The Lipases from *Candida antarctica*:

Cloning, Expression and their Application in the Synthesis of Structured Lipids

Von der Fakultät Geo- und Biowissenschaften der Universität Stuttgart

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Vorgelegt von

Jan Christoph Pfeffer

aus Aalen (Ostalbkreis)

Hauptberichter: Prof. Dr. Rolf D. Schmid

Mitberichter: Prof. Dr.-Ing. Matthias Reuss

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe.

Stuttgart, den

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Abbreviations

ABA	Triglyceride with fatty acids A and B (A ≠ B)
Amp	Ampicillin
AOX	Alcoholoxidase from *P. pastoris*
ARA	Arachidonic acid
bp	base pair
C. antarctica	*Candida antartica*
CalA	*Candida antartica* lipase A
CalB	*Candida antartica* lipase B
1,2(2,3)-DAG	1,2(2,3)-Diacylglyceride
1,3-DAG	1,3-Diacylglyceride
Da	Dalton
DMSO	Dimethylsulphoxide
DNA	Desoxyribonucleic acid
DHA	Docosahexaenoic acid
EA	Erucic acid
E. coli	*Escherichia coli*
EPA	Eicosapentaenoic acid
EtOH	Ethanol
FAME	Fatty acid methyl ester
GC	Gas chromatography
GC/MS	Gas chromatography coupled with Mass Spectrometry
h	hour
HPLC	High Performance Liquid Chromatography
IPTG	Isopropyl-β-D-thiogalactopyranosid
l	litre
LB	Luria Bertani
LC/MS	Liquid Chromatography coupled with Mass Spectrometry
M	Mol, molar
1(3)-MAG	1(3)-Monoacylglyceride
2-MAG	2-Monoacylglyceride
min	Minute
1-MP	1-Monopalmitate

Abbreviations

2-MP	2-Monopalmitate
MTBE	Methyl-*tert*-butylether
OA	Oleic acid
OD_{600}	Optical density measured at a wave length of 600 nm
OPO	1,3-Dioleolyl-2-palmitoylglyceride
PA	Palmitic acid
PAGE	Polyacrylamide gelelectrophoresis
P. pastoris	*Pichia pastoris*
PCR	Polymerase Chain Reaction
PFL	*Pseudomomas fluorescens* lipasi
RAL	*Rhizopus arrhizus* lipase
RML	*Rhizomucor mihei* lipase
SDS	Sodiumdodecylsulfate
TAG	Triacylglyceride
TC	Tricaprylin
TO	Triolein
U	Unit
% w/w	percent by weight
% v/v	percent by volume

1. Zusammenfassung

Die vorliegende Arbeit wurde im Rahmen der Kooperation „Structured Lipids by Bio-Engineering“ zwischen dem Institut für Technische Biochemie an der Universität Stuttgart und dem Nestlé Research Center in Lausanne durchgeführt. Im Rahmen des Projekts wurden neue Möglichkeiten zur enzymatischen Synthese von Lipiden definierter Zusammensetzung und Struktur (strukturierte Lipide) sowie 2-Monoacylglyceriden entwickelt und die Synthese auf einen technischen Produktionsmaßstab (> 100 g) gebracht. Darüber hinaus wurde eine für die Synthese von strukturierten Lipiden viel versprechende Lipase im technischen Maßstab (5 l Fermenter) exprimiert, aufgereinigt und charakterisiert sowie die Grundlagen für eine gerichtete Evolution zur Optimierung des Biokatalysators geschaffen.

Strukturierte Lipide sind ein wichtiger Bestandteil der menschlichen Ernährung. Einerseits dienen Fette und Lipide als wichtiger und effektiver Energielieferant, andererseits werden wertvolle, mehrfach ungesättigte Fettsäuren in Form von 2-Monoacylglyceriden im Körper absorbiert, die essentielle physiologische Funktionen übernehmen, wie z.B. das Senken des Cholesterinspiegels im Blut oder die Entwicklung des zentralen Nervensystems von Neugeborenen.

Die chemische Synthese von 2-Monoacylglyceriden benötigt mehrere Reaktionsstufen, eine sehr aufwändige Aufreinigung und hat mit relativ geringen Ausbeuten zu kämpfen. Dagegen sind 2-Monoacylglyceride und strukturierte Triacylglyceride unter dem Einsatz von Lipasen relativ leicht in hohen Ausbeuten und geringem technischen Einsatz herstellbar.

Als viel versprechendes Enzym zur direkten Synthese von 2-Monoglyceriden schien sich die Lipase A aus *Candida antarctica* (CalA) zu erweisen. Dieses Enzym besitzt die seltene Eigenschaft einer Präferenz für die Position *sn*2 des Glycerinrückgrats (*sn*2-Päferenz), und unter den bereits beschriebene Enzymen mit dieser Eigenschaft weißt sie die höchste Präferenz für dieses Position auf.

Im Rahmen der Arbeit wurde das für CalA codierende Gen in einen *Pichia pastoris*-Expressionsvektor kloniert und sowohl eine Fermentation im Zufütterungsverfahren als auch eine semi-kontinuierliche Fermentation durchgeführt. Während die Proteinkonzentrationen in beiden Verfahren nahezu gleich waren, war die Gesamtaktivität im semi-kontinuierlichen Prozess um 25 % höher. Anschließend konnte die Lipase mittels Kationentausch-Chromatographie aufgereinigt und charakterisiert werden. CalA zeigt bei 50 °C die höchste Hydrolyseaktivität. Das pH-Optimum liegt bei pH 7.0. Kurzkettige Triglyceride (C4-C8)

werden bevorzugt gespalten; gegenüber Triolein (C18:1) zeigt die Lipase nur noch eine geringe Aktivität, ebenso gegenüber längerkettigen Estern.

Die exprimierte und immoblisierte CalA zeigte nur eine sehr geringe Aktivität in Veresterungsreaktionen und die Veresterungsreaktion verlief – im Gegensatz zur Hydrolyse, in der CalA eine *sn*2-Präferenz aufwies – unspezifisch. Da auch verschiedene Parametereinstellungen (Temperatur, Lösungsmittel, Wasseraktivität, Substrate verschiedener Kettenlänge, Einsatz von ionischen Flüssigkeiten) keine Verbesserung der Reaktivität und der Spezifität brachten, wurde eine Optimierung des Enzyms ins Auge gefasst.

Da für CalA keine Kristallstruktur vorliegt und auch die Homologie zu Lipasen mit bekannter 3D-Struktur sehr gering ist, blieb als einzige Möglichkeit das Enzym durch gerichtete Evolution zu optimieren, d.h. die Anfertigung einer Mutantenbibliothek durch error-prone PCR (epPCR) und eines anschließenden Hochdurchsatzscreenings mit einem geeigneten Nachweis-Verfahren, d.h. der Entwicklung eines Assay-Systems.

Oftmals können Enzyme aus eukaryontischen Mikroorganismen nicht funktionell in prokaryontischen Expressionssystem, wie z.B. *E. coli,* exprimiert werden. Meist sind fehlende oder unzureichende posttranslationale Modifikationen, wie Glykosilierung, proteolytische Prozessierung oder das nicht-oxidative Milieu im *E. coli* Cytoplasma, das eine Ausbildung von Disulfidbrücken erschwert, die Ursache. So kommt es meist zur Bildung von Einschlusskörpern – so genannten „inclusion bodies“, die das nicht-funktionelle und unlösliche Protein enthalten.

Da eine Expression von CalA in einem schnell wachsenden und leicht kultivierbaren Mikroorganismus wie *E. coli* eine Voraussetzung für die gerichtete Evolution ist, musste die Expression der Lipase in *E. coli* optimiert werden. Da mit dem Standardvektor pUC18 kein aktives Protein hergestellt werden konnte, wurde auf spezielle Expressionsplasmide der pET und pCold-Reihe zurückgegriffen und die Faltung durch Co-Expression verschiedener Faltungshelfer – sogenannter Chaperone – unterstützt. Zur Expression wurden *E. coli* Stämme der Origami™-Reihe verwendet, in denen eine Reihe von cytoplasmatisch lokalisierten Reduktasen genetisch ausgeschaltet wurden, um die Ausbildung von Disulfidbrücken im Cytoplasma zu ermöglichen. Durch ein Absenken der Expressionstemperatur konnte der Anteil von löslichem und funktionellem Protein letztendlich noch einmal deutlich gesteigert werden. Die Kombination des pColdIII-Plasmides und des Gro7-Chaperones zeigten hierbei die besten Ergebnisse und CalA wies dabei eine spezifische Aktivität von 13,1 U/mg im löslichen Gesamtprotein auf. Damit war die größte Hürde für eine gerichtete Evolution von CalA überwunden und im Rahmen einer auf dieser Arbeit aufbauenden Diplomarbeit konnten

Mutanten von CalA mit erhöhter *sn*2-Präferenz hergestellt werden. Dabei zeigte besonders eine vierfach Mutante eine um 10 % erhöhte Präferenz für die *sn*2-Position in Hydrolyse-Reaktionen.

Parallel zu dem CalA-Ansatz wurde ein Verfahren zur Synthese von 2-Mono- und strukturierten Triacylglyceriden entwickelt, welches den Einsatz von Wildtyplipasen erlaubt. Bei diesem Verfahren sind allerdings zur Synthese von 2-Monoacylglyceriden zwei Schritte notwendig. Die erste Reaktion ist eine Veresterung von Glycerin und freien Fettsäuren, die von einer unspezifischen Lipase katalysiert wird. Am Ende der ersten Reaktion erhält man ein homogenes Triacylglycerid, welches in einer Alkoholyse mit einer *sn*1,3-spezifischen Lipase umgesetzt wird und das 2-Monoacylglycerid bildet. In einer möglichen dritten Reaktion können nun die äußeren Positionen des Glycerin-Rückgrates mit kurz- oder mittelkettigen Fettsäuren besetzt werden, um mögliche Acylwanderungen der Fettsäure von der *sn*2-Position zu einer endständigen Position zu verhindern. Da vor allem die Synthese von 2-Monoacylglyceriden von mehrfach ungesättigten, langkettigen Fettsäuren vorgesehen war, mussten Lipasen gefunden werden, die ein räumlich geeignetes aktives Zentrum aufweisen, um die Substrate umzusetzen. Ein Screening verschiedener Lipasen zeigte, dass der gesamte zweistufige Prozess zur Synthese von 2-Monoacylglyceriden unter dem Einsatz einer einzigen Lipase möglich war: der Lipase B aus *Candida antarctica* (CalB), der zweiten Lipase aus *Candida antarctica*. Dieses Enzym zeigt einerseits in der Veresterungsreaktion ein unspezifisches Verhalten, so dass ein homogenes Triacylglycerid entsteht und andererseits ändert sich in der Alkoholyse durch den Ethanolgehalt des Lösungsmittelgemisches seine Spezifität und die Lipase spaltet das Triacylglycerid *sn*1,3-spezifisch. So können durch den Einsatz eines einzigen Biokatalysators zwei völlig unterschiedliche Reaktionen mit unterschiedlicher Spezifität durchgeführt werden.

Nachdem die Reaktionen erfolgreich und mit hohen Ausbeuten durchgeführt werden konnten, wurde der Maßstab des Prozesses auf eine vorindustrielle Produktionsstufe vergrößert. Dies wurde in der Miniplant des Instituts für Bioverfahrenstechnik durchgeführt. Dort konnte in einer zweistufigen Reaktion aus Tripalmitin – durch Alkoholyse mit anschließender Veresterung mit Ölsäure – der Muttermilchersatz 1,3-Dioleoyl-2-palmitoylglycerol (OPO) hergestellt und damit der 2-Stufen-Prozeß erfolgreich vom Labor- in den Technikumsmaßstab übertragen werden.

2. Summary

This work was part of the cooperation “Sturctured Lipids by Bio-Engineering“ between the Institute of Technical Biochemistry at the University of Stuttgart and the Nestlé Research Center in Lausanne, Switzerland. In the framework of this project new enzymatic methods for the synthesis of structured triacylglycerides and 2-monoacylglycerides were established and scaled up to a technical scale (> 100 gram product). Additionally a lipase showing high *sn*2-preference which is an ideal prerequisite for the direct synthesis of 2-monoacylglycerides and structured lipids was expressed in technical scale (5 litre fermenter), purified and generally characterised. For this lipase the prerequisites for an error-prone PCR (epPCR)-based mutagenesis aiming at improvement of the biocatalyst were generated.

Structured lipids are an important and essential component of human nutrition. On the one hand these fats are an efficient energy reservoir and on the other hand they act as carrier to transport valuable, polyunsaturated fatty acids (PUFA) in the form of 2-monoacylglycerides into the body. After absorption of the 2-monoglycerides in the small intestine these PUFAs take care of several physiological functions, e.g. lowering the blood cholesterol level or stimulating the development of the central nervous system of infants.

The chemical synthesis of 2-monoacylglycerides is challenging due to several reaction steps, a costly purification and often results in very low yields. In contrast 2-monoacylglycerides and structured triacylgylcerides are relatively easy to synthesise using lipases with regard to yield and purity.

Lipase A from *Candida antarctica* seemed to be a promising catalyst for the direct esterification of fatty acids and glycerol yielding in 2-monoacylglycerides. This enzyme displays the highest preference for the *sn*2-position published in scientific literature.

In the framework of this project the CalA-coding gene was expressed in the methylotrophic yeast *Pichia pastoris*. Two fermentation strategies were performed and compared: a fed-batch process and a semi-continuous fermentation. While the protein concentration was more or less equal in both processes, the total activity in the semi-continuous process was 25 % higher. The lipase could be purified via cation exchange chromatography and was generally characterised. CalA showed highest hydrolysis activity at 50 °C and has its pH optimum at 7.0. Short-chain (C4-C8) triacylglycerides are hydrolysed preferentially; against triolein (C18:1) and long-chain-esters CalA showed a very low activity.

The immobilised enzyme showed only a very weak esterification potential and the esterification was – in contrast to the hydrolysis, where CalA showed a clear *sn*2-preference – unspecific. Different parameters (temperature, solvent, water activity, substrates of different chain lengths, and use of ionic liquids) were changed, but no improvement in activity or specificity could be observed. Therefore optimisation of CalA via directed evolution was planned.

As the crystal structure of CalA is not solved yet and also the homology with other lipases is quite low, the only possibility to optimize the enzyme was directed evolution: design of a mutant library by epPCR followed by a high-throughput-screening using a versatile assay system.

Often enzymes from eukaryotic microorganisms can not be functionally expressed in prokaryotic hosts like *E. coli*. The main reasons are the lack of necessary posttranslational modifications, as glycosylation, proteolytic processing or the non-oxidative milieu in the *E. coli* cytoplasm hindering the formation of disulfide bonds. Often the consequence are inclusion bodies consisting of wrong-folded and insoluble protein.

As the functional expression of CalA in a fast-growing and easy-to-handle-microorganism is a major prerequisite for directed evolution, the expression of the lipase had to be adapted to *E. coli*. With pUC18 – a standard vector for *E. coli* – no active protein was expressed. Therefore special expression vectors of the pET- and pCold-series were chosen and the protein folding was supported by co-expression of special folding proteins – so-called chaperones. For expression strains of the Origami™-series lacking several reductases located in the cytoplasm for improved formation of disulfide bonds, were used. Additionally a lower expression temperature increased the amount of soluble and functional protein significantly. The combination of the pColdIII-plasmid and the Gro7-chaperone showed best results for hydrolysis activity of CalA yielding in an specific activity of 13.1 U/mg of total soluble protein. Consequently the major bottleneck for directed evolution of CalA was overcome and a following diploma thesis based on the methods developed here was able to create new mutants of CalA showing an increased *sn*2-preference. Especially a quadruple mutant showed an increased *sn*2-prefernce for hydrolysis reactions.

In parallel to the CalA-approach, a new process for the synthesis of 2-mono- (2-MAG) and structured triacylgylcerides (TAG) allowing the application of wild-type enzymes was established. However in this process two steps for the synthesis of 2-MAG are necessary. The first reaction step is an esterification of glycerol and free fatty acids catalysed by an unspecific lipase. The product of this first reaction is a homogeneous TAG which is in the second

reaction – an alcoholysis reaction – converted to a 2-MAG catalysed by an *sn*1,3-specific lipase. In a possible third reaction step the outer positions of the glycerol backbone can be blocked with a short- or medium-chain fatty acid to avoid acyl migration of the fatty acid from the *sn*2 position to an outer position. As the project was mainly focused on the synthesis of 2-MAG of polyunsaturated fatty acids (PUFA), the lipases catalysing the process had to be able to convert such bulky fatty acids. A screening of different lipases showed that the whole process can be catalysed by just one lipase: the lipase B from *Candida antarctica* (CalB). This enzyme shows an unspecific manner in the esterification reaction, but converts the TAG *sn*1,3-specifically in an ethanolysis reaction. So by the use of just one biocatalyst two totally different reactions can be performed with different specificity.

After the reactions were performed successfully and with high yields, the process was up-scaled in the miniplant of the Institute of Biochemical Engineering (IBVT). In this scale tripalmitin was converted in a two-step process – alcoholysis to the corresponding 2-MAG followed by esterification with oleic acid – to 1,3-Dioleoyl-2-palmitoylglycerol (OPO), which is a major component of mother milk.

3. Introduction

3.1 Lipases: Members of the α/β-Hydrolase Family

3.1.1 Introduction

The enzyme class of hydrolases (E.C. 3.X.X.X) contains a large number of different enzymes: acetylcholinesterases (E.C. 3.1.1.7), amidases (E.C. 3.5.1.X), amylases (E.C. 3.2.X.Y), carboxylesterases (E.C. 3.1.1.1), cellulases (E.C. 3.2.1.4), esterases (E.C. 3.1.X.X), hydantoinases (E.C. 3.5.2), lipases (3.1.1.3), serin-proteases (E.C. 3.4.16 + 3.4.21) and a lot more.

Lipases are ubiquitous enzymes that have been isolated from a wide variety of mammalian and microbial sources, even several lipases from archaea are known. Generally there is very little homology among the known sequences, the most conserved feature is the consensus sequence GxSxG found in the substrate binding site. This is common to all lipases and esterases, as is the α/β-hydrolase fold (Ollis et al., 1992). This means that the enzymes have an α/β-folding motif – a structurally conserved core - which consists of eight parallel β-sheets, which are surrounded by α-helices on both sides (figure 1).

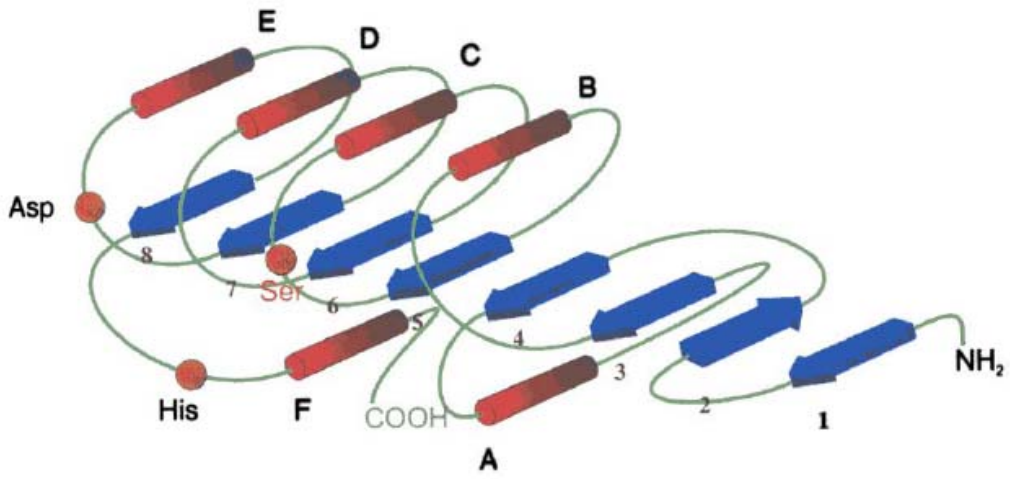

Figure 1: α/β-hydrolase fold of lipases. The eight β-sheets (1-8) are drawn as blue arrows and the red cylinders represent the α-helices (A-F). Also the distribution of the amino acids (Ser, Asp, His) belonging to the catalytic triad are shown (Ollis et al., 1992).

Nearly all lipases have the catalytic triad Ser-Asp(Glu)-His (in some exceptions like the *Geotrichum candidum* or *Candida rugosa* lipases, the aspartate is replaced by a glutamate).

Hydrolases are able to reversibly catalyse the hydrolytic cleavage of covalent bonds. Among hydrolases lipases hydrolyse carboxyl ester bonds, e.g. in triacylglycerides. Besides hydrolysis reactions further industrially relevant reactions can be catalysed by lipases: in a water-free environment the esterification of carboxylic acids with an alcohol (esterification) or with an alcohol residue of a second ester (interesterification) can be performed. In an interesterification reaction in the absence of water also an acyl residue of an ester is transferred to another ester. In a transesterification reaction an acyl residue is either transferred to an alcohol (alcoholysis) or to a carbonic acid (acidolysis). The product of an ammonolysis reaction is an amide and an alcohol (figure 2).

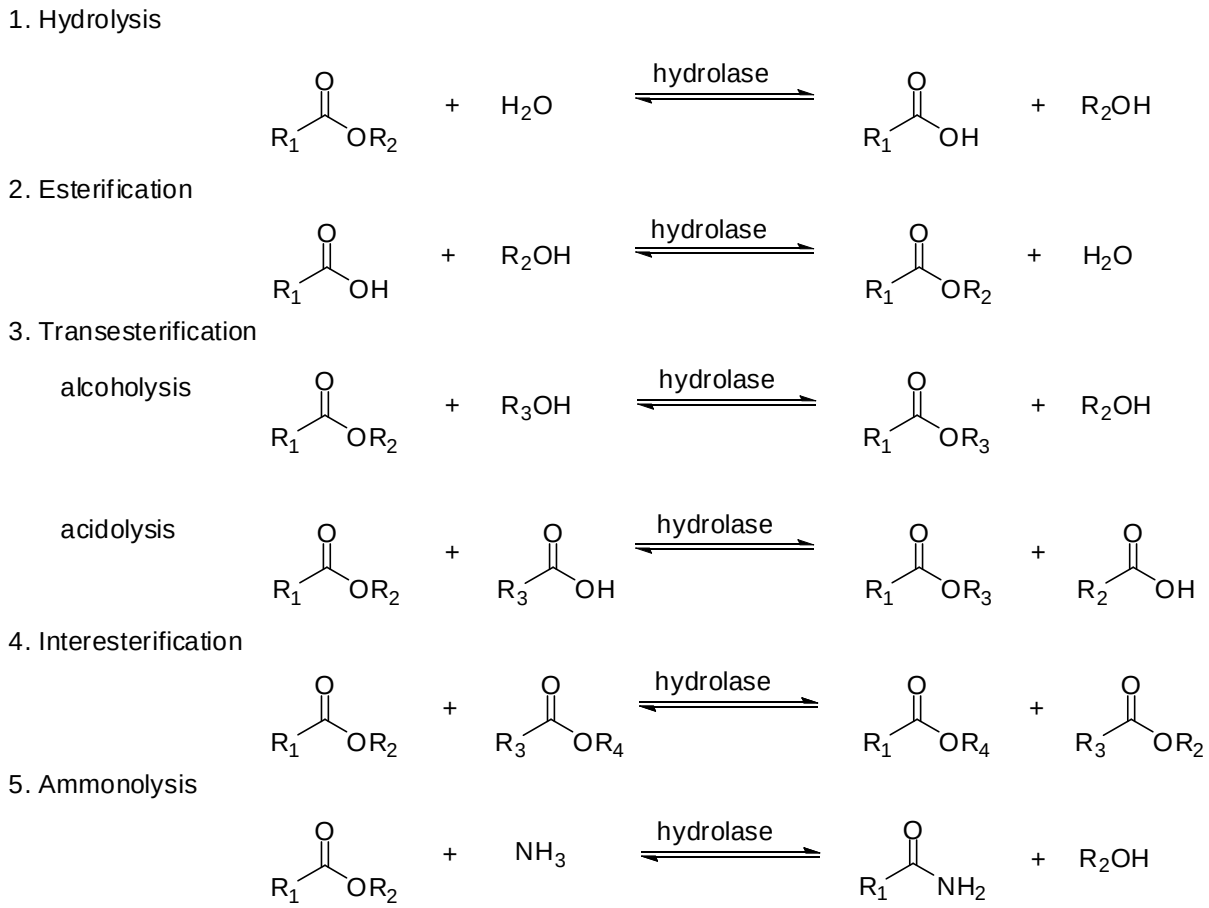

Figure 2: Possible reactions that are catalysed by lipases. Generally lipases catalyse the cleavage of carboxyl ester bonds. This reaction can be performed with water as substrate or product (hydrolysis and esterification), with an alcohol (alcoholysis), with an organic acid (acidolysis) or between two different esters (interesterification). In an ammonolysis reaction the ester reacts with ammonia leading to an alcohol and an amide.

A large number of lipases possess a kind of a *flap* or *lid* which is covering the active site of the enzyme. Because of this structural phenomenon the outstanding feature of interfacial activation which all lipases possessing a lid are showing can be explained. At very low substrate concentrations and in an aqueous environment the lipases are inactive. At substrate concentrations (the so-called critical micelles concentration (cmc)) high enough to form lipid micelles (an oil-water interface) the enzymes become catalytically activated. This observation can be explained as follows: lipases can occur in two different conformations. In the closed conformation the active site is covered by a structural element (mostly a helix) – the so-called lid. In the open conformation the active site is exposed allowing the substrate to access the catalytic centre. The oil-water interface initiates a movement of the lid to expose the active site.

Favoured substrates of lipases are water-insoluble glycerides, e.g. olive oil. Towards water-soluble substrates, lipases containing a lid show a significantly lower activity because the lid remains closed (figure 3).

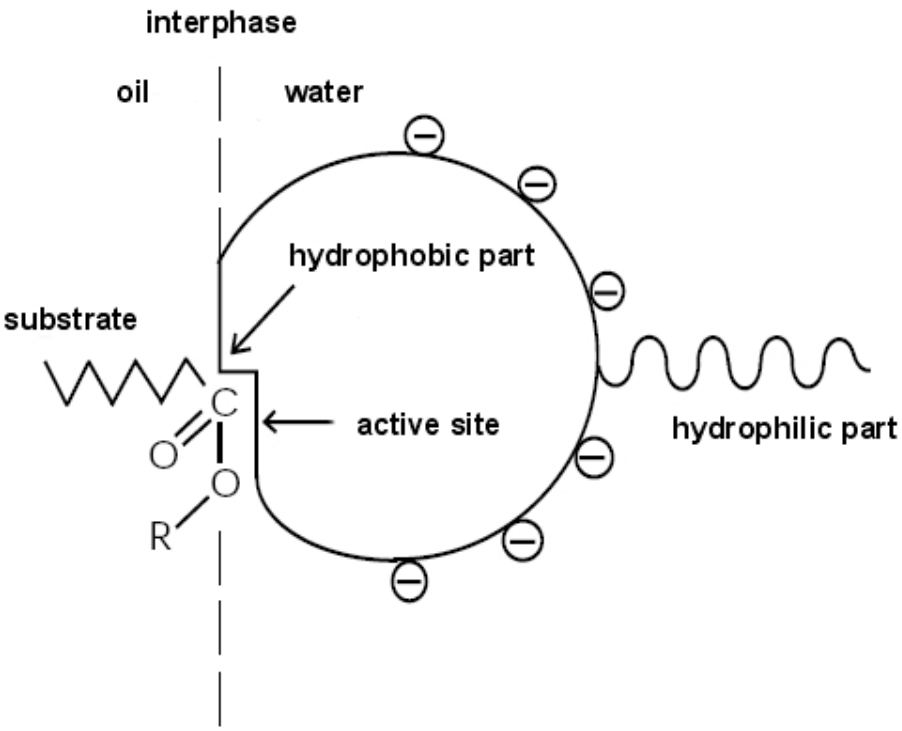

Figure 3: Ester cleavage catalysed by a lipase at the oil-water interphase. At the oil-water interphase the lipase is activated by the opening of the lid. Then the substrate (ester) can enter the active centre where the catalytic triad is located and the substrate is cleaved (Pleiss, 2004).

3.1.2 Reaction Mechanism of Lipases

The hydrolysis of esters is a five-step process (figure 4). As prerequisite for hydrolysis, a carboxylic acid ester binds via a hydrogen bond to the residues of the oxyanion hole. The Michaelis complex is formed.

In the resting state of the enzyme, the side chain hydroxyl proton of the active serine is part of a hydrogen bonding network comprising the catalytic triad. The oxygen of serine thus becomes more nucleophilic and can attack the sp^2-hybridised carbon atom of the substrate, which will hybridise to sp^3. The negative charge of the serine is further moved to the ester oxygen and stabilised by H-bonds from different amino acid residues of the oxyanion hole. The resulting positive charge is located at the catalytic histidine and stabilised by the active site aspartate or glutamate, respectively. As a result, the first tetrahedral transition state is formed. This first tetrahedral transition state is supposed to be the rate limiting step in the hydrolysis reaction.

The active site histidine transfers the intermediately bound proton to the ester oxygen. The negative charge is transferred from the oxyanion to the ester oxygen. The ester bond is cleaved leading to an acylated enzyme and an alcohol molecule. Afterwards the alcohol in the binding site is replaced by a water molecule. Inverting the first steps, the water molecule binds to the acyl enzyme. A second tetrahedral intermediate is formed with partial charge distribution as in the first tetrahedral intermediate. The active site histidine transfers its proton to the serine. The catalytic cycle is finished. An ester has been cleaved (figure 4).

Lipases from microorganisms, as from bacteria and fungi have received a considerable interest as catalysts in many industrial applications (Björkling et al., 1991; Schmid et al., 1998) such as ester synthesis (Okumura, Iwai et al. 1979; Nakano, Kitahata et al. 1991), optical resolution (Fukusaki et al. 1999; Sakaki and Itoh 2003), transesterification (Kaieda et al. 2004; Matsumoto et al. 2004) or washing processes (Kojima et al. 1994).

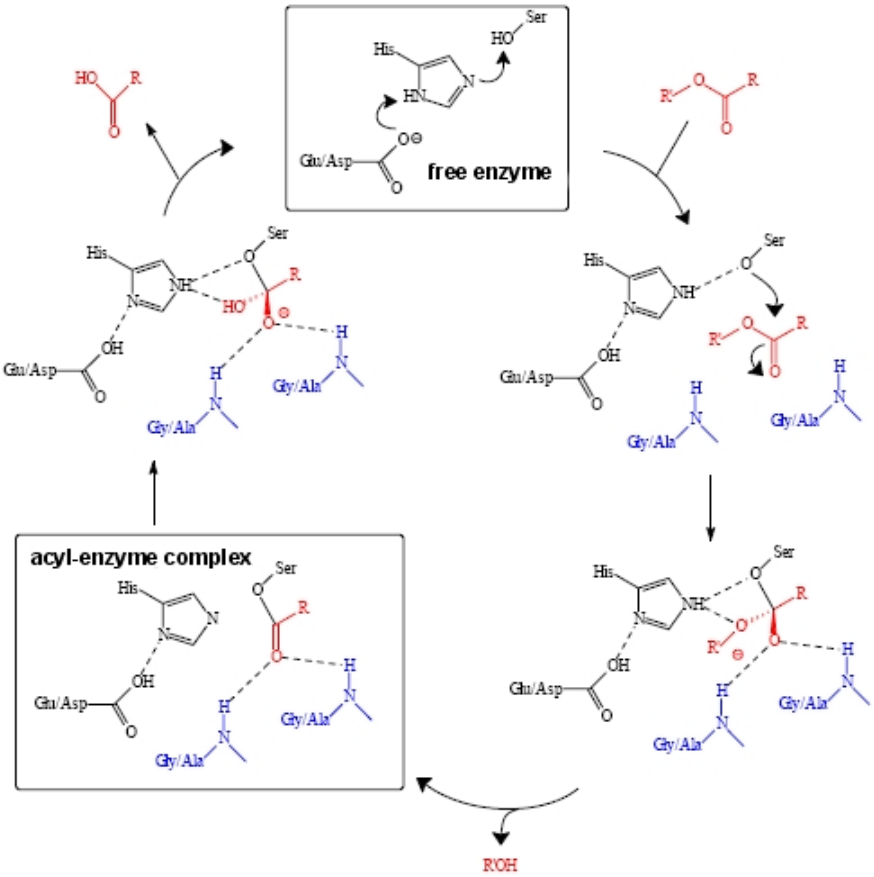

Figure 4: Illustration of the five step mechanism of substrate hydrolysis catalyzed by lipases (Rusnak, 2004). The process starts with the activation of the serine by the histidine residue followed by the nucleophilic attack towards the ester bond of the substrate. Finally after the release of an alcohol the ester bond is cleaved and a carbonic acid is released from the active center.

3.1.3 Specificity of Lipases

Lipases show different specificities towards fats and oil. These can be divided into five different categories (Rogalska et al., 1993):

1) Substrate specificity

Triacylglycerides are not the only substrates for lipases; diacylglycerides, monoacylglycerides and phospholipids can also be cleaved. Some lipases show a preference for special acylglycerides. The lipase from *Penicilium camembertii* for example has an increased activity towards mono- and diacylglycerides, but not towards triacylglycerides.

2) Regioselectivity

Regioselectivity is the ability of lipases to distinguish between the two outer positions (*sn*1 or *sn*3-position) and the inner position (*sn*2-position) of glycerides. *sn*1,3-specific lipases hydrolyse preferentially the *sn*1 and *sn*3-position, e.g. the lipases from *Rhizopus oryzae* and *Rhizomucor mihei*. So far no lipases are known showing a clear *sn*2-specifictiy, just very few lipases show a preference for this position (e.g. lipase A from *Candida antarctica*, the lipase from *Candida parapsilosis* or a lipase from the bovine rumen metagenome).

3) Non-specific lipases

Some lipases neither show any regiospecificity nor substrate specificity. These enzymes hydrolyse all ester bonds equally. Such non-specific lipases are known from *Candida rugosa* and *Pseudomonas cepacia*.

4) Fatty acid-specific lipases or acyl-selective lipases

Some lipases are highly specific for special fatty acids, e.g. the lipase from *Geotrichum candidum*, which is highly specific towards Δ9-unsatturated fatty acids (e.g. oleic acid). Other lipases of this group show enhanced specificity towards short-chain fatty acids like lipases from *Penicilium roquefortii*.

5) Stereospecific lipases

These lipases can distinguish between the *sn*1 and *sn*3 position in triglycerides, e.g. the human tongue lipase and some other lipases from *Pseudomonas fluorescens* and *Humicola languinosa*.

3.2 Fats and Oils

3.2.1 Introduction

Fats and oils are collective names for solid, semisolid and liquid – more or less viscous - products of plants and animals, consisting mostly of triacylglycerides with fatty acids with an even-numbered carbon number. Fats are solid or semisolid at 20 °C whereas oils are liquid.

Fats and oils are immiscible with water and show a lower density than water. The physical properties of fats and oils are determined by their fatty acid chain lengths and the number of double bonds within the fatty acids. Long-chain saturated fatty acids show a higher melting point than short-chain unsaturated fatty acids. Plant fats are usually rich in unsaturated fatty acids and consist of even-numbered fatty acids only and the double bonds have the Z-

configuration (cis-form). The most common fatty acids occurring in glycerolesters are dodecanoic acid (lauric acid), tetradecanoic acid (myristic acid), octadecanoic acid (stearic acid), hexadecanoic acid (palmitic acid) and oleic acid (table 1).

Table 1: Fatty acid composition of important plant fats and oils (Gunstone et al.,1994)

Fat/Oil	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3	20:1	22:1
Rapeseed Oil	-	-	0.5	4	1	60	20	9	2	2
Sunflower Oil	-	-	-	6	4	28	61	-	-	-
Coconut fat	7	48	17	9	2	7	1	-	-	-
Palm Kernel Oil	5	50	15	7	2	15	1	-	-	-
Palm Oil	-	-	2	42	5	41	10	-	-	-
Soy Oil	-	-	-	10	3	41	36	-	1	-

During the last 60 years the worldwide production of fats and oils was doubled (2006/07 more than 100 million tons) (table 2). Especially the production of rapeseed oil (*Brassica napus*), soy oil (*Glycine max*), palm fats and sunflower oil (*Helianthus annuus*) is increasing, whereas the amount of fish oil decreased. In Germany preferentially soy oil, butter and cattle fat is consumed.

Table 2: World production of oils and fats (million tons) in the quinquennia 1978-82 and 1986-90 and predicted for 2003-07 (Gunstone et al., 1994)

	1978 - 82	1986 - 90	2003 - 07
Soy	12.4	15.3	23.0
Palm	4.5	9.1	23.0
Groundnut	10.7	14.6	15.7
Greases	11.2	11.9	15.7
Rapeseed	3.9	7.5	8.4
Olive	7.3	8.3	8.4
Coconut	3.4	4.3	7.3
Others	2.8	4.6	3.0
Total	**56.2**	**75.6**	**104.5**

Triacylglycerides are highly concentrated energy reservoirs because they are reduced and water-free because of their low polarity. In a complete oxidation fatty acids yield about 38-39

kJ/g, compared to 17-19 kJ/g with carbohydrates or proteins. The reason is that fatty acids are much more reduced. Proteins and carbohydrates are much more polar and highly hydratised.

Fats and oils are stored in the seeds or progenies of plants as tiny oil drops. The drops are synthesized in the cytoplasm according to the Kennedy-pathway.

The variation of the fatty acid composition within a species is very low. Therefore the pattern is used for general taxonomic classification, e.g. bacteria prefer fatty acids of C_{14}-C_{18} chain lengths.

Usually fats and oils are obtained by extraction with organic solvents, e.g. n-hexane or forcing off vegetable, animal or microbial sources (oil mill).

In the human body the consumed fats and oils are cleaved enzymatically in the small intestine or liver. In the intestine lumen the already absorbed fatty acids are converted with glycerol-1-phosphate and fatty acid esters of Coenzyme A to human body fats. The other fatty acids are used for energy recovery in the blood or are in the liver converted to tissue-specific fats.

Besides the food-technical applications, fats and oils are used as renewable sources for the production of cosmetics, soaps, lubricants and biodiesel.

In recent years polyunsaturated fatty acids (PUFA) are reaching the focus of interest. The polyunsaturated fatty acids with non-conjugated double bonds are classified into ω-6 and ω-3 fatty acids.

ω-3 fatty acids have several positive health effects, e.g. in the case of cardiovascular diseases, dysfunctions of the immune system, inflammations, kidney diseases and allergic reactions (Koletzko et al., 1989; Koletzko et al., 1996). A major source of these PUFA is fish oil (table 3).

Table 3: Summary of several important ω-6 and ω-3 fatty acids and their sources

Fatty Acid	Chain length and double bond position	Source
	ω-6-family	
Linoleic Acid	18:2 (9, 12)	Poppy seed-, Sunflower-, Soy-, Cotton- and Corn Oil
γ-Linolenic Acid	18:3 (6, 9, 12)	Evening Primrose Oil
Arachidonic Acid (AA)	20:4 (5, 8, 11, 14)	Meat, Liver
	ω-3-family	
α-Linolenic Acid	18:3 (9, 12, 15)	Linseed Oil
Eicospaentaenoic Acid (EPA)	20:5 (5, 8, 11, 14, 17)	Fish Oil
Docosahexaenoic Acid (DHA)	22:6 (4, 7, 10, 13, 16, 19)	Fish Oil

3.2.2 Metabolism of Fats and Oils

For efficient absorption of fats and oils (dietary value), the position of the fatty acids on the glycerol backbone thus the structure of the triglyceride is important.

The initial digestion takes place in mouth and stomach catalysed by the tongue- and stomach-lipase. These *sn*3-specific lipases hydrolyse preferentially short-chain fatty acids yielding in a mixture of free fatty acids and 1,2-diacylglycerides. A main problem during the digestion of lipids is their immiscibility in an aqueous system leading to a rather small lipid-water interface and therefore reducing the digestion efficiency of lipases. To overcome this problem bile acids are secreted which form a lipid-water emulsion entering the small intestine. The mixture of tri-, diacylglycerides and free fatty acids enters the small intestine, where the pancreatic lipase hydrolyses the fatty acids in the *sn*1-position. Additionally the pancreatic carboxylester hydrolase hydrolyses tri- and diacylglycerides preferring long-chain polyunsaturated fatty acids like AA, DHA or EPA yielding in 2-monoacylglycerides. The resulting 2-monoacylglycerides are absorbed highly efficient at the small intestine lumen (Koletzko et al., 1989; Müller et al., 2007; Oxley et al., 2007).

After absorption the 2-monoglycerides are reacylated to triacylglycerides by the monoacylglycerol transferase and the diacylglycerol transferase. The fatty acid in the *sn*2-position remains constant. Afterwards the lipids are secreted into the lymph system and distributed in the whole body. The triacylglycerides can bind to special receptors, e.g. at the surface of blood vessels and there be converted again. The resulting products can enter different metabolic pathways (figure 5).

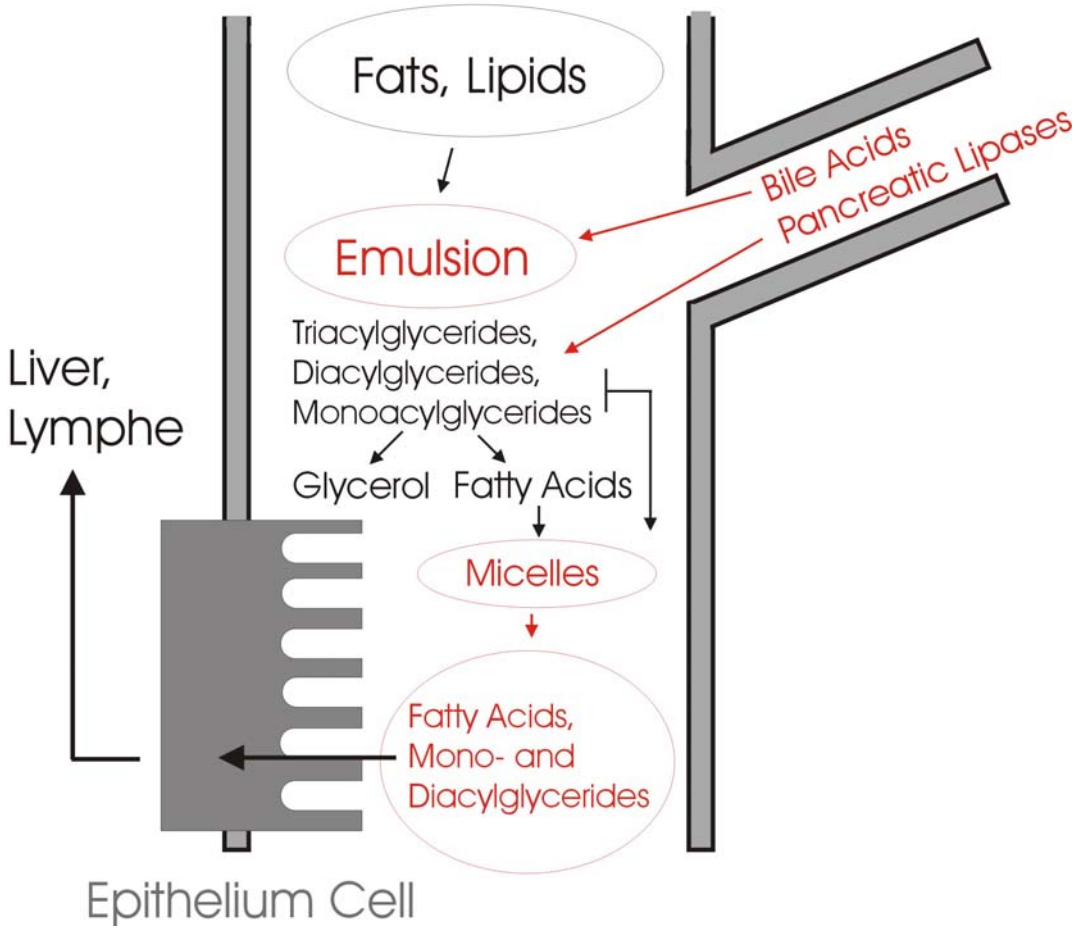

Figure 5: Lipid conversion during digestion. Before entering the small intestine the partially pre-digested fats and lipids form together with different bile acids an emulsion which is converted by the pancreatic lipase to a mixture of mono-, di-, triacylglycerides, glycerol and free fatty acids. After forming micelles mono-, diacylgylcerides and partially free fatty acids are absorbed by epithelium cells of the intestine lumen, reacylated and transported via the lymph system to the liver entering different pathways (Müller et al., 2007).

Of special importance in fat digestion is the distribution and position of the fatty acids at the glycerol backbone. An important example is the mother milk fat. 1,3-oleoyl-2-palmitoylglycerol is the main component of the mother milk fat. The position of the palmitic acid is tremendously important, because only glycerides with palmitic acid in *sn*2-position are absorbed highly efficient. Cleavage of the palmitic acid in *sn*1 or *sn*3 position of the glyceride is leading to insoluble calcium soaps. In this case dyspepsia and loss of calcium happen. So the better absorption of mother milk compared to cow milk can be explained although the general fatty acid composition of both milks is very close, but mother milk has a significantly higher amount of palmitic acid in *sn*2-position (Müller et al., 2007).

3.3 Heterologous Expression of Proteins

3.3.1 Introduction

The production of proteins via heterologous expression is a major task in modern biotechnology. Nowadays gene technology has invaded huge-scale production processes of a large number of proteins, e.g. antibodies for pharmaceuticals.

At the beginning of this technique only a very small number of microorganisms was employed for such processes, namely *Escherichia coli*, as a representative of prokaryotic microorganisms and the baker's yeast *Saccharomyces cerevisiae*. During the last decades the number of expression systems was constantly increasing leading now to a huge platform of different host systems for protein expression (table 4). The application of such expression platforms including the use of "broad-host-range-vectors" and the improvement of fermentation conditions is a major business for several biotech start-up companies, e.g. MedArtis Pharamceuticals GmbH, Aachen, Germany.

Table 4: Some characteristics of different expression systems (Gelissen, 2005)

Expression system	Classification	Disulfide bonds	Gycosylation	Secretion	Processes developed	Products on market
Mammalian cells	higher eukaryote	yes	yes	possible	industrial scale	yes
Plant cells	higher eukaryote	yes	yes	possible	pilot scale	no
Yarrowia lipolytica	dimorphic yeast	yes	yes	possible	lab scale	no
Pichia pastoris	methylotrophic yeast	yes	yes	possible	industrial scale	yes
Pseudomonas fluorescens	gram-negative bacterium	in the periplasm	no	periplasmic secretion	pilot scale	no
Escherichia coli	gram-negative bacterium	in the periplasm	no	periplasmic secretion	industrial scale	yes

In this introduction I will focus on two different expression systems which had huge impact in gene technology since many years: for prokaryotes the well-known *Escherichia coli* system and for eukaryotes the methylotrophic yeast *Pichia pastoris*. Whereas the expression of eukaryotic lipases is simple in an eukaryotic yeast, the production in a bacterial system is much more challenging, especially in the case of glycosylation and the formation of disulfide-bonds which is inefficient in *E. coli* and the major bottleneck in heterologous bacterial expression leading to the formation of aggregates of wrong-folded, insoluble proteins (inclusion bodies). To overcome such problems special sets of vectors and chaperones –

which are facilitators of folding - for co-expression have been developed by companies like TaKaRa Inc., Kyoto, Japan.

3.3.2 *Escherichia coli* as an Expression System

Escherichia coli is a gram-negative bacterium described first in 1885 by T. Escherich. It was the first microorganism which was employed for genetic engineering and recombinant protein production. From a model lab-organism *E. coli* has evolved during the last years into an industrial microorganism which is now the most frequently used expression system and besides many drawbacks (instability of vectors during large-scale fermentation, inefficient translation start and elongation, instability of mRNA and toxicity of gene products) also the standard organism for production of enzymes for diagnostic and analytical purposes.

Many *E. coli* strains were developed specifically either for the production of proteins in the cytoplasm or in the periplasm. Besides the strains, hundreds of different expression vectors with specially regulated promoters and tags for efficient protein purification have been constructed.

A typical *E. coli* expression vector contains an origin of replication (*ori*), an antibiotic resistance marker, a multiple cloning site (MCS) with an expression cassette for regulated transcription and translation of a target gene. Additional features can include plasmid stability functions, and genes or DNA structures for mobilization and transfer to other strains. Up-to-now the most frequently used vector system is derived from the ColE1-like plasmid pMB1 (Betlach et al., 1976)

Blattner and colleagues first published the genome sequence for the K-12 strain (Blattner et al., 1997). Today whole-genome arrays are available and used as a tool to identify changes on the gene expression level in the field of system's biology (Lemuth, 2006).

The cultivation of *E. coli* was first established in lab-scale. During the years *E. coli* developed into an industrial microorganism reaching now the high-cell-density (HCD) cultivation which is today's standard.

One of the major problems in heterologous protein expression in *E. coli* using strong promoters is misfolding and aggregation of proteins in inclusion bodies. In some cases the subsequent refolding to an active and correctly-folded conformation can be a first important purification step, e.g. for pharmaceutical proteins. For products with a lower commercial value, such as bulk enzymes, the costs would be too high.

A very efficient way to avoid inclusion body formation is to lower the expression temperature to 20 °C or even lower. Of course the lower temperature will also lead to a significantly

reduced protein production rate. Another possibility is the co-expression of chaperones which facilitate de novo protein folding, inhibit aggregation of denatured proteins or dissolve aggregated proteins.

Disulfide bond formation can only occur in the periplasm but not in the cytoplasm due to the presence of reducing components such as glutathione and thioredoxines. Additionally the enzymes which oxidize pairs of cysteines are only located in the periplasm. This problem is solved by using mutant strains which lack the thioredoxin reductase (TrxB) or increased production of the disulfide isomerase DsbC (Bessette et al., 1999).

Although there are several drawbacks in heterologous protein production in *E. coli* there numerous examples of pharmaceuticals produced with this bacteria (table 5). Also for the application in directed evolution, *E. coli* is still the system of choice.

Table 5: Pharmaceutical products produced by *Escherichia coli* strains, approved in Germany (selection) (Gellissen, 2005)

Class	Product	Brand name
Human insulin	insulin	Berlinsulin®
	insulin	Huminsulin®
	Insulin lispro (variant)	Humalog® 100
Pituitary hormones	somatotropin	Genotropin®
		Humatrope®
		Norditropin®
		Zomacton®
Cytokines	Aldesleukin, IL2	Proleukin®
	Interferon α-2a	Roferon®-A
	Interferon α-2b	Intron A®
	Interferon β-1b	Betaferon®
	Interferon γ-1b	Imukin®
	Molgramostim, rhGM-CSF	Leucomax®
	Filgrastim, r-metHuG-CSF	Neupogen®
Fibrinolytics	Reteplase (t-PA variant)	Rapilysin®

3.3.3 *Pichia pastoris* as an Expression System

The methylotrophic yeast *Pichia pastoris* is a highly successful system for expression of a large number of heterologous proteins. In general the *Pichia* system has several important advantages: an extremely strong and very tightly regulated and easy to manipulate promoter derived from the alcohol oxidase I gene (AOX1) from *Pichia pastoris*. The techniques for the genetic manipulation are quite simple. *Pichia* is able to perform most post-translational modifications like glycosylation, disulfide bond formation and proteolytic processing. The expression system is available as a kit by Invitrogen company.

The conceptional basis for the expression of heterologous proteins in *Pichia pastoris* is its ability to grow on methanol as sole carbon and energy source (Gregg et al., 2000). The enzymes for the methanol metabolism are only expressed in presence of methanol. This fact led to the concept of the methanol-inducible AOX1-promoter. Very common is the use of Zeocin and the Zeocin resistance gene as selection marker. But also biosynthetic markers like HIS4 (histidinol dehydrogenase), ARG4 (argininosuccinate lyase) or URA3 (orotidine 5´-phosphate decarboxylase) have been described (Gellissen, 2005).

Like most expression systems the *Pichia pastoris* expression system is an *Escherichia coli*-based shuttle-vector system. Generally the expression vectors have an expression cassette which is composed of 0.9-kb fragment from AOX1. Between the AOX1 promoter and the terminator sequence a multiple cloning site (MCS) is inserted. Additionally secretion signals, e.g. the α-mating factor from *Saccharomyces cerevisiae* can be fused in-frame to the heterologous gene. By secretion of the heterologously expressed genes the down-stream-processing can be simplified because cell lysis and extraction from the cytosol is not necessary.

In contrast to *E. coli* the *P. pastoris* expression vectors are designed to integrate into the genome. This increases the stability of the expression vectors which is significantly high compared to *E. coli*.

P. pastoris is a poor fermentor which is an important advantage relative to *S. cerevisiae*. With a high preference for respiratory growth, *P. pastoris* can be cultured at extremely high cell densities (150 g L^{-1} dry well weight) leading also to very high protein yields concentrated in the supernatant.

Up to now several pharmaceutical proteins, e.g. a hepatitis B surface antigen, produced in *P. pastoris* are in use or observed in clinical trials.

4. Results and Discussion

4.1 Cloning, Expression, Purification and Characterisation of CalA (see also 5.1.4)

Lipase A from *Candida antarctica* (CalA) is reported to show several outstanding characteristics. Most interestingly is its *sn*2-preference in hydrolysis reactions (Rogalska et al., 1993). The first aim of the thesis was to express and characterise this catalyst generally and test its application in the synthesis of structured lipids and 2-MAG.

4.1.1 Sequence Analysis

The gene sequence of calA was already published (Hoegh et al., 1995). A multisequence alignment using BLAST showed that CalA shares highest similarity to the lipases from *Kurtzmanomyces* sp. I-11 (74 %) and *Ustilago maydis* (64 %), respectively (Kakugawa et al., 2002a,b). No other known lipase – not even from the Candida family – showed significant similarity to CalA. The typical consensus pentapeptide (-Gly-X-Ser-X-Gly-) is conserved in the gene and alignment tools suggest that CalA is an α/β-hydrolase (Ollis et al., 1992).

4.1.2 Cloning and Expression in Shake Flask Scale

The lipase-encoding gene was amplified from genomic DNA isolated of *Candida antarctica* (DSM 70725) using specific primers. Afterwards the purified gene was cloned into pPicZαA – a strong *Pichia pastoris* expression vector (Invitrogen, 1997). A first expression round in shake flask scale was performed to pick out the clone showing highest expression rate. Due to the fact that the transformed gene can be inserted several times at several different positions in the *Pichia* genome, different specific activities has to be expected from different clones. The best clone was showing an specific activity of 215 U/mg after five days of expression and was chosen for all further experiments.

4.1.3 Fermentation and Purification

Two different expression strategies were applied: a fed-batch-fermentation and a semi-continuous process.

The fed-batch process reached a final specific activity of 635 U/mg and a CalA concentration of 0.88 g/l in the supernatant after 12 days of fermentation. On the other hand the semi-

continuous process reached a final specific activity of 380 U/mg and a lipase concentration of 0.55 g/l in the supernatant after 15 days of fermentation. The lower lipase concentration can be explained due to the fact that the culture broth was diluted every day with fresh media. The total activity was some 15 % higher in the semi-continuous process.

The CalA concentration in both processes is significantly higher, compared to the yield reached by expressing *Candida rugosa* lip4 lipase in *Pichia pastoris* (0.1 g/l) (Tang et al., 2001). For comparison, an expression of a CalB-fusion protein in *Pichia pastoris* involving pure oxygen led to an lipase concentration of 1.5 g/l (Jahic et al., 2002). With a temperature-limited feeding strategy this value was increased to 2 g/l (Jahic et al., 2003). Therefore it seems possible with changes in aeration and different feeding strategies to even further increase the CalA concentration and the yield of the process.

CalA did not show any binding to HIC- (hydrophobic-interaction-chromatography) or anion-exchange chromatography columns. Usually HIC are the columns of choice for lipase purification, as lipases bind to these materials due to the hydrophobic patches on their surface. CalA only showed binding to a cation-exchange column. The silver-stained SDS-PAGE confirmed a nearly homogenous, pure lipase. The purification factor was 3.1 and the specific activity after the purification was 2,033 U/mg (the commercial available CalA from Novozymes has an activity of 6,000 U/g).

With densitometric analysis the quality of the purification process was determined. In the supernatant the lipase was 33 % pure and this value increased to 85 % after cation exchange chromatography.

4.1.4 Characterisation of CalA

CalA showed highest hydrolysis activity at approximately 50 °C, with very similar activities between 50 and 70 °C. The temperature optima of CalA and CalB are very similar (Otto et al., 2000). As many industrial applications, such as acidolysis or the generation of new ester compounds (Sahin et al., 2005), require high temperature, CalA and CalB might be excellent biocatalysts. The pH optimum of CalA was ~ pH 7.0. CalA showed high activity between pH 7.0 and 9.0. In contrast to the publication of Solymár (Solymár et al., 2002) the addition of $CaCl_2$ did not show any positive effect on the enzyme activity.

CalA hydrolyses preferentially triacylglycerides with short chain lengths (C4-C8). Towards hydrophilic esters with chain lengths between C4 and C10 only a low conversion rate was detectable, which indicates that CalA is a true lipase. Surprisingly the *Kurtzmanomyces* lipase, which has highest homology to CalA, hydrolyses long-chain fatty acids more efficiently.

Therefore in spite of the high sequence homology, the structures of the substrate-binding sites of both enzymes seem to be different.

Via QuikChange™ mutations the three amino acids belonging to the active site were determined: serine at position 174, asparatate at 288 and histidine at 320.

The *sn*2-preference of CalA could be reproduced in hydrolysis reactions using tricaprylin and triolein as substrates (Rogalska et al., 1993). The 1,3-DAG proportion in the product mixture was about 66 % and therefore the enzyme is showing a clear preference of the *sn*2-position.

CalA was successfully immobilised on several carriers and applied in esterification and interesterifciation reactions. Generally the reactivity is much lower compared to the hydrolysis reaction. Changing several reaction parameters (solvent, chain length of substrates, temperature, and application of molecular sieves) did not show significant improvements in reactivity. Only the application of ionic liquids showed an increase in reactivity. Esterification and interesterification catalysed by CalA did not show any specificity at all. Therefore the wild-type CalA seems not to be suited for synthesis of structured lipids via esterification or interesterification.

4.2 Functional Expression of CalA in the Cytoplasm of *Escherichia coli* – a Prerequisite for Directed Evolution (see also 5.2.4)

Due to the fact that the wild-type CalA did not show *sn*2-preference in esterification reactions and additionally catalyses this reaction type only very weakly, the aim was to improve the selectivity of the enzyme by directed evolution. As a prerequisite the enzyme has to be expressed functionally in a fast-growing and easy-to-handle microorganism. Therefore the expression of CalA in the cytoplasm of *Escherichia coli had* to be established.

4.2.1 Expression of CalA Using Different Vectors

The CalA gene was cloned into three different vectors: pUC18, pET-32b(+) and pColdIII. Two different strains with a deficiency in the thioredoxin and glutathione reductase were selected for expression. These deficiencies are reported to enhance the disulfide bond formation in *E. coli* which is the major bottleneck for expression of eukaryotic enzymes in prokaryotic hosts (Prinz et al., 1997). Maybe due to this reason DH5α cells transformed with *pUC18-calA* did not show any halo formation on tributyrin agar plates.

CalA activity in the Origami^{TM} B lysate was very weak; the hydrolysis activity against tributyrin was about 0.7 U/mg of total soluble protein.

For enhancing the yield of active enzyme, the *calA* gene was fused to the extremely soluble thioredoxin tag (Trx·Tag^{TM}) using the vector pET-32b(+) and expressed in *E. coli* Origami^{TM}2(DE3). Expression of Trx-CalA was performed at 30 °C and yielded 0.6 U mg^{-1} lipase activity. This expression strategy resulted in a strong overexpression of CalA. The insoluble fraction consisted of 25 % CalA. This fits with the general observation that high expression levels can lead to an increase in the proportion of incorrectly folded protein. Finally expression of the *Trx-calA* construct at 15 °C yielded 1.7 U mg^{-1} and 22 % CalA content in the insoluble fraction. A high yield expression protocol for functional lipases in *E. coli* cells using the T7 *lac* promoter has recently been reported (Kim et al., 2000; Nthangeni et al., 2001). However, in these expression experiments, most of the target proteins were prokaryotic enzymes with no need for folding assistance and posttranslational modifications as required by most eukaryotic enzymes.

A further increase of functional CalA expression level to 9.6 U mg^{-1} was achieved using the temperature-inducible pColdIII vector system using Orgami™B cells. Still analysis by SDS-PAGE and densitometry showed a high amount of insoluble CalA (18.5 %).

Decreasing the expression temperature increased the yield of functional enzyme slightly in the case of pET32-b(+) and strongly in the pColdIII system. The positive influence of decreased expression temperature on the yield of functionally expressed enzyme was already reported (Liao, 1991; Torre et al., 2002). This dependency of the yield of correctly folded enzyme on the expression temperature can be theoretically explained: Whereas folding rate is only slightly decreased by a temperature switch from e.g. 37 to 15 °C, the rate of transcription and translation in *E. coli* is significantly reduced. These facts provide sufficient time for protein refolding, yielding active enzyme and avoiding the formation of insoluble aggregates of misfolded protein (Makrides, 1996).

The fusion of an enzyme to a tag, like TrxA can lead to changes in catalytic properties (Mutsuda et al., 2003). This can explain the fact that compared to the pColdIII results, the activity of the enzymes expressed in the pET-32b(+) clones are low. Therefore screening of mutants carrying a TrxA tag would not be suitable for the detection of improved variants of the native CalA. From this point of view the use of the pColdIII system seems to be more favourable than utilising pET-32b(+).

4.2.2 Functional Lipase Expression Using Co-Expression of Molecular Chaperones

A strong increase in soluble heterologous protein concentration and yield of active enzyme in the *E. coli* cytoplasm by co-expression of chaperones has already been reported (Nishihara et al., 1998; Nishihara et al., 2000; Ikura et al., 2002). The pColdIII and the pET-32b(+) constructs both were co-expressed with several combinations of chaperones from the TaKaRa Chaperone Plasmid Set.

CalA was most efficiently expressed in the pColdIII vector with co-expression of pGro7 resulting in 13.1 U/mg. Generally expression from the pColdIII constructs resulted in higher specific activity than expression from the pET-32b(+) constructs. The specific activity of the pET-32b(+) constructs expressed at 30 °C was lower than 1 U mg^{-1}. Enzyme expression at 15 °C increased the specific activity significantly. Still activity was lower than with the pColdIII constructs. Also among the pET-32b(+) constructs, the best activity value was reached co-expressing pGro7 (3.4 U mg^{-1}).

Co-expression using the pGro7 construct surpassed the specific activity of the expression of CalA-pColdIII without chaperones. All other expression experiments using co-expression of chaperones yielded lower activity.

Generally co-expression of chaperones increased the amount of soluble CalA (10 – 22 % of total soluble protein) compared to expression without chaperones (0.22 – 0.35 % of total soluble protein).

With both expression systems CalA was most efficiently expressed when co-expressing Gro7, indicating strong impact of the GroES/GroEL chaperone system on the correct folding of CalA. It is known that these molecular chaperones play an important role in folding α/β structures enriched in hydrophobic and basic residues. This is underlined by the fact, that the molecular mass of CalA (43 kDa) fits with the size of GroES/GroEL´s most preferred substrates (proteins < 60 kDa) (Baneyx et al., 2004). Recently a lipase with the molecular weight of 33 kDa was also functionally expressed in *E. coli* using co-expression of Gro7 (Liu et al., 2006). In combination with *trxB gor* suppressor cells (e.g. Origami™) the yield of properly disulfide-bonded proteins was increased by the co-expression of these folding modulators (Bessette et al., 1999; Levy et al., 2001; Jurado et al., 2002). Co-expression of the trigger factor (encoded by pG-Tf2 and pTf16) did not show any positive effect in functional enzyme expression and co-expression of DnaK-DnaJ-GrpE showed only a slight increase of functional CalA expression. These results fit to the observation that a positive influence of the DnaK-DnaJ system is mostly restricted to target proteins with higher molecular weight than 60 kDa (Mujacic et al., 2004).

The expression of CalA was consequently down-scaled to microtitre plate scale and successfully tested for screening of a large mutant library. A diploma thesis starting from this point aiming at the enhancement of the *sn*2-specificty of CalA was able to generate mutants showing a significant increasing specificity using the reported expression system of CalA in *E. coli* (Wilhelmi, 2007, see also 5.4.4).

4.3 Enzymatic synthesis of 2-Monoacylglycerides and Structured Lipids on a Technical Scale (see also 5.3.4)

Nevertheless the tools had been established to tailor CalA with regard to its *sn*2-specificity and its esterification potential the generated mutants still do not show the desired characteristics. So far there is no enzymatic one-step process for the direct synthesis of structured lipids or 2-MAG possible. Therefore a two step process was established to synthesise each desirable 2-MAG or structured lipid: the first reaction is an esterification of glycerol and fatty acids leading to a homogenous TAG which is converted in an alcoholysis reaction to the corresponding 2-MAG. The very interesting finding of this work was, that CalB was able to catalyse both steps. In the esterification reaction the lipase acts unspecifically and afterwards in the presence of alcohol the TAG is cleaved *sn*1,3-specifically.

This process was first verified in lab scale to synthesize 2-MAG of different PUFA (ARA, DHA and EPA) and afterwards up-scaled to produce 2-MAG and TAG (e.g. OPO) in miniplant scale.

4.3.1 Enzymatic Synthesis of 2-MAG of PUFA in Lab-Scale

The potential of CalB for synthesis of triglycerides in an unspecific manner was already described (Medina et al., 1999). A glycerol:PUFA ratio of 1:3 was found to be the optimum for the reaction. Excess of glycerol or fatty acids is inappropriate because of the production of large amounts of MAG and diacylglycerols (DAG) decreasing the yield of TAG.

For the *sn*1,3-specific alcoholysis several lipases classified as *sn*1,3-specific were tested: *Candida antarctica* B lipase (CalB), *Penicilium camembertii* lipase, *Pseudomonas fluorescens* lipase, *Rhizomucor mihei* lipase, *Rhizopus javanicus* lipase and *Rhizopus oryzae* lipase

(Bornscheuer et al., 1999). Only CalB was able to convert all three TAG of PUFA to the desired 2-MAG indicating the substrate binding pocket of CalB is the only one that can accept glycerides of bulky and unsaturated fatty acids. It is possible that the addition of ethanol converts CalB from an unspecific to a more *sn*1,3-specific lipase. A possible reason for this behaviour might be decreased flexibility of the tertiary structure of CalB caused by ethanol, hindering the substrate from accessing the catalytic binding pocket with the acyl group in the middle position (Irimescu et al., 2002). Consequently CalB should be classified as an unspecific lipase whose specificity can be tailored by the co-solvent.

The 2-MAG of the three model PUFA (ARA, DHA, EPA) were synthesized in gram quantity with purities higher than 85 % and yields up to 96 %.

The same process was proven to be usable for the synthesis of 2-MAG of short and middle-chain fatty acids like caprylic- (C8), palmitic- (C16) and oleic acid (C18:1). So the substrate spectra of the 1,3-specific alcoholysis reaction with CalB covers all chain lengths from short to long and saturated or polyunsaturated.

4.3.2 Synthesis of Triglycerides in the Miniplant

For up-scaling the esterification process, erucic acid, a cheap model substrate was chosen. The CalB-catalysed esterification yielded in 352 gram of triglycerides of erucic acid yielding in 97 % of the theoretically possible yield.

4.3.3 Synthesis of OPO in the Miniplant

OPO was synthesized in the miniplant starting with tripalmitate as substrate. In the first step tripalmitate was converted to 2-monopalmitate (2-MP) in an alcoholysis reaction, followed by esterification of 2-MP with oleic acid to OPO. So this process was vice-versa the process leading to the 2-MAG of PUFA. The yield of 2-MP was 73 % corresponding to 84 g (77 % purity). The esterification led to 198 g of OPO (yield 90 %) with a purity of 95 %.

Yield and purity of OPO synthesized in the miniplant using the process described here were slightly lower than a similar process catalyzed by *Rhizopus* lipases (Schmid et al., 1999). Still this process was performed in a laboratory scale whereas in our case we already reached a technical scale (> 100 gram of product). Processes for OPO synthesis have been described using two different lipases with distinct regioselectivity for the ethanolyis and esterification reaction (Chen et al., 2004). Use of only one enzyme as suggested in our work will facilitate industrial applications.

The potential of our approach is further underlined by comparison of the results to the latest literature on 2-MAG synthesis. A lab-scale process for the enzymatic synthesis (ethanolysis) of 2-MAG described by Shimada and colleagues yielded only in 28 – 29 mol% of 2-MAG content (Shimida et al., 2003). Yang and colleagues described a CalB-driven glycerolysis process yielding 2-MAG of PUFA (Yang et al., 2005). Still the yield in the stirred tank just reached 70 % and unfortunately they had to work with a multiphase-system.

With respect to process development and further up scaling, the use of only one lipase for the unspecific esterification and the *sn*1,3-selective alcoholysis is a big advantage of the process described herein. Reusability of immobilized CalB in the batch processes indicates that establishment of continuous production processes is possible. Thus new promising applications of this outstanding biocatalyst might arise.

4.4 Directed Evolution of Candida antarctica Lipase A: towards enhanced *sn*2-specificty (see also 5.3.5)

After the expression of CalA in *E. coli* was successfully established, the development of a high-throughput screening assay was a second prerequisite for a directed evolution increasing the *sn*2-preference. The principle of the assay (TwinAssy) is two hydrolysis reactions which are performed in parallel: the hydrolysis of tricaprylin and the hydrolysis of 1, 3-dicaprylin. By calculating the reaction rate of both reactions the specificity of the mutant can be determined.

The assay was verified by lipases with known specificity: CalA (*sn*2-preference), RML and RAL (both 1,3 –specific) and PFL (non-specific).

A mutant library including about 5000 clones was generated using error-prone PCR which was screened using the new TwinAssay system. Finally a quadruple mutant showing a 10 % increased *sn*2-prefernce was found and characterized.

5. Publications

5.1 High yield expression of Lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* and its purification and characterisation

Jan Pfeffer, Sven Richter, Jens Nieveler, Carl-Erik Hansen [1], Rachid Bel Rhlid [1], Rolf D. Schmid, and Monika Rusnak*

J. Pfeffer, S. Richter, J. Nieveler, R. D. Schmid, M. Rusnak
Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

C. E. Hansen, R. B. Rhlid
[1] Nestlé Research Center Lausanne, Nestec Ltd., Vers chez les blanc, P.O. Box 44, 1000 Lausanne 26, Switzerland

*** Author for correspondence (Fax: (+49)-711-685-3196; E-mail: Monika.Rusnak@gmx.de)**

Key words:

Lipase, *Candida antarctica*, fed-batch, semi-continuous fermentation, *Pichia pastoris*

5.1.1 Abstract:

The current investigation focuses on shedding further light on the characteristics of lipase A from *Candida antarctica* (CalA), which has attracted growing attention in its suitability for industrial applications. CalA was functionally expressed in the methylotrophic yeast *Pichia pastoris*, purified and characterised. A classical fed-batch-process and a semi-continuous-process were developed and tested with regard to their yield capacity. Lipase concentrations

of 0.88 and 0.55 g l^{-1} were obtained using the fed-batch and semi-continuous process respectively. Fed-batch fermentation resulted in a lipase concentration of 0.88 g l^{-1} and in 0.55 g l^{-1} using the semi-continuous process. Nevertheless the total activity of the semi-continuous-process reaches a total activity of 10,233,000 Units and so surpasses the fed-batch process reaching 7,530,000 Units.

The purified enzyme showed highest activity between 50 to 70 °C at pH 7.0 and a preference for short-chain triglycerides (C4 – C8). Significantly reduced activity was observed in the presence of hydrophilic esters.

5.1.2 Introduction:

Lipases [triacylglycerol-hydrolases (EC 3.1.1.3)] play an important role in several industrial applications. They catalyse the hydrolysis of triacylglycerols at the interface between water and the hydrophobic substrate. Besides the hydrolysis of triacylglycerols, lipases also catalyse the enantio- and regioselective hydrolysis or synthesis of many natural and synthetic esters (Björkling et al. 1991; Bornscheuer et al. 1998; Schmid and Verger 1998). Lipases are found in animals, plants and microorganisms like bacteria, yeasts or archeae (Rusnak et al. 2005) (Schmidt-Dannert 1999). Lipases from microorganisms have received a considerable interest as catalysts in many industrial applications such as ester synthesis (Okumura, Iwai et al. 1979; Nakano, Kitahata et al. 1991), optical resolution (Fukusaki et al. 1999; Sakaki and Itoh 2003), transesterification (Kaieda et al. 2004; Matsumoto et al. 2004) or washing processes (Kojima et al. 1994).

Two lipases, lipase A (CalA) and B (CalB), were previously isolated from *Candida antarctica*: (Hoegh et al. 1995), in which CalB was characterised in greater depth. It reveals high enantioselectivity against secondary alcohols and, due to its extraordinary stability in organic solvents and at high temperature, has become one of the most frequently used enzymes in industrial applications (Anderson et al. 1998). Fewer data are available for CalA, a thermostable lipase (Solymár et al. 2002) that might also have a high potential for industrial applications. CalA is the only known lipase with an *sn*2-preference towards triglycerides (Rogalska et al. 1993) and therefore opens up a plethora of possible applications including the synthesis of symmetric triglycerides. Nevertheless, this preference is not sufficiently pronounced in order to enable the selective synthesis of 1,3-diglycerides or 2-monoglycerides

(Dominguez de Maria et al. 2005) and CalA was consequently rated as a non-selective biocatalyst with regard to industrial interesterifications (Heldt-Hansen et al. 1989).

CalA is a calcium-dependent lipase that might prove suitable for the resolution of heteroaromatic β-amino esters (Solymár et al. 2002). It exhibits a high activity towards sterically hindered alcohols, including both secondary and tertiary alcohols (Kirk and Christensen 2002; Krishna et al. 2002) and hydrolyses several tertiary-butyl esters of protected amino acids (Schmidt et al. 2005). This indicates that CalA might be a perfect tool for the conversion of highly branched and bulky substrates that cannot be hydrolysed by most other lipases. Compared to the free enzyme, immobilized CalA has an extraordinary substrate selectivity. For example, it esterifies elaidic acid about 15 times faster than oleic acid (Borgdorf and Warwel 1999). The enzyme tends to preferentially esterify the *trans*-isomer in the presence of *cis/trans* unsaturated fatty acid isomers, while most other lipases favour the esterification of *cis*-fatty acids. Recently, it has been discovered that CalA together with *Candida rugosa* lipases, pig liver esterase and esterase from *Bacillus subtilis* shows activity towards tertiary alcohols (Henke et al. 2002; Henke et al. 2003).

In contrast to CalB, CalA shows interfacial activation, but the activation is not as prominent as observed for *Humicola lanuginosa* lipase (HLL). The protein structure of CalA is not resolved yet and hence it is difficult to say what might be the reason for this (Martinelle et al. 1995).

In 1995, CalA was functionally expressed in the filamentous fungus *Aspergillus oryzae* (Hoegh et al. 1995), no information is, however, available on the heterologous expression of CalA in other expression systems, including yeast, bacteria or other fungi.

The methylotrophic yeast *Pichia pastoris* has gained high popularity as expression system due to the high yields of soluble protein, obtained by intracellular as well as secreted expression. A couple of proteins were expressed in very high protein yields (up to 10 g l^{-1}) (Cregg et al. 2000; Daly and Hearn 2003). In total, over 350 proteins have already been heterologously expressed in this yeast.

The alcohol oxidase gene I is the most frequently used promoter for high yield protein expression in this yeast system. *Pichia pastoris* is able to perform a lot of posttranslational modifications, like proteolytic processing, disulfide bond formation and glycosylation. One of the advantages of *Pichia pastoris* over the common beakers´ yeast *Saccharomyces cerevisiae* is the glycosylation pattern which is much closer to the human, so that even human Insulin was functionally expressed in *Pichia pastoris*.

A recently published review on CalA (Dominguez de Maria et al. 2005) stated an increasing interest in this biocatalyst and its potential for industrial applications. The current investigation was undertaken in order to characterise the enzyme in even further depth and provide a solid basis for further investigations, including the crystallisation and structural resolution as well as the mutagenesis of the enzyme, which will eventually help tailor the enzyme to the needs as required by the industry. The CalA enzyme was functionally expressed in *Pichia patoris* in a 5-litre scale using two different fermentation strategies, purified using a cation-exchange column and characterised.

First a classical fed-batch process was performed to reach high protein yields in a short operation time. Afterwards a semi-continuous process was established which allows daily harvest of protein over a longer time period. This is a quite simple process which diluted the accumulation of metabolic by-products in the fermentation broth by daily harvesting and adding fresh media. Finally a higher total activity was expected with this process.

5.1.3 Material and methods:

I. Cloning of the lipase gene:

Candida antarctica (DSM 70725) was cultivated in Universal Medium For Yeasts (yeast extract 3 g l^{-1}, malt extract 3 g l^{-1}, peptone from soybeans 5 g l^{-1}, glucose 10 g l^{-1}) at 30 °C and 180 rpm. The cells were harvested after 3 days and disrupted with glass beads (0.75 – 1.00 mm) using a Retsch-mill (MM2000, Retsch, Haan, Germany) for 30 minutes. The proteins were precipitated with SDS (2 % vol.) at 65 °C. After centrifugation, the genomic DNA was precipitated with 0.3 M Na acetate and 2-propanol and finally resuspended in 100 µl TE (Tris 100 mM, EDTA 10 mM, pH 8.2) supplemented with RNase (0.1 % vol.).

The lipase gene was amplified by PCR using DyNAzyme™ EXT DNA polymerase (Finnzymes, Heidelberg, Germany) and primers containing the restriction sites for *Eco*RI and *Not*I (CalA-*Eco*RI-f: 5´-CCGGAATTCGCGGCGCTGCCCAACC-3`, CalA-*Not*I-r: 5`-TTTTCCTTTTGCGGCCGCCTAAGGTGGTGTGATGGGGC-3`). The PCR product was cloned into the pPicZαA vector (Invitrogen, Karlsruhe, Germany) using standard procedures and *E. coli* DH5α cells (Clontech, Heidelberg, Germany) were transformed with the construct. The construct was used to transform *Pichia pastoris* X 33 cells (wildtype, Invitrogen) (Cregg, Cereghino et al. 2000; Daly and Hearn 2003).

II. Expression of CalA:

Recombinant *Pichia pastoris* cells were cultivated at 30 °C and 180 rpm in 10 ml BMGY-Zeocin (10 g l^{-1} yeast extract, 20 g l^{-1} Bacto Peptone, 100 mM potassium phosphate buffer, 10 % (v/v) glycerol, 0.0 2 % (w/v) biotin, 100 mg l^{-1} Zeocin) media until an OD_{600} of 5 – 15. The cells were pelleted by centrifugation and resuspended in BMMY media (10 g l^{-1} yeast extract, 20 g l^{-1} Bacto Peptone, 100 mM potassium phosphate buffer, 0.02 % (w/v) biotin, 0.5 % (v/v) methanol) adjusting an OD_{600} of 0.5. During the expression process, every day 0.5 % (v/v) methanol was added for induction. The cells were subsequently pelleted by centrifugation and the supernatant was analysed by SDS-PAGE (Laemmli 1970) and pH stat (Metrohm, Filderstadt, Germany).

Fed-batch fermentation conditions:

A 50 ml shake flask with 5 ml buffered complex glycerol medium (BMGY, containing 1 % (w/v) yeast extract, 2 % (w/v) peptone, 1 % (v/v) glycerol, 5 µL (100 mg ml^{-1}) Zeocin, $4x10^{-5}$ (w/v) biotin, 100 mM potassium phosphate, pH 6.0) was inoculated with 50 µL stock seed. The flask was incubated for ~48 h at 30 °C on an orbital shaker at 140 rpm. Afterwards, 2L shake flasks with 200 ml of BMGY medium were inoculated with the pre-culture and incubated for ~48 h at 30 °C on an orbital shaker at 160 rpm. 0.5 % glycerol were added to each shake flask and incubated at the same conditions until cell density reached OD_{600} > 50. The bioreactor cultivation was seeded with the content of the shake flask cultures up to an OD_{600} of 0.5. Fermenter cultivation was performed in a 7.5 L reactor (Infors, Bottmingen, Switzerland) containing 5 L basal salt medium made of 45.5 g K_2SO_4, 37.5 g $MgSO_4 \cdot 7H_2O$, 31 g KOH, 2.35 g $CaSO_4 \cdot 2H_2O$, 66.75 ml H_3PO_4 (85 %), 250 g glycerol, 0.5 ml Antifoam 286 (Sigma, Deisenhof, Germany), 4.35 mg Biotin (filter sterilized) and 1.5 ml PTM_1 trace salts (filter-sterilised). One litre PTM_1 contains 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3.0 g $MnSO_4 \cdot H_2O$, 0.5 g CoCl, 20.0 g Zn Cl, 0.02 g H_3BO_3, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 65.0 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 30 ml 6N H_2SO_4. The temperature was kept constant at 30 °C and the pH was maintained at 6.8 using NH_4OH (28 %) and H_3PO_4 (10 %). The airflow was maintained at 10 L min^{-1} and the stirrer speed was adjusted between 800 and 950 rpm.

When the initial methanol concentration of 0.5 % (v/v) in the culture broth was depleted (indicated by an abrupt increase in dissolved oxygen (DO)), 20 g of 100 % methanol solution containing 1.2 % (v/v) PTM_1 was automatically added. During the fermentation process, another 12 ml Antifoam were added.

Methanol consumption was monitored using a balance (BP 4100, Sartorius, Goettingen, Germany) that was interfaced with the IRIS process control system (Infors, Bottmingen, Switzerland).

Semi-continuous fermentation conditions:
The semi-continuous fermentation was performed in the same way and using the same equipment like the fed-batch process. But after 5 days daily harvests of 500 ml were started. After each partial harvest 500 ml of fresh batch media was added.

Cell density measurement:
The optical density was measured at 600 nm (spectrophotometer Ultrospec 3000, Amersham Biosciences, Freiburg, Germany) three times. After centrifugation (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 4600g and 10 °C for 6 min the pellet was washed in 0.9 % (w/v) NaCl solution and the wet cell weight (WCW) of a 5 ml cell suspension sample was determined gravimetrically (RC 210 D, Sartorius, Germany).

Determination of protein concentration:
Protein concentration was determined using the Bradford assay (Bio-Rad, Munich, Germany (Bradford et al. 1976)).

III. Purification of CalA:
After expression, the cells were pelleted by centrifugation of the culture broth at 10,800 g for 10 minutes in a Sorvall RC5C Plus centrifuge (Sorvall, Langenselbold, Germany). The lipase containing supernatant was filtrated by using a cross-flow application using a Milipore set-up according to the manufacturer's manuscript. A 100 kDa membrane (Pall, Dreieich, Germany) was used to remove cellular particles and large contaminants, and the permeate was filtered using a 10 kDa membrane (Pall). The retentate was dialysed overnight using 20 mM citrate buffer and applied on a 25 ml CM-Sepharose column (Amersham Biosciences, Freiburg, Germany) equilibrated with 20 mM citrate buffer (pH 4.5). The lipase was eluted from the column with a linear salt gradient using 1 M NaCl (pH 8).
The purity of the lipase was determined using SDS-PAGE on a 12.5 % separation gel under the conditions developed by Laemmli (Laemmli 1970). A Minigel-Twin cell (Whatman, Biometra, Göttingen, Germany) was used for electrophoresis. The molecular weight marker was purchased from Amersham Biosciences. The gels were silver stained as described by

Blum (Blum et al. 1987) and the percentage of soluble protein was measured densitometrically using the program Scion Image.

IV. Characterisation: The hydrolytic activity during fermentation and purification was determined using a pH-stat device (Metrohm) with tributyrin as substrate at 30 °C (pH 7.5). Tributyrin (5 % (v/v)) was emulsified in distilled water containing 2 % (w/v) gum Arabic using a homogeniser for 7 minutes at maximum speed (Ultraturrax T25, Janke and Kunkel, Staufen, Germany). Twenty millilitres of the substrate emulsion were heated to the reaction temperature and the pH was adjusted using 0.1 M NaOH or 0.1 M HCl. Liberated fatty acids were titrated automatically with 0.1 M NaOH in order to maintain a constant pH. One unit (U) of lipase activity was defined as the amount of lipase that liberates 1 µmol fatty acids per minute.

For the substrate spectra different substrates were used in the concentration given above. For the pH and temperature optima the assay was performed using tributyrin with different reaction temperatures or pH conditions.

The kinetic parameters were determined by hydrolysing tributyrin under the pH and temperature values determined before.

All measurements were done in triplicates.

5.1.4 Results:

I. Multisequence alignment using BLAST:

The amino acid sequence of CalA shows highest similarity to the lipases from *Kurtzmanomyces* sp. I-11 (74 %) and *Ustilago maydis* (64 %), respectively (Kakugawa et al. 2002). None of the other lipases – not even from the *Candida* family – had a significant similarity with CalA. For example, the enzyme only displayed 31 % similarity with the lipase/acyltransferase of *Candida parapsilosis* (Neugnot et al. 2002)(Neugnot et al. 2002). The typical lipase consensus pentapeptide (-Gly-X-Ser-X-Gly-) is also conserved in CalA. Homology searches and alignment tools such as Pfam (data not shown) (Bateman et al. 2000) suggest that CalA is actually an α/β-hydrolase (Ollis et al. 1992).

II. Cloning and expression of CalA in shake flask scale:

The CalA gene was amplified from genomic *Candida antarctica* DNA and cloned into the pPicZαA vector. The construct was sequenced (Sanger et al. 1977) and compared with the published CalA sequence (Hoegh et al. 1995) for confirmation. In the obtained construct, CalA was fused to the gene of the alpha factor from *Saccharomyces cerevisiae* which allows the secretion of the expressed lipase into the medium.

Since different patterns of homologous recombination lead to varying expression rates in individual clones, several clones were tested for their hydrolytic activity against tributyrin. A clone revealing a hydrolytic activity of 215 U mg^{-1} (related to the total protein concentration in the supernatant) after 5 days of incubation in a shake flask was chosen for all further experiments.

III. Fermentation and purification of CalA:

Two different fermentation protocols were used, involving a classical fed-batch-fermentation and a repeated-fed-batch (semi-continuous) process, and the protein yields compared.

The fed-batch process continued over a period of 12 days (Fig. 1). The cells were induced with methanol after 27 hours of batch processing. The dry cell weight (DCW) increased continuously and reached a final value of approximately 110 g l^{-1}. The OD_{600} also grew continuously and reached a final value of approximately 500. The activity increased slowly for the first 100 hours after induction and then started increasing more strongly and finally reached a value of 653 U mg^{-1} (1802,0370 U ml^{-1}). The final volume was 5.2 litres and the volume of the supernatant amounted to 3.7 litres. The final total protein concentration was 3.12 g l^{-1} and the lipase concentration was 0.88 g l^{-1}. The wet cell weight (WCW) and the OD_{600} both and the dry cell weight (DCW) both increased continuously over the fermentation time and reached 320 g l^{-1} and 110 g l^{-1}500, respectively (data not shown). The expression of CalA was controlled by SDS-PAGE. The protein band on the gel, which corresponds to the theoretical molecular weight of CalA at 43 kDa, was getting stronger over the time, reflecting the increasing amount of the enzyme (data not shown). After 12 days, a total activity of 7,530,000 units was reached.

The repeated fed-batch fermentation continued over a period of fifteen days. After 5 days of batch-processing, every day 10 % (v/v) of the fermentation broth was harvested and the same volume replaced by fresh batch media, thus establishing a semi-continuous fermentation process (Fig. 2). The DCW first increased slowly and after the first harvest strongly reaching

a value of 160 g 1^{-1} and then decreasing slowly to a constant final value of 120 g l^{-1}. The OD_{600} increased dramatically during the batch (approximately the first 27 hours of fermentation) and fed-batch phases. After each harvest, the OD_{600} decreased significantly and increased again to the pre-harvest value within 24 hours (data not shown). At first, the activity increased only slowly but took up speed 70 hours after induction. The WCW course was virtually parallel to the DCW and reached finally 250 g l^{-1} The curse of the DCW and WCW course were virtually parallel and decreased after 210 hours (Fig. 2).

After a fermentation time of 115 hours, the first of ten harvests was taken. The OD_{600} ranged between 270 and 370 during the harvesting periods. The final concentration of total protein in the supernatant was 2.97 g l^{-1}, the CalA concentration 0.55 g l^{-1} and the final activity was 380 U mg^{-1} (1,127 U ml^{-1}). The final culture volume on day 15 of fermentation was 5.2 litres and the volume of the supernatant was 4.15 litres; the total activity was 10,230,000 units (Table 1).

Several hydrophobic interaction chromatography columns (HIC) and ion exchange chromatography columns were tested in order to determine the best suitable column for purification. CalA only showed binding bound to the matrix of the cation exchange chromatography column. Following cross-flow filtration and cation exchange chromatography, SDS-PAGE confirmed a nearly homogenous, pure CalA. The purification factor for the lipase expressed in the fed-batch process was 3.1; after purification, the specific activity was 2,033 U mg^{-1}. The gel revealed contaminations in the supernatant and the concentrated lipase – especially at low molecular weights; the band of the purified enzyme was thick and clear and contained hardly any contaminants. The commercial available CalA from Novozymes (Lipase Novozym® 735) has an activity of 6,000 U g^{-1}.

With densitometry (Scion Image) analysis the quality of the purification process was determined. In the supernatant the lipase was 33 % pure and this value increased to 85 % after the cation exchange chromatography (Fig. 3).

IV. Characterisation of CalA:

Temperature and pH optimum, kinetic constants:

CalA showed maximal activity at approximately 50 °C, with similar activities between 50 and 70 °C. At temperatures over 70 °C, the activity decreased dramatically; the activity increased continuously at temperatures from 10 to 50 °C (Fig. 4).

The pH optimum of CalA was ~ pH 7.0. CalA showed high activity between pH 7.0 and pH 9.0; enzyme activity decreased very fast at higher or very low pH values (Fig. 5).
In contrast to the suggestion of Solymár (Solymár et al. 2002), the addition of $CaCl_2$ (0,5 % (w/v)) did not have any effect on the hydrolytic activity of the enzyme under investigation (data not shown).
The kinetic constants of CalA using tributyrin as a substrate were determined as K_M = 4,483 µM, k_{cat} = 1,496,020 s^{-1} and $k_{cat}\ k_M^{-1}$ = 334 $\mu M^{-1}\ s^{-1}$.

Substrate spectra:

CalA preferentially hydrolysed triglycerides with short chain lengths (C4 – C8). No activity was measured for tripalmitin (C16) while the enzyme exerted low activity when using triolein (C18) and olive oil as substrates.
Different esters were tested and hydrolytic activity observed for vinyl acetate (C2). Esters with chain lengths of between C4 – C10 only enticed a low conversion rate (Table 2).

5.1.5 Discussion:

Sequence analysis:
The amino acid sequences of the *Candida antarctica* lipases A and B differ substantially and are not very similar to other known lipases. Both lipases seem to be unique and although they come from the same species, *C. antarctica*, they do not only differ extremely in sequence but also in their characteristics. Similar observations were reported previously and could be explained by a distribution of biological tasks within the organism like utilizing different substrates (Rusnak et al. 2005).

Fermentation and purification:
A semi-continuous fermentation process was implemented involving a process that allowed every-day harvesting of expressed CalA over a long time. The protein concentration and the activity in the finally harvested culture supernatant were higher in the fed-batch process. However, the total activity of the harvested and pooled CalA harvests of the semi-continuous process repeated-fed-batch CalA yield was higher than in the fed-batch process. After 12 days of fed-batch processing, the total activity was some 15 % higher in the semi-continuous

process. The lower lipase concentration in the semi-continuous process can be explained because the culture was diluted every day with fresh media.

The CalA concentration in the supernatant of the fed-batch-process was 0.88 g l^{-1}. This is substantially higher compared to the yield reached by expressing *Candida rugosa* lip4 lipase in *Pichia pastoris* (0.1 g l^{-1}) (Tang et al. 2001). For comparison, the expression of a CalB-fusion protein in *Pichia pastoris* in a fermentation processes involving the aeration with pure oxygen led to a lipase concentration of 1.5 g l^{-1} (Jahic et al. 2002). With a temperature-limited feeding strategy this value could be increased to 2 g l^{-1} (Jahic et al. 2003). Therefore it is possible that pure oxygen and a temperature-dependant strategy might be able to lead also to an increase in the protein yield of CalA.

Silver-stained SDS-PAGE confirmed the purity of the protein. The purification factor was however significantly lower than the purification factor observed for proteins expressed in *Escherichia coli.* For example, a fermentation protocol for a *Archaeoglobus fulgidus* lipase in *E. coli* which also included cell disruption reached a purification factor of 317 (Rusnak et al. 2005). In contrast to proteins expressed in the *E. coli* cytoplasm, CalA was secreted into the medium and consequently separated from the cellular proteins. This explains the low purification factor achieved in our investigation.

CalA did not bind to the HIC or anion-exchange chromatography columns. This indicates that the surface of the protein is predominantly constituted of positively charged amino acids. The majority of lipases can easily be purified using HIC columns because they have hydrophobic patches on their surface.

The observed binding behaviour as well as the homology searches indicate that CalA is an extraordinary enzyme.

Characterisation:

The temperature optima of lipases A and B from *Candida antarctica* are very similar. CalB is highly thermostable and has its highest activity between 60 and 80 °C (Otto et al. 2000). CalA shows a plateau of high activity between 50 and 70 °C. Since many industrial applications such as acidolysis or the generation of new ester compounds (Sahin et al. 2005) require a high temperature, CalA might therefore be an excellent converter (Tsuchiya et al. 2005).

The pH optimum of CalB (pH 6.0) is lower than that of CalA (Kirk and Christensen 2002). The lipase from the glycolipid-producing yeast *Kurtzmanomyces* sp. I-11 (49 kDa), which has 74 % sequence similarity to CalA, has its pH optimum in the acidic pH range (pH 1.9 – 7.2).

This enzyme is also a thermophilic enzyme. It displays maximum activity at temperatures from 65 °C to 85 °C and therefore higher than that of CalA (Kakugawa et al. 2002).
CalA displays a much higher conversion rate towards triglycerides than towards hydrophilic esters, which indicates that CalA is a true lipase. The enzyme's catalytic activity towards triglycerides decreases with chain length. The conversion rate of tripalmitin is virtually zero. This indicates that only short-chain substrates can dock to the substrate-binding site of the enzyme. Surprisingly, the *Kurtzmanomyces* lipase hydrolyses long-chain fatty acids more efficiently than short-chain fatty acids. Therefore, in spite of the high sequence homology the structures of the substrate- binding sites of these two lipases seem to be different.

5.1.6 Conclusion:

Two different fermentation protocols were developed and tested with regard to the ability of expressing lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* in high yields. The lipase was purified by cation-exchange chromatography. The purified enzyme had the highest activity at 50 °C and pH 7.0 and prefers triglycerides with short chain lengths over fatty acids with longer chains.

The results show that the tested protocols lead to sufficiently high enzyme yields. This work allows further investigations and applications of this potentially applicable enzyme in our effort to increase the number of enzymes suitable for industrial applications.

Acknowledgement:

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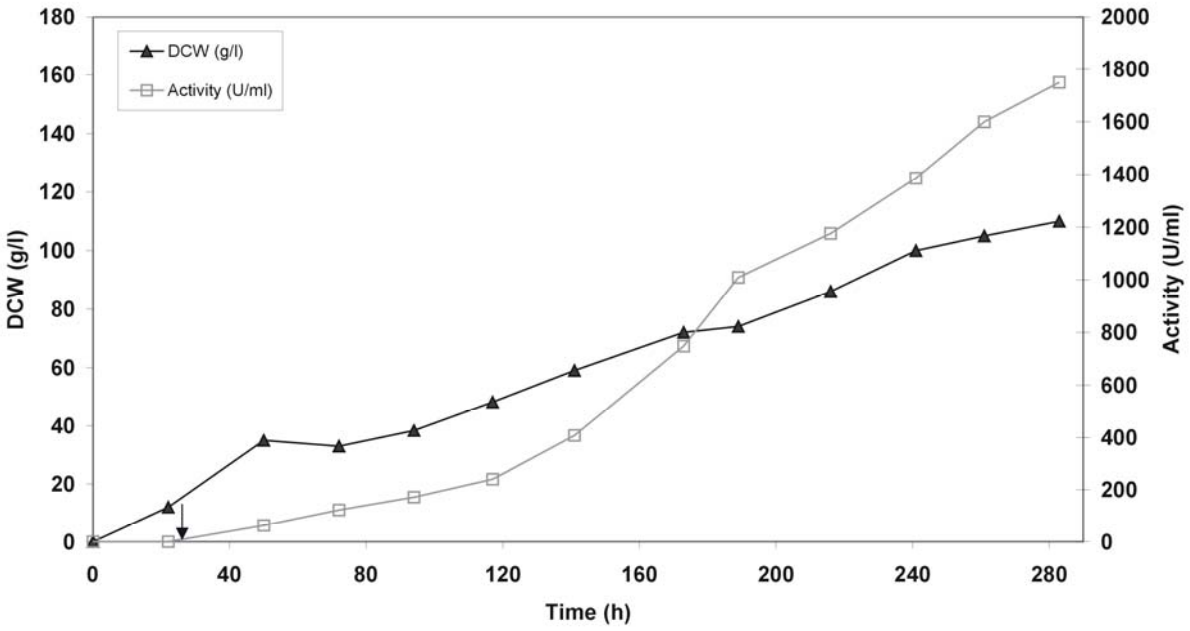


Figure 1: Fed-batch fermentation of *Pichia pastoris* cells secreting lipase A from *Candida antarctica*. Secretion of the enzyme was induced through the addition of methanol after 27 hours of batch processing (arrow).

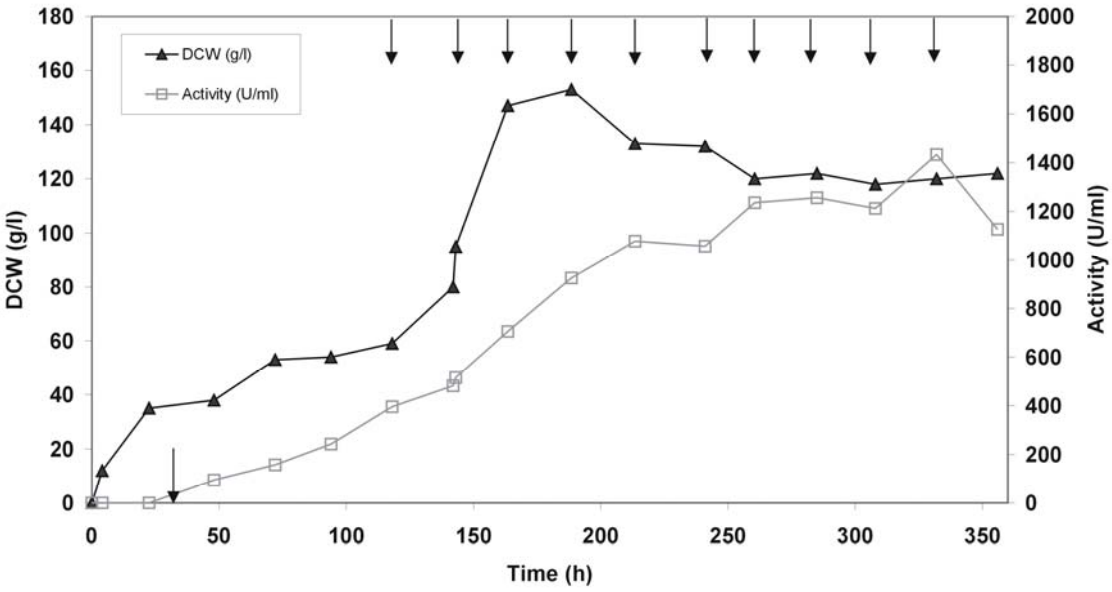


Figure 2: Repeated fed-batch fermentation (semi-continuous process) of *Pichia pastoris* cells expressing lipase A from *Candida antarctica*. The arrows mark the point in time when 500 ml fermentation broth were harvested. The first arrow marks the start of methanol feeding.

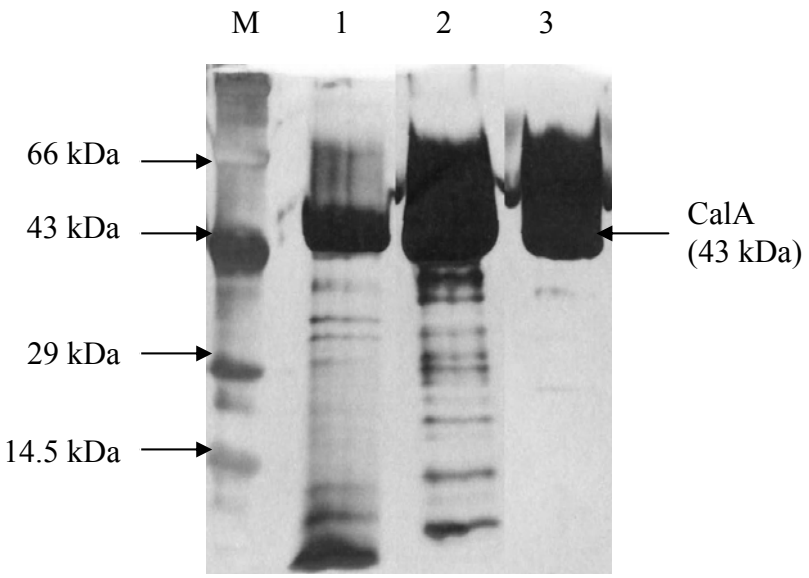


Figure 3: Silver-stained SDS-PAGE of lipase A samples (M: marker, lane 1: supernatant, lane 2: concentrated supernatant after cross-flow-filtration, lane 3: purified lipase A after cation-exchange chromatography).

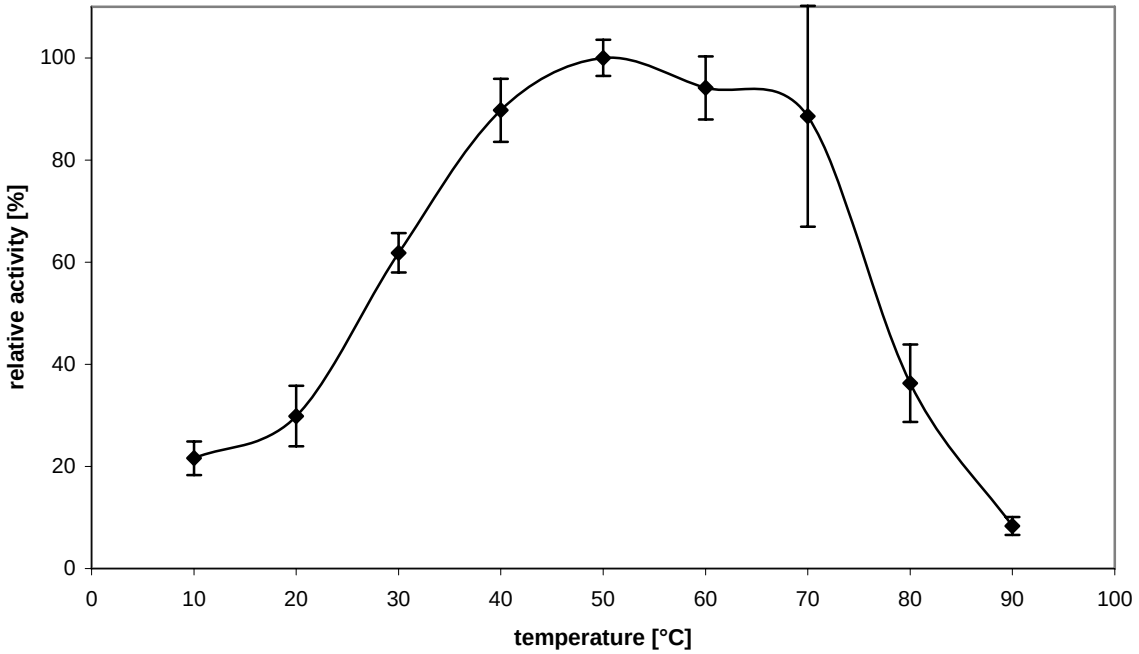


Figure 4: Temperature profile of lipase A activity. Activity at 50 °C is set as 100 % (4,275 U ml^{-1}). All measurements were repeated three times at pH 7.

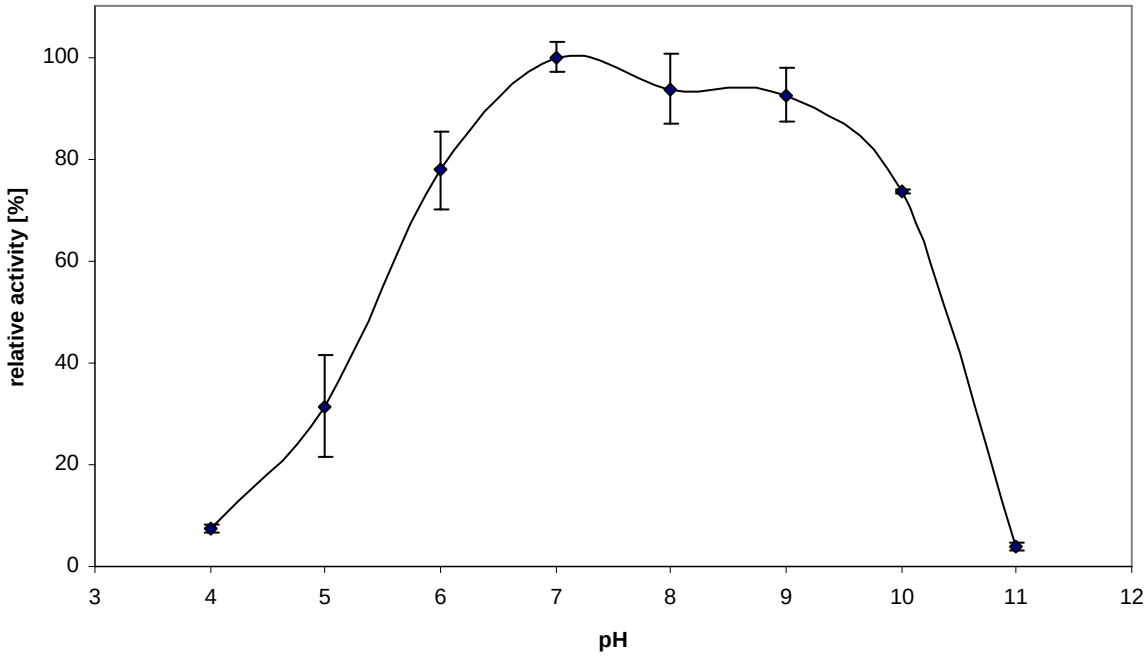


Figure 5: pH profile of CalA activity. The activity at pH 7 is set as 100 % (4,091 U/ml). All measurements were repeated three times at 30 °C.

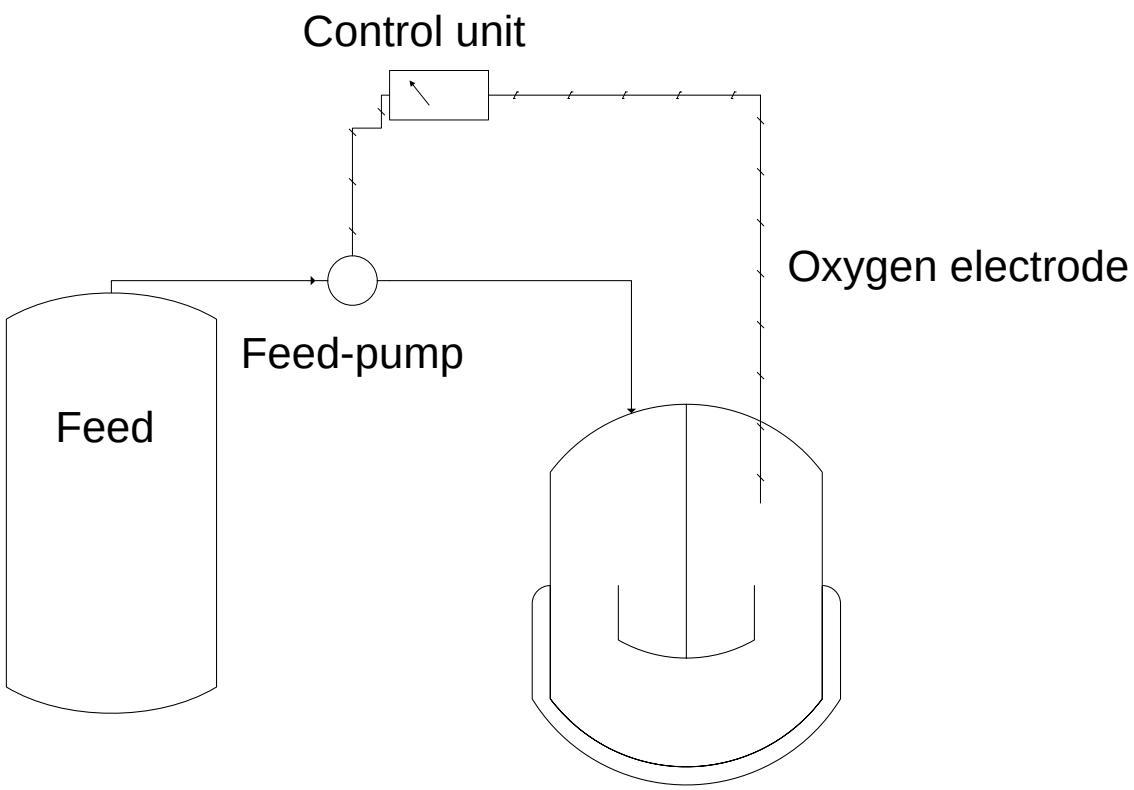
Table 1: Overview of the results obtained with different lipase A expression strategies of and the subsequent purification of the enzyme.

	Shake-flasks	**Fermentation I (fed-batch)**	**Fermentation II (semi-continuous)**
Processing-time [days]	5	12	15
Protein concentration (supernatant) [g/ l^{-1}]	0..6	3..1	3.0
Specific activity (supernatant) [U/ mg^{-1}]	215	653	380
Specific activity (after purification) [U /mg^{-1}]	-	2033	-
Purification factor	-	3.1	-
Total activity [U]	12,900	7,530,000	10,233,000

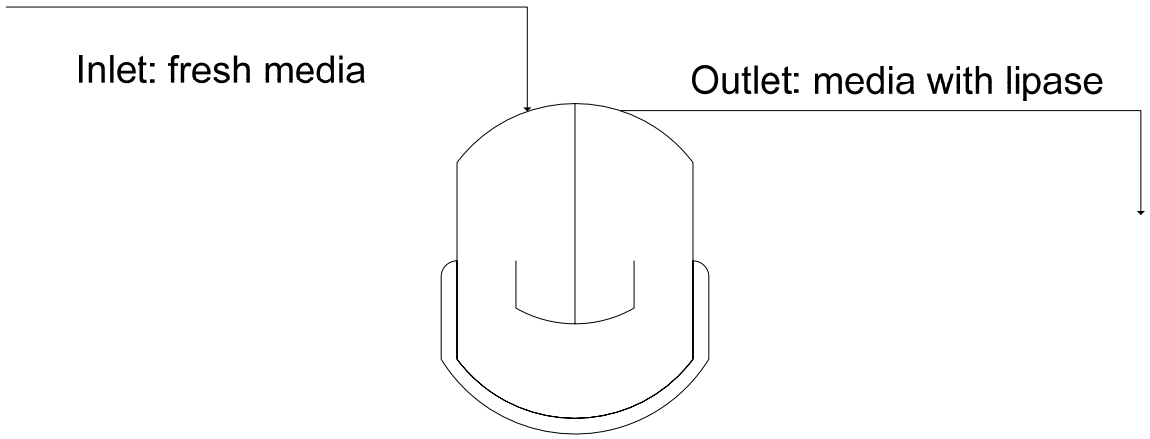
Table 2: Substrate spectrum of lipase A at pH 7 and 50 °C. 25 µl (28 µg) of purified enzyme solution was added to start the reaction. All measurements were repeated times.

Substrate	**Specific activity of purified enzyme [U mg^{-1}]**	**Relative activity of purified enzyme [%]**
Tributyrin (C4)	2,742	100
Tricaproin (C6)	2,149	78
Tricaprylin (C8)	1,369	50
Tripalmitin (C16)	0	0
Triolein (C18)	222	8
Olive oil	373	14
Vinylacetate (C2)	608	22
Methylbutyrate (C4)	164	6
Methylcapronate (C6)	78	3
Methylcaprylate (C8)	83	3
Methyldecanoate (C10)	101	4

Appendix: The priciple of the two fermentation strategies



Principle of the fed-batch process: If the solved oxygen in the broth reaches an adjusted value (all methanol is metabolized) the electrode gives a signal to the control unit which activated the feed-pump and fresh feed (methanol) is added to the bioreactor.



Principle of the semi-continuous process (repeated fed-batch process): Every 24 hours 500 ml of media with lipase are harvested via the outlet. Afterwards 500 ml of fresh prepared media is added. So the concentration of possible toxic compounds (from cell lysis) is kept on a low level

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5.2 Functional expression of Lipase A from *Candida antarctica* in *E. coli* – a prerequisite for high-throughput screening and directed evolution

Jan Pfeffer [1], Monika Rusnak [1], Carl-Erik Hansen [2], Rachid Bel Rhlid [2], Rolf D. Schmid [1], and Steffen C. Maurer [1] *

[1] ***Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany***

[2] ***Nestlé Research Center Lausanne, Nestec Ltd., Vers-Chez-Les-Blanc, 100 Lausanne 26, Switzerland***

*** Author for correspondence (Fax: (+49)-711-685-3196; E-mail: steffen.maurer@itb.uni-stuttgart.de)**

Key words:

Lipase, *Candida antarctica*, functional expression, *E. coli*, chaperone co-expression

5.2.1 Abstract

We report for the first time the functional and heterologous expression of Lipase A from *Candida antartica* (CalA) in the cytoplasm of *Escherichia coli* Origami^{TM} B cells. Expression under control of the *lac* promoter in the pUC18 vector yielded 0.7 U mg^{-1} lipase activity, whereas expression of a thioredoxin-CalA fusion protein using the pET-32b(+) vector yielded 1.7 U mg^{-1}. The native enzyme was most efficiently expressed under control of the *csp*A promoter (9.63 U mg^{-1}) using the pColdIII vector.

Co-expression of various chaperones led to a significant increase in formation of active protein (up to 13.1 U mg^{-1}).

This expression strategy was validated in microtitre plates and therefore is suitable for high-throughput screening of large gene libraries and applications including directed evolution of CalA.

5.2.2 Introduction

Lipases [triacylglycerol-hydrolases (EC 3.1.1.3)] play an important role in several industrial applications. They catalyse the hydrolysis of triacylglycerols at the interface between water and the hydrophobic substrate. Besides the hydrolysis of triacylglycerols, lipases also catalyse the enantio- and regioselective hydrolysis or synthesis of a wide range of natural and unnatural esters [1-3]. Especially lipases from microorganisms received a lot of interest because they are useful catalysts for many industrial applications such as ester synthesis [4,5], optical resolution [6,7], transesterification [8] or washing processes [9].

From *Candida antarctica* two lipases were isolated: lipase A and B [10]. Lipase B shows high enantioselectivity against secondary alcohols and due to its high stability in organic solvents and at high temperature it is one of the most frequently used enzymes for industrial applications nowadays [11].

Lipase A is reported to be a thermostable lipase [12] and thus could be interesting for industrial use. Moreover, CalA is described to be the only lipase displaying a preference for position *sn*-2 of triglycerides [13] opening a wide range of possible applications in synthesis of structured triglycerides. Recently CalA was expressed in high yields in the methylotrophic yeast *Pichia pastoris*, purified and generally characterised [14].

Furthermore the enzyme was found to exhibit high activity towards sterically hindered alcohols, including both secondary and tertiary alcohols [15] and hydrolyses a range of tertiary-butyl esters of protected amino acids [16]. These results indicate that CalA could be applied for the conversion of highly branched and bulky substrates where most other lipases fail to display any activity. In the patent literature lipase A was claimed to be able to esterify tertiary alcohols. Unfortunately the protein structure of CalA is not solved yet and thus rational protein design is impossible. Consequently the only way to tailor the enzyme is "directed evolution" [17] which requires functional expression in a fast-growing and easy-to-handle organism.

Up to now CalA has not been functionally expressed in *E. coli*, the standard expression system for high-throughput screening of large mutant libraries.

Expression of eukaryotic proteins in *E. coli* is challenging as the cellular environment, folding machinery and conformational quality-control checkpoints of prokaryotes are different from those of eukaryotes [18]. Generally expression problems can occur during transcription of the encoding gene, translation of the mRNA, protein folding and posttranslational modification e.g. formation of disulfide bonds. Misfolding often leads to proteins displaying hydrophobic patches on the surface, thus favouring aggregation and insolubility of proteins. There are

several methods to improve the solubility of proteins expressed in *E. coli*: domain optimisation [19], optimisation of translation initiation regions [20], introduction of solubilising amino acids [21] or co-expression of chaperones as reported in this work.
A recently published review on CalA [22] documented the increasing interest this unique biocatalyst attracts and the multitude of applications the enzyme can be used for. Our work represents the basis for optimisation of CalA by directed evolution aiming to improve regioselectivity and to enhance the substrate spectra or other properties of the enzyme. In this context we summarised the use of different expression strategies and the impact of co-expressed chaperones.

5.2.3 Material and methods

Cloning of the lipase gene

The yeast *Candida antarctica* (DSM 70725) was cultivated in Universal Medium For Yeasts (DSMZ) (Yeast extract 3 g l^{-1}, Malt extract 3 g l^{-1}, Peptone from soybeans 5 g l^{-1}, Glucose 10 g l^{-1}) at 30 °C and 180 rpm. After cultivation for 3 days the cells were harvested and disrupted with glass-beads (0.75 – 1.00 mm) using a Retsche-mill for 30 minutes. Cellular proteins were precipitated with SDS (2 % vol.) at 65 °C. After centrifugation the genomic DNA was precipitated with 3 M Na-acetate and 2-propanol and finally resuspended in 100 µl TE (Tris 100 mM, EDTA 10 mM) supplemented with RNase (0.1 % vol.).
The lipase gene was amplified by PCR using DyNAzyme™ EXT DNA Polymerase (Finnzymes) and primers containing the restriction sites for *Eco*RI and *Not*I for cloning into pUC18 (MBI Fermentas, St. Leon-Rot, Germany), *Eco*RI and *Not*I for cloning into pET-32b(+) (Novagen, Darmstadt, Germany) and *Nde*I and *Eco*RI for cloning into pColdIII (Takara, Otsu, Japan). The PCR-products were cloned into the corresponding vector using standard procedures and *E. coli* Origami™ B or Origami™2(DE3) (Novagen, Darmstadt, Germany) cells were transformed with the construct. Table 1, 2 and 3 give an overview of the strains, plasmids and primers used.

CalA expression and co-expression of chaperones

pUC18 expression:

Origami^{TM} B cells were transformed with CalA-pUC18 constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 µg ml^{-1}) up to an optical density OD_{600} of 0.4 – 0.6 at 180 rpm and 37 °C. The expression of the lipase was induced by adding IPTG (final concentration 1 mM). Cells were grown for additional 24 hours at 180 rpm and 30 °C and were harvested by centrifugation.

pET-32b(+) expression:

Origami^{TM} 2(DE3) cells were transformed with CalA-pET-32b(+) constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 µg ml^{-1}) up to an optical density OD_{600} of 0.4 – 0.6 at 180 rpm and 37 °C. The expression at 30 and 15 °C, induction and harvest were done as described before.

pColdIII expression:

Origami^{TM} B cells were transformed with CalA-pColdIII constructs. *E. coli* cells were grown in 100 ml LB media containing ampicillin (final concentration 100 µg ml^{-1}) up to an optical density OD_{600} of 0.4 – 0.6 at 180 rpm and 37 °C. The expression at 15 °C, induction and harvest were done as described before.

Co-expression of chaperones using pET-32b(+) and pColdIII constructs:

CalA-pET-32b(+) and CalA-pColdIII clones were co-transformed with chaperone plasmids from the Takara Kit and selected on LB agar containing ampicillin (final concentration 100 µg ml^{-1}) and chloramphenicol (final concentration 34 µg ml^{-1}).

For expression, cells were grown in LB media containing the corresponding antibiotics and 1 mg ml^{-1} L-arabinose (in case of pGro7, pKJE7 and pTf16), 5 ng ml^{-1} tetracycline (in case of pG-Tf2) or L-arabinose and tetracycline (in case of pG-KJE8) in concentrations given above at 180 rpm and 37 °C up to an optical density OD_{600} of 0.4 – 0.6. Lipase expression was induced by adding IPTG (final concentration 1 mM). Cells were grown for additional 24 hours at 180 rpm and 37 °C (pUC18), 30 °C (pUC18, pET-32b(+)) or 15 °C (pET-32b(+), pColdIII). Afterwards they were harvested by centrifugation.

All expression experiments were done three to fourfold; therefore all published data are mean values.

Cell disruption, pH-stat assay, SDS-PAGE and densitometric analysis

Cell disruption was performed using three times 30 seconds sonification in 50 mM sodium phosphate buffer pH 7.5. The cell debris (insoluble fraction) was removed by centrifugation at 20,000 *g* for one hour.

Lipase activity was determined using a pH-stat application with tributyrine as substrate at 50 °C (pH 7.5). Tributyrine (5 % (v/v)) was emulsified in distilled water containing 5 % (w/v) gum Arabic as stabiliser using a homogeniser for 7 minutes at maximum speed. Twenty mililiters of the substrate were heated to the reaction temperature and the pH was adjusted using 0.01 M NaOH. Liberated fatty acids were titrated automatically with 0.01 M NaOH to maintain a constant pH. One unit (U) of lipase activity is defined as the amount of lipase that liberates 1 µmol fatty acids per minute.

For protein-concentration determination, the Bradford-assay (Bio-Rad, Muenchen Germany) according to the instructors´ manual was used [23].

For determination of the amount of solubly and insolubly expressed lipase, the supernatant and the pellet fraction after cell disruption were analysed using SDS-PAGE on a 12.5 % separation gel under the conditions developed by Laemmli [24]. A Minigel-Twin cell (Whatman, Biometra, Goettingen, Germany) was used for electrophoresis. Molecular weight marker was purchased from Amersham Biosciences. After electrophoresis the gels were stained using Coomassie Brilliant blue.

The percentage of soluble and insoluble CalA on the total cell protein was measured densitometrically using the software “Scion Image”.

Tributyrin agar plate assay

For this assay agar plates containing 1 % emulsified tributyrin and the appropriate antibiotics were used. After cell growth for 24 hours at 37 °C, the agar plates were covered with soft agar (0.6 % agar in water) containing 1 mM IPTG and incubated at 30 °C for expression. Expression of functional lipase was indicated by formation of halos around the colonies.

5.3.4 Results and discussion

In this publication we compare the activity and solubility of lipase CalA heterologously expressed in *E. coli* using different expression strategies.

Cloning of calA gene

The *calA* gene dispensed from the N-terminal pre-pro-peptide sequence [14] was amplified from genomic *Candida antarctica* DNA using the appropriate primers (table 1) and afterwards cloned into *E. coli* expression vectors (table 2). Successful cloning was confirmed by sequencing [25] of the isolated plasmids.

Lipase expression using three different vector systems

CalA has not been functionally expressed in *E. coli* so far. Two *E. coli* strains (table 3) were used for expression experiments: *E. coli* Origami^{TM} B and Origami^{TM} 2(DE3). Common to both strains is the deficiency of their thioredoxin and glutathione reductase genes. These deficiencies are reported to enhance disulfide bond formation in the *E. coli* cytoplasm [26].

DH5α cells transformed with *pUC18-calA* did not show any halo formation on tributyrin agar plates, but Origami^{TM} B cells transformed with the same plasmid showed halo formation. The CalA activity in the Origami^{TM} B lysate was very weak; the hydrolysis activity against tributyrin was about 0.7 U mg^{-1} of total soluble protein (figure 1). The fact that *pUC18-calA* showed halo formation in Origami^{TM} B cells, but not in DH5α cells indicates that the formation of correct disulfide bonds is a major bottleneck in functional expression of CalA in the *E. coli* cytoplasm.

In SDS-PAGE analysis, the corresponding band of CalA (43 kDa) was not detectable in the soluble fraction, while the CalA content in the insoluble fraction was 13 % of whole insoluble cell proteins (data not shown).

To enhance the yield of active enzyme, the *calA* gene was fused to a thioredoxin tag (Trx·Tag^{TM}) using the vector pET-32b(+) and expressed in *E. coli* Origami^{TM}2(DE3). Cultivation at 37 °C did not result in any detectable lipase activity using the pH stat assay (data not shown). Expression of Trx-CalA at 30 °C yielded 0.6 U mg^{-1} lipase activity in the cleared cell lysate (figure 1). This expression protocol resulted in a strong overexpression of CalA. The insoluble fraction consisted of 25 % CalA. This fits with the general observation that high expression levels can lead to an increase in the proportion of incorrectly folded protein. Finally expression of the *Trx-calA* construct at 15 °C yielded 1.7 U mg^{-1} (figure 1) and 22 % CalA content in the insoluble fraction (table 4). A high yield expression protocol for

functional lipases in *E. coli* cells using the T7 *lac* promoter has recently been reported [27,28]. However, in these expression experiments, most of the target proteins were prokaryotic enzymes with no need for folding assistance and posttranslational modifications as required by most eukaryotic enzymes. By fusion to the extremely soluble TrxA protein several eukaryotic enzymes, e.g. mammalian cytokines and growth factors were functionally expressed in the *E. coli* cytoplasm [29]. Using both, the T7 *lac* promoter and the *trxA* fusion tag provided in the vector system pET-32b(+), the murine interleukin-2 (IL-2) was expressed highly soluble in *E. coli* [30].

A further increase of functional CalA expression level to 9.6 U mg^{-1} was achieved using the temperature-inducible pColdIII vector system (figure 1). Nevertheless, analysis by SDS-PAGE and densitometry still showed a high amount of insoluble CalA (18.5 %) (table 4).

Decreasing the expression temperature increased the yield of functional enzyme slightly in the case of pET32-b(+) and strongly in the pColdIII system. The positive influence of decreased expression temperature on the yield of functionally expressed enzyme was reported previously [31,32]. This dependency of the yield of correctly folded enzyme on the expression temperature can be theoretically explained: Whereas folding rate is only slightly decreased by a temperature switch from e.g. 37 to 15 °C, the rate of transcription and translation in *E. coli* is significantly reduced. These facts provide sufficient time for protein refolding, yielding active enzyme and avoiding the formation of insoluble aggregates of misfolded protein [33].

The fusion of an enzyme to a tag, like TrxA can lead to changes in catalytic properties [34]. This may explain the fact that compared to the pColdIII results, the activity of the enzymes expressed in the pET-32b(+) clones are low. Therefore screening of mutants carrying a TrxA tag would not be suitable for the detection of improved variants of the native CalA. From this point of view the use of the pColdIII system seems to be more favourable than utilising pET-32b(+). Moreover, expression using pColdIII led to higher amounts of soluble CalA indicating a positive effect of the cold-responsive promoter *cspA* on efficient gene expression at reduced temperatures as already reported [35].

Functional lipase expression using co-expression of molecular chaperones

A strong increase in soluble heterologous protein concentration in the *E. coli* cytoplasm by co-expression of chaperones has already been reported [36-38]. The pColdIII and the pET-32b(+) constructs both were co-expressed with several combinations of chaperones from the TaKaRa Chaperone Plasmid Set (table 2) [39]. The specific activity (figure 1) and the amount of soluble protein (table 4, figure 2 and 3) expressed by the various constructs differed

significantly. Increased yield of active enzyme by co-expression of different chaperones was reported recently [36-38].

CalA was most efficiently expressed in the pColdIII vector with co-expression of pGro7 resulting in 13.1 U mg^{-1}. Generally expression from the pColdIII constructs resulted in higher specific activity than expression from the pET-32b(+) constructs. The specific activity of the pET-32b(+) constructs expressed at 30 °C was lower than 1 U mg^{-1}. Enzyme expression at 15 °C increased the specific activity significantly, still activity was lower than with the pColdIII constructs. Also among the pET-32b(+) constructs, the best activity value was reached co-expressing pGro7 (3.4 U mg^{-1}) (figure 1).

Co-expression using the pGro7 construct surpassed the specific activity of the expression of CalA-pColdIII without chaperone. All other expression experiments using co-expression of chaperones yielded lower activity (figure 1).

Generally co-expression of chaperones increased the amount of soluble CalA (10 – 22 % of total soluble protein) compared to expression without chaperones (0.22 – 0.35 % of total soluble protein) (table 4, figure 2 and 3).

With both expression systems CalA was most efficiently expressed when co-expressing Gro7, indicating strong impact of the GroES/GroEL chaperone system on the correct folding of CalA. It is known that these molecular chaperones play an important role in folding α/β structures enriched in hydrophobic and basic residues. This is underlined by the fact, that the molecular mass of CalA (43 kDa) fits with the size of GroES/GroEL most preferred substrates (proteins < 60 kDa) [18]. Recently a lipase with the molecular weight of 33 kDa was also functionally expressed in *E. coli* using co-expression of Gro7 [40]. In combination with *trxB gor* suppressor cells (e.g. Origami™) the yield of properly disulfide-bonded proteins was increased by the co-expression of these folding modulators [41-43]. Co-expression of the trigger factor (encoded by pG-Tf2 and pTf16) did not show any positive effect in functional enzyme expression and co-expression of DnaK-DnaJ-GrpE showed only a slight increase of functional CalA expression. These results fit to the observation that a positive influence of the DnaK-DnaJ system is mostly restricted to target proteins with higher molecular weight than 60 kDa [44].

We reported the first expression system for soluble and active CalA variants in a prokaryotic host. This is a prerequisite for a high-throughput-screening system for CalA. The *E. coli* system has some major advantages compared to other high throughput systems like for example *S. cerevisiae*. Besides lower costs and easier handling, the total amount of clones that can be screened daily is much higher in *E. coli*. The total cultivation period in microtiter

plates is 48 hours, while the *S. cerevisiae* system needs a 9 day period. Therefore the described *E. coli* expression system is a very important achievement for directed evolution of CalA. The method described here is a useful approach for the expression of proteins which up-to-now failed to be functionally expressed in *E. coli*.

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Table 1: Primers used for the amplification of the calA gene

Primer	Sequence (5´ - 3´)	Function
CalA_EcoRI-fw	ccggaattcggcggcgctgcccaacccc	Cloning into pUC18 and pET-32b(+)
CalA-NotI-r	ttttccttttgcggccgcctaaggtggtgtgatggggc	Cloning into pET-32b(+)
CalA_Nde1-r	gggaattccatatgctaaggtggtgtgatggg	Cloning into pUC18
CalA_pColdIII_Nde1-fw	gggaattccatatggcggcgctgcccaacccc	Cloning pColdIII
CalA_pColdIII_EcoRI-r	ccggaattcctaaggtggtgtgatggg	Cloning pColdIII

Table 2: Plasmids used for the expression and co-expression

Plasmid	Gene of interest	Promoter	Inducer	Ori	Resistance marker	Supplier
pUC18/calA	calA	Lac	IPTG	pBR322	ampicillin	MBI Fermentas
pColdIII/calA	calA	cspA	Cold shock + IPTG	ColE1	ampicillin	TaKaRa
pET32-b(+)/calA	Trx-calA	T7	IPTG	pBR322	ampicillin	Novagen,
pKJE7	dnaK-dnaJ-grpE	araB	L-arabinose	pACYC	chloramphenicol	TaKaRa,
pG-KJE8	dnaK-dnaJ-grpE groES-groEL	araB Pzt1	L-arabinose tetracyclin	pACYC	chloramphenicol	TaKaRa,
pGro7	groES-groEL	araB	L-arabinose	pACYC	chloramphenicol	TaKaRa,
pG-Tf2	groES-groEL-tig	Pzt1	tetracycline	pACYC	chloramphenicol	TaKaRa,
pTf16	tig	araB	L-arabinose	pACYC	chloramphenicol	TaKaRa,

Table 3: Strains used for the expression experiments

Strains	Genotype	Reference
DH5α	supE44 ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 end A1 gyrA96 thi1relA1	Clontech (Heidelberg, Germany)
Origami™ B	Δara-leu7697 ΔlacX74 ΔphoAPvull phoR araD139 ahpC galE galK rpsL F´[lac^+ ($lacI^q$)pro] gor522::Tn10 (Tc^R) trxB::kan	Novagen 2004
Origami™ 2(DE3)	Δ(ara-leu)7697 ΔlacX74 ΔphoA Pvull phoR araD139 ahpC gale galK rpsL F´[lac^+ ($lacI^q$)pro] (DE3) gor522::Tn10 trxB (StrR, TetR)	Novagen 2004

Table 4: Densitometric analysis of CalA contents in the soluble and insoluble fractions of cell extracts

Clone	Soluble [%]	Insoluble [%]
pColdIII/calA	0,22	8,5
pColdIII/calA+pKJE7	19,2	22,8
pColdIII/calA+pKJE8	14,4	17,8
pColdIII/calA+pGro7	22,4	18,5
pColdIII/calA+pG-Tf2	17,2	17,8
pColdIII/calA+pTf16	14,8	22,7
pET32-b(+)/calA	0,35	22,3
pET32-b(+)/calA+pKJE7	12,1	9,4
pET32-b(+)/calA+pKJE8	17,8	12,9
pET32-b(+)/calA+pGro7	18,9	19,7
pET32-b(+)/calA+pG-Tf2	11,7	11,3
pET32-b(+)/calA+pTf16	10,3	9,3

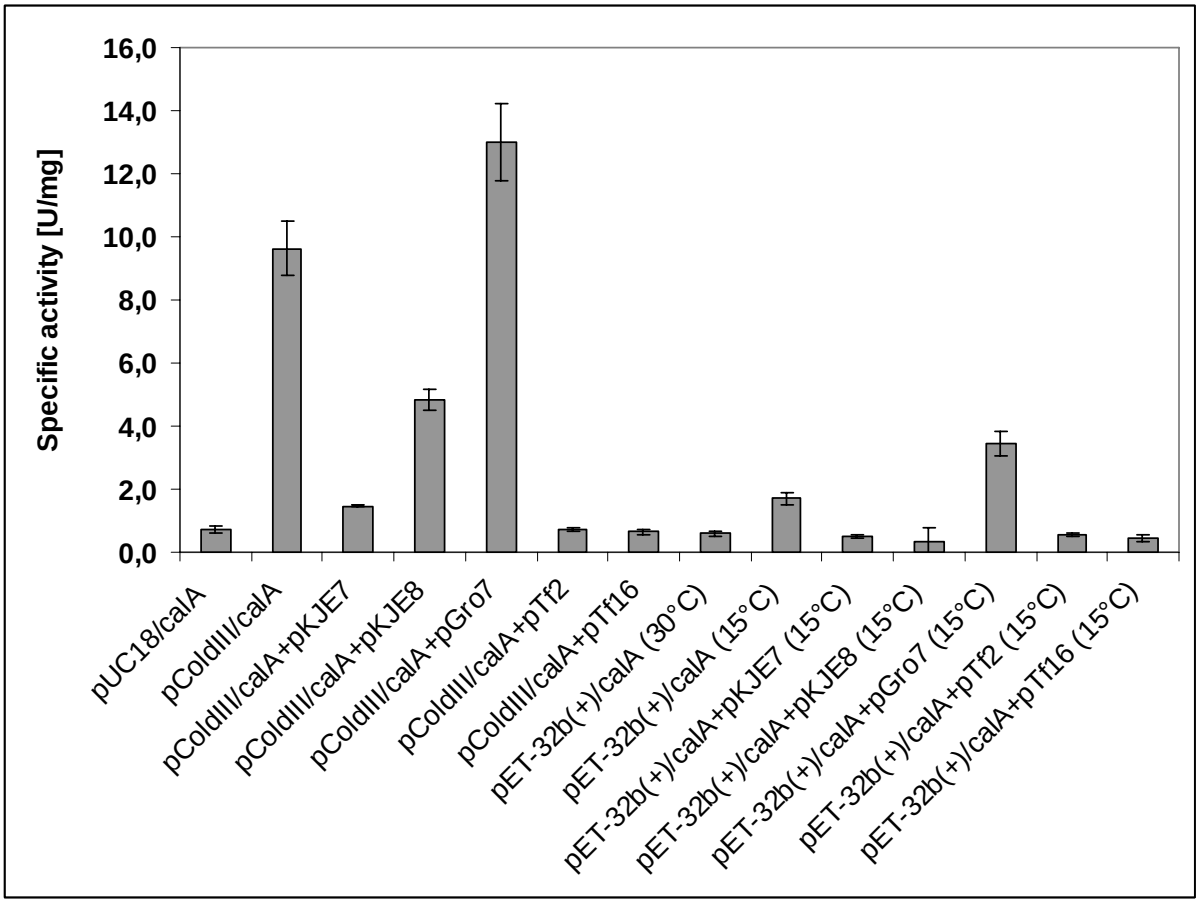

Figure 1: Specific lipase activity yielded in the various expression experiments. The hydrolytic activity was determined using the pH stat assay with the cleared cell lysates from *E. coli* Origami B cells (pUC18 and pColdIII constructs) and Origami 2(DE3) (pET32-b(+) constructs). The error bars show the standard deviation of three independent experiments.

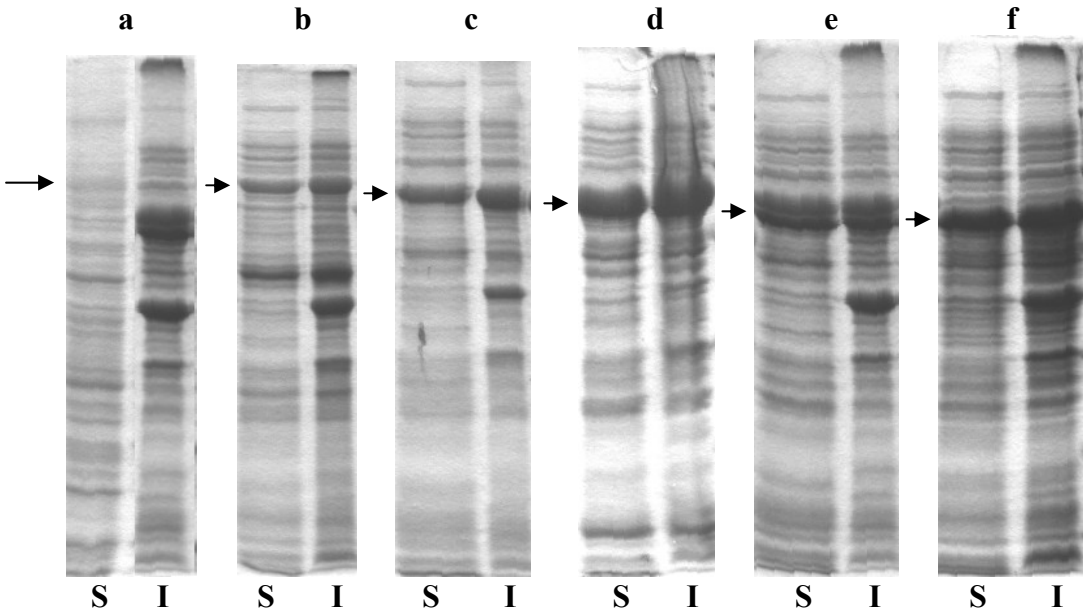

Figure 2: SDS PAGE analysis of soluble (S) and insoluble (I) fractions obtained from various CalA expression experiments at 15 °C using pColdIII constructs in Origami B cells. The CalA bands (43 kDa) are marked with arrows. Abbreviations for constructs: a: pColdIII/calA (without chaperones), b: pColdIII/calA+pKJE7, c: pColdIII/calA+pKJE8, d: pColdIII/calA+pGro7, e: pColdIII/calA+pG-Tf2, f: pColdIII/calA+pG-Tf16

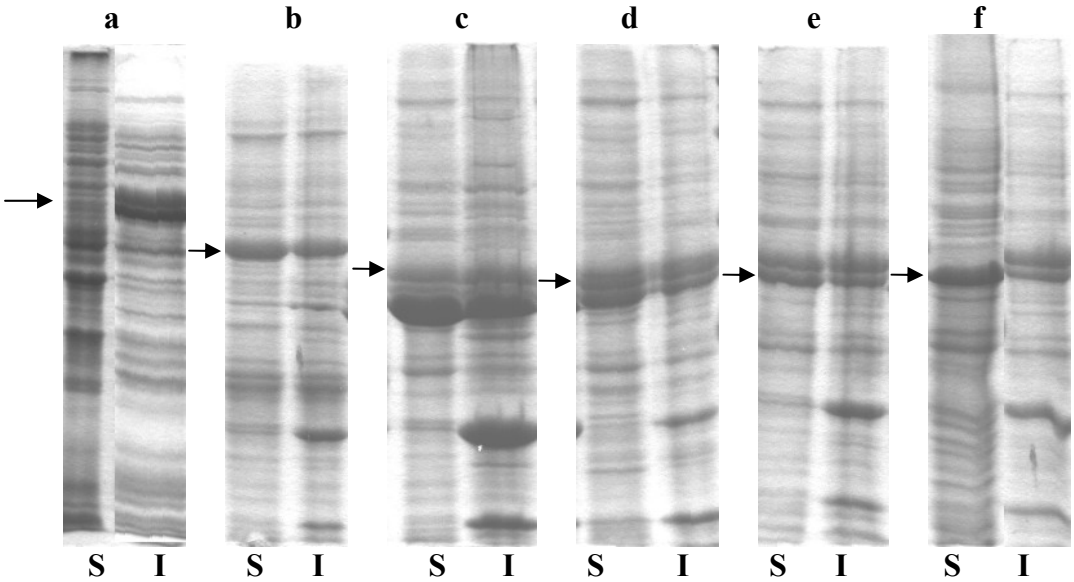

Figure 3: SDS PAGE analysis of soluble (S) and insoluble (I) fractions obtained from various CalA expression experiments at 15 °C using pET-32b(+) constructs in Origami 2(DE3) cells. The Trx-CalA fusion protein bands (55 kDa) are marked with arrows. Abbreviations for constructs: a: pET-32b(+)/calA (without chaperones), b: pET-32b(+)/calA+pKJE7, c: pET-32b(+)/calA+pKJE8, d: pET-32b(+)/calA+pGro7, e: pET-32b(+)/calA+pG-Tf2, f: pET-32b(+)/calA+pG-Tf16. The first SDS PAGE was performed under different conditions than the five others; therefore the Trx-CalA band is shifted to the top.

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5.3 Highly Efficient Enzymatic Synthesis of 2-Monoacylglycerides and Structured Lipids and their Production on a Technical Scale

Jan Pfeffer[1], Andreas Freund[2], Rachid Bel-Rhlid[3], Carl-Erik Hansen[3], Matthias Reuss[2], Rolf D. Schmid[1] and Steffen C. Maurer[1] *

[1] Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
[2] Institute of Biochemical Engineering, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany
[3] Nestlé Research Center, Lausanne, Nestec Ltd., Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland

*** Author for correspondence (Fax: (+49)-711-685-63196; Phone: (+49)-711-685-69985; E-mail: steffen.maurer@itb.uni-stuttgart.de)**

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2-monoacylglycerides, lipase, miniplant technology, OPO, polyunsaturated fatty acids (PUFA), up scaling

5.3.1 Abstract

We report here a two-step process for the high-yield enzymatic synthesis of 2-monoacylglycerides (2-MAG) of saturated as well as unsaturated fatty acids with different chain lengths.

The process consists of two steps: first the unselective esterification of fatty acids and glycerol leading to a triacylglyceride followed by an *sn*1,3-selective alcoholysis reaction yielding 2-monoacylglycerides. Remarkably, both steps can be catalyzed by lipase B from *Candida antarctica* (CalB). The whole process including esterification and alcoholysis was scaled up in a miniplant to a total volume of 10 litres. In this volume a two-step process catalyzed by CalB for the synthesis of 1,3-oleoyl-2-palmitoylglycerol (OPO) using tripalmitate as starting material was established.

In lab scale we obtained gram quantities of the synthesized 2-monoacylglycerides of polyunsaturated fatty acids like arachidonic-, docosahexaenoic- and eicosapentaenoic acid and up to 96.4 % of the theoretically possible yield with 95 % purity. In technical scale (>100 g of product, > 5 l of reaction volume), 97 % yield was reached in the esterification and 73 % in the alcoholysis and a new promising process for the enzymatic synthesis of OPO was established.

5.3.2 Introduction

Arachidonic acid (ARA or C20:4n-6), docosahexaenoic acid (DHA or C22:6n-3) and eicosapentaenoic acid (EPA or C20:5n-3) are polyunsaturated fatty acids (PUFA) involved in a wide range of biologically relevant functions and have strong effects on human health [1, 2]. ARA is an essential fatty acid in human nutrition and a major component of mature human milk and necessary for cognitive development of infants. ARA is also a precursor of biologically active prostaglandins and leucotrienes, involved in inflammatory processes [3-5]. DHA is important for the development of the central nervous system [3]. EPA shows significant effects in preventing heart diseases and lowering blood cholesterol level and thus reduces the risk of arteriosclerosis [6, 7]. The fatty acid distribution on the glycerol backbone influences the adsorption and tissue uptake of glycerides [8, 9]. Generally, 2-monoacylglycerides (2-MAG) are most readily absorbed through the intestinal mucosa and are also most rapidly absorbed among PUFA derivatives. 2-MAG have very good emulsifying properties and are physiologically essential molecules involved in lipid absorption. In vertebrates 2-MAG and not glycerol is preferentially utilised for triacylglycerol and phosphatidylcholine biosynthesis [10]. Up to now these molecules have only limited industrial applications because of difficulties in their synthesis. The chemical synthesis takes several steps, a costly purification and often results in very low yields [11]. Enzymatic synthesis of 2-MAG usually depends on two types of lipases: an unspecific enzyme for the esterification and an *sn*1,3-specific lipase for the alcoholysis (usually ethanolysis) reaction to the 2-MAG.

The type of fatty acid in the 1,3-position of triglycerides [12] also influences the intestinal adsorption as 2-MAG after the TAG are regiospecifically hydrolyzed in mouth, stomach and small intestine. Symmetrically structured triglycerides incorporate the same fatty acids in *sn*1 and *sn*3 position (ABA type). ABA with medium chain fatty acid groups (C_6-C_{10}) in the outer positions and a PUFA in the middle position have excellent dietary and absorption characteristics. Additionally the PUFA residue is protected against oxidation by the two saturated fatty acid residues protecting the more oxidizable PUFA in the middle position from oxygen (sterical reasons). Several experiment by Endo and colleagues proofed that 1,2-dipalmitoyl-3-PUFA-glycerol is oxidised much more readily than 1,3-dipalmitoyl-2-PUFA-glycerol [13, 14]. The medium-chain fatty acids are easily hydrolyzed in the gastrointestinal tract by the pancreatic lipase. Resulting 2-MAG are absorbed readily and used either as a high-energy resource or for several health-protecting actions [8, 15, 16].

Fish oil especially tuna and salmon oils are well-known and inexpensive natural sources of PUFAs. A large variety of health products are made of or contain fish oil-derived compounds, e.g. the encapsulation of these oils in gelatine as a health food supplement interesting in the prevention and treatment of cardiovascular diseases, neurodegenerative disorders and cancer [9, 17].

1,3-oleoyl-2-palmitoylglycerol (OPO) is an important ABA-type TAG in infant nutrition. Human milk fats contain palmitic acid predominantly in the *sn*2-position of TAG. Often infant formulas contain palmitic acid in *sn*1,3-positions, which leads to the formation of calcium soaps after their release. These soaps are only poorly absorbed by the intestine, which results in indigestion and loss of calcium [18].

We developed a two-step process leading to 2-monoacylglycerides which is enzymatically catalyzed by lipases. Lipases [triacylglycerol-hydrolases (EC 3.1.1.3)] catalyze the hydrolysis of triacylglycerols at the interface between water and the hydrophobic substrate. Besides the hydrolysis of triacylglycerols, lipases also catalyze the enantio- and regioselective hydrolysis or synthesis of a wide range of natural and unnatural esters [19, 20]. Especially lipases from microorganisms received a lot of interest because they are useful catalysts for many industrial applications [21, 22] such as ester synthesis, optical resolution [23-25], transesterification or washing processes [26]. In the process described here the same lipase was used for both steps - the esterification as well as the following alcoholysis: Lipase B from *Candida antarctica* (CalB) [27]. CalB reveals high enantioselectivity against secondary alcohols and, due to its extraordinary stability in organic solvents and at high temperature, has become one of the most frequently used enzymes in industrial applications [28]. For hydrolysis or transesterification of TAG, CalB is classified as an *sn*1,3-specific lipase. On the other side it is known that in esterification CalB forms homogeneous TAG (AAA type). Starting from this knowledge our strategy was first to synthesize a homogeneous TAG of PUFAs followed by the conversion to the desired 2-MAG. In the present work we synthesized the 2-MAG of three different PUFA: ARA, DHA and EPA all in gram quantities and high purities. We also showed that 2-MAG of short and medium chain length can be synthesized by the same method.

After this process was successfully established in lab scale it was transferred and scaled up to a miniplant (total reaction volume up to 10 litres). A miniplant is a minimized production line involving all processing steps including downstream-processing regarding different parameters like temperature, pH value and mass transfer (glycerol/hexane mixture).

For the synthesis of OPO a CalB-driven two-step miniplant process was established. Starting with tripalmitate, the substrate was first converted to the corresponding 2-monopalmitate in an alcoholysis reaction followed by an esterification with oleic acid. Again only CalB was used as biocatalyst.

5.3.3 Material and methods

Lipases and Chemicals

Lipase CalB (Novozym 435) was from Novozymes (Bagsvaerd, Denmark). The lipase gene was derived from *Candida antarctica* and transferred to the host organism *Aspergillus oryzae* in which the lipase was expressed. The purified lipase was immobilized on a macroporous acrylic resin. All lipases were purchased from Fluka. Immobilized CalB was removed after the reaction by filtration, washed three times, dried in an desiccator and afterwards could be used again. All chemicals except the PUFAs and solvents used were of analytical reagent grade and purchased from common suppliers (Sigma, Fluka and Riedel de Haën). PUFAs were supplied by Nu-Chek Prep (Elysian, USA). References of MAG, DAG and TAG for GC/MS analysis were also supplied by Nu-Chek Prep.

Esterification Reaction

Purified 2-MAG (100 mg, obtained from alcoholysis reactions) or glycerol and fatty acids (molar ratio 1:3) [29] were dissolved in 20 ml *n*-hexane. The water which is generated during the reaction was removed by addition of 200 mg activated molecular sieve (pore diameter 3 Å, bead diameter ~2 mm, UOP Type 3A, Fluka, Buchs, Switzerland). The mixture was stirred magnetically (level 10 on a RCTbasic, IKAlabortechnik, Staufen, Germany) and incubated at 50 °C. The reaction was started by adding 100 mg immobilized lipase (CalB). After 24 hours the reaction was stopped by removing the lipase (figure 1) as described above.

Alcoholysis Reaction of Triglycerides

The homogeneous TAG of PUFA or other TAG (100 mg) were mixed with pure ethanol (molar ratio TAG:ethanol is 1:4) and emulsified for 30 minutes at 25 °C. The reaction was started by adding CalB (100 mg) and stopped after 4-5 hours by filtration of the catalyst (figure 1). The reaction process was monitored using TLC.

Reactions on a Technical Scale (reaction volume: 8 litre)

The miniplant was built by the Institute of Biochemical Engineering (IBVT, University of Stuttgart, Germany) within a Bosch-Rexroth frame (UTZ Ratio Technik, Korb, Germany). Process control equipment and software was supplied by National Instruments Germany GmbH, München and National Instruments (Labview®, Austin, TX, USA), respectively. The enzyme reactions were performed in a 10 litre glass-vessel (Type BDAV, HWS Labortechnik, Mainz, Germany). The reaction mixture was stirred by air-driven stirrers (Gebr. Buddeberg GmbH, Mannheim, Germany). The temperature was controlled by a thermostat (F33, Julabo, Seelbach, Germany) [30].

The reactions in the miniplant were performed as batch processes under nitrogen atmosphere. The amount of chemicals and lipase was calculated to the working volume of the miniplant which was 8 litres.

Because of the high price of highly purified PUFA, erucic acid (EA or C22:1n-9), a cheaper, long-chain unsaturated fatty acid was used as a model substrate for the esterification with glycerol. 8 l *n*-hexane, 304 g erucic acid, 37 g glycerol, 60 g immobilized CalB and 500 g molecular sieves (pore diameter 3 Å, beads diameter ~2 mm, UOP Type 3A, Fluka, Buchs, Switzerland) were applied.

In the alcoholysis reaction triplamitate was converted to 2-monopalmitate. TAG and ethanol were emulsified in a molar ratio of 1:10 and then the vessel was filled up with acetone to the working volume (8 l acetone, 283 g tripalmitate, 161 g ethanol and 60 g immobilized CalB).

This product was afterwards esterified with oleic acid leading to OPO (8 l *n*-hexane, 84 g 2-monopalmitate, 260 g oleic acid, 60 g immobilized CalB and 500 g molecular sieves; figure 2).

Purification of 2-Monoacylglycerides and Triglycerides

For purification of 2-MAG of PUFA, the solvent was evaporated and the concentrate was dissolved in acetonitrile/water (95:5 v/v, 10-fold volume compared to the concentrate) and washed three times with the same volume of hexane for ethyl ester removal [31]. The pure 2-MAG are found in the acetonitrile/water phase.

2-Monopalmitate was purified via crystallization. After filtering off the catalyst, the excess solvent was evaporated and the residue was dissolved in *n*-hexane:methyl-*t*-butylether (MTBE) (70:30 v/v, 10-fold volume compared to the concentrate) and stored for 1 h at – 25 °C. After this period, the white crystals formed were collected by filtration at – 25 °C. The supernatant containing ethyl esters, fatty acids, and a small amount of diglycerides was

discarded. The 2-MP was recrystallized until the TLC plate showed only one band of 2-MP. The purity of 2-MP was confirmed by GC/MS analysis.

For purification of TAG, the solvent was evaporated and the TAG purified by silica gel column chromatography eluted with *n*-hexane/methyl-*t*-butylether (MTBE) (70:30 v/v). The TAG are eluted first followed by ethyl ester, fatty acids, di- and finally monoacylglycerides.

TLC Analysis

For rapid analysis during the process, thin-layer-chromatography (TLC) was performed using aluminium sheets with silica gel (Merck KGaA, Darmstadt, Germany) as stationary phase; *n*-hexane/MTBE (70:30 v/v) was the mobile phase. The TLC were developed in a staining solution (10 g cer(IV)-sulphate, 2 g molybdatophosphoric acid, 10 ml concentrate sulphuric acid, 100 ml water) for 30 seconds and afterwards dried with a heat gun. The spots were identified using the corresponding references.

GC/MS Analysis

For GC/MS analysis, samples were derivatized with 1 % of trimethylchlorosilane in N,O-bis(trimethylsilyl)trifluoroacetamide. The derivatization reagent converts all hydroxyl- and carboxyl groups to the corresponding trimethylsilyl ethers and -esters. After addition of the derivatization solution, samples were incubated for 30 minutes at 60 °C.

Products were identified on a Shimadzu GC/MS-QP2010 (Shimadzu Corporation, Kyoto, Japan) equipped with a 30 m FS-Supreme-5 column (5 % diphenyl polysiloxane / 95 % dimethyl polysiloxane, internal diameter 0.25 mm, film thickness 0.25 µM) using helium as carrier gas at linear velocity of 30 cm s^{-1}. Analysis of Mono-, Di- and Triglycerides was performed using the following program: 200 °C followed by heating (8 °C/min) to 360 °C. Using the GC/MS software GC/MS-solution® (Shimadzu Corporation, Kyoto, Japan) the amounts of substrate and products as well as the yield were calculated. For product identification the respective references were measured on the GC/MS and the reaction products compared in terms of retention time and mass spectra.

5.3.4 Results

Synthesis of PUFA 2-Monoacylglycerides

So far direct esterification of PUFA and glycerol or an adequate interesterification leading to 2-MAG of PUFA is impossible because there is no *sn*-2 specific lipase known. Therefore we developed a two-step process including esterification of glycerol and PUFA leading to TAG followed by ethanolysis resulting in the desired 2-MAG (figure 1). The potential of CalB for synthesis of triglycerides in an unspecific manner was already described [29]. A glycerol:PUFA ratio of 1:3 was found to be the optimum for the reaction. Excess of glycerol or fatty acids is inappropriate because of the production of large amounts of MAG and diacylglycerols (DAG) decreasing the yield of TAG.

For the *sn*1,3-specific alcoholysis several lipases classified as *sn*1,3-specific were tested: *Candida antarctica* B lipase (CalB), *Penicilium camembertii* lipase, *Pseudomonas fluorescens* lipase, *Rhizomucor mihei* lipase, *Rhizopus javanicus* lipase and *Rhizopus oryzae* lipase [32]. Only CalB was able to convert all three TAG of PUFA to the desired 2-MAG. The lipase of *Rhizopus oryzae* was able to convert the TAG of ARA to the 2-MAG, but failed in the case of TAG of DHA (DDD) and EPA (EEE). For most lipases the substrates seem to be too sterically demanding for their substrate-binding-site and therefore can not be converted. Interestingly CalB which acts in the first reaction as an unspecific catalyst is able to convert the substrates regioselectively to 2-MAG.

The alcoholysis reaction was very fast (figure 3). After 240 minutes the reaction was completed and almost no TAG was detected by TLC anymore. The reaction rate was constant from the beginning till the end of the reaction after 240 minutes. No DAG or free fatty acids were detectable. Fatty acid ethyl ester could be completely removed by liquid-liquid extraction. No isomerization of MAG was observed. TLC-analysis revealed a single spot of 2-MAG.

No acyl migration was observed when the alcoholysis reaction was performed in a temperature range from 25 to 30 °C. Temperatures higher than 40 °C led to increasing acyl migration and partial deacylation to glycerol.

The 2-MAG of the three model PUFA (ARA, DHA, EPA) were synthesized in gram quantity with purities higher than 85 % and yields up to 96 % (table 1).

The same process was proven to be usable for the synthesis of 2-MAG of short and middle-chain fatty acids like caprylic- (C8), palmitic- (C16) and oleic acid (C18:1). The corresponding 2-MAG were not purified as these studies were only performed to explore the

range of 2-MAG accessible by the technology described herein. So the substrate spectra of the 1,3-specific alcoholysis reaction with CalB covers all chain lengths from short to long and saturated or polyunsaturated.

Synthesis of Triglycerides in the Miniplant

For up scaling erucic acid was chosen as a cheap substrate, available in large quantities and high purity. The CalB-catalyzed esterification yielded 352 g of triglyceride of erucic acid corresponding to 97 % of the theoretically possible yield (table 2). The glycerol:fatty acid ratio of 1:3 led to a highly efficient esterification and less than 5 % of by-products like mono- and diglycerides.

Synthesis of OPO in the Miniplant

OPO was synthesized in the miniplant starting with tripalmitate as substrate. In the first step tripalmitate was converted to 2-monopalmitate (2-MP) in an alcoholysis reaction, followed by esterification of 2-MP with oleic acid to OPO (figure 2). The yield of 2-MP was 73 % corresponding to 84 g (77 % purity). The esterification led to 198 g of OPO (yield 90 %) with a purity of 95 % (table 2).

During the alcoholysis reaction no acyl migration was detectable.

In general immobilized CalB could be reused after filtration, washing and drying at least three times without any loss of activity.

5.3.5 Discussion

We showed in our study that it is possible within a two-step process to synthesize several 2-MAG without limitations regarding chain-length and saturation degree with just one biocatalyst. The reactions were successfully scaled up to 8 litre scale and also a two-step process for the synthesis of OPO was established.

The easiest and most effective route towards 2-MAG would be the application of an *sn*2-specific lipase which would allow to synthesize these desired molecules in a one-step reaction. With such an outstanding catalyst it would be possible to perform a direct esterification of glycerol and fatty acids (glycerolysis) leading to the 2-MAG or an interesterification reaction between two homogeneous triglycerides leading to an ABA type TAG. Such an enzyme would be an outstanding innovation in the field of enzyme technology. Up to now only very few lipases like Lipase A from *Candida antarctica* (CalA) [33] have

been described to show significant preference for position *sn*2 of glycerides. For other enzymes this characteristic is discussed like for the lipases from *Vernonia anthelminitica* [34] or *Candida parapsilosis* [35, 36]. Recently the expression of CalA in *E. coli* in microtiter plate scale was described allowing the directed evolution of this lipase [37]. So the prerequisites for a possible tailoring of CalA towards an *sn*2-specifc lipase via directed evolution are fulfilled.

The results of the esterification and alcoholysis reactions showed that CalB displays either an unspecific or a very strict *sn*1,3-specific behaviour depending on the reaction conditions. We also observed that after complete conversion of TAG to 2-MAG the catalyst starts to hydrolyze the 2-MAG leading to glycerol and ethyl esters. So the reaction has to be stopped by removing the lipase directly after the TAG are converted quantitatively. These results indicate that CalB is not a “classical” or “totally” *sn*1,3-specific lipase because it is also able to convert 2-MAG. 2-MAG are not hydrolyzed at all by highly *sn*1,3-specific lipases e.g. the *Rhizomucor mihei* lipase. It is probable that the addition of ethanol converts CalB from an unspecific to a more 1,3-regiospecific enzyme. A possible reason for this behaviour might be decreased flexibility of the tertiary structure of CalB caused by ethanol, hindering the substrate from accessing the catalytic binding pocket with the acyl group in the middle position [31]. This increased rigidity should also be favoured by decreasing the reaction temperature to 25 °C. Thus CalB should be classified as an unspecific lipase which can be tailored by reaction engineering to a more *sn*1,3-specific enzyme. The most important advantage over all other *sn*1,3-specific lipases tested in our work is the ability of CalB to convert the sterically demanding TAG of PUFAs which do not fit into the substrate binding pockets of other lipases (e.g. the *Rhizopus* lipases).

Reduced acyl migration has been reported when lowering the water content of the reaction mixture or adjusting the water activity [38]. In the reactions described here the water content was adjusted to a low level by addition of molecular sieve. Even though in other processes the water activity is exactly adjusted [39] we did not detect any effect of acyl migration in our miniplant reactions. Accordingly the temperature was the most determinant parameter for acyl migration.

In a first attempt the alcoholysis reaction in the miniplant was performed in pure ethanol as solvent and co-substrate which ended in very weak product formation and low yield. Ethanol inhibits the lipase activity: after a certain amount of time the reaction stopped although most of tripalmitate was not converted. After filtration of the lipase and removing the ethanol, the enzyme was active again. This indicates a kind of inhibitory complex that is formed between

the enzyme and ethanol. Still the alcoholysis reaction in pure ethanol worked fine in small scale. This may be due to significantly higher lipase concentration in small scale compared to the technical scale. Consequently the amount of ethanol was decreased in the miniplant reactions. In this case no inhibition of the lipase was detectable.

Inhibition of the alcoholysis reaction by ethanol in the miniplant was surprising. It is even more surprising that the reaction performed well in an acetone/ethanol mixture although this kind of mixtures has been described to inactivate lipases [40, 41]. The results presented here show that high-yield production of 2-monopalmitate in acetone is possible. As acetone is regarded as food-safe, the process is suitable for the production of nutrition-related materials.

The purification taking advantage of high solubility of 2-MAG in acetonititrile/water (95:5 v/v), while more hydrophobic by-products are more soluble in *n*-hexane allowed an easy and fast purification of the 2-MAG of PUFA. Monoacylglycerides and ethyl esters could be clearly separated. Usually the purification of 2-MAG is performed via crystallization as in the case of 2-MP. This is unsuitable in the case of PUFA because of their very deep freezing point. A possible alternative that also has been tested is the purification via a silica gel column treated with boric acid. In that case a high amount of organic solvents was necessary and significant acyl migration was detected (up to 40 %).

Yield and purity of OPO synthesized in the miniplant with CalB was slightly lower than in a similar process catalyzed by *Rhizopus* (*Rhizopus delemar*, *Rhizopus oryzae* and *Rhizomucor mihei*) lipases [39]. In the alcoholysis reaction a yield of 85 % with a purity > 95 % was described. Still this process was performed in lab scale whereas in our case we reached a technical scale (> 100 g of product) indicating further up scaling for industrial production is possible. Processes for OPO synthesis have been described using two different lipases with distinct regioselectivity for the ethanolyis and esterification reaction [42]. Use of only one enzyme as suggested in our work will facilitate industrial applications.

The potential of our approach is further underlined by comparison of the results to the latest literature on 2-MAG synthesis. A lab-scale process for the enzymatic synthesis (ethanolysis) of 2-MAG described by Shimada and colleagues yielded only in 28 – 29 mol% of 2-MAG content [43]. Yang and colleagues described a CalB-driven glycerolysis process yielding 2-MAG of PUFA [44]. Still the yield in the stirred tank just reached 70 % and unfortunately they had to work with a multiphase-system.

With respect to process development and further up scaling, the use of only one lipase for the unspecific esterification and the *sn*1,3-selective alcoholysis is a big advantage of the process described herein. Reusability of immobilized CalB in the batch processes indicates that

establishment of continuous production processes is possible. Thus new promising applications of this outstanding biocatalyst might arise.

Acknowledgement

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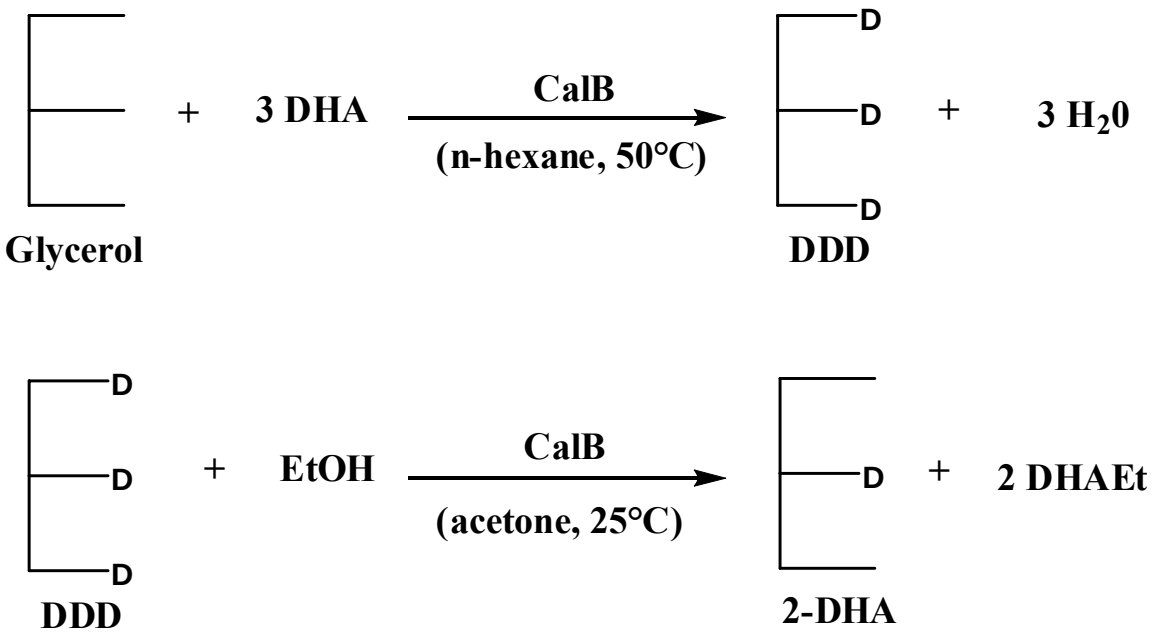

Figure 1: Two-step synthesis of 2-Monoacylglycerides of DHA catalyzed by CalB. The process consists of two steps. First esterification of glycerol and free fatty acids leads to homogeneous TAG and water. TAG are further converted in an alcoholysis reaction to the corresponding 2-MAG and ethyl esters. The ethyl esters are removed in the following purification step in a two-phase (acetonitril/water and *n*-hexane) system via a separatory funnel. Both steps are catalyzed by CalB which acts unspecific in the esterification and *sn*1,3-specific in the following alcoholysis. Synthesis of the 2-MAG of ARA and EPA was achieved according to the same scheme (D, DHA: Docosahexaenoic acid; DHAEt: DHA ethyl ester).

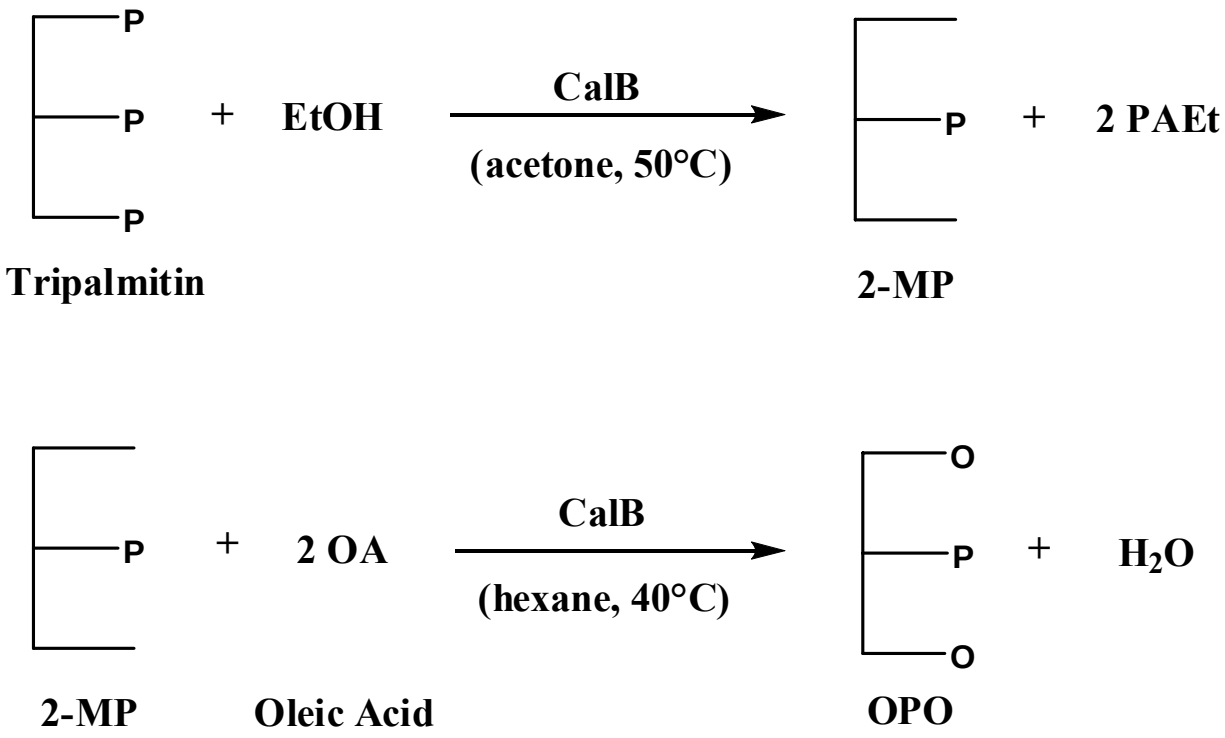

Figure 2: Two-step synthesis of OPO catalyzed by CalB. Starting substrate for OPO synthesis is tripalmitate which is converted to 2-monopalmitate in an alcoholysis reaction and afterwards purified by crystallization. The following reaction consisted in esterification of 2-monopalmitate with oleic acid to OPO which was purified via a silica-gel column. Both steps were catalyzed by CalB (PAEt: Palmitic acid ethyl ester).

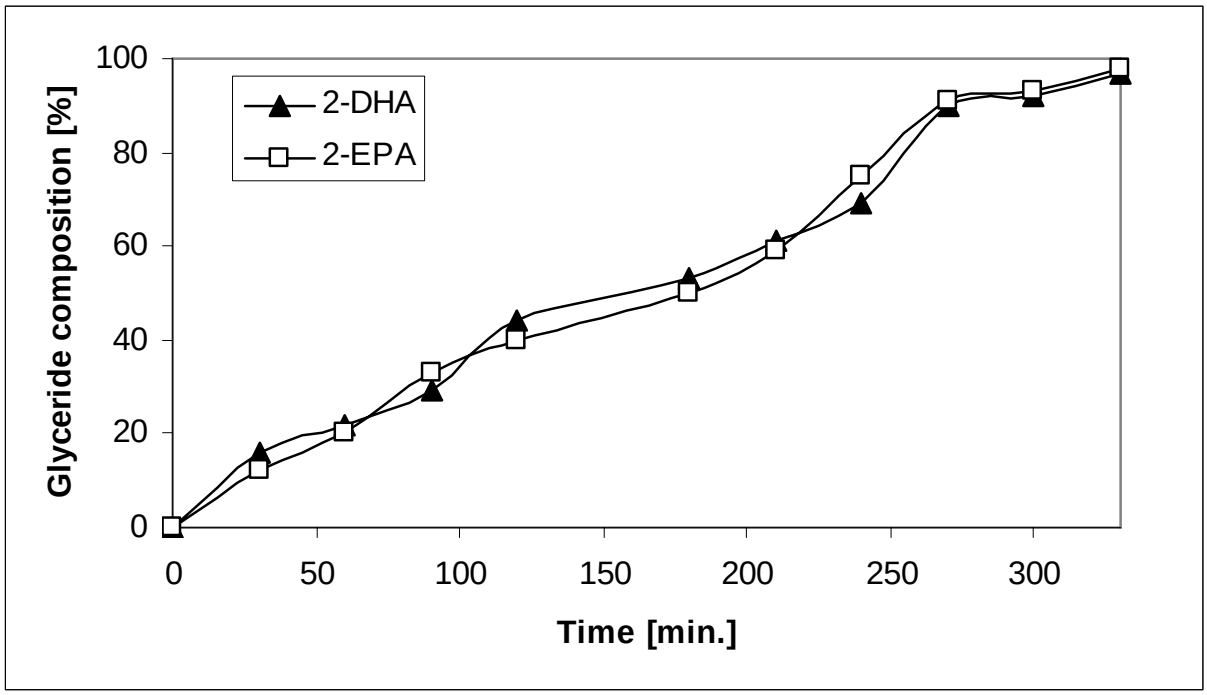

Figure 3: Product formation during the alcoholysis reaction of TAG of DHA (DDD) and EPA (EEE). The reaction is catalyzed by CalB and the product formation proceeded continuously until 240 minutes where the amount of product reached its final plateau.

Table 1: Mass, purity and yields of PUFA-2-monoacylglycerides performed in lab scale.

2 – Monoacylglyceride of	Isolated yields [g]	Purity [%]	Yield [%]
Arachidonic Acid (ARA)	**1,4**	**86**	**84**
Docosahexaenoic Acid (DHA)	**1,2**	**86**	**72**
Eicosapentaenoic Acid (EPA)	**1,6**	**95**	**96**

Table 2: Mass, purity and yields of products from miniplant reactions.

Reaction	Isolated yield [g]	Purity [%]	Yield [%]
Esterification of Glycerol with Erucic Acid (TAG Synthesis)	**352**	**95**	**97**
Alcoholysis of Tripalmitate (Synthesis of 2-MP)	**84**	**77**	**73**
Esterification of 2-MP with Oleic Acid (OPO Synthesis)	**198**	**95**	**90**

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5. 4 Directed Evolution of *Candida antarctica* Lipase A: towards enhanced *sn*2 specificity

Jörg Wilhelmi [1], Jan Pfeffer [1], Marko Kirtz [1], Sabine Eiben [1 *], Rachid Bel Rhlid [2], Carl-Erik Hansen [2], Rolf D. Schmid [1], and Steffen C. Maurer [1]

[1] ***Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany***

[2] ***Nestlé Research Center Lausanne, Nestec Ltd., Vers-Chez-Les-Blanc, 1000 Lausanne 26, Switzerland***

*** Author for correspondence (Fax: (+49)-711-685-3196; E-mail: sabine.eiben@itb.uni-stuttgart.de)**

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Lipase, *Candida antarctica*, directed evolution, *sn*2-preference, 2-monoacylglyceride, high throughput assay

5.4.1 Abstract

We report here a new high throughput assay for screening lipases with regard to a preference for position *sn*2 of the glycerol backbone and its application for improvement of *Candida antarctica* lipase A (CalA). The assay determines the ratio of activity towards 1,3-dicaprylin and tricaprylin. Calculating these ratios the differences in expression of mutants which is often a problem in high throughput screening can be neglected. The assay was validated with the *sn*1,3-specific lipases (*Rhizopus arrhizus* lipase (RAL), *Rhizomucor mihei* lipase (RML)), the non-specific *Pseudomonas fluorescens* lipase (PFL) and a lipase with *sn*2-preference (CalA). In the following the assay was used to screen a CalA error prone library for enhanced *sn*2 preference. Several improved mutants were identified in microtiter plate formate. The most promising mutant exhibiting four amino acid exchanges (W129L, I159N, L243I, H366Y) was expressed in *Pichia pastoris* and investigated in more detail. Product analysis revealed that this mutant had indeed an increased *sn*2-preference (~10 % higher) compared to the wildtype. Surprisingly the H366 is considered to be part of the catalytic triad.

5.4.2 Introduction

Lipases [triacylglycerol-hydrolases (EC 3.1.1.3)] play an important role in several industrial applications. They catalyse the hydrolysis of triacylglycerols at the interface between water and the hydrophobic substrate. Besides the hydrolysis of triacylglycerols, lipases also catalyse the enantio- and regioselective hydrolysis or synthesis of a wide range of natural and unnatural esters (Björkling *et al.* 1991; Bornscheuer *et al.* 1998; Schmid and Verger 1998). Especially lipases from microorganisms received a lot of interest because they are useful catalysts for many industrial applications such as ester synthesis (Nakano *et al.* 1991; Okumura *et al.* 1979), optical resolution (Kirchner *et al.* 1985; Langrand *et al.* 1985), transesterification (Kaufman *et al.* 1971) or washing processes (Kojima *et al.* 1994).

The yeast *Candida antarctica* expresses two lipases: lipase A (CalA) and B (CalB) (Hoegh *et al.* 1995). CalB shows high enantioselectivity against secondary alcohols and due to its high stability in organic solvents and at high temperature it is one of the most frequently used enzymes for industrial applications nowadays (Anderson *et al.* 1998).

CalA is reported to be a thermostable lipase (Solymár *et al.* 2002) and thus could also be interesting for industrial use. CalA was expressed in high yields in the methylotrophic yeast *Pichia pastoris*, purified and generally characterised (Pfeffer *et al.* 2006a). CalA shows high activity towards sterically hindered alcohols, including both secondary and tertiary alcohols (Kirk and Christensen 2002) and hydrolyses a range of tertiary-butyl esters of protected amino acids (Schmidt *et al.* 2005). A review on CalA published lately documents the increasing interest this unique biocatalyst attracts and the multitude of applications the enzyme can be used for (Dominguez de Maria *et al.* 2005). Furthermore CalA is described to be the only lipase displaying a preference for position *sn2* of triacylglycerides (Rogalska *et al.* 1993). That opens up a wide range of possible applications in synthesis of structured triacylglycerides.

The protein structure of CalA was published recently (Ericsson *et al.* 2008). To this date rational protein design was impossible. Consequently the only way to tailor the enzyme was directed evolution (Arnold *et al.* 2001) which requires functional expression in a fast-growing and easy-to-handle organism. Recently the functional expression of CalA in the cytoplasm of *E. coli*, the standard expression system for high-throughput screening (HTS) of large mutant libraries, was reported (Pfeffer *et al.* 2006b). Thus the prerequisites for directed evolution are available. Here we report use of this expression system in combination with a newly designed assay to enhance *sn*2-preference of CalA.

HTS assays have been developed, especially for lipases and esterases. Many of these HTS assays are based on spectrophotometric or fluorimetric measurements, because they are fast, efficient, easy to handle and reliable for identification of favored enzyme variants. Lipase activity can be determined e.g. by hydrolysis of *para*-nitrophenyl esters and measurement of the absorption at 405-410 nm. It was first used by Reetz et al. to screen for enantioselective lipases using optically pure (*R*)- or (*S*)-2-methyl-decanoate-*p*-nitrophenyl esters as substrates (Reetz *et al.* 1997). GC and HPLC methods are costly and time consuming methods and therefore not suitable for HTS. They are an add-on for further investigations of promising enzyme variants identified by an easy-to-use and cheap HTS. HTS assays can provide information on enzyme kinetics, activities and selectivities and allow screening of thousands of mutants per day (Schmidt and Bornscheuer 2005). When using HTS assays the reaction used for screening should be as close to the real problem as possible. Surrogate substrates might lead to optimized enzymes with high activity towards the surrogate substrate but not necessarily towards the favoured substrate.

We designed an assay for fast determination of the positional specificity of lipases towards the glycerol backbone. This assay was validated using *sn*1,3-specific, unspecific and *sn*2-preferring lipases. The assay was further used for screening an epPCR library of CalA.

5.4.3 Material and methods

Chemicals

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma-Aldrich (Taufkirchen, Germany) and Roth GmbH (Karlsruhe, Germany) in analytical quality unless otherwise specified. 1,3-Dicaprylate (1,3-DC) was purchased from Nu-Chek (Elysian, USA)

Strains and plasmids

E. coli Origami™ B cells were purchased from Novagen (Darmstadt, Germany). The plasmid pColdIII was purchased from Takara (Otsu, Japan).

Cloning of calA variants

CalA wildtype was modified by error prone PCR (epPCR) using the Gene Morph™ II Random Mutagenesis Kit (Stratagene, La Jolla, USA), then cloned into vector pColdIII using primers containing the restriction sites for *Eco*RI and *Nde*I.

Expression in microtiter plates and activity assay

E. coli Origami™ B cells were transformed with pColdIII constructs as described before (Pfeffer, Rusnak *et al.* 2006b; TaKaRa). Cells were grown in 200 µl LBamp at 37 °C and 180 rpm up to an optical density (λ = 600 nm) of 0.4–0.6. Afterwards, cultures were chilled on ice for 30 min. Lipase expression was induced by adding IPTG (c_{end} = 0.5 mM). Cells were grown for an additional 24 h at 15 °C and 180 rpm and were harvested by centrifugation (3220 x g). Cells were lysed by adding 50 µl lysis buffer (1 mM sodium phosphate, pH 7.5, 1 mg ml^{-1} lysozyme). Lysates were incubated for 45 min at 37 °C and 600 rpm and chilled on ice for 30 min. After incubation for 1 h at −80 °C, lysates were thawed at RT, and cell debris was removed by 30 min centrifugation at 3220 g and 4 °C.

High throughput screening (HTS) using the TwinAssay

Every well of a 96-well microtiter plate contained 100 µl sodium phosphate buffer pH 7(c = 1 mM), 10 µl bromocresol purple (stock solution c = 600 µg/ml) and 20 µl cell lysate containing CalA. Reactions were started by adding 10 µl substrate solution (1,3-DC or tricaprylate, 10 mM). Reaction rates were measured at 590 nm for 30 min. In an identical setup lysates from the same clones were incubated with tricaprylate and the respective reaction rates were determined. After a lag phase of 5 min the velocity was calculated over the whole time course.

For automation of screening the BioPick™, colony picker from BioRobotics, and Janus™, a pipetting robot from Perkin Elmer coupled to a SpectraMax 384 mtp photometer were used. A mastermix of buffer and colour indicator was filled into each well. After addition of cell lysate the reaction was started by adding the respective substrate.

Enzyme characterisation

The best mutant and the wildtype CalA were cloned into pPICZαA using *Eco*RI and *Not*I restriction sites which were introduced by specific primers.

The lipases were expressd in Pichia pastoris X33 according to the user manual (Invitrogen 1997)

The hydrolysis reaction with tricaprylin as substrate was performed at 30 °C (pH 7.5). Tricaprylin (5 % (v/v)) was emulsified in distilled water containing 2 % (w/v) gum arabic using a homogeniser for 7 minutes at maximum speed (Ultraturrax T25, Janke and Kunkel, Staufen, Germany). Each hour samples were taken, extracted with *n*-hexane and the organic phase was dried over magnesium sulphate. Finally the samples were analysed with GC/MS.

Product analysis by GC/MS

Using GC/MS analysis the ratio of 1,2-dicaprylate (1,2-DC) versus 1,3-DC was determined. The ratio of regioisomeric monocaprylates was not taken into account due to the risk of misinterpretation because of acyl migration in 2-monoacylglycerides (Boswinkel *et al.* 1996). GC/MS samples were first extracted twice with *n*-hexane followed by trimethylsilylation through addition of a mixture of *N, O-bis*(trimimethylsilyl)trifluoroacetamide and 0.1 % trimethylsiloxane and heating for 20 min at 80 °C.

Products were identified by comparison to authentic standards. The ratio of 1,2-DC versus 1,3-DC was calculated on basis of the peak areas.

In silico analysis

The orientation of the mutations introduced was visualised in the crystal structure of CalA, pdb file 2VEO (Ericsson, Kasrayan *et al.* 2008), using Pymol (DeLano 2005; http://www.pymol.org).

5.4.4 Results

Establishment and validation of the screening system

A classical way to determine ester hydrolysis by lipases is the pH stat assay. The release of protons during catalysis is monitored by titration with diluted NaOH. Although a very accurate method to analyse lipase activity it is very time-consuming. High throughput assays frequently use the change in colour of a pH indicator to visualise the lipase mediated decrease in pH. Therefore the first challenge was to identify a pH indicator sensitive in the investigated pH range. Three indicators were taken into account: phenol red (pH 6.5-8.2), bromothymol blue (pH 3-4.5), and bromocresol purple (pH 5.4-6.8).

While for the first two indicators the protonated and deprotonated forms could not be easily distinguished spectrophotometrically, bromocresol purple turned out to be an adequate pH-indicator for the chosen assay setup. It shows purple colour (pH 7.5) in basic and yellow colour (pH 6) in slightly acidic media. Loss in absorption was followed at 590 nm in a microtiter plate photometer. During lipase catalysed hydrolysis of tricaprylin a constant decrease of absorption could be measured. In the same assay it could be shown that tricaprylin is not susceptible to autohydrolysis under the conditions chosen (Figure 1). Other important parameters were buffer strength and substrate concentration. Both have influence on the change of pH during the reaction. Buffer strength of the lysis buffer had to be decreased to 1 mM. Substrate concentration had to be adjusted to a value where the release of protons was high enough to induce a pH shift in a short time while the substrate was still soluble. While a change of 0.4 absorption units took one hour at a final substrate concentration of 0.84 mM, it took only 15 minutes with 3.5 mM substrate, resulting in disturbances of the linear decrease in absorption due to substrate precipitation. A compromise was found by using 1.4 mM (10 mM stock solution) of substrate with 30 minutes reaction time.

Principle of sn2-preference determination

The positional specificity of hydrolases on the glycerol backbone can be estimated based on reaction rate comparison for hydrolysis of tricaprylate versus 1,3-DC. Reaction rates were measured separately for each substrate with the same amount of a chosen lipase. Thus the experiment described above allows to discriminate whether a lipase is *sn*2-specific, *sn*1,3-specific or non-specific. (Figure 2)

Equal concentrations of 1,3-DC and TC were used. To validate the stability of the assay the influence of cell lysates (*E. coli* cell lysate with pColdIII plasmid without a lipase), and the chosen solvent (DMSO) was investigated. None had a negative influence on the stability of the assay.

Validation of the hydrolysis assay with specific and non-specific lipases

The *sn*2-assay with tricaprylate and 1,3-dicaprylate was validated using commercially available lipases. *Candida antarctica* Lipase A (CalA) is a lipase with significant preference for position *sn*2 on triacylglycerides (~ 60 %), whereas lipases from *Rhizopus arrhyzus* (RAL) and *Rhizomucor mihei* (RML) are both *sn*1,3-specific (Bornscheuer 1999). As non-specific lipase the lipase from *Pseudomonas fluorescens* (PFL) was used (Bornscheuer 1999).

By calculating the ratio of the hydrolysis reaction rates of tricaprylate versus 1,3-DC at equal substrate concentration it is possible to discriminate *sn*2-specific, 1,3-specific and non-specific lipases. Unspecific lipases should hydrolyse tricaprylate as quick as 1,3-DC, in consequence resulting in a ratio of 1 (Formula 1) while *sn*1,3-specific lipases show a ratio of < 1 and *sn*2-preferring lipases should lead to a ratio of > 1.

$$\text{ratio Q} = \frac{\text{hydrolysis reaction rate of tricaprylate}}{\text{hydrolysis reaction rate of 1,3 - dicaprylate}} \qquad \text{(Formula 1)}$$

In figure 3 the results of the assay validation using lipases displaying different positional specificity on the glycerol backbone of triacylglycerides are summarised. As expected the ratio for CalA was higher than 1 while both *sn*1,3 specific lipases exhibit ratios distinctively lower than 1. Interestingly the PFL, generally considered to be unspecific, shows a value of Q = 0.8 indicating a slight preference for *sn*1,3-position in the assay. By applying four different lipases the assay was proven to be suitable for determination of regio-specificities in triacylglyceride hydrolysis.

Mutant library creation

Random mutagenesis was performed by epPCR using the Gene Morph II Kit (Stratagene). Three approaches with low (20 µl plasmidic DNA, 50 ng/µl), medium (10 µl plasmidic DNA, 50 ng/µl) and high (5 µl plasmidic DNA, 50 ng/µl) mutation rate were tested. Mutation rate was verified by sequencing. Medium (1.4 mutations per 1000 bp) and high (2.5 mutations per 1000 bp) mutation rate showed the desired amount of mutations for directed evolution (1 - 3 bp per 1000 bp). 5000 colonies were separated in 96-well MTPs.

It has to be noted that the first published DNA sequence from Hoegh et al. (Hoegh, Patkar *et al.* 1995) is not the same as the sequence we obtained, which is identical to the sequence of the crystallised CalA although we removed the signal sequence for cloning.

Screening of mutant library

As CalA is a thermostable enzyme while the substrates showed autohydrolysis at temperatures above 40 °C the reaction temperature was set to 37 °C. Screening of the epPCR mutant library led to identification of some mutants with higher *sn*2-specificity than CalA wildtype. Nevertheless most mutants exhibited equal or lower values than the wildtype. The mutant showing the most significant increase in ratio (5) was sequenced. Four amino acid

exchanges could be detected: W129L, I159N, L243I and H366Y further referred to as CalA_LNIY (Figure 4).

Characterisation of mutants by GC/MS product spectra

To get larger quantities of enzyme, the best mutant CalA_LNIY was cloned and expressed in *Picha pastoris* X33 using standard procedures. To verify the screening results hydrolysis of tricaprylin and subsequent GC/MS for product identification were performed. The ratio of 1,2-dicaprylate (1,2-DC) versus 1,3-DC was analysed in dependency of the reaction turnover. Formation of 1,3-DC and 1,2-DC was calculated by integration of the peaks of both products. Ratio R was calculated by forming the proportion of 1,3-DC divided by 1,3-DC plus 1,2-DC (Formula 2).

$$\text{ratio R} = \frac{\text{amount of 1,3 - DC}}{\text{amount of 1,3 - DC} + \text{amount of 1,2 - DC}} \cdot 100 \qquad \text{(Formula 2)}$$

CalA_LNIY showed an increase of sn2-preference in comparison to the wildtype of 10 % at all time points measured (Table1), therefore confirming the data from the HTS screening. However although equal protein concentrations were applied activity of the mutant was only 25 % of wildtype activity resulting in 20 % conversion of tricaprylin after six hours in comparison to 80 % as was determined for CalA wildtype.

5.4.5 Discussion

Up to now there is no process for direct synthesis of structured triacylglycerides known, that would use glycerol and fatty acids for synthesis of 2-monoacylglycerides (2-MAG). The same is true for interesterification of triacylglycerides with fatty acids or esters thereof. All these routes might open up new approaches to structured triacylglycerides of the ABA type.

Nowadays the access to 2-monoacylglycerides takes a two-step route: starting from a homogeneous triacylglyceride of the AAA-type that is built up with a non-specific lipase and subsequently hydrolysed with an *sn*1,3-specific lipase (Schmid 1999). In a third reaction 2-MAG can be esterified with the desired fatty acids in positions *sn*1 and *sn*3. It is clear, that this process is complex and costly, because all these steps require specific reaction conditions. Lipases showing *sn2*-selectivity would offer the easiest access to 2-monoacylglycerides. Starting e.g. from triolein and palmitic acid ethyl ester, structured triacylglycerides from the ABA-type such as OPO could be synthesised directly in one step (Pfeffer *et al.* 2007). Enzymes described in literature with *sn*2-preference are CalA (Rogalska, Cudrey *et al.* 1993) and lipase from *Candida parapsilosis* (Briand *et al.* 1995; Neugnot *et al.* 2002). Nevertheless none of them is *sn*2-specific.

Recently the expression of CalA succeeded in *E. coli* (Pfeffer, Rusnak *et al.* 2006b) and was transferred to microtiter plate scale for growth and expression. Expression in microtiter plates opened up the possibility for directed evolution of the enzyme and HTS of enzyme variants. In the present work we used epPCR and developed a new screening system for selection of mutants with increased *sn*2-preference.

It could be shown that the preferences of different lipases towards the three ester bonds in triacylglycerides can be unambiguously discriminated using the described assay. This opens up the possibility to screen more than thousand mutants per day to find lipases with enhanced regiospecificity.

The assay is stable, reliable and not dependent on expression levels and therefore especially useful when the expression levels of mutants varies.

Finally we found the quadruple mutant CalA_LNIY with significant higher preference for *sn*2-position compared to the wildtype (Rogalska, Cudrey *et al.* 1993). To have a closer look at the exchanged positions we introduced these mutations into the pdb file 2VEO (Figure 5). As L243I is situated farthest from the active site and because the change from leucine to isoleucine is just a minor one this mutation might contribute least to the observed enhancement in *sn*2-selectivity. Both W129L and I159N are also located far away from the active site. However the substitution of a very big aromatic amino acid to a smaller one (W→L) as well as from a hydrophobic to a polar amino acid (I→N) can have tremendous long range effects on the structure of an enzyme (Wang *et al.* 2006). The most unusual finding was that the fourth amino acid exchange was found to be located at the catalytic histidine H366Y. Interestingly it was reported by Bäckvall et al. that exchange of this histidine to alanine did not result in a complete loss of activity towards *para*-nitrophenol palmitate but was in the same range as exchange of H330 to alanine (Kasrayan *et al.* 2007). It seems that CalA might be able to utilise both histidine residues for catalysis. Alternatively another basic group might take the part of the catalytic histidine. This phenomenon was never observed for other lipases and needs to be investigated by further analysis.

Up to now only diacyl- and triacylglycerides with the chain length of C8 were investigated. Subsequent experiments could explore the substrate spectrum of CalA_LNIY. Further rounds of epPCR starting with this mutant could be used to identify additional mutants with even higher *sn*2-specifity. One might also start from other enzymes reported to display *sn*2-preference, creating variants and testing them with this newly established assay.

5.4.6 References

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Figure List

Figure 1. Hydrolysis and autohydrolysis of Tricaprylin. Autohydrolysis is diagrammed in diamonds. Hydrolysis is diagrammed in rectangles. Autohydrolysis is zero and stable – it has no influence on the enzymatic reaction shown in rectangles.

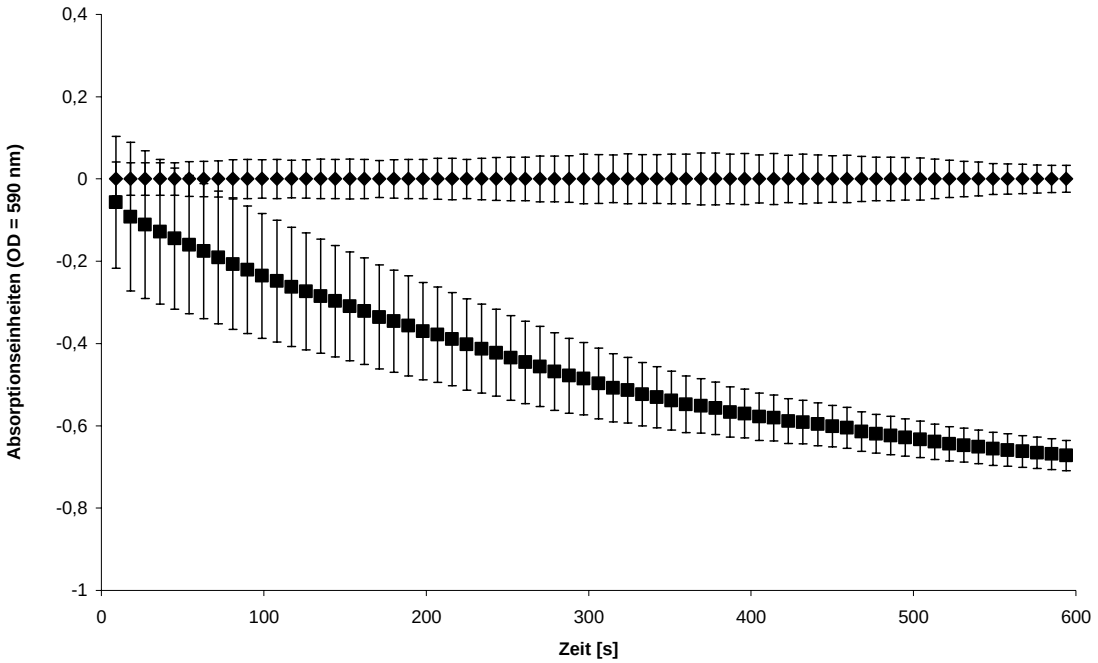

Figure 2. Reaction scheme of Hydrolysis of TC and 1,3-DC. An *sn*2-specific lipase hydrolyse TC faster than 1,3-DC.

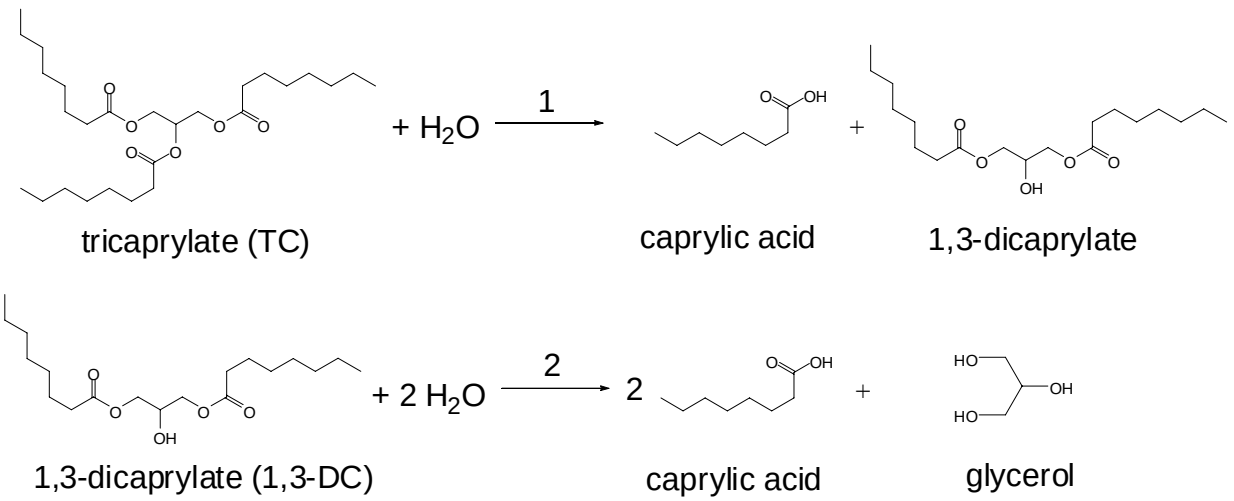

Figure 3. Validation of *sn*2-assay for non-specific and regiospecific lipases. CalA (*Candida antarctica* lipase A) with preference for *sn*2-position. RAL (*Rhizopus arrhizus* lipase) and RML (*Rhizomucor mihei* lipase) are *sn*1,3-specific. PFL (*Pseudomonas fluorescens* lipase) is non-specific. * marks expectancy value for a non-specific lipase. Measurements were repeated at least three times.

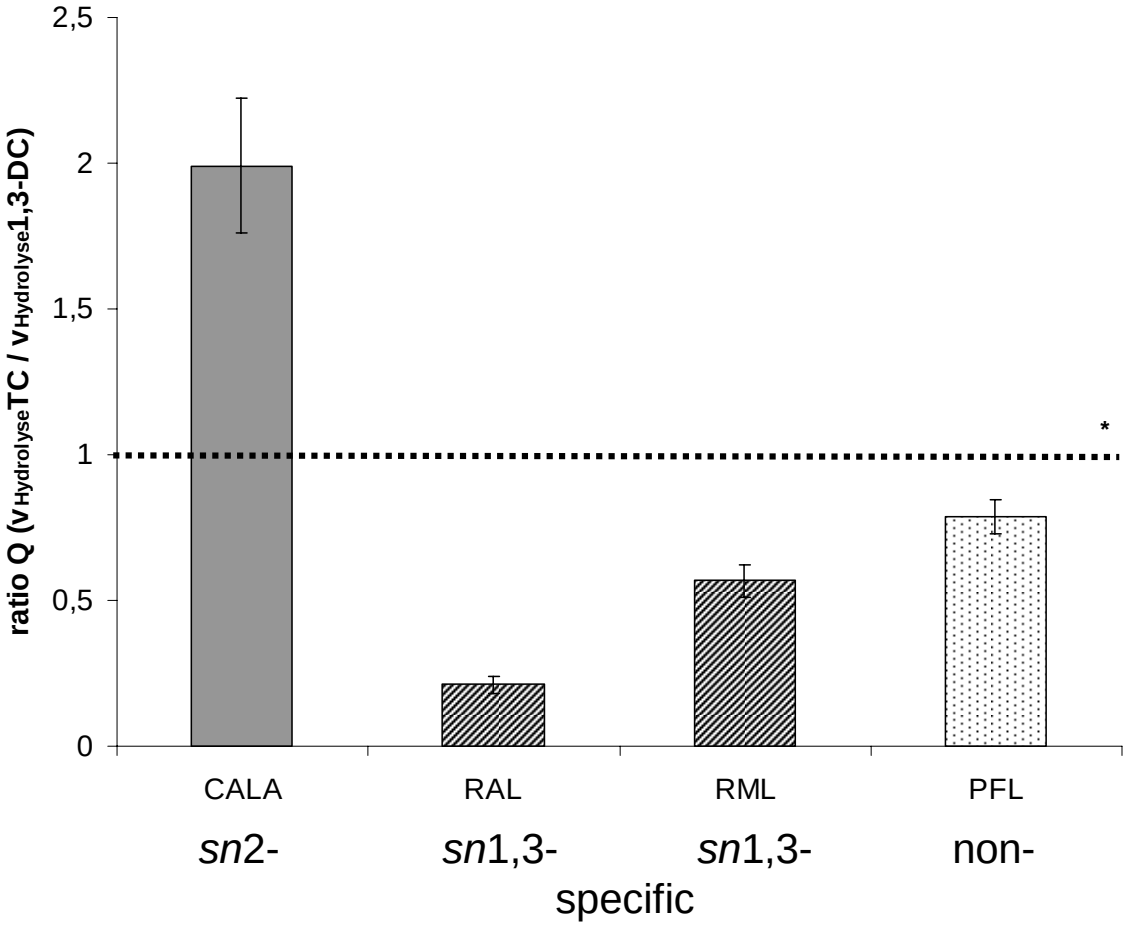

Figure 4. Alignment of CalA_LNIY with the 2VEO crystal structure sequence. The mutations are annotated by asterisks, while the catalytic triad (Ser184, Asp334 and His366) is shown in bold and underlined. The putative alternative histidine is marked by a hash.

```
2VEO        APATETLDRRAALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTDIGNANNAASFQLQYR 60
CalAmut     ---------MAALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTDIGNANNAASFQLQYR 51

2VEO        TTNTQNEAVADVATVWIPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGLDQPNKVTAV 120
CalAmut     TTNTQNEAVADVATVWIPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGLDQPNKVTAV 111

2VEO        LDTPIIIGWALQQGYYVVSSDHEGFKAAFIAGYEEGMAILDGIRALKNYQNLPSDSKVAL 180
CalAmut     LDTPIIIGLALQQGYYVVSSDHEGFKAAFIAGYEEGMANLDGIRALKNYQNLPSDSKVAL 171
                     *                                  *
2VEO        EGYSGGAHATVWATSLADSYAPELNIVGASHGGTPVSAKDTFTFLNGGPFAGFALAGVSG 240
CalAmut     EGYSGGAHATVWATSLADSYAPELNIVGASHGGTPVSAKDTFTFLNGGPFAGFALAGVSG 231

2VEO        LSLAHPDMESFIEARLNAKGQQTLKQIRGRGFCLPQVVLTYPFLNVFSLVNDTNLLNEAP 300
CalAmut     LSIAHPDMESFIEARLNAKGQQTLKQIRGRGFCLPQVVLTYPFLNVFSLVNDTNLLNEAP 291

2VEO        IAGILKQETVVQAEASYTVSVPKFPRFIWHAIPDEIVPYQPAATYVKEQCAKGANINFSP 360
CalAmut     IAGILKQETVVQAEASYTVSVPKFPRFIWHAIPDEIVPYQPAATYVKEQCAKGANINFSP 351
                                            #
2VEO        YPIAEHLTAEIFGLVPSLWFIKQAFDGTTPKVICGTPIPAIAGITTPSADQVLGSDLANQ 420
CalAmut     YPIAEYLTAEIFGLVPSLWFIKQAFDGTTPKVICGTPIPAIAGITTPSADQVLGSDLANQ 411
                 *
2VEO        LRSLNGKQSAFGKPFGPITPP 441
CalAmut     LRSLNGKQSAFGKPFGPITPP 432
```

Figure 5. Distribution of the mutations of CalA_LNIY in the structure of CalA (2VEO). The right site shows an enhanced view of the active site with Ser184, Asp334 and the mutations depicted as black stick models. Additionally His 330 is also marked in black.

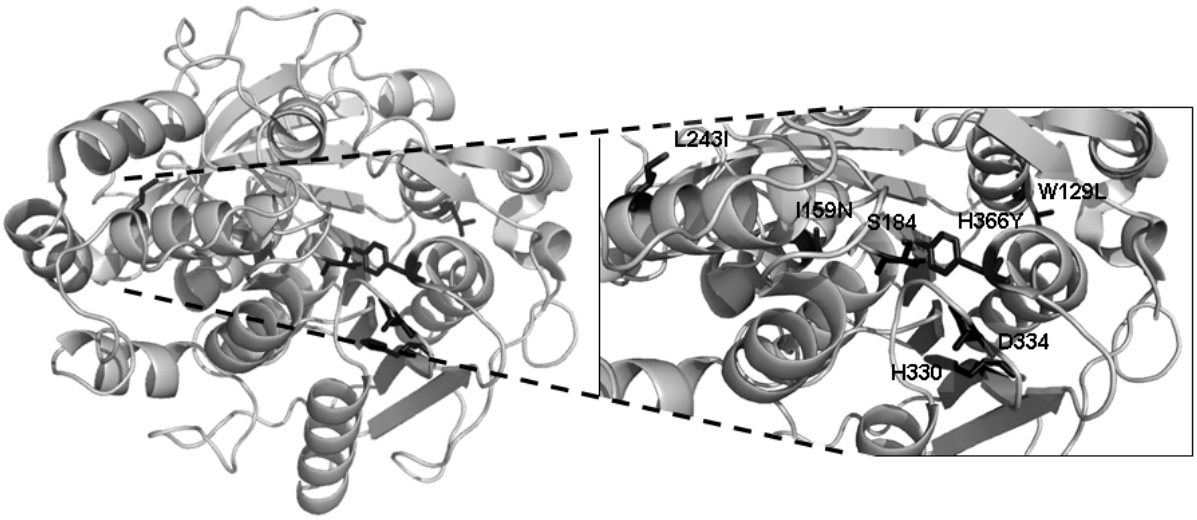

Table list

Table 1. Percentage amount of 1,3-DC of total diacylglyceride in hydrolysis of tricaprylate (TC). Calculated from GC/MS data.

Reaction time [h]	[% 1,3-DC]	[% TC]	[% 1,3-DC]	[% TC]
	CalA wildtype		CalA LNIY	
1	60	77	68	88
2	59	33	73	85
3	62	21	70	84
4	62	20	72	84
5	61	18	73	82
6	59	18	72	79

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7. Curriculum vitae

Personal details

Name: Jan Christoph Pfeffer

Place of birth: Aalen (Germany)

Date of birth: 31st October 1978

Education

PhD thesis at the "Institute of Technical Biochemistry", University of Stuttgart, Germany — **March 2005 – October 2007**

Title: The Lipases from *Candida antarctica*: Cloning, Expression and their Application in the Synthesis of Structured Lipids

Master thesis at the "Institute of Technical Biochemistry", University of Stuttgart, Germany — **July 2004 – March 2005**

Title: Establishment of a FRET system for the protein-protein-interaction of human nuclear receptors

Curriculum "Technical Biology" — **October 1999 – July 2004**

Master of Science

University of Stuttgart, Germany

High-school graduation — **July 1989 - August 1998**

Theodor-Heuss-Gymnasium, Aalen, Germany

Work experience

Internship at Roche Pharmaceuticals, Ltd. Shanghai, China — **December 2002-August 2003**

Different projects in production- and quality control-department

Publications

Pfeffer J, Richter S, Nieveler J, Hansen CE, Rhlid RB, Schmid RD, Rusnak M (2006) High yield Expression of Lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* and its purification and characterisation. Appl Microbiol Biotechnol, 72:931-8

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