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An acetyl esterase of *Trichoderma reesei* and its role in the hydrolysis of acetyl xylans

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Summary. An acetyl esterase was purified from *Trichoderma reesei* by cation and anion exchange chromatography. The enzyme had a molecular weight of 45 000 as determined by SDS-electrophoresis, or 67 000 as determined by gel filtration. In chromatofocusing the enzyme was shown to consist of two isoenzymes with isoelectric points of 6.8 and 6.0. The enzyme showed activity towards naphthyl acetate, triacetin and glucose- and xylose acetates. However, it liberated acetic acid from acetylated xylo-oligomers only to a small extent. The liberation of acetic acid from the oligomeric substrate was enhanced by addition of endoxylanase and β-xylosidase.

Introduction

The glucuronoxylans of hardwoods are acetylated: 60 to 70% of the xylose residues are esterified at the hydroxyl group of carbon 2 or 3 in the xylopyranose ring. The galactoglucomannan of softwoods is also acetylated, but to a lesser extent (Lindberg et al. 1973a, b). The acetyl groups of hemicellulose are readily liberated when wood is processed in alkaline conditions. In acid hydrolysis of birchwood xylan the rates of deacetylation and xylose production were essentially the same (Maloney et al. 1985). In a steaming process, most of the acetyl groups remained bound to the solubilized and partially hydrolyzed xylan and were not removed until enzymatic hydrolysis by *Trichoderma reesei* enzymes (Poutanen et al. 1986).

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Biely et al. (1985, 1986) first reported the presence of acetyl xylan esterases in fungal cellulolytic systems. As compared with plant and animal esterases, the fungal esterases had high specific activities towards acetylated xylan. The partially purified acetyl xylan esterases of *Schizophyllum commune* acted synergistically with xylanases to produce deacetylated xylo-oligomers, xylose and acetic acid (Biely et al. 1986).

The capability of unpurified enzyme preparations to hydrolyze acetylated carbohydrates was first reported over 20 years ago (Frohwein et al. 1963). Cellobiose octa-acetate has been used as an inducer in the production of cellulases. The esterases of growing micro-organisms were reported to hydrolyze the acetate ester slowly to yield the true inducer, cellobiose (Reese et al. 1969).

Extracellular esterase activity was also detected in hemicellulolytic bacteria isolated from the rumen of sheep (Williams and Withers 1981). It has been suggested that acetylation could be an important factor influencing the digestibility of plant cell walls in ruminants (Morris and Bacon 1977). Chemical deacetylation of the xylan of ryegrass cell walls had a significant effect on the rate but not on the extent of hydrolysis by xylanases and cellulases of *Trichoderma koningii* (Wood and McCrae 1986).

We have previously reported high acetyl xylan esterase activity of *Trichoderma reesei* as compared with the xylanolytic system of *Aspergillus awamori* (Poutanen et al. 1986). The versatility of the xylanolytic enzyme system of *T. reesei* was also demonstrated in comparison with some other hemicellulolytic micro-organisms (Poutanen et al. 1987). In this paper the characteristics of an acetyl esterase of *T. reesei* are described, and its role as a part of the xylanolytic system of the fungus is discussed.

Materials and methods

Enzyme production

Trichoderma reesei Rut C 30 was cultivated in a laboratory bioreactor at 30° C for 4 d in a medium containing 6% Solka Floc cellulose and 3% distiller's spent grain. The culture filtrate was used as raw material for purification of the esterase activity.

Enzyme activity assays

Acetyl xylan esterase activity was determined using steamed birchwood hemicellulose as substrate according to the method of Poutanen et al. 1987. 0.5 ml of the enzyme sample was incubated with 0.5 ml of 5% substrate in 50 mM citrate buffer pH 5.3 at 50° C for 60 min and the acetic acid released was determined enzymatically. The activity was expressed as katals.

Acetylesterase (NA-esterase) activity was determined using 1 mM α-naphthylacetate (NA, Sigma N-8505) in 50 mM citrate-phosphate buffer, pH 5.3 as substrate. The enzyme sample (0.2 ml) was incubated with 1.8 ml of substrate at 50° C for 10 min. Thereafter, 1 ml of 0.01% Fast Corinth V Salt (Sigma-F-6383) in 1 M sodium acetate buffer, pH 4.3 containing 10% Tween 20 was added. The absorbance at 535 nm was read exactly 10 min after addition of the dye. The activity was expressed as katals using α-naphthol as standard.

Xylanase activity was assyed using beechwood xylan as substrate and β-xylosidase using *p*-nitrophenyl-β-D-xyloside as substrate as described previously (Poutanen 1988).

Enzyme purification

1000 ml of culture filtarate was buffered by gel filtration (Sephadex G-25, Pharmacia, Uppsala (Sweden), packed in a column with diameter 113 mm and height 400 mm). The sample in 25 mM sodium citrate buffer, pH 4.0 was then applied to a cation exchange resin (CM-Sepharose FF, Pharmacia, packed in a column with diameter 50 mm and height 100 mm) with a linear flow rate of 1.5 m h^{-1}. Elution was performed at the same flow rate, first with the starting buffer to remove unadsorbed protein and thereafter with a linear salt gradient up to 0.4 M NaCl. 100 ml fractions were collected and assayed for NA-esterase, xylanase and β-xylosidase.

The fractions containing esterase were pooled, concentrated, buffered by ultrafiltration (Amicon PM 10000) and applied to an anion exchanger (DEAE-Sepharose FF, Pharmacia, packed in a column with diameter 15 mm and height 100 mm) equilibrated in 5 mM phosphate buffer, pH 7.0. The esterase which passed through the column unadsorbed was pooled. This enzyme preparation was used for characterization of the esterase activity.

The endoxylanase preparation used to study synergism between esterase and depolymerizing enzymes was partially purified from *T. reesei* culture filtrate by gel filtration in a Sephacryl S-200 SF column as described by Lappalainen (1986). The *T. reesei* β-xylosidase used was purified by cation and anion exchange chromatography (Poutanen and Puls 1988).

Electrophoresis in polyacrylamide gels

Electrophoresis in denaturating conditions was carried out in 10% polyacrylamide gel slabs at pH 8.3 using 25 mM TRIS-glycine buffer containing 0.1% sodium dodecyl sulphate (Laemmli 1970). The low molecular weight calibration mixture (Pharmacia) was used as standard.

Gel filtration

The molecular weight of the purified enzyme was also determined by gel filtration in a Sephacryl S-200 SF column (Pharmacia, 16 × 900 mm). A 2 ml enzyme sample containing 0.3 mg protein was eluted with 50 mM phosphate buffer, pH 6.8 containing 0.5 M sodium chloride at a linear flow rate of 100 mm h^{-1}. 3.5 ml samples were collected and assayed for NA-esterase. The low molecular weight calibration mixture (Pharmacia) was used as standard.

Chromatofocusing

The isoelectric point of the purified enzyme was determined by chromatofocusing in a PBE-94 anion exchange resin (Pharmacia), packed in a 10 × 300 mm column. 0.8 mg of the purified enzyme in 25 mM Tris-acetic acid buffer, pH 8.3 was applied to the column which was pre-equilibrated in the same buffer, and a pH gradient was created by eluting with a mixture of polybuffer 94 and polybuffer 74 (3:7, diluted 1:10), pH 5.0 (Pharmacia). The pH and NA-esterase activity of the fractions were determined.

pH- and temperature optima

For determination of the pH optimum the substrate of the activity assay (1 mM naphthyl acetate) was dissolved in 50 mM citrate-phosphate buffers, pH 2.8—7.2 and the incubation was performed at 50° C. For determination of the temperature optimum the incubation of the activity assay (10 min) was performed at pH 5.3 at different temperatures (30°—65° C).

pH- and temperature stability. For determination of pH stability the purified enzyme preparation was diluted 1:10 in 50 mM citrate-phosphate buffers at pH 3—8 and incubated at room temperature and at 45° C. Samples were withdrawn after 1 h and 24 h and analyzed immediately for esterase activity. For determination of temperature stability the purified enzyme preparation in 50 mM citrate buffer, pH 4.5 was incubated at different temperatures (20° C—60° C) and the samples withdrawn were assayed for residual esterase activity.

Substrate specificity

The capability of the purified enzyme to release acetic acid from β-D-xylose tetraacetate (Sigma X-5500), β-D-glucose pentaacetate (Sigma G-5375), triacetin (Sigma T-5376) and steamed birchwood xylo-oligomers (Puls et al. 1985) was studied. Partly purified acetylesterase from orange peel (Sigma A 4530) and carboxyl esterase from porcine liver (Sigma E 3128) were used as reference enzymes. The substrates were suspended in 50 mM sodium citrate buffer, pH 5.3 and were incubated with the enzyme at 45° C with magnetic stirring. Substrate concentration was 10 mg ml^{-1} and enzyme concentration 5 nkat ml^{-1} NA-esterase.

Other analyses

Enzyme protein was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard,

Table 1. Purification of acetyl esterase from *Trichoderma reesei* culture filtrate (1000 ml)

Purification step	Acetyl esterase (μkat)	Activity yield (%)	Protein (mg)	Specific activity (nkat mg^{-1})	Purification factor
Culture filtrate	123	100	14600	8	—
Step 1. CM-Sepharose	48	39	91	530	66
Step 2. DEAE-Sepharose	35	29	19	1840	230

or by reading the absorbance at 280 nm. Acetic acid was determined enzymatically (Boehringer Test Combination 148261).

Results and discussion

Enzyme purification and characteristics

The culture filtrates of *Trichoderma reesei* showed esterase activity both as assayed with α-naphthyl acetate as substrate and also in the hydrolysis of steamed, ultrafiltrated birchwood xylan, in which the release of acetic acid from a natural hemicellulosic substrate was assayed. In the latter assay, the presence of other xylanolytic enzymes was observed to influence the result, and naphthyl acetate esterase activity was therefore used to monitor purification of the esterase.

The purification was started with adsorption to CM-Sepharose FF at pH 4.0, at which value most of the enzyme protein of *Trichoderma reesei* was not bound. The β-xylosidase and acetyl esterase were eluted one after another in a linear sodium chloride gradient between 0.2 and 0.4 M (Poutanen and Puls 1988). The specific activity of the pooled acetyl esterase was about 66-fold as compared with that of the starting material (Table 1). The esterase-enriched prepartion was then concentrated and applied to a DEAE-Sepharose column in 5 mM phosphate buffer, pH 7.0. The esterase activity passed through the column unadsorbed, whereas a large part of the overall enzyme protein was adsorbed on the resin. The specific activity of the pooled acetyl esterase was 880 nkat mg^{-1} (purification factor 230) and the overall yield of esterase activity after the two ion exchange steps was 29%. The purified enzyme protein represented less than 1% of the starting material (Table 1).

The purified enzyme showed a single band in SDS-electrophoresis (Fig. 1) and had an apparent molecular weight of 45000 as determined by SDS-electrophoresis, or 67000 as determined by gel filtration. Chromatofocusing revealed the presence of two isoenzymes with exactly the same mobility in SDS-electrophoresis. Their isoelectric points were pI 6.8 and pI 6.0.

The acetyl esterase was most active at pH values between 5 and 6, the optimum being at pH 5.5 (Fig.2). At room temperature the enzyme retained

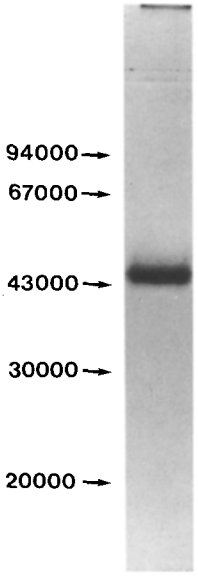

Fig. 1. SDS-electrophoresis of the purified acetyl esterase. Sample size 2 μg protein. Protein staining with silver stain. The standards are indicated by *arrows* showing their molecular weights

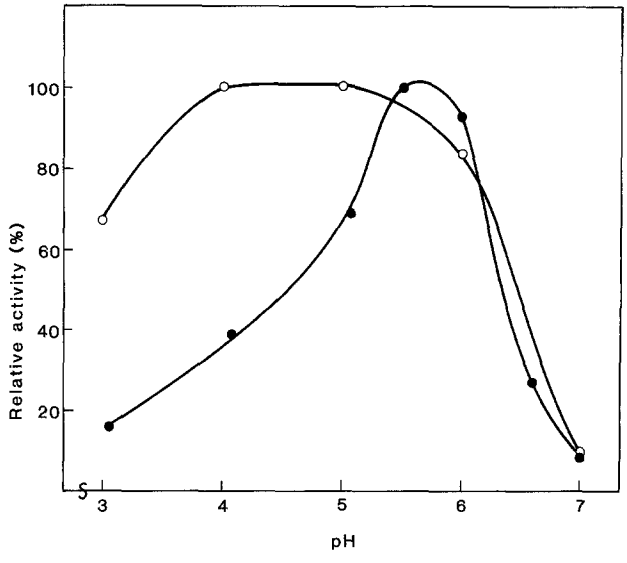

Fig. 2. Effect of pH on the activity (● — ●) and stability (○ — ○) of the acetyl esterase of *T. reesei*

Table 2. Characteristics of the purified acetyl esterase of *Trichoderma reesei*

MW	pI[c]	pH-optimum[d]	Temperature optimum[d]
45000[a] 67000[b]	6.8, 6.0	5.5	50° C

[a] as determined by SDS-electrophoresis
[b] as determined by gel filtration
[c] as determined by chromatofocusing
[d] α-naphthylacetate as substrate, 10 min reaction time

all its activity for 24 h at pH values between 4 and 7 (results not shown), but at 45° C it was only stable at pH 4—5 (Fig. 2). The temperature optimum of the enzyme was 50° C in the 10 min assay (Table 2), but the activity decreased rapidly when the enzyme was incubated for longer periods at temperatures of 50° C or more (Fig. 3).

Little information is available on microbial extracellular esterases. Okumura et al. (1983) and Iwai et al. (1983) purified four extracellular carboxylic esterases of *Aspergillus niger* with isoelectric points from 3.6 to 3.9. These esterases hydrolyzed short-chain aliphatic esters and acetyl esters of phenols. Another esterase of *A. niger* was reported to hydrolyze triacetin and α-naphthyl acetate (Schöbel 1980).

Substrate specificity

When the whole culture filtrate of *Trichoderma reesei* was used as source of enzymes, the liberation of acetic acid from acetylated xylo-oligomers in the xylan acetyl esterase assay was linear up to enzyme dosages of at least 70 NA-esterase units/g of substrate (7 mg total protein/g). The purified enzyme, however, liberated very little acetic acid when similar amounts of NA-esterase units were used in the assay (Fig. 4). It was obvious that the capability of the enzyme alone to liberate acetic acid from xylo-oligomers with a DP_n of over 10 was very limited.

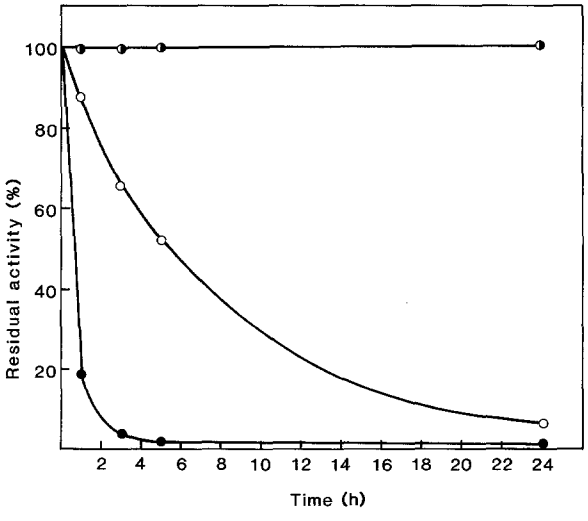

Fig. 3. Effect of temperature on the stability of the acetyl esterase of *T. reesei*. ◑—◑ 45° C, ○—○ 50° C, ●—● 55° C

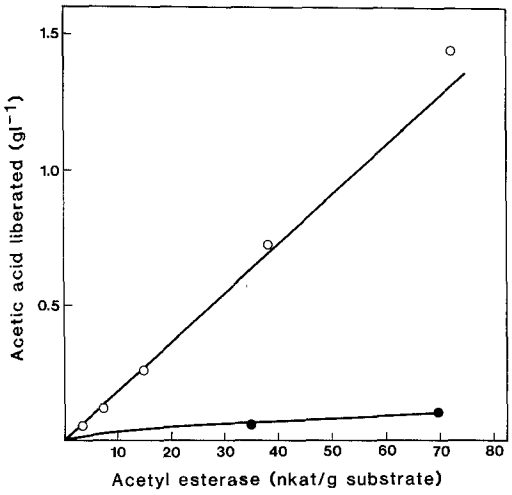

Fig. 4. Formation of acetic acid in the xylan acetyl esterase assay. Substrate: steamed acetylated xylo-oligomers, 25 mg ml^{-1}, initial pH 5.3, temperature 50° C, hydrolysis time 60 min. ○—○ culture filtrate of *T. reesei*, ●—● purified naphthyl-acetyl esterase

The liberation of acetic acid was enhanced by addition of partly purified endoxylanase and β-xylosidase (Fig. 5). The amount of NA-esterase activity units use in all experiments was the same. A surprising phenomenon was the liberation of acetic acid by the xylanase preparation alone. The endo-xylanase preparation partly purified by gel filtration was free from β-xylosidase and NA-esterase activities and showed only traces of acetyl xylan esterase activity, the latter being determined by a 1 h incubation with the steamed, acetylated xylan. The release of acetic acid in prolonged incubation was most probably due to the presence of some esterase as a minor impurity in the endoxylanase preparation, and will be studied further. A mixture of the purified acetyl esterase and the xylanase preparation produced more acetic acid than the sum of the amounts produced by the invididual prepartions separately.

The results clearly indicate that the acetyl esterase has specificity towards short xylo-oligomers and that synergism with depolymerizing enzyme(s) is needed to achieve decetylation and

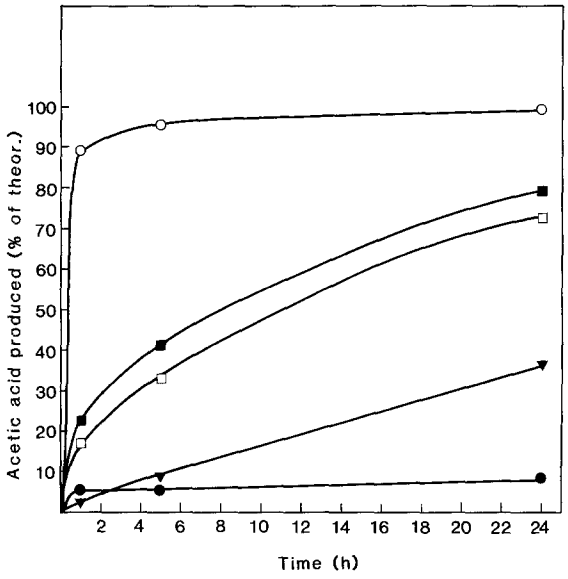

Fig. 5. Formation of acetic acid in the hydrolysis of steamed acetylated xylo-oligomers by enzymes of *T. reesei*. Substrate concentration 10 mg ml^{-1}, initial pH 5.0, temperature 45° C. ●—● purified naphthylacetyl esterase (5 nkat ml^{-1}), ▼—▼ partly purified endoxylanase preparation (150 nkat ml^{-1} xylanase), □—□ esterase and xylanase (5 and 150 nkat ml^{-1}, respectively); ■—■ esterase, xylanase and β-xylosidase (5, 150 and 5 nkat ml^{-1}, respectively), and ○—○ culture filtrate (0.5 mg ml^{-1} protein, 5 nkat ml^{-1} esterase, 5 nkat ml^{-1} β-xylosidase, 150 nkat ml^{-1} xylanase.

production of xylose. There are two possible explanations for the slow and incomplete deacetylation by the purified enzymes as compared with that by *T. reesei* culture filtrate at equal activity levels (Fig. 5). It is probable that some xylanolytic enzymes (α-glucuronidase, another endoxylanase) needed to complete the synergism were missing. The other possibility is that the fungal culture filtrate contained other esterases with higher substrate specificity towards the hemicellulosic substrate. Biely et al. (1986, 1987), indicated the occurrence of multiple esterases in *T. reesei* and reported that their partially purified esterase also showed activity towards long xylo-oligosaccharides, although short ones were the preferred substrate.

The activity of both the culture filtrate of *Trichoderma reesei* and the purified naphthyl acetyl esterase towards some other acetylate compounds was further examined. For comparison, two commercial esterase preparations were used also. The purified esterase hydrolyzed triacetin to glycerol and acetic acid and also liberated acetic acid from xylose tetraacetate and glucose pentaacetate (Fig. 6a). The use of the whole culture filtrate as enzyme source did not remarkably enhance the liberation of acetic acid from these substrates (Fig. 6b).

The capability of *T. reesei* esterase to hydrolyze triacetin and acetylated sugars resembled that of a partly purified acetyl esterase (E.C.3.1.1.6.) from orange peel when compared at equal activity levels towards naphthyl acetate (Table 3). The esterase (E.C.3.1.1.1.) of porcine liver showed little activity towards triacetin, and did not hydrolyze the sugar acetates in the reaction conditions used.

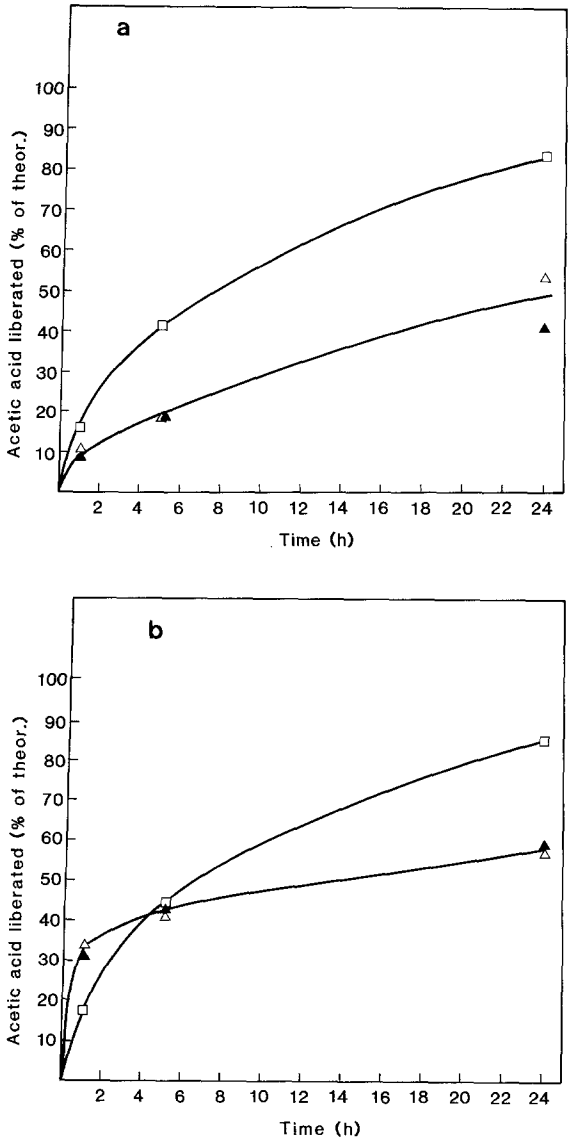

Fig. 6 a, b. Liberation of acetic acid from different acetylated substrates by the purified naphthylacetyl esterase **(a)** and by the culture filtrate of *Trichoderma reesei* **(b)**. Substrate concentration 10 mg ml^{-1}, enzyme concentration 5 nkat ml^{-1} NA-esterase, initial pH 5.3, temperature 45° C. △—△ xylose tetraacetate, ▲—▲ glucose pentacetate, □—□ triacetin

Table 3. Liberation of acetic acid from acetylated substrates by esterases of different origin. The enzymes were dosed according to their NA-esterase activity (500 nkat per gram of substrate dry weight). Substrate concentration 10 mg ml^{-1}, initial pH 5.5, temperature 45 °C

Enzyme	Acetic acid formed (% of theoretical) from:					
	Triacetin		Xylose tetraacetate		Glucose pentaacetate	
	1 h	24 h	1 h	24 h	1 h	24 h
Acetyl esterase *(T. reesei)*	16	83	11	54	9	41
Acetyl esterase (orange peel)	9	22	29	70	6	16
Esterase (porcine liver)	4	14	1	1	<1	<1

The esterases are classified according to their substrate specificity (Enzyme Nomenclature 1973). Due to the rather wide substrate specificity of many esterases this classification is not very clear. The enzyme purified in this study belongs to the group carboxylic ester hydrolases, E.C.3.1.1. and resembles acetylesterase (E.C.3.1.1.6.). As the enzyme is active towards naphthyl acetate, it could also be considered as an arylesterase (E.C.3.1.1.2.). However, further research would be needed for a more detailed classification.

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