Biol. Chem., Vol. 386, pp. 435–440, May 2005 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2005.052

Short Communication

An extracellular carboxylesterase from the basidiomycete *Pleurotus sapidus* hydrolyses xanthophyll esters

Holger Zorn[1,*], Henning Bouws[1], Meike Takenberg[1], Manfred Nimtz[2], Rita Getzlaff[2], Dietmar E. Breithaupt[3] and Ralf G. Berger[1]

[1] Zentrum Angewandte Chemie, Institut für Lebensmittelchemie der Universität Hannover, Wunstorfer Straße 14, D-30453 Hannover, Germany
[2] Department of Structural Biology GBF, German Research Centre for Biotechnology, Mascheroder Weg 1, D-38124 Braunschweig, Germany
[3] Universität Hohenheim, Institut für Lebensmittelchemie, Garbenstraße 28, D-70599 Stuttgart, Germany

* Corresponding author
e-mail: zorn@lci.uni-hannover.de

Abstract

An extracellular enzyme capable of efficient hydrolysis of xanthophyll esters was purified from culture supernatants of the basidiomycete *Pleurotus sapidus*. Under native conditions, the enzyme exhibited a molecular mass of 430 kDa, and SDS-PAGE data suggested a composition of eight identical subunits. Biochemical characterisation of the purified protein showed an isoelectric point of 4.5, and ideal hydrolysis conditions were observed at pH 5.8 and 40°C. Partial amino acid sequences were derived from N-terminal Edman degradation and from mass spectrometric *ab initio* sequencing of internal peptides. An 1861-bp cDNA containing an open reading frame of 1641 bp was cloned from a cDNA library that showed ca. 40% homology to *Candida rugosa* lipases. The *P. sapidus* carboxylesterase represents the first enzyme of the lipase/esterase family from a basidiomycetous fungus that has been characterised at the molecular level.

Keywords: carotenoids; cDNA library; fungi; lipase.

Due to their radical scavenging properties and preventive impact on certain features of age-related macular degeneration (Landrum and Bone, 2001), xanthophylls are currently attracting enormous interest as active compounds in so-called functional foods. In producer plants such as *Tagetes erecta* (marigold) or *Capsicum annuum* L. (paprika), xanthophylls typically occur esterified with fatty acids of varying chain length and degree of unsaturation (Breithaupt et al., 2002). Presuming improved bioavailability of free xanthophylls compared to xanthophyll esters (Hencken, 1992), the technological carotenoid work-up procedure usually includes a base-catalysed saponification step. To finally substitute chemical saponification by an enzymatic process, numerous commercial lipases were screened for their potential to hydrolyse xanthophyll esters in a recent study (Zorn et al., 2003). Amongst the enzymes tested, only lipases from *Candida* species were capable of at least partial ester hydrolysis. Surprisingly, highly effective cleavage of the ester bonds was observed with culture supernatants of the edible basidiomycete *Pleurotus sapidus*. Enormous efforts have been made to characterise the lignolytic enzyme systems of basidiomycetous fungi, but little has been learned about their lipolytic properties (Rajarathnam et al., 1998). Bio-

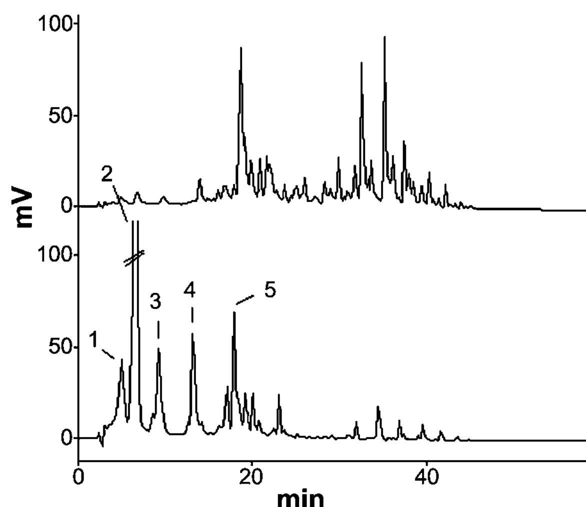

Figure 1 HPLC-DAD chromatograms (450 nm) of samples obtained by treatment of xanthophyll esters derived from *Capsicum annum* with heat-inactivated (top) or active (bottom) enzyme samples purified by FPLC.
Peak assignment: 1, (*Z*)-capsanthin; 2, all-(*E*)-capsanthin; 3, zeaxanthin; 4, β-cryptoxanthin; 5, all-(*E*)-β-carotene. Peaks between 20 and 45 min correspond to xanthophyll mono- and diesters. Identification of the xanthophylls was based on LC-MS data as reported by Breithaupt and Schwack (2000). Experimental conditions: 7.1 mg of paprika oleoresin, 1.2 g of Triton X-100 (Sigma, Deisenhofen, Germany), 8.8 ml of mineral salt medium, 0.2 ml of 1 M $CaCl_2 \cdot 2H_2O$ solution, and 448 μl of active (200 mU) or inactivated enzyme sample were mixed. The pH was adjusted to 6.8 with 1 M NaOH and the mixture was incubated overnight at 24°C and 150 rpm. The mixture was extracted with methanol/*n*-hexane/ethyl acetate (1:1:1 by volume), and the solvent was evaporated under reduced pressure (40°C, 20 kPa). The residue was dissolved in 5 ml of *tert*-butyl methyl ether/methanol/butylated hydroxytoluene (1:1:0.01 v/v/w) and subjected to HPLC analysis. Column, 120-5 RP-18, 250×4 mm, 5 μm (Macherey Nagel, Düren, Germany). The mobile phase consisted of mixtures of methanol/acetonitrile/dichloromethane/*n*-hexane (A, 10:85:2.5:2.5 and B, 10:45:22.5:22.5 by volume), starting with 100% A (5 min), followed by a gradient to obtain 100% B after 40 min and isocratic 100% B from 40 to 60 min at a flow rate of 1 ml min^{-1}. The injection volume was 20 μl.

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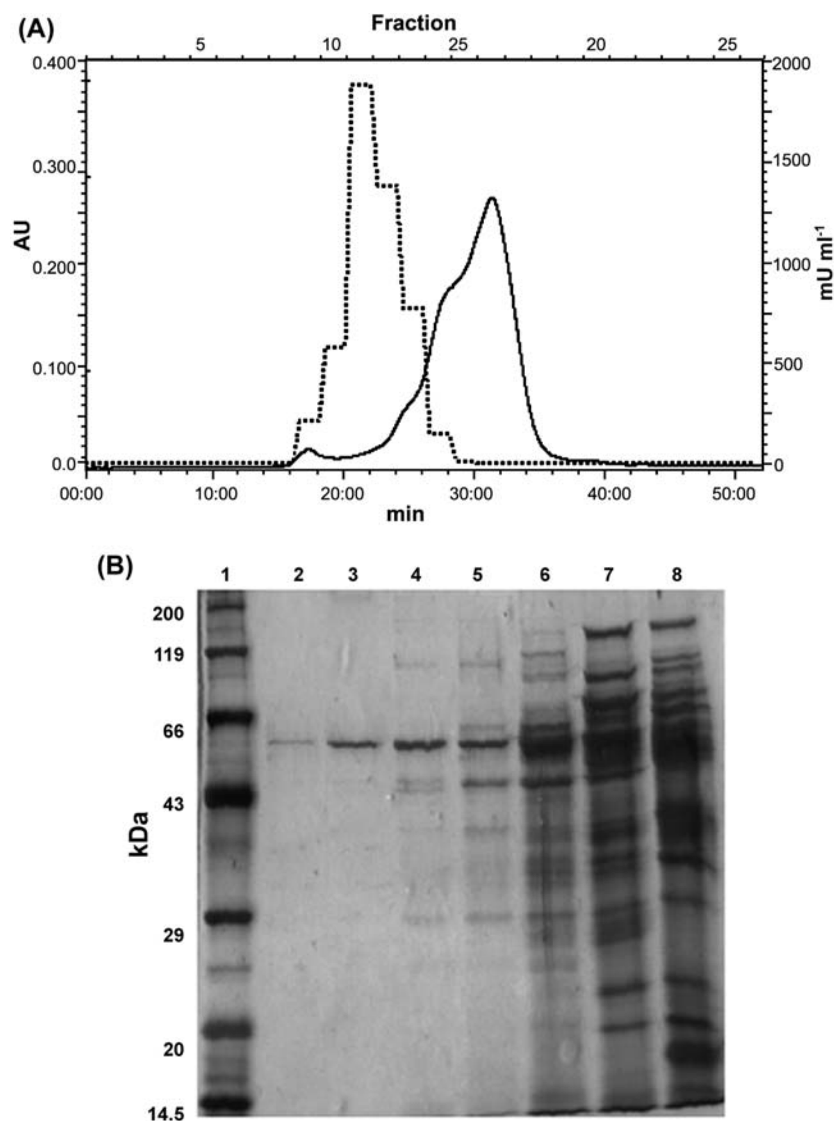

Figure 2 Purification of *P. sapidus* extracellular carboxylesterase.
(A) SEC elution profile of total protein (-) and esterolytic activity (…). Column, Superdex 200 10/600 GL (Amersham, Uppsala, Sweden); elution buffer, 20 mM Tris-HCl, 1 mM $CaCl_2$, pH 6.7; injection volume, 200 μl; flow rate, 0.5 ml min^{-1}; fraction volume, 1 ml.
(B) SDS-PAGE (12%) (Laemmli, 1979) of protein samples purified by ultrafiltration, IEX, and SEC. Lane 1, marker proteins; lanes 2–8, SEC fractions 9–15. Fraction 10 was used for amino acid sequencing by Edman degradation and ESI-tandem-MS.

chemical characteristics of extracellular lipases have been reported, e.g., for the basidiomycetes *Agaricus bisporus* (Holtz and Smith, 1978), *Bjerkandera adusta* (Gutierrez et al., 2002), *Pleurotus sajor caju* (Nair et al., 1990), and *Tyromyces sambuceus* (Hädrich-Meyer and Berger, 1994), but no basidiomycetous lipase/esterase has been cloned and sequenced to date. The aim of the present investigation was to purify and characterise the *P. sapidus* enzyme capable of xanthopyll ester hydrolysis from culture supernatants, and to isolate the corresponding cDNA from a cDNA library.

For enzyme production, *Pleurotus sapidus* (8266 DSMZ) was grown in a mineral salt medium as described by Zorn et al. (2003). Paprika oleoresin was added to the main cultures to induce enzyme secretion, and a photometric assay (Lipase-PS; Sigma Diagnostics, Deisenhofen, Germany) was used to monitor the formation of esterolytic activity in the culture broth. Maximum hydrolytic activity was detected on the third culture day. Thus, the mycelium was removed by centrifugation after a cultivation period of 48 h, and the supernatant was concentrated by ultra-filtration. A similar secretion profile has been reported for *Phanerochaete chrysosporium*, which produced lipase activity predominantly during the primary phase of growth until the fifth culture day (Asther et al., 1987). Ion exchange chromatography on a preparative DEAE column served as a first chromatographic purification step. The active fractions were pooled, concentrated, and subjected to size exclusion chromatography (SEC). By comparison to reference compounds, a molecular mass of 430 kDa was assigned to the fraction capable of xanthophyll ester hydrolysis. While nearly complete ester hydrolysis was achieved with this fraction, no saponification was observed with heat-inactivated samples and with fractions of lower molecular mass (Figure 1). Denaturing SDS-PAGE analysis revealed a single protein band corresponding to a molecular mass of 54.9 kDa (Figure 2). When the heating and reduction

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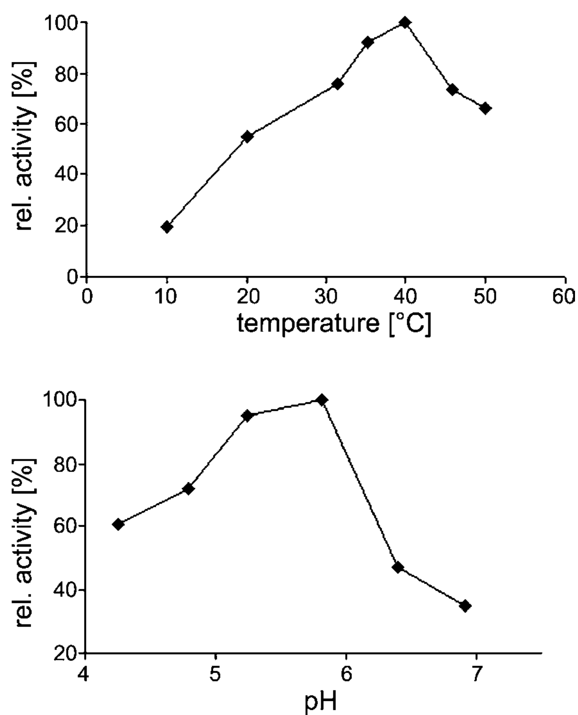

Figure 3 Temperature optimum and pH dependence of *P. sapidus* carboxylesterase.
For experimental conditions see Figure 1.

steps in sample preparation for SDS-PAGE were omitted, a molecular mass of 52.6 kDa was calculated. Considering the molecular mass of 430 kDa determined under native conditions by SEC, the active enzyme represents a homo-octamer. Lipases isolated from various yeast and lower fungi species (e.g., *Candida rugosa, Pythium ultimum*, and *Neurospora crassa*) have been described as oligomeric enzymes, typically comprising two, three, four, or six subunits (Saxena et al., 2003). Monomers associate facing their active-site regions, and thus create a hydrophobic cavity shielded from the solvent (Mancheno et al., 2003). Due to the high molecular weight of the native enzyme, agarose gels (IsoGel; Cambrex, East Rutherford, USA) were chosen for determination of the isoelectric point. The less restrictive matrix compared to polyacrylamide makes agarose gels especially suitable for the analysis of high-molecular-weight proteins. The samples were applied twice, on both sides of an agarose IEF gel, alongside marker proteins. For detection, the gel was cut concentrically, with one half subjected to Coomassie blue and the other to activity staining. Lipolytic activity was visualised by a Ca^{2+}- and Tween 80-containing agarose gel overlay (modified after Nuero et al., 1994). Clouding, attributed to the formation of poorly soluble calcium-fatty acid precipitates, indicated an isoelectric point of 4.5 for the *P. sapidus* xanthophyll ester hydrolase. Further biochemical characterisation of the purified enzyme comprised the determination of pH and temperature optima. Most efficient hydrolysis of paprika oleoresin derived xanthophyll esters occurred at 40°C and pH 5.8 (Figure 3). Optimum lipolytic activity in the acidic pH range has also been described for the basidiomycete *P. sajor caju*, a close relative of *P. sapidus* (Nair et al., 1990).

Edman sequencing of the purified enzyme yielded the N-terminus H_2N-(A)NSVTLDSATFTGTT(S)GRVTKF(D)(G)I, in which amino acids in parentheses were tentative calls. Additional sequence information from tryptic peptides was obtained from ESI-tandem-MS analyses, as indicated in Figure 4. To identify the genetic information of the new enzyme, total RNA was isolated from *P. sapidus* on the point of maximal specific activity, and a cDNA library was constructed in the λ vector TriplEx2 (Clontech, Palo Alto, USA). A polymerase chain reaction (PCR)-based approach was used to screen the library. Primer sets were designed according to the N-terminus and according to degenerate reverse-translated internal peptides. By means of primer walking, an 1861-bp cDNA was cloned and sequenced. The sequence obtained contains an open reading frame (ORF) of 1641 bp, corresponding to a protein of 546 amino acids. A 23-aa signal peptide precedes the N-terminus of the enzyme, and a molecular weight of 56.7 kDa was calculated for the mature protein. These data are in good agreement with those obtained from SDS-PAGE analysis. Sequence similarity searches against public databases (Pearson and Lipman, 1988; Gish, 2004) returned lipases of the yeast *Candida rugosa* as the best matches, thus confirming recent findings (Zorn et al., 2003). Table 1 compares biochemical characteristics of the *P. sapidus* carboxylesterase to those of related enzymes of the type B carboxylesterase/lipase family. X-ray structures are available for EstA from *Aspergillus niger* (Bourne et al., 2004), *C. rugosa* lipase 1 (Grochulski et al., 1994), lipase 3 (Ghosh et al., 1995), and lipase 2 (Mancheno et al., 2003), which exhibited the highest degree of sequence homology to the *P. sapidus* enzyme (43% identity). As the sequence alignment identified the *P. sapidus* enzyme as a new member of the type B carboxylesterase/lipase family (EC 3.1.1.X), a 3-D model was calculated using the X-ray structure of *C. rugosa* lipase 2 as a template (Guex and Peitsch, 1997; Schwede et al., 2003) (Figure 5). The protein scaffold is characterised by an 11-stranded mixed β-sheet, 16 helical structure elements, and a three-stranded N-terminal

Table 1 Comparison of *P. sapidus* extracellular carboxylesterase with related type B carboxylesterase/lipase family enzymes.

Organism	Enzyme	Accession number	M (kDa)	Subunits	AA per subunit	pI	Reference
Pleurotus sapidus	Carboxylesterase	Q5W281	430	8	523	4.5	This paper
Candida rugosa	Lipase 3	P32947	60, 120	1 and 2	534	4.9	Ghosh et al., 1995; Kaiser et al., 1994
Geotrichum candidum	Lipase 2	P22394	66	1	544	4.3	Sugihara et al., 1990
Aspergillus niger	EstA	Q6ED33	57	1	522	4.7	Bourne et al., 2004

AA per subunit, amino acids in the mature protein per subunit.

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P.s.  MFFVLQAVLGLFAIQTAATPLPRANSVTLDSATFTGTTSGRVTKFLGIPYAQPPTGDRRF 60
C.r.  ------MKLCLLALGAAVAAAPTATLANGD--TITGLNAIVNEKFLGIPFAEPPVGTLRF 52
              * *:*: :*.:. * *. .. *  *:** .:    ******:*:**.*  **

P.s.  RLPEPIPPYTGTVRATAFGPACPQQSARLPLPDGLASDVVDLIVN-TAYKAVFPDNEDCL 119
C.r.  KPPVPYSASLNGQQFTSYGPSCMQMNPMGSFEDTLPKNARHLVLQSKIFQVVLPNDEDCL 112
      : * * ..   .  : *::**:* * ..  .: * *..:. .*::: . ::.*:*::****

P.s.  SINVVVPTSATPTSKLPVAVWIFGGGFELGSPSLYDGGLIVERSIQLGEPVIYVSMNYRL 179
C.r.  TINVIRPPGTRASAGLPVMLWIFGGGFELGGSSLFPGDQMVAKSVLMGKPVIHVSMNYRV 172
      :***: *..: .:: *** :**********..**: *. :* :*: :*:***:******:

P.s.  SAFGFLASQEVKDTGVGNLGLQDQREALRWIQKYISSFGGDPTKVTIWGESAGAISVALH 239
C.r.  ASWGFLAGPDIQNEGSGNAGLHDQRLAMQWVADNIAGFGGDPSKVTIYGESAGSMSTFVH 232
      :::****. :::: * ** **:*** *::*: . *:.*****:****:*****::*. :*
                                                    ↑
P.s.  MVANNG----NHEGLFRGAFMQSGSPIPVG--DISHGQTYYDAIAAETGCSSAADTLACL 293
C.r.  LVWNDGDNTYNGKPLFRAAIMQSGCMVPSDPVDGTYGTEIYNQVVASAGCGSASDKLACL 292
      :* *:*    * : ***.*:****. :* .  * ::*   *: :.*.:**.**:*.****

P.s.  RSVPYATLKTAVDRTPFIFDYQSLALARIPRADGVFLTDNPQRLVQAGKVANVPFVTGDC 353
C.r.  RGLSQDTLYQATSDTPGVLAYPSLRLSYLPRPDGTFITDDMYALVRDGKYAHVPVIIGDQ 352
      *.:.  **  *.. ** :: * ** *: :**.**.*:**:   **: ** *:**.: **

P.s.  DDEGTLFSLANLNVTTTSQVRTYIKTFFMPQSTNAELDQMLNHYPLDLTQGSPFDTGILN 413
C.r.  NDEGTLFGLSSLNVTTDAQARAYFKQSFIHAS-DAEIDTLMAAYTSDITQGSPFDTGIFN 411
      :******.*:.***** :*.*:*:*  *:  * :**:* ::  *. *:**********:*
        ↑
P.s.  ALSPQFKRLAAFQGDAVFQAPRRFFLQQRSGKQNTWAFLSKRFKVAPFLGSFHASDILNV 473
C.r.  AITPQFKRISALLGDLAFTLARRYFLNYYQG-GTKYSFLSKQLSGLPVLGTFHGNDIIWQ 470
      *::*****::*: ** .*  .**:**:  .*  ..::****::.  *.**:**..**:
                                                      ↑
P.s.  YFGGELGDYLIN-----FVNNLDPNGQGRGINWPKYTTSSPNLVTFNDNLLFPVTITQDT 528
C.r.  DYLVGSGSVIYNNAFIAFANDLDPNKAGLWTNWPTYTSSSQSGNNLMQINGLGLYTGKDN 530
      :    *. : *     *.*:****  *   ***.**:** .  .: :   : :   :*.

P.s.  FRTDAINFLTGVTLANPL 546
C.r.  FRPDAYSALFSNPPSFFV 548
      **.** . * . . :  :
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Figure 4 Alignment of the translated nucleotide sequence of the *P. sapidus* carboxylesterase gene with the *Candida rugosa* lipase 2 precursor.

EMBL database accession number of the *P. sapidus* cDNA sequence, AJ810032; accession number of the *C. rugosa* sequence, P32946. The sequence alignment was performed with ClustalW (Higgins et al., 1994). The N-terminal sequence of the mature *P. sapidus* protein (determined by Edman degradation) is printed in bold; peptides sequenced by ESI-tandem-MS are underlined. A potential N-glycosylation site is depicted in italics, and active site residues are marked by arrows. For *ab initio* sequencing of tryptic peptides, the protein band was excised from a Coomassie-stained SDS-PAGE gel and digested with trypsin. For collision-induced dissociation experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyser into the collision cell. The resulting daughter ions were separated by an orthogonal time-of-flight mass analyser, and the MS spectra acquired were enhanced and used for the *ab initio* sequencing of tryptic peptides.

β-sheet. Besides *C. rugosa* lipases, this class of evolutionarily related hydrolases includes cholinesterases, mammalian liver microsomal carboxylesterases, and mammalian bile-salt-activated lipases (Lockridge, 1988; Cygler et al., 1993; Wang and Hartsuck, 1993). All members of this family with catalytic activity share the consensus sequence F-[GR]-G-x(4)-[LIVM]-x-[LIV]-x-G-x-S-[STAG]-G, where S is the active site residue of the serine esterases (Falquet et al., 2002). As a second signature pattern, the conserved region [ED]-D-C-L-[YT]-[LIV]-[DNS]-[LIV]-[LIVFYW]-x-[PQR], located in the N-terminal section, contains a cysteine involved in the formation of a disulfide bond. Different from the other members of this family, a serine residue replaces the tryptophan or threonine in position 5 of this motif (Figure 4). A putative N-glycosylation site [NVT] is located at position 366–368 (Combet et al., 2000). While the overall α/β-hydrolase fold and the side chains directly involved in the catalytic machinery are highly conserved (Benjamin and Pandey, 1998; Ollis et al., 1992), significant differences in the assumed flap region, the substrate binding pocket, and the hydrophobic tunnel distinguish the *P. sapidus* enzyme from other carboxylesterase B/lipase type enzymes. The proposed hinge point for the opening of the flap in *C. rugosa* lipases (M80 in lip2, E80 in lip1 and lip3) is replaced by an arginine residue (R88) in *P. sapidus*, which may result in different functional properties of the flap. In the contact region between the flap and the substrate binding pocket, aromatic amino-acid side chains (F83 in lip2 and lip3, W83 in lip4, Y83 in lip1) stabilise the flap structure via ring-stacking interactions and van der Waals' contacts in *Candida* lipases. Replacement of this aromatic side chain by an aliphatic leucine residue in *P. sapidus* could cause reduced flap stability. For *C. rugosa* lipases, it has been reported that residue 464 modulates the enzyme specificity for triacylglycerol esters (G464 in lip2) or cholesteryl esters (A464 in lip3) (Ghosh et al., 1995). An alanine (A467) in the *P. sapidus* carboxyl esterase indicates a closer relationship of the new enzyme to cholesteryl esterases. In both cholesteryl and xanthophyll

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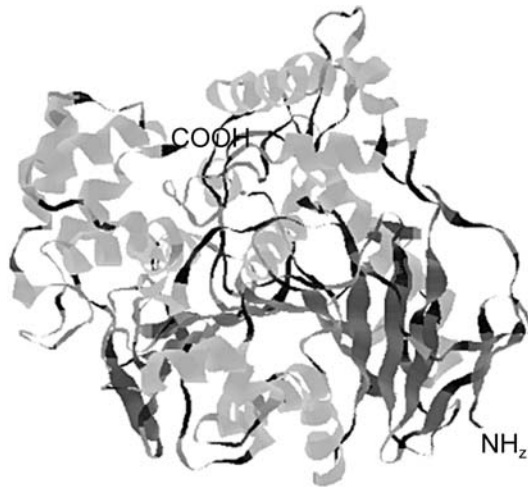

Figure 5 Ribbon presentation of the *P. sapidus* carboxylesterase generated with SWISS-MODEL (Guex and Peitsch, 1997; Schwede et al., 2003).

esters, a secondary hydroxyl function attached to a cyclohex(en)yl moiety has to be released from the ester bond.

Future biochemical investigations, especially elucidation of the 3-D structure by X-ray analysis, will help better understanding of the different catalytic properties of the *P. sapidus* carboxylesterase. Evaluation of the substrate spectrum of the new enzyme in hydrolytic and synthesis reactions may broaden the wide field of applications of microbial lipases in biotechnology (Pandey et al., 1999). Although numerous lipases and esterases have been cloned and sequenced from a wide variety of organisms, this is the first representative from a basidiomycetous species that has been characterised at the molecular level.

Acknowledgments

This project is part of the joint initiative ‘Biologisch aktive Naturstoffe – Chemische Diversität’ at the University of Hannover, Germany.

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Received February 9, 2005; accepted March 22, 2005

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