Purification and Characterization of Thermophilic and Alkalophilic Tributyrin Esterase from *Bacillus* Strain A30-1 (ATCC 53841)[1]

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An extracellular esterase (EC 3.1.1.1) from a thermophilic *Bacillus* A30-1 (ATCC 53841) was purified 139-fold to homogeneity by sodium chloride (6 M) treatment, ammonium sulfate fractionation (30–80%) and phenyl-Sepharose CL-6B column chromatography. The native enzyme was a single polypeptide chain with a molecular weight of about 65,000 and an isoelectric point at pH 4.8. The optimum pH for esterase activity was 9.0, and its pH stability range was 5.0–10.5. The optimum temperature for its activity was 60°C. The esterase had a half-life of 28 h at 50°C, 20 h at 60°C and 16 h at 65°C. It showed the highest activity on tributyrin, with little or no activity toward long-chain (12–20 carbon) fatty acid esters. The enzyme displayed K_m and K_{cat} values of 0.357 mM and 8365/min, respectively, for tributyrin hydrolysis at pH 9.0 and 60°C. Cyclodextrin (α, β, and γ), Ca^{2+}, Co^{2+}, Mg^{2+} and Mn^{2+} enhanced the esterase activity, and Zn^{2+} and Fe^{2+} acted as inhibitors of the enzyme activity. The enzyme activity was not affected by ethylenediaminetetraacetic acid, p-chloromercuribenzoate and *N*-bromosuccinimide.

KEY WORDS: Alkalophilic, *Bacillus*, esterase, thermostable.

There are two types of fatty acid esterase—one acts on substrates in solution (EC 3.1.1.1), and the other acts predominantly on undissolved substrates (EC 3.1.1.2) (1). With the expanding application in stereospecific hydrolysis and synthesis of esters, the importance of esterases in biotechnology is growing rapidly. We have isolated a thermophilic and alkalophilic *Bacillus* strain A30-1 (ATCC 53841) that produces lipase (2–3). The lipase was optimally active at pH 9.5 and 60°C, over a broad range of long-chain fatty acid esters. We found that, in addition to lipase, the organism produced another enzyme, which showed maximum activity at 60°C on tributyrin (C4:0) and had little or no activity on long-chain fatty acid esters. In this paper, we report the purification and characterization of this novel tributyrin esterase from this organism.

MATERIALS AND METHODS

Organism, cultivation and preparation of enzyme. The microorganism used was thermophilic *Bacillus* strain A30-1 (deposited with American Type Culture Collection, Rockville, MD; ATCC 53841) isolated from the Yellowstone National Park hot springs area. It was maintained in 10% glycerol vials (at −80°C) or on agar slants at 4°C. For enzyme production, the organism was inoculated into several 1-L flasks, containing 500 mL of a medium containing corn oil (0.5%), yeast extract (0.5%), glucose (0.5%) and potassium monobasic phosphate (0.05%), and incubated at 60°C for 15–24 h with shaking (200 rpm). The culture broth with cells was concentrated by ultrafiltration (hollow-fiber cartridge, 30 K cut-off) and centrifuged to remove cells. The concentrated supernatant solution was used as the source of esterase.

Purification of esterase. All operations were performed at 4°C unless otherwise mentioned. The concentrated supernatant solution was treated with 6 M NaCl, kept at room temperature for 2 h and centrifuged at 45,000 × *g* for 20 min to remove the oil phase. It was then dialyzed against 4 L of 50 mM Tris-HCl buffer, pH 9.0 (buffer A) for 24 h with two changes. Solid ammonium sulfate was added to the dialyzed enzyme solution to give 35% saturation, and the mixed solution was left for 2 h. The precipitate formed was removed by centrifugation (45,000 × *g*, 25 min) and discarded. More ammonium sulfate was added to the supernatant solution to give a final concentration of 80% saturation, and the mixed solution was left overnight. The precipitate formed was collected by centrifugation, dissolved in buffer A (100 mL) and dialyzed against the same buffer (4 L) overnight. The dialyzed enzyme solution was adjusted to 35% saturation with ammonium sulfate. It was then applied to a phenyl-Sepharose CL-6B column (ϕ 2.6 × 25 cm) (Pharmacia LKB Biotechnology, Piscataway, NJ) which was pre-equilibrated with buffer A containing 35% ammonium sulfate. Then, the column was washed with buffer A to zero absorbance at A_{280} (3–5 bed vol), and a linear Triton X-100 gradient (0–1.5%, 500 mL each) in Tris-HCl buffer (50 mM, pH 8.2), containing 1 M NH_4Cl, was applied to elute the esterase. The active esterase peak fractions were combined, concentrated by ultrafiltration and dialyzed against buffer A overnight. The dialyzed enzyme solution was used for subsequent studies.

Enzyme assay. Esterase activity was routinely assayed by monitoring the butyric acid released from tributyrin. The reaction mixture (1.1 mL), containing tributyrin (9%), Tris-HCl buffer (50 mM, pH 9.0) and the enzyme solution, was incubated at 60°C for 1 h. The reaction was stopped by adding 0.1 mL of 10 M H_3PO_4. The butyric acid formed was measured by gas chromatography. One unit of esterase activity is defined as the amount of enzyme that liberates one micromole butyric acid per minute under the above assay conditions.

Other methods. Protein was estimated by the method of Lowry *et al.* (4), with bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (5). The molecular weight of native esterase was estimated by gel filtration on a Sephacryl S-200 (Pharmacia LKB Biotechnology) as described by Andrews (6). The isoelectric point was determined by running samples in a Servalyt Precote isoelectric focusing gel (pH 3-10) (SERVA Fine Biochemicals Inc., Westburg, NY) at constant power of 1 W in an LKB (Broma, Sweden) ultraphore isoelectric focusing apparatus, and by staining the gel with Serva Blue W (Serva). For amino acid composition determination, samples were prepared and analyzed by the PICOTAG method (Waters Associates,

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Milford, MA). The K_m was determined by the Lineweaver-Burk (7) method. The K_{cat} number was determined according to the method described by England and Singer (8).

RESULTS AND DISCUSSION

Purification of esterase. Table 1 summarizes the purification steps involved and the recovery of the esterase activity at each step from the culture supernatant of *Bacillus* strain A30-1. The final enzyme preparation was purified 139-fold over the crude enzyme, with an overall recovery of 61%, and had a specific activity of 55.5 units/mg protein. The NaCl (6 M) treatment was essential to allow protein in the concentrated crude culture broth to become precipitable by ammonium sulfate. The phenyl-Sepharose CL- 6B step removed most of the contaminating protein before the esterase was eluted (with a Triton X-100 gradient) (Fig. 1). The purified esterase showed a single protein band by SDS-PAGE when stained with Coomassie Brilliant Blue (Fig. 2).

Molecular properties. The molecular weight of the purified esterase was estimated at 64,000 by SDS-PAGE (Fig. 2) and 66,000 by gel filtration on Sephacryl S-200 (Fig. 3). Thus, it was a monomeric protein. The purified enzyme migrated as a single protein band on isoelectric focusing, and had a pI of 4.8 by comparison with commercial standards. Amino acid composition analysis of the purified enzyme indicates that the enzyme was rich in acidic and hydrophobic amino acid but low in methionine and histidine.

Physicochemical properties. The optimum temperature for the action of the purified esterase on tributyrin was 60°C, with about 65% activity at 50°C and 43% activity at 70°C (Fig. 4). The half-life of the enzyme was 28 h at 50°C, 20 h at 60°C and 16 h at 65°C without any substrate and/or co-factor. Thus, the esterase from the *Bacillus* strain A30-1 was fairly thermostable. Figure 5 shows that the esterase exhibited optimum activity at pH 9.0, with about 90% activity at both pH 10.0 and 8.0. The enzyme had a broad pH stability range (pH 5.0-10.5). Thus, the esterase was thermophilic and alkalophilic.

Catalytic properties. The purified enzyme showed highest activity on tributyrin, with only 43% activity on triacetin and 25% on tricaproin (Table 2). It showed no activity on trilaurin and other, longer chain fatty acid esters (with the exception of a 2% activity on cholesterol butyrate). Table 2 also compares the substrate specificity of the purified esterase from *Bacillus* strain A30-1 with that from porcine liver (Sigma Chemical, St. Louis, MO). The general enzyme kinetic features of *Bacillus* strain

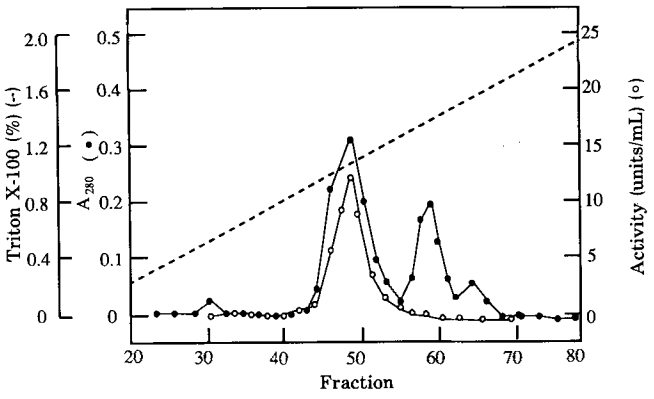

FIG. 1. Phenyl-Sepharose CL-6B column chromatography of esterase from *Bacillus* strain A30-1. The dialyzed enzyme solution after step 3 (see Table 1) was adjusted to 35% ammonium sulfate saturation and applied to a column (2.6 × 25 cm) pre-equilibrated with buffer A containing 35% ammonium sulfate. The column was washed with the same buffer and eluted with a gradient of 0-1.5% Triton X-100 in 50 mM Tris-HCl buffer, pH 8.2. Fraction volume, 10 mL.

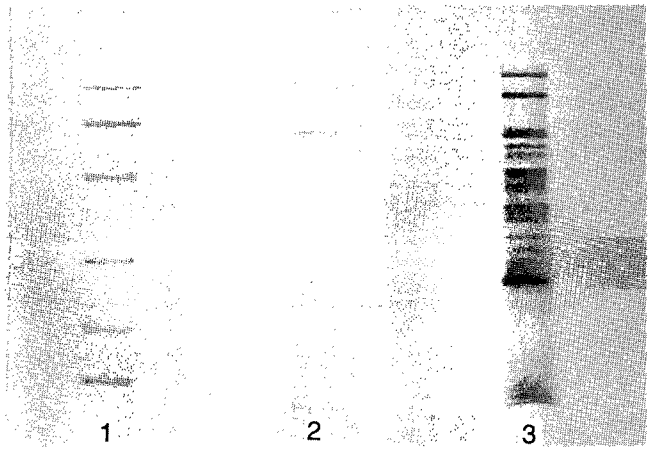

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of esterase. The enzyme was electrophoresed at pH 8.3 on 12% acrylamide gel and stained with Coomassie Brilliant Blue R-250. Lane 1, standard marker proteins; 2, purified esterase and 3, crude esterase preparation. The standards used were: rabbit muscle phosphorylase B (97,400), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (29,000), soybean trypsin inhibitor (21,500) and lysozyme (14,400).

TABLE 1

Purification of Esterase from *Bacillus* Strain A30-1 (ATCC 53841)

Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Recovery (%)
1) Concentrated broth	620	247	0.4	100
2) NaCl treatment (6 M)	600	188	0.3	76
3) Ammonium sulfate (35-80%)	275	169	1.5	68
4) Phenyl-Sepharose CL-6B	2.7	150	55.5	61

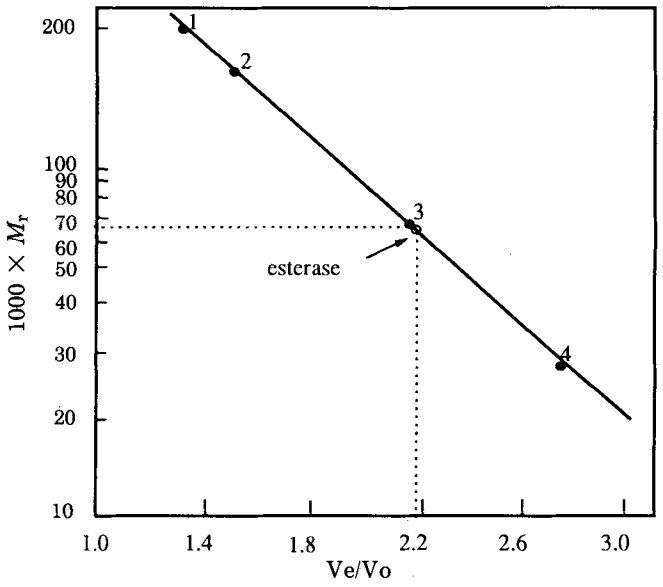

FIG. 3. Molecular weight (M_r) determination of native esterase by Sepharyl S-200 gel filtration. A column (1.5 × 150 cm) was used with a flow rate of 5 mL/h. Standard: 1, sweet potato β-amylase (200,000); 2, yeast alcohol dehydrogenase (150,000); 3, bovine serum albumin (66,000); and 4, carbonic anhydrase (29,000). Ve, elution volume of each protein. Vo, void volume of the column determined by Blue dextrian (2,000,000).

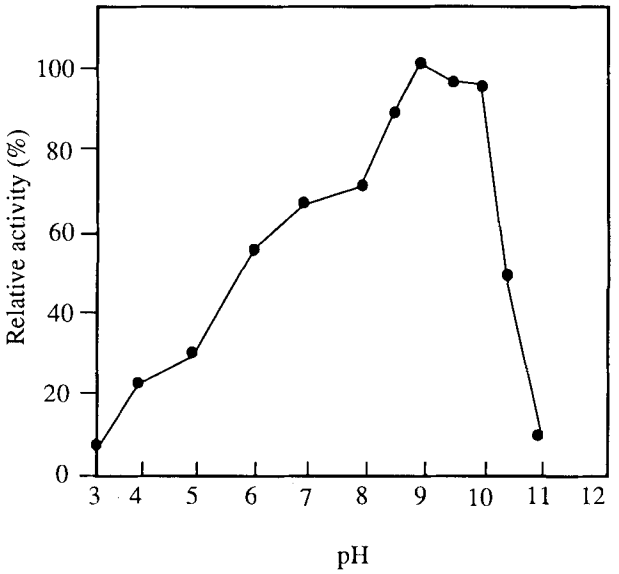

FIG. 5. Effect of pH on the activity of purified esterase. Buffers (50 mM) used: glycine-HCl (pH 3.0–4.0), phosphate (pH 5.0–8.0), Tris-HCl (pH 8.5–9.0) and glycine-OH (pH 9.5–11.0). Enzyme used was 1.0 unit.

A30-1 esterase were determined with tributyrin as a substrate at 60°C and pH 9.0. The enzyme had K_m and K_{cat} values of 0.357 mM and 8365/min, respectively.

Table 3 shows the effects of various metal ions and other inhibitors/activators on esterase activity. Cyclodextrins, Ca^{2+} Co^{2+}, Mg^{2+}, and Mn^{2+} enhanced the hydrolysis rate of tributyrin, and Fe^{2+} and Zn^{2+} were inhibitors of the enzyme activity. The enzyme activity was not affected by ethylenediaminetetraacetic acid, p-chloromercuribenzoate and *N*-bromosuccinimide.

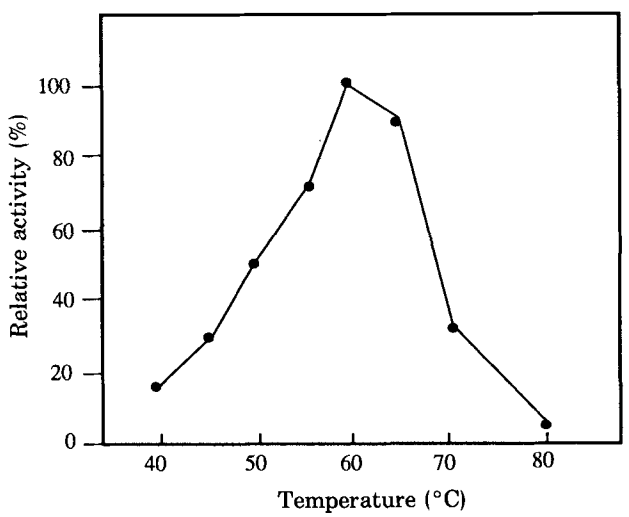

FIG. 4. Effect of temperature on purified esterase activity. Enzyme activity was assayed at various temperatures according to standard method. Enzyme used was 1.0 unit.

We have purified to homogeneity and characterized a thermophilic and alkalophilic esterase from *Bacillus* strain A30-1 grown on corn oil. The enzyme was recovered from the culture broth and, therefore, is an extracellular esterase. As mentioned earlier, the strain also produced a thermophilic and alkalophilic lipase. The esterase activity is distinctly different from the lipase activity in that it had no activity on long-chain fatty acid esters. A similar esterase from soybeans, which had highest activity on triacetin, was reported by Yokoyama and Hirai (9). Although a large number of microbial esterases have been reported from *Bacillus* (10–14), *Pseudomonas* (15–17), *Aspergillus* (18) and *Sulfolobus* (19), only two thermophilic esterases were found to be from thermophilic *Bacillus* (13–14). These thermophilic esterases, however, were extracted after the cells were disrupted by lysozyme (13) or

TABLE 2

Comparison of Substrate Specificity of Pure Esterase from *Bacillus* Strain A30-1 with That from Porcine Liver

Substrate	Relative rate of hydrolysis (%)[a]	
	Bacillus strain A30-1	Porcine liver
Triacetin (C2:0)	43	85
Tributyrin (C4:0)	100	100
Tricaproin (C6:0)	25	43
Tricaprylin (C8:0)	8	2
Tricaprin (C10:0)	4	0
Trilaurin (C12:0)	0	0
Tripalmitin (C16:0)	0	0
Cholesterol acetate	0	0
Cholesterol butyrate	2	0
Choline acetate	0	0

[a]100% Activity corresponds to 1.0 unit of esterase under standard assay condition.

TABLE 3

Effect of Various Enzyme Affectors on Purified Esterase Activity

Affector	Concentration (mM)	Relative activity[a] (%)
None	—	100
$CaCl_2$	10	117
$MgCl_2$	10	114
$MnCl_2$	10	134
$CoCl_2$	10	125
$ZnCl_2$	10	56
$FeCl_2$	10	63
EDTA	10	98
pCMB	0.02	120
DTT	10	110
Acarbose	0.03	96
α-Cyclodextrin	10	143
β-Cyclodextrin	10	137
γ-Cyclodextrin	10	128
NBS	10	106

[a]100% Activity corresponds to 1.0 unit of esterase activity assayed under standard assay condition. EDTA, ethylenediaminetetracetic acid; DTT, dithiothreitol; NBS, *N*-bromosuccinimide.

by ultrasonication (14), and, therefore, they were intracellular enzymes.

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REFERENCES

1. Hofstee, B.H. in *The Enzymes*, edited by P.D. Boyer, H. Hardy and K. Myrback, Academic Press Inc., New York, 1960, pp. 485-500.
2. Shen, G.J., K.C. Srivastava, Y. Wang and H.Y. Wang, U.S. Patent 5,093,256 (1992).
3. Shen, G.J., K.C. Srivastava, Y. Wang and H.Y. Wang, U.S. Patent 5,166,069 (1992).
4. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem. 193*:265 (1951).
5. Laemmli, U.K., *Nature (London) 227*:680 (1970).
6. Andrews, P., *Biochem. J. 91*:222 (1964).
7. Lineweaver, H., and D.J. Burk, *J. Am. Chem. Soc. 56*:658 (1934).
8. England, S., and T.P. Singer, *J. Biol. Chem. 187*:213 (1960).
9. Yokoyama, Z., and H. Hirai, *Agric Chem. 51*:259 (1987).
10. Robert, T.L., and H. Rosenkrantz, *Can. J. Biochem. 44*:671 (1966).
11. Bott, K.F., *J. Bacteriol. 108*:720 (1971).
12. Higerd, T.B., and J. Spizizen, *J. Bacteriol. 114*:1184 (1973).
13. Matsunaga, A., N. Koyama and Y. Nosoh, *Arch. Biochem. Biophys. 160*:504 (1974).
14. Owusu, R.K., and D.A. Cowan, *Enzyme Microb. Technol. 13*:158 (1991).
15. Ohkawa, I., S. Shiga and M. Kageyama, *J. Biochem. 86*:643 (1979).
16. Nakagawa, A., T. Tsujita and H. Okuda, *Ibid. 95*:1047 (1984).
17. Shum, A.C., and A.J. Markovetz, *J. Bacteriol. 118*:880 (1974).
18. Okumura, S., M. Iwai and Y. Tsujisaka, *Agric. Biol. Chem. 47*:1869 (1983).
19. Sobek, H., and H. Gorisch, *J. Biochem. 250*:453 (1988).

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