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STUDIES ON THE LIPASE OF *CHROMOBACTERIUM VISCOSUM*

III. PURIFICATION OF A LOW MOLECULAR WEIGHT LIPASE AND ITS ENZYMATIC PROPERTIES

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SUMMARY

One of the lipases (glycerol ester hydrolase, EC 3.1.1.3) of *Chromobacterium viscosum* was purified from the original crude powder by approx. 1000-fold to give a yield of 18%. The purification method consisted of chromatography on Sephadex G-100, CM-cellulose and DEAE-Sephadex A-50. The homogeneity of the preparation was demonstrated by disc electrophoresis.

A molecular weight of 27 000 was obtained by gel filtration and the isoelectric point in pI 6.9 was determined by isoelectric focusing. The optimum pH for the hydrolysis of olive oil was 6.5 and the optimum temperature was 70 °C in the standard assay system. The purified enzyme was stable in the range of pH 4–11 and below 40 °C in the absence of substrate. The lipase had a resistance for the inactivation by various metal ions and chemical reagents. Neither acceleration nor inhibition of the activity by bile salts was observed.

INTRODUCTION

There are many reports on the isolation and purification of lipase from molds [1–5] or yeasts [6–8]. However, only a few lipases are known from bacteria, which are *Pseudomonas* [9], *Staphylococcus* [10] and others.

In order to develop a new lipase preparation that has stability and unique specificity, screening was carried out. As the result, a bacteria which belongs to *Chromobacterium* was isolated and the properties of the crude enzyme were elucidated [11].

The crude enzyme preparation contains more than two molecular species of lipase. In the previous paper [12] of this series, the purification and characterization of a lipase which has a high molecular weight (lipase A) has been reported.

The present paper deals with the purification and characterization of a lipase which has a low molecular weight (lipase B).

EXPERIMENTAL

Enzyme

The crude enzyme which was obtained by ethanol precipitation of the culture filtrate of *Chromobacterium viscosum*, was supplied by the Toyo Jozo Co., Ltd. Some data for the lipase production were previously reported [11].

Materials

Sephadex G-100 and DEAE-Sephadex A-50 were obtained from Pharmacia Uppsala, Sweden, and CM-cellulose was obtained from Brown Co., Ltd. Polyvinyl alcohol 117 and polyvinyl alcohol 210 were supplied by Kurashiki Rayon Co., Ltd. The other chemicals used were of special or reagent grades.

Assay of lipase activity

Dole's method [13] was adapted for the assay of lipase activity. The olive oil emulsion used as a substrate, was prepared as follows: 25 ml of olive oil and 75 ml of aqueous polyvinyl alcohol solution (2% solution of a 9:1 mixture of polyvinyl alcohol 117 and polyvinyl alcohol 210) was emulsified in a homogenizer. The reaction mixture, consisting of 1 ml of olive oil emulsion, 1 ml of McIlvaine buffer (pH 7.0) and 0.5 ml of enzyme solution, was incubated at 37 °C for 20 min. The enzyme reaction was terminated by adding 5 ml of the mixture of isopropanol, *n*-heptane and 2 M H_2SO_4 (40:10:1, by vol.), and then 2 ml of water and 3 ml of *n*-heptane, and this was left after vigorous stirring. 3 ml of the upper layer was taken from and titrated with 0.01 M ethanolic KOH solution, using thymol blue as an indicator. One unit of lipase was defined as the amount of enzyme which enabled the liberation of 1 μmole of free fatty acid per min.

Disc electrophoresis

Disc electrophoresis was carried out with the 7.5% polyacrylamide gel. For pH 9.4, glycine–Tris buffer [14] was used and electrophoresed at the constant current of 3 mA/tube for 90 min. For pH 4.0, β-alanine–acetic acid buffer [15] was employed at the constant current of 3 mA/tube for 150 min. Staining was carried out using amido black 10B.

Isoelectric focusing

Isoelectric focusing was carried out as described by Vesterberg and Svensson [16] with the use of 1% carrier ampholite (pH 3–10) at the constant voltage of 800 V for 48 h.

RESULTS

Purification of low molecular weight lipase

The crude enzyme (100 g) was extracted with 0.1 M citrate buffer (pH 4.0) and the extract was precipitated with a 0.5 saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in 20 ml of water and then the enzyme solution was applied onto a column of Sephadex G-100 equilibrated with 10 mM citrate buffer at pH 6.5. As the result, shown in Fig. 1, a lipase which has a lower molecular weight (lipase B) was separated

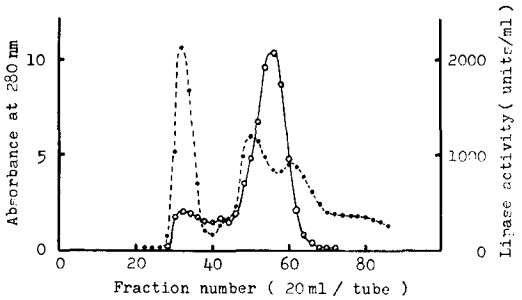

Fig. 1. Gel-filtration by Sephadex G-100. The Sephadex G-100 column (5.0 cm × 100 cm) was equilibrated with and eluted by 10 mM citrate buffer, pH 6.5. Flow rate, 60 ml/h. ●---●, protein; ○—○, lipase activity.

from a lipase of higher molecular weight (lipase A). In these studies lipase B (Fraction No. 45–65) was collected and was further purified.

After the dialysis, the enzyme solution was adsorbed onto a column of CM-cellulose equilibrated with 10 mM citrate buffer at pH 4.0. The adsorbed enzyme was eluted by a linear gradient of NaCl and the chromatogram was shown in Fig. 2.

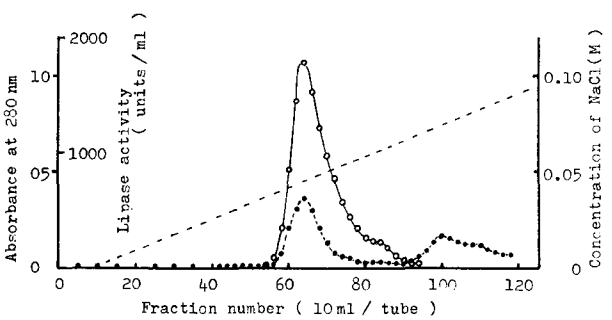

Fig. 2. Chromatography of lipase B on CM-cellulose. The column of CM-cellulose (2.2 cm × 25 cm) was equilibrated with 10 mM citrate buffer, pH 4.0 and eluted with a linear concentration gradient of NaCl (0–0.1 M). Flow rate, 40 ml/h. ●---●, protein; ○—○, lipase activity ---------, concentration of NaCl.

The active fractions obtained above were passed through a column of DEAE-Sephadex A-50 equilibrated with 10 mM citrate buffer at pH 7.0 and then purified by the rechromatography on CM-cellulose. After the dialysis, the purified enzyme solution was lyophilized. The results of the purification procedure are summarized in Table I.

Lipase B was purified from the crude enzyme preparation approx. 1000-fold giving a yield of 18% and the purified enzyme was homogeneous on disc electrophoresis as shown in Fig. 3.

Molecular weight

The molecular weight of lipase B was determined as the method of Whitaker [17] by gel filtration on Sephadex G-100. From the result, as shown in Fig. 4, the value of the molecular weight was calculated to be 27 000.

TABLE I

PURIFICATION PROCEDURE OF LIPASE B

	Total activity (10^3 units)	Specific activity (units/$A_{280\ nm}$)
Original powder	620	7.7
Extract with buffer	510	12.0
$(NH_4)_2SO_4$ fractionation	450	50
Sephadex G-100 gel-filtration	335	530
CM-cellulose chromatography	230	5200
DEAE-Sephadex A-50 chromatography	180	6800
CM-cellulose rechromatography	112	7400

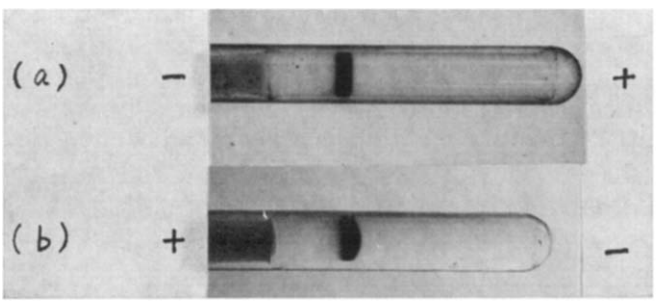

Fig. 3. Disc electrophoretic patterns of purified lipase B. Purified lipase B was subjected to electrophoresis at pH 9.4 (a) and pH 4.0 (b). Conditions are described in Experimental.

Isoelectric point

The isoelectric point was determined by isoelectric focusing using a carrier ampholyte (pH 3–10). As the result, the lipase activity and protein appeared in a single peak at pH 6.9. The homogeneity of lipase B was further proven from the data.

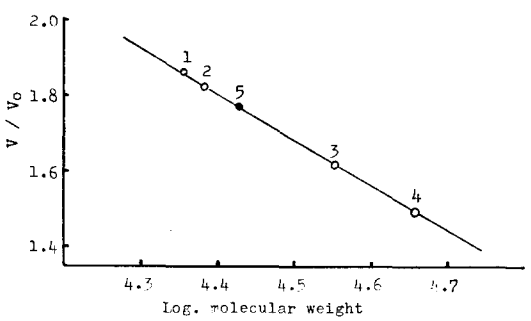

Fig. 4. Determination of the molecular weight of lipase B by gel filtration on Sephadex G-100. A column (1.8 cm × 180 cm) of Sephadex G-100 was equilibrated with 10 mM citrate buffer, pH 6.5 and eluted by the same buffer. 1, α-chymotrypsin (mol. wt 21 600); 2, trypsin (mol. wt 23 300); 3, pepsin (mol. wt 35 500); 4, egg albumin (mol. wt 45 000); 5, lipase B.

Effect of pH on the activity and stability

As shown in Fig. 5, lipase B had an optimum pH at 6.5 in the standard assay system and exhibited a high activity between pH 4 and 9. The effect of pH on the stability of the enzyme was examined. Under the condition of 37 °C for 20 min, more than 80% of the original activity remained at the pH range of 4–11.

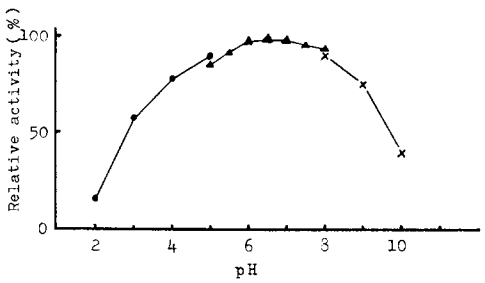

Fig. 5. Effect of pH on the activity of lipase B. Olive oil emulsions, made up at various pH values with buffer solution, were incubated with lipase for 20 min at 37 °C and enzyme activity was measured as described in text. Buffers were as follows; ●—●, 0.1 M citrate buffer; ▲—▲, 0.1 M phosphate buffer; ×—×, 0.1 M ammonium buffer.

Effect of temperature on the activity and stability

Lipase B was stable below 40 °C but the activity was completely lost at 60 °C within 20 min. On the other hand, the lipase had a maximum activity at 70 °C in the standard assay system and more than 50% of the maximum activity was observed at 80 °C. From these results, it is presumed that the enzyme was stabilized to a large extent by the substrate.

Effect of metal salts and chemicals on the enzyme

The solution of lipase B was incubated with metal salt or chemicals at 37 °C for 20 min. After the mixture was diluted 10-fold with a buffer solution, the remaining activity was assayed, and was found to be strongly inhibited by 0.1 mM of $ZnCl_2$, 1 mM of $CuSO_4$ and 10 mM of $FeCl_3$, however, the other heavy metal salts, $HgCl_2$, $AgNO_3$ and $PbCl_2$, did not inhibit the activity.

The effect of the chemicals, EDTA, DFP, *p*-chloromercuribenzoate, *N*-bromosuccinimide, 2-mercaptoethanol and cysteine, on the lipase activity was examined. It was observed that lipase B was not inhibited by any chemicals used in this study at the concentration of 1 mM.

Effect of bile salts on the activity

The activity of lipase B was assayed in the presence of bile salts at the concentration of 0.1–100 mM and neither a large activation nor inhibition of the activity was observed.

DISCUSSION

Newly isolated bacteria, *Chromobacterium viscosum* produces more than two molecular species of lipases. Studies on a lipase which has a high molecular weight

(lipase A) were previously reported [12], and in this paper, the purification and characterization of a low molecular weight lipase (lipase B) were studied.

Lipase B was purified with a good yield, and the homogeneity was proven by disc electrophoresis and isoelectric focusing. The purified lipase B was found to differ from lipase A in molecular weight, isoelectric point and the influence of bile salts. Lipase B was stable and active in a wide range of pH values as was the lipase from *Candida cylindracea* [7]. The thermal stability of the enzyme was similar to the stabilities from *Penicillium crustosum* [18] and *Aspergillus niger* [5]. However, in the presence of the substrate lipase B was more stable and active at a higher temperature compared with a lipase from *Humicola lanuginosa*, which is a thermophilic fungus. Various lipases are well known which are activated by Ca^{2+} [20], bile salts [1] or serum protein [21], but lipase B was not affected by these chemicals.

The substrate specificity and the other physical properties will be described in the subsequent report.

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