Properties and Substrate Specificities of Four Esterases from *Aspergillus niger* NRRL 337[†]

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Properties and substrate specificities of four esterases (Esterase-I, -II, -III, -IV) from *Aspergillus niger* were studied. Esterase-I and Esterase-II were found to be markedly stable to heat. When Esterase-I was assayed at 35°C using methylacetylsalicylate as a substrate, even after heating at 100°C for 15 min 60% of its activity remained. However, Esterase-I scarcely hydrolyzed the substrate at 70°C or over, because of a reversible change in conformation by heating as found by CD measurement. The maximum activity of Esterase-I was found at 55°C at 20 min of reaction time. Esterase-II was stable up to 80°C and had an optimum temperature for reaction at 80°C, but was irreversibly inactivated by heating for 15 min at 90°C.

The four esterases hydrolyzed aliphatic esters of short chain fatty acids and acetyl esters of phenols, but neither methyl esters of aromatic carboxylic acids nor acetyl esters of aromatic alcohols.

Many studies on carboxylesterase (EC 3. 1. 1. 1) from animal sources have been carried out, and it has been reported that plural esterases are present in liver,^{1,2)} serum³⁾ or other tissues,^{4,5)} as so-called isoenzymes. Recently, characterization of individual esterases belonging to isoenzymes from animal sources has been attempted, to clarify the mechanism of formation of complicated isoenzymes.^{1~3)} However, no detailed studies of isoenzymes or multiple formes of microbial esterases have been presented.

In the preceding paper, we described the purification of esterases from *Aspergillus niger* NRRL 337 and showed that *Aspergillus niger* produced four kinds of esterases.⁶⁾ In the present paper, characterization of these four esterases from *Aspergillus niger* is described as a further contribution to clarify the mechanism of the production of multiple forms of microbial esterases.

MATERIALS AND METHODS

Esterase preparations. Four esterases (Esterase-I, -II, -III, -IV) were purified to homogeneity according to

the procedures described in the preceding paper.⁶⁾

Esterase assay. Esterase activity was assayed as described previously⁶⁾ and 1 unit (U) was defined as the activity which liberated 1 μ mol equivalent of methylsalicylate per min from methylacetylsalicylate.

Circular dichroism measurement. Circular dichroism (CD) spectra in the 200 to 250 nm region were measured on a model J-500 with DP-500 dataprocessor (JASCO, Japan) using a water-jacketed cell of 0.5 mm path length. Optical density at 280 nm of the sample was 0.3/cm.

Analysis of reaction products. Products released from methylacetylsalicylate by the action of the esterases were separated using a high performance liquid chromatography (HPLC) RP-18 column (E. Merck, 4 $\times$ 150 cm, methanol-1% acetic acid 4:6 v/v) and detected by UV absorption at 290 nm.

Hydrolysis of various esters. A reaction mixture consisting of 0.5 g of ester, 5 ml of 0.02 M phosphate buffer (pH 7) for Esterase-I and Esterase-II or 5 ml of 0.05 M acetate buffer (pH 5) for Esterase-III and Esterase-IV, and 1 ml of enzyme solution (1 U) was stirred at 500 rpm for 1 hr at 45°C. The reaction was stopped by adding 20 ml of ethanol and liberated acid was titrated with 0.05 M KOH.

Reagents. Various esters were products of Tokyo Kasei Kogyo Co. Other reagents were purchased from Wako Pure Chemicals Co.

[†] Studies on microbial esterases. Part II. For Part I, see ref. 6.

TABLE I. PROPERTIES OF FOUR ESTERASES FROM *Aspergillus niger*

The activity was assayed on methylacetylsalicylate as described in METHODS.

	Esterase-I	Esterase-II	Esterase-III	Esterase-IV
Optimum pH (35°C, 20 min reaction) ^a	7.0	7.5	5.5	4.5
pH stability (30°C, 16 hr incubation) ^b	6~12.3	3~9	4~8	4~8
Optimum temperature (20 min reaction at opt. pH)	55°C	80°C	55°C	45°C
Thermal stability ^c (15 min incubation at opt. pH)	55°C (100%) ^d 100°C (60%)	80°C (100%) 90°C (0%)	55°C (100%) 70°C (0%)	45°C (100%) 60°C (0%)

^a 0.2 M Na₂HPO₄-0.1 M citric acid buffer was used.^b 0.2 M Na₂HPO₄-0.1 M citric acid buffer (pH 2.5~8) and 0.1 M glycine-0.1 M NaOH buffer (pH 8.5~13) were used.^c After the heat treatment, the enzyme solution was cooled at 0°C for 5 min, and activity was measured.^d Relative residual activity after treatment at each temperature is shown in parentheses.

RESULTS AND DISCUSSION

Enzymatic properties

The effects of pH and temperature on enzyme activity were examined using methylacetylsalicylate as substrate, and the results are summarized in Table I. Esterase-I and Esterase-II were stable in the wide ranges of pH 6~12.6 and pH 3~9, respectively. The maximum activities of Esterase-I and Esterase-II were found at around neutral pH, while those of Esterase-III and Esterase-IV were found at acidic pHs.

Esterase-II was not inactivated by temperatures up to 80°C during incubation for 15 min, and had an optimum temperature of 80°C. In contrast, Esterase-I had an optimum temperature of 55°C and scarcely hydrolyzed the substrate over 70°C. However, 60% of its activity remained when it was measured at 35°C after heating at 100°C for 15 min. It is presumed that this is because of a reversible change of conformation. To prove this assumption, the change in conformation of Esterase-I at high temperature was examined by CD measurement. As shown in Fig. 1-A, a marked change in conformation of the enzyme was observed during the rise of temperature from 15°C to 75°C, that is, a random coil was formed as a result of loosening of α -helix by heating.

FIG. 1. CD Spectra of Native and Heat-treated Esterases.

A Esterase-I: —, measured at 15°C; ----, measured at 75°C; ·····, measured at 15°C after heating at 75°C for 15 min.

B Esterase-II: —, measured at 15°C, ----, measured at 15°C after heating at 90°C for 15 min.

However, cooling the enzyme solution from 75°C to 15°C, the spectrum coincided with that of native enzyme. In addition, the remaining activity of Esterase-I which had been cooled after heating at 75°C for 15 min was 95% of that of native enzyme. From the results described above, we concluded that Esterase-I changed its conformation by heating at 75°C and so was inactivated, but the conformation and activity are immediately restored by cool-

ing. The CD spectra of Esterase-II (Fig. 1-B) showed that Esterase-II was irreversibly denatured by heating at 90°C for 15 min.

As it is well known that carboxylesterases from animal sources have serine residues at their active site,⁵⁾ the effects of diisopropyl fluorophosphate (DFP) on the activities of the four esterases were investigated. A mixture consisting of 0.5 μ mol of DFP and 0.02 μ mol of enzyme was incubated at 20°C. After 20 min, remaining activity was assayed. Activities of all the esterases tested were strongly inhibited by DFP. The results suggest that a serine residue is present in the a-

ctive site or substrate binding site of the esterases, as in the esterases of animal tissues.

Substrate specificity

The action of the four esterases on methyl esters of fatty acids and triacylglycerols are shown in Table II. They hydrolyzed only the esters of short chain fatty acids (C2–C4) except that Esterase-II also hydrolyzed methyl esters of medium chain fatty acids (C6–C10). None of the esterases hydrolyzed esters of long chain fatty acids, e.g. olive oil, tristearin, methyloleate or methylpalmitate.

Table III summarized the extent of hy-

TABLE II. HYDROLYSIS OF FATTY ACID ESTERS BY FOUR ESTERASES
FROM *Aspergillus niger*

Experimental conditions are described in the text. After the reaction, liberated fatty acids were titrated with 0.05 M KOH. End point of titration was pH 8.

Substrate	Esterase-I	Esterase-II	Esterase-III	Esterase-IV
0.05 M KOH (ml)				
Methylacetate	0.5	0.1	0.1	1.2
Methylpropionate	1.0	0.1	0.9	0.2
Methylbutyrate	0.6	0.1	0.2	0.1
Methylcaproate	0.4	0.4	0	0
Methylcaplyrate	0.1	2.1	0	0
Methylcaprate	0	0.6	0	0
Triacetin	2.0	0.2	1.6	3.4
Tripropionin	1.9	1.1	0.4	0.1
Tributylin	0.3	1.4	0.4	0.1
Tricaproin	0	0.3	0.2	0
Tricaplyrin	0	0	0	0
Tricaprin	0	0	0	0

TABLE III. HYDROLYSIS OF AROMATIC ESTERS BY FOUR ESTERASES
FROM *Aspergillus niger*

Experimental conditions are described in the text. After the reaction liberated fatty acids were titrated with 0.05 M KOH. End point of titration was pH 7.

Substrate	Esterase-I	Esterase-II	Esterase-III	Esterase-IV
0.05 M KOH (ml)				
Methylacetylsalicylate	1.1	3.5	2.7	1.0
Phenylacetate	2.0	2.1	1.5	0.9
α -Naphthylacetate	0.5	0.3	0	0
β -Naphthylacetate	0	0	0	0
Benzylacetate	0	0	0	0
β -Phenethylacetate	0	0	0	0
Methylsalicylate	0	0	0	0
Methylbenzoate	0	0	0	0

drolysis of various aromatic esters by the esterases. All four esterases hydrolyzed acetyl esters of phenols (phenylacetate, methylacetylsalicylate) but not methyl esters of aromatic carboxylic acids (methylbenzoate, methylsalicylate) or acetyl esters of aromatic alcohols (benzylacetate, β -phenethylacetate). Esterase-I and Esterase-II hydrolyzed α -naphthylacetate but Esterase-III and Esterase-IV did not. The action of Esterase-I and Esterase-II on several substrates other than the compounds shown in Table II was investigated. Among them, *o*-cresolacetate, *m*-cresolacetate, *p*-cresolacetate, *p*-nitrophenylacetate and resocinoldiacetate were hydrolyzed by Esterase-I and Esterase-II, as well as phenylacetate. Acetylcholine and *p*-nitrophenylphosphate were not hydrolyzed by any of the four esterases.

The specificity of each esterase on the two ester bonds of methylacetylsalicylate was investigated. Each esterase was allowed to react with methylacetylsalicylate, and part of the reaction mixture was injected directly onto HPLC at different reaction times. The products were identified by their retention times. Only methylsalicylate was detected, and acetylsalicylate and salicylic acid were not found at any stage of the reaction. In this manner, it was revealed that the four esterases cleaved only the acetyl bond of methylacetylsalicylate and did not cleave the methyl bond. This result agreed with the data shown in Table III.

As described above, the four esterases from

Aspergillus niger hydrolyzed not only aliphatic esters (Table II) which are the usual substrates for carboxylesterases (EC 3. 1. 1. 1) but also aromatic esters (Table III) which are the substrates for arylesterases (EC 3. 1. 1. 2). This is an example of the ambiguity of the present classification of esterases, as pointed out also by Krish.⁵⁾ However, according to the presently-used nomenclature system, these four esterases from *Aspergillus niger* appear to be classified as carboxylesterases, because most of the animal esterases which can hydrolyze both aliphatic and aromatic esters have been so classified.

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