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CHARACTERIZATION OF INTRACELLULAR ESTERASE A FROM *BACILLUS SUBTILIS*

JOHN F. RIEFLER III and THOMAS B. HIGERD

Department of Basic and Clinical Immunology and Microbiology, Medical University of South Carolina, Charleston, S.C. 29401 (U.S.A.)

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Summary

Esterase A (EC 3.1.1.1) obtained by sonic disruption of *Bacillus subtilis* SR22 (*spoA12, trpC2*) was purified approximately 400-fold by differential chemical and heating precipitation, DEAE-cellulose chromatography, and Bio-Rad P-150 gel filtration chromatography, with an overall yield of 59%. The purified enzyme hydrolyzed both aliphatic and aromatic acetate esters at substrate concentrations of 0.25 M but did not hydrolyze amino acid esters. Aliphatic alcohols did not inhibit the hydrolysis of *p*-nitrophenyl acetate; the most potent inhibitors of esterase activity were mercuric chloride, diisopropylfluorophosphate, eserine, and sodium fluoride.

Introduction

Carboxylesterases (EC 3.1.1.1) of multiple forms have been found in bacteria, fungi, plants, and animals, but only a few have been fractionated and characterized. Previous studies [1,2] with *Bacillus subtilis* indicate the existence of two intracellular esterases. Esterase A, with lower electrophoretic mobility, is relatively heat stable and has a molecular weight of about 160 000 [2]. This enzyme is produced during the exponential growth phase (Higerd, T.B., unpublished) and is present in early blocked asporogeneous mutants [2]. The esterolytic activity of esterase A does not appear to be involved in sporulation, because a mutant of *B. subtilis* that lacks esterase A and is able to sporulate at the wild-type frequency has been isolated (Higerd, T.B. unpublished).

It has not been shown whether *B. subtilis* esterase A is similar to the *Pseudomonas cepacia* undecyl acetate esterase described by Shum and Markovetz [3], which apparently hydrolyzes an acetate ester intermediate in the catabolic pathway for 2-tridecanone, or whether it has similarities to mammalian carboxylesterases. Therefore, we have examined the catalytic properties of esterase A from *B. subtilis* by measuring the hydrolysis rates of esters with different

acyl or alkyl groups and by determining which compounds inhibit its esterolytic activity.

Materials and Methods

B. subtilus strain SR22 (*spoA12, trpC2*) growing in the sporulation medium of Greenleaf and Losick [4] supplemented with 0.5% glucose was harvested from a 350-l fermentor culture immediately after logarithmic phase. The cells were washed, resuspended in 0.02 M potassium phosphate buffer, pH 7.5, at a concentration of 0.2 g wet weight per ml of buffer, and stored frozen at −20° C.

Purification of esterase A was performed at 4° C unless noted otherwise. After the cells were sonicated for 14 min and centrifuged, $MnCl_2$ was added to the clarified sonicate (final concentration 0.05 M) and the pH was adjusted to 7.5. After centrifugation at 27 000 × *g* for 15 min, the resulting supernatant was treated with 40% acetone and left over night at −15° C. The sediment was centrifuged and the precipitate was dissolved in 0.5 M phosphate buffer, pH 7.5. The solution was heated at 70° C for 10 min and centrifuged, and the supernatant was dialyzed against 0.02 M phosphate buffer.

The dialyzed preparation was chromatographed on a DEAE-cellulose column (5 cm × 39.5 cm) previously equilibrated with 0.02 M phosphate buffer. Esterase activity was eluted on a linear potassium phosphate concentration gradient. Fractions containing esterase activity were pooled and mixed with an equal volume of 2.0 M phosphate buffer. The solution was heated at 80° C for 10 min and centrifuged. The supernatant was dialyzed against 50 vols. of distilled water and concentrated 20-fold by lyophilization. Aliquots of 1 ml were applied to a Bio-Rad P-150 column (1.5 cm × 90 cm) previously equilibrated with 0.02 M phosphate buffer, pH 7.5. The fractions with the highest esterase activities were pooled and used as the source of enzyme throughout the study.

A 400-fold purification of esterase A activity was achieved, with approx. 60% recovery. The purification procedure was that of Higerd and Spizizen [2], modified to give a higher percent yield. From densitometer tracings of acrylamide gels stained for protein with Coomassie brilliant blue and for esterase activity with β-naphthyl acetate, this partially purified preparation was estimated to be about 20% pure.

Esterolytic activity was assayed by two methods. The first assay, with *p*-nitrophenyl acetate as the substrate, was a modification of the procedure of Huggins and Lapides [5]. The reaction was conducted in 0.02 M potassium phosphate buffer, pH 7.5, at room temperature and was monitored with a Cary 118C spectrophotometer. This assay was used principally for inhibitor studies. The second method measured esterolytic activity against a variety of substrates by the pH-stat method. The reaction mixture (2.5 ml) contained 0.25 M substrate in 0.01 M potassium phosphate buffer, pH 7.5, with 0.1 M ethanol and 50 µl of the esterase preparation. The liberated acid was continuously back-titrated with 0.04 M NaOH to pH 7.55, employing an autotitrator TTT60 with automatic burette and recorder (Radiometer; London, Ohio). For both methods, 1 unit of activity was defined as 1 mmol of substrate hydrolyzed per min.

Determination of protein in esterase samples was based on the Lowry method [6]. Bovine serum albumin was used to prepare the standard curve.

A variety of potential inhibitors were dissolved in 0.02 M potassium phosphate buffer, pH 7.5, distilled water, acetonitrile, or methanol at a concentration permitting solubilization. None of the four solvents showed inhibition in controls with solvent alone. The mixture of inhibitor and enzyme preparation was incubated for 10 min at 37° C and assayed for remaining activity.

Preparations of *N*-heptyl acetate, undecyl acetate, and 2-tridecanone were kindly supplied by Dr. A.J. Markovetz, University of Iowa. β-Naphthyl acetate was recrystallized from dilute ethanol. DEAE-cellulose (Cellex D) was purchased from Bio-Rad Laboratories. All other reagents were commercial preparations of the highest purity available.

Results

Aliquots of the partially purified (400-fold) preparation of esterase A were incubated at several buffered pH values for 16 h, and the solutions were adjusted to pH 7.5 prior to assay for residual esterase activity. Esterase A activity was relatively stable above pH 6 but decreased significantly after incubation at lower values (Fig. 1A). A similar profile was obtained when esterase A preparations were assayed for activity at various pH values (Fig. 1B).

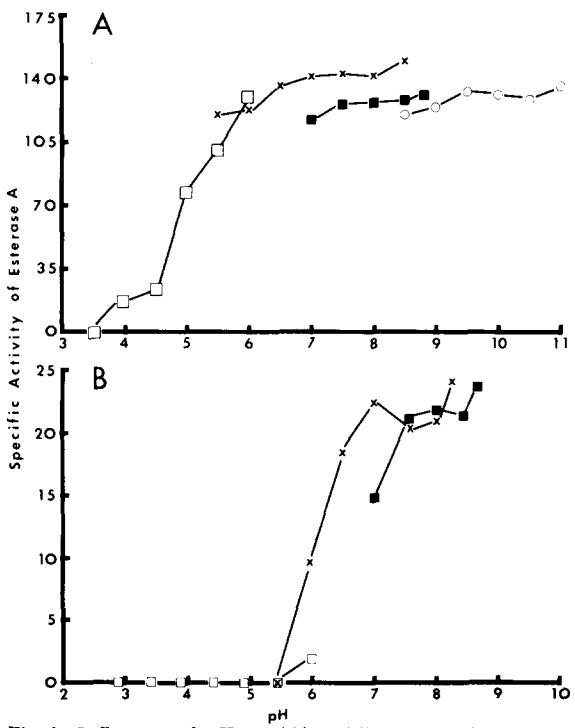

Fig. 1. Influence of pH on (A) stability and (B) activity of esterase A. The buffers were maintained at 0.05 M and consisted of citrate (□———□), phosphate (X———X), barbitol-HCl (■———■), and carbonate (○———○) buffers. For determining pH stability, aliquots of the same esterase A preparation were refrigerated for 16 h in buffers of various pH values and then returned to pH 7.5 by dilution prior to assay. Residual esterase activities were measured spectrophotometrically and by the pH-stat method in (A) and (B) respectively.

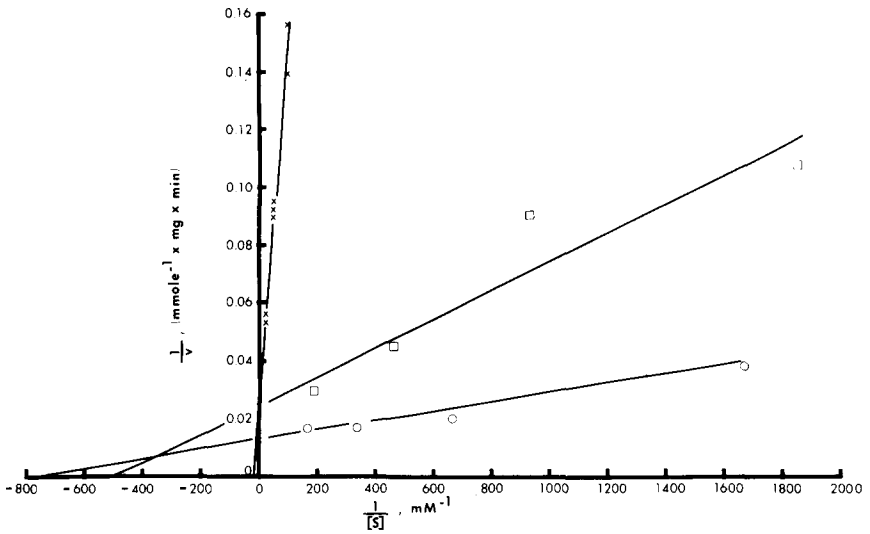

Fig. 2. Lineweaver-Burk plot for the hydrolysis of *p*-nitrophenyl acetate (V, 83.9 units/mg; K_m, 0.0013 mM) (○———○), β-naphthyl acetate (V, 41.7 units/mg; K_m, 0.0021 mM) (□———□), and ethyl acetate (V, 34.1 units/mg; K_m, 0.0435 mM) (X———X) by esterase A as measured by the pH-stat method. The apparent K_m and V constants were approximated by the method of least squares.

Assuming that the apparent K_m value reflects the affinity of the enzyme for the substrate, apparent K_m values were determined for *p*-nitrophenyl acetate, β-naphthyl acetate, and ethyl acetate. Under the conditions tested, esterase A gave a typical Michaelis-Menten curve with all three substrates and showed a preferential affinity for *p*-nitrophenyl acetate (Fig. 2).

Earlier results in our laboratory [2] indicated that esterase A is not reactive against casein. In this study, with substrate concentration of 1.33 mM, no hydrolysis of the amino acid esters that are commonly used as "specific"

TABLE I

INFLUENCE OF THE CHAIN LENGTH OF THE ACYL GROUP OF *p*-NITROPHENYL AND ETHYL ESTERS ON ENZYME ACTIVITY

Concentrations of *p*-nitrophenyl and ethyl esters were 0.1 and 60 mM respectively. Enzymatic activity for *p*-nitrophenyl derivatives determined spectrophotometrically and for ethyl derivatives determined by the pH-stat method were compared with values obtained for *p*-nitrophenyl acetate and ethyl acetate respectively.

Substrate	Activity (%)
p-Nitrophenyl acetate	100
p-Nitrophenyl propionate	13
p-Nitrophenyl butyrate	0
p-Nitrophenyl caproate	0
p-Nitrophenyl caprylate	0
p-Nitrophenyl caprate	0
Ethyl acetate	100
Ethyl propyionate	11
Ethyl butyrate	0

substrates for the serine proteases could be demonstrated by the pH-stat method. The substrates tested were *N*-acetyl-L-phenylalanine, *N*-acetyl-L-tyrosine, and *p*-tosyl-L-arginine methyl esters and *N*-benzoyl-L-arginine and *N*-benzoyl-L-tyrosine ethyl esters.

In an attempt to determine the specificity of esterase A toward the acid moiety of ester substrates, several derivatives of *p*-nitrophenyl acetate and ethyl acetate were tested (Table I). No hydrolytic activity was observed when the acyl portion of the ester contained four or more carbon atoms. In order to determine the influence of the alkyl residue upon esterase activity, the acyl group was kept constant and the alcohol moiety was varied from C_2 to C_{11} (Table II). In general, as the alkyl chain length increased, the activity decreased, becoming negligible beyond C_8.

Compounds that effectively inhibited esterase A activity are listed in Table III. A significant loss of esterase activity occurred during purification with DEAE-cellulose columns when NaCl gradients were applied to elute the activity. When concentrations greater than 0.2 M NaCl and KCl were added directly to the assay mixture, an immediate and significant loss of activity (about 40% reduction) resulted. Conversely, when the concentration of potassium phosphate buffer was increased to 1 M, there was an apparent increase in esterase activity (data not shown).

Discussion

The catalytic behavior of *B. subtilis* esterase A is similar to that of mammalian carboxylesterases, in that catalysis is exhibited only toward simple ali-

TABLE II

INFLUENCE OF THE CHAIN LENGTH OF THE ALKYL GROUP OF ACETATE ESTERS ON ENZYME ACTIVITY

The concentration of esters was 0.25 M. Enzymatic activity was measured by the pH-stat method and compared with values obtained for ethyl acetate.

Substrate	Activity (%)
Ethyl acetate	100
n-Propyl acetate	71
i-Propyl acetate	50
n-Butyl acetate	66
i-Butyl acetate	64
t-Butyl acetate	1
n-Pentyl acetate	43
n-Hexyl acetate	24
n-Heptyl acetate	16
n-Octyl acetate *	17
n-Nanyl acetate *	0
n-Decyl acetate *	0
n-Undecyl acetate *	0
Phenyl acetate *	94
Indoxyl acetate *	74

* These esters were not fully solubilized under the conditions of the assay.

TABLE III

EFFECTIVE INHIBITORS OF ESTERASE A ACTIVITY

Enzyme and inhibitor were incubated at 37°C for 10 min. The remaining activity was measured spectrophotometrically using *p*-nitrophenyl acetate as substrate and comparing the activity value to the control without inhibitor.

Inhibitor	Concentration (M)	Activity remaining (%)
Mercuric chloride	10^{-3}	0
	10^{-4}	49
	10^{-5}	86
	10^{-6}	99
Diisopropylfluorophosphate	10^{-3}	3
	10^{-4}	69
	10^{-5}	99
Phenylmethyl sulfonylfluoride	10^{-2}	85
	10^{-3}	94
	10^{-4}	96
Eserine	10^{-2}	7
	10^{-3}	61
	10^{-4}	90
Sodium fluoride	10^{-1}	36
	10^{-2}	83
	10^{-3}	101

phatic and aromatic esters. Esterase A can be clearly differentiated from the "acidic protease" and alkaline proteases recovered from the culture medium of *B. subtilis* [7—10], since it does not attack the amino acid esters hydrolyzed by the extracellular proteases.

Esterase A shows many similarities to pig and rat liver carboxylesterases. Like pig liver carboxylesterase at noninhibiting concentrations of substrate [11], the bacterial enzyme has a broad pH optimum; and like rat liver esterase, it is less active against the butyrate derivative than against the acetate derivative of *p*-nitrophenol [12] and prefers the *n*-isomer of the alkyl group over the branched isomers of propyl and butyl acetate esters. In addition, esterases from *B. subtilis* and rat liver are catalytically more sensitive to changes in the length of the acyl group than to changes in the length of the alkyl chain of the substrate.

On the other hand, in contrast to the typical Michaelis-Menten curve reported for the bacterial esterase, both pig and rat liver carboxylesterases show substrate activation kinetics [11,12]. Further, Dabich et al. [13] reported that rat brain esterase has a narrow pH optimum (pH 7.0 to 7.5), and that there were 7- and 34-fold reductions of adult rat brain esterase activity with β-naphthyl propionate and β-naphthyl butyrate as substrates, respectively.

Esterase A from *B. subtilis* does not appear to be similar to the undecyl acetate esterase from *P. cepacia* reported by Shum and Markovetz [14]. Al-

though the undecyl acetate esterase inhibition patterns and Michaelis-Menten curves for the two enzymes are similar, no hydrolysis of undecyl acetate by the *B. subtilis* esterase could be detected. In addition, attempts to inhibit esterase A activity with unbranched alcohols from C_1 and C_{11} (not listed) were unsuccessful. Undecyl acetate esterase was inhibited by undecanol, and the culture of *P. cepacia* was able to grow with undecyl acetate and 2-tridecanone as the sole carbon source. Attempts to culture *B. subtilis* 168 on 2-tridecanone were unsuccessful.

Thus, although apparent similarities exist in substrate specificities between esterase A and the liver carboxylesterases, esterase A does not exhibit substrate activation and does not hydrolyze L-tyrosine ethyl ester. Its broad specificity, as well as its sensitivity to organophosphate inhibition, tentatively classifies esterase A from *B. subtilis* as a non-specific esterase of the B type (EC 3.1.1.1).

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