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Heterologous expression of an extra-cellular lipase from the basidiomycete *Pleurotus sapidus*

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ABSTRACT

The 1641 bp cDNA encoding an extra-cellular lipase of the basidiomycete *Pleurotus sapidus* (Lip2) was cloned from a cDNA library. Expression of the cDNA in *Escherichia coli*, with and without signal sequence, led to the production of recombinant Lip2, mainly as inclusion bodies with low catalytic activity. Refolding yielded catalytically active protein. A C-terminal His tag was used for purification and immunochemical detection. The recombinant lipase hydrolysed xanthophyll esters with high efficiency, and omitting the signal sequence did not alter the catalytic properties. The *P. sapidus* lipase represents the first enzyme of the lipase/esterase family from a basidiomycetous fungus characterised on the molecular level and expressed in a manageable host.

1. Introduction

Lipases (EC 3.1.1.3) accept biotic and abiotic substrates and may transform them in water, organic solvents, or in ionic liquids to products of industrial importance [1]. However, only few enzymes are have become known to catalyse xanthophyll ester hydrolysis [2–4]. Xanthophylls are widely used as colorants and antioxidants in the feed, food, pharmaceutical, and cosmetics industries. Most of the naturally occurring xanthophylls are present as mono- and diesters of fatty acids in plant sources, such as *Tagetes erecta* (marigold) and *Capsicum annuum* (paprika) [5]. The conventional method to liberate free xanthophylls from their ester precursors is saponification by hot (80 °C) and concentrated sodium hydroxide solution. This process results in the formation of side products, causes high energy costs, and imposes high requirements concerning safety issues and waste disposal. Enzyme technology could provide a green process alternative.

Screening basidiomycete species, an extra-cellular lipase from *Pleurotus sapidus* (Lip2) with the distinct property to convert xanthophyll esters into free xanthophylls was found, purified and sequenced [6]. The novel enzyme represented an octamer with a molecular mass of 430 kDa, an isoelectric point of 4.5, and worked best at pH 5.8 and 40 °C. Its active site contained the well-known Ser-Glu-His catalytic triad. A 23-aa signal peptide preceded the N-terminus of the enzyme [6] (EMBL accession number AJ810032). This paper reports on the purification, refolding and characterisation of Lip2 expressed in *E. coli*.

2. Materials and methods

2.1. Cultivation and lipase production

Mycelia of *P. sapidus* [8266 DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen)] were maintained as described previously [7].

2.2. RNA purification and cDNA library construction

Mycelium of the submerged culture (5th culture day) was separated via centrifugation (9000 × *g*, 15 min, 4 °C). To purify RNA, 100 mg of mycelium were chopped off with a scalpel, and ground in a mortar under liquid nitrogen. Total RNA was extracted with the RNeasy-Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. A cDNA library was constructed using the RNA as template with the SMART PCR cDNA Library Construction Kit (CLONTECH, Saint-Germain-en-Laye, France).

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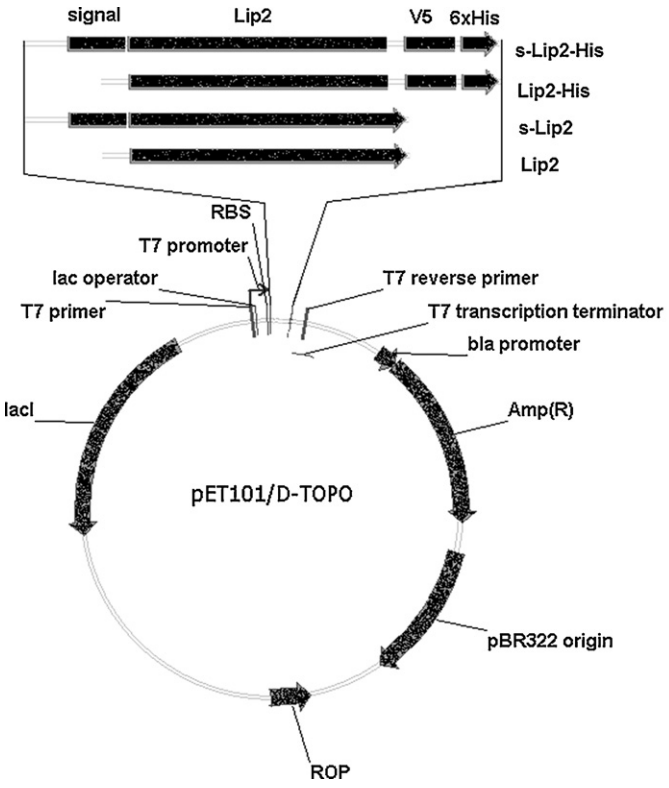

Fig. 1. Plasmid for the expression of Lip2 variants. A pET101/D-TOPO® vector was used for construction of the different variants. Expression of the gene of interest was under the control of the T7 promoter for a high-level, IPTG-inducible expression [22,23]. The vector contained C-terminal fusion tags, a V-5 epitope and a polyhistidine tag for detection and purification of recombinant fusion proteins. An ampicillin resistance gene was used for selection.

2.3. Cloning of expression plasmids

The plasmid pET101/D-TOPO (Invitrogen, Karlsruhe, Germany) with a strong RBS and a C-terminal His tag was used (Fig. 1). Four different constructs of Lip2 were cloned. The *lip2* cDNA was amplified from a cDNA library using the following primers. For s-Lip2-His and s-Lip2 the forward primer included the signal peptide sequence of *lip2* (5′-CACCATGTTCTTCGTTCTACAGGC-3′). For Lip2-His and Lip2 the sequence of the signal peptide was omitted (5′-CACCATGGCAAACAGCGTAACGCTTG-3′). All forward primers contained the sequence CACC at the 5′ end of the primer, the overhang sequence of the vector used. For s-Lip2 and Lip2, the reverse primer included the stop codon TGA (5′-TCAAAGTGGATTTGCCAGAGT-3′). A reverse primer without stop codon (5′-AAGTGGATTTGCCAGAGTCAC-3′) was used to generate s-Lip2-His and Lip2-His. The PCR fragments were purified by gel electrophoresis and extracted from the gel using the Nucleospin Extract II Kit (Macherey-Nagel). The purified DNA was cloned into the pET101/D-TOPO plasmid *via* the TOPO cloning reaction, yielding the plasmids pET101s-*lip2*-His, pET101*lip2*-His, pET101s-*lip2* and pET101*lip2*.

2.4. Expression of Lip2 variants

E. coli BL21(DE3) (Invitrogen) was transformed with the four plasmids encoding the different variants of Lip2. Expression was performed at 27 or 37 °C and 250 rpm, using 50 ml of LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl) containing 50 mg ml^{-1} ampicillin. Expression was induced using 1 mM isopropyl-L-dithiogalactopyranosid (IPTG) at an OD_{600} of 1.5. The cells were harvested 4 h after induction by centrifugation at 6000 × *g* and 4 °C for 15 min.

2.5. DNA sequencing and alignment

The plasmid-DNA was sequenced by MWG-BIOTECH AG (Ebersberg, Germany) and aligned with clustalW [8] against the *lip2* cDNA sequence (AJ810032).

2.6. SDS-PAGE and Western blot analysis

Cell extracts were prepared using ice-cold lysis buffer (50 mM sodium phosphate, 300 mM NaCl, pH 8.0, 0.1% DNAse, 1 mg ml^{-1} lysozyme) for 30 min at 4 °C. After four freeze and thaw cycles the crude extract was centrifuged for 30 min at 12 000 × *g* and 4 °C. The supernatants as well as the pellets were mixed or re-suspended, respectively, in loading buffer (Laemmli loading dye). SDS-PAGE was performed according to Laemmli [9] using 4% (w/v) polyacrylamide in the stacking gels and 14% (w/v) polyacrylamide in the resolving gels. Proteins were stained with 0.1% (m/v) Coomassie Brillant Blue G-250 or silver [10]. PageRuler pre-stained protein ladder plus (FERMENTAS, St. Leon-Rot, Germany) was used for the preparation of a calibration curve for determination of molecular masses. After transferring the proteins to a nitrocellulose transfer membrane (Schleicher & Schuell, Dassel, Germany) the clones carrying the pET101s-*lip2*-His or the pET101*lip2*-His plasmid were immunoblotted using an anti-His(C-term)-AP antibody (Invitrogen).

2.7. Isoelectric focusing (IEF)

IEF polyacrylamide gel electrophoresis was performed on a Multiphor II system (Pharmacia LKB) using ServalytTM PrecotesTM precast gels with an immobilised pH gradient of pH 3–6 (Serva, Heidelberg, Germany). The p*I* of the lipase was estimated using a low-p*I* (2.8–6.5) calibration kit (Amersham Biosciences, Freiburg, Germany). Gels were silver stained [10]. For activity staining the Lipase overlay assay (Section 2.9) was used.

2.8. Refolding and purification

Cell extracts were prepared with ice-cold lysis buffer as described under Section 2.6. The inclusion bodies were pelleted by centrifugation (12 000 × *g*). After washing with 0.5% (v/v) Triton X-100, the inclusion bodies were dissolved in 20 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 8 M urea, 1 mM EDTA and 10 mM dithiothreitol (DTT). After an incubation time of 1 h at room temperature, the suspension was diluted to 200 ml with 20 mM Tris–HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ and 1 mM EDTA and incubated for 10 days at 4 °C [11]. For protein purification under native conditions the Ni-NTA Spin Kit (QIAGEN) was used.

2.9. Lipase overlay assay

60 ml of a solution containing 0.008 g Tris–HCl and 0.025 g NaCl in 100 ml H_2O and 1.2 g agar agar were heated to 100 °C for 1 min (modified after Nuero et al. [12]). After cooling to about 65 °C, 60 ml of a solution containing 1 g polyoxyethylene (20) sorbitan monooleate (Tween 80) and 0.02 g $CaCl_2$ in 100 ml H_2O (pH adjusted to 6.8 with 0.1N NaOH) were added. The IEF gel was covered with the mixture and after incubation at 35 °C over night the plates were covered with 0.1N NaOH. Formation of a whitish precipitate indicated active fractions.

```
A        1 MFFVLQAVLGLFAIQTAATPLPRANSVTLDSATFTGTTSGRVTKFLGIPYAQPPTGDRRF
B        1 MFFVLQAVLGLFAIQTAATPLPRANSVTLDSATFTGTTSGRVTKFLGIPYAQPPTGDRRF
           ************************************************************

A       61 RLPEPIPPYTGTVRATAFGPACPQQSARLPLPDGLASDVVDLIVNTAYKAVFPDNEDCLS
B       61 RLPELIPPYTGTVRATAFGPACPQQSARLPLPDGLASDVVDLIVNTAYKAVFPDNEDCLS
           **** ********************************************************

A      121 INVVVPTSATPTSKLPVAVWIFGGGFELGSPSLYDGGLIVERSIQLGEPVIYVSMNYRLS
B      121 INVVVPTSATPTSKLPVAVWIFGGGFELGSPSLYDGGLIVERSIQLGEPVIYVSMNYRLS
           ************************************************************
                                                                    ↓
A      181 AFGFLASQEVKDTGVGNLGLQDQREALRWIQKYISSFGGDPTKVTIWGESAGAISVALHM
B      181 AFGFLASQEVKDAGVGNLGLQDQREALRWIQKYISSFGGDPTKVTIWGESAGAISVALHM
           ************ ********************************************

A      241 VANNGNHEGLFRGAFMQSGSPIPVGDISHGQTYYDAIAAETGCSSAADTLACLRSVPYAT
B      241 VANNGNHEGLFRGAFMQSGSPIPVGDISHGQTYYDAIAAETGCSSAADTLACLRSVPYAT
           ************************************************************
                                                                  ↓
A      301 LKTAVDRTPFIFDYQSLALARIPRADGVFLTDNPQRLVQAGKVANVPFVTGDCDDEGTLF
B      301 LKTAVDRTPFIFDYQSLALAWIPRADGVFLTDNPQRLVQAGKVANVPFVTGDCDDEGTLF
           ******************* ****************************************

A      361 SLANLNVTTTSQVRTYIKTFFMPQSTNAELDQMLNHYPLDLTQGSPFDTGILNALSPQFK
B      361 SLANLNVTTTSQVRTYIKTFFMPQSTNAELDQMLNHYPLDLTQGSPFDTGILNALSPQFK
           ************************************************************
                                                              ↓
A      421 RLAAFQGDAVFQAPRRFFLQQRSGKQNTWAFLSKRFKVAPFLGSFHASDILNVYFGGELG
B      421 RLAAFQGDAVFQAPRRFFLQQRSGKQNTWAFLSKRFKVAPFLGSFHASDILNVYFGGELG
           ************************************************************

A      481 DYLINFVNNLDPNGQGRGINWPKYTTSSPNLVTFNDNLLFPVTITQDTFRTDAINFLTGV
B      481 DYLINFANNLDPNGQGRGINWPKYTTSSPNLVTFNDNLLFPVTITQDTFRTDAINFLTSV
           ****** ***************************************************** *

A      541 TLANPL
B      541 TLANPL
           ******
```

Fig. 2. Alignment of the translated nucleotide sequence of the cloned *P. sapidus* lipase gene *lip2* (B) with the amino acid sequence of Lip2 (EMBL accession number CAH17527) (A). The sequence alignment was performed with ClustalW [8]. The N-terminal sequence of the mature *P. sapidus* protein is printed in bold. Active site residues are marked. Sequences of *lip2* are equal except for the underlined signal peptid in all four constructs and united in sequence (B).

2.10. p-NPP lipase activity assay

Lipase activity was determined using *p*-nitrophenyl palmitate (*p*-NPP) [13]. Solution A contained 30 mg *p*-NPP (Sigma) dissolved in 10 ml propane-2-ol. Solution B contained 0.207 g sodium deoxycholate (Sigma) and 0.1 g gum arabic (Sigma) dissolved in 90 ml 0.2 M Tris/HCl buffer (pH 7.5). Solution A was added in small quantities to Solution B while stirring continuously until complete dissolution. This substrate solution was prepared daily, pre-warmed to 37 °C, and dispensed in 200 μl quantities into test plates. Absorbance of duplicates was measured at 410 nm against an appropriate blank.

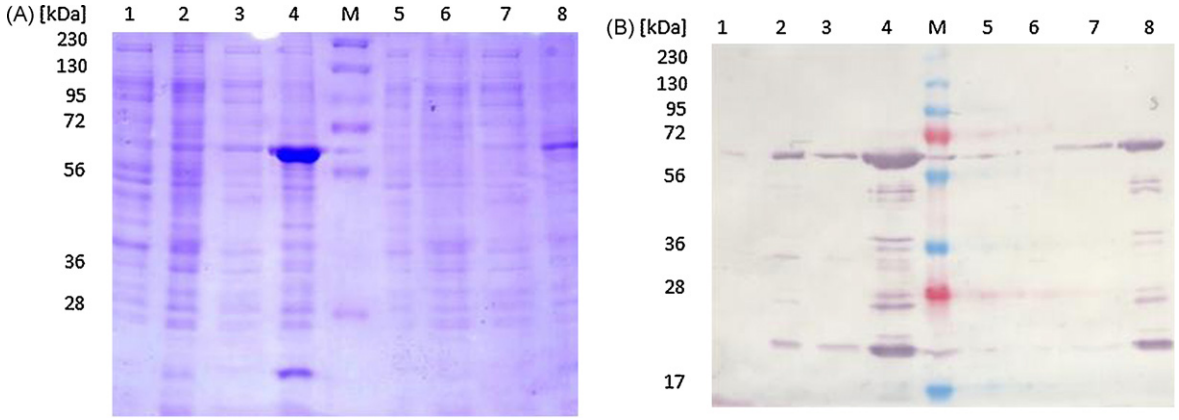

Fig. 3. Expression of Lip2 variants containing a His tag in *E. coli* BL21(DE3): Coomassie blue stained SDS-PAGE gel (A) and Anti-His blot (B). *E. coli* bearing Lip2-His (1: not induced, soluble; 2: not induced, insoluble; 3: induced, soluble; 4: induced, insoluble) and *E. coli* bearing s-Lip2-His (5: not induced, soluble; 6: not induced, insoluble; 7: induced, soluble; 8: induced, insoluble) were cultivated over night. Recombinant protein production was induced using IPTG for a cultivation time of 4 h.

2.11. Xanthophyll ester cleaving activity

The hydrolytic activity of the recombinant protein was tested by HPLC-DAD analysis using a xanthophyll ester isolate of *Capsicum annum* [6].

2.12. Protein concentration

The protein concentration of duplicate samples was estimated by the method of Lowry et al. [14] using *DC*-Protein-Assay (Bio-Rad, Hercules, USA) and bovine serum albumin as a standard. In the range used the calibration curve was linear with a regression coefficient of $R^2 = 0.998$.

2.13. MALDI peptide mapping

Protein bands separated by 1D SDS-PAGE were excised, washed, reduced and carboxamidomethlyated, and tryptically digested. The resulting peptides were extracted, desalted and analyzed on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) as described [15]. The peptide fingerprints were then subjected to database searches (NCBI, Fungi) using the Mascot system.

3. Results

Lip2 is an extra-cellular protein containing a 23 amino acid signal peptide, which targets the protein to the extra-cellular compartment of *P. sapidus*. As nothing is known about the processing of basidiomycetous pro-proteins and about the influence of the signal sequence on the correct protein folding in *E. coli*, two different constructs of the N-terminus were cloned as described (Section 2.3): s-Lip2-His and s-Lip2 containing the complete pro-protein, and Lip2 and Lip2-His missing the signal sequence. The constructs s-Lip2-His and Lip2-His were cloned with a C-terminal His tag to facilitate detection and purification. S-Lip2 and Lip2 were cloned with the native stop codon to compare their activities with those of the two hybrid proteins.

The plasmid-DNA of the clones was sequenced and compared with the published sequence of Lip2 (EMBL accession number CAH17527) (Fig. 2). The same five differences were detected on the protein level in all four constructs. These differences are supposed not to result from amplification mistakes, but from an isoenzyme sequence. The supernatant of the *P. sapidus* strain, which was used as source for the construction of the cDNA library, showed good activity against paprika oleoresin indicating that the sequence to be amplified should code for a functional protein. Cell lysates of all *E. coli* clones showed a very low activity against *p*-NPP as substrate when incubated at 27 or 37 °C. IEF analysis with activity overlay showed lipase activity at the application point only. The soluble and insoluble fractions were analysed by SDS-PAGE (Fig. 3A), and the s-Lip2-His and Lip2-His clones were also blotted and detected by anti-His-antibodies (Fig. 3B). SDS-PAGE and Western blot showed one strong band with the expected size in the induced insoluble fraction in both s-Lip2-His (63 kDa) and Lip2-His (60 kDa). Some weaker bands

(A)
1 MFFVLQAVLG LFAIQTAATP LPR**ANSVTLD SATFTGTTSG RVTKFLGIPY**
51 **AQPPTGDRR**F R**LPEPIPPYT GTVRATAFGP ACPQQSAR**LP LPDGLASDVV
101 DLIVNTAYKA VFPDNEDCLS INVVVPTSAT PTSKLPVAVW IFGGGFELGS
151 PSLYDGGLIV ER**SIQLGEPV IYVSMNYRLS AFGFLASQEV KDTGVGNLGL**
201 **QDQR**EALRWI QK**YISSFGGD PTK**VTIWGES AGAISVALHM VANNGNHEGL
251 FRGAFMQSGS PIPVGDISHG QTYYDAIAAE TGCSSAADTL ACLRSVPYAT
301 LKTAVDR**TPF IFDYQSLALA RIPRADGVFL TDNPQR**LVQA GK**VANVPFVT**
351 **GDCDDEGTLF SLANLNVTTT SQVR**TYIKTF FMPQSTNAEL DQMLNHYPLD
401 LTQGSPFDTG ILNALSPQFK R**LAAFQGDAV FQAPRR**FFLQ QRSGK**QNTWA**
451 **FLSK**RFKVAP FLGSFHASDI LNVYFGGELG DYLINFVNNL DPNGQGRGIN
501 WPKYTTSSPN LVTFNDNLLF PVTITQDTFR TDAINFLTGV TLANPLKGEL
550 RGHPFEGKPI PNPLLGLDST RTGHHHHHH

(B)
1 MADL**ANSVTL DSATFTGTTS GR**VTK**FLGIP YAQPPTGDRR** FR**LPEPIPPY**
51 **TGTVRATAFG PACPQQSAR**L PLPDGLASDV VDLIVNTAYK AVFPDNEDCL
101 SINVVVPTSA TPTSKLPVAV WIFGGGFELG SPSLYDGGLI VER**SIQLGEP**
151 **VIYVSMNYRL SAFGFLASQE VKDTGVGNLG LQDQR**EALRW IQKYISSFGG
201 DPTKVTIWGE SAGAISVALH MVANNGNHEG LFRGAFMQSG SPIPVGDISH
251 GQTYYDAIAA ETGCSSAADT LACLRSVPYA TLKTAVDR**TP FIFDYQSLAL**
301 **ARIPRADGVF LTDNPQR**LVQ AGK**VANVPFV TGDCDDEGTL FSLANLNVTT**
351 **TSQVR**TYIKT FFMPQSTNAE LDQMLNHYPL DLTQGSPFDT GILNALSPQF
401 KR**LAAFQGDA VFQAPRRFFL QQR**SGK**QNTW AFLSK**RFKVA PFLGSFHASD
451 ILNVYFGGEL GDYLINFVNN LDPNGQGRGI NWPK**YTTSSP NLVTFNDNLL**
501 **FPVTITQDTF R**TDAINFLTG VTLANPLKGE LRGHPFEGKP IPNPLLGLDS
551 TRTGHHHHHH

Fig. 4. MALDI-MS peptide mapping of s-Lip2-His (A) and Lip2-His (B): peptide sequences which fit to the translated nucleotid sequence of the cloned *Pleurotus sapidus* lipase genes *s-lip2-His* (A) and *lip2-His* (B) are printed bold. Differences to the published sequence of Lip2 (EMBL accession number CAH17527) are underlined.

may represent partially hydrolysed proteins with an intact C-terminus.

The bands with the expected size were excised from the SDS-gel and analysed by MALDI-mapping. The data showed similarity to the translated plasmid-DNA sequence of *lip2* and reinforced three sequence differences to the published sequence of Lip2 (Fig. 4). The variants with the signal sequence (s-Lip2-His) were larger than the construct without (Lip2-His), thus, the pro-peptide of s-Lip2-His was not cleaved in *E. coli*. No expression was detected by SDS-PAGE for the clones S-Lip2 and Lip2, neither in the insoluble nor in the soluble fraction (data not shown). In s-Lip2-His and Lip2-His most of Lip2 was expressed as inclusion bodies.

The protein located in the insoluble fraction was refolded as described (Section 2.8). Contaminants of the insoluble enzyme fraction of s-Lip2-His and Lip2-His were removed using a Ni^{2+}-NTA column (Fig. 5). The refolded fractions of the clones s-Lip2-His and Lip2-His showed activity against *p*-NPP (Table 1), while those of the clones s-Lip2 and Lip2 were inactive. HPLC-DAD analyses of xanthophyll esters from *C. annum* incubated with recombinant and refolded s-Lip2-His or Lip2-His showed the same product spectrum as was obtained with native Lip2 (Fig. 6). As with the native enzyme from *P. sapidus* supernatant (Zorn et al., 2005), the chromatograms were dominated by the end product of hydrolysis, capsanthin, while the concentrations of mono- and diesters were significantly decreased.

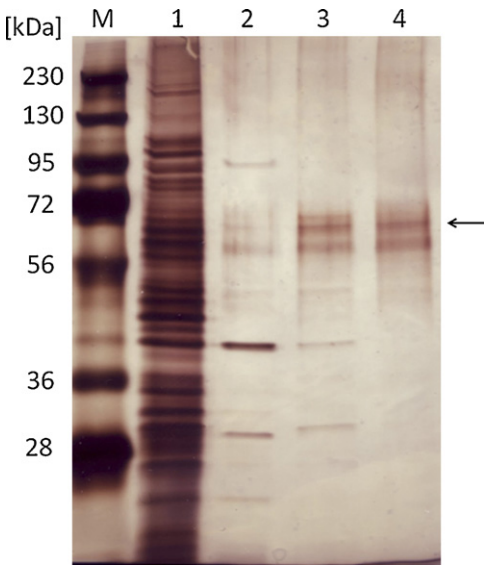

Fig. 5. Purification of Lip2-His with a Ni^{2+}-NTA column: 1: insoluble enzyme fraction after refolding; 2: flow through; 3: washing with 40 mM imidazole, 4: elution with 250 mM imidazole, silver stained SDS-PAGE gel.

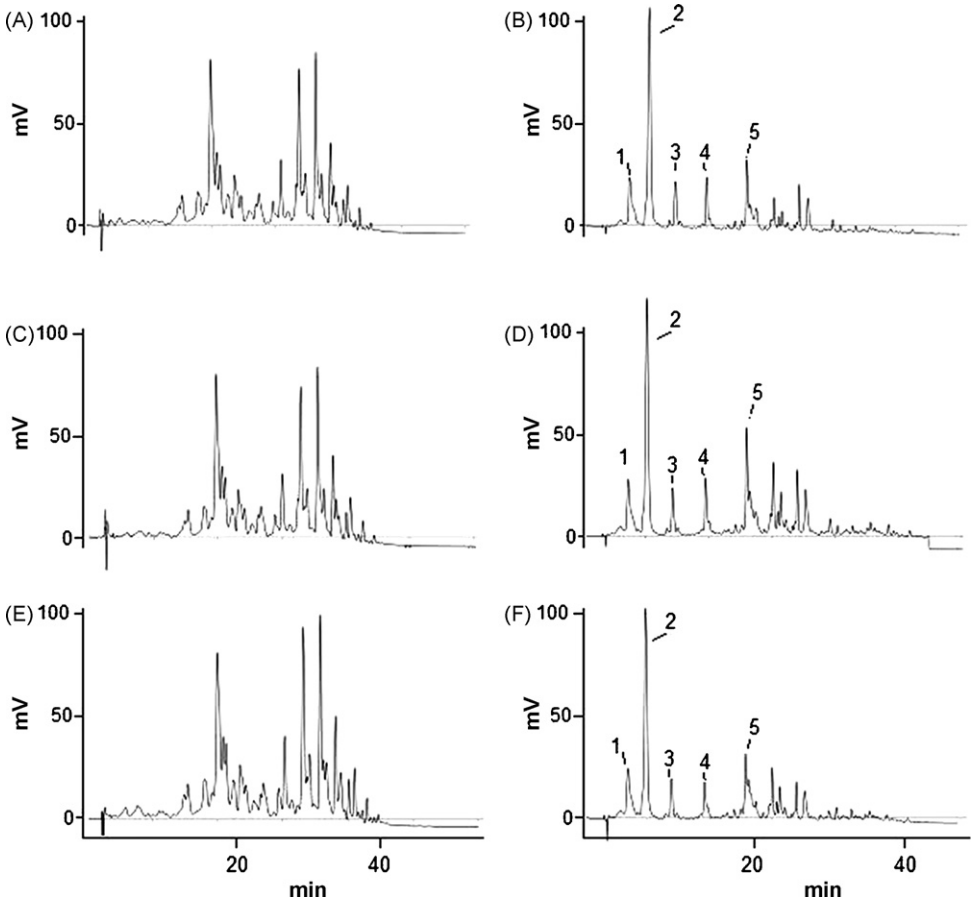

Fig. 6. HPLC-DAD chromatograms (450 nm) obtained with refolded s-Lip2 (A), refolded s-Lip2-His (B), refolded Lip2 (C), refolded Lip2-His (D), inactivated wild-type Lip2 (E) and wild-type Lip2 (F) used to hydrolyze xanthophyll esters derived from *Capsicum annum*. Peak assignment: 1 (*Z*)-capsanthin; 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. Reactivated or inactivated enzyme was mixed with the assay substances as described by Zorn et al. [6].

Table 1
Specific activity of recombinant Lip2 variants

	Specific activity *p*-NPP[a] (U/mg(mU/ml culture))	Hydrolysis of xanthophyll esters[b]
s-Lip2	0 (0)	–
s-Lip2-His	31.4 ± 0.9 (111.0 ± 3.3)	+
Lip2	0 (0)	–
Lip2-His	30.0 ± 2.9 (116.6 ± 11.3)	+

[a] Activity was measured in duplicate after refolding of the protein with *p*-NPP as substrate.

[b] Ability to hydrolyse xanthophyll esters detected after refolding of the protein with a HPLC based assay.

4. Discussion

Numerous esterases and lipases from a large variety of organisms have been functionally expressed in heterologous hosts, but only one lipase from a basidiomycetous yeast has recently been cloned and expressed in *E. coli* [16]. Lip2 represents the first basidiomycetous fungus lipase that has been functionally expressed in a heterologous host.

Refolding yielded soluble and functional proteins for s-Lip2-His and Lip2-His. Apparently, neither the particular codon usage of *P. sapidus* nor the lacking glycosylation restricted the active expression of Lip2 in *E. coli* (Table 1). Both variants had similar catalytic activity against *p*-NPP and paprika oleoresin, indicating that the structure of the active sites was not significantly altered. The signal sequence was neither essential nor prejudicial for *E. coli* to produce active enzyme. This was shown for other lipases, for example for lipase B from *Candida antarctica* [17]. However, the C-terminal tag was necessary to express Lip2 in *E. coli*. No activity was found in the experiments with s-Lip2 and Lip2. SDS-PAGE indicated that no recombinant protein was produced with these constructs. A localization sequence in the C-terminus of this enzyme that interfered with functional expression in *E. coli* similar to peroxisomal targeting sequence motives [18] could explain this result. An improvement of active expression of Lip2 in *E. coli* could be achieved by using other vector constructs, for example one which contains a thioredoxin fusion sequence [19] or one with an *E. coli* specific signal sequence for expression in the periplasm [17]. Work in this direction has started.

With the annotation of the *Coprinopsis cinerea* genome [20] many putative lipase sequences with a low homology to known microbial and yeast lipases and a high homology to Lip2 have been revealed. These sequences may serve as starting points to screen for other lipases with likewise unusual properties.

The functional expression of Lip2 in *E. coli* opens the possibility to modify the present enzyme by site directed mutagenesis. Fusion proteins may be designed without a loss of activity to support (directed) immobilisation. Investigation of the substrate specificity of Lip2 for both hydrolytic and synthetic reactions may lead to new applications which are not feasible with current microbial or yeast lipases [21]. No information about the 3D structure of Lip2 is available yet. Heterologous expression can produce enough material for X-ray analysis, which in turn may contribute to explain the uncommon catalytic properties of Lip2.

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