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ORIGINAL PAPER

Separation of Extracellular Esterases from Pellet Cultures of the Basidiomycete *Pleurotus sapidus* by Foam Fractionation

Diana Linke · Manfred Nimtz · Ralf G. Berger · Holger Zorn

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Abstract Two extracellular esterases were produced in submerged cultures of the basidiomycete *Pleurotus sapidus*. A foam fractionation device was designed and employed for the isolation of the esterolytic enzymes. The recovery of enzyme activity in the liquefied foam strongly depended on the superficial gas velocity. High purification and enrichment factors ($E_a = 62.0$, $P = 15.5$) were achieved using nitrogen at 1.87 cm min^{-1} within 100 min. Increasing the superficial gas velocity to 2.49 cm min^{-1} improved the recovery of total esterase activity in the foam to >95% at the expense of reduced enrichment and purification factors. Differences in their physicochemical characteristics resulted in differing foaming properties of the two esterases secreted by *P. sapidus*. By variation of the pH value of the culture medium and addition of Triton X-100, both esterases were successively and quantitatively transferred into the foam in a two-step fractionation process.

D. Linke · R. G. Berger (✉)
Institute of Food Chemistry, Zentrum Angewandte Chemie, Gottfried Wilhelm Leibniz University of Hannover, Callinstr. 5, 30167 Hannover, Germany
e-mail: rg.berger@lci.uni-hannover.de

M. Nimtz
Department of Biophysical Analysis, Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany

H. Zorn
Institute of Food Chemistry and Food Biotechnology, Justus-Liebig-University Gießen, Heinrich-Buff-Ring 58, 35392 Gießen, Germany

Keywords Esterase · Foam fractionation · Downstream processing · Basidiomycete · *Pleurotus sapidus*

Abbreviations

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E_a	Enrichment factor of activity, see "Experimental Procedure"
E_p	Enrichment factor of protein, see "Experimental Procedure"
ESI	Electrospray ionization
IEF	Isoelectric focusing
pI	Isoelectric point
P	Purification factor, see "Experimental Procedure"
R_a	Recovery of activity, see "Experimental Procedure"
R_p	Recovery of protein, see "Experimental Procedure"
SEC	Size exclusion chromatography
SNL	Standard nutrient liquid
U	Units of enzyme activity

Introduction

Efficient and cheap techniques for the isolation of secreted enzymes from microbial production systems are required to meet the growing demand of the "White Biotechnologies" [1]. Traditional protein purification procedures typically comprise multistep operations, which are often accompanied by significant losses of enzyme activity. Optimized concepts should thus be based on the minimization of

processing steps, while preserving or even improving the purification selectivity and decreasing the activity losses. Alternative downstream processing strategies have to be both ecologically and economically attractive.

In recent years, foam fractionation has become an interesting separation tool [2, 3]. It is a simple and cost-effective method, which may conserve the biological activity of secreted enzymes. Foam fractionation is particularly suitable for diluted protein solutions as they typically occur in microbial culture supernatants.

Foams represent two-phase systems, composed of gas bubbles dispersed in a liquid. By injecting gas into a solution, surface-active molecules like proteins adsorb to the gas–liquid interface of the gas bubbles and form stable foams [4, 5]. Different physicochemical properties of the proteins allow the development of optimized separation and purification foam fractionation protocols, with the isoelectric point being the most important parameter. On the basis of differences in net charge caused by the respective pH value of the bulk solution, natural materials like proteins, flavokavines, alkaloids, and terpenes have been isolated [6–9]. In recent years, a special focus has been set on the isolation of enzymes by foam fractionation. Surprisingly, a number of enzymes have thus been isolated and enriched without significant loss of activity. These enzymes' tertiary structure is obviously rigid enough to prevent denaturing by unfolding when adsorbed to the gas–liquid interface [6, 10, 11].

In general, a successfully adapted laboratory purification method may be difficult to scale up, and high costs of equipment may arise. Previous experiments on a laboratory-scale showed the suitability of the foaming process for the nearly quantitative recovery (95%) of an extracellular lipase from submerged cultures of *Pleurotus sapidus* [12]. The present work reports on a successful enlargement (2.6 fold with respect to the crude culture volume) of this process, and on a stepwise transport of two different extracellular esterases from *P. sapidus* by varying the pH and the detergent which generates the foam.

Experimental Procedure

Materials

Tris hydrochloride (>99%), glycerol (>99.5%), dithiothreitol (p.a.), and agar agar were obtained from Roth (Karlsruhe, Germany). Manganese sulfate monohydrate (>98%), bovine serum albumin (>96%), azocasein, and dipotassium hydrogen phosphate (p.a.) were from Fluka (Seelze, Germany). D-(+)-Glucose monohydrate (puriss), L-asparagine monohydrate (>99%), yeast extract (for microbiology), potassium dihydrogen phosphate (p.a.), sodium hydroxide (p.a.), hydrochloric acid (p.a.), Coomassie blue G250 (pure), bromophenol blue (for electrophoresis), and ethylene diamine tetraacetic acid (>99%) were from Merck (Darmstadt, Germany). Tween 80 (Ultra grade), magnesium sulfate (>99%), ferrous(III) chloride × $6H_2O$ (>99%), calcium chloride × $2H_2O$ (99%), sodium dodecyl sulfate (SDS) (Ultra pure >99%), trichloroacetic acid (p.a.), and copper sulfate × $5H_2O$ (99%) were from Riedel-de Haën (Seelze, Germany). Triton X-100 (Ultra grade) was from Sigma (Deisenhofen, Germany). Zinc sulfate × $7H_2O$ (99.5%) and phenolphthalein (ACS) were from Baker B·V. (Deventer, The Netherlands). Methanol, *n*-hexane, ethyl acetate, *tert*-butyl methyl ether, butylated hydroxytoluene, acetonitrile and dichloromethane were all HPLC grade and obtained from Fisher Scientific GmbH (Schwerte, Germany). Ethanol (96%) was purchased from Kraul & Wilkening und Stelling (Hannover, Germany). High-purity water was prepared with an E-pure water purification system (Barnstead, Dubuque, Iowa, USA).

Organism

The white-rot fungus *P. sapidus* (8266 DSMZ) was obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH", Braunschweig, Germany.

Cultivation of *Pleurotus sapidus*

The strain was kept on standard nutrition liquid (SNL) agar plates containing 30.0 g L^{-1} D-(+)-glucose × H_2O, 4.5 g L^{-1} L-asparagine × H2O, 1.5 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgSO_4$, 3.0 g L^{-1} yeast extract, 15.0 g L^{-1} agar agar, 1.0 mL L^{-1} trace element solution (0.005 g L^{-1} $CuSO_4{\cdot}5H_2O$, 0.08 g L^{-1} $FeCl_3{\cdot}6H_2O$, 0.09 g L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.03 g L^{-1} $MnSO_4{\cdot}H_2O$, and 0.4 g L^{-1} EDTA; the pH was adjusted to 6.0 with 1 N NaOH prior to sterilization). Media and equipment were autoclaved prior to use, and sterile techniques were applied throughout the procedures. Main cultures of *P. sapidus* were grown as described previously in SNL without glucose plus 0.4% (v/v) Tween 80 [12]. After 5 days, the cultures were subjected to the foam fractionation process. The total esterolytic activity of the culture supernatants at this point of time was 420 U L^{-1} with Units (U) defined below.

Foam Fractionation

Previous experiments were carried out on a 70-mL laboratory scale [12]. For enlarging the process, the culture volume (pH 7.0) was increased to 200 mL, and a larger fractionation device was developed [column ($l = 105$ cm, $d = 3.2$ cm), glass bowl ($V = 500$ mL)] (Fig. 1). The superficial nitrogen velocity was adjusted from 1.87 to

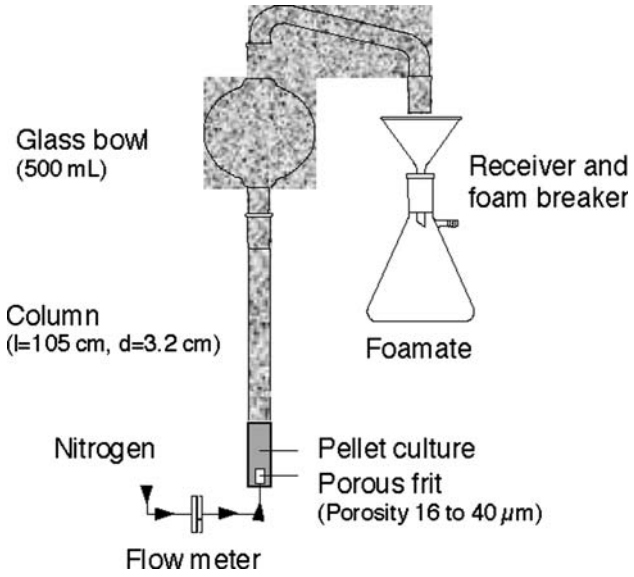

Fig. 1 Scheme of the up-scaled foam fractionation device

2.49 cm min^{-1}, and the foaming period was set to 100 min. For the isolation of Esterase B out of the retentate, 0.44 mM Triton X-100 Ultra grade was added to the solution. Experiments were carried out for 50 min at pH 7.0 and 5.4. All foaming processes were performed batchwise at room temperature.

Determination of Enzyme Activity and Protein Concentration

For the determination of esterase activity, an assay mix was prepared by adding 2.16 g Tween 80 to 5 mL 50 mM Tris/HCl, pH 7.0. The reaction was started by addition of a 2-mL sample. Incubation was performed at 37 °C and 160 rpm for 30–60 min, depending on the enzyme activity. Hydrolytic activity was quantified by monitoring the free fatty acids released from Tween 80 by titration with 50 mM NaOH using 2% ethanolic phenolphthalein as an indicator. One unit of esterase activity was defined as the amount of enzyme that released 1 μmol of free fatty acids per min under standard conditions.

Peptidolytic activity was assayed in a 2-mL tube at 40 °C for 15 min using 100 μL of 0.5% (w/v) azocasein in water, 300 μL of 0.1 M Tris/HCl (pH 7.0), and 100 μL of the enzyme solution. The reaction was stopped by adding 1.5 mL of 3% (w/v) trichloroacetic acid, followed by incubation for 10 min on ice, and centrifugation for 10 min at 13,600*g*. Blanks were prepared with heat inactivated enzyme solutions. The absorbance of the supernatant was determined at 366 nm, and peptidase activity was calculated from the absorbance difference of the blank and the sample. One arbitrary unit of peptidase activity was defined as the activity which produced an absorbance of 0.01 per hour under the specified conditions.

Protein concentration was determined according to Lowry et al. [13], using bovine serum albumin used as standard.

Calculations

Enrichment (E), purification (P), and recovery (R) were calculated for each foam fractionation using the following equations:

$$\text{Recovery of activity } (R_a) = \frac{\text{activity [U] in the foamate}}{\text{activity [U] in the initial solution}} \times 100 \quad (1)$$

$$\text{Recovery of protein } (R_p) = \frac{\text{protein mass [mg] in the foamate}}{\text{protein mass [mg] in the initial solution}} \times 100 \quad (2)$$

$$\text{Purification factor } (P) = \frac{\text{specific activity [U mg}^{-1}\text{] in the foamate}}{\text{activity [U mg}^{-1}\text{] in the initial solution}} \quad (3)$$

$$\text{Enrichment factor ofactivity } (E_a) = \frac{\text{activity [U mL}^{-1}\text{] in the foamate}}{\text{activity [U mL}^{-1}\text{] in the initial solution}} \quad (4)$$

$$\text{Enrichment factor of protein } (E_p) = \frac{\text{protein mass [mg mL}^{-1}\text{] in the foamate}}{\text{protein mass [mg mL}^{-1}\text{] in the initial solution}} \quad (5)$$

"U" thereby refers either to esterase or peptidase activity, respectively. All quantitative data represent average values of at least duplicate analyses.

Determination of Surface Tension

Surface tension of the culture supernatants at pH 4.5, 5.0, 6.0, 7.0, and 8.0 was measured according to standard protocols of the De Noüy ring method at 20.0 °C using a digital tensiometer K10ST (Krüss GmbH, Hamburg, Germany). Measurements were carried out in fourfold replication.

Electrophoresis

Isoelectric focusing polyacrylamide electrophoresis was performed on a Multiphor II system (Pharmacia LKB) using Servalyt™ Precotes™ precast gels with an immobilized pH gradient from 3 to 6 (Serva, Heidelberg, Germany). Isoelectric points were estimated using a low pI calibration kit from 2.8 to 6.55 (Amersham Biosciences, Freiburg, Germany). Gels were Coomassie stained and

activity stained using Tween 80 as substrate [12]. For the transfer of protein spots from IEF to SDS gels, unstained enzymes were excised from the IEF gels and prepared for the SDS-PAGE as described below.

SDS-PAGE analysis was performed using 12% polyacrylamide separation gels [14]. 20 µL of sample or gel pieces excised from IEF gels were prepared by adding 20 µL of loading buffer (0.1 M Tris/HCl pH 6.8, 0.2 M DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue) and boiling for 10 min. For molecular mass determination, marker proteins from 10 to 150 kDa (Serva, Heidelberg, Germany) were used. Gels were stained with Coomassie blue G250.

ESI-Tandem MS

Coomassie stained protein bands were excised from SDS-PA gels, dried, and digested with trypsin. The resulting peptides were extracted and purified according to standard protocols. A Qtof II mass spectrometer (Micromass, Manchester, UK) equipped with a nanospray ion source and gold-coated capillaries (Protana, Odense, Denmark) was used for electrospray ionization (ESI) MS of peptides. For collision-induced experiments, multiple charged parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell (25–30 eV). The resulting daughter ions were separated by an orthogonal time-of-flight mass analyzer. The acquired MS–MS spectra were enhanced (Max. Ent. 3, Micromass) and used for the ab initio sequencing of tryptic peptides.

Size Exclusion Chromatography (SEC)

Two hundred microliter-samples of the culture supernatant, of the liquefied foam, and of the retentate were applied to a Superose 6-column 10/300 GL (24 mL, 5,000–5 × 10^6 Da, Amersham, Uppsala, Sweden), pre-equilibrated with 200 mM Tris/HCl buffer, pH 7.5. The flow rate was set to 0.5 mL min^{-1} (superficial velocity of 0.1 m s^{-1}) and 1-mL fractions were collected. Molecular masses were calculated by comparison to reference proteins.

Hydrolysis of Xanthophyll Esters Derived from *Capsicum annuum*

Enzymatic hydrolysis of paprika oleoresin and HPLC analyses were performed according to Zorn et al. [15]. 8.5 mg of paprika oleoresin and 1.2 g of Triton X-100 Ultra grade were mixed with 8.8 mL of 0.5 M KH_2PO_4/K_2HPO_4 buffer (pH 6.0) and 0.25 mL of 1 M $CaCl_2 \cdot 2H_2O$ solution. The mixture was preheated to 40 °C. To start the reaction, 50 mU of active enzyme sample were added to 2.5 mL of the substrate solution in a 50-mL Erlenmeyer flask. The reaction mixture was incubated at 40 °C and 100 rpm in a water bath for 24 h. Blanks were prepared with heat-inactivated enzyme solutions. 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.

HPLC Analysis

For HPLC separation, an analytical column (120-5 RP-18, 250 × 4 mm, 5 µm, Macherey Nagel, Düren, Germany) was used. The mobile phase consisted of mixtures of methanol/acetonitrile/dichloromethane/*n*-hexane (10:85:2.5:2.5 [A] and 10:45:22.5:22.5 (B), 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. Carotenoids were monitored at 450 nm (DAD; reference: 550 nm).

Results and Discussion

Enlargement of the Fractionation Process

Extracellular esterases were produced in submerged cultures of *P. sapidus* in a standard nutrient medium without glucose, using Tween 80 as an inducer. The cultures were harvested at the point of maximum esterase activity, and the submerged cultures were directly submitted to the foaming procedure without upstream filtration or centrifugation. In previous investigations, >90% of total esterase activity were recovered in the foam within 50 min under optimized conditions (pH 7.0 and 1.87 cm min^{-1} N_2) with $P = 11.6$ and $E_a = 28$ using a small-scale laboratory foaming device [12]. To prepare for an industrial process, the foaming device was enlarged (Fig. 1), and the foaming period was extended to 100 min. The recovery of enzyme activity in the foamate was strongly influenced by the superficial gas velocity. At a nitrogen flow of 1.87 cm min^{-1}, 63.2% of total esterase activity was transported into the foam phase with exceptional high purification and enrichment factors ($E_a = 62.0$, $P = 15.5$, $E_p = 3.5$). The remarkably higher enrichment and purification factors that were achieved in this study concerning the small-scale foaming device may be ascribed to the increased coalescence and drainage by the lengthened foaming column [16]. Traditional multistep downstream processes like ultrafiltration, precipitation,

and chromatography typically result in considerable lower purification factors (1.6–11.9), and additionally may cause high losses of enzymatic activity [17–21]. Thus, foam fractionation allows an almost quantitative isolation of esterases with high E and P factors in a single-step purification procedure.

While the recovery of enzyme activity increased with higher superficial nitrogen velocities, enrichment and purification factors decreased concomitantly (Table 1). Reduced drainage effects at increased superficial gas velocities caused wetter foams, and hydrophilic contaminants were carried along with the interstitial liquid phase. Hence, apart of esterases, peptidase activities of up to 61 U mL^{-1} were determined in the culture supernatants of *P. sapidus*. While no peptidases were transferred into the foam at a superficial nitrogen velocity of 1.87 cm min^{-1}, higher superficial gas velocities came along with a partial transport of peptidases (Table 1). The presence of Tween 80 in the culture medium has been reported to stimulate the secretion of peptidases [22]. According to Jacques et al. [23] and Henriette et al. [24], the mechanism of action of Tween 80 could be explained by an interaction of the detergent with the cell membrane, resulting in an improved substrate transport into the mycelium, and a facilitated release of enzymes and secondary metabolites into the medium. As discussed above, esterase preparations essentially free of peptidase activity were obtained at a superficial gas velocity of 1.87 cm min^{-1}, thus preventing an enzymatic degradation of the target enzymes.

SDS-PAGE analysis and IEF electrophoresis with activity staining indicated the presence of two enzymes with esterase/lipase activity in the supernatants of *P. sapidus*. The one called Esterase A hydrolyzed *p*-nitrophenyl palmitate, diacyl glycerol (Lipase PS test, Sigma), and Tween 80; the enzyme, unlike any other lipase tested also efficiently hydrolyzed xanthophyll esters [15]. The second enzyme, Esterase B, hydrolyzed Tween 80 only (data not shown). Esterase A was highly enriched by the foaming process, and represented the main protein in the liquefied foam. It showed a molecular weight of ~57 kDa and an isoelectric point of 4.5 (Fig. 2, lane 2). By means of electrospray ionization tandem mass spectrometry, two tryptic peptides (LPEPLPPYTGTVR and ATAFGPACPQQSAR) were sequenced ab initio from the protein band excised from the denaturing SDS-PAGE gel. Homology searches against public databases (NCBI Blast, program *blastp*) identified the enzyme as a member of the type B esterase/lipase family [15]. When *P. sapidus* was cultured in a mineral salt medium [25], this enzyme was characterized as an octamer with a calculated molecular mass of 430 kDa.

Table 1 Recovery, enrichment, and purification factors of esterases and peptidases in the foam phase after foam fractionation of submerged cultures of *P. sapidus* (200 mL submerged culture, pH 7.0, 100 min, 24 °C)

Superficial gas velocity (cm min^{-1})	Recovery (%) of			E_a (Esterases)	P (Esterases)
	Esterolytic activity	Peptidolytic activity	Protein		
1.87	63.2 ± 1.6	0.0	4.8 ± 1.6	62.0	15.5
2.17	89.3 ± 2.0	7.2 ± 1.5	6.0 ± 1.7	36.0	16.0
2.49	95.7 ± 1.4	24.7 ± 1.9	12.0 ± 2.1	18.0	8.5

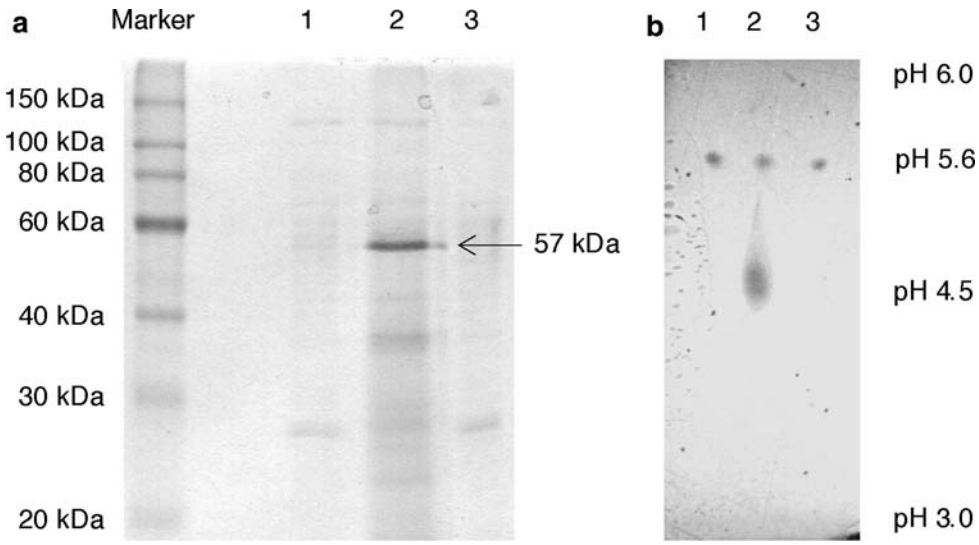

Fig. 2 SDS-PAGE analysis (**a**) after Coomassie staining and IEF electrophoresis (**b**), with activity staining of the culture supernatant *1*, the liquefied foam *2*, and in the retentate *3*. Esterase A (57 kDa, pI 4.5) was strongly enriched protein in the foam phase

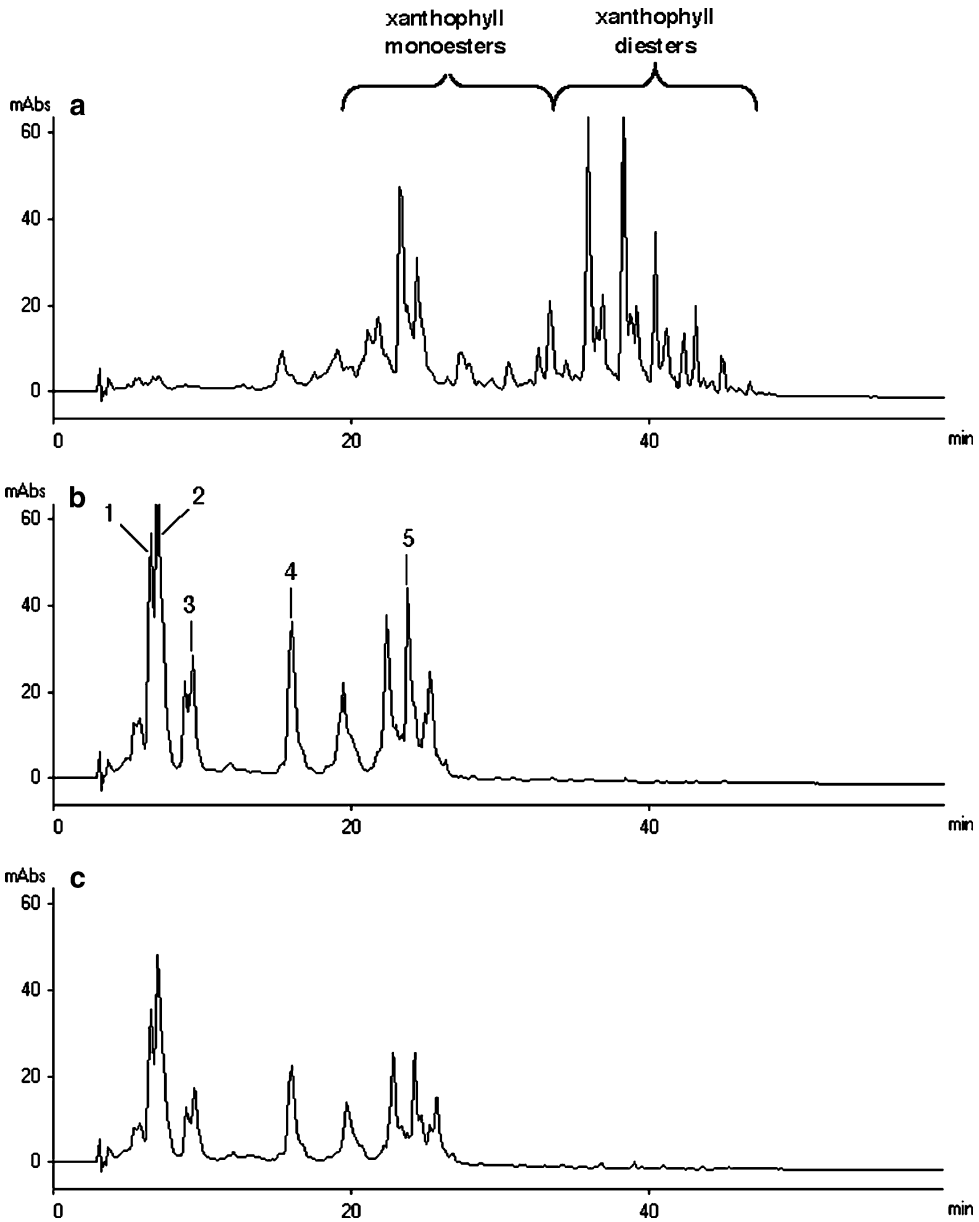

Fig. 3 HPLC-DAD chromatograms (450 nm) of samples obtained by treatment of *Capsicum annuum*-derived xanthophyll esters with heat-inactivated sample of culture supernatant (**a**), active sample of culture supernatant (**b**), and active liquefied foam sample (**c**). Peak assignment: (*Z*)-capsanthin *1*, all-(*E*)-capsanthin *2*, zeaxanthin *3*, *β*-cryptoxanthin *4*, all-(*E*)-*β*-carotene *5*

Surprisingly, a molecular weight of ~60 kDa, corresponding to the monomeric enzyme, was determined by size exclusion chromatography under the conditions of this study (data not shown). Presumably, the presence of the detergent Tween 80 is responsible for the disaggregation of the oligomer. To investigate the monomer's enzyme characteristics, hydrolysis of xanthophyll esters was performed using the culture supernatant and the liquefied foam. Both fractions yielded nearly quantitative ester hydrolysis with paprika oleoresin as substrate (Fig. 3). No ester hydrolysis was observed with parallel analyzed heat-inactivated samples (blanks). Thus both, monomer and octamer are catalytically active.

Though surface activity and therefore an optimal adsorption of a protein to the gas–liquid interface are maximal at its pI [26–28], the most efficient transport of Esterase A was attained at pH 7.0, far removed from its isoelectric point [12]. The surface tension of the culture supernatant possessed a sloping curve from pH 4.5 (34.2 mM m^{-1}) to pH 8.0 (28.8 mN m^{-1}) with a flat area between pH 7.0 and 8.0. Thus, the foaming capacity was highest at the last-mentioned pH values. The presence of Tween 80, which served as a substrate to induce esterase secretion and additionally contributed to the formation of a stable foam, could be responsible for this phenomenon. Loha et al. [29] observed a low recovery of cellulase

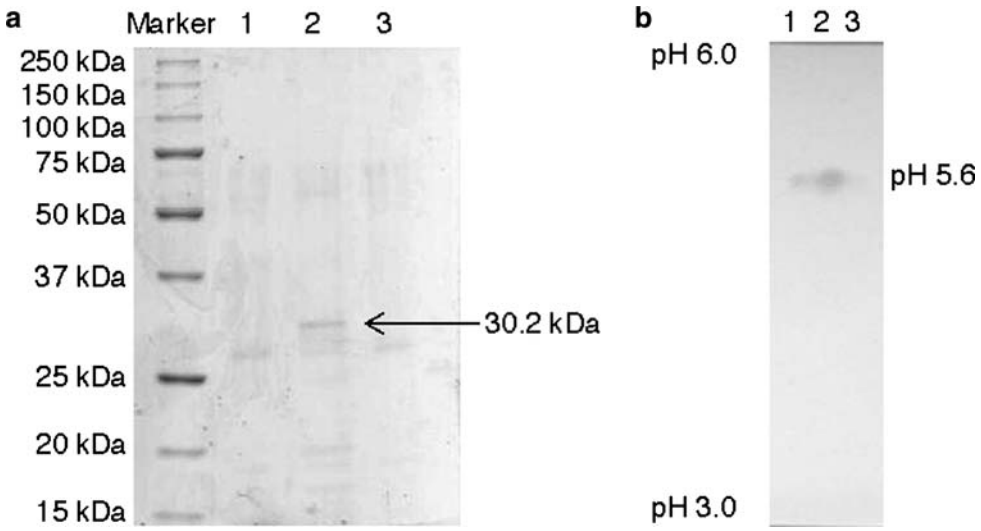

Fig. 4 SDS-PAGE (**a**) and visualization of the esterolytic activities after isoelectric focusing by activity staining (**b**); retentate of the first fractionation step *1*, liquefied foam phase after the second foaming step *2*, and retentate after the second fractionation step *3*. Esterase B (30.2 kDa, pI 5.6) was enriched into the foam phase under the given conditions

activity at its isoelectric point but its highest recovery at a difference of four pH units. The authors ascribed this phenomenon to a change in the molecular structure.

Different from Esterase A, the transfer of Esterase B into the foam was incomplete under the foaming parameters used, as indicated by the activity staining (Fig. 2b, lane 3). After the foam fractionation, the active band of Esterase B (pI 5.6) was still present in the retentate. The visualized esterolytic activity at pI 5.6 in the foam phase was caused by the non-adsorbed transport with the interstitial liquid phase.

Fractionated Foaming of Esterase A and B

Differences in its surface polarity probably resulted in differing foaming properties of *P. sapidus* Esterase B. To completely separate Esterase B out of the submerged cultures, the retentate of the first fractionation step was mixed with 0.44 mM Triton X-100 (Ultra grade) and re-fractionated at pH 7.0 and 1.87 cm N_2 min^{-1} for 40 min. Thus, 71% of the remaining activity were enriched in the foam with $E_a = 8.3$ ($E_p = 1.4$) and $P = 2.3$. Drainage was affected with a 250-mL-glass bowl. An optimal adsorption of a protein to the interfacial region of a foam system is typically observed at its isoelectric point [26–28]. Therefore, the pH of the retentate was adjusted to 5.6, the pI of Esterase B. Additionally, by using a 500-mL-glass bowl to improve the drainage effect, the recovery of activity was increased to 97% with $E_a = 31$ ($E_p = 1.7$) and $P = 18$. The efficiency of the fractionation process of Esterase B was evaluated by SDS-PAGE and IEF analysis (Fig. 4). In the foam phase (lanes 2), an enriched esterase band was visualized, reflecting the fractionation results. Esterase B was characterized by an isoelectric point of 5.6, and a molecular weight of ~30 kDa. Two peptides (GTPVVFVSINYR and IGPIGFPQGQEAHDR) were sequenced ab initio from the protein excised from the denaturing SDS polyacrylamide gel (Fig. 4a, lane 2) by ESI tandem MS. Database searches (NCBI Blast, program *blastp*) returned homologies to a hypothetical protein of the lipase/esterase family of *Coprinopsis cinerea* as the best hit.

In conclusion, foam fractionation was efficiently upscaled to recover esterase activity out of basidiomycete pellet cultures. Superficial gas velocity, pH value, and the choice of the detergent proved to be the most critical process parameters. Because of the varying optimal parameters, the process has to be optimized for each protein individually.

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Conflict of interest statement The authors declare that they have no competing financial interests.

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