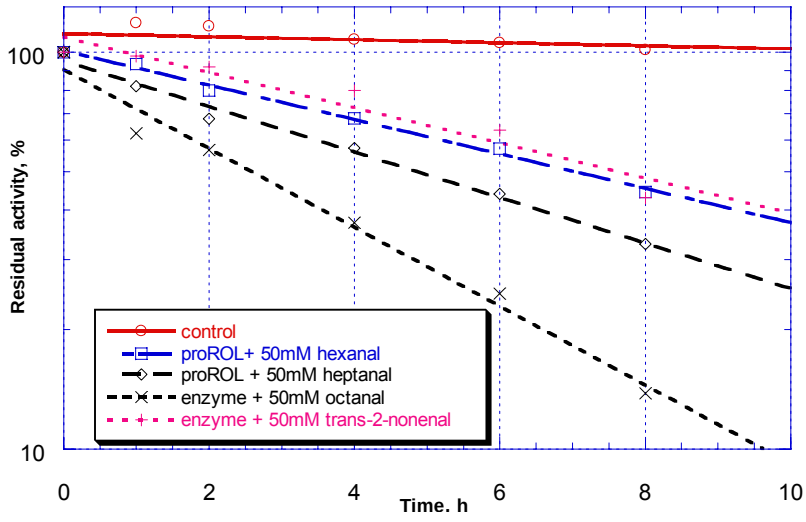


Figure 4.19. Incubation of the proROL with several aldehydes. Initial enzyme activity 50 mU/ml, dilution of the enzyme solution after cell distruption 1:100, incubation temperature 20°C

Within the time-span of our experiments, the kinetics are first order. Thus, a quantitative comparison among mutant stabilities in the high-throughput screening can be performed by comparing the deactivatio constant. Among all aldehydes tested, octanal was the best candidate for initial screening of the libraries. The stability screening settings are summarized in Table 4.9.

Table 4.9: conditions fixed for the stability screening

Incubation temperature	20°C
Dilution of the enzyme solution, after cell disruption	From 1:50 to1:100
Deactivator	Octanal
Deactivator concentration	50mM
Incubation time	~ 6h

4.4.2 IMPROVEMENT OF THE HIGH-THROUGHPUT SCREENING

Generally, the method utilized for the high-throughput screening of enzyme libraries is of a critical importance. Prior to the generation of genetic diversity, one should carefully consider which screening procedure would be more suited with respect to the handling capacity, sensitivity, and achievement of the envisioned goal. It is also preferred to ensure that the screening procedure directly measures the property that one wishes to improve, particularly for directed evolution applications where it is well known that “you only get what you screen for” (Schmidt-Dannert et al., 1999).
In this Thesis, the high-throughput screening method was established by starting from the conditions optimized for the assay in 1.5ml tubes and initially it was developed by considering only the wild type proROL, expressed in MTP with the optimized conditions described in section 4.3.4.
First of all, the optimal enzyme dilution after cell disruption needed to be determined (Figure 4.20). Due to smaller cell and protein productivity, this factor was lower than in the expression in flask. In the latter case in fact, a dilution factor up to 100 could be used, but when the expression was performed in MTP the maximum dilution of the enzyme solution was only 5-fold.

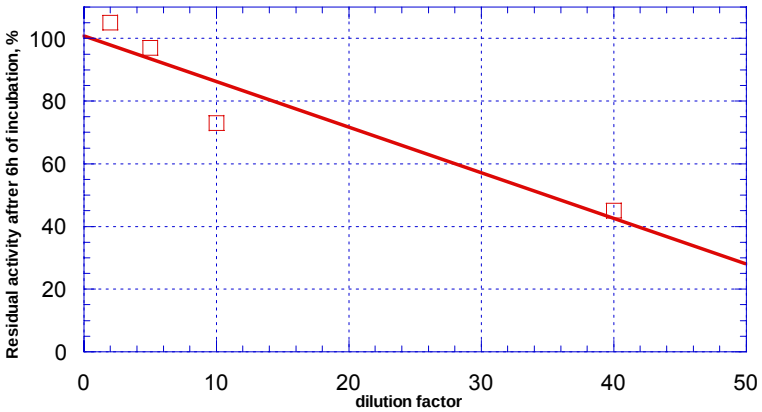


Figure 4.20: Effect of the lysate dilution on the proROL stability in the case of expression in MTP. Points represent the average of the residual activity in 96 wells after 6h of incubation. Initial activity, average: 50 mU/ml. Temperature of incubation: 20°C

The scheme followed in the high-throughput expression and screening in MTP was discussed in the materials and methods section (Figure 3.10, page 35). When the enzyme solution obtained from the cell disruption as described in

Table 4.7 was incubated with octanal at two different concentrations, no activity loss took place (Figure 4.20). This might be explained in two ways: i) there is a loss of octanal; ii) the high overall concentration level of lysozyme and of DNAse used in for the enzymatic lysis of the cells stabilize lipase.

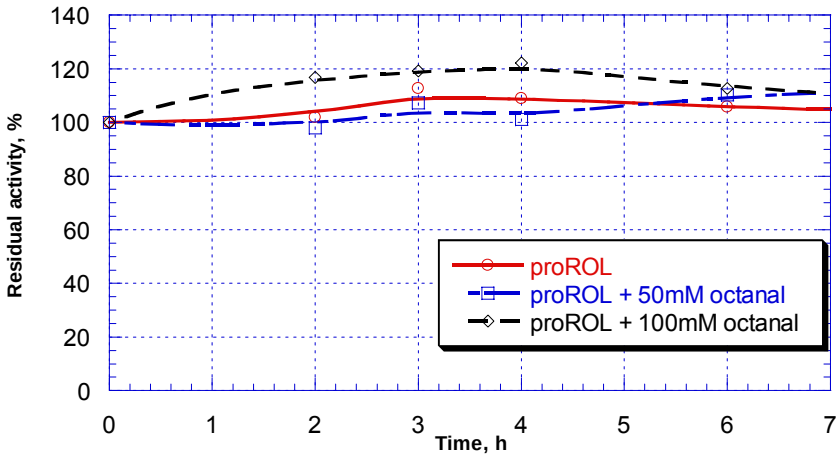


Figure 4.20: Incubation of the proROL wild type with a 50mM and 100mM octanal solution. Initial enzyme activity 50 U/ml, dilution of the enzyme solution after cell disruption 1:5, incubation temperature 20°C

Loss of aldehyde was evidenced by loss of adherence in the adhesive film covering the microtiter plates during incubation, probably due to solvent evaporation and aldehyde volatility. To circumvent this problem, the incubation was performed in strips of PCR tubes, which provided an airtight reaction environment. Moreover, since the solubility of the octanal in water is very low, variability in the aldehyde concentration in each well might occur, leading each time, to variability in the incubation conditions. In order to avoid this inconvenience, three different organic solvents for the aldehyde stock solution, were considered instead of phosphate buffer: ethanol, DMSO and acetone. The amount of each solvent in the well was kept to a maximum of 5% (v/v) in order to reduce interactions with the enzyme to a minimum. The influence of these solvents on the assay system and the effect on the enzyme stability were investigated as well.
When ethanol and acetone were used, the results were unsatisfactory because of their high volatility (data not shown). On the contrary, DMSO was a good alternative to phosphate buffer as its volatility is low and it had no effects on the proROL stability, at the concentration considered. When in fact the proROL was incubated with a 5% solution of DMSO no changes in the proROL activity were observed (Figure 4.22).
The stabilizing effect of high, overall protein concentration on enzymes, is well known(Greco jr. et al., 1991; Toscano et al., 1994). Specific tests were carried out, in order to optimize the cell disruption procedure, in terms of lower amounts of lysozyme and DNase.
A way to reduce the DNase and the lysozyme concentration could have been to reduce the volume of buffer utilized for the resuspension, thus also increasing the relative proROL concentration. Unfortunately, in that case the viscosity of

the suspension would have increased to the point of difficulty pipetting and liquid handling.
Analysing the lysis assay utilized (as described in the Methods), it was assumed that since the optimal working temperature required by the lysozyme is normally 37°C , the incubation at 4°C could not have allowed the enzyme to work at its best, so that the cells disruption might have been brought about mainly by the freezing step at -80°C. This led to the investigation of the influence of the lysozyme concentrations in the lysis buffer on the enzyme activity.
A range of lysozyme concentrations varying from 0 to1 mg/ml was then considered.
In each case a virtually constant enzymatic activity was observed, with only minor variations even when lysozyme was not present in the lysis buffer.
The lysates, obtained with different lysozyme concentration in the lysis buffer, were afterwards incubated with a 50mM and a 100mM octanal solution. As expected, lysozyme interfered with the assay, the deactivation effect of the aldehyde increasing with decreasing lysozyme concentration. The highest proROL deactivation was produced when no lysozyme was present in the lysis buffer (Figure 4.21)..

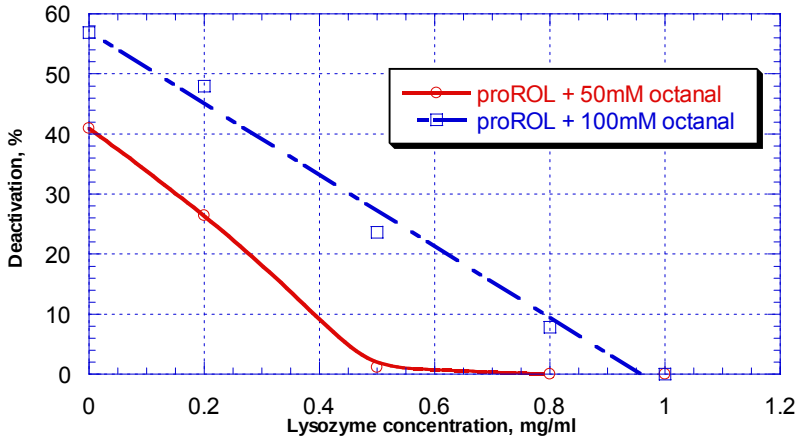


Figure 4.21. Effect of the lysozyme concentration in the lysis buffer on the proROL deactivation after 6h of incubation with two different octanal solutions. Temperature of incubation 20°C

With a 100mM octanal concentration, the relationship between the deactivation achieved at a fixed incubation time of 6 h and the amount of lysozyme was linear. On the contrary, with 50mM octanal concentration, the deactivation is very low and a sort of saturation effect was observed: This implies that a 0.5mg/ml lysozyme concentration is high enough as to produce a stabilization effect that reduces proROL deactivation to nil. In the absence of lysozyme, even at 100mM octanal concentration, proROL deactivation occurring in 6 h of incubation was only 60%, whereas, in 1.5ml tubes, after 6 hours of incubation with 50mM octanal, the protein deactivation was of 80%. Thus, the conclusion is that the presence of DNase in the lysis buffer, essential to reduce the lysate viscosity, stabilizes the lipase. On the other hand, the total aldehyde concentration cannot be increased, because 100mM is the upper limit for octanal solubility in the enzyme solution.

The three different methods considered to perform the cell disruption were compared in order to better understand their differences. The table 4.10 shows the results concerning the proROL expressed in flask.
The highest activity was reached when the cells were disrupted by sonication, and the lowest with the freeze/thaw method, meaning that among all methods, the latter has the worst disruption efficiency. Moreover, the proROL deactivation was much higher when sonication was the method utilized. These results are logical since with the sonication, not extra proteins that compete in the interaction with octanal, are introduced and in the case of the freeze/thaw, only DNAse is added. Therefore, to observe the same deactivation increasing aldehyde concentrations should be employed.

Table 4.10. Comparison of the three different methods utilized for the cell disruption (wild type proROL expressed in 100ml). The residual activity is referred to a 6h incubation time.

Lysis method	Initial activity [U/ml]	residual activity Control [%]	residual activity 50mM octanal [%]
Sonication	113	89	4
Freeze/thaw	63	83.	15
Lysozyme	99	87	54

The results of the proROL incubation with octanal in the case of no lysozyme in the lysis buffer, are shown in the Figure 4.22. The quality of the curves is different from the one obtained with the 1.5ml tubes assay. The shape of the deactivation curves is different from that of those obtained with the 1.5ml tubes assay. They show a fairly typical, two-step behaviour often occurring in enzyme thermal and chemical inactivation.
A 100mM octanal concentration was the most suitable for the high-throughput mutant screening, the enzyme half-life being in this case already of approximately 2 hours and the difference with the residual activity of the control, of 60%.

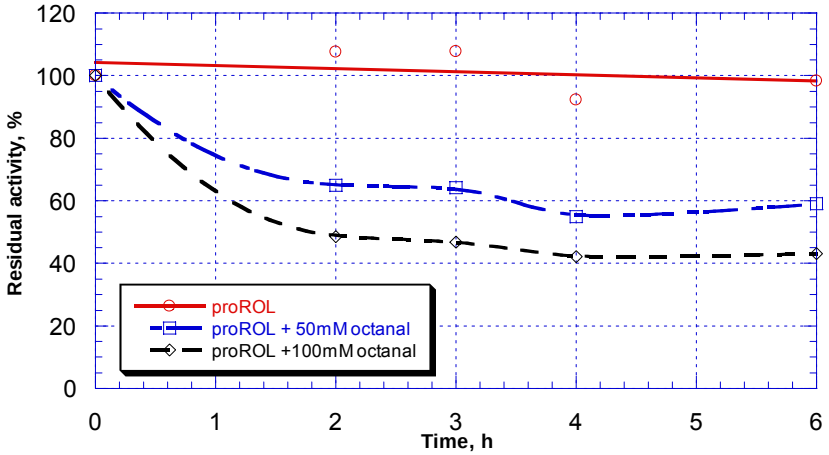


Figure 4.22. Incubation of the enzyme solution obtained after cells distruption, in the case of no lysozyme, with 50mM octanal and 100mM. Initial enzyme activity : 47 U/ml.

Temperature of incubation 20°C. Solvent for the octanal stock solution: DMSO. Total DMSO amount per well : 5% The control also contains 5% DMSO

The fixed high-throughput screening settings are summarized in Table 4.11.

Table 4.11: conditions fixed for the high-throughput stability screening

Incubation temperature	20 °C
Dilution of the enzyme solution, after cell disruption	1:5
Deactivator	Octanal
Deactivator concentration	100mM
Incubation time	~ 6h

4.4.3 LIBRARY SCREENING

Even after the high-throughput screening was successfully optimized, a complete control of the MTP system was difficult to achieve. However, it proved satisfactory for a high-throughput initial screening of the mutants. In a second stage, deeper analysis of the mutant selected would be performed by considering the assay in the 1.5ml tubes, under tightly controlled conditions.
Two replicates were considered for each library plate. The residual activity of all the mutants after 6h of incubation in the conditions given in Table 4.11, were plotted on a graph, in order to select the variants with a residual activity higher than the wild type proROL (a threshold of at least 40% residual activity difference between putative hits and wild-type was considered). The mutants, whose stability was confirmed by the two replicates, were sequenced to confirm the presence of a mutation in the desired position, and to identify the nature of the residue introduced. Figure 4.22 shows an example of the criteria utilized for the selection.

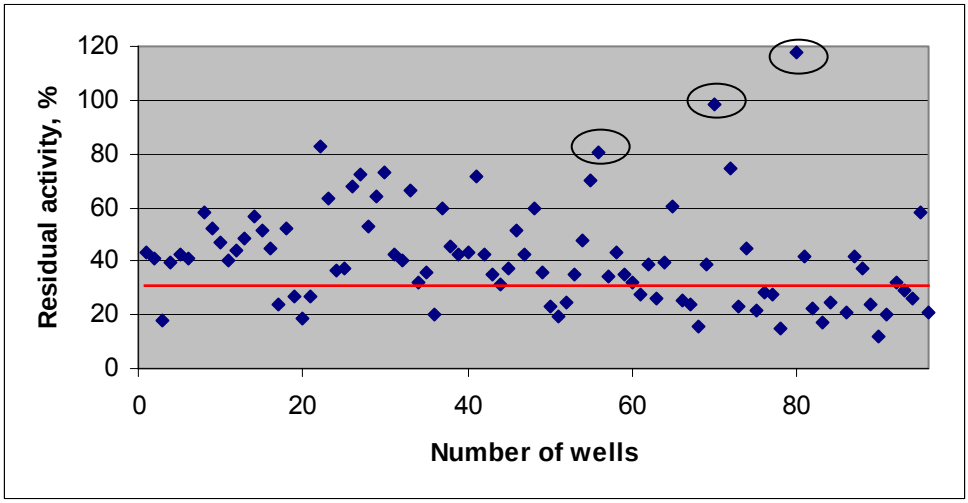


Figure 4.22. Selection of stable mutants in a His201 library.The red line represents the residual activity percentage of the wild type proROL . The variants marked are the ones confirmed by the replication.

Tables 4.12 and 4.13 summarize the overall results obtained from the screening of all the libraries, concerning respectively the mutation of the histidine and of

the lysine residues. For each position modified, the number of variants selected with the method described above, and the identity of the substitution introduced, are reported. Tables 4.12 and 4.13 also indicate, for each position modified, the frequency with which a variant carrying a specific substitution was selected, calculated by relating the number of times in which this variant was selected with the total number of variants selected for that specific position mutated.
The frequency values were particularly interesting for the libraries concerning the histidine residues close to the active site. For these residues, in fact it is very likely that only few mutations would lead to an active proROL and that their relative libraries contained many copies of the same variants. This hypothesis appears to be confirmed in the case of the His201 libraries, since of 20 variants selected, 50% were His201Ser, or in the case of His109 for which the 5 variants selected were all carrying the same substitution. Moreover, the fact that a variant with the same substitution is selected more than once, also in the case of the library generated with the high-throughput plasmid purification (protocol III), is not in contrast with the assumption that the libraries created with this assay are of high quality, but should be related with the fact that different triplet combinations can codify for the same side chain.
In the case of His144 and His218 no hits were found. These positions have already shown, with the activity staining test, to be essential for the proROL activity.

Table 4.12. Results from the high-throughput screening of the His libraries

Library	Num. of variants selected	Nature of the substitution	Frequency [%]
His109	5	Ile	100
His134	7	Cys	14.3
		Phe	85
His144	None		
His201	20	Ser	50
		Gln	10
		Ala	30
		Asn	5
		Tyr	5
His208	4	Asn	75
His218	None		

Table 4.13. Results from the high throughput screening of the Lys libraries

Library	Num. of variants selected	Nature of the substitution	Frequency [%]
Lys 168	20	Leu	30
		Ser	60
		Ile	5
		Phe	5
Lys 101	10	Ala	45
		Ile	10
		Gln	10
		Asn	30
Lys 104	2	Leu	50
		Thr	50
Lys 37	2	Ser	50
		Asp	50
Lys 202	1	Phe	100
Lys 5	4	Ser	50
		Gly	25
		Leu	25

4.4.4 CONFIRMATION OF THE SELECTED MUTANTS

The positive hits resulting from the screening of the histidine and the lysine libraries, were expressed in flask and subjected to stability screening, according to the previously optimized semi-micro scale assay, (see paragraph 4.4.1). For each variant two replicates were considered. The test included always the screening of the wild type enzyme as well, in order to compare the variants characteristics with a control (performed in exactly the same working conditions) so that slight changes of the operative conditions, would have been always included. The screening results were then interrelated as percentages of the variant relative stability with respect to the control. The results are reported in the tables 4.14 and 4.15.
For some variants, the high-throughput screening results were confirmed: a higher stability was observed. On the contrary, other variants were shown to be as stable as the wild type, or in some cases, even less stable. This was not especially surprising, since it was already known that the high-throughput screening presented inevitable variability, accumulated throughout all the

protein production, cell lysis, and aldehyde inactivation processes. Additionally, the introduction of mutations can cause changes in the solubility of the protein that could render the protein insoluble, affecting the value obtained in the assay. The best results were obtained for the histidine substitution and in particular, for position 201. For mutants His201Ala and His201Ser, a stability increase close to 50% could be reached. Moreover, the data show that, among all the histidine substituted, the less effective on the stability is His134, which is the only histidine residue far from the active site. According to this data then, it seems that to increase the proROL stability, the substitution of the histidine residues close to the active site would cause greater effects. Indeed, study of mutations that improve enzyme properties revealed that in many, but not all, cases closer mutations are more effectively than distant ones. Therefore, depending on the enzyme property to be improved, focusing mutation near the substrate-binding site might increase the success rate in many directed evolution experiments and avoid the screening of large libraries (Morley et al., 2005).
As regards lysine residues, the mutagenesis effect on the protein stability was much lower. An increase of 17% in residual acticvitywas achieved only for the Lys101Ile. The effect of one single lysine mutation was therefore, not as strong as that of a single histidine one. Indeed, the mutation of just one lysine cannot prevent covalent interactions between the other 14 lysine residues distributed on the proROL surface and the deactivating molecules. According to these considerations, in order to improve the overall proROL stability, mutants with more then one lysine residue substituted should be generated.
The relative lysine content of a lipase was previously related to its aldehyde-stability. A higher stability was observed for lipases having lower lysine content, such as *Mucor javanicus*, *Mucor miehei* and *Pseudomonas* sp. lipases (Weber et al., 1995).
It could be interesting to further accumulate the found substitutions, especially in the case of the lysine residues. To that extent, protocols for multi-site mutagenesis have been recently developed and made available, such as QuikchangeMulti™ from Stratagene. However, it is questionable if such a protocol will be able to handle all the positions simultaneously with reasonable yield or if, on the contrary, a stepwise strategy should be implemented to introduce 3-4 changes at a time. In a similar way to the Protocol established in this Work, multi-site mutated libraries could be generated with the low over-representation of variants, and thus, a higher quality.
Moreover, the single mutation of all the lysine residues considered, did not affect markedly the enzyme activity, as in the case of the histidine libraries: generally all the mutants of a lysine library were active. It is likely therefore, that the mutation of more than one lysine may not affect the protein activity. However, it should be considered that the lysine groups, distributed on the protein surface, play surely a role in determining the protein solubility. An evaluation of the number of lysine substitutions allowed, should then be performed to avoid undesired effects on the enzyme solubility and also, on the tertiary structure, since some of the proROL lysine residues are involved in H-bonds.

Table 4.14: Stability increase values of the single histidine mutants selected from the high-throughput screening

Mutant	Stability increase [%]
His201Ala	47
His201Ser	45
His201Gln	41
His201Tyr	-28
His201Asn	32
His134Phe	13
His134Cys	-10
His109Ile	6
His208Asn	3

Table 4.15: Stability increase values of the single lysine mutants selected from the high-throughput screening

Mutant	Stability increase [%]
Lys168Leu	5
Lys168Ser	12
Lys168Ile	8
Lys168Phe	0.5
Lys101Ala	-8
Lys101Gln	9
Lys101Asn	14
Lys101Ile	17
Lys5Ser	13
Lys5Leu	-5
Lys5Gly	3
Lys104Leu	5
Lys104Thr	-4
Lys202Phe	6
Lys37Asp	2
Lys37Ser	4

In order to improve the results obtained for proROL stability, a recombination of some of those mutants showing higher stability than the wild type proROL was also considered. The objective was to achieve higher stability by means of an additive effect, generating a mutant with two or more mutations. Unfortunately, only few experiments on recombinant mutants have been carried out so far. However, the first results, concerning the recombination of the variant His201Ser with the Lys168X variants led to promising results (Table 4.16). A theoretical rough stability of the double mutant was calculated by adding the

stability increases of the respective single variant. This was compared with the real stability increase of the variant. As reported in Table 4.16, the variant H201S/K168S a stability increase of 55.4% was observed, that is very close to the predicted one.

Table 4.16: Comparison between the theoric and the real stability increase of the His201Ser/Lys168X double variants.

Mutant	**Theoretical stability increase [%]**	**Experimental stability increase [%]**
H201S/K168S	57	55
H201S/K168P	45	40
H201S/K168I	53	44

Further experiments in recombination of stable variants could be performed by conventional DNA shuffling (Stemmer, 1994b; Stemmer, 1994a; Stemmer, 1994c) or rather, simplified with a high-fidelity method for the recombination of point mutations (multiplex-PCR-based recombination), recently developed (Eggert et al., 2005). This method has also the disadvantage, in comparison with the multiple site-directed mutagenesis mentioned above, to generate large, diluted libraries, thus increasing the number of variants to be screened.

5 CONCLUSIONS

The deactivation of Novozym® 435 was investigated only in the presence of soybean oil containing specific triglycerides oxidation products, in order to determine the effect of single components (Pirozzi, 2003). In this Work the overall effect on the Novozym® 435 of soybean oil, oxidized on air for several days or subjected to deep frying for some hours, was investigated. Both a batch and a continuous reactor were considered for the incubation. The activity decay increased with the oil oxidation grade, being higher when incubation was performed in a continuous reactor and ,in this case, with increasing input flow.
A tentative Novozym® 435 treatment was performed by utilizing site-blocking agents, such as PNP and DTT, that specifically interact with, respectively, the ε-amino groups of lysine and SH-bonds of cysteine. The pre-treatment led to a more stable enzyme for prolonged time. A drawback was the tendency of the ionic resin onto which this commercial lipase is immobilized, to trap water during the treatment, which was not totally removed even after dehydration.

The second, and much more developed, part of this PhD Thesis was related to the genetic modification of the fungal lipase from *Rhizopus oryzae* in order to improve its stability.
Much of the Work regarded the prolipase (proROL) less toxic than the ROL and therefore, produced in higher amount. A satisfactory proROL production in a soluble and active form was performed when *E. coli* Origami was used as expression host.
Optimal conditions for high protein yield and high enzymatic activity were found when pET22 was utilized as vector, and the incubation was performed at 20°C and an OD_{600} of induction equal to 0.5. In these conditions, the specific activity was 110 U/mg. Moreover, it was shown that the presence of a His-tag in the N-terminal position negatively influences the protein activity.

A site-directed saturation mutagenesis strategy was addressed to the six histidine groups, present in the protein and to some lysine groups, involved in up to two hydrogen bonds.
An obstacle in the mutant libraries generation was represented by the impossibility of directly using *E. coli* Origami as cloning strain, after the mutation, but *E. coli* DH5α had to be employed. In the case of the histidine residues close to the active site, this problem was overcome by reducing the library size with a previous selection of the active clones, performed in solid agar plates. This allowed the screening of a large numbers of variants in a short time. His144 and His218 had a percentage of active clones per plate of less than 10%.
On the contrary, in the case of the lysine residues and of the His134 far from the active site, an high-throughput plasmid purification and transformation in *E. coli* Origami were performed, leading to a high quality library.
The high-throughput expression of the libraries was optimized, by considering the results already obtained for the expression in flask. Under such conditions the wild type proROL activity was 50mU/ml.

The stability screening assay was previously developed in semi-micro scale for the wild type proROL. When 50mM octanal was used as a deactivator, at 20°C, the protein half life was approximately of 4 hours.
For the subsequent high-throughput screening of mutant libraries, some problems, connected with octanal loss in MTPs and the stabilizing effect of DNAse and lysozyme, employed to perform cell lysis, had to be solved. Even though the optimized high-throughtput screening assay presented an intrinsic variability, a very fast selection of variants showing higher stability than wild type proROL, could be performed.
The selected variants were analysed in-depth, under controllable conditions (expression in flask and semi-micro scale assay)
The increased stability of the variants was thus confirmed, apart very few cases in which it was lower than wild type proROL.
Moreover, the substitution of the residues close to the active site influences protein stability to a greater extent. The best results were in fact obtained in the case of the variants His201Ser and His201Ala, with a stability increase of approximately 50%. Another interesting variant was represented by His201Gln for which a stability increase of 40% was reached. These results indicate that position 201 is crucial for enzyme stability.
In the case of the lysine residues very small stability increases were observed, the best case was Lys101Ile, with a stability increase of 17%. As regards lysine residues, it can be concluded that one single mutation is not enough to improve proROL stability.
The recombination of some stable variants was also performed, so far leading only to few, even though promising, results. When some of the His201Ser/Lys168X variants were screened, an additive effect on protein stability was observed.
Much work has still to be done in this direction. Variants with more then one mutation should be generated, either with a multiple site directed mutagenesis, or a multiplex-PCR-based recombination. In any case, a deeper analysis with purified protein needs to be still performed.
Moreover, it would be interesting to analyse why, in the case of histidine 201, only specific substitutions, such as serine and alanine, improved the stability.
It would be also necessary to investigate the effect of the incubation of the improved enzymes with an oxidized oil, and whether or not these mutations affect the enzyme (trans)esterification activity.

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7 APPENDIX

COMMUNICATIONS AND PUBLICATIONS

Domenico Pirozzi, **Mirella Di Lorenzo** and Guido Greco jr (2004) "Improvement of the lipase stability in the presence of commercial triglycerides", III Euro Fed Lipid Congress, 5-8 September, Edinburgh, United Kingdom

Mirella Di Lorenzo, Aurelio Hidalgo, M. J. Haas and U.T. Bornscheuer (2005). "Heterologous production of functional forms of *Rhizopus oryzae* lipase in *Escherichia coli*." Applied and Environmental Microbiology **12** (17): 8974-8977.

Mirella Di Lorenzo, Aurelio Hidalgo, Domenico Pirozzi, Guido Greco jr, M. J. Haas and U.T. Bornscheuer (2005) "Active expression of lipase forms from *Rhizopus delemar* in *E. coli*", BioPerspectives 2005, 10-12 May, Wiesbaden

Mirella Di Lorenzo, Domenico Pirozzi, Guido Greco jr and Aurelio Hidalgo (2005) "Stabilization of a lipase from *Rhizopus delemar* in the presence of commercial triglycerides", BioPerspectives 2005, Wiesbaden, Germany

Mirella Di Lorenzo, Aurelio Hidalgo, M. J. Haas and U.T. Bornscheuer (2005) "Heterologous Production of Functional *Rhizoupus oryzae* forms in *Escherichia coli*", 4-8 October 2005, Rimini, Italy

Mirella Di Lorenzo, Domenico Pirozzi and Guido Greco jr (2006) "Lipases in the Oleochemical Industry", 17th International Congress of Chemical and Process Engineering, 27-31 August 2006, Praha, Czech Republic

Mirella Di Lorenzo, Aurelio Hidalgo, Domenico Pirozzi, Guido Greco jr and Uwe T. Bornscheuer (2006) "Stabilization of Lipase from *Rhizopus oryzae* in the Presence of Commercial Triglycerides", Symposium on protein design and evolution for biocatalysis, 30 August-1 September, Greifswald, Germany

Mirella Di Lorenzo, Aurelio Hidalgo, Domenico Pirozzi, Guido Greco jr and Uwe T. Bornscheuer (2006) "Stabilization of lipase from *Rhizopus oryzae* in the presence of commercial triglycerides", Third Internation Congress on Biocatalysis, Biocat 2006, 3-7 September 2006, Hamburg, Germany

Domenico Pirozzi, Farina Vincenzo and **Mirella Di Lorenzo** (2006) "Use of lipases for the separation of byproducts of the vegetable oil refinement," First Mediterranean Congress on the Chemical Engineering for Environment (MCCEE I), 4-6 October, Venezia, Italy.

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Heterologous Production of Functional Forms of *Rhizopus oryzae* Lipase in *Escherichia coli*

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To date, expression of the lipase from *Rhizopus oryzae* (ROL) in *Escherichia coli* always led to the formation of inclusion bodies and inactive protein. However, the production of active ROL and its precursor ProROL in soluble form was achieved when *E. coli* Origami(DE3) and pET-11d were used as expression systems.

Lipases (triacylglycerol ester hydrolases; E.C. 3.1.1.3) have multiple applications in a wide range of biotechnological processes (11, 13, 19, 20, 25). Lipases from the genus *Rhizopus* are attractive catalysts in lipid modification processes, since they are active only against esters of primary alcohols and positionally selective, acting only at the *sn1* and *sn3* locations (2). Both the structure (5, 22) and function of the lipase from the fungus *Rhizopus oryzae* ATCC 34612 (formerly *Rhizopus delemar*) (18) have been deeply investigated. The *Rhizopus delemar* lipase is initially synthesized as a preproenzyme, consisting of the 269 amino acids of the mature enzyme, a 97-amino-acid propeptide fused to its amino terminus, and a 26-amino-acid-long export signal peptide at the amino terminus of the propeptide (8). Additionally, it contains six cysteine residues, which form three disulfide bridges (6). Since *Escherichia coli* lacks the necessary proteases to process fungal maturation signals, the *Rhizopus delemar* lipase cDNAs were previously expressed in *E. coli* for both the unprocessed lipase precursor and the mature product in insoluble form (10). The production of an active mature *Rhizopus* lipase has been performed in *Pichia pastoris* (4, 14) and in *Saccharomyces cerevisiae* (23).

In this work we present, for the first time, the expression of the *Rhizopus oryzae* lipase gene in *E. coli* to yield a correctly folded product, present only in the cytoplasm fraction.

Methods, results, and discussion. Cloning of the cDNA coding for the prolipase and mature lipase from the fungus *Rhizopus delemar* (renamed as *Rhizopus oryzae* in accordance with the literature [18]) in pET11-d has been previously reported (15). *E. coli* strain DH5α [*supE44* d*lac*U169(ϕ80*lac*ZΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*] was used as host for genetic manipulation of plasmids. *E. coli* BL21(DE3) [F^- *ompT hsd*S_B(r_B^- m_B) *gal dcm* (DE3)], Rosetta(DE3)[F^- *ompT hsd*S_B(r_B^- m_B) *gal dcm* (DE3) pRARE^2 (Cm^r)] and Origami(DE3) [Δ*ara-leu7697* d*lacX74* Δ*phoAPvuII phoR araD139 ahpC galE galK rpsL* (Sm^r)$^4F^1$ [lac^+(*lacI*^{q}) *pro*] *gor522*::Tn*10* (Tc^r) *trx*B::kan (DE3)] strains were used for the overexpression of proteins. The *E. coli* strains were grown in Luria-Bertani medium containing 100 mg/liter ampicillin, 30 mg/liter kanamycin, 10 mg/liter tetracycline, 34 mg/liter chloramphenicol, as required. Plasmids pET-11d, pET-15b, pET-28b(+), and pET-22b(+) (Novagen) were used for cloning and protein expression. Transformation of *E. coli* was performed as described previously (9). All molecular biology protocols were performed using standard methods (17).

For PCR amplification of the genes of interest, the following oligonucleotides were used: 1F (5′-AAGGAGATATCATATGGTTCCTGT-3′), 2F (5′-GAGATATCATATGGATGGTGGTA-3′), and 3R (5′-AACACGTCAAGAATTCTTCAAACA-3′) (underlined portions of sequences are NdeI restriction sites introduced for cloning purposes). To obtain an N-terminal His-tagged product, the prolipase and mature lipase genes were amplified by PCR by using, respectively, oligonucleotides 1F or 2F and the T7 terminator primer. To obtain a C-terminal His-tagged product, the amplification by PCR was also performed with primer 1F or 2F and primer 3R.

The PCR products were purified and digested with NdeI and EcoRI and ligated into pET-28b(+) and pET-22b(+) vectors. The pET-28b(+) constructs carrying the prolipase and mature lipase genes were digested with NdeI and XhoI, purified, and ligated into the empty pET-15b vector. To evaluate the influence of the His tag on expression, the prolipase gene preceded by a six-His tag sequence was cloned into pET-11d. For this purpose the pET-15b vector containing the prolipase gene was digested with NcoI and BamHI, and the fragment was purified and ligated into the pET-11d vector.

The *E. coli* strains harboring the pET recombinant plasmids were grown in 100 ml Luria-Bertani medium supplemented with the required antibiotics using isopropyl-β-D-thiogalactopyranoside as inducer to a final concentration of 0.1 mM. At different time intervals, aliquots (equivalent to 5 ml at an optical density at 600 nm [OD_{600}] of 1) were centrifuged for 10 min at 800 × *g* to harvest the cells. The cells were then resuspended in 300 μl 50 mM phosphate buffer, pH 7.5, and disrupted by sonication (20 s, 50% pulse). The soluble fraction and the particulate material were separated by centrifugation, and 10 μl from these preparations was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% polyacrylamide gels and stained with Coomassie blue, as described by Laemmli

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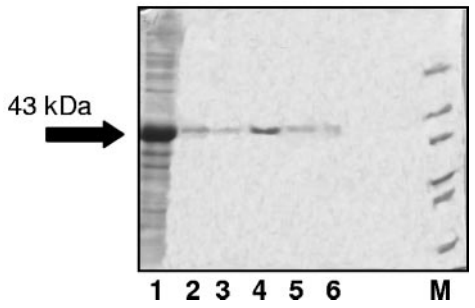

FIG. 1. Purification of the prolipase from inclusion bodies with a cobalt-based resin. Lane 1, pellet solution; lanes 2 and 3, washings; lanes 4, 5, and 6, elution; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa). Ten-microliter aliquots of the soluble and insoluble fraction samples, prepared as described in the text, were loaded onto each lane. The arrow indicates the prolipase.

(12). The soluble fraction was also subjected to activity measurement by monitoring the amount of *p*-nitrophenol released upon hydrolysis of a 1 mM solution of *p*-nitrophenyl butyrate in 50 mM phosphate buffer, pH 7.5 at room temperature. Aliquots (100 μl) of the cell fraction assayed were added to 900 μl of the reaction mixture, and the increase in absorbance at 410 nm was measured for 1 min. One unit of hydrolase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per min at room temperature. The protein concentration of the samples was determined according to the method of Bradford (3).

The expression of the pET-11d prolipase and mature lipase constructs in *E. coli* BL21(DE3) or Rosetta led to an insoluble and inactive product (data not shown). The insoluble protein pellet obtained from a 100-ml culture was resuspended in 15 ml of 0.1% (vol/vol) Triton X-100, incubated at 37°C for 10 min, centrifuged at 16,000 × *g*, and washed with 20 ml of 50 mM phosphate buffer, pH 7.5. The inclusion bodies were resuspended in 2 ml of sodium phosphate buffer, pH 7, containing 8 M urea and incubated at 37°C for 20 min. The total sample volume was then purified with 2 ml of Talon cellThru IMAC resin (BD Biosciences, Palo Alto, Calif.) according to the manufacturer's instructions in the presence of 8 M urea (Fig. 1). The eluted fractions containing the target protein were pooled, concentrated, and refolded according to a previously described protocol (7). The purified prolipase was refolded in the presence of cysteine, leading to an active enzyme preparation (0.645 U/ml). Unfortunately, the enzyme was inactivated after storage of the refolded protein either at −20°C, at 4°C, or when lyophilized.

When the *E. coli* Origami(DE3) strain was used, the expression was successful and the target protein was expressed as soluble and active forms (Fig. 2A and B). Although their specific activities are comparable, the yield of prolipase was higher than for the mature lipase, probably due to the toxicity of the latter towards the host cells (8). The prosequence has been reported to modulate the enzyme activity of the mature lipase so that it can be synthesized without damaging the host, in this case as a result of a decreased affinity of the prolipase for phospholipids in comparison to the mature lipase (1). This modulation also causes the prolipase and the

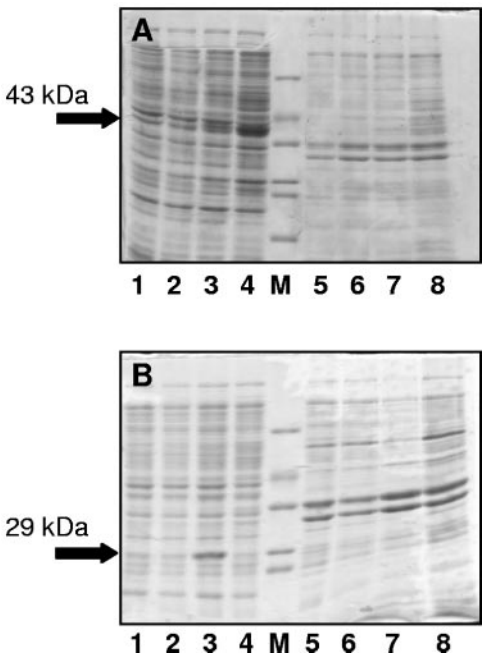

FIG. 2. A. Expression at 25°C of *E. coli* Origami harboring the pET11-d prolipase. Lane 1, prolipase before induction, supernatant; lane 2, prolipase after 1 h of induction, supernatant; lane 3, prolipase after 3 h of induction, supernatant; lane 4, prolipase after 24 h of induction, supernatant; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa); lane 5, prolipase before induction, pellet; lane 6, prolipase after 1 h of induction, pellet; lane 7, prolipase after 3 h of induction, pellet; lane 8, prolipase after 24 h of induction, pellet. Ten microliters of the soluble and insoluble fraction samples, prepared as described in the text, was loaded onto each lane. The arrow indicates the prolipase. B. Expression at 25°C of *E. coli* Origami harboring the pET11-d mature lipase. Lane 1, lipase before induction, supernatant; lane 2, lipase after 1 h of induction, supernatant; lane 3, lipase after 3 h of induction, supernatant; lane 4, lipase after 24 h of induction, supernatant; lane M, molecular mass marker (66, 45, 36, 29, 24, and 20.1 kDa); lane 5, lipase before induction, pellet; lane 6, lipase after 1 h of induction, pellet; lane 7, lipase after 3 h of induction, pellet; lane 8, lipase after 24 h of induction, pellet. Ten microliters of the soluble and insoluble fraction samples, prepared as described in the text, was loaded onto each lane. The arrow indicates the mature lipase.

mature lipase to have different affinities for their substrates (24), although whether this is caused by an interaction between the prosequence and the peptide lid that sits atop the active site in an inactive form of the mature enzyme (5) has not been confirmed, since a resolved crystal structure of the prolipase is not available. However, the fact that the pro-

TABLE 1. Influence of growth temperature and OD_{600} of induction on yield of prolipase from *R. oryzae* produced in *E. coli* Origami (pET 11-d construct)

Growth temp (°C)	OD_{600} at induction	Activity[a] (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)
20	0.5	40.7	0.7	60.4
20	1	166	1.5	110.7
25	0.5	45.1	0.95	47.3
25	1	28.4	0.7	42.4

[a] Determined using *p*-nitrophenyl butyrate as substrate.

TABLE 2. Influence of vector and OD_{600} at induction time on activity of prolipase from *R. oryzae* expressed in *E. coli* Origami at 20°C

Vector	OD_{600} at induction	Activity[a] (U/ml)	Protein concn (mg/ml)	Sp act (U/mg)
pET11	0.5	40.7	0.7	60.4
pET11	1	166	1.5	110.7
pET15	0.5	53.1	1.6	32.5
pET15	1	35.7	0.875	40.8
pET11 + His tag	0.5	34	1.64	20.7
pET11 + His tag	1	33	1.75	18.8
pET22	0.5	82	0.7	116
pET22	1	50	0.7	70.7

[a] Determined using *p*-nitrophenyl butyrate as substrate.

lipase is active implies that the part of the expression product corresponding to the mature peptide is already correctly folded.

In order to improve the amount of prolipase produced, several temperatures and cell densities at the time of induction were analyzed (Table 1). Only at 25°C and 20°C was an active product obtained, and under optimal conditions, the expression of *E. coli* Origami pET-11d prolipase gave 110.7 U/mg, at a growth temperature of 20°C with induction at an OD_{600} of 1. Although the functional expression of *Rhizopus* sp. lipase has been already performed in *Pichia pastoris* (14–16) and *Saccharomyces cerevisiae* (21, 23), we hesitate to compare the data, as different activity assays with different substrates and conditions have been used. In addition, the productivity is difficult to compare, as a system using *Pichia pastoris* has the advantage—in contrast to an *E. coli* expression system—that the lipase is in the supernatant and cell disruption is not necessary, but the enzyme is highly diluted. On the other hand, high-cell-density cultivation of *E. coli* can also yield large amounts of recombinant protein, and no background lipase (or esterase) activity is present in crude cell extracts. Thus, a purification of the lipase is not necessary.

The influences of several vectors in the prolipase production were studied too. pET15 and pET22 were considered for a simplified purification of the His-tagged, recombinant product. The expressions were performed at 20°C and at OD_{600} values of 1 and 0.5. A deeper evaluation of the influence of the His tag on the expression was carried out by cloning the prolipase gene preceded by a six-His tag sequence into vector pET-11d and comparing the expression results between this construct and that without an N-terminal His tag. Table 2 shows that the His tag in the N-terminal position negatively influenced the protein activity. On the other hand, the His tag in the C-terminal position did not influence the activity. The fact that the C-terminal position of the His tag does not have an effect on activity, while the N-terminal markedly does, may be related to the role of the N-terminal prosequence as an intramolecular chaperone assisting in the folding of the mature peptide. Although no structural data on the prolipase are available, it seems logical that the environment of the prosequence should be kept as unmodified as possible, since it has been previously reported to influence the formation of disulfide bonds (1).

In summary, we have demonstrated that the lipase from *Rhizopus oryzae* can now be functionally expressed in *E. coli* without the need for inclusion body purification and a tedious refolding process. The prolipase could be efficiently produced in high yield at high specific activity.

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