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An enzyme from *Auricularia auricula-judae* combining both benzoyl and cinnamoyl esterase activity

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Abstract

Benzoic acid esterases and ferulic acid esterases (FAE) are enzymes with different profiles of substrate specificity. An extracellular esterase (EstBC) from culture supernatants of the edible basidiomycete fungus *Auricularia auricula-judae* was purified by anion exchange chromatography, followed by preparative isoelectric focusing and hydrophobic interaction chromatography. EstBC showed a molecular mass of 36 kDa and an isoelectric point of 3.2 along with broad pH and temperature windows similar to fungal FAEs. However, EstBC exhibited also characteristics of a benzoic acid esterase acting on both benzoates and cinnamates, and most efficiently on methyl and ethyl benzoate, methyl 3-hydroxybenzoate and methyl salicylate. Feruloyl saccharides as well as lipase substrates, such as long chain fatty acids esterified with glycerol, polyethoxylated sorbitan and *p*-nitrophenol were not hydrolyzed. Protein database analyses with tryptic peptides of EstBC solely yielded hits regarding hypothetical proteins belonging to the *alpha/beta* hydrolase family. The uncommon substrate specificity of EstBC concomitant with a lack of sequence homology to known enzymes suggests a new type of enzyme.

Keywords

Fungi, basidiomycete, enzymatic hydrolysis, benzoyl esterase, cinnamoyl esterase, feruloyl esterase

1. Introduction

Esterases belong to the class of carboxylic ester hydrolases (EC 3.1.1.) that catalyze both the hydrolysis and synthesis of ester bonds [1], and are characterized by an *alpha/beta* hydrolase fold and a highly conserved catalytic triad [2, 3]. Many of them, such as benzoic acid esterases and ferulic acid esterases (FAE) accept bulky substrates which has evoked growing industrial interest [4, 5]. FAEs (EC 3.1.1.73) hydrolyze ferulic acid (FA) and other cinnamic acid (CA) derivatives [6]. The hydrolysis of FA from arabinoxylan, hemicellulose and lignocellulosic material decreases the degree of cross-linking and thus improves enzymatic saccharification for the production of biofuels [7, 8]. The product of FAEs, ferulic acid, is a valuable precursor for the biotechnological production of natural aroma compounds such as vanillin and 4-vinylguaiacol [9]; in addition, FA and other cinnamic acid derivatives are of wide interest to the food, pharmaceutical and cosmetic industry due to their antioxidative and photo-protective properties [10, 11].

In contrast to FAEs, benzoyl esterases are hardly described in literature. There is no consistent nomenclature for benzoyl esterases. They are reported as enzymes hydrolyzing benzoyl esters of simple sugars [12], as lipases acting on oleyl benzoate [13] and esterases cleaving 4-hydroxybenzoic acid esters [14]. Applications of benzoyl esterases are the enantioselective synthesis of flavors, fine chemicals and pharmaceuticals [4, 15].

Both, benzoyl esterase and FAEs were only marginally identified in the large division of basidiomycetes. The first biochemically characterized FAE of these fungi was recently published [16], and only one basidiomycete benzoyl esterase hydrolyzing benzoyl-tartrate was described [17]. Enzymes acting on both benzoates and

cinnamates (FAE substrates) were neither described for basidiomycetes nor any other organism.

This work presents the purification and biochemical characterization of an esterase named EstBC from the Agaricomycete *Auricularia auricula-judae* (Aaj). A comprehensive substrate profile was mapped using a combination of benzoates and cinnamates. In addition, peptide fragments for EstBC were identified and aligned with sequences of known hydrolases.

2. Materials and methods

2.1. Chemicals and enzymes

All chemicals were of highest purity grade and purchased from Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Sigma-Aldrich (Taufkirchen, Germany). Benzoates and cinnamates were bought from aforementioned suppliers apart from following: ferulic acid methyl and ethyl ester were from Alfa Aesar (99%, Karlsruhe, Germany); 4-nitrophenyl ferulate was synthesized according to [18]; methyl caffeate, coumarate, sinapate were synthesized according to [19]. Feruloylated saccharides (5-O-trans-feruloyl-L-arabinofuranose(F-A); β -D-xylopyranosyl-(1 $\rightarrow$ 2)-5-O-trans-feruloyl-L-arabinofuranose (F-AX); α -L-galactopyranosyl-(1 $\rightarrow$ 2)- β -D-xylopyranosyl-(1 $\rightarrow$ 2)-5-O-trans-feruloyl-L-arabinofuranose(F-AXG)) were prepared by M. Bunzel (KIT, Karlsruhe, Germany). The substrates were purified and characterized as described [20]. Arabinoxylan was a gift from Jäckering Mühlen- und Nahrungsmittelwerke (Hamm, Germany), sugar beet pectin was from Herbstreith & Fox (Neuenbürg, Germany). Wheat bran was Alnatura (Bio Weizenkleie, Bickenbach, Germany). De-starched wheat bran (DSWB) was produced by treatment with 0.25% potassium

acetate at 90 °C for 15 min followed by extensive washing with water to remove starch. *Aspergillus niger* feruloyl esterase AnFaeA was provided by Sternenzym (Ahrensburg, Germany). Xylanase (*Trichoderma viridae*) was purchased from Sigma-Aldrich.

2.2 Cultivation of *Auricularia auricula-judae* (Aaj)

The basidiomycete strain *A. auricula-judae* (Aaj) used in this study was purchased from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany; No. 11326). The strain was inoculated on standard nutrient liquid (SNL)-agar and maintained at 4 °C. The identity of Aaj was confirmed by 18S ribosomal DNA gene internal transcribed spacer (ITS) sequencing.

SNL was prepared on the basis of Sprecher medium: D-(+)-glucose-monohydrate 30.0 g/L, L-asparagine monohydrate 4.5 g/L, yeast extract 3.0 g/L, KH₂PO₄ 1.5 g/L, MgSO₄ 0.5 g/L; trace element solution (1.0 mL/L (FeCl₃ x 6 H₂O 0.08 g/L, ZnSO₄ x 7 H₂O 0.09 g/L, MnSO₄ x H₂O 0.03 g/L, CuSO₄ x 5 H₂O 0.005 g/L, Titriplex III (EDTA) 0.4 g/L); adjusted to pH 6.0 with 1 M NaOH. For preparing SNL-agar medium, agar (15 g/L) was added to SNL.

To prepare submerged cultures, 500 mL Erlenmeyer flasks containing 200 mL SNL were inoculated with 10 x 10 mm agar plugs of mycelium and homogenized using an Ultra Turrax (Micra D-9, Müllheim, Germany). Cultures were kept at 24 °C on a rotary shaker (Infors, Bottmingen, Switzerland) at 150 rpm. Every day methyl cinnamate (CA-ME) and methyl ferulate (FA-ME) hydrolyzing activity was measured in the culture supernatant after removing the mycelia by centrifugation.

2.3. Purification

500 milliliter culture supernatant, harvested after five days of cultivation, were centrifuged at 10,000 g for 30 min (Sorvall RC 28S, Wilmington, USA) and subsequently filtered under reduced pressure using a 0.45 µm polyether sulfone (PES) membrane (Merck Millipore, Billerica, USA). The filtrate was diluted 1:1 with 50 mM acetate buffer pH 4.75 (equilibrating buffer A) and applied on a 25 mL self-casted Q Sepharose FF column (GE Healthcare, Buckinghamshire, England). A linear NaCl gradient (from 0 to 1 M) in equilibrating buffer A was used to elute the bound proteins (3 mL/min), which were collected and monitored for FA-ME activity. Active fractions were pooled, concentrated with an ultrafiltration module (PES membrane, molecular mass cut off 10,000; Sartorius, Göttingen, Germany), and 1 mL was applied on a preparative isoelectric focusing system (Rotofor, Bio-Rad, Hercules, USA) using pH 2-4 ampholytes (SERVA, Heidelberg, Germany). Fractions were gathered after stabilization of voltage (three hours, 1200 volt). Active FA-ME fractions were pooled and desalted with 10 mM phosphate buffer pH 6.5 using a HiTrap Desalting column (GE Healthcare; 2 mL/min). Samples were freeze dried (VaCo 2, ZIRBUS technology, Bad Grund, Germany), resolved in 1 mL 25 mM phosphate buffer pH 6.5 with 1 M ammonium sulfate (equilibrating buffer B), and applied on a 1 mL HiTrap Phenyl Sepharose HP column (GE Healthcare). A linear ammonium sulfate gradient (from 1 to 0 M; 1 mL/min) in 25 mM phosphate buffer pH 6.5 was used to receive electrophoretically pure EstBC fractions.

2.4. Activity assays

The esterase activity of benzoic and cinnamic acid ester derivatives was determined by HPLC. The reaction mixture (500 μ L) of the samples contained 1.5 mM substrate, 5% DMSO (resulting from substrate stock solution) and 75 mM phosphate buffer at pH 6.5. Crude culture supernatant samples contained additionally 1 mM of sodium azide for laccase inhibition. The reaction mixture was incubated at 37 °C for one hour. The assay was terminated by doubling the reacting volume (500 μ L) with methanol/acetic acid (95:5 v/v); an aliquot was analyzed by HPLC.

Enzymatic hydrolysis of polysaccharides was performed with 5% arabinoxylan, 2% DSWB and 1% sugar beet pectin solutions in 75 mM phosphate buffer at pH 6.5 (1 mL). The reaction was started by the addition of 50 μ L EstBC (corresponding to 165 U/L FA-ME activity) and was incubated for one hour and 18 hours, respectively, at 37 °C. Positive control samples were realized using equi-active AnFaeA instead of EstBC. The assay was terminated by doubling the reacting volume with methanol/acetic acid (95:5 v/v) followed by centrifugation at 15,000 g for 30 min and 4 °C; an aliquot was analyzed by HPLC. All polysaccharide assays were also performed by the addition of 50 μ L xylanase (1.2 U) solution. The endogenous FA content of plant material was determined after alkaline hydrolysis. Aforementioned polysaccharide solutions (1 mL) were incubated with 1 mL 4 N sodium hydroxide at 37 °C for two hours. The mixture was acidified by the addition of 500 μ L 50% (v/v) acetic acid and 2.5 mL methanol. The samples were centrifuged at 15,000 g for 30 min and 4 °C and analyzed by HPLC.

Kinetic parameters were determined from the 45 min hydrolysis rate of methyl benzoate (BA-ME), CA-ME and FA-ME using eight different substrate concentrations from 0.01 mM to 2.5 mM at 37 °C and pH 6.5 (Fig. S1). The other experimental conditions were according to the aforementioned HPLC esterase activity assay.

K_m/k_{cat} values were calculated with non linear regression using SigmaPlot 10.0 (Systat Software Inc., Chicago, USA). HPLC assay were performed at least in duplicates. Blanks were performed for all assays using denatured enzyme instead of active enzyme. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of free acid per min under the experimental conditions used.

Alkyl esterase activity was determined using 4-nitrophenyl butanoate (*p*-NPB) and palmitate (*p*-NPP) as a substrate. The reaction mixture contained the sample and 1.25 mM substrate in 88 mM phosphate buffer at pH 6.5 and 2% Triton X-100 in a total volume of 200 μ L. The formation of 4-nitrophenol was measured in a microtiter plate at 37 °C at 410 nm using a Synergy 2 Microplate reader (BioTek, Winooski, USA). One unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mol of 4-nitrophenol per min under the experimental conditions used. Photometric activity assays were performed at least in triplicates, and standard deviation was found below 10% if not mentioned otherwise.

For determination of Tween 80 and triglyceride (olive oil) hydrolyzing activity (triacylglycerol lipase EC 3.1.1.3), an assay mix (2 mL) was prepared containing 10% Tween 80 and 5% olive oil with 5% gum arabic, respectively, in 75 mM phosphate buffer pH 6.5. Incubation was performed at 37 °C and 150 rpm for 60 min. The assay was terminated by doubling the reaction volume (2 mL) with ethanol/acetone (50:50 v/v). Hydrolytic activity was quantified by monitoring the free fatty acids released from Tween 80 and olive oil by titration with 0.1 M NaOH to pH 9.5 using a titrator (Titro line easy, Schott instruments, Mainz, Germany). One unit of esterase activity was

defined as the amount of enzyme that released 1 μmol of free fatty acids per min under the experimental conditions used. Titrimetric activity assays were performed in quadruplicates, and standard deviation was found below 10%.

2.5. HPLC

HPLC was performed using a Chromolith Performance RP-18e 100-4.6 reverse-phase column (Merck, Darmstadt, Germany). Substrates were separated from the products using a step-wise gradient at a flow velocity of 1.5 mL/min: 20 μL sample was loaded in 90% buffer A (0.1% formic acid), 10% buffer B (acetonitrile); 10 to 36% buffer B in 3.5 min; 36 to 66% buffer B in 1.5 min; 66 to 100% buffer B in 0.5 min; 100% buffer B for 1.5 min; 100 to 10% buffer B in 0.5 min; re-equilibrate with 10% buffer B for 2 min; total run time 9.5 min.

Substance elution was detected with two different programs using a HP 1050 multiple wavelength detector (79854A MWD, Hewlett-Packard, Waldbronn, Germany): 232 nm, 275 nm, 323 nm were used for benzoic, cinnamic, ferulic, tannic, caffeic, chlorogenic, sinapinic and gallic acid; the wavelengths 232 nm, 254 nm, 306 nm were used for 3-hydroxybenzoic, 4-hydroxybenzoic, vanillic, salicylic, and coumaric acid.

2.6. ESI tandem MS of tryptic peptides

The amino acid sequences of tryptic peptides were manually deduced from MS² and MS³ spectra using an Orbitrap Velos Pro mass spectrometer (Thermo Scientific, Bremen, Germany). The procedure was described in detail elsewhere [[16](#)].

2.7. pH optimum

The effect of pH on esterase activity was determined between pH 3.5 and 11.5. Different buffers, such as acetate (pH 3.5–5.5), Bis-Tris (pH 5.5–7.5), Tris (pH 7–9) and borate (pH 8.5–11.5) at a concentration of 75 mM were used. Incubation was performed with CA-ME and FA-ME for 60 min at 37 °C. The other experimental conditions were according to the HPLC esterase activity assay.

2.8. Temperature optimum and stability

Enzyme was incubated at a temperature range from 25 to 95 °C with CA-ME and FA-ME at pH 6.5 (75 mM phosphate buffer) for 45 min to determine the effect of temperature on the enzyme activity. Thermal stability was tested via preincubation of enzyme at temperatures from 15 to 95 °C for 60 min in a 75 mM phosphate buffer pH 6.5; residual enzyme activity was determined via incubation of the substrates CA-ME and FA-ME for 60 min at 37 °C. The other experimental conditions were according to the HPLC esterase activity assay.

2.9. Denaturing SDS-PAGE and isoelectric focusing

SDS-PAGE was performed on a 12% polyacrylamide separation gel. Samples were prepared by mixing 20 µL of enzyme solution and 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. After electrophoresis at 15 mA per gel, the gels were stained with

silver or InstantBlue™ Coomassie (Expedeon, Cambridgeshire, England). Marker proteins from 10 to 250 kDa (Bio-Rad) were used for molecular determination. Isoelectric focusing (IEF) polyacrylamide gel electrophoresis was performed on a Multiphor II system (GE Healthcare) using Servalyt™ Precotes™ precast gels with an immobilized pH gradient from 3 to 6 (SERVA) for 3,500 V h. The determination of isoelectric point was based on a protein test mixture from pI 3 to 10 (SERVA) and on pH control of EstBC active preparative IEF fractions. Gels were Coomassie stained as described above.

3. Results

3.1. General properties of EstBC

The purification of the Aaj esterase EstBC was accomplished by combining an anion exchange chromatography (lane 4, Fig. 1), followed by preparative IEF (lane 5) and hydrophobic interaction chromatography (lane 1). EstBC showed a molecular mass of 36 kDa on a denaturing SDS-PAGE. The isoelectric point of 3.2 was determined via analytical and preparative IEF. The relatively low pI facilitated good purification using anion exchange chromatography and preparative IEF. Hydrophobic interaction chromatography (HIC) was added for polishing the protein.

3.2. pH, temperature optimum and thermal stability

The biochemical characterization was carried out for the substrates CA-ME and FA-ME. For both substrates EstBC showed the same pH optimum at 6.5 (Fig. 2). The enzyme was active in a broad pH range hydrolyzing CA-ME from pH 3.5 to 8.0 with

more than 85% and FA-ME from pH 4.5 to 8.5 with more than 80% of relative activity (Fig. 2). Alkaline auto hydrolysis was not detectable up to pH 8.5 for CA-ME and 9.5 for FA-ME.

Optimal reaction temperature was determined at 66 °C for CA-ME and 61 °C for FA-ME (Fig. 3). The temperature profile of both substrates was similar with the curve of FA-ME parallel displaced to 5 °C lower values (Fig. 3). EstBC exhibited good thermal stability. The values were comparable for both, CA-ME and FA-ME (Fig. 3). 50% residual activity was measured after preincubation for one hour at 65 °C. No activity was detectable at temperatures higher than 86 °C (Fig. 3).

3.3. Hydrolysis of FAE substrates

A comprehensive substrate profile was mapped. Cinnamate and benzoate derivatives were tested besides a number of aliphatic esters (Table 1). CA-ME was found to be the ester hydrolyzed best out of the group of cinnamate derivatives with a specific activity of 5.1 ($\pm$ 0.03) U/mg (No. 1, Table 1). This activity was set as 100%, with all other relative activities found in Table 1 being calculated on this basis. EstBC showed hydrolysis of all typical synthetic FAE substrates, such as FA-ME (40% relative activity), methyl caffeate (7.8%), methyl coumarate (21%), and methyl sinapate (14%; Table 1, No. 3-5, 7). These substrates were usually used to classify the FAEs according to [Crepin et al. \[21\]](#). Hydrolysis of all four substrates would result in a type C or D FAE; exact classification would additionally require sequence information. The classification system of FAEs was extended by [Benoit et al. \[22\]](#) and [Udatha et al. \[23\]](#) on the basis of phylogenetic and descriptor analysis, respectively. Other synthetic FAE substrates, such as ethyl ferulate (12%) as well as methyl

(100%) and ethyl cinnamate (70%) were also hydrolyzed by EstBC (Table 1, No. 1, 2, 6). Besides the synthetic substrates, natural FAE substrates, such as arabinoxylan, DSWB and sugar beet pectin containing 0.93, 1.53 and 7.79 mg esterified FA per dry matter, were incubated with EstBC, but hydrolysis was not detected. Long-term incubation (18 hours) and co-incubation with xylanase yielded analogous results. Smaller natural substrates, such as feruloylated mono-, di- and trisaccharides (F-A, F-AX, F-AXG) and chlorogenic acid did likewise not result in liberation of ferulate (Table 1, No. 8, 10-15).

K_m and k_{cat} were determined for the substrates CA-ME and FA-ME from the initial hydrolysis rates. K_m was calculated for FA-ME to 0.04 (± 0.002) mM and k_{cat} to 1.4 (± 0.01) s⁻¹ which resulted in a k_{cat}/K_m value of 38 mM⁻¹ s⁻¹. CA-ME was the preferred substrate for EstBC compared to FA-ME, as the kinetic parameters were almost doubled. K_m was determined for CA-ME to 0.02 (± 0.002) mM and k_{cat} to 3.5 (± 0.12) s⁻¹ which resulted in a k_{cat}/K_m value of 175 mM⁻¹ s⁻¹.

3.4. Benzoic acid esterase activity vs. FAE activity

Benzoate derivatives were generally better hydrolyzed than the analogous cinnamates: CA-ME (100%) vs. BA-ME (2171%), FA-ME (40%) vs. methyl vanillate (131%) and methyl coumarate (21%) vs. methyl 4-hydroxybenzoate (34%; Table 1). Kinetic studies for EstBC with the substrate BA-ME resulted in a K_m value of 0.15 (± 0.017) mM and a k_{cat} value of 88 (± 0.13) s⁻¹ (k_{cat}/K_m value of 600 mM⁻¹ s⁻¹). The turnover was 25- to 63-fold higher for BA-ME compared to CA-ME (3.5 s⁻¹) and FA-ME (1.4 s⁻¹), yet the affinity of the enzyme for BA-ME (0.15 mM) was 4- to 8-fold

lower compared to FA-ME (0.04 mM) and CA-ME (0.02 mM). EstBC is the first characterized enzyme, which efficiently hydrolyzed both, benzoates and cinnamates.

3.5. Substrate profile of EstBC

Best EstBC activities with all tested benzoates and cinnamates were observed for non- substituted phenyl rings (BA-ME, 2171% and CA-ME, 100%; Table 1). Further investigation of the substrate profile of EstBC revealed that maximum hydrolysis was achieved with ortho-hydroxy substrate (methyl salicylate, 1404%), followed by the meta (methyl 3-hydroxybenzoate, 530%) and para position (methyl 4-hydroxybenzoate, 34%). Starting from the phenolic hydroxyl group in the fourth position, the activity increased with an additional methoxy group in the third position (methyl 4-hydroxybenzoate, 34% vs. methyl vanillate, 131% and methyl coumarate, 21% vs. FA-ME, 40%). Adding a second methoxy group to the phenol decreased the activity (methyl sinapate, 14%). Elongation of the alkyl chain of the alcohol yielded decreasing activities in all experiments (compare methyl/ethyl cinnamate, ferulate, benzoate and vanillate). Analogous results were obtained for the series from methyl to propyl 4-hydroxybenzoates with no detectable residual activity for the butyl ester (Table 1).

As mentioned above, natural FAE substrates (No. 10-15, Table 1) and chlorogenic acid were not hydrolyzed by EstBC. Furthermore, the synthetic substrate *p*-nitrophenyl ferulate, usually used for photometric FAE assays, was not hydrolyzed in the HPLC and photometric esterase assay. The natural polybenzoate tannic acid did not serve as substrate, whereas the monomer methyl gallate was cleaved by EstBC (No. 24 and 27, Table 1). Lipase substrates, such as long chain fatty acids esterified

with glycerol (olive oil), polyethoxylated sorbitan (Tween 80) and *p*-nitrophenol were not hydrolyzed. However, EstBC showed a side activity for the short chain substrate *p*-nitrophenol butanoate (0.44 U/mg, No. 28, Table 1).

3.6. Partial amino acid sequence of EstBC

Tryptic peptides of EstBC were sequenced *ab initio* by means of ESI-MS/MS. Six peptide fragments with 93 amino acids in total were deduced. Homology searches of EstBC fragments 1 to 6 using NCBI BLAST (program blastp) yielded hits regarding hypothetical proteins belonging to the *alpha/beta* hydrolase family. Multiple sequence alignments of EstBC fragments against the top BLAST hits resulted in the fragment order 3-6-2-1-4-5, in which fragments 6-2 as well as 4-5 were joining (Fig. 4). Most frequent and best ranked hit was the hypothetical protein AURDEDRAFT_160157 from *Auricularia delicata* TFB-10046 SS5 (gi|393243500, GenBank: EJD51015; Fig. 4). No specific enzyme activity was assigned to EJD51015 apart from the presence of the conserved domain Abhydrolase_6 and MhpC (*alpha/beta* hydrolase family, Conserved Domain Database, CDD). The active sites were predicted as Ser174, His298, Asp333 (pfam.sanger.ac.uk), and the “nucleophilic elbow” consensus motif G-x-S-x-G (x: any amino acid) around the active serine of the catalytic triad was found (Fig. 4) [23, 24].

4. Discussion

The molecular mass (36 kDa) and pI (3.2) of EstBC was found to be within the typical literature values of fungal FAEs and benzoyl esterases [14, 17, 25]. The pH optima of

FAEs described in literature was between 4.5 and 7.6 [26]. However, the pH optimum of benzoyl esterases was found to be at higher pH values ranging from 7.0 to 9.0 [14, 17, 25]. The pH optimum of EstBC at 6.5 corresponded most to the literature values of FAEs. The temperature optimum exceeded with 66 °C the optimum of previously published FAEs and benzoyl esterases. These ranged from 37 to 60 °C [26]. The thermal stability properties of EstBC were with 50% residual activity at 65 °C greater than other FAEs and benzoyl esterases [14, 17, 25, 26].

[Topakas et al. \[27\]](#) compared kinetic parameters of seven different FAEs from *Aspergillus niger* (AnFaeA, AnFaeB), *Fusarium oxysporum* (FoFaeA, FoFaeB), *Sporotrichum thermophile* (StFaeB, StFaeC) and *Talaromyces stipitatus* (TsFaeC) for several cinnamates. The turnover number of EstBC for CA-ME (3.5 s^{-1}) and FA-ME (1.4 s^{-1}) was at the lower end of literature FAEs, but the affinity to the substrate (Michaelis-Menten constant, CA-ME: 0.02 mM; FA-ME: 0.04 mM) was at the top end.

All of the FAE enzymes compared by [Topakas et al. \[27\]](#) (apart from AnFaeA) acted on CA-ME. Particularly the type B FAEs AnFaeB, FoFaeB and StFaeB exhibited with 267, 359 and 255 s^{-1} high k_{cat} values for CA-ME [27-29]. The catalytic efficiency (k_{cat}/K_m) of all of these type B FAEs was higher for CA-ME than for FA-ME. EstBC showed the same type B characteristics. However, the hydrolysis of substrates no. 3, 4, 5 and 7 (Table 1) represents type C and D FAE characteristics [21].

FAEs usually prefer feruloylated saccharides compared to the synthetic FA-ME. The FAE Est1 from the basidiomycetous fungus *Pleurotus sapidus*, for instance, preferred the substrate F-AX, followed by F-A, F-AXG and finally FA-ME [16]. FaeA and FaeB from the ascomycete *Aspergillus niger* hydrolyzed F-A with a higher activity than FA-ME [30]. In contrast to other fungal FAEs, EstBC did not work on the feruloylated

saccharides F-A, F-AX and F-AXG. Taken together, the biochemical properties of EstBC resembled fungal FAEs; however, the substrate specificity of EstBC differed from FAEs.

Benzoate derivatives were generally better hydrolyzed by EstBC than the analogous cinnamates (Table 1). Studies in context with FAEs and benzoates are rare in the literature. [de Vries et al. \[31\]](#) analyzed methyl gallate using FAEs of *A. niger*, but neither faeA nor faeB were able to hydrolyze this compound. Within an extensive substrate screening of 26 methyl phenylalkanoates the benzoates methyl 4-hydroxybenzoate and methyl vanillate were tested for seven FAEs (AnFaeA, AnFaeB, FoFaeA, FoFaeB, StFaeB, StFaeC and TsFaeC) – none of the FAEs acted on these benzoates either [\[27-29\]](#). In the present study AnFaeA was incubated with the benzoates of Table 1, and no activity was detected likewise. In contrast to FAEs, only few publications described benzoyl esterases. They hydrolyzed benzoates, however, unlike FAEs they did not act on cinnamates [\[12, 14, 17\]](#). One trait is shared with FAEs and benzoate esterases – both usually exhibit side activities for short chain *p*-nitrophenolates, a typical esterase substrate [\[12, 14, 17, 27-29\]](#). This characteristic was in line with the *p*-nitrophenyl butanoate esterase side activity of EstBC (0.44 U/mg).

There are no indications of esterases with a substrate specificity comparable to EstBC in the literature. FAEs hydrolyze cinnamates, especially esterified with saccharides, which is usually arabinose in natural substrates. However, FAEs are not capable of acting on benzoates. Chlorogenate hydrolase (EC 3.1.1.42) substrates are hydroxycinnamates, such as chlorogenic acid, but not benzoates. Tannases (EC 3.1.1.20) hydrolyze tannic acids and hydroxybenzoates, such as gallates, however they do not work on benzoic acid esters, methoxy derivatives of benzoic acid and

cinnamates. Substrates of benzoyl esterases are benzoates, but not cinnamates. EstBC acted on all tested benzoates and cinnamates efficiently, as long as the alcohol moieties were not too bulky. Lipase activity could be excluded from EstBC as no long chain fatty acids were hydrolyzed from alcohols.

The results of the experimental data were in line with the sequence analyses. Protein database analyses with tryptic peptides of EstBC solely yielded hits regarding hypothetical proteins belonging to the *alpha/beta* hydrolase family; however, the hypothetical proteins had no defined enzyme activities. The best hit of the EstBC fragments was a hypothetical protein (GenBank: EJD51015) from *A. delicata*. The “nucleophilic elbow” consensus motif G-x-S-x-G (G-H-S-Y-G) around the active serine of the catalytic triad Ser174-His298-Asp333 was detected. [Udatha et al. \[23\]](#) stated that the presence of the catalytic triad with the serine containing nucleophilic elbow was an essential criterion for being a putative FAE. The order of the predicted catalytic triad of FAEs was mostly characterized by Ser-Asp-His (77% out of 321 sequences), followed by Asp-Ser-His (12%) and His-Ser-Asp (11%) [\[23\]](#). The differing catalytic triad order Ser-His-Asp of EJD51015 as well as the missing homology to benzoyl esterases, FAEs and other enzymes confirmed EJD51015, and thus also EstBC to be a novel enzyme type.

The biological function of EstBC is not clear yet. Although methyl esters and other alkyl esters of cinnamates are not the natural substrates of FAEs, these substances are naturally occurring. CA-ME, for instance, accounts for up to 99% of the *Eucalyptus* and up to 70% of the basil (*Ocimum*) essential oil [\[32, 33\]](#). Methyl salicylate, which is rapidly hydrolyzed by EstBC, is abundantly present in many essential oils and other plant materials e.g. birch (*Betula*) and alder (*Alnus*) [\[34\]](#). As

A. auricula-judae may infest trees of the order *Fagales*, the fungus might have evolved EstBC to metabolize alkyl benzoates and cinnamates.

EstBC is the first characterized esterase exhibiting both benzoate and cinnamate derivative activity combined with broad pH and temperature windows. The unique substrate profile and the lack of sequence homology to known enzymes established EstBC as a member of a new class of enzymes of possible industrial relevance.

Future applications may be the hydrolysis as well as the synthesis of benzoylated and feruloylated compounds, such as flavors, fine chemicals and pharmaceuticals [4, 15].

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Figure legends

Fig. 1. SDS-PAGE (12%) of EstBC purification; staining of protein was performed with InstantBlue™ Coomassie. Lane 1: concentrated purified EstBC after hydrophobic interaction chromatography; lane 2: marker proteins; lane 3: Aaj crude culture supernatant; lane 4: EstBC after anion exchange chromatography; lane 5: EstBC after preparative isoelectric focusing.

Fig. 2. Effect of pH on EstBC activity tested with the substrates methyl cinnamate (solid line) and methyl ferulate (dotted line). Various buffer solutions were used: acetate pH 3.5–4.5 (filled circle), Bis-Tris pH 5.5–7.5 (open triangle down), Tris pH 7–9 (filled square) and borate pH 8.5–11.5 (open diamond).

Fig. 3. Temperature profile (solid line) and thermal stability (dotted line) of EstBC for the substrates methyl cinnamate (CA-ME, filled circle) and methyl ferulate (FA-ME, open triangle down). For thermal stability, EstBC was preincubated at temperatures from 15 to 95 °C for 60 min in a 75 mM phosphate buffer pH 6.5.

Fig. 4. Multiple sequence alignment (Clustal O 1.1.0) of EstBC protein fragments 1-6 (leucine and isoleucine were not distinguishable via ESI tandem MS technique; 93 aa) and EJD51015 (hypothetical protein AURDEDRAFT_160157 from *Auricularia delicata* TFB-10046 SS5, GenBank: EJD51015; 357 aa). The serine esterase consensus motif GxSxG (“nucleophilic elbow”) around the active Ser174 (gray shadowed) of the catalytic triad was underlined.

Fig. S1. Kinetics of EstBC for the substrates methyl benzoate (**a**), cinnamate (**b**) and ferulate (**c**). Kinetic parameters were determined from the 45 min hydrolysis of eight different substrate concentrations from 0.01 mM to 2.5 mM at 37 °C and pH 6.5.