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Purification and Properties of Extracellular Esterases of *Aspergillus oryzae* which synthesize Ethyl Caproate

LEE, JONG-HOON*, TOSHITSUGU SATO, YURI KAWAI AND HITOSHI ENEI

Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami-shi, Iwate 024, Japan

Ethyl caproate, one of the major flavor compounds deciding the quality of sake (Japanese wine), is produced during the brewing by the action of alcohol acyltransferase and esterases of sake yeast and koji mold. Extracellular esterases of *Aspergillus oryzae* required for ethyl caproate synthesis were purified partially. The enzymes had different optimum pH and affinity toward substrates. Substrate preferences and inhibition features showed the three enzymes to be B-type esterases or carboxylesterases (EC 3.1.1.1).

Ester compounds such as ethyl caproate and isoamyl acetate roduced during brewing are important determinants of sake flavor. Alcohol acetyltransferase by which isoamyl acetate is obtained from isoamyl alcohol and acetyl-CoA has been purified and some of its properties have been reported (1, 5, 11, 13). Ethyl caproate is produced by alcohol acyltransferase from ethanol and caproyl-CoA by esterase from ethanol and caproate. Kuriyama *et al.* (6, 7) found esterases in sake yeast and koji mold for ethyl caproate synthesis and hydrolysis. In sake brewing, ethyl caproate was also noted to be produced mainly by sake yeast in moromi (unrefined sake) and not produced by koji mold (8). This mold possess enough esterase activity for the synthesis of ethyl caproate in the existence of ethanol and caproate. In order to understand the roles of the esterases produced by koji mold which synthesize ethyl caproate, the extracellular esterases produced by koji mold were partially purified in this study and their characteristics were studied.

MATERIALS AND METHODS

Strains and Culture Conditions

Aspergillus oryzae IFO 30102 purchased from the Institute for Fermentation Osaka (Japan) was used as koji mold. The strain was grown in YEPD agar consisting of 1% yeast extract, 2% polypeptone, 2% glucose, and 1.6% agar at 25°C. For enzyme production, approximately 10^3 spores were suspended in 100 ml YEPD medium in 500 ml Sakaguchi flasks and incubated at 25°C on a reciprocal shaker at 100 rpm for 6 days.

*Corresponding Author
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Enzyme Assay

1.5 ml reaction mixture for assay of esterase activity contained 0.1 mM sodium caproate, 3% ethanol, and an appropriate amount of enzyme in 0.1 M acetate buffer (pH 4.5). In a 20-ml sample vial with silicon rubber stopper, 1.5 ml reaction mixture was incubated for 60 min at 27°C. The reaction was terminated by heating at 60°C for 10 min after adding, as the internal standard, *n*-butanol. The activity of esterase was determined by head space gas chromatography. The production of ester compounds from alcohols and fatty acids other than ethanol and sodium caproate by esterase was determined as previously reported with 0.1 mM fatty acids or their sodium salts and 3% alcohols instead of sodium caproate and ethanol. One unit of esterase for the synthesis activity of ester compounds was defined as that amount of enzyme that would produce each ester compound at 1 ppm in 60 min.

Gas Chromatography

After stopping the reaction by heating, the vial was incubated for 30 min at 55°C and the volatile compounds were injected into a gas chromatograph with a Headspace sampler (Perkin Elmer). A Shimadzu GC-17A gas chromatograph (Shimadzu, Japan) with a FID, equipped with a 30-m×0.25 mm i.d. DB-Wax column (d_f=0.25 µm, bonded carbowax 20-M) (J&W Scientific, Inc.) was used. Helium was provided as the carrier gas at a flow rate of 2 ml/min (40:1 split vent ratio), and oven temperatures held isothermal at 80°C for 2 min and then programmed to increase at 5°C/min to 150°C. The injector and detector temperatures were the same, 250°C.

Molecular Weight Determination

A Superose 12 HR 10/30 gel filtration column (Pharmacia) was equilibrated with 1/30 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and calibrated with a gel filtration standard (Bio-Rad).

Electrophoresis and Staining

SDS-PAGE was conducted by the method of Laemmli (9), using a 5-20% gradient gel (Atto, Japan). Proteins were detected using a silver staining kit from Daiichi Pure Chemicals Co. (Japan), or by immersion in 0.25% Coomassie brilliant blue. Sugar chains were detected by PAS staining (4).

Protein Determination

Protein was estimated using a Bio-Rad protein assay kit calibrated with bovine serum albumin.

Purification of Esterases for the Synthesis of Ethyl Caproate

After 6-day incubation of *A. oryzae* IFO30102 in 2-l of YEPD medium, the cells were filtered off through cotton gauze, and the filtrate was used as the crude enzyme for the purification of extracellular esterases possessing activity for ethyl caproate synthesis. The filtrate was concentrated by salting out with 90% saturated ammonium sulfate. The precipitate was dissolved in a small volume of 1/45 M phosphate buffer (pH 7.0) containing 50 mM NaCl, followed by dialysis against the same buffer overnight. The dialysate was applied onto a Q-Sepharose column (2.6×10 cm, Pharmacia) previously equilibrated with the same buffer. The column was washed with 160 ml of the same buffer and eluted with 240 ml linear gradient of 0.05-0.8 M NaCl in 1/45 M phosphate buffer (pH 7.0) at a flow rate of 2.0 ml/min (Fig. 1). Ethyl caproate synthesis activity was detected primarily in fractions which passed through the column and eluted by the buffer containing 0.2-0.6 M NaCl. Two peaks were noted and designated as SI and SII based on their elution profiles (Fig. 1). SI fractions were concentrated with a Centriprep-10 (Amicon) and applied onto a Phenyl-Superose HR 5/5 column (Pharmacia) equilibrated with 1/30 M phosphate buffer (pH 7.0) containing 1.4 M ammonium sulfate after buffer exchange with a Column PD-10 (Pharmacia) equilibrated with the same buffer. After being washed with the buffer, the Phenyl-Superose column was eluted with 50 ml linear gradient of ammonium sulfate from 1.4 to 0 M. The active fractions were pooled and concentrated with Centriprep-10 and applied onto a Mono-Q HR 5/5 column (Pharmacia) equilibrated with 0.02 M Bis-Tris-HCl buffer (pH 6.0) after buffer exchange with a Column PD-10 equilibrated with the same buffer. After being washed with the buffer, the Mono-Q column was eluted with linear gradient of 0 to 0.3 M NaCl in 90 ml of the buffer. Two active peaks were eluted by the buffer containing about 0.05 M NaCl and the buffer containing from 0.12 to 0.22 M NaCl (Fig. 2). The former peak was designated SI-a and the latter, SI-b. After concentrating the active fractions of the two peaks with a Centricon-10 (Amicon), the samples were applied onto a Superose 12 HR 10/30 gel filtration column equilibrated with 1/30 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and eluted with the same buffer.

The active peak SII fractions were concentrated with a Centriprep-10, and applied onto a Superdex 200 column (1.6×60 cm, Pharmacia) equilibrated with 1/30 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. The active fractions were concentrated and applied onto a Hydroxyapatite column (Econo-

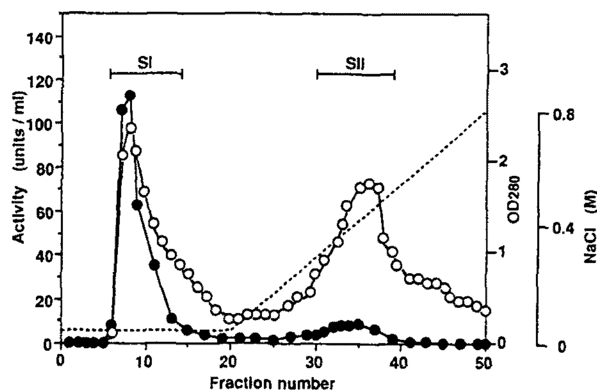

Fig. 1. Q-Sepharose column chromatography of the protein fraction obtained by ammonium sulfate precipitation from the filtrate of the cultured medium.
The effluent from the column was collected as an 8 ml fraction and absorbance at 280 nm (-○-) and ethyl caproate synthesis activity (-●-) were measured. The dotted line indicates NaCl concentration.

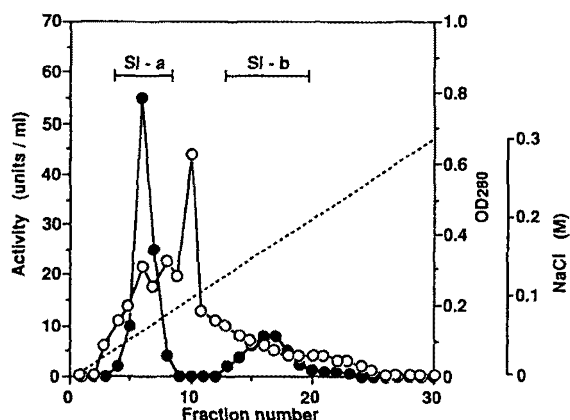

Fig. 2. Mono-Q column chromatography of esterase SI.
The effluent from the column was collected as a 3 ml fraction and absorbance at 280 nm (-○-) and ethyl caproate synthesis activity (-●-) were measured. The dotted line indicates NaCl concentration.

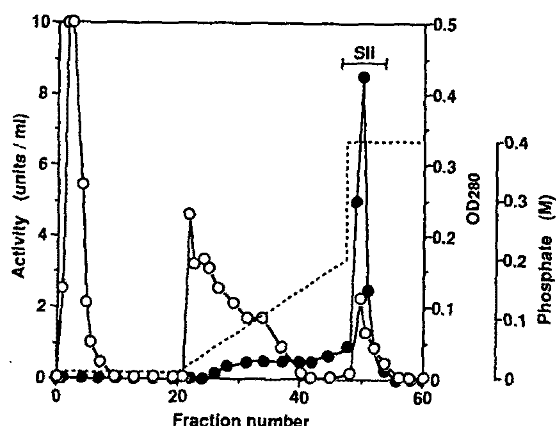

Fig. 3. Hydroxyapatite column chromatography of esterase SII.
The effluent from the column was collected as a 2 ml fraction and absorbance at 280 nm (-○-) and ethyl caproate synthesis activity (-●-) were measured. The dotted line indicates the concentration of phosphate.

Pac HTP cartridge, Bio-Rad) equilibrated with 0.002 M phosphate buffer (pH 7.0) containing 0.05 M NaCl after buffer exchange with a Column PD-10 equilibrated with the same buffer. After washing the Hydroxyapatite column with the buffer, the Hydroxyapatite column was eluted with 60 ml linear gradient of the phosphate buffer (pH 7.0) concentration ranging from 0.002 to 0.2 M containing 0.05 M NaCl and then eluted with 0.4 M phosphate buffer (pH 7.0) containing 0.05 M NaCl (Fig. 3). The active fractions eluted with the 0.4 M phosphate buffer containing 0.05 M NaCl were pooled. After concentrating the active fractions with a Centricon-10, the sample was applied onto a Superose 12 HR 10/30 gel filtration column equilibrated with 1/30 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and eluted with the same buffer.

Inhibition

Inhibition by several compounds was assessed by incubating 1 unit esterase while adding the inhibitor at each concentration to the reaction mixture. Activity was determined by the standard assay as previously reported.

RESULTS

Purification of Esterase SI-a, Esterase SI-b, and Esterase SII

After salting out the culture filtrate with 90% ammonium sulfate, total activity of esterase for ethyl caproate synthesis increased 1.34 fold, possibly as a result of decreased ethyl caproate hydrolysis activity in the culture filtrate. As shown in Fig. 1, the elute from the Q-Sepharose column was separated into two fractions possessing enzyme activity and designated as SI and SII. SI and SII were individually purified as described in materials and methods (Table 1). The SI-a and SI-b peaks appeared after chromatography of the SI fraction with a Mono-Q column (Fig. 2). The former active peak was designated as esterase SI-a and the latter, esterase SI-b. The purified enzyme from the SII fraction was designated esterase SII. After gel filtration, esterase SII appeared to be homogeneously purified with about 0.2% yield (Table 1), but esterase SI-a and SI-b were not homogeneous. The homogeneity of the preparations was confirmed by SDS-PAGE (Fig. 4).

Table 1. Purification of extracellular esterases of *A. oryzae* which synthesize ethyl caproate.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Cuture filtrate	58.8	6300	107	100
Ammonium sulfate (90%)	43.0	8428	196	134
Esterase SI-a				
Q-Sepharose	6.1	5016	822	80
Phenyl-Superose	3.6	2843	790	45
Mono-Q	0.5	857	1714	14
Gel filtration	0.3	575	1916	9.1
Esterase SI-b				
Q-Sepharose	6.1	5016	822	80
Phenyl-Superose	3.6	2843	790	45
Mono-Q	0.4	228	570	3.6
Gel filtration	0.06	130	2167	2.1
Esterase SII				
Q-Sepharose	12.9	373	29	5.9
Gel filtration	1.5	125	83	2.0
Hydroxyapatite	0.19	19	100	0.3
Gel filtration	0.005	10	5000	0.2

Homogeneity of the Preparations

From the results of gel filtration chromatography and SDS-PAGE (Fig. 4), the molecular weight of purified esterase SII was 700 kDa and would appear to be composed of 12 subunits. SDS-PAGE demonstrated the molecular weight of a 61 kDa protein band after silver staining. PAS staining of the purified protein after SDS-PAGE (Fig. 4) indicated a PAS positive broad band with a very large molecular weight. It then follows that, esterase SII is not a homopolymer of the 61 kDa protein with 12 subunits but a complex with very large molecular weight sugar chains (Fig. 4). The molecular weights of partially purified esterase SI-a and esterase SI-b determined by gel filtration were approximately 30 kDa and 44 kDa, respectively. SDS-PAGE of partially purified esterase SI-a and esterase SI-b is shown in Fig. 4. Discordance of the molecular weight of esterase SI-b

between the results of gel filtration and SDS-PAGE was possibly originated from contamination of sugar or some other compounds. As the enzymes were not homogeneously purified, we could not determine the exact size of enzymes. But the difference of characteristics suggests the existence of three different enzymes.

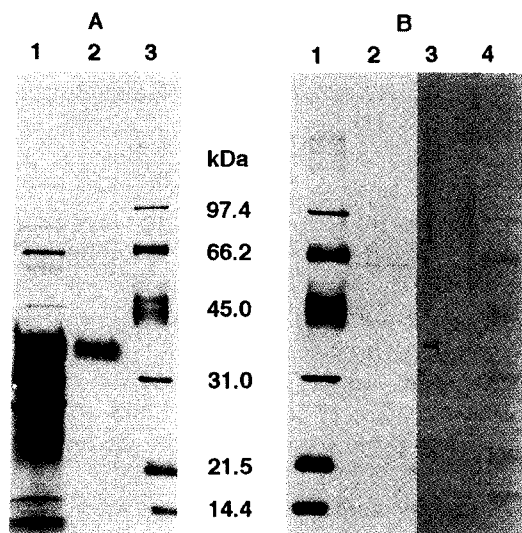

Fig. 4. SDS-PAGE of partially purified extracellular esterases of *A. oryzae* which synthesize ethyl caproate.
Panel A shows silverstained esterase SI-a and esterase SI-b (Lanes: 1, esterase SI-a; 2, esterase SI-b; 3, molecular weight standards). Panel B shows the migration profile of esterase SII (Lanes: 1, silver-stained molecular weight standards; 2, silver-stained esterase II; 3, PAS-stained esterase SII after Coomassie blue staining; 4, PAS-stained molecular weight standards after Coomassie blue staining). Molecular weight standards are specified between panel A and B.

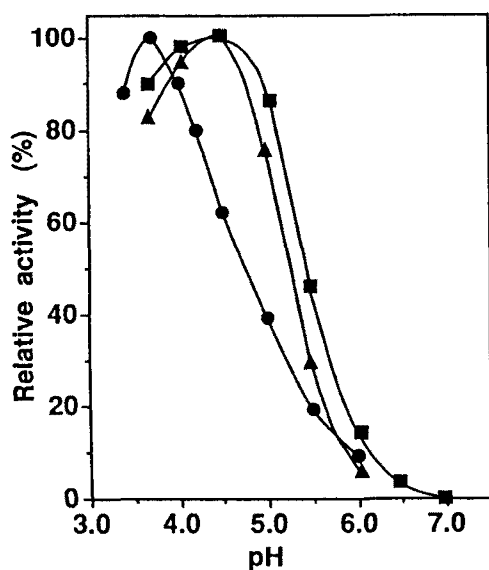

Fig. 5. Effects of pH on ethyl caproate synthesis activity of esterase SI-a (-▲-), esterase SI-b (- ● -), and esterase SII (- ■ -).
Activity at optimal pH of each enzyme was defined as 100%.

Effects of pH

The enzyme activity of esterase SI-a, esterase SI-b, and esterase SII was determined at various pH. 0.1 M acetate buffer (pH 3.4, 3.8, 4.2, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0) was used. Optimal pH of esterase SI-a, esterase SI-b, and esterase SII was around 4.5, 3.8, and 4. 5, respectively (Fig. 5).

Substrate Specificity

The activity of the esterases toward fatty acids and alcohols was determined (Tables 2 and 3). Ethanol and sodium caproate served as the standard substrates for this calculation. In either case, the activity of the esterases producing ethyl caproate from sodium caproate and ethanol was 100%. Esterase SI-a, esterase SI-b, and esterase SII each showed broad substrate specificity toward alcohols and fatty acids.

Inhibitors

Diisopropyl fluorophosphate strongly inhibited the activity of esterase SI-a, esterase SI-b, and esterase SII (Table 4). That of esterase SI-a and SI-b was inhibited by PCMB and $HgCl_2$, while esterase SII activity was not inhibited by PCMB or $HgCl_2$.

Table 2. Substrate specificity of esterases for alcohols.

Alcohols	Carbon number	Relative activity (%)		
		SI-a	SI-b	SII
Methyl alcohol	1	61	90	40
Ethyl alcohol	2	100	100	100
n-Propyl alcohol	3	80	210	190
iso-Propyl alcohol	3	3	9	3
n-Butyl alcohol	4	9	74	30
iso-Butyl alcohol	4	9	116	52
n-Amyl alcohol	5	0.2	1	0.1
iso-Amyl alcohol	5	20	119	20

Sodium caproate was used as the standard substrate to determine activity toward alcohols. Activity for the synthesis of ethyl caproate from sodium caproate and ethanol was defined as 100%. Activity of esterase toward *n*-propyl alcohol was calculated from the standard activity curve for *iso*-propyl alcohol.

Table 3. Substrate specificity of esterases for fatty acids.

Fatty acids	Carbon number	Relative activity (%)		
		SI-a	SI-b	SII
Valeric acid	5	10	94	30
Caproic acid	6	100	100	100
Heptanoic acid	7	33	210	130
Octanoic acid	8	20	118	17
Nonanoic acid	9	5	41	3
Capric acid	10	2	35	2

Ethanol was used as the standard substrate to determine activity toward fatty acids. Activity for the synthesis of ethyl caproate from sodium caproate and ethanol was defined as 100%.

Table 4. Effects of inhibitors on esterase activity.

Inhibitor	Concentration (mM)	Relative activity (%)		
		SI-a	SI-b	SII
Eserine	1	110	108	98
Phenylmethylsulfonyl fluoride (PMSF)	1	120	110	80
Diisopropyl fluorophosphate (DFP)	1	16	33	48
p-Chloromercuribenzoic acid (PCMB)	0.1	70	50	100
Neostigmine bromide	1	100	96	98
Dithiothreitol (DTT)	1	126	120	123
$HgCl_2$	1	35	17	93
EDTA	10	102	98	102

DISCUSSION

Kuriyama *et al.* (7) consider that there may be isozymes of *A. oryzae* possessing ethyl caproate synthesis activity. In the present study, three esterases which synthesize ethyl caproate from the culture medium of *A. oryzae* were partially purified and their features were compared. Partially purified esterase SI-a, esterase SI-b, and esterase SII showed optimal activity around pH 4. Optimum pH of the esterases reported by Kuriyama *et al.* (7) which synthesize ethyl caproate was 5-6. Difference in measurement conditions may possibly be the reason for this difference in pH. Esterase SI-a, esterase SI-b, and esterase SII showed similar substrate specificity toward alcohols and fatty acids but different substrate affinity. Generally, these enzymes have a high affinity for short chain fatty acids and low molecular weight alcohols. No definite results in this regard could be obtained for substrates, propionic acid or butyric acid with our gas chromatography condition, but the esterases showed no reactivity toward acetate (data not shown). Reactivity of the three enzymes toward alcohols possessing more than 5 carbons and fatty acids with more than 10 carbons was not examined here owing to the difficulty of analysis. The results in Tables 2 and 3 show the enzymes possibly have affinity toward these substrates but the affinity of the enzymes decreases as the carbon number of substrates increases. The enzymes were thus concluded to differ from lipases more reactive toward fatty acids with long chains. Esterase SI-a, esterase SI-b, and esterase SII activity was strongly inhibited by DFP. Esterase SI-a and esterase SI-b were inhibited by sulfhydryl group reagents, PCMB and $HgCl_2$, while esterase SII was not. According to the classification of esterases based on substrate specificity and sensitivity towards various inhibitors (2, 3), arylesterases or A-esterases are not inhibited by organophosphates, such as paraoxon, and hydrolyze aromatic esters. Carboxylesterases or B-esterases are typically inhibited by organophosphates and have a wide substrate specificity, hydrolyzing aliphatic and aromatic esters. C-esterases are inhibited by organophosphates and eserine and hydrolyze choline esters. However, esterases have been shown to exhibit overlapping substrate specificity (3), leading to problems of identification and classification (12). The substrate specificity and sensitivity toward various inhibitors of esterase SI-a, esterase SI-b, and esterase SII show them to be carboxylesterases or B-esterases (EC 3.1.1.1). Inhibition by DFP indicates the possible presence of a reactive serine residue which may function as an active site in the same manner as that of serine hydrolyase. The sensitivity of esterase SI-a and esterase SI-b toward the sulfhydryl group of reagents suggests the involvement of the sulfhydryl group for esterase activity. The present data alone are not sufficient to confirm the presence of two active sites, serine and sulfhydryl residues due to impurities present in esterase SI-a and esterase SI-b. Matsunaga *et al.* (10) reported an esterase with two active sites, one serine and one sulfhydryl group residue. It thus follows that esterase SI-a and esterase SI-b may possess two active sites, a serine and a sulfhydryl residue.

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