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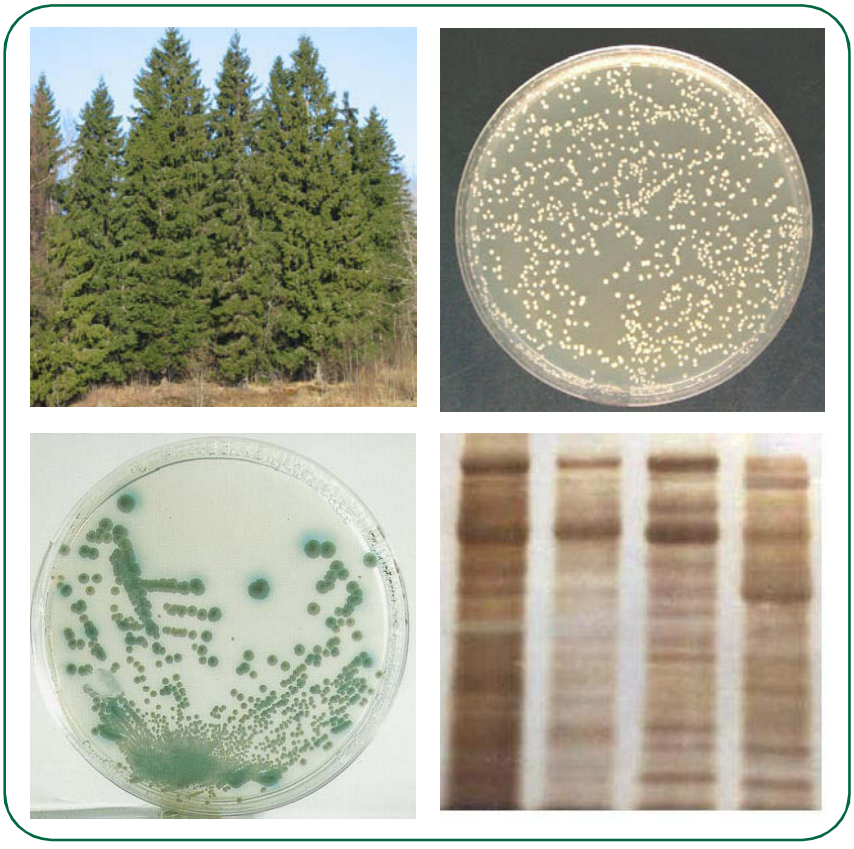

Hanna Kontkanen

Novel steryl esterases as biotechnological tools

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Hanna Kontkanen

VTT

Faculty of Mathematics and Science
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University of Jyväskylä

ACADEMIC DISSERTATION

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Abstract

Esterases form a class of enzymes catalysing the hydrolysis of different types of esters. Steryl esterases are a sub-class of esterases that primarily catalyse the hydrolysis of fatty acid esters of sterols, i.e. steryl esters. Steryl esterases may also be active against other substrates containing ester linkages, such as triglycerides, and thus they can also be classified as lipases. Steryl esterases and lipases capable of modifying different types of esters have potential applications in the food, textile, and pulp and paper industries. In this work novel steryl esterases were characterised and their usefulness in modification of fibre products was preliminarily evaluated.

Commercial lipase preparations were tested for their ability to degrade steryl esters. Lipases from yeast *Candida rugosa* and bacteria *Chromobacterium viscosum* and *Pseudomonas* sp. were shown to have the highest steryl esterase activities, and they were able to hydrolyse steryl esters totally in the presence of surfactant. Up to 80–90% of the steryl esters were also degraded by lipases from *C. rugosa* and *Pseudomonas* sp. in the absence of surfactant. The steryl esterase of *C. rugosa* lipase preparation was purified and identified as the lipase LIP3, a well-known lipase having steryl esterase activity. LIP3 was found to be highly active against plant-derived steryl esters, especially in the presence of surfactant. In aqueous dispersions, all the different steryl esters were hydrolysed equally but total hydrolysis of steryl esters was not achieved.

A novel steryl esterase from filamentous fungus *M. albomyces* was purified and biochemically characterised. The enzyme had broad substrate specificity for different steryl esters, *p*-nitrophenyl esters and triglycerides. The pH optimum was dependent on the substrate and varied within the pH range 5–7. The enzyme was more stable at lower pH than at alkaline values. It had a half-life of 2 h at

70°C, and thus was rather thermostable. The enzyme was highly active on steryl esters and triglycerides in the presence of surfactant, whereas only triglycerides were degraded in the absence of surfactant.

The gene encoding *M. albomyces* steryl esterase (*ste1*) was isolated in order to express the protein heterologously in *Pichia pastoris* and *Trichoderma reesei*. The mature *M. albomyces* steryl esterase with a length of 545 amino acids appeared to be significantly related to other lipases and esterases. The level of amino acid sequence identity of the *M. albomyces* steryl esterase was 47% with the *C. rugosa* lipase LIP3. The production level in *P. pastoris* was very low, and a significant proportion of the total activity was found to be present intracellularly. Heterogeneous overglycosylation of the intracellular enzyme indicated that STE1 enters the secretory pathway but is not fully secreted.

The *M. albomyces* steryl esterase was also expressed in the filamentous fungus *T. reesei* under the inducible *cbh1* promoter. In a laboratory-scale fermenter, the main portion of activity was in the culture supernatant and a production level of 280 mg l^{-1} was achieved. The recombinant steryl esterase (rSTE1) was characterised and its properties were compared to those of the native enzyme. The recombinant enzyme was shown to be a dimer with a molecular weight of 120 kDa, whereas the native enzyme has a tetrameric structure with a molecular weight of 238 kDa. The recombinant enzyme was somewhat less stable at higher temperatures and had slightly lower specific activities against various substrates than the native enzyme.

The effects of rSTE1 on wood extractives, as well as on polyethylene terephthalate (PET) fabric, were preliminarily evaluated. The tensile strength and hydrophilicity of the paper were increased by rSTE1 due to the hydrolysis of esters. The rSTE1 treatment increased significantly the polarity of PET fabric. Dyeing of PET with methylene blue was also slightly improved after rSTE1 treatment. Improved textile properties of PET by steryl esterase treatment showed that esterases are interesting tools for the modification of synthetic materials in addition to their natural substrates.

Preface

This work was carried out at VTT Biotechnology during the years 2000–2004. I wish to thank Professor Juha Ahvenainen, Technology Director of VTT Biotechnology Cluster and Research Professor Liisa Viikari for providing me excellent working facilities. Research Professor Johanna Buchert and Technology Manager Richard Fagerström are thanked for their supportive attitude towards my research. I am grateful to Professor Helena Nevalainen and Professor Annele Hatakka for reviewing the manuscript of my thesis and for their constructive feedback and valuable suggestions. Financial support from Raisio Group Foundation, Jenny and Antti Wihuri Foundation and VTT Biotechnology is gratefully acknowledged. I also thank Professor Christian Oker-Blom at the University of Jyväskylä for his support during the final stages of my studies.

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for all their help in scientific issues as well as for sharing the many joys and sorrows along the way and, very importantly, for reminding me that life is not so serious, after all.

My parents and closest relatives deserve my thanks for their continuous support and valuable help in everyday life throughout this work. All my friends are thanked for their patience and understanding, and for arranging every now and then some funny activities for me. Finally, I wish to express my warmest gratitude to my husband Tapio for all his support and patience, and our son Aapo for his unquestioning love and for teaching me to enjoy many little things in my life.

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Publications I–V

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List of publications

This thesis consists of a summary of the field and of the following 5 publications, which are referred in the text by the Roman numerals I–V:

I. Kontkanen, H., Tenkanen, M., Fagerström, R. and Reinikainen, T. (2004) Characterisation of steryl esterase activities in commercial lipase preparations. Journal of Biotechnology 108:51–59.

II. Tenkanen, M., Kontkanen, H., Isoniemi, R., Spetz, P. and Holmbom, B. (2002) Hydrolysis of steryl esters by a lipase (Lip 3) from *Candida rugosa*. Applied Microbiology and Biotechnology 60:120–127.

III. Kontkanen, H., Tenkanen, M. and Reinikainen T. (2006) Purification and characterisation of a novel steryl esterase from *Melanocarpus albomyces*. Enzyme and Microbial Technology. In press.

IV. Kontkanen, H., Reinikainen, T. and Saloheimo, M. (2006) Cloning and expression of a *Melanocarpus albomyces* steryl esterase gene in *Pichia pastoris* and *Trichoderma reesei*. Biotechnology and Bioengineering. In press.

V. Kontkanen, H., Saloheimo, M., Pere, J., Miettinen-Oinonen, A. and Reinikainen T. (2006) Characterization of *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei* and modification of fibre products with the enzyme. Applied Microbiology and Biotechnology. In press.

Abbreviations

AOX1	alcohol oxidase 1
BLAST	basic local alignment search tool
BMMY	buffered methanol-complex medium
CAE	carboxyl esterase
cbh1	gene encoding cellobiohydrolase I (CBHI)
CE	cholesteryl esterase
DEAE	diethyl aminoethyl; anion exchanger
DCS	dissolved and colloidal substances
GC	gas chromatography
HIC	hydrophobic interaction chromatography
LIP3	lipase isoform produced by *Candida rugosa*
MALDI-TOF	matrix assisted laser desorption/ionisation -time of flight
MTBE	methyl *tert*-butyl ether
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PET	polyethylene terephthalate
pI	isoelectric point
p-NPB	*p*-nitrophenyl butyrate
p-NPC	*p*-nitrophenyl caprate
PSE	mixture of plant steryl esters
rSTE1	recombinant *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei*
SDS	sodium dodecyl sulphate
SE	steryl ester
STE1	*Melanocarpus albomyces* steryl esterase
ste1	gene encoding *M. albomyces* steryl esterase
TG	triglyceride
TMP	thermomechanical pulp
WSE	mixture of wood steryl esters

1. Introduction

Lipids comprise a wide variety of different structures, most of which are fatty acid esters. Esters occur widely in all plants since they have a physiological role in membranes and as storage lipids. Some esters may impair the industrial processing of plant materials due to their hydrophobic character. Thus, modification of the ester compounds would improve the processing and exploitation of several natural materials, and would reduce disposal of co-product wastes. Processing of waste fractions and of some low-value esters could be exploited as a source of more valuable compounds.

Wood contains lipophilic extractives, in which triglycerides and steryl esters are the major esters. These compounds cause production problems in pulp and paper manufacturing when released together with carbohydrates and lignans into the process waters during mechanical pulping. These dissolved and colloidal substances (DCS) can have a negative impact on paper machine runnability and paper product quality. The problems can be partially controlled by traditional methods, such as debarking, seasoning of wood chips prior to pulping, and addition of chemicals. Alternatively, wood extractives can be modified biologically by lipase treatment or by microbial pre-treatment (Farrell *et al*., 1997). However, none of these methods is fully effective, e.g. only triglycerides can be degraded by the current lipase treatment. Thus, novel esterases acting on a broader range of esters, especially on steryl esters, will be potential tools for biological pitch control.

Esterases, as well as lipases affecting ester bonds, are found in various organisms including animals, plants, fungi and bacteria. Several microbial lipases have been cloned and the crystal structures of many lipases have been solved (Schmidt-Dannert, 1999; Sharma *et al*., 2001). Microbial lipases are very diverse in their enzymatic properties and substrate specificities, which makes them attractive for industrial applications (Pandey *et al*., 1999). They are used in the detergent, food and oleochemical industries as well as in the high technology production of fine chemicals and pharmaceuticals. Steryl esterases, a subclass of esterases, have been extensively examined in a number of mammalian tissues primarily because of their involvement in cholesterol metabolism. However, steryl esterases are a less well known group of esterases in microorganisms, and

only a small number of microbial steryl esterases have been identified and characterised. Microbial steryl esterases are primarily used for diagnostic measurement of total serum cholesterol (Allain *et al.*, 1974), and hitherto none of them have been produced for industrial applications. Knowledge on the biochemical properties of microbial steryl esterases as well as the chemistry of potential substrates could lead to the development of tools to tailor plant esters, resulting in added value of the product or, alternatively, in improved processes.

1.1 Lipolytic esterases

1.1.1 Classification of esterases

Esterases (EC 3.1.1.) are hydrolases that catalyse the cleavage of ester linkages by the addition of a water molecule. Esterases usually have broad substrate specificity and they fall into several classes. Lipases (triacylglycerol lipase EC 3.1.1.3) are esterases that hydrolyse water-insoluble substrates such as long-chain triglycerides at the interface between the substrate and water. By contrast, typical esterases such as carboxylesterase (EC 3.1.1.1) are restricted to water-soluble esters of short-chain carboxylic acids. Esterases that have the ability to hydrolyse fatty acid esters of sterols, i.e. steryl esters, are classified as steryl esterases (sterol esterase, cholesterol esterase, EC 3.1.1.13). Most of the esterases display hydrolytic activity towards a broad variety of esters, e.g. lipases often have other activities such as steryl esterase, phospholipase (EC 3.1.1.4) or cutinase (EC 3.1.1.74). Thus, esterases cannot be classified solely based on their substrate specificities; analyses of their sequence and structure are also needed.

1.1.2 Steryl esterases

Steryl esterases are defined as enzymes that catalyse the hydrolysis of fatty acid esters of sterols (Figure 4D, p. 28). Steryl esterase activity is typically assayed using different cholesteryl esters, such as cholesteryl oleate and cholesteryl linoleate, as substrates and thus the term “cholesteryl esterase” is generally used to describe this enzymatic activity. The term “steryl esterase” has a broader meaning since it describes enzymatic activity against any fatty acid esters of sterols, e.g. plant-derived steryl esters.

Steryl esterase activities have been examined in a variety of mammalian tissues principally because of their involvement in the absorption and metabolism of cholesterol in living organisms. Microbial steryl esterases have been detected and characterised e.g. from filamentous fungus *Ophiostoma piceae* (Calero-Rueda *et al*., 2002), yeasts *Candida rugosa* (Kaiser *et al*., 1994), *Fusarium oxysporum* (Okawa and Yamaguchi, 1977; Madhosingh and Orr, 1981) and *Saccharomyces cerevisiae* (Taketani *et al*., 1981), and bacteria *Pseudomonas aeruginosa* (Sugihara *et al*., 2002), *Pseudomonas fluorescens* (Uwajima and Terada, 1976) and *Streptomyces lavendulae* (Kamei *et al*., 1979) (Table 1). Steryl esterases, such as those of *O. piceae* and *P. aeruginosa*, generally have broad substrate specificities and are capable of hydrolysing different triglycerides in addition to steryl esters (Calero-Rueda *et al*., 2002; Sugihara *et al*., 2002). However, some steryl esterases, such as those from *P. fluorescens* (Uwajima and Terada, 1976) and *S. cerevisiae* (Taketani *et al*., 1981), are specific for steryl esters and do not possess lipase activity.

1.1.3 Reactions catalysed by lipases

Lipases catalyse particularly the hydrolysis of the ester bond of tri-, di- and monoacylglycerols (glycerides). Generally lipases have a preference for tri- and diglycerides rather than monoglycerides (Svendsen, 2000). Lipases are often 1,3-regioselective, acting rather on the positions *sn*-1 and *sn*-3 (end positions) than on *sn*-2 (mid position). Although naturally occurring triglycerides are preferred substrates, lipases also catalyse the hydrolysis or synthesis of a rather broad range of other substrates containing ester linkages, such as aliphatic, alicyclic, bicyclic and aromatic esters (Schmid and Verger, 1998). Lipases are also effective biocatalysts for the acylation and deacylation of a wide range of synthetic substrates, while still showing high regioselectivity and chiral recognition. The catalytic action of lipases is affected by the water content of the reaction mixture. In aqueous environments lipases catalyse the hydrolysis reactions, whereas in organic solvents they catalyse several other types of biotransformations, e.g. the formation of esters (esterification) and the exchange of ester bonds (transesterification) (Figure 1).

Table 1. Biochemical properties of microbial steryl esterases.

Organism	Mw (kDa)	pI	Substrate preference[a]	pH optimum	pH stability	Thermostability	Reference
Fungi							
Fusarium oxysporum	n.d.	n.d.	18:2	7.0	4–10 (25°C, 20 h)	50°C (90% after 90 min)	Okawa and Yamaguchi, 1977
Fusarium oxysporum	60 (I)[b]; 15 (II)[b]; 75 (III)[b]	n.d. n.d. n.d.	18:2	4.8 (I), 8.0 (II), 7.0 (III)	n.d. n.d. n.d.	n.d. n.d. n.d.	Madhosingh and Orr, 1981
Ophiostoma piceae	56.5[c]	3.3	18:2	6–8 (*p*-NPB)	3.5–6.5	30°C (80% after 24 h)	Calero-Rueda *et al.*, 2002
Yeasts							
Candida rugosa	56	5.2		7–8	3–7	50°C (pH 5, 10 min)	Meito Sangyo, product information
Saccharomyces cerevisiae	70[b]	n.d.	18:1	4.4 and 6.8	n.d.	n.d.	Taketani *et al.*, 1978; 1981
Bacteria							
Pseudomonas aeruginosa	58[c]; 53[b]	3.2	18:2	5.5–9.5	5–10 (25°C, 19 h)	53°C (pH 7, 30 min)	Sugihara *et al.*, 2002
Pseudomonas fluorescens	128[b] (I and II)	3.8 (I), 4.9 (II)	18:2	7.3	5–12 (30°C, 30 min)	60°C (pH 7, 30 min)	Uwajima and Terada, 1976
Staphylococcus aureus	25.5[c]; 175[b]	9.1	n.d.	n.d.	n.d.	n.d.	Harvie, 1977
Streptomyces lavendulae	29[c]	n.d.	18:2	6.0	5.5–8.0 (37°C, 1 h)	n.d.	Kamei *et al.*, 1977

n.d., not determined
a) Fatty acid specificity in hydrolysis of cholesteryl esters
b) Molecular weight determined by gel filtration
c) Molecular weight determined by SDS-PAGE

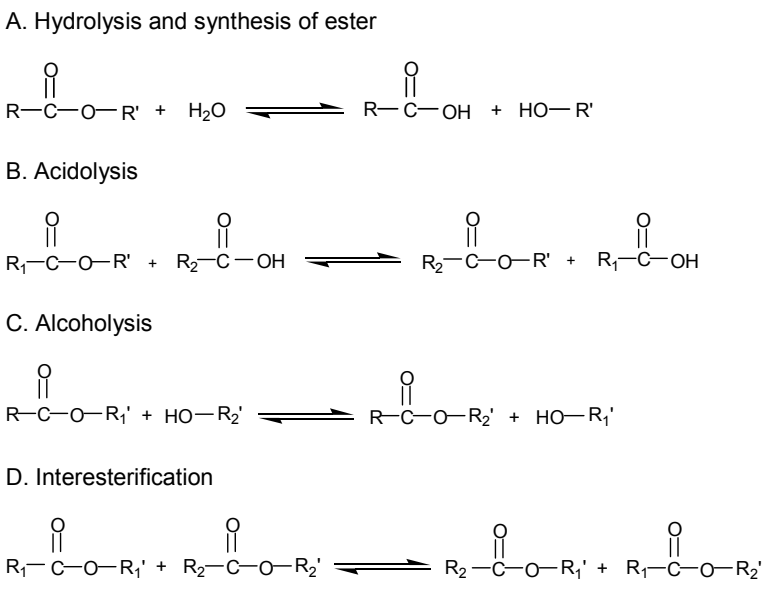

Figure 1. Reversible lipase-catalysed reactions. (A) Hydrolysis and synthesis reactions. Reactions B, C and D are trans-esterification reactions: (B) transfer of an acyl group to a fatty acid, (C) transfer of an acyl group to an alcohol, (D) transfer of an acyl group to a fatty acid ester (ester exchange).

1.1.4 Structure and hydrolysis mechanism of esterases

All known lipases, as well as other esterases and hydrolytic enzymes, belong to the family of α/β-hydrolases, which share a characteristic fold of their central catalytic domain (Ollis *et al*., 1992). The α/β-hydrolases share a common fold composed of a central hydrophobic eight-stranded β-sheet packed between two layers of amphiphilic α-helices, and a common catalytic mechanism composed of five subsequent steps (Cygler *et al*., 1994). The active sites of lipases are composed of serine, aspartate/glutamate and histidine residues, which form a catalytic triad similar in arrangement to those of serine proteases (Brady *et al*., 1990; Schmid and Verger, 1998). After binding of the substrate, oxygen of the serine attacks the carbonyl group of the substrate and a transition state is reached. This enzyme-substrate complex is stabilised by two or three hydrogen bonds within a pocket on the enzyme called the oxyanion hole (Cygler *et al*., 1994; Cygler and Schrag, 1999). Subsequently, the ester bond of the substrate is

cleaved, the alcohol moiety leaves the enzyme and a new ester linkage between the carbonyl carbon and the enzyme is formed. In the last step, the ester linkage of the acyl-enzyme intermediate is hydrolysed and the enzyme is regenerated. Aspartate/glutamate and histidine are needed to mediate the nucleophilic attack of the catalytic serine. Many lipases and esterases possess a short consensus sequence around the active serine residue, Gly-X-Ser-X-Gly (Antonian, 1988; Derewenda and Sharp, 1993; Pleiss *et al*., 2000). However, a novel sub-family of lipolytic enzymes has recently been shown to have a different motif (Gly-Asp-Ser-Leu) (Akoh *et al*., 2004).

The activity of lipases is low on monomeric (dispersed) substrates but strongly enhanced on aggregated triglycerides (e.g. emulsion or micelles) formed above the saturation limit (Figure 2). This enhanced activity, a phenomenon known as interfacial activation (Sarda and Desnuelle, 1958; Schmid and Verger, 1998), distinguishes lipases from esterases acting only on water-soluble carboxylic ester molecules. The first two three-dimensional (3D) structures of lipase, human pancreatic lipase (Winkler *et al*., 1990) and *Rhizomucor miehei* lipase (Brady *et al*., 1990), revealed that a surface loop, called the lid or the flap, covers the active site serine. Binding of lipase to a lipid-water interface induces a conformational change in the surface loop of the enzyme, rendering the active site accessible to the substrate (Brzozowski *et al*., 1991; Cygler and Schrag, 1997; Jaeger and Reetz, 1998; Schmid and Verger, 1998). Not all lipases, e.g. those from *Pseudomonas glumae*, *P. aeruginosa* and *Candida antarctica* B, show interfacial activation although they have an amphiphilic lid covering the active site (Verger, 1997). Lipases may also show activation only with certain substrates (van Oort *et al*., 1989).

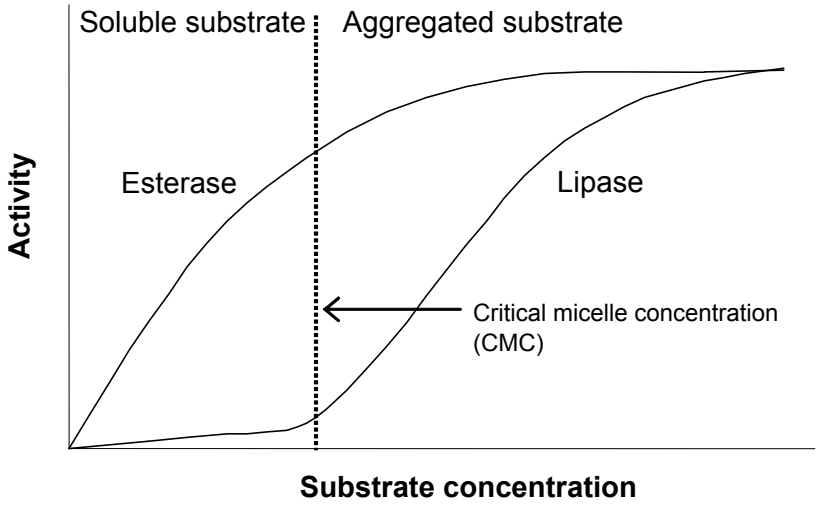

Figure 2. Interfacial activation mechanism of lipases (Verger, 1997).

Esterases and lipases share similar overall architecture and catalytic mechanism of ester hydrolysis, but they have different substrate specificities for the acyl moiety (Pleiss *et al*., 1998; Fojan *et al*., 2000). This can be explained by the size of their acyl binding sites. Lipases have a large, hydrophobic scissile fatty acid binding site for medium and long chain fatty acids, whereas esterases have a small acyl binding pocket, in which the acyl moiety of their substrates fits optimally. Lipases can be sub-divided into three sub-groups according to the geometry of the binding site: a crevice-like binding site near the surface (*Rhizomucor miehei* family), a funnel-like binding site (*Burkholderia cepacia* family, *Candida antarctica* lipase and *Fusarium solani* cutinase) or a tunnel-like binding site (*Candida rugosa* family) (Pleiss *et al*., 1998; Schmidt-Dannert, 1999). The length of the scissile fatty acid binding site varies between 7.8 Å in *F. solani* cutinase and 22 Å in *C. rugosa* and *R. miehei* lipases, and the length was observed to correlate with the fatty acid chain length profiles of the lipases (Pleiss *et al*., 1998). In addition, only a small number of amino acid residues line the scissile fatty acid binding site inside the binding pocket. Common to all lipases is the arrangement of the hydrophobic residues around the active site, which has been suggested to be crucial for the attachment of the lipase to the aggregated substrate (Fojan *et al*., 2000). Despite the high sequence homology between *C. rugosa* lipases (LIP1 and LIP3), having different substrate specificities, the different amino acids were not homogeneously distributed but were concentrated either near the active site, at the dimer interface or within the

hydrophobic core of LIP3 (Lotti *et al*., 1994; Pernas *et al*., 2001). These changes enhance hydrophobicity in the binding pocket of LIP3 and increase the flexibility of its flap, and thus account for the high tendency of LIP3 to dimerize (Pernas *et al*., 2001).

1.1.5 Biochemical properties of steryl esterases and lipases

Most microbial lipases exhibit maximum activity in the temperature range 30–40°C (Malcata *et al*., 1992). Thermostable lipases, that are desired for many industrial applications, have been isolated from microorganisms including bacteria *Pseudomonas* sp. KWI-56 (Iizumi *et al*., 1990), *P. fluorescens* AK102 (Kojima *et al*., 1994) and *Bacillus thermocatenulatus* (Schmidt-Dannert *et al*., 1994) and filamentous fungi *Thermomyces lanuginosus* (Ibrahim *et al*., 1987) and *Aspergillus niger* (Sugihara *et al*., 1988). These enzymes retain activities typically in the temperature range 50–65°C for varying incubation times (1–24 h). Many characterised steryl esterases have also been reported to be stable at temperatures up to 50–60°C (Table 1). Some lipases, such as these of *Bacillus* sp. A30-1 and *Thermus thermophilus*, have been shown to be stable at even higher temperatures, 75–85°C (Wang *et al*., 1995; Fuciños *et al*., 2005). The thermal stability of a lipase is obviously related to its structure, e.g. a single amino acid substitution in the lid has been shown to have a significant impact on the thermal stability, secondary structure and activity of *T. lanuginosus* lipase (Zhu *et al*., 2001). Thermal stability is also affected by environmental factors such as pH and the presence of metal ions or substrate, as well as by immobilisation (Xu *et al*., 1995; Reetz *et al*., 1996; Sharma *et al*., 2001).

Maximum activity of most microbial lipases is displayed in the pH range 5.6–8.5, and maximum stability in the neutral pH range (Malcata *et al*., 1992). The pH-optima for most characterised steryl esterases also vary around 6–8 (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Calero-Rueda *et al*., 2002). Some clearly alkaline lipases having pH optima around 9.5 have been found e.g. from *Bacillus* and *Pseudomonas* species (Watanabe *et al*., 1977; Wang *et al*., 1995; Ghanem *et al*., 2000). The steryl esterase from *P. aeruginosa* has also been shown to be highly active even at pH 9.5 (Sugihara *et al*., 2002). Several *Bacillus* lipases are stable at pH 9–11 (Wang *et al*., 1995; Schmidt-Dannert *et al*., 1996).

Biochemical characterisation of lipases and steryl esterases typically involves studying the effects of metal ions and surfactants on enzyme action. According to literature data the bile acid salts, e.g. cholate and taurocholate, typically increase the lipase and steryl esterase activities (Rúa *et al*., 1997; Hiol *et al*., 2000; Sugihara *et al*., 2002). Non-ionic surfactant, Triton X-100, also activates several lipases and steryl esterases (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Rúa *et al*., 1997). Salts of heavy metals (Fe^{2+}, Zn^{2+}, Hg^{2+}, Fe^{3+}, Cu^{2+}, Ag^{+}) typically strongly inhibit lipases, suggesting that metal ions are able to alter the enzyme conformation (Sharon *et al*., 1998; Hiol *et al*, 2000). Several cations, such as Na^{+}, Mg^{2+}, Mn^{2+}, and especially Ca^{2+}, have been reported to have enhancing effect (Höfelmann *et al*., 1985; Gao and Breuil, 1998) or no effect on the lipolytic activity (Kojima *et al*., 1994; Mase *et al*., 1995). However, an inhibitory action of calcium has also been reported (Ohnishi *et al*., 1994; Wu *et al*., 1996). Calcium has been shown to have only a slight effect on microbial steryl esterases (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Sugihara *et al*., 2002).

Certain microbial species produce multiple lipases, which differ in molecular weights or substrate specificities and may have different pH and thermal stability properties. The multiple forms are due both to post-transcriptional processing such as proteolytic degradation and glycosylation, and to the synthesis of truly different lipases (Sugihara *et al*., 1990). It is known that microorganisms, particularly yeasts and filamentous fungi such as *Candida rugosa*, *Geotrichum candidum*, *Aspergillus niger* and *Rhizopus niveus*, secrete different lipase isoforms in varying amounts depending on the culture conditions (Höfelmann *et al*., 1985; Sugihara *et al*., 1990; Chang *et al*., 1994; Kohno *et al*., 1994). *C. rugosa* secretes a mixture of lipase isoforms encoded by separate genes that are expressed constitutively or regulated by the fatty acid content of the medium (Benjamin and Pandey, 1998; Lotti *et al*., 1998; Lee *et al*., 1999). Five of them (LIP1-LIP5) have been fully characterised and they have been shown to have high sequence identity, ranging between 77 and 88% (Longhi *et al*., 1992; Lotti *et al*., 1993; Lotti *et al*., 1994). They have differences in their biochemical properties, such as in substrate specificities (Lotti *et al.*, 1994). Only LIP3 has been reported to be capable of hydrolysing cholesteryl esters, and thus it is also classified as a steryl (cholesteryl) esterase (Kaiser *et al*., 1994).

1.1.6 Molecular biology of steryl esterases and lipases

Microbial steryl esterase genes have been cloned and characterised only from yeast *Candida rugosa* and bacterium *Streptomyces lavendulae* (Lotti *et al*., 1993; Nishimura and Sugiyama, 1994). However, many genes encoding mammalian steryl (cholesteryl) esterases have been cloned and sequenced, e.g. human (Nilsson *et al*., 1990), bovine (Kyger *et al*., 1989), rat (Kissel *et al*., 1989) and mouse (Mackay and Lawn, 1995) pancreatic steryl esterases. They have a typical lipase/esterase-related consensus sequence Gly-X-Ser-X-Gly. The genes of several bacterial and fungal lipases have been cloned and the corresponding protein products have been experimentally characterised. Some of these lipase genes are shown in Table 2. In addition to these genes, several lipase genes have been found in genome sequencing projects and have been annotated on the basis of sequence homology with known lipases but not characterised at the enzyme level.

Lipases can be classified into four main families according to their similarities (Schmidt-Dannert, 1999). Fungal lipases are classified into *C. rugosa* and *R. miehei* families, and bacterial lipases into *Burkholderia cepacia* and *Staphylococcus* families (Table 2, Figure 3). Due to the growing lipase sequence data several extensions of the original classification scheme have recently been proposed (Pleiss *et al*., 2000; Jaeger and Eggert, 2002).

Table 2. Examples of lipase genes encoding well-known biochemically characterised lipase proteins.

Organism		Protein (gene)		Mature protein encoded by the gene		Reference
		Name	EMBL Acc. No.	Length (aa)	Mw (kDa)[1]	
I.	*Candida rugosa* family, formerly *Candida cylindracea*					
	Candida rugosa	LIP1 (*lip1*)	P20261	534	60	Longhi *et al*., 1992; Benjamin and Pandey, 1998
		LIP2 (*lip2*)	P32946	534	60	
		LIP3 (*lip3*)	P32947	534	60	Lotti *et al*., 1993, 1994; Benjamin and Pandey, 1998
		LIP4 (*lip4*)	P32948	534	60	
		LIP5 (*lip5*)	P32949	534	60	
	Geotrichum candidum	LIP1 (*lip1*)	P17573	544	64	Sugihara *et al*., 1990; Nagao *et al*., 1993
		LIP2 (*lip2*)	P22394	544	66	
II.	*Rhizomucor miehei* family					
	Rhizomucor miehei	RML-A	P19515	269	32	Huge-Jensen *et al*., 1987; Boel *et al*., 1988
	Rhizopus oryzae	LIP	P61872	269	32	Haas *et al*., 1991; Beer *et al*., 1998; Hiol *et al*., 2000
	Thermomyces lanuginosus, formerly *Humicola lanuginosa*	HLL	O59952	269	39	Ibrahim *et al*., 1987; Boel *et al*., 1998
III.	*Burkholderia cepacia* family, formerly *Pseudomonas cepacia*					
	Pseudomonas aeruginosa	(*lipA*)	P26876	285	30	Jaeger *et al*., 1992; Reetz and Jaeger, 1998
	Burkholderia glumae, formerly *Chromobacterium viscosum*	LIPA (*lipA*)	Q05489	319	33	Taipa *et al*., 1995; Reetz and Jaeger, 1998
IV.	*Staphylococcus* family					
	Bacillus thermocatenulatus	BTL2	No entry	388	43	Schmidt-Dannert *et al*., 1996
	Staphylococcus epidermidis	- (*gehSE1*)	Q02510	386	43	Simons *et al*., 1998
V.	Others					
	Aspergillus oryzae	L3 (*tglA*)	No entry	224	25	Toida *et al*., 2000
	Pseudomonas fluorescens	LIPA	P26504	426	50	Chung *et al*., 1991; Reetz and Jaeger, 1998

1) determined by SDS-PAGE

		1	2	3	4	5	6	7	8	9
Candida rugosa (LIP1)	1	100	63	47	48	48	44	47	43	44
Geotrichum candidum (LIP1)	2		100	43	48	48	44	46	40	40
Burkholderia glumae	3			100	89	61	47	49	42	40
Burkholderia cepacia	4				100	60	43	49	41	40
Pseudomonas fragi	5					100	52	49	41	40
Staphylococcus hyicus	6						100	69	46	45
Staphylococcus aureus	7							100	42	45
Thermomyces lanuginosus	8								100	56
Rhizomucor miehei	9									100

Figure 3. Pairwise similarity (%) of microbial lipases (Svendsen, 1994). Similarity > 50% are shown boxed.

The size of the smallest fungal lipases is in the range of 20–25 kDa whereas the largest are 60–65 kDa (Table 2). Lipases from *R. miehei*, *C. rugosa* and *G. candidum* are composed of 269-, 534- and 544-amino acid residues, respectively. The protein-encoding regions of *C. rugosa* and *G. candidum* lipase genes contain no introns. *R. miehei* lipase is intervened by one intron of 74 basepairs in length. The bacterial lipases are generally proteins of around 300–400 amino acids. Fungal lipases typically contain a signal peptide of about 20 amino acids, whereas the lengths of bacterial signal peptides often vary between 30 and 40. Some fungal lipases contain cleavable propeptides from only a few to several dozens of amino acids. Propeptides of bacterial lipases, such as the *Staphylococcus* lipases, may be even longer.

Dimerisation that has an effect on enzyme kinetics has been shown to be an intrinsic property of *C. rugosa* isoenzyme LIP3 (Kaiser *et al*., 1994; Ghosh *et al*., 1995; Pernas *et al*., 2001). Formation of enzyme aggregates, which are composed of subunits identical in molecular weight, is typical for both bacterial and fungal lipases due to hydrophobic interactions between lipase monomers (Dünhaupt *et al*., 1992; Sztajer *et al*., 1992; Taipa *et al*., 1994; Rúa *et al*., 1997; Brush *et al*., 1999). Different surfactants and organic solvents have typically been used to break up high molecular weight lipase aggregates.

1.1.7 Heterologous production of lipases

Lipases have been studied extensively, as they are potential enzymes for many industrial applications (Jaeger and Reetz, 1998; Schmid and Verger, 1998;

Benjamin and Pandey, 1998; Pandey *et al*., 1999). There are several commercial lipases on the market that have been produced heterologously by recombinant microorganisms (Schmid and Verger, 1998). Purification of native enzymes is often laborious due to the hydrophobic nature of lipases and the presence of several similar isoforms. Thus, the use of suitable expression systems enables the effective production of large amounts of individual active lipases. In 1988, Novo Nordisk (currently Novozymes) introduced the first commercial recombinant lipase 'Lipolase', which originated from the filamentous fungus *Thermomyces lanuginosus* and was expressed in *Aspergillus oryzae* (Boel *et al*., 1996). At present, the genes of several bacterial and fungal lipases have been cloned, and efficient heterologous expression systems have been set up for their production (Table 3).

Table 3. Lipase production in heterologous hosts.

Lipase	Production host	Lipase production (mg l^{-1})	Reference
Candida antarctica (LIPB)	*Pichia pastoris*	25	Rotticci-Mulder *et al*., 2001
Candida antarctica (LIPA, LIPB)	*Aspergillus oryzae*	-[a]	Hoegh *et al*., 1995
Candida rugosa (LIP1)	*Pichia pastoris*	-[a]	Brocca *et al*., 1998
Candida rugosa (LIP4)	*Pichia pastoris*	100	Tang *et al*., 2001
Geotrichum candidum (LIPI, LIPII)	*Pichia pastoris*	60	Holmquist *et al*., 1997
Geotrichum candidum (LIPA, LIPB)	*Pichia pastoris*	538[b]	Catoni *et al*., 1997
Rhizopus oryzae (ROL)	*Saccharomyces cerevisiae*	28	Ueda *et al*., 2002
Rhizopus oryzae (ROL)	*Pichia pastoris*	59, 156[b]	Minning *et al*., 1998, 2001
Rhizopus oryzae (ROL)	*Pichia pastoris*	16 730[bc], 19[bd]	Resina *et al*., 2004
Rhizopus niveus (RNL)	*Saccharomyces cerevisiae*	200–300	Kohno *et al*., 1999
Rhizomucor miehei (RML)	*Aspergillus oryzae*	-[a]	Huge-Jensen *et al*., 1989
Thermomyces lanuginosus (HLL)	*Aspergillus oryzae*	-[a]	Boel *et al*., 1996
Bacillus thermocatenulatus (BTL2)	*Pichia pastoris*	13[b]	Quyen *et al*., 2003
Bacillus thermocatenulatus (BTL2)	*Escherichia coli*	660 000[c]	Rúa *et al*., 1998
Burkholderia cepacia (PCL)	*Escherichia coli*	314 000[c]	Quyen *et al*., 1999

a) Expression level has not been reported
b) The reported expression levels have been obtained in a laboratory scale fermenter, other values have been obtained in shake flask cultivations
c) Yield of active lipase per gram of biomass (U g^{-1})
d) Lipolytic activity (U ml^{-1})

C. rugosa obeys a non-universal codon usage where the triplet CUG (a universal codon for leucine) is read as serine. The use of synthetic codon-optimised nucleotide sequences has enabled the functional overexpression of *C. rugosa* lipases, LIP1 and LIP4 e.g. in *Pichia pastoris* (Brocca *et al*., 1998; Tang *et al*., 2001). Several other lipases, from e.g. *G. candidum*, *C. antarctica*, *Rh. oryzae* and *B. thermocatenulatus*, have also successfully been expressed in *P. pastoris* (Table 3). Production of larger amounts of lipases have been obtained by fermentations using optimal operating conditions and feeding strategies (Catoni *et al*., 1997; Minning *et al*., 2001). Recombinant lipases produced in *P. pastoris* generally have well-preserved enzymatic characteristics (Minning *et al*., 1998; Holmquist *et al*., 1997; Rotticci-Mulder *et al*., 2001; Quyen *et al*., 2003).

The filamentous fungus *Aspergillus oryzae*, which is a well-known industrial production host for heterologous proteins, has also been used for expression of lipases at production levels far exceeding those in the donor organism (Huge-Jensen *et al*., 1989; Hoegh *et al*., 1995; Yaver *et al*., 2000). The lipases of *C. antarctica* and *T. lanuginosus*, which have successfully been secreted from *A. oryzae*, were shown to differ from the native lipases in having more extensive glycosylation, but the enzymatic characteristics were unaltered (Hoegh *et al*., 1995; Boel *et al*., 1996). *Saccharomyces cerevisiae* has been used successfully for expression of *Rhizopus* lipases (Kohno *et al*., 1999; Ueda *et al*., 2002).

Pseudomonas and *Burkholderia* lipases are among the most important lipases in biotechnological applications (Jaeger *et al*., 1994; Jaegert and Eggert, 2002). However, many of them are not amenable to conventional heterologous expression systems since their folding and secretion are complex and highly regulated processes requiring a specific chaperone (Reetz and Jaeger, 1998; Rosenau and Jaeger, 2000). Typically, these lipases have been expressed homologously in their original hosts. Co-expression of lipase-specific foldases has enhanced lipase secretion in homologous expression systems, or enabled heterologous production of enzymatically active lipase, e.g. in *Escherichia coli* (Jaeger *et al*., 1997; Gerritse *et al*., 1998; Reetz and Jaeger, 1998; Jaeger and Eggert, 2002).

1.2 Lipophilic compounds of plants

1.2.1 Biological functions of lipids in plants

Lipids are a chemically and functionally diverse group of compounds which share pronounced hydrophobicity as a common feature. Typically, lipids are fatty substances with long hydrocarbon chains and often contain ester linkages in the molecule (Harwood, 1980; Lehninger *et al*., 1993). Plant lipids can be divided into three classes according to their structure and role: storage, membrane and cuticular lipids. Fats and oils, in which three fatty acids are attached to a glycerol molecule by ester bonds, are the principal forms of long-term energy store. Triglycerides are found in the seeds of most plants which are sources of vegetable oils. Glycolipids and phospholipids are important membrane lipids. In phospholipids a polar group is joined through a phophodiester bond to the backbone, whereas glycolipids have one or more sugars connected to the backbone. Sterols also form an important group of membrane lipids. The amphipathic property of membrane lipids directs their packing into lipid bilayers. Cuticular lipids are a complex mixture of waxy compounds which protects the cell from environmental damage and pathogenic invasion during growth (Kolattukudy, 1980). Esters of long-chain aliphatic acids and alcohols form waxes, and interesterified long chain hydroxy and epoxy fatty acids form biopolyester (cutin). In addition, other lipids such as hundreds of steroids and isoprenoids act as enzyme cofactors, electron carriers, light-absorbing pigments, hydrophobic anchors, emulsifying agents, hormones and intracellular messengers (Lehninger *et al*., 1993).

1.2.2 Wood extractives

The main components of wood are cellulose, hemicelluloses and lignin, but wood also contains minor amounts of extractives. The amount and composition of extractives vary between different wood species and even different wood parts and their proportion is typically 0.2–5% of dry wood (Allen, 1988; Fengel and Wegener, 1989). Wood extractives include a large number of different compounds which are poorly soluble in water and are commonly referred to as wood resin or wood pitch. The main lipophilic (hydrophobic) compounds are free fatty acids, resin acids, sterols (and other triterpenyl alcohols) and different fatty acid esters, of which triglycerides and steryl esters are the most abundant

(Figure 4; Örså and Holmbom, 1994). In addition, lipophilic extractives include a large group of several terpenes, terpenoids and waxes (Fengel and Wegener, 1989; Back and Ekman, 2000). Due to their lipophilic character, pitch compounds are extractable with neutral solvents such as dichloromethane, diethyl ether, ethanol and hexane (Sjöström, 1993; Holmbom, 1998). In addition to the lipophilic compounds, wood extractives also include water-soluble substances, e.g. carbohydrates and inorganic compounds, as well as phenolic compounds, such as lignans, tannins and flavonoids (Fengel and Wegener, 1989; Sjöström, 1993). Extractives are predominant contributors to wood colour, fragrance and durability, and they protect trees from microbial damage (Fengel and Wegener, 1989; Umezawa, 2001).

In woody plants, the extractives occur in ray parenchyma (storage) cells and in resin canals or resin pockets only (Back, 2000). The composition of canal resin differs significantly from that of resin in the living parenchyma cells. Canal resin is generally an amorphous mixture of cyclic terpenes and terpenoids, such as resin acids and volatile terpenes. Parenchyma resin is composed of fatty acid esters, such as glycerides, steryl esters, and some alkanol esters (Back, 2000). The sterols occur as steryl esters in the living wood parenchyma cells of all common pulpwoods (Back and Ekman, 2000).

1.2.3 Chemical structures of lipophilic wood extractives

Isoprene is the basic unit (C_5H_8) of terpenes, which in turn form the body of several extractives (Fengel and Wegener, 1989). Resin acids, which are for example built from four isoprene units (C_{20}), are diterpenes or diterpenoids, i.e. diterpene incorporated with an oxygen-containing functional group, such as carboxyl (Figure 4A). Sterols are biosynthetically derived from squalene, which is composed of six isoprene units (C_{30}), and form a group of triterpenes (triterpenoids). The steroid nucleus consisting of four fused rings, three with six carbons and one with five, forms the characteristic structure of sterols (Figure 4B). The steroid nucleus is almost planar and relatively rigid, i.e. the fused rings do not allow rotation about C-C bonds. All wood sterols have a hydroxyl group at the C-3 position and a flexible side chain of variable length at the C-17 position (Back and Ekman, 2000). Sterols can have methyl groups and double bonds at various positions in the steroid nucleus or the side chain (Figure 5A). Most plant sterols are alkylated at the C-24 position (Bramley, 1997), whereas

alkyl side chain does not exist in cholesterol. In most wood sterols, the number of carbon atoms varies between 28 and 32 (Back and Ekman, 2000). Triterpenyl alcohols are similar to sterols but have a non-steroidal, typically pentacyclic, ring system (Figure 5B). The most common sterols and stanols (saturated derivatives of sterols) both in softwood and hardwood resin are sitosterol, campesterol, sitostanol, campestanol (4-desmethyl sterols), citrostadienol (4-methyl sterol) and cycloartenol, 24-methylene cycloartenol and butyrospermol (4,4-dimethyl sterols) (Ekman and Holmbom, 2000). Hardwoods, such as birch and aspen, contain significant amounts of triterpenyl alcohols (betulinol, amyrin and lupeol), which all carry two methyl substituents at the C-4 position (Figure 5B).

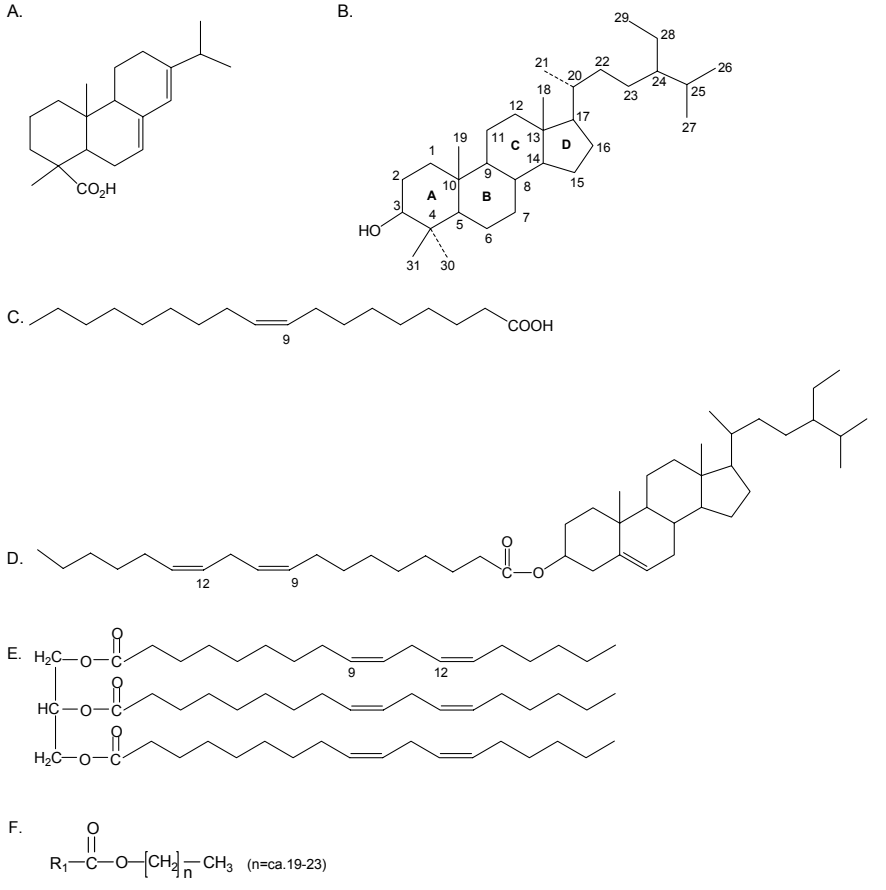

Figure 4. Structures of major wood pitch components. A) Abietic acid; resin acid, B) basic sterol nucleus, C) oleic acid; fatty acid, D) sitosteryl linoleate; steryl ester, E) trilinolein; triglyceride, and F) general formula of "wax", in which R_1 is a fatty acid chain.

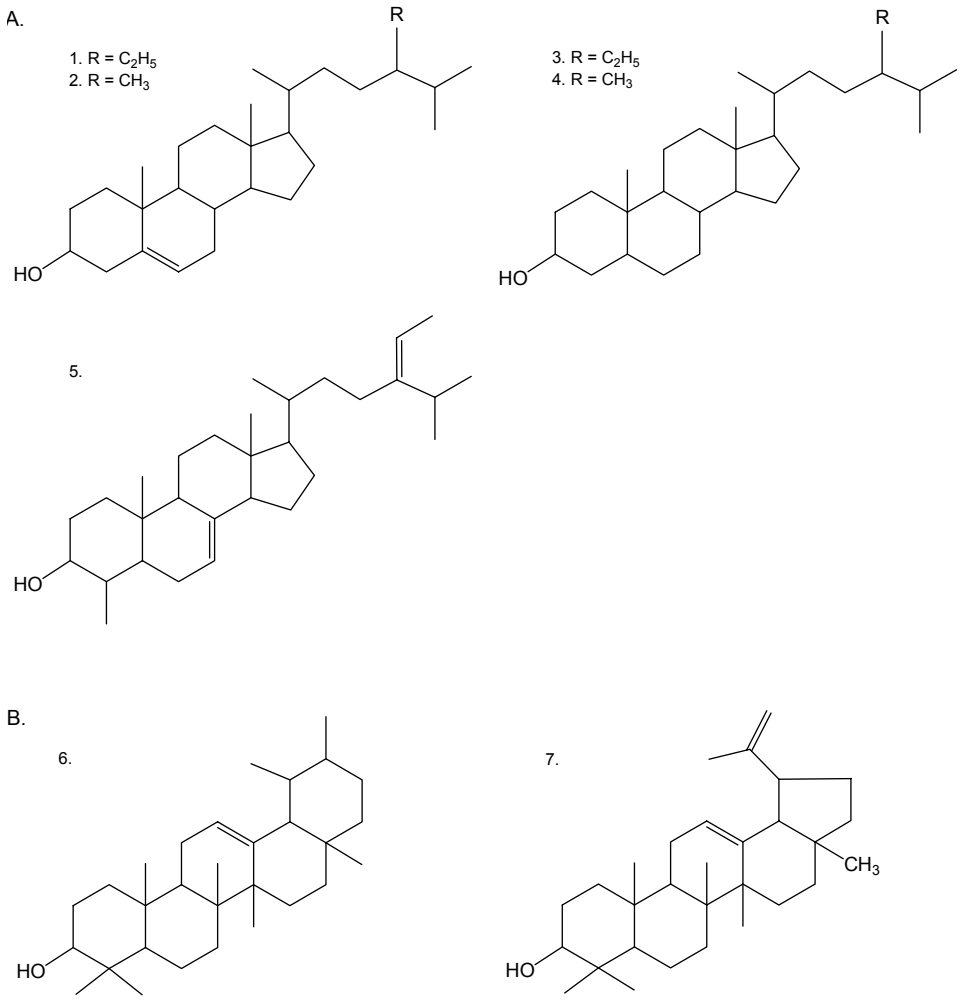

Figure 5. Structures of some common A) sterols and stanols and B) nonsteroidal triterpenyl alcohols occurring in wood. (1) Sitosterol, (2) campesterol, (3) sitostanol, (4) campestanol, (5) citrostadienol, (6) α-amyrin, and (7) lupeol.

The C-3 hydroxyl group of free sterols (and triterpenyl alcohols) may be esterified by a fatty acid and form steryl esters (and triterpenyl esters) (Figure 4D). Glycerides (fats) are esters of saturated and unsaturated monocarboxylic fatty acids with glycerol (Figure 4E). Most fats are triglycerides; only minor amounts of mono- and diglycerides exist. Waxes are long-chain alcohols ($>C_{20}$) and their esters, which are primarily fatty acid esters (Figure 4F). These alcohols can exist as hydroxyl fatty acids (Back and Ekman, 2000). Alkanes and other linear hydrocarbons are also included in waxes.

Aliphatic straight-chain saturated and unsaturated monocarboxylic fatty acids with 16–24 carbon atoms are the most important fatty acids in woody plants (Ekman and Holmbom, 2000). Fatty acids exist both as esters and in free form (Figure 4C–F). The most common fatty acids both in softwood and hardwood are the unsaturated C18 acids, of which oleic (9-18:1), linoleic (9,12-18:2) and linolenic (9,12,15-18:3) acids are major components. Saturated palmitic (16:0), 14-methyl hexadecanoic acid (17:0ai), stearic (18:0), behenic (22:0) acids, and varieties of unsaturated fatty acids, such as vaccenic (11-18:1) acid are also present in wood.

1.2.4 Role and occurrence of plant sterols and steryl esters

Sterols are ubiquitous in nature and have been detected in fungi, algae, plants and animals (Nes and McKean, 1977). Over 250 different plant sterols (phytosterols) and related compounds have been reported to exist in different plants, and they have been observed in all types of plant tissue (Akihisa *et al*., 1991; Dyas and Goad, 1993). Plant lipids contain 0.15–0.9% sterols, in which sitosterol is the main component followed typically by campesterol and stigmasterol (Belitz and Grosch, 1999; Piironen *et al*., 2000; Abidi, 2004).

Sterols have a structural role in plant cell membranes and they also act as the precursors to several bioactive steroids (Nes, 1989; Dyas and Goad, 1993). Some plant sterols have a specific function in signal transduction (Piironen *et al*., 2000). Sterols occur in plants in free form but also as steryl conjugates, such as steryl esters, steryl glycosides and acylated steryl glycosides (Dyas and Goad, 1993). Plant steryl esters are located intracellularly and they represent mostly a storage form of sterols comparable to cholesteryl esters in mammalian cells (Dyas and Goad, 1993; Piironen *et al*., 2000).

Nutritionally, vegetable oils are the richest natural sources of sterols and steryl esters. Cereals, nuts and some vegetables are also rich in sterols (Piironen *et al*., 2000). Dietary plant sterols, and especially stanols (the saturated form of sterols), are known to have a cholesterol-lowering effect both in the free form and as fatty acid esters. Thus several studies concerning the nutritional and therapeutic effects of phytosterols and phytostanols have been reported (Ling and Jones, 1995; Gylling and Miettinen, 2000).

1.3 Behaviour and control of pitch in the pulp and paper industry

1.3.1 Pitch problems

Norway spruce (*Picea abies*) wood contains about 1% non-volatile lipophilic extractives which are mainly located in resin ducts and in parenchyma cells, as is typically the case in softwoods (Ekman *et al*., 1990). The fatty acid esters such as steryl esters and triglycerides are located in the parenchyma cells of sapwood i.e. the living part of the wood (Back and Ekman, 2000). During mechanical pulping of softwood, most of the extractives are dispersed as colloidal droplets and released into the process water because of the strong mechanical shear forces and the high temperature (Allen, 1975; Örså and Holmbom, 1994; Örså *et al*., 1997). The major components of lipophilic extractives in unbleached thermomechanical pulp (TMP) produced from Norway spruce are triglycerides, steryl esters and resin acids accounting for 47, 25 and 18% of the total amount of lipophilic extractives, respectively (Mustranta *et al*., 2001). The dispersed colloidal particles, ranging in size from 0.15 to 0.4 μm, are sterically stabilised by polysaccharides, i.e. galactoglucomannan, and are susceptible to aggregation and form deposits when their stability is disturbed (Figure 6; Nylund *et al*., 1993; Sundberg *et al*., 1994, 1996b; Sihvonen *et al*., 1998; Otero *et al*., 2000). Since mechanical pulp is not commonly washed, the dissolved and colloidal substances (DCS) are carried over to the paper machine (Örså *et al*., 1997). Decreased fresh water intake and increased process water circulation by closure of water systems leads to the accumulation of DCS, and thus increases the problems caused by extractives (Lindström *et al*., 1977; Zhang *et al*., 1999; Rundlöf *et al*., 2000a).

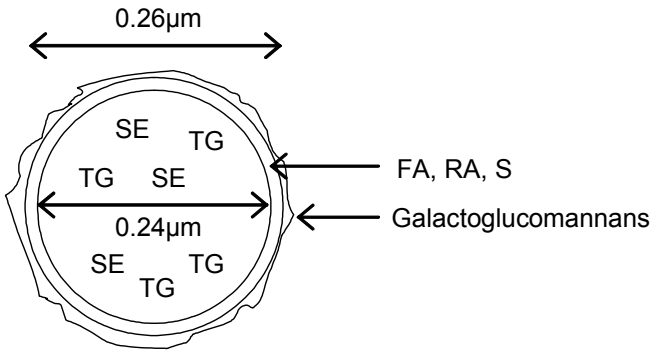

Figure 6. Sterically stabilised pitch particle. SE; steryl esters, TG; triglycerides, FA; fatty acids, RA; resin acids, S; sterols. (Adapted from Sundberg et al., 1996b; Qin et al., 2003).

The presence of DC substances during paper making can cause runnability problems, such as deposit formation in the different parts of the paper machine and in the paper. This in turn results in decreased screening, cleaning and dewatering efficiency, and interference with process chemicals (Allen, 1980; Allen, 1988; Pelton *et al*., 1980; Linhart *et al*., 1987; Dreisbach and Michalopoulos, 1989). Retention of DCS onto fibres affects paper product quality by decreasing the strength and optical properties of the paper (Lindström *et al*., 1977; Wearing *et al*., 1985; Sundberg *et al*., 2000). Particularly the adsorption of colloidal particles onto TMP fines decreases the tensile strength by preventing formation of bonds between the fibres (Rundlöf *et al*., 2000b; Francis and Ouchi, 2001). As individual compounds, triglycerides, fatty acid and steryl esters with long hydrocarbon chains have the greatest negative effect on tensile strength (Kokkonen *et al*., 2002). The presence of extractives can also result in odour and taste problems in food packaging materials. Some wood extractives may also be detrimental to the environment when released into the wastewaters (Leach and Thakore, 1976).

In typical kraft (chemical) pulping, many extractives, including triglycerides and some steryl esters, are hydrolysed (saponified) and then dissolved (Douek and Allen, 1978; Peng *et al*., 1999). However, some of the steryl esters and waxes, as well as free sterols, do not form soluble soaps under the alkaline conditions used in kraft pulping and are thus susceptible to be deposited and to cause pitch problems (Allen, 1988; Chen *et al*., 1995). Generally, extractives create more

problems in hardwood (such as aspen and eucalyptus) kraft pulps than in softwood pulps due to high amounts of the unsaponifiable extractives in hardwoods (Allen, 1988; Chen *et al*., 1995; Peng *et al*., 1999). Although significant amounts of extractives are modified during pulping and subsequent bleaching, a large proportion of free sterols, triterpenyl alcohols and fatty acids and their derivatives remain in the pulp. Therefore, the removal of detrimental compounds should be carried out before pulping (Allen, 1988; Chen *et al*., 1995; Peng *et al*., 1999).

1.3.2 Methods for pitch control

Traditional methods for reducing pitch problems include careful debarking, seasoning of wood chips prior to pulping, and addition of chemicals (Allen, 1980; Allen, 1988; Hassler, 1988; Shetty *et al*., 1994). Alternatively, biological pitch control by enzymatic treatment or by microbial pretreatment has been developed. Microbial pretreatment is based on the acceleration of natural wood seasoning by spraying selected microorganisms onto wood chips or logs. Fungi and bacteria are able to use extractives as carbon source and consume the resulting compounds (Blanchette *et al*., 1992; Leone and Breuil, 1999). Wood pretreatment with a colourless isolate of *Ophiostoma piliferum*, marketed as Albinex™ (formerly Cartapip™) has been shown to decrease triglycerides efficiently, but not steryl esters or waxes (Blanchette *et al*., 1992; Farrell *et al*., 1993; Brush *et al*., 1994). A number of fungi have also been screened and some of them have been shown to reduce the amounts of all lipophilic extractives, including steryl esters (Chen *et al*., 1994; Rocheleau *et al*., 1998; Leone and Breuil, 1999; Martínez-Iñigo *et al*., 1999; Gutiérrez *et al*., 1999, 2000; Dorado *et al*., 2001). Bacterial pretreatments have also been studied for reduction of wood extractives, including steryl esters, from woody raw materials (Kallioinen *et al*., 2003, 2004). The drawbacks of these methods are the rather long treatment time and the necessary adjustment of conditions, as well as decreased pulp yield and paper optical quality (Fischer *et al*., 1994; Farrell *et al*., 1997; Gutiérrez *et al*., 2001).

Enzymes are potential tools for DCS modification due to their specificity. Lipases can hydrolyse efficiently the triglycerides of extractives (Fujita *et al*., 1992; Fischer and Messner, 1992; Fischer *et al*., 1993; Mustranta *et al*., 1995; Hata *et al*., 1996). A commercial recombinant lipase expressed in *Aspergillus*

oryzae, Resinase^{R} (Novozymes), has been used for the treatment of red pine mechanical pulps for several years in Japan (Fujita *et al*., 1992; Chen *et al*., 2001). Despite the broad substrate specificity of lipases, the effects of Resinase and other industrial lipases studied for enzymatic pitch control are restricted to triglycerides (Mustranta *et al*., 1995, 2001; Hata *et al*., 1996; Fleet and Breuil, 1998). Resinase has not been shown to have any effect on steryl esters (Mustranta *et al*., 2001) having a significant negative impact on paper production due to their physical properties (Kokkonen *et al*., 2002). Simultaneous enzymatic degradation of triglycerides and steryl esters by novel esterases might provide a means of controlling pitch deposit formation in paper production. Steryl esterase from *Ophiostoma piceae* has recently been characterised and its potential for pitch biocontrol has been evaluated (Calero-Rueda *et al*., 2002, 2004). In addition to esterases/lipases, other enzymes such as cellulases, hemicellulases and laccases have also been studied for DCS modification (Kantelinen *et al*., 1995; Karlsson *et al*., 2001; Zhang, 2000; Zhang *et al*., 2000). The use of cellulases and laccases is based on destabilisation of colloidal particles, and polymerisation (and probably partial degradation) of the low molecular weight extractives, respectively.

1.4 Applications of lipolytic esterases

1.4.1 Lipases as versatile tools in biotechnology

Lipases are widely used in different industrial applications due to their broad substrate specificity and outstanding regio- and stereoselectivity under mild reaction conditions. Moreover, lipases often exhibit very good stability, remain enzymatically active in a wide range of organic solvents and do not usually require cofactors (Jaeger and Eggert, 2002). Lipases are used in the detergent, food, and pulp and paper industries as well as in the production of fine chemicals, pharmaceuticals and cosmetics (Vulfson, 1994; Mustranta, 1995; Schmid and Verger, 1998; Pandey *et al*., 1999).

In the food industry lipases have been used e.g. for the production of desirable flavours in dairy products, and for the modification of physical properties and of the nutritional and sensory value of products (Jaeger and Reetz, 1998). The synthesis of structured lipids, such as enrichment of lipids with n-3 type

polyunsaturated fatty acids (PUFA), has been exploited by the pharmaceutical industry, as well as by the health food industry as food supplements (Haraldsson, 2000). Lipases can also be used for the production of mono- and diglycerides for food emulsifiers, and for the generation of fatty acids from natural oils. Lipases can be used in the synthesis of medicines as biocatalysts for the preparation of chiral compounds in enantiomerically pure form (Pandey *et al*., 1999). Lipase-catalysed synthesis of various steryl esters used as ingredients of cosmetics and food products has also been studied (Jonzo *et al*., 1997; 2000; Shimada *et al*., 1999; Villeneuve *et al*., 2005). Lipases have been used for bioremediation purposes, e.g. in wastewater treatment (Pandey *et al*., 1999). Production of high-value chemicals and pharmaceuticals with novel synthetic routes is one of the most potential lipase applications in future (Jaegert and Eggert, 2002).

Commercial steryl esterases are primarily used for diagnostic measurement of total serum cholesterol and synthesis of optically active alcohols and carboxylic acids (White and White, 1997). Therefore they are available only as highly purified and expensive preparations. Recently, the use of a novel *P. aeruginosa* steryl esterase as a contact lens cleaner was proposed (Sugihara *et al*., 2002).

1.4.2 Novel substrates for lipolytic enzymes

In addition to the present industrial applications, lipolytic enzymes have been proposed as potential tools in different industrial fields due to their activity towards a broad variety of esters, from soluble to insoluble, and from natural to non-natural substrates. In recent years, lipolytic enzymes have shown promise in the modification of both natural and synthetic polymers. Degradable, biocompatible and biofunctional polymeric compounds are of interest in the biomedical and pharmacological, as well as environmental sectors. The possibility to use lipases and esterases in biodegradation of synthetic polymers has recently been reported (Howard, 2002; Marten *et al*., 2005; Yang *et al*., 2005; Tserki *et al*., 2006). The use of lipases and esterases as natural catalysts for synthesis and functionalisation of polymers is also a rapidly expanding area and is showing outstanding promise (Gross *et al*., 2001, Kobayashi *et al*., 2001; Gübitz and Cavaco-Paulo, 2003).

Polyethylene terephthalate (PET, Figure 7) is the most important synthetic fibre in the textile industry. It is used in many textile products due to its favourable characteristics, such as resistance to wrinkling, stretching, shrinking, abrasion and many chemicals (Yoon *et al*., 2002). However, PET is relatively hydrophobic, which impedes its processability such as finishing and coloring. Furthermore it has high tendency to pill and is resistant to removal of oil and grease stains due to its hydrophobic nature and inactive surface (Andersen *et al*., 1999; Yoon *et al*., 2002; Buschle-Diller, 2003). Lipases, esterases and cutinases, so-called polyesterases, have all shown promise for the surface modification of PET (Hsieh and Cram, 1998; Andersen *et al*., 1999; Yoon *et al*., 2002; Miettinen-Oinonen *et al*., 2005).

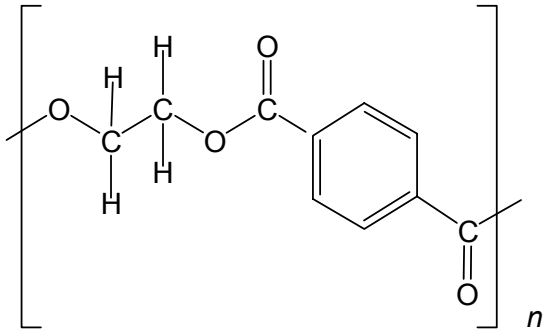

Figure 7. Structure of polyethylene terephthalate, PET (Buschle-Diller, 2003).

Lipolytic enzymes may also be potential tools for modification of natural textile fibres, such as cotton. Cotton contains different amounts of impurities, such as lipids and waxes, located on the surface layers of the fibres (Hartzell and Hsieh, 1998). The proportion of cotton wax, which contains e.g. sterols and related terpenoids, is typically 0.6% of the fibre weight and 17% of the cuticle (Li and Hardin, 1997; Hardin and Kim, 1998). The waxy compounds as well as other impurities are conventionally removed chemically using harsh conditions, e.g. by boiling with sodium hydroxide. Enzymatic removal of cotton impurities has been studied as a specific and environmentally friendly alternative to scouring (Hardin and Kim, 1998; Hartzell and Hsieh, 1998; Degani *et al*., 2002).

Processing of fruits and berries leads to the formation of waste fractions which contain cutin and other insoluble polymers. The use of lipolytic enzymes, as well as other hydrolytic enzymes, could enable the exploitation and recovery of these

valuable polyfunctional polyesters and cutin monomers by physical and chemical processes.

1.5 Aims of the present study

The overall aim of the present work was to identify and characterise novel esterases acting primarily on wood steryl esters and triglycerides. The work also aimed at utilising a heterologous production system for a novel steryl esterase in order to produce the enzyme in large scale for application studies. The effects of steryl esterase on the properties of paper sheets and polyester were evaluated.

The specific aims were:

- to study steryl esterase activities in commercial lipase preparations
- to purify and characterise a novel *Melanocarpus albomyces* steryl esterase
- to isolate the gene encoding the *Melanocarpus albomyces* steryl esterase
- to produce *Melanocarpus albomyces* steryl esterase in a heterologous host
- to study the use of steryl esterases in the treatment of extractives in thermomechanical pulp and in the treatment of polyester.

2. Materials and methods

A summary of the materials and methods in this work is presented in this section. More detailed information is given in the original publications I–V.

2.1 Substrates

The substrates used in this work are presented in Table 4.

Table 4. Substrates used in this work.

Substrate	Acyl residue	Supplier	Use (Publication)
Cholesteryl palmitate	16:0	Sigma	Substrate specificity studies (I, III, V)
Cholesteryl stearate	18:0	Sigma	Substrate specificity studies (I, III, V)
Cholesteryl oleate	18:1	Sigma	CE activity assay (I–V)
Cholesteryl linoleate	18:2	Sigma	Substrate specificity studies (I, III, V)
Cholesteryl linolenate	18:3	Sigma	Substrate specificity studies (I, III, V)
p-nitrophenyl acetate (*p*-NPA)	2:0	Fluka	Substrate specificity studies (III, V)
p-nitrophenyl propionate (*p*-NPP)	3:0	Sigma	Substrate specificity studies (III, V)
p-nitrophenyl butyrate (*p*-NPB)	4:0	Sigma	Substrate specificity studies (III, V)
p-nitrophenyl caprylate	8:0	Fluka	Substrate specificity studies (III, V)
p-nitrophenyl caprate (*p*-NPC)	10:0	Sigma	CAE activity assay (I–V)
p-nitrophenyl myristate	14:0	Fluka	Substrate specificity studies (III, V)
p-nitrophenyl stearate	18:0	Sigma	Substrate specificity studies (III, V)
Olive oil	18:1[a]	Bertolli	Lipase activity assay (I, II, III, V)
Mixture of plant steryl esters (PSE)	18:1[a]	Raisio Chemicals	Steryl esterase activity assay, hydrolysis experiments (I, II, III)
Mixture of wood steryl esters (WSE)	18:2[a]	Åbo Akademi	Hydrolysis experiments (I, II)
Mixture of extractives (simulating TMP resin)		Åbo Akademi (Qin *et al*., 2003)	Hydrolysis experiments (III)
DCS water (produced from TMP)		VTT	Hydrolysis experiments
Thermomechanical pulp (TMP), Norway spruce		Paper mill (Rauma)	Application studies (V)
Polyethylene terephthalate (PET)		Rhodia Industrial Yarns, Switzerland	Application studies (V)

a) main component

2.2 Activity assays

The assay used for determination of cholesteryl esterase (CE) activity was based on spectrophotometric determination of liberated cholesterol using cholesteryl oleate as substrate (II). Activities against different cholesteryl esters or steryl ester mixtures were measured using the same assay (I). Carboxyl esterase (CAE) activity was determined by measuring the liberated *p*-nitrophenol spectrophotometrically after hydrolysis of *p*-nitrophenyl caprate (*p*-NPC) (II). Determination of CAE activity provided a rapid and straightforward method to follow non-specific esterase activity during cultivation and purification procedures. Activities against different *p*-nitrophenyl esters were measured using the same assay (III). Lipase activity was based on the spectrophotometric determination of liberated fatty acids using olive oil as substrate (I).

2.3 Protein purification

Melanocarpus albomyces steryl esterase (STE1), recombinant *M. albomyces* steryl esterase (rSTE1) and *Candida rugosa* steryl esterase (LIP3) were purified with two chromatographic steps: hydrophobic interaction chromatography (HIC) and anion exchange chromatography (DEAE as exchanger), using different binding and elution conditions. Finally, Triton X-100 was removed by the anion exchange chromatography. The native STE1 was purified from Triton X-100 extract of *M. albomyces* mycelium (III), whereas the rSTE1 was purified from the culture filtrate concentrate (V). Steryl esterase activity of *C. rugosa* was purified from a commercial lipase preparation (Amano) (II).

2.4 Biochemical characterisation of steryl esterases

The molecular weights of purified steryl esterases were determined with SDS-PAGE and analytical gel filtration chromatography. The isoelectric points were determined by isoelectric focusing over the pH range 3.5–9.5 using silver staining (III). Enzyme stabilities were determined at different pH-values and temperatures for 24 h (I, III, V). The residual enzyme activities were measured against cholesteryl oleate or *p*-NPC. The pH optima were determined using cholesteryl oleate, *p*-NPC and olive oil as substrates (I, III, V). The effects of

various surfactants (Triton X-100, Tween 80, Polidocanol, Zwittergent 3-14, cholic acid and taurocholic acid) on the action of steryl esterase were determined by measuring the activity against *p*-nitrophenyl butyrate (*p*-NPB) (III). Interfacial activation was investigated by measuring the activity on triacetin, tripropionin and tributyrin below and above the solubility limit (III).

2.5 Hydrolysis of model substrates

Degradation of plant (PSE) and wood (WSE) steryl esters was investigated in aqueous dispersions as well as in the presence of surfactant using different substrate concentrations (I, II, III). The effects of enzyme dosage, pH and temperature on hydrolysis of steryl esters were evaluated. Simultaneous hydrolysis of steryl esters and triglycerides was also investigated in different reaction conditions (III). The hydrolysis products were determined by gas chromatography (GC) or by spectrophotometry.

Hydrolysis of steryl esters and triglycerides was also investigated in model DCS water containing dissolved and colloidal substances. DCS fraction was prepared by diluting the unbleached TMP with distilled water to 1% consistency and agitated at 60°C for 3 h according to Örså and Holmbom (1994). Separated DCS fraction was treated with enzymes at temperatures of 40, 50 and 60°C at pH 7 for 18 h. Enzymes were dosed as lipase activities of 5000 nkat l^{-1} of DCS water.

2.6 Isolation of the genomic *Melanocarpus albomyces ste1* gene

DNA manipulations were performed according to standard methods (Sambrook and Russel, 2001). The genomic library of *M. albomyces* was constructed into the SuperCos I cosmid (Kiiskinen and Saloheimo, 2004), and clones from the library were hybridised with a *Neurospora crassa* gene (IV). The cosmid DNA isolated from strongly hybridising colonies was mapped by restriction enzyme digestions followed by Southern hybridisation to find a suitable fragment for sub-cloning. The *M. albomyces* steryl esterase gene was sequenced from sub-cloned plasmids by the primer walking technique. Similarities between the deduced amino acid sequence of *M. albomyces* steryl esterase and other

esterases were searched using various BLAST programs found at http://au.expasy.org/tools/#similarity. To obtain the protein-coding sequence for steryl esterase, the intron was removed by overlap extension PCR. Additionally, a seqment encoding a 6×His tag was joined to the C-terminus of steryl esterase. The resulting fragment was cloned into pDONR^{TM}221 to generate an entry clone (pHAK3).

2.7 Heterologous expression of *Melanocarpus albomyces* steryl esterase in *Pichia pastoris*

The expression clone was generated between the entry clone and destination vector pJT10 to create the plasmid pHAK5 for *P. pastoris* transformation (IV). The positive transformants were cultivated in shake flasks and steryl esterase production was monitored by measuring extra- and intracellular CAE and CE activities. In addition, the steryl esterase produced by *P. pastoris* was studied by Western blotting of samples from the culture supernatants and yeast cell lysates.

2.8 Heterologous expression of *Melanocarpus albomyces* steryl esterase in *Trichoderma reesei*

The entry clone pHAK3 was recombined with a *T. reesei* destination vector pMS186 carrying the *cbh1* promoter and terminator (IV). *T. reesei* was transformed with the resulting expression vector (pHAK4) essentially as described by Penttilä *et al*. (1987), and transformants were selected for hygromycin resistance for three successive rounds. In order to test steryl esterase production, a piece of mycelium grown on minimal medium plates with lactose and hygromycin was incubated with a reaction mixture containing cholesteryl oleate and reagents needed for enzymatic determination of cholesterol (Allain *et al*., 1974). Selected transformants producing steryl esterase were purified through conidia and steryl esterase production was studied in shake flask cultivations. In order to study the presence of bound and aggregated enzyme, the mycelium was extracted with Triton X-100 solution. Extracellular steryl esterase production was analysed by Western blotting.

The *T. reesei* transformant which produced the highest level of steryl esterase in shake flasks was cultivated in a laboratory-scale fermenter on a whey-spent grain medium that was most favourable for rSTE1 production in the shake flask cultivation (V). Steryl esterase production was monitored by measuring CAE and CE activites from culture supernatant and Triton-extracted samples.

2.9 Application studies

2.9.1 TMP treatments

Unbleached thermomechanical pulp (TMP) produced from Norway spruce was obtained from a Finnish paper mill. Hot disintegrated TMP was treated for 20 h at 1% consistency with rSTE1 and commercial lipase preparation Resinase A2X (Novozymes, Denmark) (V). The treatments were performed at 50°C and at the natural pH of TMP (pH 5) using a lipase dosage of 500 nkat g^{-1} TMP. The used lipase dosage of the rSTE1 preparation corresponds to a CE dosage of 25 nkat g^{-1} of pulp. The rSTE1 preparation contained 0.1% Triton X-100 in order to stabilise the enzyme (corresponding 0.0009% Triton X-100 percentage in the pulp suspension). Pulp treatments were also performed in the presence of 0.05% Triton X-100. After the treatments the suspensions were filtered twice and washed with ion-exchanged water. The pulp was homogenised by cold-dispersion before preparation of handsheets using wire cloth. The sheets were wet-pressed and dried in a rotary drier. Technical sheet properties (ISO-brightness, light scattering coefficient, tensile index, tear index) and the contact angle of water on the wire side of handsheets were determined (Helsinki University of Technology, Department of Forest Products Technology). The chemical compositions of extractives in the TMP waters were analysed by GC after extraction with methyl *tert*-butyl ether (MTBE) (Örså and Holmbom, 1994).

2.9.2 Polyester treatments

Polyester fabric (Rhodia Industrial Yarns, Switzerland) was washed with detergent and extracted with dichloromethane, whereafter the fabric was treated with rSTE1 at 40°C and pH 7 using a fabric to liquid ratio of 1:30 and a lipase dosage of 1000 nkat g^{-1} (V). After treatment the fabrics were rinsed twice with

ion-exchanged water and dried flat at room temperature. The wetting behaviour of polyester was analysed by measuring contact angle, penetration time of water and rising height. Colour values were measured spectrophotometrically after dyeing with 0.1% methylene blue.

3. Results and discussion

3.1 Steryl esterase activities in commercial lipase preparations (I)

Because of the capability of lipases to hydrolyse different fatty acid esters, steryl esterase activities were characterised from the commercial lipase preparations presented in Table 1/I. However, these preparations may have contained several lipases and esterases with different properties, and therefore no detailed comparison could be made without purifying the different enzymes. The substrate specificities of the lipase preparations clearly differed from each other (Table 2/I). Some lipases had very high lipase activity but no activity on steryl esters. The lipase preparations from *Candida rugosa*, *Chromobacterium viscosum*, *Pseudomonas* sp., and *Rhizopus oryzae* were able to degrade various steryl esters under the conditions used. Thus, they were further characterised in detail. Lipases from *C. rugosa* (LIP3), *P. cepacia*, *P. pseudoalcaligenes* and *Ophiostoma piceae* have previously been shown steryl esterase activity (Kaiser *et al.*, 1994; Svendsen *et al*., 1995; Gao and Breuil, 1998).

3.1.1 Substrate specificities

C. rugosa (Amano) lipase preparation had high steryl esterase activity in comparison to the lipase activity. The *Ch. viscosum* preparation also had high activity on steryl esters, and it showed the highest specific activity on olive oil and *p*-NPC. Steryl esterases from *C. rugosa* and *Rh. oryzae* were shown to be similar to the steryl (cholesteryl) esterases from *P. fluorescens*, *S. lavendulae* and *F. oxysporum*, having higher activity on unsaturated and more hydrophilic long-chain fatty acid esters of cholesterol (Uwajima and Terada, 1976; Kamei *et al*., 1977; Madhosingh and Orr, 1981). Steryl esterases from *Pseudomonas* sp. and *Ch. viscosum* had highest activities on cholesteryl palmitate and lowest on cholesteryl stearate, whereas unsaturated cholesteryl esters were moderate substrates.

3.1.2 pH and temperature characteristics

The effects of pH and temperature on the activity and stability of steryl esterases from *C. rugosa* (Biocatalysts), *Pseudomonas* sp. (Seppim), *Ch. viscosum* and *Rh. oryzae* were measured using cholesteryl oleate as substrate. The pH optimum of *C. rugosa* lipase was rather narrow and at acidic pH, but other preparations showed activity over a broad pH range (Figure 1/I). Lipases from *Pseudomonas* sp. and *Ch. viscosum* were highly active even at pH 10. Enzyme preparations, with the exception of that of *Rh. oryzae*, were stable over a broad pH range (Figure 3/I). The results showed that the preparations of *Ch. viscosum* and *Pseudomonas* sp. might contain two or more lipases active against steryl esters and having different pH optima and stabilities.

C. rugosa and *Rh. oryzae* steryl esterases had their highest activity at 50°C (Figure 2/I). *Ch. viscosum* steryl esterase was clearly more active at higher temperature, showing maximal steryl esterase activity at 60°C. *Pseudomonas* sp. steryl esterase had the broadest temperature range. Over 80% of the maximum activity was observed between 30 and 70°C. Steryl esterases of *Pseudomonas* sp. and *Ch. viscosum* were the most thermostable, retaining 80% of their activity at 50°C after 3 h (Figure 4/I). Their half-lives were 2 h at 60°C. *C. rugosa* steryl esterase had a half-life of 7 h at 50°C, but was clearly unstable at 60°C. *Rh. oryzae* steryl esterase was not stable at temperatures above 40°C.

3.1.3 Hydrolysis experiments

Commercial lipase preparations from *C. rugosa*, *Ch. viscosum* and *Pseudomonas* sp. were shown to degrade PSE mixture very effectively in the presence of 0.1% surfactant in 24 h incubation at 40°C and pH 7 (Figure 5/I). The reaction rates were clearly increased when surfactant was present, except in the case of *C. rugosa* enzyme. Total hydrolysis could be obtained even in 30 min with the lipase preparation of *Ch. viscosum*. In the absence of surfactant the enzymes could maximally hydrolyse 70–80% of PSE.

The effects of pH and temperature on the hydrolysis of aqueous dispersions were studied in 24 h incubation (Figure 6/I). The highest degree of hydrolysis was obtained at pH 7. Up to 90% of PSE were degraded with the lipase preparations

of *C. rugosa* and *Pseudomonas* sp. at 50°C. In addition to accelerated reaction rate of the enzyme, higher temperature may make the substrate more accessible to the enzyme due to liquefaction and thus lead to a higher degree of hydrolysis.

The effects of *C. rugosa* and *Pseudomonas* sp. lipase preparations on the extractives of 1% DCS water were also investigated (Table 5). Triglycerides were effectively degraded and the amount of steryl esters was also clearly decreased. Hydrolysis of triglycerides and steryl esters is supported by simultaneous increase in amounts of fatty acids and sterols. Somewhat higher hydrolysis yields were obtained at 50°C than at 40°C with both enzyme preparations.

Table 5. Effects of esterase treatments on the extractives of 1% DCS water. Treatments were performed using a lipase dosage of 5000 nkat l^{-1} of DCS water at pH 7 for 18 h.

Treatment	Steryl esters (mg l^{-1})		Triglycerides (mg l^{-1})		Fatty acids (mg l^{-1})		Sterols (mg l^{-1})	
	40°C	50°C	40°C	50°C	40°C	50°C	40°C	50°C
Reference	9.1	9.0	16.1	16.3	1.1	0.9	1.2	1.2
Candida rugosa	6.8	5.6	1.3	1.3	5.0	2.8	1.8	2.2
Pseudomonas sp.	8.0	6.7	1.2	0.8	4.2	2.4	1.5	1.7

On the basis of PSE treatments it can be concluded that at higher temperatures, which is an important consideration in industrial applications, *Pseudomonas* sp. lipase preparation was the most potent. However, the lipase preparation of *C. rugosa* that degraded PSE efficiently at 50°C appeared to be most effective in hydrolysis of steryl esters in DCS water. However, the preparations may contain several lipases and esterases with different properties, and therefore no detailed comparison could be made without purifying the different enzymes.

3.2 Enzymatic properties of steryl esterases from *Melanocarpus albomyces* STE1 and *Candida rugosa* LIP3 (II, III)

3.2.1 Sources of STE1 and LIP3 steryl esterases

Steryl esterase production by the thermophilic ascomycete *Melanocarpus albomyces* (VTT D-96490, originally isolated from soil in Saudi Arabia) was investigated in this work because *M. albomyces* had previously been reported to produce many industrially interesting enzymes, including cellulases, xylanases and laccase (Vehmaanperä *et al*., 1996; Prabhu and Maheshwari, 1999; Kiiskinen *et al*., 2002). *M. albomyces* was observed to produce steryl esterase, and a sufficient amount of protein was obtained for purification when the fungus was cultivated in a medium containing olive oil as an inducer (75 mg l^{-1} of culture supernatant). The *M. albomyces* steryl esterase (STE1) had a high affinity to the fungal mycelium and was purified from Triton X-100 solution after extraction of separated mycelium.

C. rugosa steryl esterase (LIP3) was purified from a commercial lipase preparation (Amano) with high steryl esterase activity compared with lipase activity. STE1 and LIP3 could be purified by two chromatographic steps: hydrophobic interaction chromatography and anion exchange chromatography. In order to investigate the pH- and temperature characteristics and the effect of surfactants, Triton X-100 was removed from some of the preparations by the anion exchange chromatography.

3.2.2 Substrate specificity

The enzymatic activities of purified STE1 and LIP3 were characterised using cholesteryl oleate, *p*-NPC and olive oil as substrates. In addition to the steryl esterase activity, both enzymes had carboxyl esterase activity and high lipase activity (Table 6, p. 56). Specific carboxyl and cholesteryl esterase activities of LIP3 were over three times higher than those of STE1. However, specific lipase activities of both preparations were approximately at the same level. Some steryl esterases, such as those from *Ophiostoma piceae* and *Pseudomonas aeruginosa*, have previously been reported to have both lipase and steryl esterase activity

(Calero-Rueda *et al*., 2002; Sugihara *et al*., 2002). On the other hand, some steryl esterases have been found to be specific for steryl esters and to possess no lipase activity (Uwajima and Terada, 1976; Taketani *et al*., 1981).

One of the *C. rugosa* lipases (LIP3) had previously been found to show CE activity and it is therefore also classified as a steryl (cholesteryl) esterase (Kaiser *et al*., 1994). Comparison of the N-terminal amino acid sequences among the *C. rugosa* lipases has revealed that LIP3 differs from other lipases. The N-terminal amino acid sequence of the purified *C. rugosa* enzyme in this study was identical to that of LIP3 (Lotti *et al*., 1993), and thus it was concluded that the purified steryl esterase was LIP3 and was not a new esterase of *C. rugosa*.

Substrate specificities of the native *M. albomyces* STE1 were compared using cholesteryl esters with long-chain fatty acids resembling wood-derived steryl esters and different *p*-nitrophenyl esters as substrates (Table 3/III). Activities against cholesteryl esters clearly differed from each other. STE1 had the highest activity on cholesteryl palmitate and the lowest on cholesteryl stearate. Unsaturated cholesteryl esters were moderate substrates. In general, long-chain and unsaturated fatty acid esters are hydrolysed more effectively than the short and saturated esters (Uwajima and Terada, 1976; Kamei *et al*., 1977; Madhosingh and Orr, 1981). The length of the fatty acid chain had only little effect on the *p*-nitrophenyl esters as substrates. Only the activity on *p*-nitrophenyl acetate differed clearly from that on other substrates. Despite the high lipase activity of STE1 measured against olive oil, containing triglycerides with long-chain fatty acids, the enzyme showed no activity on triglycerides with short-chain fatty acids (triacetin, tripropionin, tributyrin). Thus interfacial activation, i.e. enhanced activity of lipases on aggregated substrate, could not be demonstrated. The steryl esterase of *Ophiostoma piceae* has been shown to have activity on tripropionin and tributyrin below their solubility limits, and thus it is a typical esterase showing no interfacial activation (Calero-Rueda *et al*., 2002).

Despite similar structural features of esterases and lipases and their similar catalytic mechanism of ester hydrolysis, these two enzyme groups have different substrate specificities for the acyl moiety of the substrate (Pleiss *et al*., 1998). Due to their large fatty acid binding sites, lipases display a much broader substrate range than the esterases (Pleiss *et al*., 1998; Fojan *et al*., 2000). In addition to the fatty acid binding site, lipases have been shown to have

differences in the alcohol binding site. For example, the binding site structure that is lined with small amino acids suggests that lipases are specific for esters of large alcohols (Kazlauskas, 1994). Activity of STE1 and LIP3 on steryl esters indicate that they have relatively large alcohol binding sites.

3.2.3 Hydrolysis of steryl esters and triglycerides

STE1 and LIP3 were found to be highly active on steryl ester mixtures in the presence of non-ionic surfactant (Polidocanol). Maximum hydrolysis of 80–100% was reached when plant (PSE) or wood-derived (WSE) steryl ester mixtures were treated for 24 h at different substrate concentrations (0.1 or 1 g l^{-1}) using a CE dosage of 500 or 1000 nkat g^{-1} (Figure 8). However, the enzymatic action was more restricted when the substrate was present as colloidal droplets in aqueous dispersion. When PSE was treated with LIP3 at a concentration of 0.02 g l^{-1} (pH 7, 40°C) using an enzyme dosage of 500 nkat g^{-1}, 30–50% hydrolysis was achieved in 2 h (Figure 4/II). Prolonged incubation or addition of fresh enzyme did not significantly increase the degree of hydrolysis. However, LIP3 was found to be able to hydrolyse all the different steryl esters equally well (Figure 5/II), and thus none of the individual components were responsible for the incomplete hydrolysis. PSE was treated with STE1 in conditions similar to those used for LIP3, with the exception of a five-fold substrate concentration of 0.1 g l^{-1}. Only 20% of the substrate was hydrolysed by STE1 in 24 h treatment in the absence of surfactant. The degree of hydrolysis was lower than that obtained with LIP3, probably as a consequence of the higher substrate concentration.

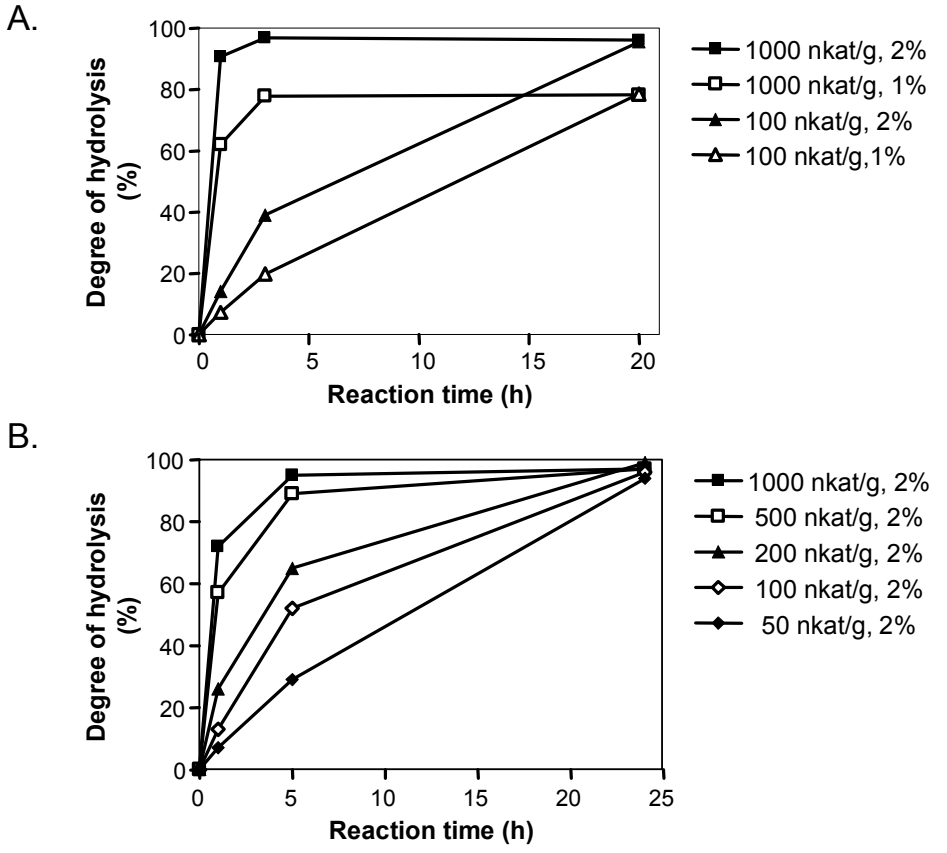

Figure 8. Hydrolysis of a plant steryl ester mixture (1 mg ml^{-1}) by STE1 (A) and LIP3 (B) in the presence of 1% or 2% Polidocanol (Na-phosphate buffer pH 7, 40°C).

In order to monitor the hydrolysis of steryl esters and triglycerides simultaneously, a mixture of extractives was treated with STE1 (Figure 6/III). The results indicated that 60–80% of triglycerides were hydrolysed in 24 h even in the absence of surfactant. When surfactant was added, triglycerides were hydrolysed almost completely. Steryl esters were not degraded in the absence of surfactant, but addition of surfactant improved the degree of hydrolysis considerably. At Polidocanol concentrations of 0.1% and 1%, the degree of hydrolysis in 24 h was 70% and 85–100%, respectively, depending on the pH and temperature. When hydrolysis of steryl esters and triglycerides was investigated as a function of time, it was observed that treatment for 3 h was not sufficient to reach maximal degradation of substrates (Figure 7/III). It is noteworthy that hydrolysis

of steryl esters by steryl esterases, such as those from *O. piceae* and *P. aeruginosa*, has also been shown only in the presence of a surfactant (Calero-Rueda *et al*., 2002, 2004; Sugihara *et al*., 2002).

3.2.4 pH dependence

The pH-optimum of STE1 was measured using cholesteryl oleate, *p*-NPC and olive oil as substrates. The pH optimum of lipase activity was at neutral pH, whereas carboxyl and cholesterol esterase activities had optima around pH 5–5.5 (Figure 9 and Table 6, p. 56). Different pH optima might be caused by the effect of pH on ionisation of other than catalytic amino acids either inside or outside the active site, which affects the stability of the active conformation of the enzyme and interaction between the enzyme and different substrates. The steryl esterase of *P. aeruginosa* has been shown to have a very broad pH-optimum from 5.5 to 9.5 against cholesteryl linoleate (Sugihara *et al*., 2002). Commonly the pH-optima of steryl esterases have been reported to vary in the range 6–8 when measured on various cholesteryl esters or *p*-NPB (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Calero-Rueda *et al*., 2002). The pH stability of STE1 was measured on *p*-NPC. STE1 was most stable within the pH range of 3.5–7 (24 h), and clearly less stable at more alkaline pH (Figure 3/III). The steryl esterase of *O. piceae* has been shown to retain its activity in the same pH range (Calero-Rueda *et al*., 2002). The pH stability of the commercial Resinase A2X preparation was also investigated using *p*-NPC as substrate. The preparation was most stable within the pH range of 4–10 (24 h).

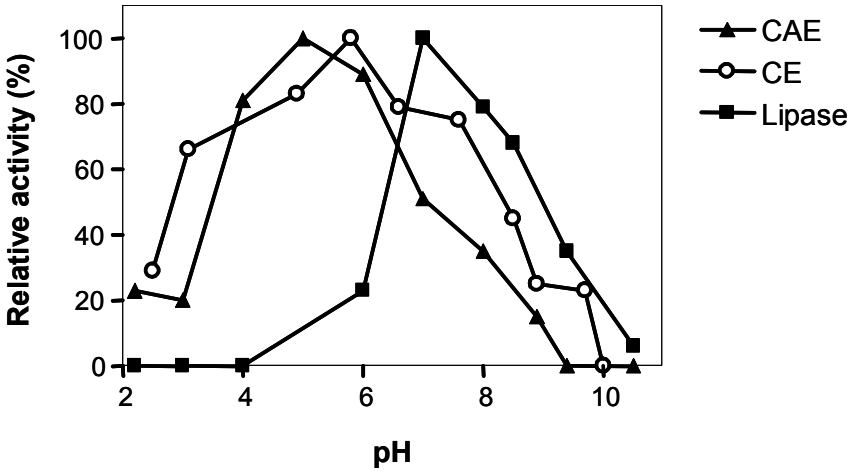

Figure 9. pH optimum of Melanocarpus albomyces *steryl esterase.* p-*Nitrophenyl caprate (▲), cholesteryl oleate (○) and olive oil (■) were used as substrates.*

LIP3 showed the highest cholesteryl esterase activity at pH 5.0–7.5, similarly to that reported previously (Rúa *et al*., 1993; Pernas *et al*., 2000). This supports the conclusion that the purified steryl esterase was LIP3.

3.2.5 Temperature dependence

M. albomyces steryl esterase was shown to have good thermostability (Figure 10). STE1 retained over 70% of its CE activity after 5 h at 50°C and had half-lives of 4 h at 60°C and 2 h at 70°C. Characterised steryl esterases have been reported to be generally stable at temperatures up to 50–60°C for 30 min (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Sugihara *et al*., 2002). The steryl esterase of *O. piceae* has been shown to retain over 80% and 25% of its activity after 24 h incubation at 30°C and 45°C, respectively (Calero-Rueda *et al*., 2002). The effect of temperature on the action of LIP3 was determined using a 20 min reaction time at pH 7. The highest CE activity was obtained at 50°C, and about 20% of the maximal activity was obtained at 60°C. According to the manufacturer, Resinase A2X showed highest lipase activity at 60°C and was still active at 80°C. However, due to insufficient stability of Resinase at temperatures 60–70°C, more stable Resinase variants have recently been developed for enzymatic pitch control (Blanco *et al*., 2005).

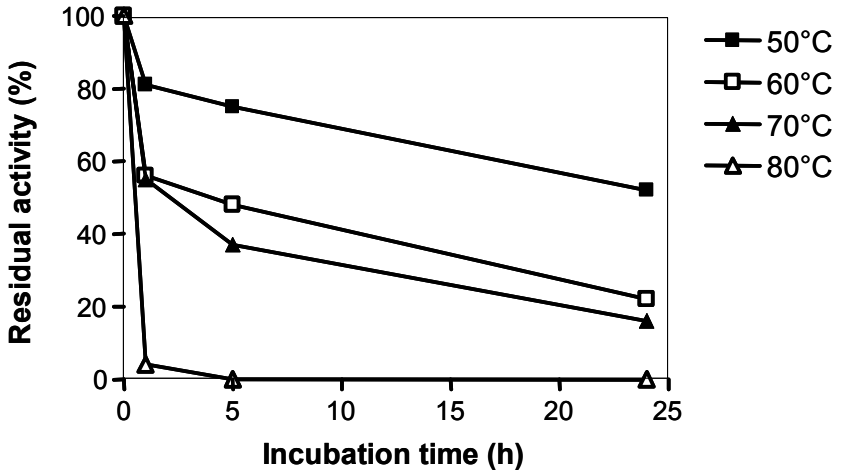

Figure 10. Thermostability of purified Melanocarpus albomyces *steryl esterase at pH 5. Residual activities were measured against cholesteryl oleate.*

3.2.6 Effects of surfactants

The effects of surfactants on the action of STE1 were investigated using *p*-NPB as substrate (Table 4/III). The activity of STE1 clearly increased in the presence of the surfactants studied. The optimal concentration for Triton X-100, Tween 80 and Zwittergent 3–14 was 0.1%, whereas bile acid salts and Polidocanol had concentration optima of 0.01% and 0.05%, respectively. The optimum surfactant concentrations have been found to vary between 0.1 and 0.3% when activities have been measured with various cholesteryl esters (Uwajima and Terada, 1976; Madhosingh and Orr, 1981) or *p*-NPB (Calero-Rueda *et al*., 2002). Further increase in concentrations has been shown to reduce the activities of steryl esterases. However, treatment of PSE mixture with STE1 showed that a Polidocanol concentration of 2% resulted in a higher degree of hydrolysis than 1% (Figure 8A, p. 50). Higher surfactant concentration probably increases the interface area between the insoluble substrate and the enzyme located in the water phase. This facilitates the access of enzyme to the substrate and leads, in turn, the higher degree of hydrolysis since activity is directly correlated with the total substrate area (Verger, 1997).

Interestingly, steryl esterase and lipase activities of *Pseudomonas aeruginosa* were not affected by Triton X-100, but were increased by bile salts, such as cholate and taurocholate, which were thus proposed to have a significant role in opening the active site of the enzyme (Sugihara *et al*., 2002). Bile salts are also able to form soluble mixed micelles with released fatty acids. These accelerate the diffusion of fatty acids away from the hydrophobic interface and thus enhance the catalytic activity of the enzyme (Malcata *et al*., 1992). In addition to direct activation or inactivation of the enzyme, lipase activity is strongly dependent on deaggregation of the enzymes caused by surfactants, which leads to the exposure of more active sites (Dünhaupt *et al*., 1992; Rúa *et al*., 1997). Surfactant may also alter the hydrophobicity of the enzyme, and affect micelle formation and the ratio of free and micellar substrate and thus the availability of substrate to enzyme (Helistö and Korpela, 1998).

3.2.7 Effect of calcium ions

The effect of Ca^{2+} ions on the action of STE1 was investigated. No effect on CE activity was detected, but lipase activity decreased as a function of calcium concentration. About 90, 60 and 50% of lipase activity was retained at 0.5, 5 and 50 mM $CaCl_2$ concentrations, respectively. The action of calcium as a lipase inhibitor has also been shown previously (Ohnishi *et al*., 1994; Wu *et al*., 1996), and only a slight effect on microbial steryl esterases has been observed (Uwajima and Terada, 1976; Okawa and Yamaguchi, 1977; Sugihara *et al*., 2002). Generally, calcium is known to increase the catalytic activity of several microbial lipases (Ibrahim *et al*., 1987; Gao and Breuil, 1998; Sharon *et al*., 1998; Simons *et al*., 1998). The formation of insoluble soaps, resulting from the interaction of Ca^{2+} ions with released fatty acids, might effectively accelerate the diffusion of fatty acids away from the hydrophobic interface and thus decrease the inhibitory effect of the product (Gao and Breuil, 1998). Svendsen *et al*. (1995) assumed that the bound calcium in some of the *Pseudomonas* lipases influences the geometry of active site residues or may also participate in the catalytic activity.

3.2.8 Domain structure

SDS-PAGE of the STE1 preparation showed a major band of 64 kDa and a minor band of 53 kDa which could not be separated by chromatographic purification (Figure 1/III). The bands gave identical peptide sequences and it was concluded that the smaller unit represents a proteolytic fragment originating from the full-length protein (Table 2/III). Analytical gel filtrations performed in the presence of 0.1 or 1% Triton X-100 showed only one peak with a molecular weight of 238 kDa, and thus the active enzyme was concluded to form a tetramer under the conditions used (Table 6).

The purified LIP3 showed one band in SDS-PAGE with a molecular mass of 65 kDa (Figure 2/II), which is close to the values (60–66 kDa) reported previously for LIP3 (Rúa *et al*., 1993; Kaiser *et al*., 1994; Sánchez *et al*., 1999; Jonzo *et al*., 2000; Pernas *et al*., 2000). Structural studies of LIP3 have shown that the active form of the enzyme is a dimer which is stabilised by the substrate (Kaiser *et al*., 1994; Ghosh *et al*., 1995). In the dimeric association, two monomers face each other with their flaps open, shielding the hydrophobic surfaces of the two catalytic triads from the aqueous environment.

Steryl esterases of *O. piceae* and *P. aeruginosa* have been shown to be monomers, with molecular weights of 56.5 and 58 kDa, respectively (Calero-Rueda *et al*., 2002; Sugihara *et al*., 2002). Molecular weights up to 300 kDa have been shown by analytical gel filtration for the enzymes of various *Pseudomonas* species, indicating multimeric structures (Uwajima and Terada, 1976; White and White, 1997). The steryl esterase of *Fusarium oxysporum* is reported to consist of two subunits having molecular weights of 15 and 60 kDa (Madhosingh and Orr, 1981). Some lipases are also known to exist as multimeric structures, containing two, four or six subunits (Malcata *et al*., 1992).

Table 6. Biochemical properties of native and recombinant Melanocarpus albomyces *steryl esterases (STE1 and rSTE1) and* Candida rugosa *steryl esterase (LIP3).*

Property		STE1	rSTE1	LIP3
Molecular weight, kDa	SDS-PAGE Gel filtration	64 (53) 238	60 120	65 n.d.
Length of mature protein (aa)		545	n.d.	534
Specific activity (nkat mg^{-1})	CAE CE Lipase	1925 125 3770	2020 300 2800	7270 382 3840
pH optimum	CAE CE Lipase	5.0 5.5 7.0	5.2 5.5–6 7–8	n.d. 5–7.5 n.d.
Thermostability (pH 5)	T½ 50°C T½ 60°C T½ 70°C	> 24 h (60%) 4 h 2 h	> 24 h (90%) 1 h unstable	n.d. n.d. n.d.
pH stability (24 h, 37°C)		3.5–7	3–7	n.d.

n.d. not determined

3.2.9 Physical properties

It was observed during the purification that a surfactant (1% Triton X-100) was required to maintain the stability of the STE1 preparation, i.e. to prevent the formation of enzyme aggregates. The proportion of hydrophobic amino acid residues (Phe, Leu, Ile, Tyr, Trp, Val, Met, Pro) of STE1 is high (41.1%), which probably encourages aggregation because regions rich in hydrophobic amino acids tend to shield from the aqueous environment. The aggregation of steryl esterases and lipases is well documented in the literature, and is explained by the strong hydrophobic character of these enzymes (Dünhaupt *et al*., 1992; Rúa *et al*., 1997; Brush *et al*., 1999; Fojan *et al*., 2000). Various surfactants and solvents have typically been used to prevent the formation of enzyme aggregates, by manipulating either interactions between the lipase monomers or between monomers and solvent molecules. Strong negative correlations between aggregation of *Pseudomonas cepacia* and *Bacillus thermocatenulatus* lipases and their activities have been shown, and thus it has been concluded that the active sites of lipase molecules are involved in aggregation (Dünhaupt *et al*., 1992; Rúa *et al*., 1997). However, regardless of the presence of 1% Triton

X-100, LIP2 and LIP3 from *C. rugosa* have been reported to be able to form active aggregates (Pernas *et al*., 2000). Temperature has been shown to have a clear effect on the oligomeric state of *Thermus thermophilus* esterase/lipase (Fuciños *et al*., 2005). Dissociation of the trimer (108 kDa) into the monomer (34 kDa) was observed to start at temperatures higher than 60°C. In addition to higher temperature, elevation of pH or ionic strength by addition of a single salt might enhance the solubility of the protein (Schlieben *et al*., 2004). Naturally, increase in protein concentration dramatically increases the aggregation tendency of lipase monomers.

STE1 was found to be tightly bound to the fungal mycelium or solid components of culture medium, or existed as aggregates, and could be released with 0.1% Triton X-100 extraction. The Triton-extract was enriched with steryl esterase and therefore it was used as starting material for chromatographic purification (Table 1/III). Property to bound to the fungal mycelium can also be exploited by employing mycelium-bound lipases directly as naturally immobilised enzymes without any further isolation, purification and immobilisation procedures. Mycelium-bound lipases have been characterised previously e.g. from *Aspergillus* and *Rhizomucor* species, and their ability to catalyse ester formation or the exchange of ester bonds in different applications has been studied (Long *et al*., 1998; Molinari *et al*., 2000; Liew *et al*., 2001; Antczak *et al*., 2004).

3.3 Cloning of the *Melanocarpus albomyces ste1* gene (IV)

The purified *M. albomyces* steryl esterase STE1 was subjected to sequencing of its N-terminus and internal peptides. A search with the obtained peptide sequences among the existing sequence databases showed that one internal peptide sequence had very high identity with a hypothetical protein of *Neurospora crassa* (NCBI: EAA30450, EMBL: Q7S4M5) that showed overall similarity to known esterase sequences. The N-terminal sequence of STE1 also had some similarity with the putative N-terminal sequence of the hypothetical protein of *N. crassa* (Table 2/III). Therefore, the gene encoding this hypothetical *N. crassa* protein was used as a heterologous hybridisation probe in isolation of the gene encoding STE1. Screening of *M. albomyces* genomic cosmid library (Kiiskinen and Saloheimo, 2004) resulted in the identification of two individual

fragments hybridising with the probe, a 2 kb *Kpn*I and a 4 kb *Spe*I fragment, which were sub-cloned and sequenced. Both the N-terminal and the three internal peptide sequences obtained from the purified STE1 were identified in the amino acid sequence deduced from the gene sequence. This confirmed that the cloned gene encoded the purified and characterised STE1.

The *M. albomyces ste1* gene encodes a protein consisting of 576 amino acids. The gene contains an open reading frame of 1,728 bp in length and is interrupted by one intron of 73 bp. The first 18 N-terminal amino acids consisted of a predicted signal sequence typical for secreted eukaryotic proteins (Nielsen *et al*., 1997). The signal sequence is followed by a cleavable propeptide of 13 amino acids. The mature protein has a length of 545 amino acids, which is typical for lipases of the *C. rugosa* family, usually containing approximately 550 amino acid residues (Cygler *et al*., 1993).

A search with the deduced STE1 amino acid sequence in the existing databases showed that STE1 is significantly related to lipases and other esterases. STE1 showed the closest identity with the hypothetical protein of *Neurospora crassa*, the gene of which was used as a hybridisation probe (Table 7). STE1 also showed high identity with several ascomycete lipases. Significant similarity with fungal lipases of the *R. miehei* family or with bacterial lipases was not observed. Only 27% identity with different mammalian cholesteryl esterases (bile-salt-activated lipases) was shown.

Table 7. Results of a BLAST search for sequence similarity showing the percentage of residues which are identical (identity) or conserved (similarity) with Melanocarpus albomyces *steryl esterase (EMBL accession number AJ971405).*

Organism	Protein	Accession No	Identity	Similarity
Neurospora crassa	hypothetical protein	Q7S4M5[a]	74%	84%
Gibberella zeae PH-1	hypothetical protein	EAA67628[b]	61%	75%
Magnaporthe grisea 70–15	hypothetical protein	EAA48656 [b]	62%	76%
Botrytis cinerea	lipase	Q5XTQ4 [a]	59%	72%
Candida rugosa LIP4	lipase LIP4	P32948 [a]	46%	63%
Candida rugosa LIP3	lipase LIP3	P32947 [a]	47%	63%
Geotrichum candidum	lipase	Q12614 [a]	39%	53%

Sequence accession numbers are for the EMBL database (a) or for the NCBI database (b).

STE1 showed identity of 47% with LIP3 from *C. rugosa* (Figure 1/IV). Comparison of the primary structures of STE1 and LIP3 suggested that essential amino acids for catalytic activity and for structure are well conserved. The catalytic triad of STE1 was deduced to be composed of the residues Ser208, His453 and Glu340, and a consensus sequence typical for lipase and esterase active sites, GESAG, was found at positions 206–210. Based on sequence similarity with LIP3, the residues forming the oxyanion hole and two disulphide bridges were also predicted (Cygler and Schrag, 1999). The deduced STE1 contains two potential N-glycosylation sites (Asn-X-Thr/Ser) at residues 45 and 350.

3.4 Production of *Melanocarpus albomyces* STE1 in heterologous hosts (IV, V)

The production levels of steryl esterase by native *M. albomyces* were rather low and the productive cultivations were very difficult to reproduce. Only a very small amount of enzyme could be produced in fermenter cultivations and thus the highest production level, CAE activity of 140 nkat ml^{-1} corresponding to 75 mg l^{-1} (based on specific CAE activity), was obtained in shake flasks. In addition to the unreproducible production, steryl esterase production occurred at a very late phase of cultivation in the native host, i.e. maximum steryl esterase yield was reached in 12 d. In order to obtain a more reproducible production and higher yields, the enzyme was expressed heterologously in the well-known protein production hosts *Pichia pastoris* and *Trichoderma reesei*. Steryl esterase production in *M. albomyces*, *P. pastoris* and *T. reesei* is presented in Figure 13 (p. 64).

3.4.1 Heterologous expression of STE1 in *Pichia pastoris*

The methylotrophic yeast *P. pastoris* has many of the advantages of eukaryotic expression systems, such as protein processing, folding and post-translational modification, while being relatively easy to manipulate (Wegner, 1990; Cregg and Higgins, 1995; Faber *et al*., 1995). *P. pastoris* generally does not hyperglycosylate foreign proteins, and has therefore an advantage over *Saccharomyces cerevisiae*. The length of oligosaccharide (mannose) chains added posttranslationally to proteins in *P. pastoris* (average 8–14 mannose

residues per side chain) is much shorter than those in *S. cerevisiae* (50–150 mannose residues). In addition, *P. pastoris* secretes very low levels of endogenous proteins, which is a major advantage in protein purification. It is known that yeasts are generally not able to splice introns from genes of filamentous fungi, and therefore an intronless version of the *ste1* gene was made by PCR and cloned by *in vitro* recombination to an entry vector of the Gateway recombination system (Invitrogen). After generating the entry clone (pHAK3), the expression clone (pHAK5) was created and used for transformation of *Pichia pastoris*. DNA encoding *M. albomyces* steryl esterase was expressed in *P. pastoris* under the *AOX1* promoter. A single crossover event between the *AOX1* locus and the *AOX1* region in the vector preserves the functionality of *AOX1* gene product and generates a recombinant strain able to metabolise methanol (Mut^+).

Steryl esterase production of *P. pastoris* transformants was studied in shake flasks on BMMY medium using methanol as an inducer. The maximum CAE activity produced was 18 nkat ml^{-1}, considerably lower than the yields obtained from *M. albomyces*. Due to lower sensitivity of the cholesteryl esterase activity assay, CE activity was predominantly detected after prolonged incubation time. Extraction of transformant cells with 0.1% Triton X-100 in 0.05 M sodium phosphate buffer yielded no activity, suggesting that STE1 protein was not bound to the cell surface. However, a large proportion of the total activity, 30–85% depending on the transformant, was found to be present intracellularly (Figure 2/IV). Different steryl esterase secretion between transformants were also clearly seen by Western blotting (Figure 11). The intracellular enzyme showed a smear of several bands with molecular weights between 85 and 95 kDa, indicating that the protein is overglycosylated by *P. pastoris*. Thus, it is likely that STE1 enters the secretory pathway but is not secreted efficiently. This could be due to general incompatibility of the protein with the folding and secretion machinery of *Pichia*.

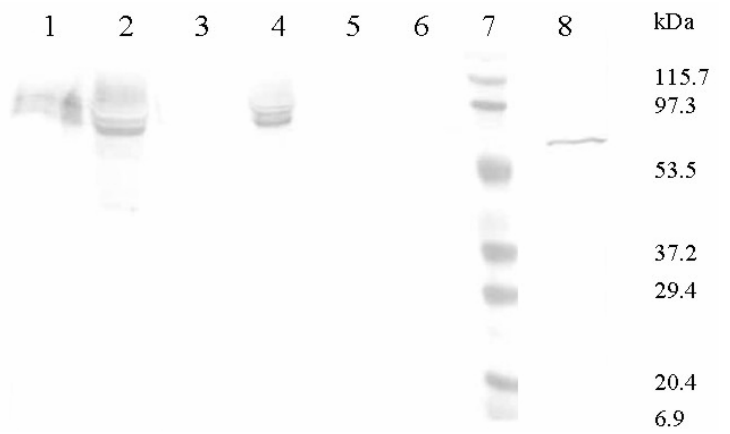

Figure 11. Western blot analysis of culture supernatants (lanes 1, 3 and 5) and cell lysates (lanes 2, 4 and 6) of Pichia pastoris *after 6 days of induction with methanol. Transformant pHAK5/1 (lanes 1–2), transformant pHAK5/7 (lanes 3–4), negative control* P. pastoris *GS115 (lanes 5–6), molecular weight marker (lane 7), 400 ng of native STE1 (lane 8).*

Several lipases have successfully been expressed and secreted in the yeast *P. pastoris*. In shake flasks, the production levels for lipases from *C. antarctica*, *G. candidum* and *C. rugosa* (LIP4) have been 25, 60 and 100 mg l^{-1}, respectively (Rotticci-Mulder *et al*., 2001; Holmquist *et al*., 1997; Tang *et al*., 2001), and the level of *Rh. oryzae* lipase was maximally 156 mg l^{-1} in a fermenter cultivation (Minning *et al*., 1998; 2001). If the steryl esterase produced in *P. pastoris* in this work has the same specific CAE activity as the native *M. albomyces* steryl esterase, the highest steryl esterase production level in *P. pastoris* culture supernatant should correspond to about 9 mg l^{-1}. Thus the STE1 was produced at a relatively low level in *P. pastoris*. The properties of the heterologous lipases have generally been similar to those reported for the native enzymes (Minning *et al*., 1998; Holmquist *et al*., 1997; Rotticci-Mulder *et al*., 2001; Quyen *et al*., 2003).

3.4.2 Heterologous expression of STE1 in *Trichoderma reesei*

In order to improve the steryl esterase production, STE1 was expressed in *T. reesei*, a well-known filamentous fungus capable of producing high amounts of hydrolytic enzymes extracellularly (Keränen and Penttilä, 1995; Mäntylä

et al., 1998). Lipases, such as those from *Candida antarctica* and *Thermomyces lanuginosus*, have successfully been produced heterologously in another well-known filamentous fungus, *Aspergillus oryzae* (Huge-Jensen *et al*., 1989; Hoegh *et al*., 1995; Boel *et al*., 1996). Furthermore, the heterologous expression of a lipase from *Penicillium allii* has been demonstrated in *T. reesei* (Bradner *et al*., 2003).

DNA encoding *M. albomyces* steryl esterase was expressed in *T. reesei* under the strongly inducible promoter of the major cellulase gene *cbh1*, and the transformants were selected for hygromycin resistance. Transformants indicating the highest CE activities were cultivated in shake flasks in minimal medium supplemented with 3% lactose and 1.5% spent grain. The highest CAE activity in the culture supernatant after a preliminary screening cultivation was 15 nkat ml^{-1}. Due to the binding property and aggregation tendency of native STE1, *T. reesei* mycelium was also extracted with Triton X-100. Two transformants produced clearly higher CAE activities than other transformants, and about 80–90% of their total CAE activity was recovered after Triton-extraction (Figure 4A/IV). The CE activities detected were relatively low due to lower sensitivity of the CE activity assay as compared to the CAE activity assay. However, a similar distribution between secreted and bound enzyme was observed on the basis of CE activities.

Steryl esterase production in *T. reesei* was studied on growth media containing cellulose or lactose (or whey) as carbon sources since they are known to be good inducers for genes expressed under the *cbh1* promoter in the RutC-30 strain. Both of the tested transformants yielded the highest production level on medium containing 4% whey (contains about 75% lactose) and 2% spent grain. Maximum CAE and CE activities of 99 nkat ml^{-1} and 7 nkat ml^{-1}, respectively, were measured from the culture supernatant of the transformant pHAK4/77 after 10d cultivation. About 60% of the activity was bound to the fungal mycelium or solid components of the medium or existed as aggregates, and about 67% of the bound activity (40% of the total activity yield) could be released by extraction with 0.1% Triton X-100 (Figure 4B/IV). The ratio between CAE and CE activities was about 14, which is close to that obtained for native STE1. *M. albomyces* was calculated to produce maximally 75 mg l^{-1} steryl esterase into the culture supernatant in shake flasks. It can be estimated on the basis of the specific CAE activity after purification of rSTE1 (2020 nkat mg^{-1}) that the steryl esterase activity produced by *T. reesei* would correspond to about 50 mg l^{-1} of

culture supernatant, and the total steryl esterase yield after Triton extraction would be 76 mg l^{-1} of culture. Despite the lower production level obtained in *T. reesei* than in the native host, the heterologous production system can be regarded as more reliable and reproducible compared to the homologous expression in *M. albomyces*.

T. reesei transformants (pHAK4/5 and pHAK4/77) cultivated in shake flasks were analysed by Western blotting. Both transformants showed a single band, whereas *T. reesei* control showed no band (Figure 12A). Western blotting indicated similar molecular weights for the native STE1 and the recombinant steryl esterase rSTE1 produced in *T. reesei* (Figure 12B). The native *M. albomyces* laccase and the recombinant laccase produced in *T. reesei* have also been shown to have very similar molecular weights when determined accurately by MALDI-TOF mass spectrometry (Kiiskinen *et al*., 2004). The difference between the molecular weights was only 0.9 kDa, suggesting that glycosylation in those two fungi is very similar.

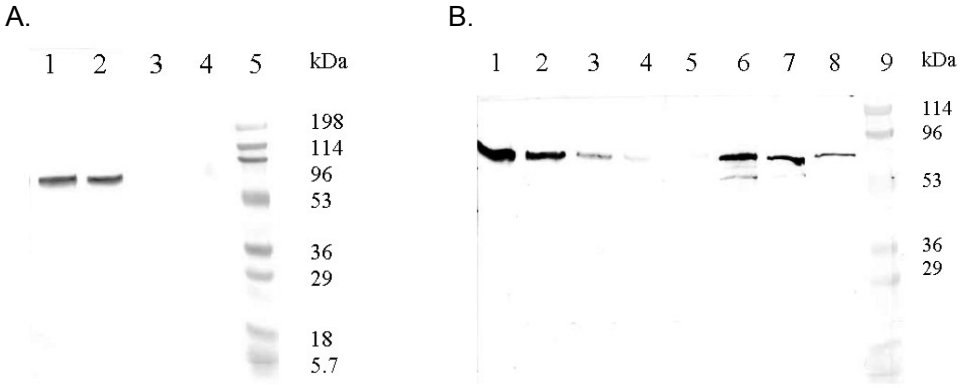

Figure 12. Western blot analysis. (A) 0.1% Triton-extract of Trichoderma transformants *pHAK4/5 and pHAK4/77 (lanes 1–2) and* T. reesei *control (lanes 3–4) cultivated for 10 d on medium containing 3% lactose and 1.5% spent grain. (B) Dilutions of 0.1% Triton-extract of* T. reesei *transformant pHAK4/77 (lanes 1–5) and native* Melanocarpus albomyces *steryl esterase: 800, 400 and 160 ng (lanes 6–8).*

In order to produce high amounts of *M. albomyces* steryl esterase, the *T. reesei* transformant producing highest steryl esterase activities in shake flask cultivations was cultivated in a laboratory-scale fermenter in whey-spent grain

medium. Maximum CAE and CE activities of 563 and 44 nkat ml^{-1}, respectively, were reached after 69 h. The main portion of activity (70%) was in the culture supernatant, and thus the cell fraction was discarded. According to the specific CAE activity immediately after purification of rSTE1 the production level in the fermenter was 280 mg l^{-1} of culture supernatant, corresponding to 325 mg l^{-1} of unseparated culture. Some lipases, such as those from *Candida antarctica*, *Rhizomucor miehei* and *Thermomyces lanuginosus*, have been reported to be secreted successfully from *Aspergillus oryzae* (Huge-Jensen *et al*., 1989; Hoegh *et al*., 1995; Boel *et al*., 1996). However, production levels have typically not been reported, with the exception of *R. miehei* lipase that was produced at 2 mg l^{-1} into the culture supernatant (Boel *et al*., 1996).

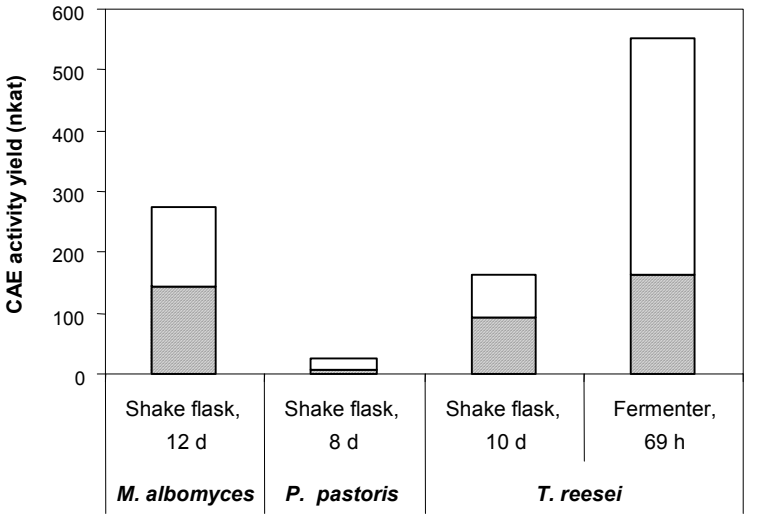

Figure 13. Steryl esterase production in Melanocarpus albomyces, Pichia pastoris (transformant pHAK5/1) and Trichoderma reesei (transformant pHAK4/77). Activity yield from 1 ml culture samples in culture supernatants (□) and in mycelium of M. albomyces and T. reesei or in P. pastoris cell lysate (▨).

In the future, steryl esterase production may be improved by engineering of the production organism. Production could be enhanced by gene fusion strategy in which the foreign protein is joined e.g. to the T. reesei cellobiohydrolase I (CBHI) that facilitate protein translocation and folding, and also protects the foreign protein from degradation (Nyyssönen et al., 1993; Keränen and Penttilä, 1995). Alternatively, co-expression of chaperones and/or foldases involved in

proper folding and assembly of subunits, may be used to increase the protein production level (Robinson et al., 1994; Valkonen et al., 2003). Other possible strategies are the increase of the copy number, the use of signal sequences from well-secreted proteins as well as classical mutagenesis of the production strain. Further optimisation of bioprocess parameters may also improve steryl esterase production.

3.5 Enzymatic properties of *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei* (V)

3.5.1 Purification of recombinant *Melanocarpus albomyces* steryl esterase

A histidine tag was fused to the C-terminus of STE1 in order to exploit a one-step purification protocol using immobilised metal affinity chromatography (IMAC). The rSTE1 enzyme did not bind to the column in the various conditions tested. This could be due to inaccessibility of the His-tag in the rSTE1 structure. This conclusion is supported by the fact that the rSTE1 also failed to react with His-tag specific antibody in Western blot analysis. Therefore, the enzyme was purified like the native STE1, using hydrophobic interaction chromatography and anion exchange chromatography. SDS-PAGE showed a single band with a molecular weight of 60 kDa for the purified rSTE1 (Figure 2/V). Interestingly, analytical gel filtration showed a single peak for the purified rSTE1 with a molecular weight of 120 kDa, indicating a dimeric structure. Under similar conditions, native STE1 showed a tetrameric structure with a molecular weight of 238 kDa. Both the native STE1 and the rSTE1 were shown by SDS-PAGE to be glycoproteins with about 5% N-linked carbohydrate. However, the native and recombinant proteins probably have slightly different glycosylation patterns, which could lead to different multimeric structures. The dimeric structure of rSTE1 might also hide the C-terminal His-tag within the interior part of the structure, thus preventing interaction between the His-tag and metal chelate. The existence of His-tag could also reduce the hydrophobicity of rSTE1 and thus affect the multimerisation structure of the protein in the conditions used.

3.5.2 Characterisation of recombinant steryl esterase

After purification the specific CAE and CE activities of rSTE1 were 2020 nkat mg^{-1} and 300 nkat mg^{-1}, respectively. During storage for one month at 4°C the activities decreased surprisingly probably due to proteolytic degradation, and residual specific activities of 1170 nkat mg^{-1} CAE and 82 nkat mg^{-1} CE were determined. Despite remarkable decrease of specific CAE and CE activities during one month storage, the preparation retained stability during long-term storage, and it was used for enzyme characterisation. Specific activities of the rSTE1 (Table 1/V) were lower than those of the native STE1 (Table 3/III) but the relative activity profiles of both enzyme preparations against different cholesteryl esters were rather similar (Figure 14). Despite lower activity levels of rSTE1 against *p*-nitrophenyl esters, both enzymes had highest specific activity against *p*-NPB and the lowest against *p*-NPA.

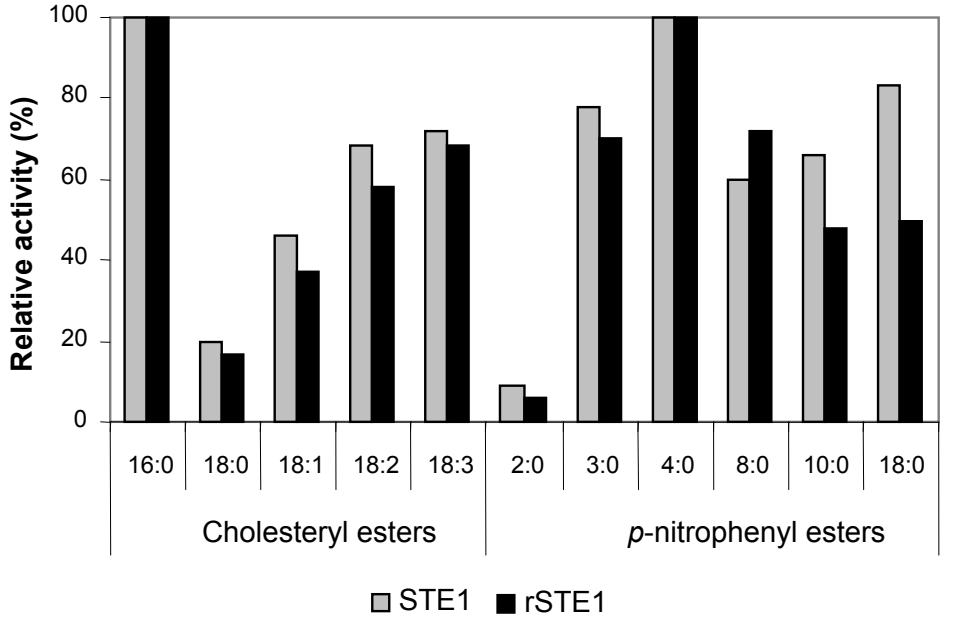

Figure 14. Relative activities of native (STE1) and recombinant (rSTE1) Melanocarpus albomyces *steryl esterases measured against different cholesteryl esters and* p-*nitrophenyl esters. Activities on cholesteryl palmitate (16:0) and* p-*nitrophenyl butyrate (4:0) represent relative activities of 100%.*

The pH optimum of the rSTE1 was studied using *p*-NPC, cholesteryl oleate and olive oil as substrates. The pH-dependence of the rSTE1 was almost identical to that of the native STE1 (Table 6, p. 56). The pH optimum of lipase activity was at pH 7–8, whereas carboxyl esterase and cholesteryl esterase activities had optima between 5 and 6 (Figure 3/V). The rSTE1 was most stable within the pH range of 3–7, and clearly less stable at pH values above 8 (Figure 4A/V). The rSTE1 retained over 90% of its CE activity after 24 h at 50°C and 50% after 1 h at 60°C, but the enzyme was inactivated at 70°C (Figure 4B/V). The native enzyme was more stable at higher temperatures, having half-lives of 4 h at 60°C and 2 h at 70°C. The residual activities were determined after incubation of the enzyme preparations in the absence of surfactant, and thus the possible differences in multimeric structures of STE1 and rSTE1 may affect the protein stabilities.

3.6 Modification of fibre products with recombinant *Melanocarpus albomyces* steryl esterase (V)

3.6.1 Effects of rSTE1 treatment on pulp extractives and handsheet properties

The hydrolysis of triglycerides of pulp by Resinase has been shown to increase paper strength properties (Fujita *et al*., 1992; Hata *et al*., 1996). In order to compare the effects of rSTE1 and the commercial lipase preparation Resinase A2X (Novozymes) on wood extractives, TMP suspension was treated with both enzyme preparations. The effects of esterase treatments on handsheet technical properties were investigated in the presence of 0.05% Triton X-100 because STE1 has previously been shown to hydrolyse model steryl esters effectively in the presence of Polydocanol, a non-ionic surfactant. The tensile index was significantly increased after both enzyme treatments when compared to the reference sample (Table 8). However, it is noteworthy that use of the surfactant in the TMP treatments might have negative implications for the binding ability of fibres, and thus decrease the tensile strength of the reference sheets. This is probably a consequence of blocking the formation of bonds between fibres due to adsorption of surfactant onto the fibre surface (Touchette and Jenness 1960). The density of the paper sheets was also lower in the presence of surfactant indicating the longer distance between fibres. The optical properties (ISO

Brightness, opacity, light scattering coefficient) and tear index were not affected by the enzymatic treatments. The tensile strength was also improved by rSTE1 in a treatment in which only the Triton X-100 of the enzyme preparation was present (0.0009%; Table 8).

Table 8. Effects of esterase treatments on physical properties of handsheets. Treatments were performed at 1% pulp consistency at 50°C and pH 5 for 20 h using a lipase dosage of 500 nkat g^{-1} pulp.

Treatment	Triton X-100 %	Grammage g/m^2	Density kg/m3	ISO Brightness %	Opacity, %	Light scattering coefficient m^2/kg	Tensile index Nm/g	Tear index Nm^2/kg
Reference	0.0009	66.8	345.0	60.2	94.8	56.6	36.3	6.27
rSTE1	0.0009	66.4	354.0	60.8	94.2	56.0	38.7	6.78
Reference	0.05	65.8	296.0	61.0	94.6	57.9	27.6	6.34
Resinase A2X	0.05	66.2	318.0	61.0	94.4	57.2	33.4	6.46
rSTE1	0.05	66.3	322.0	61.7	94.2	57.1	34.1	6.30

Extractives are known to increase the contact angle of water applied on the paper sheet surface due to their hydrophobic nature (Kokkonen *et al*., 2002). Therefore the effects of the rSTE1 and Resinase treatments of pulp on the contact angle of water on handsheets were studied. Contact angles of water on the sheets were decreased by the enzymatic treatments (results not shown). The decrease in contact angles was evidently caused by decreased hydrophobicity of the fibre and sheet surfaces. It is also possible that adsorbed extractives on the fibre surfaces affect the sheet structure and thus the contact angle (Kokkonen *et al*., 2002). The rSTE1 improved wettability somewhat more efficiently, which might be due to partial hydrolysis of steryl esters. Previously, it has been reported that steryl esters have a significant impact on the contact angle (Kokkonen *et al*., 2002; Qin *et al*., 2003), and the present results support this conclusion.

The TMP water samples were taken after enzyme treatments before filtration of the pulp suspension, in order to retain the maximal amount of extractives. The changes in the chemical composition of the extractives were analysed by GC

(Table 2/V). The triglycerides of the TMP were effectively hydrolysed to free fatty acids and glycerol by both enzyme treatments. Resinase was not able to degrade steryl esters, which has also been observed previously (Mustranta *et al*., 2001; Blanco *et al*., 2005). The amount of steryl esters was slightly decreased by rSTE1, with a corresponding increase of free sterols. Thus, it can be concluded that the hydrolysis of steryl esters is restricted in the conditions used. The treatments of model extractives with STE1 showed previously that triglycerides were hydrolysed well even in the absence of surfactant, but that hydrolysis of steryl esters requires the presence of a surfactant.

The highly hydrophobic triglycerides and steryl esters are known to form the core and the main part of the pitch droplets with an average diameter of 0.26 μm (Sundberg *et al*., 1996a; Nylund *et al*., 1998; Qin *et al*., 2003). The outer surface, the thickness of which is less than 0.01 μm, is enriched with resin acids, fatty acids and sterols with their carboxyl and hydroxyl groups extending into the water. Despite the viscous and rigid outer film, almost total hydrolysis of triglycerides can be achieved enzymatically. However, steryl esters are more hydrophobic and viscous than triglycerides (Qin *et al*., 2003), and thus they are probably less accessible. It is possible that in the presence of triglycerides and steryl esters, rSTE1 and LIP3 used in this study hydrolyse primarily triglycerides. This leads to accumulation of free fatty acids at the outer surface of the droplets, which may decrease the ability of enzymes to act on steryl esters. Fatty acids may also bind to the active site of the enzyme and thus lower the activity on steryl esters (Fleet and Breuil, 1998). Total hydrolysis of steryl esters would increase the amount of sterols, which have been shown to increase the viscosity of resin and to have a negative impact on pitch deposition tendency (Qin *et al*., 2003). Therefore it is likely that 100% hydrolysis of steryl esters would probably not be advantageous in the papermaking process. However, the physico-chemical characterisation of model pitch mixtures has shown that a combination of lipase and steryl esterase treatments, and particularly adjustment of the hydrolysis ratio of steryl esters, can positively affect pitch properties (Qin *et al*., 2003).

Modern knowledge in protein engineering may offer efficient means to develop more optimal esterases, as well as other enzymes and enzyme cocktails, for the pulp and paper industry. For example, the fusion of a cellulose-binding domain to the *Candida antarctica* lipase B was found to be an efficient way to anchor lipase near to its substrate (Rotticci-Mulder *et al*., 2001). Rational protein design

and directed evolution also provide means to optimise biocatalytic reactions by affecting the properties of lipases and esterases, such as active site, substrate specificity, stability, lid function and calcium binding (Svendsen, 2000; Bornscheuer *et al*., 2002). Especially protein engineering of hydrophobic binding surfaces around the active site, at both fatty acid and alcohol binding regions, may be crucial for modifying the activity and selectivity of lipases (Kazlauskas, 1994; Fojan *et al*., 2000; Zhu *et al*., 2001). Improvement of thermostability generated e.g. by amino acid substitutions, introduction of disulphide bridges or C-terminal extension (Svendsen, 2000) could also be advantageous from an industrial point of view, for example in enzymatic pitch control (Blanco *et al*., 2005).

3.6.2 Effects of rSTE1 treatment on polyester properties

In addition to the pulp extractives, rSTE1 was also used for modification of polyester fabric. Despite several favourable characteristics of polyester, it also has some undesired properties (Yoon *et al*., 2002). The hydrophobic nature and inactive surface of polyester make it resistant to oil and grease stain removal and difficult to dye. Polyethylene terephthalate (PET), the most important synthetic fibre in the textile industry, was treated enzymatically with rSTE1 in order to increase its hydrophilicity. The effect of rSTE1 on the textile properties of PET was evaluated by determining wetting and dyeing behaviour. Significant reduction in hydrophobicity was observed after the enzyme treatments, since the contact angle and penetration time of water were clearly decreased (Figure 15). Improved hydrophilicity is most probably caused by hydrolysis of ester bonds in the polyester backbone, leading to increase in polar carboxyl and hydroxyl groups on the surface of PET. The increased polarity on the surface enables polar interaction and hydrogen bonding with water molecules and thus increases the water wettability of the fibres (Hsieh and Cram, 1998). Similar effects have previously been shown by treatments with commercial *Pseudomonas* lipase and cutinase (Optimyze, Buckman) preparations (Hsieh and Cram, 1998; Miettinen-Oinonen *et al*., 2005).

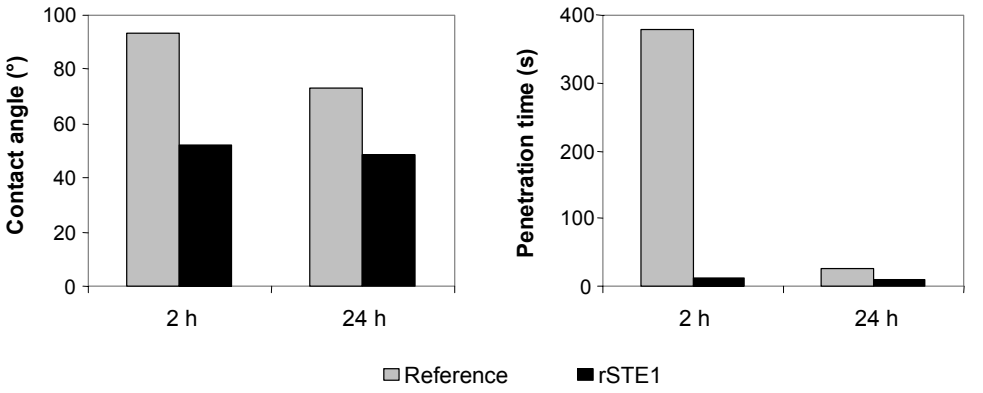

Figure 15. Contact angles and penetration times of water determined for the rSTE1-treated polyester fabric. Treatments were performed at 40°C and pH 7 using a lipase dosage of 1000 nkat g^{-1} fabric.

Treatment of PET by rSTE1 for 24 h also improved binding of methylene blue dye on the polyester surface (Table 4/V). Improved dyeing of PET has previously been demonstrated by treatments with polyesterase and cutinase, and is explained by increased amounts of carboxylic groups on the fabric surface (Yoon *et al*., 2002; Miettinen-Oinonen *et al*., 2005).

The effects of rSTE1 and *Pseudomonas* sp. lipase preparation on raw cotton woven fabric have also been preliminarily studied. The amount of released fatty acids was increased by both enzyme treatments, indicating hydrolysis of cotton waxes (A. Miettinen-Oinonen, VTT, personal communication). These results suggest that esterases may improve the wettability and thus the further processing of cotton fabrics.

4. Conclusions and future perspectives

Steryl esterases are esterases which primarily hydrolyse steryl esters, but which are able to act on a wide range of esters. In this work, steryl esterase activities were characterised from commercial lipase preparations. The work also describes biochemical characterisation and heterologous production of a novel steryl esterase from the fungus *Melanocarpus albomyces*. The effects of *M. albomyces* steryl esterase on the properties of paper sheets prepared from thermomechanical pulp and on polyethylene terephthalate fibre were also preliminarily evaluated.

Commercial lipase preparations from *Candida rugosa*, *Chromobacterium viscosum*, and *Pseudomonas* sp. were able to hydrolyse steryl esters efficiently. At temperatures above 50°C, the highest degree of steryl ester hydrolysis was achieved in the absence of surfactant with the lipase preparation of *Pseudomonas* sp., and thus it was considered as the most potential enzyme for pitch control in pulp and paper manufacturing. Steryl esterase was purified from the lipase preparation of *C. rugosa* and it was identified as the LIP3 lipase, which is known to possess steryl esterase activity. LIP3 was highly active on steryl esters in the presence of surfactant, and in aqueous dispersions about half of the steryl esters was hydrolysed.

A novel steryl esterase from *M. albomyces* was purified and biochemically characterised with respect to size, isoelectric point, substrate specificity, pH and temperature characteristics and surfactant effects. The steryl esterase had a pH optimum at slightly acidic pH with various esters and was also more stable at lower pH than at alkaline values. The steryl esterase was shown to have good thermostability, which increases its potential for many industrial applications. *M. albomyces* steryl esterase was highly active on steryl esters and triglycerides in the presence of surfactant. Triglycerides were also effectively degraded in the absence of surfactant.

In order to obtain a more reliable production and higher yields than obtained in *M. albomyces*, the steryl esterase gene of *M. albomyces* was cloned and expressed heterologously in the well-known protein production hosts *Pichia pastoris* and *Trichoderma reesei*. The production level was low in *P. pastoris*,

and a large proportion of the total activity yield was found to be present intracellularly. *T. reesei* produced a clearly higher amount of steryl esterase and a production level of 280 mg l^{-1} was achieved in a laboratory-scale fermenter without optimisation. In the future, steryl esterase production may be improved by gene fusion strategy or co-expression of genes involved in folding and secretory functions. Improvements in the product yield may also be obtained after optimisation of operating conditions and feeding strategies. Recombinant steryl esterase (rSTE1) produced in *T. reesei* was compared to the native steryl esterase (STE1). The rSTE1 was shown to be a dimer, whereas the native STE1 has a tetrameric structure. Differences in multimeric structures probably result from the different hydrophobic character of the enzymes, which also can affect enzyme activities and stabilities. The native STE1 was somewhat more stable and had slightly higher activities against various substrates than the rSTE1.

Thermomechanical pulp suspension was treated with rSTE1 in order to evaluate its effects on wood extractives and handsheet technical properties. The rSTE1 treatment clearly improved the tensile strength and wettability of the paper due to its effects on triglycerides and steryl esters. Despite the viscous and rigid outer film of pitch particles, triglycerides were hydrolysed efficiently by rSTE1 whereas the hydrolysis of steryl esters was restricted. Steryl esters are more hydrophobic and viscous than triglycerides, and thus they are probably less accessible than triglycerides. Obviously, the recombinant *M. albomyces* steryl esterase prefers the hydrolysis of triglycerides, which might also cause accumulation of free fatty acids and thus reduce the ability of the enzyme to act on steryl esters. However, total hydrolysis of steryl esters would probably not be advantageous in the papermaking process due to the negative impact of sterols on pitch deposition tendency. Thus, the combination of lipase and steryl esterase treatments, and especially adjustment of the hydrolysis ratio of steryl esters, would affect pitch properties positively. However, the actual effectiveness of steryl esterases on wood extractives can only be evaluated in white water treatments containing retention aids and other pitch-control agents present in paper mills.

The effects of the rSTE1 treatment on the textile properties of polyethylene terephthalate (PET) were evaluated. The enzyme treatment clearly increased the hydrophilicity of the fabric by hydrolysing the ester bonds in the polyester backbone. Dyeing of PET was also slightly improved after the enzyme

treatment. The results showed that steryl esterases not only have potential for the treatment of their natural substrates but that they are also interesting tools for the modification of synthetic materials. The use of steryl esterases in modification of natural polymers, such as cutin and suberin, could also provide an interesting tool for exploitation of low-value raw materials as a source for bioactive and functional components. In addition to their hydrolytic action, steryl esterases may also be exploited as specific tools in the synthesis of various esters used e.g. in food and cosmetic products.

The work presented here provides information on novel steryl esterases ranging from biochemical and molecular characterisation to heterologous expression and application studies. Microbial steryl esterases are a rather poorly characterised group of esterases, and this work has significantly increased the knowledge of the action of steryl esterases. In this work it was shown that steryl esterases are able to act on both triglycerides and steryl esters simultaneously. Further studies concerning the characteristics of steryl esterases will facilitate improvement of their usefulness in economical and environmentally friendly applications. Protein engineering may also provide methods to alter enzyme properties, such as substrate specificity by modifying the hydrophobic binding surfaces around the active site.

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PUBLICATION I

Characterisation of steryl esterase activities in commercial lipase preparations

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Characterisation of steryl esterase activities in commercial lipase preparations

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Abstract

Triglycerides, steryl esters, resin acids, free fatty acids and sterols are lipophilic extractives of wood (commonly referred to as pitch or wood resin) and have a negative impact on paper machine runnability and quality of paper. Thus, enzymes capable of modifying these compounds would be potential tools for reducing pitch problems during paper manufacture. In this work, 19 commercial lipase preparations were tested for their ability to degrade steryl esters, which may play a significant role in the formation and stabilisation of pitch particles. Six lipase preparations were shown to be able to degrade steryl esters. Lipase preparations of *Pseudomonas* sp., *Chromobacterium viscosum* and *Candida rugosa* were shown to have the highest steryl esterase activities. The enzymes were able to hydrolyse steryl esters totally in the presence of a surfactant (Thesit). Up to 80% of the steryl esters were degraded in aqueous dispersion. Preliminary characterisation of the enzymatic activities revealed that the lipase preparation of *Pseudomonas* sp. could be the most potential enzyme in industrial applications. The steryl esterase activity of this preparation was stable over a broad pH range and the enzyme was able to act efficiently at pH 6–10 and at temperatures up to 70 °C.

Keywords: Steryl esters; Steryl esterase; Cholesteryl esterase; Lipase; (Wood) extractives; Pitch problems

1. Introduction

Wood extractives include a large number of different compounds, which are poorly soluble in water. The main lipophilic compounds of pitch are triglycerides, steryl esters, resin acids, free fatty acids and sterols. In mechanical pulping of softwood, most of the extractives are dispersed as colloidal droplets and released into the process water because of the strong mechanical shear forces and the high temperature (Ekman et al., 1990; Örså and Holmbom, 1994). These colloidal particles are able to form stable aggregates together with polysaccharides present in the process water. The pitch deposits can adhere to different parts of the paper machine or may appear as sticky spots in the paper (Allen, 1980; Dreisbach and Michalopoulos, 1989). This causes interruptions in paper production, decreases paper product quality and dewatering. Problems caused by extractives are increased in closed water systems (Zhang et al., 1999).

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Traditional methods for reducing the pitch problems include debarking, seasoning of wood chips to enhance degradation of extractives, and addition of chemicals (Hassler, 1988). Alternatively, biological pitch control by enzymatic treatment and by microbial pre-treatment has been developed. Microbial pre-treatment is based on the acceleration of natural wood seasoning by spraying selected microorganisms onto wood chips or logs. A commercial product, Cartapip^{TM}, has been reported to metabolise triglycerides efficiently, but not steryl esters or waxes (Blanchette et al., 1992; Brush et al., 1994). A number of fungi have also been screened and some of them have been found to partly degrade steryl esters (Chen et al., 1994; Leone and Breuil, 1999; Gutiérrez et al., 2000). However, fungal pre-treatment is difficult to control. It normally requires wood sterilisation, adjusted conditions (aeration, temperature), rather long treatment time and can decrease the pulp yield.

Lipase treatments have been shown to reduce pulp triglycerides in laboratory and pilot scale trials (Fischer and Messner, 1992; Mustranta et al., 1995). Lipases (EC 3.1.1.3) have a broad substrate specificity. They are capable of hydrolysing water-insoluble esters typically containing long chain fatty acids. Triglycerides are the commonest substrates for lipases, but steryl esters may also act as substrates. Lipase (Resinase^{R}, Novozymes) acting on the triglycerides of wood extractives has been used by Nippon Paper Co. for treatment of red pine mechanical pulps for several years in Japan (Fujita et al., 1992; Hata et al., 1996; Chen et al., 2001). However, Resinase has not been shown to have any effect on steryl esters (Mustranta et al., 2001), which may play a significant role in the formation and stabilisation of pitch particles. Steryl esterases (EC 3.1.1.13) hydrolyse fatty acid esters of sterols. They have been studied extensively from a number of mammalian tissues primarily because of their importance in the absorption and metabolism of cholesterol. They have also been detected and characterised in microbes such as *Pseudomonas fluorescens* (Uwajima and Terada, 1976), *Streptomyces lavendulae* (Kamei et al., 1977, 1979), *Fusarium oxysporum* (Okawa and Yamaguchi, 1977; Madhosingh and Orr, 1981) and *Saccharomyces cerevisiae* (Taketani et al., 1978, 1981).

In this work, the steryl esterase activities of commercial lipase preparations were characterised in order to evaluate their potential and technical performance in the degradation of wood steryl esters, and thus further improve enzymatic methods to control pitch problems.

2. Materials and methods

2.1. Lipase preparations

Lipase preparations were obtained from Amano Enzyme Europe Ltd. (UK), Biocatalysts Ltd. (UK), Novozymes (Denmark), Asahi Chemical Industry Co., Ltd. (Japan) and Seppim Elitech (France) (Table 1).

2.2. Substrates

Cholesterol and pure cholesteryl esters (cholesteryl palmitate, -stearate, -oleate, -linoleate, -linolenate) were obtained from Sigma. Steryl ester (plant steryl ester, PSE), which was synthesised from plant sterols and fatty acids, was kindly provided by Dr. Hendrik Luttikhedde (Raisio Chemicals, Finland). It is a mixture of several steryl esters, and its purity was almost 100% (Tenkanen et al., 2002). Another steryl ester (wood steryl ester, WSE) synthesised from tall

Table 1
Lipase preparations used in the present work

Origin	Supplier	Market name
Aspergillus niger	Amano	Lipase A “Amano”6
Aspergillus niger	Biocatalysts	L018
Aspergillus sp.	Novozymes	Resinase A
Candida antarctica	Novozymes	Novozym 525L
Candida rugosa	Amano	Lipase AY “Amano”30
Candida rugosa	Biocatalysts	L034
Chromobacterium viscosum	Asahi	Lipase (LP)
Geotrichum candidum	Biocatalysts	L052
Mucor javanicus	Amano	Lipase M “Amano”10
Penicillium camembertii	Amano	Lipase G “Amano”50
Penicillium cyclopium	Biocatalysts	L055
Penicillium roqueforti	Amano	Lipase R “Amano”
Pseudomonas sp.	Amano	PS
Pseudomonas sp.	Seppim	Lipase PS-30
Rhizopus arrhizus	Biocatalysts	L057
Rhizomucor miehei	Novozymes	Palatase 20000L
Rhizopus niveus	Amano	Newlase F
Rhizopus oryzae	Amano	Lipase F-AP 15
Thermomyces lanugenousus	Novozymes	Lipozyme TI 100L

oil fatty acids and softwood sterols was provided by Peter Spetz (Åbo Akademi University). The purity of synthesised WSE was approximately 90% (Tenkanen et al., 2002).

2.3. Activity assays

Stock solutions of lipases used for activity measurements were prepared by dissolving preparations in 0.1 M sodium phosphate buffer (pH 7). Activities were measured in duplicate.

2.3.1. Cholesteryl esterase activity

The assay used for determination of cholesteryl esterase (CE) activity was based on the spectrophotometric determination of liberated cholesterol after hydrolysis of 4.3 mM cholesteryl oleate according to Tenkanen et al. (2002). Lipase preparations were diluted in 0.1 M sodium phosphate buffer (pH 7) containing 0.4% Thesit.

2.3.2. Carboxyl esterase activity

Carboxyl esterase (CAE) activity was determined using 2.5 mM *p*-nitrophenyl caprate (Sigma) as substrate according to Tenkanen et al. (2002). Lipase preparations were diluted in 0.1 M sodium phosphate buffer (pH 7) containing 0.4% Triton X-100.

2.3.3. Lipase activity

Lipase activity was assayed using olive oil emulsion as substrate according to Mustranta et al. (1993) with some modifications. The substrate solution was prepared by emulsifying 30 ml of olive oil (Bertolli) with 70 ml reagent (17.9 g l^{-1} NaCl, 0.41 g l^{-1} KH_2PO_4, 10 g l^{-1} gum arabic (Sigma) and 47% v/v glycerol in distilled water) and homogenising for 3 min. The reaction mixture consisting of 0.98 ml 0.2 M sodium phosphate buffer (pH 7.0), 0.02 ml enzyme solution and 1 ml substrate emulsion was incubated for 10 min at 40 °C. The reaction was terminated by boiling, after which 2 ml of an acetone–ethanol mixture (1:1 v/v) was added. The rather clear solution was transferred to an Eppendorf tube and centrifuged at 12 000 × *g* for 2 min. The liberated fatty acids were determined in microtiter plates using an enzymatic colorimetric method (1 383 175, Roche), and the absorbency was measured at 540 nm.

2.3.4. Activity on different cholesteryl and steryl esters

Activities on different cholesteryl esters (cholesteryl palmitate, -stearate, -linoleate, -linolenate) and steryl esters (PSE and WSE) were measured according to the assay for cholesteryl esterase activity. The substrate concentration was 4.3 mM, with the exception of cholesteryl palmitate with a concentration of 1.5 mM.

2.4. Characterisation of enzymes

2.4.1. Activity at different values of pH and temperature

Cholesteryl esterase activities of lipase preparations, dissolved in 1 mM sodium phosphate buffer (pH 7), were measured at different pH values using McIlvaine buffer (0.1 M Na_2HPO_4 and 0.05 M citric acid) at pH 2.2–8.0, Tris–HCl buffer (0.1 M Tris and 0.05 M HCl) at pH 7.2–9.1 and 0.05 M glycine–NaOH buffer at pH 8.6–10.6. The reaction time was 20 min at 40 °C. The effect of temperature was determined between 30 and 80 °C in 0.1 M sodium phosphate buffer (pH 7). The reaction time was 20 min.

2.4.2. pH and temperature stability

To investigate pH stability, the enzymes were incubated at different pH values at 40 °C for 24 h. The pH of the lipase preparations, dissolved in 1 mM sodium phosphate buffer (pH 7), was adjusted with 0.5 M citrate–phosphate buffer (2.2–8.0), 1 M Tris–HCl buffer (7.2–9.1) or 1 M ammonia–acetic acid (8.5–10.0) to obtain final buffer concentrations of 12.5 or 25 mM. The pH of hydrolysis reactions was adjusted to pH 7 with 0.5 M sodium phosphate buffer (pH 7). Temperature stability was investigated by incubating the enzymes at 30–80 °C for 0.5, 3 and 24 h in 0.1 M sodium phosphate buffer (pH 7). Protein concentrations of the reaction mixtures during both incubations were 4 mg ml^{-1}. After the incubations, enzyme activity was measured using cholesteryl oleate as substrate.

2.5. Protein determination

Protein concentrations were determined by the method of Lowry et al. (1951) with a commercial Bio-Rad DC Protein Assay kit using bovine serum albumin as standard.

2.6. Hydrolysis of plant steryl esters

Hydrolysis of the plant steryl ester mixture (PSE) was investigated in aqueous dispersions. Homogeneous dispersion was prepared by first dissolving PSE in acetone (11.5 mg ml^{-1}) after which this solution was diluted with water to obtain steryl ester concentration 100 mg l^{-1}. This dispersion was mixed at 60 °C for 3 h to evaporate the acetone. After this the pH of the dispersion was adjusted with volatile buffer (ammonium acetate for pH 5, ammonia–formic acid for pH 7, ammonia–acetic acid for pH 9) to give a final buffer concentration of 25 mM. Hydrolysis of PSE was also compared in pure aqueous dispersion and in the presence of non-ionic surfactant (polyoxyethylene-9-lauryl ether, Thesit, Sigma). After incubation with enzyme for 1–24 h, the reaction was terminated by boiling.

2.7. Chemical analysis

After termination of the hydrolysis reaction samples were lyophilised and then re-dissolved in 10% Thesit solution containing 0.9% NaCl in a water bath (60 °C). The amount of released sterols was analysed spectrophotometrically (139 050, Roche) using commercial cholesterol (Sigma) as standard and the degree of hydrolysis was calculated from the theoretical sterol yield after total hydrolysis. To confirm the reliability of sterol analysis free fatty acids were also determined spectrophotometrically (1 383 175, Roche) using linoleic acid (Fluka) as the standard.

3. Results

3.1. Enzymatic activities of lipase preparations

The enzyme preparations used in the study are listed in Table 1. The enzymatic activities were measured using different cholesteryl esters (cholesteryl palmitate, -stearate, -linoleate, -linolenate), plant steryl ester mixtures from plants (PSE) and wood (WSE), *p*-nitrophenyl caprate and olive oil (lipase activity) as substrates. The results shown in Table 2 indicate that the lipase preparations from *C. rugosa*, *Ch. viscosum*,

Table 2
Specific activities of lipase preparations (nkat mg^{-1} of protein) on different substrates

	Cholesteryl esters					Steryl ester mixtures		*p*-Nitrophenyl caprate	Olive oil
	Palmitate	Stearate	Oleate	Linoleate	Linolenate	Plant[a]	Wood[b]		
A. niger, Amano	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	4.5	320
A. niger, Biocatalysts	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	7.9	490
Aspergillus, Novozymes	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	62	27 600
C. antarctica	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	79	1 100
C. rugosa, Amano	83	20	116	115	184	97	70	3 600	5 800
C. rugosa, Biocatalysts	9.0	2.0	13	12	19	10	7.3	1 400	6 000
Ch. viscosum	130	33	80	76	77	85	75	149 000	16 900
G. candidum	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	690
M. javanicus	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.1	200
P. camembertii	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2.8	220
P. cyclopium	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2.3	730
P. roqueforti	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	260
Pseudomonas sp., Amano	5.0	0.4	2.3	3.2	3.1	2.8	2.8	3 400	1 200
Pseudomonas sp., Seppim	5.6	0.3	3.4	4.1	3.9	3.8	4.4	4 100	2 000
R. arrhizus	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	2 500
R. miehei	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	320	5 100
R. niveus	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	170
R. oryzae	0.76	0.16	0.95	0.98	1.22	0.79	0.59	6.1	3 200
T. lanugenousus	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	140	25 500

N.D.: not detected.
[a] Main components: 18:1 (53%), 18:2 (18%), 16:0 (9%), 18:3 (8%), sitosterol (46%), campesterol (26%), stigmasterol (24%).
[b] Main components: 18:2 (51%), 18:1 (25%), 18:3 (12%), sitosterol (71%), sitostanol (18%), campesterol (9%).

Pseudomonas sp. and *R. oryzae* were able to degrade steryl esters under the conditions used. Bacterial enzymes (*Ch. viscosum* and both *Pseudomonas* sp.) had the highest activities on cholesteryl palmitate and the lowest on cholesteryl stearate. The fungal enzymes (*C. rugosa* and *R. oryzae*) were more active on unsaturated fatty acid esters. The two *C. rugosa* preparations were identical in their substrate specificity. *C. rugosa* (Amano) lipase preparation had the highest relative steryl esterase activity in comparison to the lipase activity. The *Ch. viscosum* preparation also had high activity on steryl esters, and it showed the highest specific activity on olive oil and *p*-nitrophenyl caprate.

3.2. The effects of pH and temperature on enzyme activity and stability

Steryl esterases of *C. rugosa* (Biocatalysts), *Pseudomonas* sp. (Seppim), *Ch. viscosum* and *R. oryzae* were further characterised in detail. The pH-dependence and effects of temperature were studied using cholesteryl oleate as substrate. The enzymes showed activity over a broad pH range (Fig. 1). The pH optimum of *C. rugosa* was rather narrow and at acidic pH (pH 4). The shapes of the curves of *Ch. viscosum* and *Pseudomonas* sp. indicate that the preparations might contain two or more steryl esterases having different pH-optima.

The effect of temperature on the enzyme activity was determined using a 20-min reaction time at pH 7 (Fig. 2). Under these conditions the enzymes were stable during the incubation. The activity profiles of *C. rugosa* and *R. oryzae* were almost identical. Their activity decreased sharply at temperatures above 50 °C. *Ch. viscosum* steryl esterase was clearly more active at higher temperature. Maximal steryl esterase activity was obtained at 60 °C. *Pseudomonas* sp. steryl esterase had the broadest temperature range. Over 80% of the maximum activity was observed between 30 and 70 °C.

The pH stability curves are shown in Fig. 3. The enzymes were incubated at 40 °C for 24 h at different pH (2.2–10). The results indicate that steryl esterases of *C. rugosa*, *Pseudomonas* sp. and *Ch. viscosum* had broader pH stability than the *R. oryzae* enzyme. Fig. 4

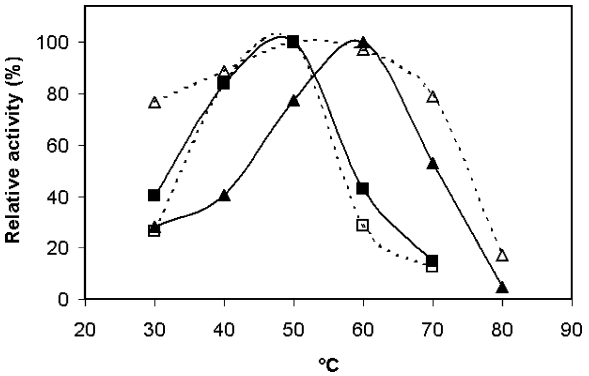

Fig. 2. Effect of temperature on cholesteryl esterase activity. The reaction mixture was incubated for 20 min at pH 7. C. *rugosa* (■), *R. oryzae* (□), *Ch. viscosum* (▲) and *Pseudomonas* sp. (△).

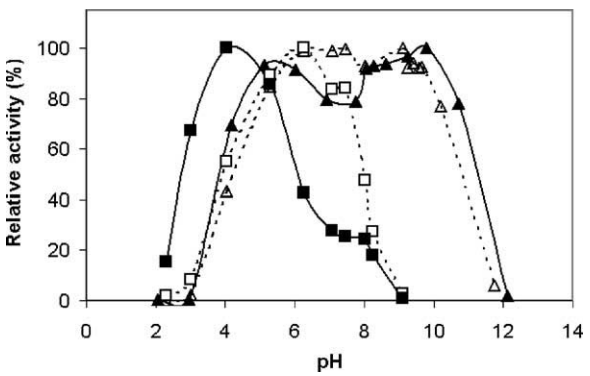

Fig. 1. Effect of pH on cholesteryl esterase activity. The reaction mixture was incubated for 20 min at 40 °C. *C. rugosa* (■), *R. oryzae* (□), *Ch. viscosum* (▲) and *Pseudomonas* sp. (△).

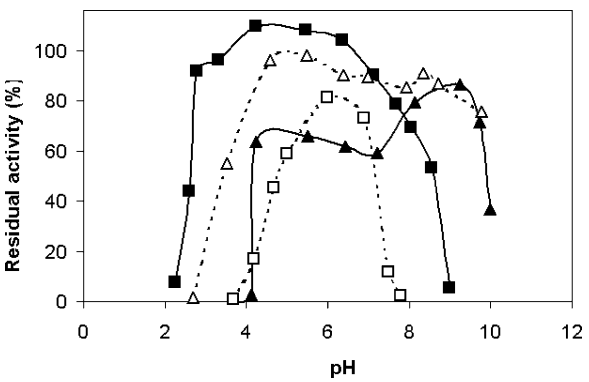

Fig. 3. pH stability of the enzymes. The preparations were incubated for 24 h at 40 °C. C. *rugosa* (■), *R. oryzae* (□), *Ch. viscosum* (▲) and *Pseudomonas* sp. (△).

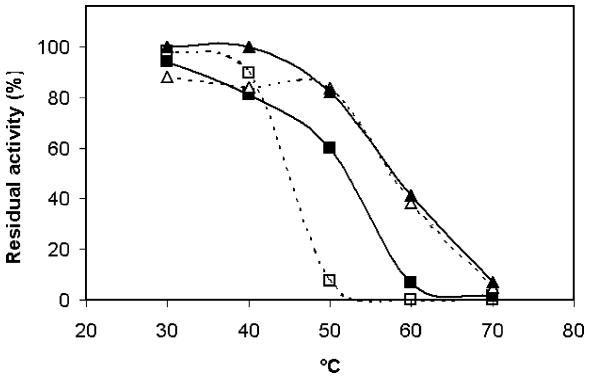

Fig. 4. Temperature stability of the enzymes. The preparations were incubated for 3 h at pH 7. *C. rugosa* (■), *R. oryzae* (□), *Ch. viscosum* (▲) and *Pseudomonas* sp. (△).

shows the temperature stability curves at pH 7 with a 3-h incubation time. Steryl esterases of *Pseudomonas* sp. and *Ch. viscosum* were the most thermostable, retaining 80% of their activity at 50 °C. The enzymes were shown to retain 100% of their activity up to 60 °C with 30-min incubation time (results not shown). *R. oryzae* steryl esterase was clearly inactivated at temperatures above 40 °C.

3.3. The effect of pH, temperature and presence of surfactant on plant steryl ester hydrolysis

The enzymes were shown to degrade plant steryl ester mixture very effectively in the presence of 0.1% surfactant (Thesit) (Fig. 5). Almost 100% hydrolysis was reached with all the enzyme preparations studied. Comparison to reactions in aqueous steryl ester dispersions without the surfactant shows that although the initial reaction rates were rather similar, the final degree of hydrolysis was only 70–80% in aqueous dispersion. The steryl esterase of *Ch. viscosum* was clearly the slowest enzyme in aqueous dispersion, but in the presence of the surfactant the initial reaction rate was the highest. In contrast to the other enzymes, the initial reaction rate of *C. rugosa* steryl esterase was lower in the presence of the surfactant than in aqueous dispersion.

The effects of pH and temperature on the hydrolysis of aqueous dispersions were studied in 24-h incubation (Fig. 6). The highest degree of hydrolysis was obtained at pH 7. The degree of hydrolysis with *Ch.*

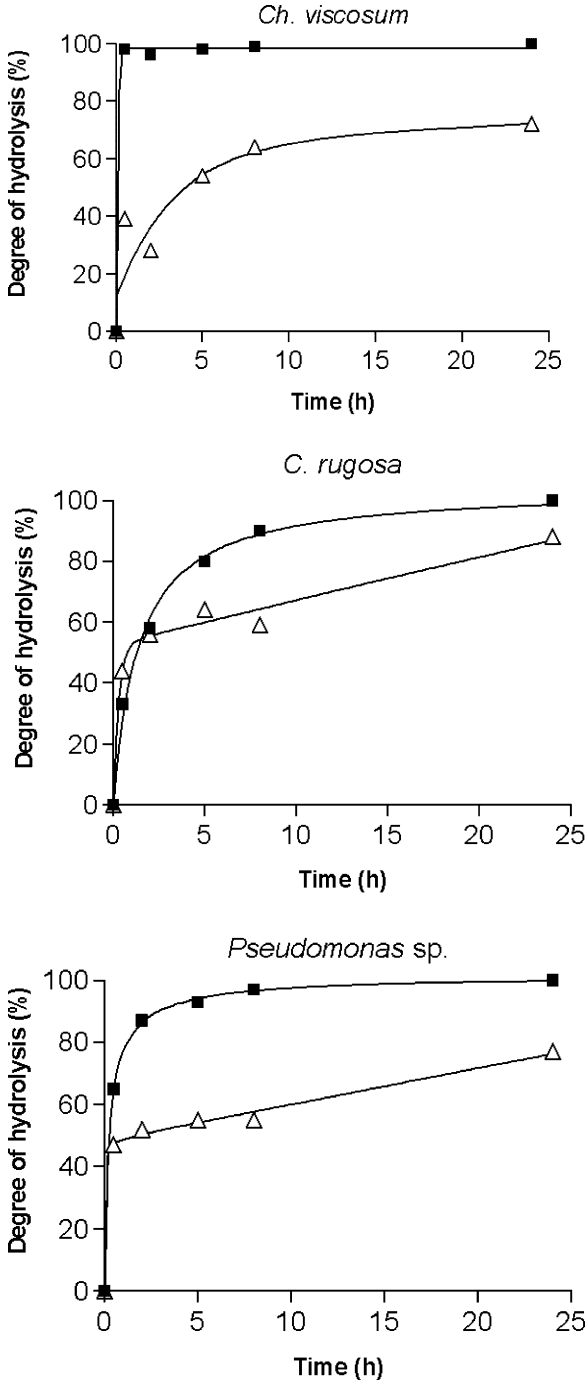

Fig. 5. Degree of hydrolysis of plant steryl esters as a function of time in aqueous dispersion (△) and in the presence of 0.1% Thesit (■). Substrate concentration 100 mg l^{-1}, enzyme dosage 500 nkat g^{-1}, pH 7, 40 °C.

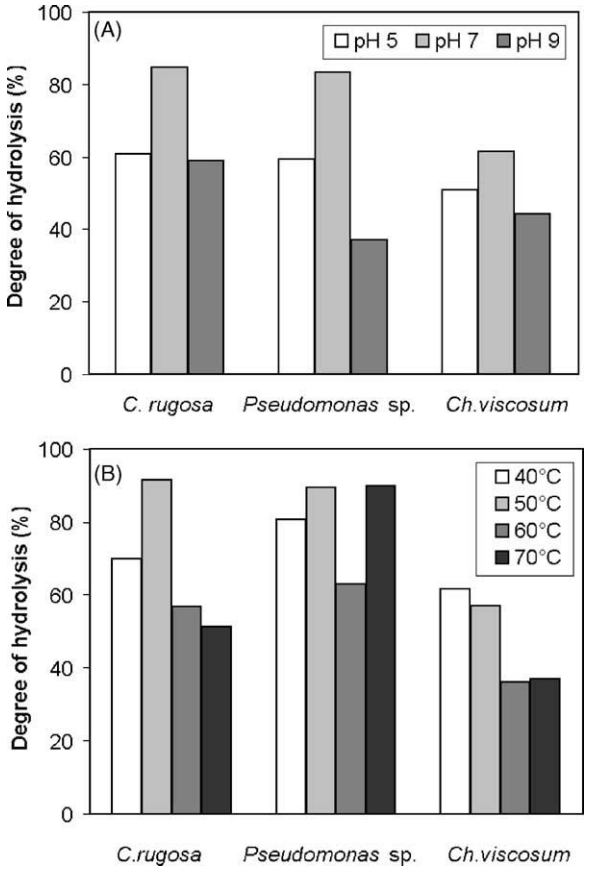

Fig. 6. Degree of hydrolysis of plant steryl esters in an aqueous dispersion as a function of pH (100 mg l^{-1}, 500 nkat g^{-1}, 40 °C, 24 h) (A) and temperature (100 mg l^{-1}, 500 nkat g^{-1}, pH 7, 24 h) (B).

viscosum steryl esterase was lower, and the pH had less effect as compared to steryl esterases of *C. rugosa* and *Pseudomonas* sp. Temperature also had a clear effect. Steryl esterase of *Pseudomonas* sp. hydrolysed almost 90% of plant steryl ester mixture at 50 and 70 °C, but surprisingly not at 60 °C. The highest degree of hydrolysis with *C. rugosa* steryl esterase was obtained at 50 °C, and a higher degree of hydrolysis was obtained at 40 rather than 60 or 70 °C. Temperature had smaller effects on the degree of hydrolysis obtained with *Ch. viscosum* steryl esterase. Substrate concentration had a clear effect on the degree of hydrolysis. At higher concentration (500 mg l^{-1}) about 30% of steryl esters were hydrolysed with all enzyme preparations (results not shown).

4. Discussion

Lipases (EC 3.1.1.3) have broad substrate specificities and are capable of hydrolysing water-insoluble esters typically containing long chain fatty acids. Some lipases also catalyse the hydrolysis of steryl esters, and thus they can also be classified as steryl esterases. Enzymes of this kind are, e.g. steryl esterases from *S. lavendulae* and *C. rugosa* (Lip 3) (Kamei et al., 1979; Tenkanen et al., 2002). There are also some cholesteryl esterases, e.g. from *P. fluorescens* and *S. cerevisiae*, which are specific for cholesteryl esters and do not possess lipase activity (Uwajima and Terada, 1976; Taketani et al., 1981).

The enzyme preparations studied clearly differed from each other. Some lipase preparations had high lipase activity but no cholesteryl esterase activity. However, the preparations used in the study may contain several lipases and esterases, and therefore no detailed comparison can be made without purifying the different enzymes. The preparations may also contain several steryl esterases with different properties.

Comparison with the substrate specificities of the steryl esterases described in the literature reveals that fungal steryl esterases (*C. rugosa* and *R. oryzae*) were similar to the cholesteryl esterases from *P. fluorescens*, *S. lavendulae* and *F. oxysporum* having higher activity on unsaturated and more hydrophilic long-chain fatty acid esters of cholesterol (Uwajima and Terada, 1976; Kamei et al., 1977; Madhosingh and Orr, 1981). Bacterial enzymes of *Pseudomonas* sp. and *Ch. viscosum* had higher activities on cholesteryl palmitate than on longer and unsaturated fatty acids. The activities on plant and wood steryl ester mixtures were comparable to activities on cholesteryl esters. This indicates that pure cholesteryl esters can be used as useful model substrates in preliminary characterisation of steryl esterases.

The pH- and temperature-stabilities of steryl esterase in the enzyme preparations were rather similar to those reported in the literature. Cholesteryl esterases of *Pseudomonas* species have been reported to be stable in the pH range 5–12 and at temperatures up to 50–60 °C for varying incubation times (Uwajima and Terada, 1976; White and White, 1997). *C. rugosa* cholesteryl esterase from Meito Sangyo has been reported to be stable for 24 h in the pH range 3–7 and

up to 50 °C (10 min), and to have a pH optimum between 7 and 8 (information from the manufacturer). The pH optimum of *C. rugosa* (Biocatalysts) enzyme was clearly lower (pH 4) than reported by the manufacturer.

The pH affects the physical properties of substrate dispersion. Wood resin is known to be completely insoluble at pH values below 6. At higher pH (pH > 7) the released fatty acids dissolve and act as emulsifiers, preventing deposition (Hassler, 1988). However, the degree of hydrolysis was not improved at pH 9 compared to pH 7, probably due to instability of enzymes in aqueous dispersion.

Temperature also has an important effect on the physical state of substrate dispersion. Higher temperature and liquefaction can be assumed to make the substrate more accessible for the enzyme. Steryl esterases of *Pseudomonas* sp. and *Ch. viscosum* were stable for 30 min up to 60 °C. Thus, higher degree of hydrolysis at 70 rather than at 60 °C was apparently a consequence of accelerated reaction rate or of more complete melting of the substrate, or both. On the basis of these hydrolysis experiments it can be concluded that at higher temperature, which is an important aspect in industrial applications, steryl esterase of *Pseudomonas* sp. was the most potential enzyme. Steryl esterase of *C. rugosa* also hydrolysed plant steryl ester mixture efficiently at temperature 40 °C and pH 7. Despite rather good stability and activity over broad ranges of pH and temperature, steryl esterase of *Ch. viscosum* did not function well in aqueous dispersion.

All steryl esterases were found to be highly active on plant steryl ester mixture in the presence of surfactant. Total hydrolysis could be obtained even in 30 min and the reaction rates were clearly increased when surfactant was present, except in the case of *C. rugosa* enzyme. However, the enzymatic action was more restricted when the substrate was present as a colloidal aqueous dispersion. The highest degree of hydrolysis obtained at 40 °C was 80% in a 24-h incubation. In our previous study, a steryl esterase of *C. rugosa* (Lip 3) was found to be able to hydrolyse all different steryl esters in the substrate equally well, and thus none of the individual components was responsible for the incomplete hydrolysis (Tenkanen et al., 2002). Thus, the physical state of the colloidal steryl ester droplet probably restrict the enzyme action and prevent the total hydrolysis of steryl esters.

In this study, up to 80–90% of the steryl esters were degraded in aqueous dispersion (100 mg l^{-1}) without any surfactants. This substrate concentration was almost 10 times higher than in process waters. In addition, extractives, carbohydrates and lignin are released during mechanical pulping and affect the formation of pitch particles. Knowledge on the potential use of other enzymes to improve the efficiency of steryl esterases in practical conditions might open up possibilities to develop new optimal enzyme cocktails for the pulp and paper industry.

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PUBLICATION II

Hydrolysis of steryl esters by a lipase (Lip 3) from *Candida rugosa*

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Hydrolysis of steryl esters by a lipase (Lip 3) from *Candida rugosa*

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Abstract

A well-known lipase, Lip 3 of *Candida rugosa*, was purified to homogeneity from a commercial lipase preparation, using hydrophobic interaction and anion exchange chromatography. Lip 3, which has been reported to act on cholesteryl esters, was also found to be active on plant-derived steryl esters. Lip 3 had optimal activity between pH 5–7 and below 55°C. It was able to hydrolyse steryl esters totally in a clear micellar aqueous solution. However, the action on a dispersed colloidal steryl ester solution was limited and only about half of the steryl esters were degraded. The degree of hydrolysis was not improved by addition of fresh enzyme. The composition of released fatty acids and sterols was, however, almost identical to that obtained by alkaline hydrolysis, showing that all the different steryl esters were hydrolysed equally and that none of the individual components were responsible for incomplete hydrolysis. Thus, it appeared that the physical state of the colloidal steryl ester dispersion limited the action of Lip 3. Wood resins contain both triglycerides and steryl esters among the hydrophobic components, which create problems in papermaking. The simultaneous enzymatic hydrolysis of triglycerides and steryl ester is therefore of considerable interest and Lip 3 is the first enzyme reported to act on both triglycerides and steryl esters.

Introduction

The main components of wood are cellulose, hemicelluloses and lignin, but wood also contains minor amounts of extractives, the amount and composition of which vary between different wood species. The major components of lipophilic extractives (also known as pitch) are free fatty acids, resin acids, sterols and triterpenyl alcohols as well as the fatty acid esters, of which triglycerides and steryl esters are the most abundant. The main fatty acids in free and esterified form are linoleic acid (9,12-18:2), oleic acid (9-18:1), palmitic acid (16:0) and pinolenic acid (5,9,12-18:3; Ekman and Holmbom 2000).

The most common sterols in woods are sitosterol, campesterol, stigmasterol and their corresponding 22-dehydroanalogues. The 4-methyl sterols (e.g. citrostadienol) and 4,4-dimethyl sterols (e.g. cycloartenol, 24-methylene cycloartenol, butyrospermol) are usually only minor components in most wood species. However, hardwoods such as birch and aspen contain significant amounts of related triterpenyl alcohols (betulinol, amyrin and lupeol), which all carry two methyl substituents at the C-4 position (Ekman and Holmbom 2000). Plant sterols, also called phytosterols, have been reported to include over 250 different sterols and related compounds in various plant and marine materials (Akihisa et al. 1991). The chemical structures of these sterols are similar to that of cholesterol.

In mechanical pulp suspensions and related paper machine waters, most of the lipophilic compounds exist in the form of emulsified colloidal droplets. As a result of colloidal destabilisation, aggregates can be formed that deposit at different sites of the paper machine, or may appear as sticky spots in the paper. During kraft pulping, triglycerides and steryl esters are hydrolysed. However, esters of the 4-methyl and 4,4-dimethyl compounds have been found to be more resistant to alkaline hydrolysis during kraft pulping than the other esters; and thus these esters can be found even after kraft pulping (Paasonen 1967). Generally, extractives create more problems in hardwood kraft pulps than in softwood pulps, due to the different composition and location of extractives in wood cells, inhibiting removal of hardwood extractives during pulping and bleaching. Extractives cause interruptions in the paper production and decrease paper product quality. The presence of extractives can also result in odour and taste problems in food-packaging materials. Problems caused by extractives increase with increased closure of the water systems.

Traditionally, pitch problems are reduced by careful barking, by ageing of wood chips prior to pulping, and by addition of specific chemicals in papermaking. New emerging biotechnical methods for pitch control include microbial pre-treatment (Farrell et al. 1993; Messner 1993; Gutiérrez et al. 1999) and enzymatic modification of pitch components (Irie et al. 1990; Fisher and Messner 1992; Fisher et al. 1993; Mustranta et al. 1995). Lipases (EC 3.1.1.3;

Enzyme Nomenclature, http://www.expasy.ch/enzyme) are already used in industrial scale in paper production to hydrolyse triglycerides, resulting in liberation of fatty acids and glycerol (Fujita et al. 1992; Chen et al. 2001). Fungal treatments of wood chips have been shown to reduce the amounts of all lipophilic extractives and also that of steryl esters (Gutiérrez et al. 1999). Enzymatic hydrolysis of steryl esters by steryl esterases (EC 3.1.1.13) might also be advantageous in paper production.

Lipases, like all esterases, are able to hydrolyse a wide range of esters. Lipases possess the unique feature of acting at an interface between the aqueous and non-aqueous phase. This feature distinguishes them from other esterases. Lipases are generally considered to go through an interfacial activation, which in many cases means that the peptide loop "lid" or "flap" closing the active site in aqueous solution opens up in the vicinity of non-polar substrate surface (Brozozowski et al. 1991; Jaeger and Reetz 1998; Schmid and Verger 1998). Lipases have been studied intensively, as they are potential enzymes for many industrial applications (Benjamin and Pandey 1998; Jaeger and Reetz 1998; Pandey et al. 1999). There are several commercial lipase preparations on the market, produced by various micro-organisms (Jaeger and Reetz 1998).

Simultaneous enzymatic degradadion of triglycerides and steryl esters might further improve the enzymatic modification of wood pitch. Commercial *Candida rugosa* preparations were found to be potential sources of the steryl esterase (SE; Kontkanen et al. 2001). The aim of this work was to isolate and characterise the main enzyme from *C. rugosa* able to degrade different steryl esters.

Materials and methods

Substrates

Cholesterol and cholesteryl oleate were purchased from Sigma. Wood sterol containing 76.4% sitosterol, 12.5% sitostanol, 7.6% campesterol and 3.5% campestanol was from UPM-Kymmene (Kaukas, Finland). Plant steryl ester, which was synthesised from plant sterols and fatty acids, was kindly provided by Dr. Hendrik Luttikhedde (Raisio Chemicals, Finland). The purity of the plant steryl ester was almost to 100%. However, it was a mixture of several steryl esters (Table 1). The sterol and fatty acid composition was analysed by GC after alkaline hydrolysis (Ekman and Holmbom 1989). The main sterol and fatty acid were sitosterol (46% of sterols) and oleic acid (56% of fatty acids), respectively. Thus, it did not totally resemble wood sterols, which normally have linoleic acid as the major fatty acid component. Another steryl ester (wood steryl ester) was synthesised by the McNeal esterification reaction from tall oil fatty acids

(Arizona Chemicals, Finland) and wood sterols Iinstructions obtained from Henrik Luttikhedde, Raisio Chemicals, Finland). The purity of the synthesised wood steryl ester was approximately 90%. The remainder were mainly dehydrated sterols. The composition of wood steryl ester was close to that of steryl esters in spruce (Table 1; Ekman and Holmbom 2000).

Table 1. Sterols and fatty acids present in plant and wood steryl esters used as substrates. The composition was analysed by gas chromatography after complete hydrolysis.

Compound	**Plant steryl ester (%)**	**Wood steryl ester (%)**
Sterols		
Sitosterol	46	71
Campesterol	26	9
Stigmasterol	24	-
Sitostanol	3	18
Campestanol	1	2
Fatty acids		
Palmitic acid (16:0)	9	-
Stearic acid (18:0)	4	3
Oleic acid (9-18:1)	53	25
Vaccenic acid (11-18:1)	5	5
Linoleic acid (9,12-18:2)	18	51
18:3 (Linolenic acid 9,12,15-18:3 + pinolenic acid 5,9,12-18:3)	8	12
Others	3	4

Activity assays

Cholesteryl and steryl esterase activity: Both qualitative and quantitative assays of cholesteryl esterase (CE) and steryl esterase (SE) activities were carried out. The substrate concentration was 1.7 mM in the qualitative thin layer chromatography (TLC) assay and 4.3 mM in the quantitative activity assay. The substrate solution was prepared by homogenising 56 mg of cholesteryl oleate (or plant steryl ester) in 1 ml Thesit (polyoxyethylene 9 lauryl ether; Sigma) and 9 ml of 0.9% NaCl. The reaction mixture for TLC contained 0.01 ml substrate solution (8.6 mM cholesteryl oleate solution), 0.02 ml 0.2 M sodium phosphate (pH 7) and 0.02 ml enzyme solution. After incubation at 40°C, the mixture was analysed qualitatively by TLC, using silica gel 60 (Merck). The eluent contained petroleum ether/diethyl ether/acetic acid (50:50:2) and visualisation was

achieved by spraying with 25% sulphuric acid, after which the plates were heated at 105°C for 5 min. Cholesterol was used as standard. The quantitative activity assay was carried out as the qualitative assay, but using 0.04 ml substrate solution. Samples were incubated at 40°C for 20 min, after which the reaction was terminated by boiling. The liberated cholesterol (or sterols) was determined in microtiter plates, according to the manufacturer's instructions using a cholesterol oxidase-based colorimetric method (kit 139 050; Roche); and the absorbance of formed lutidine dye was measured at 405 nm. According to the manufacturer, the kit was also able to assay sterols in which the hydroxyl group at carbon atom 3 is in the β position. This was verified using known amounts of wood sterols.

Carboxyl esterase activity: Carboxyl esterase (CAE) activity was determined using *p*-nitrophenyl caprate (Sigma) as substrate. The activity assay was carried out by incubating 0.02 ml of enzyme sample with 0.05 ml 5.0 mM substrate solution in 4.2% Triton-water and 100 mM sodium phosphate buffer, pH 7.0. After 10 min incubation at 40°C, the amount of free *p*-nitrophenol was analysed spectrophotometrically at 340 nm, using commercial *p*-nitrophenol (Sigma) as standard. This method enabled easy and rapid assay of esterase activity during purification.

Acetyl esterase activity: Acetyl esterase (AE) activity was determined using 1 mM α-naphthyl acetate (Sigma) as substrate in 50 mM McIlvane buffer (pH 6.0), as described previously by Poutanen and Sundberg (1988).

Lipase activity: Lipase activity was assayed using olive oil emulsion as substrate, according to Mustranta et al. (1993). The substrate solution was prepared by emulsifying 30 ml of olive oil (Bertolli) with 70 ml reagent (containing, per litre, 17.9 g NaCl, 0.41 g KH_2PO_4, 10 g gum arabic (Sigma), 47% v/v glycerol, distilled water) and homogenising for 3 min. The reaction mixture consisting of 5 ml emulsion, 4 ml 0.2 M sodium phosphate buffer (pH 7.0) and 1 ml of the enzyme solution was incubated for 10 min at 37°C. The reaction was terminated by addition of 10 ml of an acetone-ethanol mixture (1:1 v/v). The liberated fatty acids were titrated with 0.05 M NaOH solution using an automatic titrator.

All activities are presented in SI-units (katals) which correspond to the formation of 1 mol product in 1 s.

Purification

Commercial *C. rugosa* lipase (Lipase AY Amano 30) was obtained from Amano Enzyme Europe (UK); and 11 g of lipase preparation was dissolved in 360 ml

50 mM Tris-HCl, pH 7.0. The non-solubilised material was removed by centrifugation and the clear solution was applied to a phenyl-Sepharose FF column (11.3×46 cm; Pharmacia) equilibrated with 50 mM Tris-HCl, pH 7.0. The bound proteins were eluted in steps of 1 mM Tris-HCl (pH 6.8), 1 mM Tris-HCl (pH 6.8) + 1% Triton X-100 and 6 M urea, using a linear flow rate of 0.4 m/h. The main fractions containing SE activity, which were eluted with 6 M urea, were pooled (2,060 ml) and applied to a DEAE-Sepharose column (5×20 cm; Pharmacia). The column was equilibrated with 25 mM Tris-HCl (pH 7.5) +0.1% Triton X-100. The bound proteins were eluted with a linear sodium chloride gradient (0.0–0.3 M) in the equilibrating buffer, using a linear flow rate of 1.1 m/h. The SE activity divided into two peaks, of which the first, major peak was pooled (325 ml). The final purification with DEAE-Sepharose FF (in a 2.5×11 cm column) was performed in order to remove Triton X-100 from the enzyme preparation. The SE pool was diluted with 670 ml of water before feeding to the column equilibrated with 25 mM Tris-HCl (pH 7.3). Bound proteins were eluted with steps of 0.1, 0.2, 0.3 and 1 M sodium chloride in 25 mM Tris-HCl (pH 7.3), using a linear flow rate of 0.6 m/h. The fractions containing SE activity eluting with 1 M NaCl were pooled (72 ml) and used for further characterisation.

Protein properties

The molecular mass of the purified protein was determined by conventional electrophoresis in denaturating conditions, using precasted 12% SDS-PAGE gel slabs (Tris-HCl Ready Gel, Bio-Rad) and Ready Gel Cell (Bio-Rad). Proteins were stained with Coomassie staining (SimplyBlue SafeStain, Invitrogen).

The N-terminal amino acid sequence was determined by automated Edman degradation at the University of Kuopio, Finland. Before analysis, the esterase sample was buffered to 20 mM sodium phosphate (pH 6), using a ready-packed PD-10 column (Pharmacia).

CE and CAE activity assays were performed at different pH values, using McIlvane buffers with pH values between 5.1 and 7.8 (40°C) or at different temperatures between 40°C and 70°C (pH 7.0).

Hydrolysis experiments

Clear aqueous micellar solution was prepared by mixing 50 mg of synthesised plant steryl esters or wood steryl esters with 1 ml Thesit, after which 9 ml of 0.9% NaCl was added. The solution was mixed in a warm water bath until clear. In the hydrolysis experiments, 0.04 ml substrate solution was first mixed with 0.05 ml 0.1 M sodium phosphate buffer (pH 7). The reaction was started by the

addition of water and enzyme solution (50, 100, 200, 500, 1,000 nkat CE activity/g steryl esters) to give a final reaction volume of 0.2 ml. The substrate concentration in the hydrolysis experiments was 1 mg/ml. The hydrolyses were carried out at 40°C for 1, 5 or 24 h.

Homogenous steryl ester-water dispersion was prepared by first dissolving plant steryl esters in acetone (5 mg/ml), after which this solution was diluted with water to give a steryl ester concentration of 40 mg/l. This solution was mixed at 60°C for 3 h to evaporate the acetone. After this, the solution was further diluted with 50 mM sodium phosphate (pH 7), to give a final calculated steryl ester concentration of 20 mg/l. In the first experiment, 500 nkat enzyme/$g_{substrate}$ were used and samples were taken after 2 h and 20 h hydrolysis. In the second experiment, 500 nkat enzyme/$g_{substrate}$ were added at the beginning of the hydrolysis and also after 24 h and 48 h hydrolysis in order to determine whether the hydrolysis yield could be further improved.

Chemical analysis

The composition of the steryl ester solution after enzyme treatment was determined by gas chromatography (GC) after extraction with MTBE (Ekman and Holmbom 1989; Örså and Holmbom 1994).

Thesit solutions could not be injected to the GC instrument. Therefore, the amount of free sterols in Thesit solutions was determined using the same commercial cholesterol kit as that used in the CE and SE activity assays (kit 139 050; Roche). As the sensitivity of the kit was much lower than that of GC, much higher steryl ester concentrations were used in the experiments which contained Thesit solution.

Protein concentrations were estimated by the method of Lowry et al. (1951), using bovine serum albumin as standard.

Results

Determination of CE and SE activities

Measurement of CE activity has been reported by several authors (Allain et al. 1974; Uwajima and Terada 1976a; Taketani et al. 1978; Labow et al. 1983). Most methods are based on oxidation of liberated cholesterol to cholest-4-en-3-one with the cholesterol oxidase. In this reaction, hydrogen peroxide is formed, which can further react directly with chemicals or with peroxidase or catalase in order to yield chromophoric reaction products. All the reagents are added

simultaneously and the reaction is performed at 37°C in neutral pH, which are the optimal conditions for the cholesterol oxidase. In order to screen different SEs with different pH and temperature requirements, we developed a two-step method in which the hydrolysis of cholesteryl or steryl ester was performed first and terminated by boiling before addition of cholesterol oxidase to quantify the liberated cholesterol.

The assay worked well and the CE activity assay was linear up to 1.3 mg/ml of liberated cholesterol (Fig. 1). Cholesterol oxidase is also able to oxidise several free sterols (kit 139 050 Technical information; Roche). This was verified by comparing its action on cholesterol and wood sterol (results not shown). The wood sterol was fully oxidised by the cholesterol oxidase used, but the reaction was slightly slower than when cholesterol was used as substrate. Thus, the activity assay could also be performed using steryl esters as substrate. In addition, cholesterol oxidase was used to determine the amount of free sterols after the hydrolysis of steryl esters.

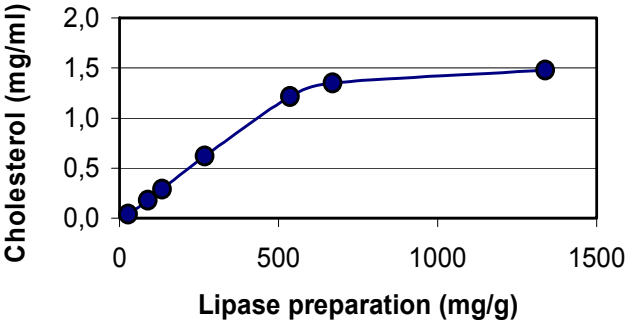

Figure 1. Linearity of the cholesteryl esterase activity assay. Different amounts of a commercial Candida rugosa *AY Amano 30 lipase preparation (x-axis shows milligrams enzyme preparation/gram substrate) were incubated with cholesterol oleate (2.8 mg/ml) as desciberd in Materials and Methods.*

Purification and protein properties of Lip 3

Several commercial lipase preparations were found in a previous screening to possess SE activity (Kontkanen et al. 2001). All *C. rugosa* preparations were able to hydrolyse steryl esters. Lipase AY Amano 30 had the highest SE activity (compared with lipase activity) and was therefore chosen as the enzyme source for the purification of SE. The purification was started by hydrophobic interaction chromatography of the clear enzyme solution. Almost all SE activity was bound tightly to the phenyl-Sepharose column, even in a dilute buffer solution (50 mM Tris-HCl, pH 7) and was eluted only with a mixture of 6 M

urea and Triton X-100. The next purification step was anion exchange chromatography, which was first performed in the presence of 1% Triton X-100 and in the next step excluding Triton X-100 in order to obtain a final enzyme preparation without Triton X-100. The SE activity divided in two peaks in the first anion exchange chromatography. The first peak, which contained ten times more activity than the second, was pooled and applied to the DEAE-Sepharose again. SE activity was bound clearly more tightly on DEAE-Sepharose without Triton X-100, as it was eluted in the first DEAE-Sepharose purification by 0.1–0.2 M NaCl, whereas 1 M NaCl was needed for elution in the second DEAE-Sepharose step. The total purification yield of SE activity was only 7% (Table 2). The yield loss in both of the first purification steps was very high. The specific activity appeared to be very low after the two first purification steps. The Triton X-100 used affected the protein determination and thus very high protein-concentration values were obtained for these samples.

Table 2. Purification of Lip 3 from the commercial Candida rugosa *preparation AY Amano 30. The starting solution was 0.03 g enzyme preparation in 1 ml of buffer. CE Cholesteryl esterase.*

Purification step	**Volume (ml)**	**Total CE activity (nkat)**	**Specific activity (nkat/mg)**	**Yield (%)**
Starting solution	355	61 570	117	100
Phenyl-Sepharose	2 060	24 390	22	40
DEAE-Sepharose	325	6 007	74	10
DEAE-Sepharose	72	4 500	382	7

The purified protein showed only one band in SDS-PAGE, with an apparent molecular mass of 65 kDa (Fig. 2). The starting enzyme solution was also rather pure lipase, showing only one main band close to this size. However, all five *C. rugosa* lipases have molecular masses close to 60 kDa and thus a single broad band is obtained from their mixtures.

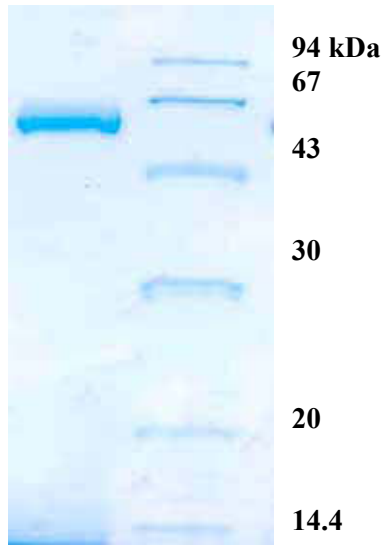

Figure 2. SDS-polyacrylamide gel electrophoresis of purified Lip 3 from C. rugosa. *The sample size was 15 µg purified enzyme protein. Staining with Coomassie stain. The molecular mass standards are indicated on the right.*

The N-terminal amino acid sequence of the purified enzyme was APTAKLANGD, which corresponds to that of Lip 3 from *C. rugosa* (Lotti et al. 1993). Therefore the name Lip 3 is used for this purified enzyme in this work. Lip 3 showed the highest activity against cholesteryl oleate at pH 5.0–7.5 (20 min incubation at 40°C). The highest CE activity was obtained at 50°C (20 min incubation at pH 7.0), above which the activity decreased. About 60% and 20% of the maximal activity was obtained at 55°C and 60°C, respectively (data not shown). Thus, Lip 3 was clearly still able to act at 60°C. When the purified enzyme was identified as Lip 3, its stability was not studied further, as Lip3 is already a well characterised enzyme (Rúa et al. 1993, LipA = Lip3).

Lip 3 showed a broad substrate specificity, having activity towards several different types of esters (Table 3). It showed the highest specific activity with *p*-nitrophenyl caprate as substrate, thus indicating high CAE activity. The lipase activity against olive oil was about half of the CAE activity. The specific CE activity was ten times lower than the lipase activity. Lip 3 showed only very low AE activity. However, the specific activities cannot be accurately compared due to the slightly different assay conditions.

Table 3. Specific activities of Lip 3 from Candida rugosa.

Component	Abbreviation	Specific activity (nkat/mg)
Steryl esterase	SE	351
Cholesterol esterase	CE	382
Lipase	-	3 837
Carboxyl esterase	CAE	7 270
Acetyl esterase	AE	24

Action of Lip 3 on steryl esters

Hydrolysis of synthetic plant and wood steryl esters was first studied in a clear micellar Thesit solution. Lip 3 was able to hydrolyse the steryl esters almost completely in 5 h, using enzyme dosages of 500 nkat/g or 1,000 nkat/g (activity measured using cholesteryl oleate as substrate; Fig. 3). Longer hydrolysis time was needed for lower enzyme dosages in order to obtain maximal hydrolysis. Plant steryl esters were degraded somewhat better than wood steryl esters, especially by the low Lip 3 dosages.

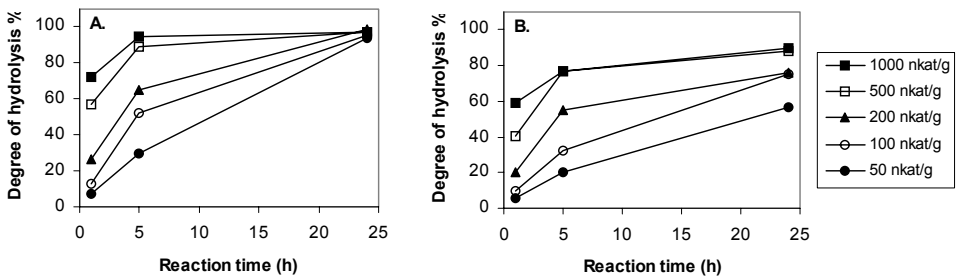

Figure 3. Hydrolysis of (A) plant steryl ester and (B) wood steryl ester (1 mg/ml) in Thesit (20 μg/ml) solution at pH 7 and 40°C with different dosages (50–1,000 nkat/g ester) of Lip 3 from C. rugosa*. The amount of liberated sterols was determined with a cholesterol kit.*

Steryl esters, along with other lipophilic compounds, are transferred into process waters as emulsified colloidal pitch droplets during mechanical pulping and integrated papermaking. Therefore, synthetic plant steryl ester was treated in water dispersion without any surface-active agent in a concentration similar to

that of steryl esters in mechanical pulp-process waters. Surprisingly, the steryl ester was only partly hydrolysed, even though the concentration of steryl ester in the water dispersion was 50 times lower than in the previous experiment using Thesit solution (Fig. 4). The degree of hydrolysis did not significantly increase after 2 h and it was only slightly improved after 24 h by the addition of fresh enzyme (data not shown).

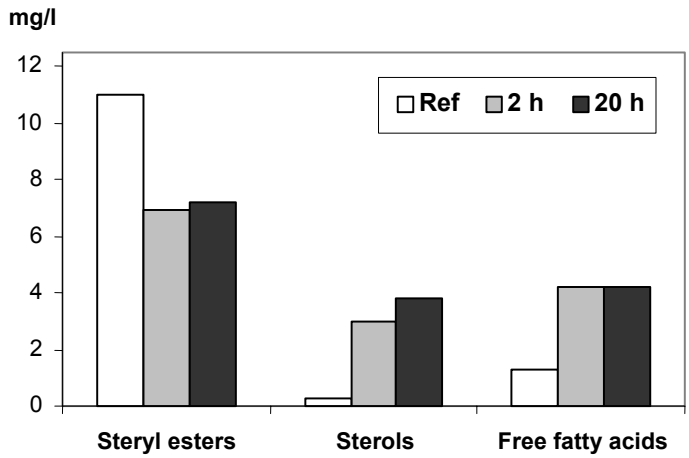

Figure 4. Hydrolysis of plant steryl esters in water dispersion (20 mg/l) at pH 7 and 40°C with Lip 3 (500 nkat/g). After hydrolysis, the amount of steryl esters, free sterols and free fatty acids was analysed by gas chromatography. Ref Reference treatment with buffer.

Surprisingly, the amount of steryl esters in the blank sample analysed by GC was significantly lower (11 mg/l, which gives a theoretical amount of 6.7 mg free sterol/l) than that originally applied in the hydrolysis experiment (20 mg/l, or 12 mg free sterols/l). Therefore, there is a great difference in the hydrolysis yields (i.e. 52% vs 29%) when calculated on the basis of analysed or applied substrate, respectively. The reason for this almost 50% difference is not known. It might be that hydrophobic, sticky steryl esters adhere to different surfaces during the experiment. However, the samples were not removed from the vials after the hydrolysis but were extracted with MTBE and handled for the GC analysis in the same vial in order to minimise losses during sample handling. However, it seems clear that Lip 3 was not able to hydrolyse colloidal steryl esters completely.

The composition of liberated fatty acids and sterols was further analysed by GC. The results showed that the composition of released fatty acids and sterols was almost identical to that obtained by alkaline hydrolysis of the synthetic steryl ester (Fig. 5, Table 1). This confirmed that all the different steryl esters were

hydrolysed equally and that none of the individual components were responsible for incomplete hydrolysis.

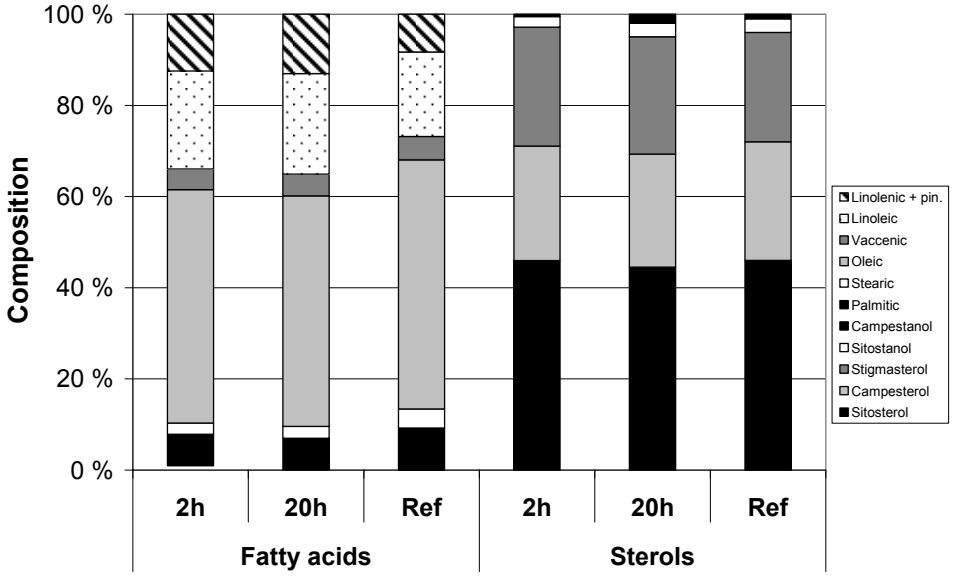

Figure 5. Composition of liberated sterols and fatty acids after hydrolysis for 2 h or 24 h with Lip 3 from C. rugosa*, as analysed by gas chromatography. pin pinolenic acid, Ref composition of substrate after alkaline hydrolysis.*

Discussion

Lipases are produced by a wide variety of organisms. A huge amount of research has been conducted with different lipases, due to their potential applications in several industrial sectors (Pandey et al. 1999; Jaeger and Reetz 1998). Most industrial enzyme producers have several lipase products on the market. The pulp and paper industry is one of the most recent industrial sectors for lipase applications (Fujita et al. 1992; Call et al. 1999; Chen et al. 2001).

C. rugosa is one of the most thoroughly studied lipase producers (Lotti et al. 1994; Benjamin and Pandey 1998). It produces several esterases, including at least five lipases (Lip 1-Lip 5), which have also been characterised in detail at the molecular level (Longhi et al. 1992; Lotti et al. 1993). Lip 1 and Lip 3 (named Lip A in some publications) have been shown to be constitutively expressed and the most abundant lipases of *C. rugosa* (Grochulski et al. 1993; Rúa et al. 1993; Lee et al. 1999; Jonzo et al. 2000; López et al. 2000). The three-dimensional structures of Lip 1 and Lip 3 have been solved (Ghosh et al. 1991, 1995; Grochulski et al. 1993). Interestingly, Lip3 has been found to show CE

activity (Kaiser et al., 1994) and thus it is also classified as a SE (CE;). Other studied microbial CEs have come from *Pseudomonas fluorescens* (Uwajima and Terada 1976b), *Streptomyces lavendulae* (Kamei et al. 1979; Nishimura and Sugiyama 1994), *Fusarium oxysporum* (Okawa and Yamaguchi 1977; Madhosingh and Orr 1981) and S*accharomyces cerevisiae* (Taketani et al. 1978; 1981). In animals CEs are primarily synthesised in the pancreas and are a component of pancreatic juice (Labow et al. 1993). Even though SEs are common in nature and are furthermore structurally very similar to CEs, there are only two short recent reports of enzymes capable of hydrolysing plant-derived steryl esters (Calero-Rueda et al. 2001; Tenkanen et al. 2001).

In a previous study, commercial *C. rugosa* lipase preparations were found to contain SE activity (Kontkanen et al. 2001). The activity might be due to Lip 3, or to a more specific enzyme, or both. Therefore the main enzyme responsible for the SE activity was isolated in this work. The first ten amino acids in the N-terminal end of the purified enzyme were the same as for Lip 3 from *C. rugosa* (Lotti et al. 1993). The molecular mass as determined by SDS-PAGE was 65 kDa, which is close to the values (60, 62, 65, 66 kDa) reported earlier for Lip 3 (Rúa et al. 1993; Kaiser et al. 1994; Sanchez et al. 1999; Jonzo et al. 2000; Pernas et al. 2000). The calculated molecular mass from the amino acid sequence is 57 kDa for all five *C. rugosa* lipases (Longhi et al. 1992; Lotti et al. 1993). The effect of pH on Lip 3 activity was also similar to that observed in this work (Pernas et al. 2000). Thus, according to all the available data, it appears that the SE purified in this work is Lip 3 and not a new esterase of *C. rugosa*.

CE activity has not been reported for other *C. rugosa* lipases. However, Jonzo et al. (2000) recently reported purification of two lipases, A and B, from *C. rugosa* and their use for esterification of cholesterol with free fatty acids. Lip A was confirmed to be the same as Lip 3. On the basis of its N-terminal amino acid sequence, Lip B could contain any of the other four lipases. Both lipase preparations showed rather similar behaviour in esterification, but Lip A (Lip 3) was slightly faster than Lip B. Thus it seems that the other lipase(s) of *C. rugosa* is also active on cholesteryl esters and possibly also on steryl esters.

Of the substrates tested, Lip 3 had the highest activity against *p*-nitrophenyl caprate. The lipase activity against triolein was clearly lower and the specific SE activity was only one-tenth of that. Thus, triglycerides would be hydrolysed much faster than steryl esters in wood pitch. The lipase and CE activities of Lip 3 have not previously been compared. Pernas et al. (2000) recently reported the specific activities of Lip 3 on different triglycerides (triacetin, tributyrin, triolein) and *p*-nitrophenyl esters with acyl chain lengths between 3 and 12. Lip 3 was found to hydrolyse best the medium acyl chain *p*-nitrophenyl esters. This is in accordance with our data, which showed higher CAE than lipase activity for Lip3. Lip 3 is reported to be a better enzyme for shorter acyl esters than Lip

1 or Lip 2, which are both better catalysts for the hydrolysis of triolein than Lip 3 (Rúa et al. 1993; Plou et al. 1997; Pernas et al. 2000). A large amount of older data is available concerning the action of *C. rugosa* lipases. However, it is not possible to say which isolated preparations contain Lip 3, as the N-terminal amino acid sequences were not reported.

The CE activity of Lip 3 has previously been assayed, using cholesteryl linoleate as a substrate (Kaiser et al. 1994). The specific activity was not reported, nor was its action on various cholesteryl/steryl esters studied. CEs from *F. oxysporum*, *P. fluorescens* and *S. lavendulae* have all been found to be most active on cholesteryl linoleate. The activity decreased with decreasing unsaturation or length of the fatty acid chain (Uwajima and Terada 1976b; Okawa and Yamaguchi 1977; Kamei et al. 1979; Madhosingh and Orr 1981). Lip 3 also seemed to have a slight preference for unsaturated fatty acids. The effects of different steryl moieties on the action of CEs have attracted less attention. According to our results, Lip 3 did not have any clear preference for the steryl moiety in plant steryl esters. Interestingly, the CEs from *P. fluorescens* and *S. cerevisiae* appear to be a totally different type of enzyme from Lip 3, as they have not showed any lipase activity (Uwajima and Terada 1976b; Taketani et al. 1981).

Lip 3 was found to be active against steryl esters in the presence of surface-active agents, but the action was clearly restricted when the substrate was present as a colloidal water dispersion. The hydrolysis yield was about 50% and was not improved by increasing the hydrolysis time or by addition of fresh enzyme. Lip 3 was found to be able to hydrolyse all the different steryl esters in the substrate equally well; and thus none of the individual components was responsible for the incomplete hydrolysis. Therefore, it seems that Lip 3 was for some reason not able to reach all the steryl esters in a colloidal substrate. Steryl esters are more hydrophobic than triglycerides. Thus, it might be that the physical state of the colloidal steryl ester droplet restricts the enzyme action. During the hydrolysis of steryl esters, both free sterols and fatty acids are formed. Liberated fatty acids can act as surface-active agents. At alkaline pH, they form sodium-soaps, which can further act as emulsifiers. In contrast, free sterols are strongly hydrophobic and have high melting points. Thus, they might form a rigid layer covering the residual steryl esters in the colloid droplet. The action of Lip 3 on steryl esters in pulp suspensions and process waters containing a mixture of several lipohilic extractives remains to be studied.

The action of CEs has almost always been analysed in the presence of surface-active agents such as Triton X-100 or Thesit. Sodium cholate or sodium taurocholate have also been added to the reaction mixture. The action of all studied CEs has been strongly dependent on the presence of surfactants. Triton X-100 has in most cases been the best surfactant tested to prepare the micellar aqueous substrate solution.

The structural details of *C. rugosa* Lip 1 and Lip 3 should provide further infomration in the future concerning their different substrate specificities. Lip 1 and Lip 3 share 89% amino acid sequence identity and have very similar three-dimensional structures (Longhi et al. 1992; Grochulski et al. 1993; Lotti et al. 1993; Gosh et al. 1995; Cygler and Schrag 1999). The structural studies of Lip 3 have shown that it exists as a dimer, which is stabilised by the substrate (Gosh et al. 1995). The two monomers have been found to associate with active-site gorges facing each other and thus shielding the hydrophobic surfaces in the formed cavity from the aqueous environment. It has been speculated that a functional role for the dimer is characteristic to CEs. The dimeric structure forms a hydrophobic cavity, which is large enough to accommodate a strongly hydrophobic cholesteryl moiety situated outside the catalytic gorge. Unfortunately, structures of specific CEs, such as the esterases from *P. fluorescens* and *S. cerivisiae*, which do not possess any lipase activity, are not yet available. Interestingly, according to inhibition studies CE from *P. fluorescens* does not appear to be a serine hydrolase, unlike Lip 3 and a CE from *S. lavendulae,* which both possess lipase activity (Uwajima and Terada 1976b; Kamei et al. 1979). Thus, it is possible that specific CEs have action mechanisms different to those of enzymes possessing both lipase and cholesteryl esterase activities. The knowledge on enzyme structures further opens up possibilities for improving catalytic efficiency or for tailoring substrate specificities of these and other lipases in order to obtain more efficient catalysts for the hydrolysis of steryl esters.

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PUBLICATION III

Purification and characterisation of a novel steryl esterase from *Melanocarpus albomyces*

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Purification and characterisation of a novel steryl esterase from *Melanocarpus albomyces*

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Abstract

A novel steryl esterase from the ascomycete *Melanocarpus albomyces* was purified using hydrophobic interaction chromatography and anion exchange chromatography, and the enzyme was biochemically characterised. The enzyme has a tetrameric structure with a molecular weight of 238 kDa. Native gel electrophoresis indicated an isoelectric point of 4.5. The enzyme had broad substrate specificity for different steryl esters, *p*-nitrophenyl esters and triglycerides with long-chain fatty acids. The pH optimum was dependent on the substrate. The pH optimum of lipase activity was at pH 7, whereas cholesteryl esterase and carboxyl esterase activities had optima at pH 5.5 and 5, respectively. The steryl esterase was more stable at lower pH than at values above pH 7. The enzyme retained over 70% of its activity after 5 h incubation at 50 °C. Activity on *p*-nitrophenyl butyrate was clearly increased by low detergent concentrations (≤0.1%). *M. albomyces* steryl esterase is considered to be an interesting tool for the enzymatic modifications of wood pitch due to its broad substrate specificity. Treatment of a model pitch simulating the TMP resin showed that both steryl esters and triglycerides were hydrolysed in the presence of a detergent, whereas only triglycerides were degraded in the absence of detergent.

Keywords: Steryl esterase; Lipase; Steryl esters; *Melanocarpus albomyces*; Purification; Characterisation

1. Introduction

Lipophilic extractives, such as triacylglycerols, steryl esters, resin acids, free fatty acids and sterols, are commonly referred to as wood resin or wood pitch. During mechanical pulping of softwood, most of the extractives are dispersed as colloidal droplets and released into the process waters in the form of stable aggregates with polysaccharides [1,2]. The presence of lipophilic extractives during papermaking can affect paper machine runnability and also the strength and optical properties of the paper [3–5].

As an alternative to traditional methods for pitch control, debarking, seasoning of wood chips and addition of chemicals [6], different biological methods using enzymatic treatment or microbial pre-treatment have been developed. Wood pre-treatment with a commercial product, Cartapip™, has been shown to metabolise triacylglycerols efficiently [7,8]. Some fungi have been found to partly degrade steryl esters [9–11]. The drawbacks of these methods are the rather long treatment time and the required adjustment of conditions.

Lipases can efficiently hydrolyse the triacylglycerols of extractives. A commercial lipase, Resinase® (Novozymes), has been used for the treatment of red pine mechanical pulps for several years in Japan [12,13]. However, Resinase has not been shown to have any effect on steryl esters [14], which may play a significant role in the formation and stabilisation of pitch particles due to their physical properties.

Steryl esterases (cholesteryl esterases) have broad substrate specificities and are capable of hydrolysing different triacylglycerols and *p*-nitrophenyl esters in addition to steryl esters. Steryl esterases from *Ophiostoma piceae* and *Pseudomonas aeruginosa* have recently been characterised. Steryl esterase of *O. piceae* has been reported to hydrolyse both triacylglycerols and steryl esters and its potential for pitch biocontrol has been discussed [15]. Steryl esterase of *P. aeruginosa* has also been shown

Abbreviations: CAE, carboxyl esterase; CE, cholesteryl esterase; DEAE, diethyl aminoethyl; *p*-NPB, *p*-nitrophenyl butyrate; *p*-NPC, *p*-nitrophenyl caprate; PSE, mixture of plant steryl esters; SE, steryl ester; TAG, triacylglycerol; TMP, thermomechanical pulp

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to have the ability to act on triacylglycerols and steryl esters simultaneously and its potential use as a contact lens cleaner has been investigated [16]. Steryl esterase activities have also been detected from commercially available lipase preparations, such as *Pseudomonas* sp., *Chromobacterium viscosum* and *Candida rugosa* [17]. The enzymes were able to hydrolyse plant-derived steryl esters totally in the presence of a detergent (Polidocanol, formerly called Thesit) and up to 80% of the steryl esters were degraded in aqueous dispersion. One of the lipases (Lip 3) from *C. rugosa* lipase preparation has been purified and simultaneous hydrolysis of steryl esters and triacylglycerols has been reported [18]. Although steryl esterases have been studied extensively from a number of mammalian tissues [19,20] and microbial sources [15–18,21–25], understanding of the potential of these enzymes in pitch control is still lacking.

In this work, a novel steryl esterase from the ascomycete *Melanocarpus albomyces* was purified and characterised. Its potential and technical performance in the degradation of wood steryl esters is discussed.

2. Materials and methods

2.1. Fungal strain and culture conditions

Melanocarpus albomyces VTT D-96490, originally isolated from soil in Saudi Arabia, was maintained on oatmeal agar (Difco, Detroit, USA). *M. albomyces* was cultivated on liquid medium as described earlier [26]. After 2 days of cultivation at 37 °C (180 rpm), the culture was homogenised and used to inoculate 0.5 l of culture medium in a 2 l Erlenmeyer flask. Culture medium, pH 5.5 adjusted with 10 M NaOH, contained (per litre) glucose 10.0 g, spent grain 15.0 g, olive oil 5.0 g, peptone 2.0 g, $(NH_4)_2SO_4$ 1.0 g, KH_2PO_4 10.0 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, and $CaCl_2 \cdot 2H_2O$ 0.5 g. Calcium chloride was autoclaved separately and added before inoculation. The flasks were incubated at 37 °C on a rotary shaker (180 rpm), and 3 ml of olive oil was added after 2 days when the glucose level had decreased below 1 g l^{-1}. Carboxyl esterase activity against *p*-nitrophenyl caprate was measured during cultivation, which was terminated when the activity reached its maximum.

2.2. Protein purification

The mycelium was separated by filtration through Whatman glass microfibre filters (GF/A). Mycelium was first washed with 0.05 M sodium phosphate buffer (pH 6.0), and the enzyme bound on the mycelium was extracted with 0.1% Triton X-100 in 0.05 M sodium phosphate buffer (pH 6.0). The steryl esterase was purified from Triton-extract using hydrophobic interaction chromatography and anion exchange chromatography. The sample was equilibrated with sodium phosphate buffer (pH 8) and chromatographed on a Phenyl Sepharose Fast Flow column (1.6 cm × 6 cm, Pharmacia, Uppsala, Sweden), which was pre-equilibrated with 0.05 M sodium phosphate buffer (pH 8). Proteins were eluted in steps of 1 mM sodium phosphate (pH 8), 1 mM sodium phosphate (pH 8) + 1% Triton X-100 and 6 M urea. Fractions containing carboxyl esterase activity were pooled and equilibrated with 0.1 M sodium acetate (pH 5.5) containing 1% Triton X-100. The pH was adjusted with acetic acid to pH 5.5 and the sample was applied to a DEAE Sepharose Fast Flow column (1.6 cm × 8 cm, Pharmacia), which was pre-equilibrated with 10 mM sodium acetate (pH 5.5) containing 1% Triton X-100. Proteins were eluted with a linear 0–0.3 M NaCl gradient (150 ml) in 10 mM sodium acetate (pH 5.5) + 1% Triton X-100, thereafter with 0.3 M NaCl in 10 mM sodium acetate (pH 5.5) + 1% Triton X-100 and 1.0 M NaCl in 10 mM sodium acetate (pH 5.5) + 1% Triton X-100, and fractions containing carboxyl esterase activity were pooled. In order to estimate the molecular size of the protein, part of the solution was applied to a gel filtration column, Superdex 200 HR 10/30 (1.0 cm × 30 cm, Amersham Pharmacia Biotech, Uppsala, Sweden), which was pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7) containing 0.15 M NaCl and 0.1% Triton X-100. Triton X-100 was removed from the pooled DEAE fractions using DEAE Sepharose Fast Flow column after pre-equilibration with 20 mM sodium phosphate (pH 6.5) containing 1% Triton X-100. Proteins were eluted in steps of 20 mM sodium phosphate (pH 6.5) and 1.0 M NaCl in 20 mM sodium phosphate (pH 6.5). Finally, the buffer of the sample was exchanged to 20 mM sodium acetate (pH 5.5) using PD-10 columns (Pharmacia).

Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad, CA) with bovine serum albumin as a standard.

SDS-PAGE (12% Tris–HCl Ready Gel, Bio-Rad) was performed according to Laemmli [27]. Protein bands were visualised by staining with Coomassie Brilliant Blue (Serva Blue R) and compared with a molecular weight marker (LMW, Pharmacia). The content of N-linked carbohydrates was estimated by SDS-PAGE after deglycosylation with endo-β-*N*-acetylglucosaminidase H (Endo H, Roche) or PNGase F (Biolabs).

2.3. Substrates

Cholesteryl esters (cholesteryl palmitate, -stearate, -oleate, -linoleate, -linolenate) were obtained from Sigma. Plant steryl ester (PSE), which was synthesised from plant sterols and fatty acids, was kindly provided by Dr. Hendrik Luttikhedde (Raisio Chemicals, Finland) [18]. A mixture of extractives simulating TMP resin [28] was kindly provided by Dr. Menghua Qin (Åbo Akademi University, Finland). *p*-Nitrophenyl acetate, caprylate and myristate were obtained from Fluka. *p*-NP propionate, butyrate, caprate and stearate were from Sigma.

2.4. Activity assays

Cholesteryl esterase (CE) activity was assayed using 4.3 mM cholesteryl oleate as substrate according to Tenkanen et al. [18]. Activities on different cholesteryl esters were measured as described earlier [17]. The reaction mixtures with a total reaction volume of 0.08 ml contained 0.02 ml enzyme solutions corresponding about 1 μg of protein.

Carboxyl esterase (CAE) activity was determined using 2.1 mM *p*-nitrophenyl caprate (*p*-NPC) as substrate as described earlier [18]. *p*-Nitrophenols esterified with acetate, propionate, butyrate, caprylate, myristate and stearate were also used. The concentrations of substrate dispersions were 5 mM. A lower concentration of *p*-nitrophenyl stearate (2.5 mM) was used due to its lower solubility. Total volume of reaction mixtures were 0.12 ml from which amount of enzyme solutions were 0.02 ml corresponding about 50 ng of protein. Determination of CAE activity provided a rapid, straightforward and sensitive measurement of enzymatic activity that correlated with hydrolysis of steryl esters. Therefore, this method enabled simple assay of steryl esterase activity during cultivation and purification.

Lipase activity was assayed by an enzymatic colorimetric method as described earlier [17]. Reactions were performed in a scale of 2.0 ml from which amount of enzyme solution was 0.02 ml corresponding about 1 μg of protein.

2.5. Hydrolysis of plant steryl esters

Hydrolysis of the plant steryl ester mixture was investigated in a clear micellar Polidocanol dispersion. Substrate dispersion (5 mg ml^{-1}) was prepared as described for cholesteryl ester dispersion using Polidocanol and 0.9% NaCl [18]. The substrate concentration of the hydrolysis reaction was 1 mg ml^{-1} and the pH was adjusted with ammonia/formic acid buffer (pH 7). Two Polidocanol concentrations (1 and 2%) and two CE dosages (100 and 1000 nkat g^{-1}) were examined. Samples were taken after 1, 3 and 20 h incubation at 40 °C. Amounts of liberated sterols were determined using a cholesterol kit (139050, Roche). The hydrolysis of PSE was also studied in water dispersions as described earlier [17].

2.6. Hydrolysis of steryl esters and triacylglycerols in mixed wood extractives

Simultaneous hydrolysis of steryl esters and triacylglycerols was investigated using a mixture of extractives as a substrate. Enzymatic treatments and

chemical analysis were performed as described previously [17] with the exception of the presence of other extractives. The extractives were first dissolved in acetone (40 mg ml^{-1}), whereafter the solution was diluted with water to obtain a final steryl ester concentration of 100 mg l^{-1} and a total amount of extractives of 400 mg l^{-1}. The effects of pH (5 and 7), temperature (40 and 50 °C) and Polidocanol concentration (0, 0.1 and 1%) on the degree of hydrolysis in 24 h incubation were examined. After incubation, the reactions were terminated by boiling. The samples were subsequently lyophilised and re-dissolved in 10% Polidocanol solution containing 0.9% NaCl. Various amounts of cholesterol (Sigma) and linoleic acid (Fluka) were treated as samples in order to prepare standard curves. The amounts of released sterols and fatty acids were analysed spectrophotometrically using commercial colorimetric assays for the determination of cholesterol (Roche) and free fatty acids (Roche). The degree of hydrolysis was calculated from the theoretical sterol and fatty acid yield after total hydrolysis of steryl esters and triacylglycerols.

2.7. Determination of the isoelectric point

The isoelectric point of *M. albomyces* steryl esterase was determined by isoelectric focusing within the pH range of 3.5–9.5 (Ampholine PAGplate 3.5–9.5, Amersham Biosciences, Uppsala, Sweden) on an LKB 2117 Multiphor II Electrophoresis Unit (LKB Pharmacia, Bromma, Sweden) according to the manufacturer's instructions. Proteins were visualised by silver staining (Silver Stain kit, Bio-Rad).

2.8. Study of the enzymatic properties

Cholesteryl oleate, *p*-NPC and olive oil were used as substrates when the pH optimum for purified steryl esterase was determined. All activities were measured using McIlvaine buffer (0.2 M Na_2HPO_4 and 0.1 M citric acid) at pH 2.2–8.0, 0.2 M Tris–HCl buffer at pH 7.2–9.1 and 0.2 M glycine–NaOH buffer at pH 8.6–10.6. The pH stability of the enzyme was determined by incubating the purified enzyme solution (without Triton X-100) at different pH values at 37 °C for 24 h. The pH of the solution was adjusted with the buffers described above (buffers diluted 1:2 with water) to obtain final buffer concentrations of 20 mM. The pH of hydrolysis reactions was adjusted to pH 7 with 0.2 M sodium phosphate buffer (pH 7) and *p*-NPC was used as substrate. Temperature stability was investigated by incubating the enzymes (without Triton X-100) at 30–80 °C for 1, 5 and 24 h in McIlvaine buffer (pH 5). After the incubations, enzyme activity was measured using cholesteryl oleate as substrate.

Triton X-100 (Fluka), Tween 80 (Fluka), Polidocanol (Polyoxyethylene 9 Lauryl Ether, Sigma), Zwittergent 3-14 (Calbiochem), cholic acid (Sigma) and taurocholic acid (Sigma) were used to study the effects of detergents on the action of steryl esterase. The use of *p*-nitrophenyl butyrate (*p*-NPB) as substrate enabled the preparation of substrate dispersion into the water in a sufficient concentration (4 mM). The detergent was added into the hydrolysis mixture in 0.1 M sodium phosphate buffer (pH 7), and the enzyme (without Triton X-100) was diluted into the same buffer containing detergent in order to prevent enzyme aggregation. The enzymatic activity was assayed as CAE activity as described above.

The effects of calcium chloride on cholesteryl esterase and lipase activities were determined in the presence of 0, 0.5, 5 and 50 mM calcium in Tris–HCl buffer (pH 7).

Interfacial activation of the enzyme was studied by measuring the specific activity as a function of concentration of triacetin (Fluka), tripropionin (Acros) and tributyrin (Fluka). Measurements were carried out using substrate concentrations below and above the solubility limit [15]. The substrates were dispersed in 1 mM Tris–HCl (pH 7), containing 0.15 M NaCl and 0.09% (v/v) acetonitrile according to Verger [29]. Activity was assayed using an enzymatic colorimetric method for released glycerol (148270, Roche).

2.9. Peptide sequencing

SDS-PAGE separated proteins were transferred by electroblotting onto a polyvinylidene difluoride (PVDF) membrane [30]. After staining with Coomassie Brilliant Blue, the protein bands of interest were cut out and subjected to N-terminal sequence analysis in a Procise 494A Protein Sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA, USA).

In order to analyse protein fragments, SDS-PAGE separated proteins were stained with Coomassie Brilliant Blue and protein bands of interest cut out of the gel. The proteins were "in-gel" digested with trypsin essentially as described previously [31]. Peptides generated by enzymatic cleavage were analysed by matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry for mass fingerprinting and electrospray tandem mass spectrometry for determining partial amino acid sequences. MALDI-TOF mass spectra of peptide fragments were acquired using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany) and electrospray ionisation quadrupole time-of-flight tandem mass spectra for de novo sequencing using a Q-TOF instrument (Micromass, Manchester, UK) as described previously [32].

3. Results

3.1. Enzyme purification

Carboxyl esterase activity in the culture supernatant reached its maximum (140 nkat ml^{-1}) after 12 days of incubation when *M. albomyces* was cultivated on culture medium containing olive oil (data not shown). A significant amount (50%) of the activity was found to be bound to fungal mycelium and 30% could be released by one extraction with 0.1% Triton X-100. According to activity tests the extract was enriched with the steryl esterase, and therefore it was used as starting material for chromatographic purification as described in Section 2.

The purification of the enzyme is summarised in Table 1. It was observed during the purification that a detergent (1% Triton X-100) was required to maintain stability of the enzyme preparation. An overall 52-fold purification was achieved, with an activity yield of 23%. SDS-PAGE showed a major band of 64 kDa and a minor band of 53 kDa, which could not be separated by chromatographic purification (Fig. 1). In order to identify the two proteins, they were first subjected to N-terminal sequence analysis. The band of 64 kDa gave a sequence AAPXVEISTG where the residue at position X could not be determined. No sequence information could be determined from the minor band, when about 5 pmol of protein was subjected for analysis.

In order to further characterise the two proteins, both protein bands were subjected for "in-gel" digestion followed by

Table 1
Purification of steryl esterase from *M. albomyces*

	CAE activity (nkat ml^{-1})	Protein (mg ml^{-1})	Specific activity (nkat mg^{-1})	Total activity (nkat)	Activity yield (%)	Purification factor
Culture filtrate	140	4.0	37	23000	100	1
0.1% Triton extract	340	0.58	590	11000	47	16
Phenyl Sepharose	180	0.20	920	8200	35	25
DEAE Sepharose	260	0.13	1900	5300	23	52

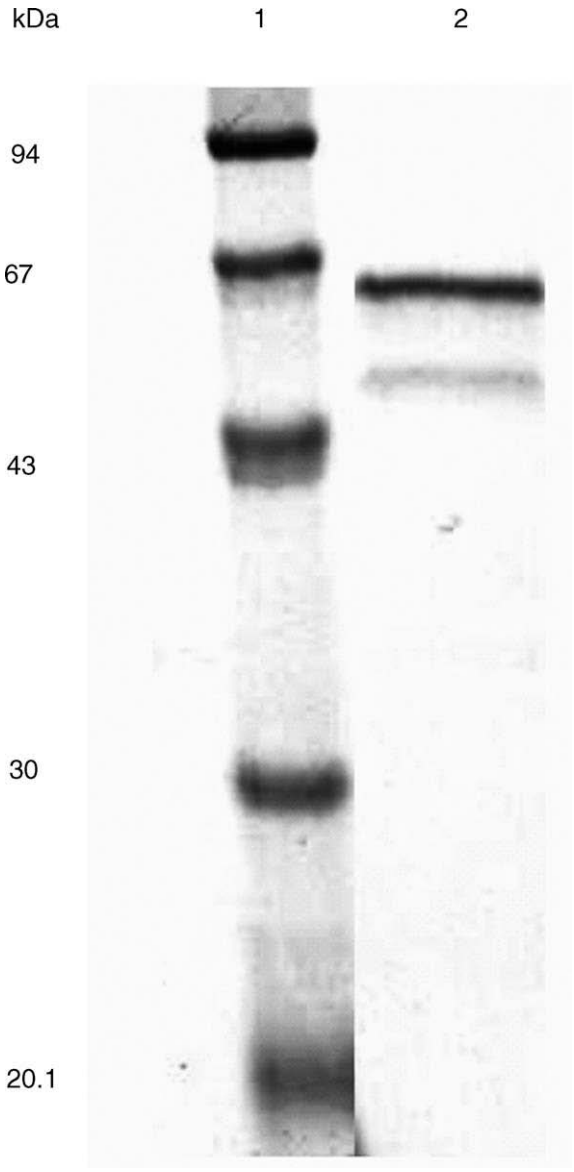

Fig. 1. SDS-PAGE of purified *M. albomyces* steryl esterase. Stained with Coomassie stain. Lane 1: molecular weight marker; Lane 2: 10 μg of *M. albomyces* steryl esterase.

peptide mass fingerprinting by MALDI-TOF mass spectrometry. The obtained mass fingerprints from the two protein bands were almost similar and essentially all peptide masses obtained from the minor band were present also in the mass fingerprint from the major band which contained also some additional peptide masses. This strongly suggests that the minor band represents a proteolytical fragment originating from the major 64 kDa protein. Further analysis of the tryptic peptides yielded partial de novo amino acid sequences as shown in Table 2. All sequences determined from the minor band were also present in the major band which further confirms their relationship. Search with the obtained peptide sequences among the existing databases showed that one of the peptides (NSGSLVPADPVDCPK) had high identity (93%) with a hypothetical protein of *Neurospora crassa* (accession number EAA30450).

Table 2
N-terminal amino acid sequence and internal amino acid sequences of *M. albomyces* steryl esterase preparation

Protein	MW (kDa)	N-terminus	Internal peptides[a]
Major	64[b]	AAPXVEISTG	CAGASDTLACLR LSEDCLTLNVLRPK NSGSLVPADPVDCPK
Minor	53[b]	No result	CAGASDTLACLR LSEDCLTLNVLRPK NSGSLVPADPVDCPK
Hp of *N. crassa*	59[c]	AAPTVTISTG	NSGSIVPAGPVDCPK

Identity with the hypothetical protein (Hp) of *Neurospora crassa* is compared.
[a] L represents either L or I which cannot be distinguished by MS/MS sequencing.
[b] Determined by SDS-PAGE.
[c] Theoretical value based on deduced amino acid sequence.

In order to investigate the possible glycosylation of the two protein forms, the purified protein preparation was treated with endoglycosidases. The resulting preparation showed two bands which were slightly smaller (about 5%) than the original units (data not shown). Analytical gel filtration showed only one peak with a molecular weight of 238 kDa, and no additional purification was achieved. Thus, the data indicated that the enzyme forms a tetramer. Isoelectric focusing indicated one broad band at pH 4.5 (data not shown).

3.2. Substrate specificity

Specific activities of *M. albomyces* steryl esterase on different substrates are shown in Table 3. Activities on cholesteryl esters clearly differed from each other. *M. albomyces* steryl esterase had the highest activity on cholesteryl palmitate and the lowest on cholesteryl stearate. Unsaturated cholesteryl esters were moderate substrates. *p*-Nitrophenyl esters differed as substrates only slightly, with the exception of *p*-nitrophenyl acetate, which

Table 3
Specific activities of purified *M. albomyces* steryl esterase on different substrates

Substrate	Acyl residue	Specific activity (nkat mg^{-1})
Cholesteryl esters		
Cholesteryl palmitate	16:0	272
Cholesteryl stearate	18:0	55
Cholesteryl oleate	18:1	125
Cholesteryl linoleate	18:2	186
Cholesteryl linolenate	18:3	196
Triacylglycerols		
Olive oil	18:1 (76.3%)[a]	3770
p-Nitrophenyl esters		
p-Nitrophenyl acetate	2:0	377
p-Nitrophenyl propionate	3:0	3370
p-Nitrophenyl butyrate	4:0	4340
p-Nitrophenyl caprylate	8:0	2620
p-Nitrophenyl caprate	10:0	2850
p-Nitrophenyl myristate	14:0	2540
p-Nitrophenyl stearate	18:0	3600

[a] Approximate value, also contains 16:0 (10.3%), 18:2 (9.7%), 18:0 (2.3%), 18:3 (0.8%).

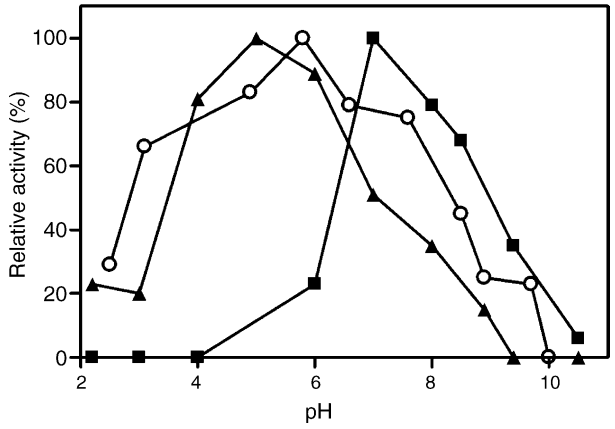

Fig. 2. pH optimum of *M. albomyces* steryl esterase as relative activities. *p*-Nitrophenyl caprate (▲), cholesteryl oleate (◯) and olive oil (■) were used as substrates.

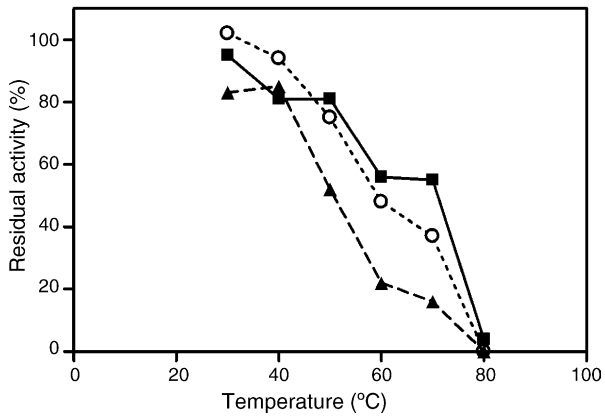

Fig. 4. Temperature stability of *M. albomyces* steryl esterase. Incubation times 1 h (■), 5 h (◯), 24 h (▲) at pH 5. Residual activity was measured using cholesteryl oleate as substrate.

was not as good a substrate as the other esters. Steryl esterase had a high activity on olive oil containing triacylglycerols with long fatty acids. Activities on triacylglycerols having short-chain fatty acids (triacetin, -propionin, -butyrin) were measured both below and above the solubility limit. However, the enzyme showed no activity, and thus the interfacial activation (enhanced activity on aggregated substrate) could not be demonstrated.

3.3. Physicochemical properties

The pH-optimum of the enzyme was measured on cholesteryl oleate, *p*-nitrophenyl caprate and olive oil (Fig. 2). The enzyme showed a pH-optimum for triacylglycerols at neutral pH, whereas the pH optima for *p*-NPC and cholesteryl oleate were 5 and 5.5, respectively. The enzyme was most stable within the pH range of 3.5–7 in 24 h incubation at 37 °C, and clearly less stable at more alkaline pH (Fig. 3). The enzyme retained nearly 80% of its CE activity after 5 h at 50 °C, but was no longer very stable at 60 °C or after prolonged incubation (Fig. 4).

The effects of detergents (Triton X-100, Tween 80, Polidocanol, Zwittergent 3-14, cholic acid and taurocholic acid) on esterase activity measured against *p*-NPB are shown in Table 4. The activity clearly increased in the presence of detergents. The optimal detergent concentration for Triton X-100, Tween 80 and Zwittergent 3-14 was 0.1%. Bile acid salts and Polidocanol had concentration optima of 0.01 and 0.05%, respectively. Bile acid salts improved the activity even at a lower concentration (0.005%). The effects of calcium chloride on CE and lipase activities were determined at concentrations of 0–50 mM. Calcium had no effect on CE activity, but lipase activity clearly decreased as a function of concentration. About 90, 60 and 50% of lipase activity was retained at 0.5, 5 and 50 mM $CaCl_2$ concentration, respectively.

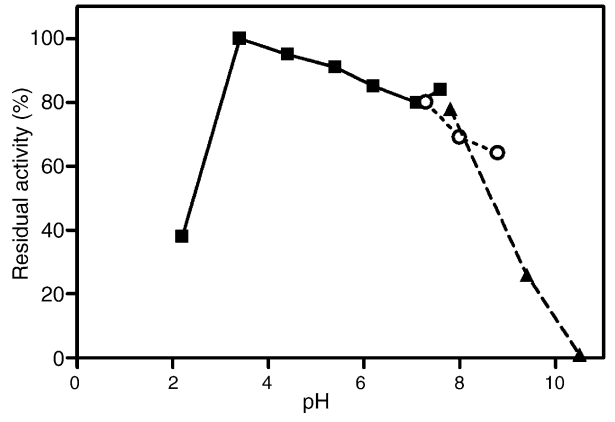

Fig. 3. pH stability of *M. albomyces* steryl esterase. Enzyme was incubated at 37 °C for 24 h in McIlvaine buffer at pH 2.2–8.0 (■), Tris–HCl buffer at pH 7.2–9.1 (◯) and glycine–NaOH buffer at pH 8.6–10.6 (▲). Residual activity was measured at pH 7 using *p*-NPC as substrate.

3.4. Hydrolysis of plant steryl esters

Hydrolysis of plant steryl ester mixture was first studied at high concentration in the presence of Polidocanol (Fig. 5). The final degree of hydrolysis was shown to be dependent on the detergent concentration. *M. albomyces* steryl esterase hydrolysed plant steryl esters completely in the presence of 2% Polidocanol when a CE dosage of 1000 nkat g^{-1} was used. A high degree of hydrolysis was obtained already in a short reaction time. Decreasing the enzyme concentration reduced the reaction velocity but not the final degree of hydrolysis.

Hydrolysis of PSE was compared in water dispersion and in the presence of 0.1% Polidocanol using a CE dosage of

Table 4
The effects of detergents on esterase activity measured on *p*-NPB (% of activity in the absence of the detergent)

Detergent	Concentration (%)						
	0	0.005	0.01	0.05	0.1	1	5
Triton X-100	100	n.d.	n.d.	229	320	300	163
Tween 80	100	n.d.	n.d.	149	212	173	100
Zwittergent 3-14	100	n.d.	n.d.	214	288	164	82
Polidocanol	100	n.d.	222	258	210	161	114
Cholic acid	100	216	264	n.d.	180	168	n.d.
Taurocholic acid	100	244	309	n.d.	279	183	n.d.

n.d., not determined.

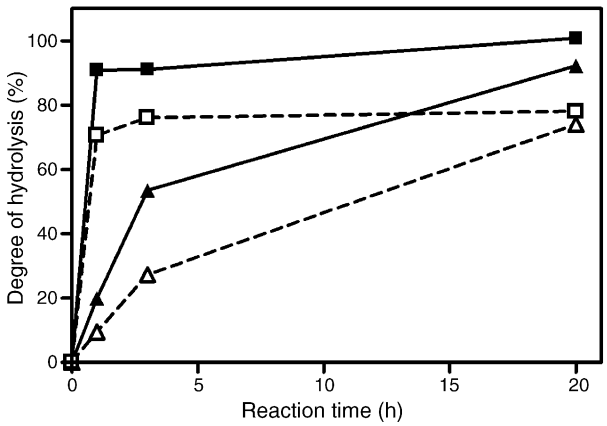

Fig. 5. Hydrolysis of plant steryl ester mixture (1 mg ml^{-1}) in the presence of the detergent (pH 7, 40 °C). 2% Polidocanol, 1000 nkat g^{-1} (■); 1% Polidocanol, 1000 nkat g^{-1} (□); 2% Polidocanol, 100 nkat g^{-1} (▲); 1% Polidocanol, 100 nkat g^{-1} (△).

500 nkat g^{-1}. After 24 h treatment (pH 7, 40 °C), about 90% of steryl esters were hydrolysed in the presence of Polidocanol, but only 20% in the absence of the detergent (results not shown).

3.5. Hydrolysis of steryl esters and triacylglycerols in mixed wood extractives

In order to monitor the hydrolysis of steryl esters and triacylglycerols simultaneously, a mixture of extractives was used as substrate. The results indicated that a large amount of triacylglycerols (58–80%) was hydrolysed even in the absence of Polidocanol (Fig. 6). When Polidocanol was added, triacylglycerols were hydrolysed almost completely in all the conditions used. Steryl esters were not degraded in the absence of Polidocanol, but addition of detergent improved the degree of hydrolysis considerably. At a Polidocanol concentration of 1%, the highest degree of hydrolysis (almost 100%) was obtained at pH 5 and at 40 °C. Treatments at pH 7 and 50 °C also resulted in a degree of hydrolysis over 83%. At 0.1% Polidocanol concentration, the degree of hydrolysis was about 70% at both pH values (5 and 7) and at both temperatures (40 and 50 °C). Degradation of steryl esters and triacylglycerols was also studied as a function of incubation time (Fig. 7). Prolonged incubation time improved the degree of hydrolysis, and 3 h was not sufficient to reach maximal degradation of substrates.

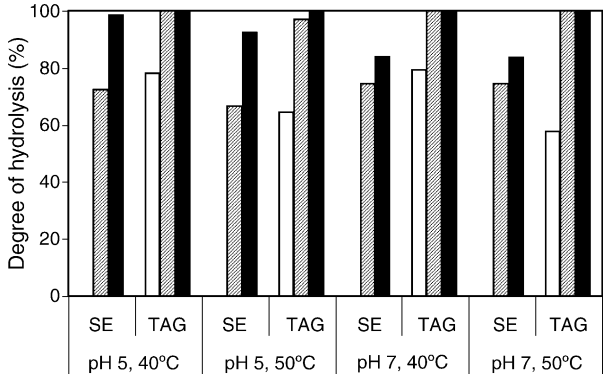

Fig. 6. Hydrolysis of steryl esters (SE) and triacylglycerols (TAG) by *M. albomyces* steryl esterase (total extractive amount 400 mg l^{-1}, including 100 mg l^{-1} steryl ester; CE dosage 1000 nkat g^{-1} steryl ester). No detergent (□), 0.1% Polidocanol (▨) and 1% Polidocanol (■).

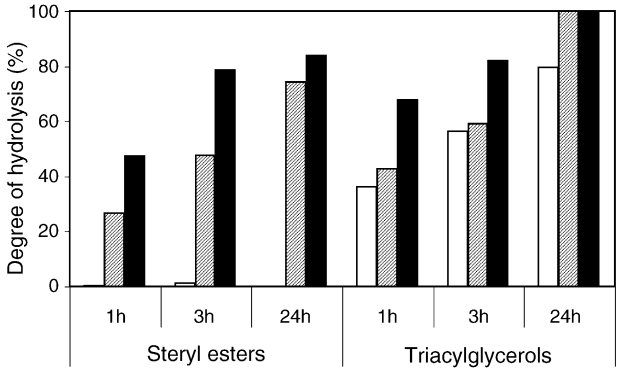

Fig. 7. Hydrolysis of steryl esters and triacylglycerols (total extractive amount 400 mg l^{-1}, including 100 mg l^{-1} steryl ester; CE dosage 1000 nkat g^{-1} steryl ester, pH 7, 40 °C). No detergent (□), 0.1% Polidocanol (▨) and 1% Polidocanol (■).

4. Discussion

Steryl esterase production by *M. albomyces* was induced on a medium containing olive oil. Production is induced in a very late phase, when the organism starts to use olive oil as the sole carbon source. The maximal activity measured in the culture filtrate was reached in 12 days. The highest esterase production on glucose medium supplemented with olive oil was also reached after 12 days cultivation with the ascomycete *O. piceae* [15]. Attempts to increase the production level were made by cultivating *M. albomyces* in a fermenter, but the level was even lower than in shake flask cultivations. Thus, the highest production level obtained into the culture supernatant was about 75 mg l^{-1}.

Steryl esterase of *M. albomyces* was found to be tightly bound to the fungal mycelium or solid components of culture medium, or existed as aggregates. As shown in Table 1, the Triton extract had 16-fold higher specific activity than the culture supernatant. Therefore, the extract was used as starting material for chromatographic purification.

The enzyme was found to be very hydrophobic. The enzyme was not bound to the Phenyl Sepharose column in the presence of Triton X-100, because the detergent disturbs the hydrophobic interaction between proteins and the phenyl ligand, but Triton X-100 was needed to elute bound steryl esterase from the column. Triton X-100 was necessary to prevent the formation of enzyme aggregates during protein binding and elution in anion exchange chromatography. The aggregation of steryl esterases and lipases is known and explained by the strong hydrophobic character of these enzymes [33,34]. The use of different detergents in the dissociation of these aggregates has previously been reported, e.g. in the purification of *O. piceae* [15], *P. aeruginosa* [16] and *C. rugosa* [18] steryl esterases.

SDS-PAGE showed a major band of 64 kDa and a minor band of 53 kDa, which could not be separated despite extensive optimisation of the purification. All internal sequences of the minor

band were also present in the major band. Thus, the proteins might differ due to differential processing, but most probably the smaller unit is a degradation product which has high affinity to the non-processed unit. Gel filtration revealed that steryl esterase has a molecular weight of 238 kDa, and thus it forms a tetramer. In higher protein concentrations and in the absence of a detergent even higher multimeric forms of the enzyme were observed. The earlier reported *O. piceae* [15] and *P. aeruginosa* [16] steryl esterases have been shown to be monomers, with molecular weights of 56.5 and 58 kDa. However, *Fusarium oxysporum* steryl esterase is known to consist of two subunits having molecular weights of 15 and 60 kDa [25]. The molecular weight of *C. rugosa* steryl esterase (Lip 3) monomer has been reported to be approximately 65 kDa [18], but the active form of the enzyme has been shown to be a dimer [35]. Isoelectric focusing indicated p*I* 4.5 for *M. albomyces* steryl esterase. This value is rather higher than the p*I* values of *O. piceae* and *P. aeruginosa*, which have been reported to be 3.3 and 3.2, respectively.

Esterases usually have broad substrate specificities and are capable of hydrolysing both water-soluble substrates typically containing short chain fatty acids, and water-insoluble substrates having long fatty acid chains. Some esterases have the ability to catalyse the hydrolysis of steryl esters, and can be classified as steryl esterases. Steryl esterases have been reported to have both lipase and steryl esterase activity, like the steryl esterases from *O. piceae* [15] and *C. rugosa* (Lip 3) [18]. Some of the enzymes are specific for steryl esters and do not possess lipase activity, e.g. steryl esterases from *Pseudomonas fluorescens* [21] and *Saccharomyces cerevisiae* [24]. Purified *M. albomyces* steryl esterase showed activity on triacylglycerols and *p*-nitrophenyl esters in addition to different steryl esters.

Substrate specificities of *M. albomyces* steryl esterase on cholesteryl esters with long-chain fatty acids resembling wood-derived steryl esters were compared. The enzyme had higher activity on cholesteryl palmitate than on longer and unsaturated fatty acid esters. In general, long-chain and unsaturated fatty acid esters are hydrolysed more effectively than the short and saturated esters [21,23,25]. The length of the fatty acid chain had little effect on the *p*-NP esters as substrates. Only *p*-nitrophenyl acetate differed clearly from other substrates. *M. albomyces* steryl esterase effectively hydrolysed bulky triacylglycerols of olive oil having long-chain fatty acids.

Interfacial activation mechanism could not be demonstrated, because *M. albomyces* steryl esterase had no activity on short chain triacylglycerols in the conditions used. Binding of lipase to a water–lipid interface induces a conformational change in the enzyme, rendering the active site accessible to the substrate [36]. This kinetic phenomenon is typical for lipases, but all lipases do not show interfacial activation although they have an amphiphilic lid covering the active site. Steryl esterase of *O. piceae* had activity against tripropionin and tributyrin, but did not exhibit the interfacial activation behaving like typical esterase [15].

The pH optimum of the *M. albomyces* steryl esterase depended on the substrate. The pH optimum of lipase activity was at neutral pH, whereas esterase activities had optima around pH 5–5.5. Different pH optima might be caused by the effect of pH on ionisation of other than catalytic amino acids either inside or outside the active site, which affect the stability of the active conformation of the enzyme and interaction between the enzyme and different substrates. The steryl esterase of *P. aeruginosa* has been shown to have a very broad pH-optimum from 5.5 to 9.5 [16], but commonly the pH-optima of steryl esterases have been reported to vary around 6–8 [15,21,22]. The *M. albomyces* steryl esterase retained activity better at pH values below 7 in 24 h incubation at 37 °C. The steryl esterase of *O. piceae* was shown to retain its activity in the same pH range [15]. The *M. albomyces* steryl esterase retained nearly 80% of its activity after 5 h at 50 °C. Characterised steryl esterases have been reported to be generally stable at temperatures up to 50–60 °C for varying incubation times [16,21,22].

Esterase activity against *p*-NPB was increased in the presence of the detergents studied. The highest activities were measured when the detergent concentration was below 0.1%. According to literature information the optimum concentrations for non-ionic detergents are usually between 0.1 and 0.3%. Further increases in concentrations have been shown to decrease the activities of steryl esterases [15,21,25]. Sugihara et al. [16] reported that addition of bile salts up to a concentration of 100 mM increased both steryl esterase and lipase activity of *P. aeruginosa*, but Triton X-100 did not have such an effect. Thus, it was suggested that the bile salt molecule has a significant role in opening the active site of the enzyme. For example binding of taurocholate near the active site of bovine pancreatic cholesteryl esterase is presumed to stabilise the open conformation of the hairpin loop [37]. In addition to direct activation or inactivation of the enzyme, detergent might alter the hydrophobicity of the enzyme and affect micelle formation and the ratio of free and micellar substrate, and thus the availability of substrate to enzyme [38]. Therefore, the effect of detergent on enzyme activity is the sum of many parameters.

Calcium (Ca^{2+}) has been shown to increase the catalytic activity of microbial lipases [39,40], but its action as an inhibitor has also been reported [41,42]. In this study lipase activity was decreased as the function of Ca^{2+} concentration, but no effect on cholesteryl esterase activity was detected at concentrations up to 50 mM. Calcium has been shown to have only a slight effect on microbial steryl esterases at a concentration of 5 mM or lower [16,21,22], but strong inhibition of some mammalian cholesteryl esterases at concentrations of 5–10 mM has been reported [43,44].

Commercial Resinase lipase has been used for pitch control for several years because of its ability to hydrolyse effectively triacylglycerols [12]. In addition to triacylglycerols steryl esters also have a negative impact on paper production [45], and thus degradation of steryl esters would improve the process. However, Resinase has no effect on steryl esters present in thermomechanical pulp (TMP) and process waters [14], or activity on different steryl ester model compounds [17]. Therefore, esterases having a broad substrate specificity have recently been examined for pitch control [15,18].

Steryl esterase of *M. albomyces* effectively hydrolysed steryl esters in the different micellar Polidocanol dispersions. In water dispersion, degradation was restricted and only 20% of PSE were

hydrolysed, and in the presence of other extractives steryl esters were not degraded at all. However, the triacylglycerols were hydrolysed well even in the absence of detergent. It is known that highly hydrophobic triacylglycerols and steryl esters remain in the interior of the pitch droplets [28,46]. Steryl esters have a much higher viscosity than triacylglycerols [28], and thus the physical state of the colloidal pitch droplet might restrict the hydrolysis of steryl esters. The interface of the pitch particle might induce lipase activity, but the steryl esters are not accessible to the enzyme. Knowledge on the action of *M. albomyces* steryl esterase can be applied in studies of different potential applications. The effects of *M. albomyces* steryl esterase on wood pitch and paper properties remain to be studied. In addition to pitch control, steryl esterases might have potential in modification of textile fibres and food products. In order to facilitate large-scale production of the *M. albomyces* steryl esterase for application studies, the enzyme must first be overproduced in a suitable heterologous host.

Acknowledgements

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PUBLICATION V

Characterization of *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei* and modification of fibre products with the enzyme

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Characterization of *Melanocarpus albomyces* steryl esterase produced in *Trichoderma reesei* and modification of fibre products with the enzyme

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Abstract

Melanocarpus albomyces steryl esterase STE1 is considered to be an interesting tool for several industrial applications due to its broad substrate specificity. STE1 was produced in the filamentous fungus *Trichoderma reesei* in a laboratory bioreactor at an estimated production level of 280 mg l^{-1}. The properties of the purified recombinant enzyme (rSTE1), such as substrate specificity, molecular mass, pH optimum and stability and thermostability were characterized and compared to the corresponding properties of the native enzyme. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed one band with a molecular weight of 60 kDa for rSTE1, whereas analytical gel filtration showed a dimeric structure with a molecular weight of 120 kDa. The rSTE1 was somewhat less stable under different conditions and had slightly lower activities on various substrates than the native STE1. The effects of rSTE1 on the properties of paper sheets and polyethylene terephthalate (PET) were preliminarily evaluated. Due to the hydrolysis of triglycerides and steryl esters by the rSTE1 treatment, the tensile strength and hydrophilicity of the paper were increased. The rSTE1 treatment increased significantly the polarity of PET by hydrolysing the ester bonds in the polyester backbone. Dyeing of PET with methylene blue was also slightly improved after rSTE1 treatment.

Introduction

Steryl esterases (EC 3.1.1.13) are a subclass of esterases which primarily hydrolyse fatty acid esters of sterols, i.e. steryl esters. Some steryl esterases also catalyse the hydrolysis or synthesis of a rather broad range of other substrates containing ester linkages, such as triacylglycerols. *Melanocarpus albomyces* steryl esterase (STE1), which was recently purified and characterized (Kontkanen et al. 2006a), has been shown to have a broad substrate specificity. The amino acid sequence deduced from the *ste1* gene was shown to have identity with known lipases, e.g. *Candida rugosa* lipases (Kontkanen et al. 2006b).

Wood extractives are known to have a negative impact on paper properties, as well as on paper machine runnability. During mechanical pulping of softwood, part of the extractives is dispersed into the white water as colloidal droplets. The negatively charged resin droplets have been shown to be stabilized by soluble polysaccharides, i.e. glucomannan, but if the colloidal stability is disturbed, they are susceptible to aggregation and may form deposits on fibre and paper machine surfaces (Sundberg et al. 1996; Sihvonen et al. 1998; Otero et al. 2000). Aggregation of lipophilic wood resin components (triglycerides, steryl esters, resin acids, free fatty acids and sterols) onto paper sheets decreased the mechanical strength of the paper by decreasing the bonding ability of fibres (Sundberg et al. 2000).

A commercial lipase preparation (Resinase, Novozymes, Denmark) has been shown to hydrolyse efficiently the triglycerides of pulp, and its ability to increase paper quality, operation stability and paper strength properties has also been reported (Fujita et al. 1992; Hata et al. 1996; Chen et al. 2001; Mustranta et al. 2001). However, Resinase contains no steryl esterase activity (Kontkanen et al. 2004), and it has been shown to have no effect on wood steryl esters (Mustranta et al. 2001), which are known to have a significant negative impact on paper quality due to their physical characteristics (Kokkonen et al. 2002). Recently, the steryl esterase of *Ophiostoma piceae* was characterized, and its potential for pitch control was discussed (Calero-Rueda et al. 2002). In addition to lipases and esterases, other enzymes such as cellulases, hemicellulases and laccases have also been tested for modification of the troublesome substances in white waters (Kantelinen et al. 1995; Zhang 2000). In addition to enzymatic treatments, microbial pretreatments of wood chips have been studied for pitch biocontrol (Chen et al. 1994; Rocheleau et al. 1998; Leone and Breuil 1999; Martínez-Iñigo et al. 2000; Kallioinen et al. 2004). The drawbacks of the microbial methods include, e.g. the rather long treatment time, difficulty of the control of microbial activity, decrease of yield and deterioration of paper optical properties (Fischer et al. 1994; Farrel et al. 1997; Gutiérrez et al. 2001).

Polyester (polyethylene terephthalate, PET) is a synthetic polymer which is used in many textile products due to its favourable characteristics, such as resistance to wrinkling, stretching, shrinking, abrasion and many chemicals. However, polyester has several undesirable properties such as a high tendency to pill, static charges and high lustre; further, it is difficult to dye and is resistant to removal of oil and grease stains (Andersen et al. 1999; Yoon et al. 2002). Lipases, esterases and cutinases have all been studied for the surface modification of polyester (Hsieh and Cram 1998; Andersen et al. 1999; Yoon et al. 2002; Gübitz and Cavaco-Paulo 2003; Miettinen-Oinonen et al. 2005; Vertommen et al. 2005), but fully satisfactory results have not been achieved. In this work, *M. albomyces* steryl esterase was produced efficiently in *Trichoderma reesei*. The recombinant enzyme was purified and characterized, and its use in the treatment of extractives in thermomechanical pulp and in the treatment of polyester was evaluated.

Materials and methods

Strains and enzymes

The steryl esterase gene originated from the fungus *Melanocarpus albomyces* (VTT D-96490). The strain used for steryl esterase production was a *cbh1* disruptant of the *Trichoderma reesei* strain Rut-C30 (Montenecourt and Eveleigh 1979). The expression construct (pHAK4) was described previously (Kontkanen et al. 2006b). Resinase A2X lipase preparation was obtained from Novozymes (Denmark).

Cultivation in bioreactor

The transformant pHAK4/77 producing the highest level of steryl esterase activity in shake flasks (Kontkanen et al. 2006b) was cultivated in a Braun Biostat C-30 bioreactor in 20 l of a medium containing (in grams per litre) whey 40.0, spent grain 20.0, KH_2PO_4 5.0 and $(NH_4)_2SO_4$ 5.0. The pH was adjusted to 5.6–6.0 with NH_4OH and H_3PO_4, and the temperature was 28°C. Dissolved oxygen level was maintained above 30% with agitation at 450 rpm, aeration at 8 l min^{-1} and 0–30% O_2 enrichment of the incoming air. A mixture of PPG 1025 (297876X, BDH) and PPG 2025 (297676Y, BDH) (1:1 v/v) was used as anti-foaming agent. The centrifuged mycelium of cultivation samples was extracted with 0.05 M sodium phosphate buffer (pH 6.0) and twice with 0.1% (w/v) Triton X-100 in the same buffer to recover all the product. Steryl esterase production was monitored by measuring carboxyl esterase (CAE) activity from the culture supernatants and the extracted samples. Cholesteryl esterase (CE) activity, lactose and total protein concentration were also measured from the samples.

The cultivation was terminated as soon as CAE activity started to decrease. The culture supernatant was recovered by centrifuging (4000 rpm, 8°C, 30 min).

Purification of recombinant steryl esterase

The recombinant steryl esterase (rSTE1) was purified by two chromatographic steps like its native counterpart (STE1) using hydrophobic interaction chromatography (HIC, Phenyl Sepharose Fast Flow) and anion-exchange chromatography (DEAE Sepharose Fast Flow) as described previously (Kontkanen et al. 2006a). To estimate the molecular size of the protein, part of the pooled DEAE fractions was applied to a gel filtration column as previously described (Kontkanen et al. 2006a). The column was pre-equilibrated with 0.1 M sodium phosphate buffer (pH 7) containing 0.15 M NaCl and 0.1 or 1% (w/v) Triton X-100. To characterize the stability of the protein, Triton X-100 was removed from pooled DEAE fractions by repeating the anion-exchange chromatography as described by Kontkanen et al. (2006a). Finally, the buffer was exchanged to 20 mM sodium acetate (pH 5.5) using PD-10 columns.

The enzyme preparation containing low Triton X-100 concentration (0.1%) was used for the application studies. HIC fractions containing CAE activity were pooled and equilibrated with 0.2 M sodium phosphate buffer (pH 6.5) containing 1% Triton X-100. The sample was applied to a DEAE Sepharose Fast Flow column (2.7×10 cm; Pharmacia, Uppsala, Sweden), which was pre-equilibrated with 0.02 M sodium phosphate (pH 6.5) containing 1% Triton X-100. Proteins were eluted with 0.02 M sodium phosphate (pH 6.5) containing 0.1% Triton X-100, and thereafter, with a linear 0–0.5 M NaCl gradient (560 ml) in the same buffer. Fractions containing CAE activity were pooled.

Protein concentrations were determined using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% Tris-HCl Ready Gel (Bio-Rad). Protein bands were visualized by staining with Coomassie Brilliant Blue (Serva Blue R) and compared with a low molecular weight (LMW) marker (Pharmacia). The content of N-linked carbohydrates was estimated by SDS-PAGE after deglycosylation with endo-β-N-acetylglucosaminidase H (Endo H, Roche) or PNGase F (Biolabs) according to the manufacturers' instructions. The rSTE1 was studied by Western blotting with a His-tag specific antibody (Dianova). The second antibody was goat anti-mouse IgG-alkaline phosphatase conjugate (Bio-Rad). Protein bands were visualized by enzymatic colour reaction and compared with a molecular weight marker (Prestained SDS-PAGE Standards, Broad Range, Bio-Rad).

Characterisation of recombinant steryl esterase

CE activity was assayed using cholesteryl oleate as substrate according to Tenkanen et al. (2002). Activities on different cholesteryl esters were measured as described earlier (Kontkanen et al. 2004). CAE activity was determined using *p*-nitrophenyl caprate (*p*-NPC) as substrate as previously described by Tenkanen et al. (2002). Activities against different *p*-nitrophenyl esters were measured as previously described (Kontkanen et al. 2006a). Lipase activity was assayed by an enzymatic colorimetric method according to Kontkanen et al. (2004).

The rSTE1 was characterized at different temperatures and pH values as previously described (Kontkanen et al. 2006a). The pH optima were determined for CAE, CE and lipase activities. The pH stability was determined by incubating the purified enzyme solution at different pH values at 37°C for 24 h. Thermal stability was determined by incubating the enzymes at 30–80°C (pH 5, 24 h). The residual enzyme activities were measured on cholesteryl oleate.

TMP treatments and preparation of handsheets

Unbleached TMP produced from Norway spruce was obtained from a Finnish paper mill. TMP was sampled after the main refining stages prior to screening. The pulp was devoid of added chemicals or contaminants from white water. The pulp consistency was about 46%, and the Canadian standard freeness (SCAN-C21:65) was 138. The pulp was stored at -20°C.

TMP was hot disintegrated according to SCAN-M 10:77. The enzyme treatments were carried out with rSTE1 and Resinase at a pulp consistency of 1%, 50°C, pH 5, using agitation of 150 rpm for 20 h. Both enzymes were dosed as lipase activity of 500 nkat g^{-1} of pulp. The used lipase dosage of rSTE1 preparation corresponds to a CE dosage of 25 nkat g^{-1} of pulp. The rSTE1 preparation contained 0.1% Triton X-100 to stabilize the enzyme. The reference treatments were performed under identical conditions but without addition of enzymes. Treatments were also performed in the presence of 0.05% Triton X-100. After the treatments, the suspensions were filtered twice and washed with ion-exchanged water. Water samples were taken from the treated pulp suspension before filtration. The pulp was homogenized by cold dispersion according to SCAN-C 18:65 before preparation of handsheets.

Handsheets (10×10 cm) were prepared according to SCAN M 5:76 using 6-µm wire cloth (Sefar) to ensure retention of fines in the sheets. The sheets were wet-pressed at 490 kPa for 4 min and dried at 80°C in a rotary drier.

Analyses of handsheets and TMP waters

Handsheet properties (ISO brightness, light scattering coefficient, tensile index, tear index) were determined according to SCAN methods. The contact angle of water on the wire side of handsheets was measured with a CAM 200 contact angle apparatus. The chemical compositions of extractives in the TMP waters were analysed by gas chromatography (GC) after extraction with methyl-*tert*-butyl ether (Örså and Holmbom 1994).

Treatment of polyester (polyethylene terephtalate) fabric

Polyester fabric (62 g/m^2; Rhodia Industrial Yarns, Switzerland) was washed with OMO detergent (Unilever, Netherlands) at 40°C, extracted with dichloromethane to remove the oligomers and cut to 8×22 cm pieces. The pretreated polyester fabric was treated with rSTE1 at 40°C and pH 7 (200 rpm) for 2 and 24 h using a fabric-to-liquid ratio of 1:30. The lipase dosage was 1,000 nkat g^{-1} of fabric. Enzyme action was terminated by incubating at 80°C for 10 min, whereafter the fabrics were rinsed twice with ion-exchanged water for 10 min and dried flat at room temperature.

Analyses of polyester fabric

Wetting rate (velocity) as rising height was measured according to the standard DIN 53924. Contact angle and penetration time of water were measured by a goniometric system, which is composed of a microlitre syringe for dosing the liquids and an optical system combined with a video recorder and computer for data analysis (Vippola 2002). Fabric pieces were dyed with 0.1% methylene blue using a fabric to liquid ratio of 1:50 at 85°C for 5 min, whereafter the dyed fabrics were rinsed with water to remove the excess dye. Colour values were measured with a Minolta CR-200 spectrophotometer.

Results

Cultivation

M. albomyces STE1 was previously expressed in *Trichoderma reesei*, and production of the enzyme in shake flask cultivations was reported (Kontkanen et al. 2006b). The transformant pHAK4/77 yielding the highest level of steryl esterase in shake flasks was cultivated in a laboratory bioreactor in a whey-spent grain medium that was most favourable for rSTE1 production in the shake flask cultivations. Steryl esterase production was detectable after about 1 day of cultivation, and the maximal CAE activity of 563 nkat ml^{-1} and maximal CE activity of 44 nkat ml^{-1} were obtained after 69 h (Fig. 1). After 71 h, the CAE

activity had decreased and the cultivation was terminated. A sample of the mycelium was extracted once with buffer and twice with 0.1% Triton X-100 to investigate the proportion of mycelium-bound enzyme. Approximately 10% of the total CAE activity yield was measured from the buffer fraction, and 20% of the activity could be obtained from the Triton X-100 extracts. However, the main part of the activity (70%) was in the culture supernatant, and therefore, the cell fraction was discarded. The culture supernatant was concentrated fivefold by ultrafiltration.

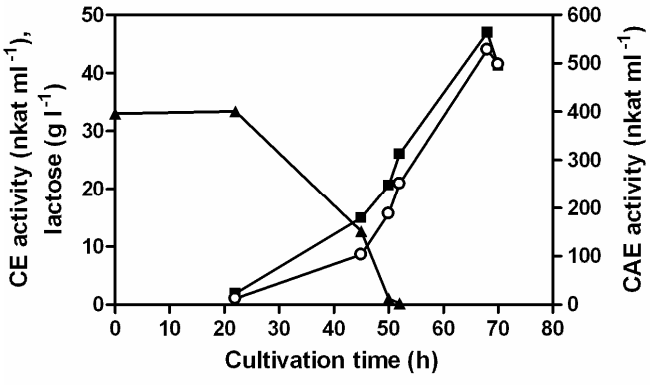

Figure 1. Bioreactor cultivation of the Trichoderma reesei *transformant pHAK4/77. Steryl esterase production was followed by measuring cholesteryl esterase (○) and carboxyl esterase (■) activities. Lactose consumption (▲) is also shown.*

Purification

A histidine tag was fused to the C terminus of *M. albomyces* steryl esterase to exploit a one-step purification protocol using immobilized metal affinity chromatography (IMAC). The rSTE1 enzyme did not bind to the Sepharose Fast Flow column to which divalent metal ions (Cu^{2+} or Ni^{2+}) had been coupled, even at several different salt concentrations and pH values in the presence of Triton X-100. This could be due to inaccessibility of the His-tag in the rSTE1 structure. This conclusion is supported by the fact that the rSTE1 also did not react with His-tag specific antibody in Western blot analysis. Finally, the enzyme was purified like the native STE1 using HIC and anion-exchange chromatography. SDS-PAGE showed a single band with a molecular weight of 60 kDa for the purified rSTE1 (Fig. 2). Analytical gel filtration showed one peak for the purified rSTE1, with a molecular weight of 120 kDa indicating a dimeric structure. Deglycosylation with the Endoglycosidase H and PNGase F suggested that rSTE1 is a glycoprotein with about 5% N-linked carbohydrate.

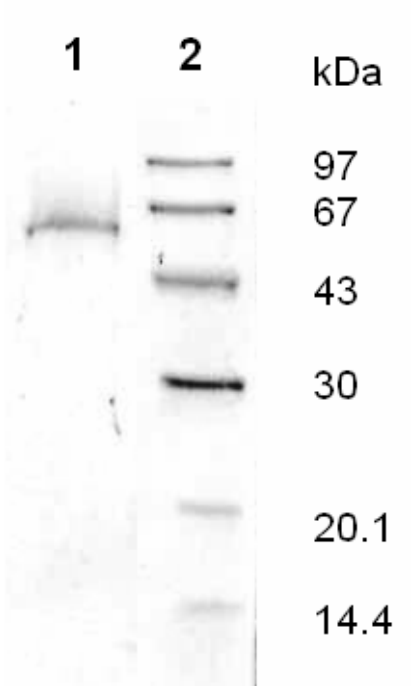

Figure 2. Coomassie-stained SDS-PAGE of purified Melanocarpus albomyces *steryl esterase. Lane 1 20 µg of rSTE1, lane 2 molecular weight marker.*

Characterization of recombinant steryl esterase

Specific activities of rSTE1 against different substrates are shown in Table 1. Specific activities of the rSTE1 were lower than those of the native STE1 determined previously (Kontkanen et al. 2006a). However, the relative activity profiles of both enzyme preparations against different cholesteryl esters were similar. The specific activity of the rSTE1 was the highest against cholesteryl palmitate, followed by the activities against cholesteryl linolenate and cholesteryl linoleate. The amount of double bonds thus corresponded to the hydrolysis rate of cholesteryl esters having C18 fatty acids. Activities of the rSTE1 against *p*-nitrophenyl esters clearly differed from each other. The specific activity was highest against *p*-nitrophenyl butyrate and lowest against *p*-nitrophenyl acetate. Activities against other *p*-nitrophenyl esters tested varied between 1,200 and 1,800 nkat mg^{-1}. The specific lipase activity of the rSTE1 preparation was 2,800 nkat mg^{-1}.

Table 1. Specific activities of recombinant Melanocarpus albomyces *steryl esterase (rSTE1) on different substrates.*

Substrate		**Acyl residue**	**Specific activity (nkat mg^{-1})**
Cholesteryl esters	Cholesteryl palmitate	16:0	219
	Cholesteryl stearate	18:0	38
	Cholesteryl oleate	18:1	82
	Cholesteryl linoleate	18:2	128
	Cholesteryl linolenate	18:3	149
p-nitrophenyl esters	*p*-nitrophenyl acetate	2:0	140
	p-nitrophenyl propionate	3:0	1710
	p-nitrophenyl butyrate	4:0	2460
	p-nitrophenyl caprylate	8:0	1780
	p-nitrophenyl caprate	10:0	1170
	p-nitrophenyl stearate	18:0	1230
Triglycerides	Olive oil	18:1 (76.3%)[a]	2800

a) Approximate value; also contains 16:0 (10.3%), 18:2 (9.7%), 18:0 (2.3%) and 18:3 (0.8%)

The pH optima of the rSTE1 were studied using *p*-NPC, cholesteryl oleate and olive oil as substrates. The pH dependence of the rSTE1 was similar to that of the native STE1 (Kontkanen et al. 2006a). The pH optimum of lipase activity was at pH 7–8, whereas CAE and CE had optima around pH 5.5 and 6.0, respectively (Fig. 3). The rSTE1 was most stable within the pH range of 3–7 and clearly less stable at pH values above 8 (Fig. 4A). The rSTE1 retained over 90% of its CE activity after 24 h at 50°C and 50% after 1 h at 60°C, but the enzyme was inactivated at 70°C (Fig. 4B). The native enzyme was more stable at higher temperatures, having half-life of 4 h at 60°C and 2 h at 70°C (Kontkanen et al. 2006a). The residual activities were determined after incubation of the enzyme preparations in the absence of surfactant, and thus, the possible differences in multimeric structures of STE1 and rSTE1 may affect the protein stabilities.

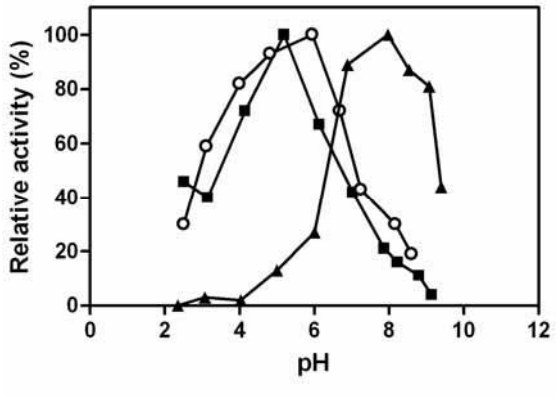

Figure 3. pH optimum of rSTE1. p-Nitrophenyl caprate (■), cholesteryl oleate (○) and olive oil (▲) were used as substrates.

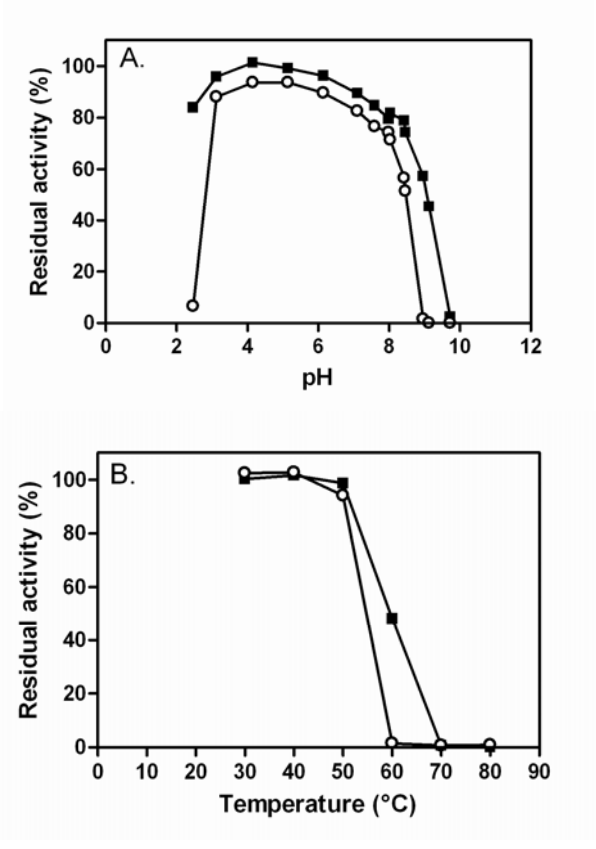

Figure 4. pH stability of rSTE1 at 37°C (A) and temperature stability of rSTE1 at pH 5 (B). Incubation times: 1 h (■) and 24 h (○). Residual activities were measured against cholesteryl oleate.

Pulp treatments

TMP suspension was treated with rSTE1 and Resinase A2X lipase in order to compare their effects on wood extractives. The TMP water samples were taken after enzyme treatments before filtration of the pulp suspension to retain the maximal amount of extractives. The changes in the chemical composition of the extractives were analysed by GC. Triglycerides were efficiently hydrolysed to free fatty acids and glycerol by both esterase treatments (Table 2). Resinase was not able to degrade steryl esters. However, the amount of steryl esters was slightly decreased by rSTE1, with a corresponding increase of free sterols.

Table 2. Effects of esterase treatments on the extractives of the thermomechanical pulp water.

Treatment	Fatty acids (mg l^{-1})	Resin acids (mg l^{-1})	Sterols (mg l^{-1})	Steryl esters (mg l^{-1})	Triglycerides (mg l^{-1})	Lignans (mg l^{-1})	Total extractives (mg l^{-1})
Reference 1	6.1	7.7	1.0	9.9	17.0	15.6	57.4
rSTE1	11.7	7.1	1.5	8.1	2.5	15.2	46.1
Reference 2	6.2	8.5	0.9	10.7	14.8	16.4	55.6
Resinase	18.7	8.9	1.1	10.8	0.0	17.4	56.9

The effects of the rSTE1 and Resinase treatments of pulp on the contact angle of water on handsheets were also studied. Both treatments increased hydrophilicity of the sheet surfaces (results not shown). The rSTE1 improved wettability somewhat more efficiently, which might be due to partial hydrolysis of steryl esters (Table 2).

The effects of rSTE1 and Resinase treatments on handsheet technical properties were investigated in the presence of 0.05% Triton X-100 because *M. albomyces* steryl esterase has been shown to hydrolyse model steryl esters effectively in the presence of polydocanol, a non-ionic surfactant (Kontkanen et al. 2006a). The tensile index was significantly increased after both enzyme treatments when compared to the reference sample (Table 3). The optical properties were not affected by the enzymatic treatments.

Table 3. Effects of esterase treatments on the properties of handsheets.

Treatment	Density (kg/m^3)	ISO brightness (%)	Opacity (%)	Light scattering coefficient (m^2/kg)	Tensile index (Nm/g)	Tear index (Nm^2/kg)
Ref	296.0	61.0	94.6	57.9	27.6	6.34
Resinase	318.0	61.0	94.4	57.2	33.4	6.46
rSTE1	322.0	61.7	94.2	57.1	34.1	6.30

Treatment of polyester

The effect of rSTE1 on the textile properties of PET was evaluated by determining wetting and dyeing behaviour. Contact angles and water penetration times of the rSTE1-treated fabrics were significantly shorter as compared to the reference fabric (Fig. 5). Thus, the hydrophilicity of the fabric was clearly improved by rSTE1 treatment. The short penetration time of water of the 24-h reference fabric indicates that Triton X-100 is ordered into the fibre surface during the longer incubation. Surprisingly, regardless of improved hydrophilicity determined by contact angle and penetration times, no differences in the rising heights of rSTE1-treated and reference fabrics was observed (data not shown). Methylene blue dyeing of polyester fabric showed better dyeing efficiency after 24-h enzyme treatment as compared to the reference fabric (Table 4). After dyeing, the lightness (*L*) of enzyme-treated fabric decreased and the blueness (*b*) increased as compared with the reference fabric.

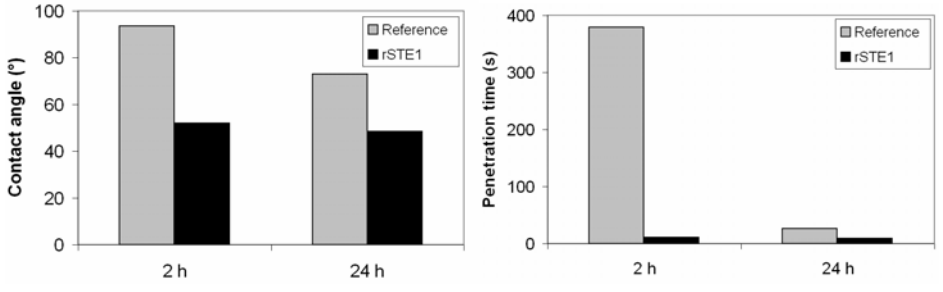

Figure 5. Contact angles (A) and penetration times of water (B) determined for the rSTE1-treated polyester fabric.

Table 4. Colour after 0.1% methylene blue dyeing.

Sample	Treatment time (h)	*L* value	*b* value
Reference	2	82.13	-7.40
rSTE1	2	85.49	-3.57
Reference	24	83.29	-5.44
rSTE1	24	81.93	-6.84

Discussion

Steryl esterases are a poorly characterized group of hydrolases, which could be of considerable importance for several industrial applications, e.g. in the pulp and paper, textile and food industries. Only a small number of microbial steryl esterases have been identified and characterized (Uwajima and Terada 1976; Kamei et al. 1979; Madhosingh and Orr 1981; Taketani et al. 1981; Calero-Rueda et al. 2002; Sugihara et al. 2002). However, steryl esterase activity has been found in some lipases (Kaiser et al. 1994; Svendsen et al. 1995; Gao and Breuil 1998) and lipase preparations (Kontkanen et al. 2004). *M. albomyces* steryl esterase has been shown to be interesting due to its ability to act on a variety of esters, such as plant-derived steryl esters (Kontkanen et al. 2006a). However, the reproducibility of productive cultivations was poor and only a very small amount of enzyme could be produced in bioreactor cultivations. In order to obtain a more reliable source for *M. albomyces* steryl esterase, the enzyme was expressed in *Trichoderma reesei* (Kontkanen et al. 2006b).

M. albomyces produced maximally 140 nkat ml^{-1} of CAE activity into the culture supernatant after 12-day cultivation in shake flasks, corresponding to about 75 mg l^{-1} (Kontkanen et al. 2006a). In shake flask cultivation of *T. reesei* transformant, the maximal production levels, CAE activity of 99 nkat ml^{-1} and CE activity of 7 nkat ml^{-1}, were measured from the culture supernatant after 10-day cultivation (Kontkanen et al. 2006b). In this work, rSTE1 was produced in a laboratory bioreactor using the same culture medium as in shake flasks. Maximum CAE and CE activities of 563 nkat ml^{-1} and 44 nkat ml^{-1}, respectively, were reached after 69-h cultivation. It can be estimated on the basis of the specific CAE activity immediately after purification that the production level in the bioreactor was 280 mg l^{-1}. In shake flasks, only approximately 40% of the total CAE activity produced by *T. reesei* was present in the culture supernatant but in the bioreactor the corresponding amount was 70%.

STE1 has previously been observed to have a tendency to form enzyme aggregates in aqueous dispersions (Kontkanen et al. 2006a, b). This phenomenon is typical for lipolytic enzymes and is explained by the strong hydrophobic

character of these enzymes (Rúa et al. 1997; Brush et al. 1999; Fojan et al. 2000). The presence of the non-ionic surfactant Triton X-100 was observed to be necessary in order to prevent aggregation of STE1 and enable successful purification (Kontkanen et al. 2006a). Therefore, 1% Triton X-100 was also used during the purification of rSTE1.

The native STE1 preparation has previously been shown to have major and minor bands with molecular weights of 64 kDa and 53 kDa, respectively (Kontkanen et al. 2006a). These bands gave identical peptide sequences, and it was concluded that the smaller unit represents a proteolytical fragment originating from the full-length protein. In this work, SDS-PAGE showed a single band with a molecular weight of 60 kDa for the purified rSTE1. Analytical gel filtration showed a single peak for the purified rSTE1, with a molecular weight of 120 kDa indicating a dimeric structure. Under similar conditions, native STE1 has been shown to have a tetrameric structure with a molecular weight of 238 kDa. Both the native STE1 and the rSTE1 were shown by SDS-PAGE to be glycoproteins with about 5% N-linked carbohydrate. However, the native and recombinant proteins probably have slightly different glycosylation patterns, which could lead to different multimeric structures. The dimeric structure of rSTE1 might also hide the C-terminal His-tag within the interior part of the structure, thus preventing interaction between the His-tag and metal chelate. The existence of His-tag could also reduce the hydrophobicity of rSTE1 and thus affect the multimeric structure of the protein in the conditions used. Differences in the multimeric structures of STE1 and rSTE1 may also affect the protein stabilities.

Wood extractives, especially triglycerides and steryl esters, are known to affect negatively the paper strength properties when retaining on the paper (Wearing et al. 1985; Sundberg et al. 2000). Resinase can efficiently hydrolyse the triglycerides of the pulp and increase the strength properties of handsheets (Hata et al. 1996; Chen et al. 2001; Mustranta et al. 2001). To compare the action of the rSTE1 and Resinase, TMP suspension was treated with both enzymes, and the effects of enzyme treatments on technical properties of handsheets were analysed. Both enzyme treatments increased the tensile strength of the paper. However, it is noteworthy that use of the surfactant in the TMP treatments might have negative implications for the binding ability of fibres, and thus, decrease the tensile strength of the reference sheets (Touchette and Jenness 1960).

Hydrophilicity of TMP fibres was increased by the enzymatic treatments, as concluded from the decreased contact angles of the handsheets. The decrease in contact angles was evidently caused by decreased hydrophobicity of the fibre and sheet surfaces. It is also possible that adsorbed extractives on the fibre surfaces affect the sheet structure, and thus, the contact angle (Kokkonen et al. 2002). The rSTE1 increased hydrophilicity more efficiently than Resinase,

which could be due to partial hydrolysis of steryl esters in addition to triglycerides. Previously, it has been reported that steryl esters have a significant impact on the contact angle (Kokkonen et al. 2002; Qin et al. 2003), and our results support this conclusion.

The triglycerides of the TMP were effectively hydrolysed to free fatty acids and glycerol by both enzyme treatments. The rSTE1 hydrolysed part of the steryl esters, but Resinase had no effect on them. Thus, it can be concluded that the hydrolysis of steryl esters is restricted in the used conditions. In our previous study dealing with the treatments of model extractives with STE1, we showed that triglycerides were hydrolysed well even in the absence of surfactant but that hydrolysis of steryl esters requires the presence of a surfactant (Kontkanen et al. 2006a). The highly hydrophobic steryl esters and triglycerides are known to form the core of the resin droplets, whereas resin acids, fatty acids and sterols are enriched at the outer surface (Nylund et al. 1998; Qin et al. 2003). Regardless of the viscous and rigid outer film, almost total hydrolysis of triglycerides can be achieved enzymatically. However, steryl esters are more hydrophobic and viscous than triglycerides (Qin et al. 2003), and thus, they are probably less accessible. Total hydrolysis of steryl esters would increase the amount of sterols, which have been shown to increase the viscosity of resin (Qin et al. 2003). Therefore, it is likely that 100% hydrolysis of steryl esters would probably not be advantageous to the papermaking process. However, the physico-chemical characterization of model pitch mixtures has shown that a combination of lipase and steryl esterase treatments, and especially adjustment of the hydrolysis ratio of steryl esters, can decrease pitch deposition tendency (Qin et al. 2003).

Despite its several favourable characteristics, polyester has many undesired properties (Yoon et al. 2002). The hydrophobic nature and inactive surface of polyester make it resistant to oil and grease stain removal and difficult to dye. To increase hydrophilicity and improve the textile properties of PET, it was treated enzymatically with rSTE1. A significant reduction in hydrophobicity was observed after the enzyme treatments since contact angle and penetration time of water were clearly decreased. Similar effects have previously been shown by treatments with commercial *Pseudomonas* lipase and cutinase (Optimyze) preparations (Hsieh and Cram 1998; Miettinen-Oinonen et al. 2005). Improved hydrophilicity is most probably caused by hydrolysis of ester bonds in the polyester backbone, leading to increase of polar carboxyl and hydroxyl groups on the surface of PET. The increased polarity on the surface enables polar interaction and hydrogen bonding with water molecules, and thus, increases the water wettability of the fibres (Hsieh and Cram 1998). Treatment of PET by rSTE1 also improved binding of methylene blue dye on the polyester surface. Improved dyeing of PET with a cationic dye has previously been demonstrated by treatments with polyesterase and cutinase, and is explained by increased

amounts of the carboxylic groups in the fabric (Yoon et al. 2002; Miettinen-Oinonen et al. 2005). These results show that esterases not only have potential for the treatment of their natural substrates but that they are also interesting tools for the modification of synthetic materials. Further studies concerning the characteristics of *M. albomyces* steryl esterase will provide possibilities to improve its usefulness in the modification of materials containing ester linkages.

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Novel steryl esterases as biotechnological tools

Abstract

Steryl esterases are esterases which primarily hydrolyse steryl esters, but which are able to act on a wide range of esters. Steryl esterases and lipases capable of modifying different types of esters have potential applications in the food, textile, and pulp and paper industries. In this work novel steryl esterases were characterised and their usefulness in modification of fibre products was preliminarily evaluated.

Commercial lipase preparations were tested for their ability to degrade steryl esters. Lipases from *Candida rugosa*, *Chromobacterium viscosum*, and *Pseudomonas* sp. were able to hydrolyse steryl esters efficiently in the presence of surfactant. Up to 80–90% of the steryl esters were also degraded by lipases from *C. rugosa* and *Pseudomonas* sp. in the absence of surfactant. The steryl esterase of *C. rugosa* lipase preparation was purified and identified as the lipase LIP3 that was found to be highly active against plant-derived steryl esters.

A novel steryl esterase from filamentous fungus *Melanocarpus albomyces* was purified and biochemically characterised. The enzyme had broad substrate specificity for different steryl esters, *p*-nitrophenyl esters and triglycerides. The steryl esterase had a pH optimum at slightly acidic pH with various esters and it was shown to have good thermostability. The enzyme was highly active on steryl esters and triglycerides in the presence of surfactant, whereas only triglycerides were degraded in the absence of surfactant.

The gene encoding *M. albomyces* steryl esterase was isolated in order to express the protein heterologously in *Pichia pastoris* and *Trichoderma reesei*. The amino acid sequence of the enzyme appeared to be significantly related to other lipases and esterases. The production level in *P. pastoris* was very low, and a significant proportion of the total activity was found to be present intracellularly. *T. reesei* produced a clearly higher amount of steryl esterase and a production level of 280 mg l^{-1} was achieved in a laboratory-scale fermenter. The recombinant steryl esterase (rSTE1) was shown to be a dimer, whereas the native STE1 has a tetrameric structure. The native STE1 was somewhat more stable and had slightly higher activities against various substrates than the rSTE1.

The effects of rSTE1 on wood extractives, as well as on polyethylene terephthalate (PET) fabric, were preliminarily evaluated. The tensile strength and hydrophilicity of the paper were increased by rSTE1 due to the hydrolysis of esters. The rSTE1 treatment increased significantly the polarity of PET fabric. Improved textile properties of PET by steryl esterase treatment showed that esterases are interesting tools for the modification of synthetic materials in addition to their natural substrates.

Keywords

Enzyme, steryl esterase, lipase, *Melanocarpus albomyces*, *Candida rugosa*, characterisation, heterologous expression, *Trichoderma reesei*, steryl esters, wood extractives, polyester

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