A NOVEL CARBOXYLESTERASE FROM *ASPERGILLUS NIGER* AND ITS HYDROLYSIS OF SUCCINIMIDE ESTERS

by

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A novel carboxylesterase (EC 3.1.1.1) from *Aspergillus niger* has been purified 1400-fold by ammonium sulphate fractionation, ion exchange chromatography, hydrophobic interaction chromatography and gel filtration. The enzyme consisted of two identical subunits, each with a molecular weight of 60,000. The isoelectric point was 4.5. The optimal pH for the hydrolysis of cinnamic acid ethyl ester and 2-furylacryloyl N-hydroxy succinimide ester was between 5 and 7. The enzyme, which had no lipase activity, catalyzed the hydrolysis of activated ester substrates where the alcohol moiety was N-hydroxysuccinimide, p-nitrophenol and phenol as well as, with lower rates, unactivated esters like ethyl and benzyl esters. The enzyme exhibited a preference for substrates with an acyl moiety containing an aryl group. The enzyme was inhibited by PMSF but not by Hg²⁺ and EDTA. It is classified as a serine carboxylesterase.

1. INTRODUCTION

Carboxyl ester hydrolases (EC 3.1.1.–), which catalyze the hydrolysis of ester bonds, are widely distributed in nature. Based on their hydrolytic specificities, these enzymes may be divided into carboxylesterases (EC 3.1.1.1), arylesterases (EC 3.1.1.2) and lipases (EC 3.1.1.3). Lipases hydrolyse triacyl-glycerols and a number of other esters, participating in hydrolysis reactions at the water/fat interface. Arylesterases

preferentially cleave aromatic esters, such as esters of phenol, naphthol and indole (4). Carboxylesterases, which are rather unspecific, catalyze the hydrolysis of ester bonds, where both the alcohol moiety and the acid moiety may be aliphatic as well as aromatic (3, 5, 11, 14, 18, 19).

Carboxylesterases are involved in a number of biotransformations: hydrolysis of xenobiotic esters, activation of prodrugs, and metabolism of extraneous compounds. Some blood esterases

Abbreviations: Ac = acetyl; Bz = benzoyl; Bzl = benzyl; Caps = 3-(cyclohexylamino)-1-propanesulfonic acid; Ches = 2-(N-cyclohexylamino)ethane sulfonic acid; Cin = cinnamoyl; DMF = N,N-dimethylformamide; DMSO = dimethylsulfoxide; EDTA = ethylenediamine tetraacetic acid; Et = ethyl; Fa = furylacryloyl; Hepes = N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid; Hex = hexanoyl; HPLC = high performance liquid chromatography; Me = methyl; Mes = 2-(N-morpholino)ethanesulfonic acid; Ph = phenyl; PMSF = phenylmethylsulfonyl fluoride; Pr = propionyl; Oct = octanoyl; ONp = p-nitrophenoxy; ONSu = succinimidooxy; SDS = sodium dodecyl sulfate; Z = carbobenzoxy.

have been found to hydrolyse ester drugs (17). In insects, a carboxylesterase has been found to be involved in the inactivation of juvenile hormone (27); administration of certain carboxylesterase inhibitors to pupae of the tobacco hornworm diminished its inactivation (2). In plants, carboxylesterases may be responsible for the hydrolysis of certain bitter tasting compounds and may thus play a role in providing flavors which attract animals to feed on the fruits. The carboxylesterases from apples have been found to hydrolyze substrates such as ethyl, propyl, pentyl and hexyl acetate (8). Carboxylesterases in microorganisms may be involved in chemotaxis which permits microorganisms to reach food sources by catalyzing demethylation and hence, activation of membrane-bound proteins capable of binding sugars and amino acids (1).

Mammalian carboxylesterases from liver (3, 11, 14, 15), kidney (6), pancreas (19), brain (12), and skin (23) have been studied. Those from liver are the best characterized; they are trimers consisting of three identical subunits with a molecular weight of 60,000. Although carboxylesterases have been detected in microorganisms, only a single one has been isolated and to some extent described (5). In recent years, carboxylesterases have been used for the synthesis of a number of esters: transesterifications of methyl and ethyl esters of acrylic and methacrylic acids (25) and of N-carbobenzoxy-L-tyrosine p-nitrophenol ester (22) have been reported.

We here describe the isolation and characterisation of an extracellular carboxylesterase (EC 3.1.1.1) from *Aspergillus niger*.

2. MATERIALS AND METHODS

2.1. Materials

Palatase A, a crude extract obtained by fermentation with *Aspergillus niger*, chymotrypsin and subtilisin A were from Novo Industri, Denmark. Pronase, lipase VI and VII were from Sigma, USA. Carboxypeptidase Y was a laboratory preparation (13). Mes, Hepes, ethyl cinnamate, phenyl acetate as well as p-nitrophenyl esters were from Sigma, USA. Z-Ala-OSu and Z-Val-OSu were from Bachem, Switzerland. Chromatographic materials were from Pharmacia, Sweden. Benzyl cinnamate and ethyl ben-

zoate were from Aldrich-Chemie, West Germany. Z-ONSu, t-cinnamic acid and 3-phenylpropionic acid were from Fluka Chemie AG, Switzerland. p-Nitrophenyl cinnamate, phenyl cinnamate and cinnamanilide was prepared in the following way: 0.01 mole cinnamic acid and 0.01 mole 4-nitrophenol, phenol, aniline or N-hydroxy-succinimide were dissolved in 20 ml tetrahydrofuran and cooled to 0 °C. 2.06 g N,N'-dicyclohexylcarbodiimide was added and left at 0 °C for 2 hours, and then at 25 °C for one hour. The reaction mixture was filtered on a sintered glass funnel to remove the precipitate. The filtrate was evaporated to dryness and crystallization was achieved from ether/benzin (p-nitrophenol cinnamate) or ethanol/water (phenyl cinnamate and cinnamanilide). The melting points of different ester compounds were determined: phenyl cinnamate, 75.5-76.5 °C; p-nitrophenyl cinnamate, 145-146 °C; cinnamanilide, 150-151 °C; Cin-ONSu, 178-179 °C.

2.2. Methods

2.2.1. Enzyme assays

During purification the enzymatic activity at 25 °C towards Fa-ONSu was determined spectrophotometrically at 337 nm using a Perkin-Elmer Lambda 17. The following routine assay mixture was used: 10 μ l 8 mM Fa-ONSu, dissolved in methanol, was added to 980 μ l 0.05 M Mes, 1 mM EDTA, pH 6.3, followed by addition of 10 μ l enzyme solution. A $\Delta E_{337} = 21200 \text{ M}^{-1} \text{CM}^{-1}$ was determined.

The activity at 25 °C towards various other substrates was assayed by using the following assay mixture: 5 μ l 25 mM substrate in DMSO was added to 985 μ l of the same buffer as described above, followed by addition of 10 μ l enzyme solution. The following wavelengths and extinction coefficients were used: $\Delta E_{305} = 7600 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Cin-OEt), $\Delta E_{270} = 2500 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Z-ONSu), $\Delta E_{310} = 15000 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Cin-ONSu), $\Delta E_{260} = 1400 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Cin-OBzl), $\Delta E_{300} = 16000 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Cin-OPh), $\Delta E_{348} = 5200 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (Cin-ONp), and $\Delta E_{348} = 5200 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (p-nitrophenyl esters) and $\Delta E_{270} = 1500 \text{ M}^{-1} \cdot \text{CM}^{-1}$ (phenyl acetate) were used. One activity unit was defined as the amount of enzyme necessary to break down one micromole of substrate per

minute.

Lipase activity was determined at 30 °C by conventional titration using tributyrin as a substrate. Substrate emulsion was prepared by blending 15 ml tributyrin (Merck), 50 ml emulsification reagent (0.3 M NaCl, 3 mM KH_2PO_4 , 54% glycerol (v/v) and 0.6% Gum Arabic) and 235 ml deionized water. The assay procedure was performed as follows: 10 ml substrate was preheated for at least 3 minutes at 30 °C, followed by the addition of 100 μl of enzyme solution (0.7 mg/ml). A control was performed using a known lipase. The butyric acid liberated was titrated with 0.05 M NaOH by means of the pH-stat technique.

The protein concentration was determined spectrophotometrically at 280 nm using A_{280} (1%) = 18.7, derived from the amino acid composition and carbohydrate content of the pure enzyme.

2.2.2. Purification

1 liter Palatase A was diluted with 2 liters of water followed by addition of ammonium sulfate to 45% saturation. Continuous stirring was maintained at 4 °C for 12 hours. The precipitate was removed by centrifugation and to the supernatant was added ammonium sulfate to 60% saturation. After 16 hours of stirring at 4 °C, the suspension was centrifuged. The precipitate was dissolved in water and diafiltrated against 0.02 M sodium acetate, pH 4.3 using a Pellicon system from Millipore. The sample was applied to a CM-52 cellulose column (10 $\times$ 25 cm), equilibrated with the same buffer, and the column was washed with 0.02 M sodium acetate pH 4.3 until A_{280} of the eluate was below 0.2. The enzyme was eluted by applying a 0-0.3 M sodium chloride gradient in the same buffer. The fractions containing activity were pooled, concentrated by ultrafiltration as described above and adjusted to 1 M ammonium sulphate. This concentrate was applied to an Octyl-Sepharose column (5 $\times$ 20 cm) and a Phenyl-Sepharose column (2.6 $\times$ 20 cm), connected in this order in series and equilibrated with 0.02 M sodium acetate, 1 M ammonium sulfate pH 4.3. The enzyme passed through the Octyl-Sepharose column and bound to the Phenyl-Sepharose column. One bed volume

of the equilibrating buffer was used to wash the two columns and after disconnecting the two columns the Phenyl-Sepharose column was washed with another two bed volumes of 0.1 M ammonium sulphate, 0.02 M sodium acetate pH 4.3. The enzyme was eluted from the Phenyl-Sepharose column with 20 mM 3-phenylpropionic acid in 0.02 M sodium acetate, at the same pH. The pooled fractions from Phenyl-Sepharose column was concentrated to 6 ml by ultrafiltration using an Amicon cell. The pH of the sample was adjusted to 6.5 with 0.5 M phosphate buffer (pH 6.5) and applied to a Sephadex G-150 column (2.6 $\times$ 90 cm), equilibrated with 50 mM KH_2PO_4 , 0.1 M NaCl, pH 6.5.

2.2.3. Homogeneity, isoelectric point and molecular weight

The purity and molecular weight of the isolated enzyme with or without reduction with dithiothreitol was determined by SDS-polyacrylamide gel electrophoresis using a 10-15% gradient gel and the Pharmacia Phast system GE-2/4LS. Coomassie blue was used for staining. The molecular weight of the native enzyme was determined by gel filtration on Sephacyl S-300 HR. The column (1.6 $\times$ 90 cm) was equilibrated with 0.05 M KH_2PO_4 , 0.1 M NaCl, pH 7.5, and calibrated with standard proteins.

The isoelectric point of the pure enzyme was determined by focusing in a polyacrylamide gel (1 mm thick, 10% glycerol slabs, Phamlyte interval 3-10 and 4.15-5.85). Coomassie blue was used for staining. The isoelectric focusing was carried out in a Pharmacia gel electrophoresis apparatus GE-2/4 LS.

2.2.4. Chemical composition

Samples were hydrolysed in 6 M HCl at 110 °C in vacuo for 24, 48 and 72 hours (21). The hydrolysates were analyzed on Durrum D-500 amino acid analyzer. Tryptophan content was determined by the procedure of GOODWIN and MORTON (9). Half-cystine was determined as cysteic acid after performic acid oxidation (10). The carbohydrate content was determined by the method of DUBOIS (7), using glucose as a standard.

2.2.5. Stability

The stability of the enzyme was estimated by incubating 25 μ l enzyme (0.7 mg/ml) in 475 μ l 0.05 M Mes, 1 mM EDTA pH 6.3 at 22 °C, 30 °C, 40 °C and 50 °C. Aliquots were taken for assay using Fa-ONSu as substrate (see section 2.2.1). The stability at different pH values was determined at 25 °C by incubating 25 μ l enzyme (1.4 mg/ml) in 475 μ l 50 mM buffer (formic acid, pH 3.3-4.2; acetic acid, pH 4.3-5.4; Mes, pH 5.5-7.0; Hepes, pH 7.1-8.0; Ches, pH 8.1-8.8; Caps, pH 9.0-11). Aliquots were taken for assay at different times. The activity towards ethyl cinnamate was assayed using the procedure described in section 2.2.1.

The influence of organic solvents on the activity was assayed in the following way: to 980 μ l assay buffer (50 mM Mes, 1 mM EDTA, pH 6.3), containing 50%, 25%, 10% or 5% of different organic solvents (dioxane, DMF, EtOH, DMSO and MeOH) was added 10 μ l of enzyme (0.118 mg/ml) and 10 μ l 8 mM Fa-ONSu in DMSO.

2.2.6. Enzymatic properties

K_m and k_{cat} values were determined graphically from Lineweaver-Burk plots. 25% DMSO was included in the assay buffer. The pH dependence was determined using the buffer system described in the previous section. Ethyl cinnamate and Fa-ONSu were used as substrates. The assay was performed as follows: 5 μ l 25 mM ethyl cinnamate in DMSO was added to 985 μ l 50 mM buffer (section 2.2.5), followed by addi-

tion of 10 μ l enzyme solution (0.24 mg/ml); with Fa-ONSu, 10 μ l 8 mM substrate in DMSO was added to 980 μ l 50 mM buffer, followed by addition of 10 μ l enzyme solution 0.031 mg/ml). The effect of PMSF was investigated by incubating the enzyme (0.04 mg/ml) in 500 μ l 50 mM Mes, 1 mM EDTA, pH 6.3, containing 0.1 mM PMSF. The activity towards Fa-ONSu (see section 2.2.1) was assayed as function of time.

The effects of mercuric ions on the enzymatic activity was investigated in the following way: 10 μ l enzyme solution (0.024 mg/ml) was added to a cuvette containing 980 μ l 50 mM Mes pH 6.3, 50 μ M $HgCl_2$, and after incubation for 20 minutes, the activity was measured by addition of 10 μ l 4 mM Fa-ONSu in DMSO. The effects of salt and EDTA on the enzymatic activity was investigated in 50 mM sodium acetate, pH 4.5 with or without EDTA and NaCl. The activity towards Fa-ONSu was determined.

3. RESULTS

A number of commercially available enzyme preparations obtained by fermentation with fungi were found to hydrolyse 2-furylacryloyl-N-hydroxy-succinimide ester (Fa-ONSu). Enzymatic hydrolysis of such activated esters, which could be monitored spectrophotometrically at 337 nm, have not previously been reported. It was initially believed that this activity was due to a proteolytic enzyme or a lipase. However, even at high concentrations (1 mg/ml), the enzymes chymotrypsin, subtilisin A, carboxypeptidase Y, pronase as well as lipases VI and VII,

Table I. Purification of carboxylesterase

Purification step	ml	A_{280}	Fa-ONSu		
			U/ml	U _{total}	U/mg
Crude extract	1000	210	6.66	6600	0.058
(NH ₄) ₂ SO ₄ (45%)	3300	73	2.19	7300	0.056
(NH ₄) ₂ SO ₄ (60%)	2000	18	2.37	4700	0.25
CM-52	1100	2.1	3.16	3500	2.8
Octyl/Phenyl-Sep.	550	0.18	3.0	1700	32
G-150	32	0.70	30.9	990	82

The assay was performed as described in section 2.1.1.

were unable to hydrolyse Fa-ONSu. This appeared to exclude the possibility of a protease or a lipase. A carboxylesterase was then considered the most probable. To establish the origin of this activity the enzyme was isolated (Table I).

It was not possible to precipitate the enzyme in the crude extract with ammonium sulfate, possibly due to the high content of glycerol in the preparation, added for stabilization. However, after a three-fold dilution in water, an ammonium sulphate fractionation (45%-60%) could be performed. In the ion exchange chromatography, we found that the capacity of the resin decreased with pH in the range 4-5. In hydrophobic interaction chromatography, a stronger binding to Octyl-Sepharose as compared with Phenyl-Sepharose would be predicted. However, with the present enzyme the opposite was the case suggesting that the binding to the Phe-

nyl-Sepharose was not purely hydrophobic by nature. This was confirmed by the inability of buffer without ammonium sulphate to elute more than 30% of enzyme; the major part remained on the column, and could be eluted with 40% ethyleneglycol, apparently due to a different type of interaction. By including 20 mM 3-phenylpropionic acid in the buffer 60% of the enzyme was eluted, suggesting that this resin to some extent behaves as an affinity resin and 3-phenylpropionic acid competes with the immobilized ligand.

The isolated enzyme showed a single band on SDS-polyacrylamide gel electrophoresis with a mobility corresponding to a molecular weight of 60,000 both in the presence and in the absence of dithiothreitol. The molecular weight of the native enzyme was 100,000 as determined by gel filtration (Sephacryl S-300 HR) indicating that the enzyme is a dimer consisting of two subunits each of molecular weights 60,000. The enzyme had an isoelectric point of 4.5. This was in good agreement with the strong pH dependence of its binding to CM-52 cellulose in the pH range 4.0-5.0 (see above). The amino acid composition of the carboxylesterase is listed in Table II.

At pH 6.3 the enzyme was stable at 22 °C and 30 °C over 24 hours. At 40 °C and 50 °C the activity was lost with half-lives of 23 hours and 1.5 hour. At 22 °C the enzyme was stable over 24 hours at pH 3.2 and 6.3 whereas at pH 9.5 the activity was lost with a half life of approximately 12 hours.

Table II. Amino acid composition of carboxylesterase

Amino acid	Residues per subunit
Asp	66
Thr	31
Ser	35
Glu	42
Pro	35
Gly	47
Ala	43
Val	28
Met	6
Ile	18
Leu	29
Tyr	30
Phe	22
His	10
Lys	15
Arg	22
Cys	8
Trp	12
Carbohydrate	4%

The experimental values were based on the amino acid analysis after hydrolysis by 6 M HCl for 24, 48, and 72 hours (21). The values of serine and threonine were determined by extrapolation to zero time. Cysteine was determined as cysteic acid after performic acid oxidation (10). Tryptophan was determined spectrophotometrically by the method of GOODWIN and MORTON (9). The carbohydrate content was determined by the method of DUBOIS (7) et al.

Table III. Effects of organic solvents on the enzymatic activity

Solvent	Concentration of solvent in assay mixture			
	50%	25%	10%	5%
Dioxan	0	2	17	29
DMF	0	20	49	59
EtOH	0	15	56	61
DMSO	0	41	63	67
MeOH	0	48	96	110

The activity (U/mg) was determined as described previously. The following assay procedure was used: to 980 μ l, 50 mM Mes, 1 mM EDTA, pH 6.3, containing 50%, 25%, 10% or 5% of different organic solvents was added 10 μ l 8 mM Fa-ONSu in DMSO, followed by addition of 10 μ l enzyme solution (0.118 mg/ml) (see section 2.2.5).

The substrates for the isolated enzyme were generally only slightly soluble in water and therefore had to be added to the aqueous buffer from a solution in a water-miscible solvent. The influence of such solvents on the activity of the enzyme was consequently studied (Table III). All the solvents tested abolished the enzymatic activity at 50% and at lower concentrations the inhibition was in the following decreasing order: dioxane > DMF > EtOH > DMSO > MeOH.

Figure 1. The influence of pH on the activity of the carboxylesterase on the hydrolysis of Cin-OEt (■) and Fa-ONSu (●). The following conditions were used: 980 μ l 50 mM buffer, 1 mM EDTA + 10 μ l 8 mM Fa-ONSu in DMSO (or 5 μ l 25 mM Cin-OEt in DMSO) + 10 μ l enzyme. For details see section 2.2.1.

Figure 2. The influence of pH on the kinetic parameters for the hydrolysis of Cin-OEt, K_m (■), k_{cat} (●), k_{cat}/K_m (□). The following conditions were used: 985 μ l 50 mM buffer, 1 mM EDTA + 5 μ l Cin-OEt (7.5–50 mM in DMSO) + 10 μ l enzyme solution. The values were determined from Lineweaver-Burke plots. For details see section 2.2.1.

The pH optima for the hydrolysis of ethyl cinnamate and Fa-ONSu were between 5.5 and 7.0 (Figure 1). The K_m value was found to be independent of pH while k_{cat} appeared to be dependent on the deprotonation of a group with a pK_a value around 5 and on the protonation of a group with a pK_a value around 8 (Figure 2). The enzyme did not hydrolyse tributyrin, strongly suggesting that it is not a lipase. However, it hydrolysed a number of esters of cinnamic acid (Table IV): the activated N-hydroxy succinimide,

Table IV. Carboxylesterase catalyzed hydrolysis of ester substrates: the influence of the alcohol moiety

Substrate	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
Cin-ONSu	23000	0.10	2.3×10^5
Cin-ONp	12000	0.10	1.2×10^5
Cin-OPh	24000	0.25	9.6×10^4
Cin-OBzl	2900	0.17	1.7×10^4
Cin-OEt	1100	0.67	1.6×10^3
Cin-NHPh	—	—	< 10

The assays were carried out by using 740 μ l 50 mM Mes, 1 mM EDTA, pH 6.3, 240 μ l DMSO (25%) and 10 μ l of substrate in DMSO, followed by addition of 10 μ l enzyme solution. The activity measurements are described in 2.2.1.

p-nitrophenyl and phenyl esters were hydrolysed with the highest k_{cat}/K_m , whereas the ethyl and benzyl esters were hydrolysed with significantly lower values. A comparison of the kinetic parameters for the hydrolysis of the structurally similar phenyl and benzyl esters suggest that the different k_{cat}/K_m values between the activated and unactivated ester primarily is due to an effect on k_{cat} . The higher k_{cat}/K_m for the hydrolysis of the benzyl ester as compared with the equally activated ethyl ester, on the other hand, is primarily due to a difference in K_m . It should be noted that the anilide of cinnamic acid is not hydrolysed by the enzyme suggesting that the enzyme is specific for ester bonds and does not hydrolyse amide bonds.

The influence of the structure of the acyl component on the kinetic parameters was investigated with a series of aromatic N-hydroxy-succinimides and p-nitrophenyl esters of different aliphatic acids. Among the latter k_{cat}/K_m increased with

the chain length and reached the highest value with the p-nitrophenyl ester of octanoic acid. The activity towards substrates of acids with longer chains could not be determined due to lack of solubility in the assay medium (25% DMSO). However, the k_{cat}/K_m values for hydrolysis of these substrates are low relative to that of the p-nitrophenyl ester of cinnamic acid, containing an aromatic group, and this is primarily due to an effect on k_{cat} . The preference for an acyl moiety containing an aromatic group is also apparent from the high k_{cat}/K_m values for the hydrolysis of various aromatic succinimide esters in Table V.

Table V. Carboxylesterase catalyzed hydrolysis of ester substrates: the influence of the acyl moiety

Substrate	k_{cat} (min^{-1})	K_m (KM)	k_{cat}/K_m ($\text{min}^{-1} \cdot \text{mM}^{-1}$)
Cin-ONSu	23000	0.1	2.3×10^5
Fa-ONSu	9300	0.06	1.6×10^5
Z-ONSu	4800	0.04	1.2×10^5
Z-Ala-ONSu	25000	0.75	3.3×10^4
Z-Val-ONSu	—	—	$<< 1$
Ac-ONp	200	0.33	6.1×10^2
Pr-ONp	330	0.37	8.9×10^2
Hex-ONp	670	0.24	2.8×10^3
Oct-ONp	500	0.04	1.3×10^4

The assays were carried out by using 740 μl 50 mM Mes, 1 mM EDTA, pH 6.3, 240 μl DMSO (25%) and 10 μl of substrate in DMSO, followed by addition of 10 μl enzyme solution. The activity measurements are described in section 2.2.1.

Other experiments showed that the enzyme did not catalyze the hydrolysis of simple esters, e.g., ethyl, butyl and propyl esters of octanoic acid.

The enzyme was completely inhibited by PMSF within 30 minutes. However, 50 μM of mercuric ions only decreased the activity by 20% and Ca^{2+} and EDTA had no influence on the activity.

4. DISCUSSION

Carboxylesterases have been found in a wide variety of higher plants (16) and animals (11,14), while only one such enzyme has been described from a microorganism (5). The present enzyme from *Aspergillus niger* was inhibited by PMSF but not by mercuric ions and EDTA. Inhibition by PMSF is indicative of the involvement of a seryl residue and an acyl-enzyme intermediate in the catalytic mechanism. Similar results have been obtained with carboxylesterases from mammalian liver (15).

Most carboxylesterases thus far described exhibit optimal activity around pH 8 (5, 26). However, JUNGE has reported that an isoenzyme of pig-liver esterase had a pH optima between 6-7 (15). In our case, the optimal pH for the hydrolysis of Fa-ONSu and ethyl cinnamate was found in the pH range of 5.5-7. The enzyme is a dimer with two identical subunits, each with a molecular weight of 60,000 as opposed to the carboxylesterases from mammalian liver which are trimers with three identical subunits of molecular weights of 60,000 (3, 14, 26).

The enzyme hydrolyses ester substrates of the type $\text{R}_1\text{-COO-R}_2$ with a preference for those with activated leaving groups (R_2), i.e., -ONSu $>$ -ONp $>$ -OPh $>$ -OBzl $>$ -OEt. The series of cinnamic acid esters are hydrolysed through the same acyl-enzyme intermediate and since the k_{cat} values vary from 1100 to 24,000 min^{-1} , it may be concluded that the acylation step is rate-limiting, at least in the hydrolysis of the two alkyl esters, i.e., Cin-OEt and Cin-OBzl.

It appears that the enzyme exhibits a preference for substrates where both R_1 and R_2 contain an aromatic group. Substrates where either of the two groups is aliphatic and the other is aromatic are also hydrolysed. However, when both groups are aliphatic, e.g. the ethyl, butyl and propyl esters of octanoic acid, no hydrolysis was observed. The ability of the enzyme to hydrolyse other than aryl esters exclude a classification as an arylesterase. Classification of the enzyme as a carboxylesterase is the most reasonable although the enzyme exhibits a preference for substrates containing aryl groups.

Among the esterases isolated from other species only those from mammalian liver have been subjected to a similar characterisation with re-

spect to substrate preference. These enzymes hydrolyse substrates where both R_1 and R_2 (see above) are aliphatic in addition to substrates where they are aromatic (15). Since they also hydrolyse the lipase substrate tributyrin (this is also the case with a number of less well characterised carboxylesterases) it is questionable whether they should be classified as carboxylesterases. In any event the enzyme from *Aspergillus* described here is much more specific.

The possibilities of utilising this enzyme for synthesis of organic compounds, especially by exploiting its activity towards N-ahydroxysuccinimides, which are important intermediates in organic chemistry, have not yet been investigated.

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