Purification, Crystallization and Some Properties of Lipase from *Geotrichum candidum* Link†

Yoshio TSUJISAKA, Mieko IWAI and Yoshio TOMINAGA

The Osaka Municipal Technical Research Institute, Osaka, Japan

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Lipase (EC 3.1.1.3) of *Geotrichum candidum* Link was purified by means of ammonium sulfate fractionation, DEAE-Sephadex column chromatography, gel-filtration on Sephadex G-100 and Sephadex G-200, and was finally crystallized in concentrated aqueous solution. It was confirmed that the crystallized preparation was homogeneous electrophoretically and ultracentrifugally.

It was estimated with the crystalline enzyme that the sedimentation constant ($s_{20,w}$) was 4.0, the isoelectric point was pH 4.33, and the molecular weight was 53,000~55,000. From the result of amino acid analysis, none of sulfur containing amino acid was detected in the enzyme. It was also recognized that the crystalline preparation contained about 7% of the carbohydrate and very small amount of lipid. It was characterized that the lipase was the most active at pH 5.6~7.0 on olive oil, at 40°C and was stable in the range of pH 4.2 to 9.8 at 30°C for 24 hr, and was stable below 55°C for 15 min.

During the course of our study on lipase, a strain of *Geotrichum candidum* Link was selected as one of potent lipase producer. As previously reported, it was revealed that this microorganism produced lipase inducibly only in the presence of the specific substance such as vegetable oil or fatty acid in a cultivation medium.¹⁾ Some reports^{2~4)} have been published on the production and the specificity of *G. candidum* lipase. Although the application of *G. candidum* lipase for the study on glyceride structure also have been described in those papers, the purification of the enzyme preparation employed in the studies has not been carried out. Therefore, its enzymatic properties in detail were still obscure except its substrate specificity.

On the other hand, the existence of a significant difference of the modes of lipase production by these microorganisms named *G. candidum* has been suggested, since *G. candidum* so far studied by other groups has no requirement of lipid for the enzyme production. Therefore, it seems to be significant to purify *G. candidum* Link lipase, to clarify the property of the purified enzyme and to

compare the properties of both lipases in further studies.

The present paper deals with the purification and the crystallization procedures and some of the enzymatic properties of *G. candidum* Link lipase.

MATERIALS AND METHODS

Cultivation. A medium used for cultivation of *G. candidum* Link was composed of 5% of corn steep liquor, 1% of soy bean oil and 0.5% of ammonium nitrate and was adjusted at pH 6.0. Ten liters of the medium was put into 30-liter jar fermentor (Marubishi Laboratory Equipment Co. Ltd.) and was sterilized at 120°C for 30 min. Inoculation was made by the addition of 70 ml of the seed culture broth obtained by 24 hr reciprocal shaking in the same medium as above in Sakaguchi flask at 28°C. The cultivation was carried out by aeration (3 liter/min) and agitation (200 rpm) at 28°C for 30 hr. After the cultivation, the cells were filtered off and the filtrate was subjected to further purification of lipase.

Assay of lipase activity. Lipase activity was assayed as described previously.⁵⁾ The activity which liberated 1 μ mole equivalent of fatty acid from olive oil per minute was defined as 1 unit (U).

Determination of protein. Protein content in an enzyme preparation was estimated by measurement of

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the absorbancy at wave length $280\text{ m}\mu$ with Hammarsten milk casein (E. Merk) as a standard. Specific activity of enzyme was expressed as units per mg of protein.

Physical measurements. Ultraviolet absorption spectrum was measured with a Beckman DB spectrophotometer. Homogeneity of purified enzyme preparation was checked by means of electrophoresis and ultracentrifugation. Analytical electrophoresis was carried out by a Hitachi HT-B Tiselius electrophoresis apparatus. Disc electrophoresis was done by the method of Davis⁶⁾ with an apparatus of M.S. Instrument Co. Analytical centrifugation was conducted by a Hitachi UC A-1 analytical ultracentrifuge. The estimation of isoelectric point of lipase was accomplished by means of isoelectric-focusing method. The carrier Ampholytes were selected to give a pH gradient from 3 to 6 and the density gradient was provided by the use of sucrose. Sample was applied in a special vertical electrophoresis column of 110 ml capacity (LKB-Productor).

Analysis of carbohydrate. Total sugar content in the crystallized lipase preparation was determined by phenol-sulfuric acid method.⁷⁾ Sugar composition was analyzed by following procedure. One milligram of the crystallized lipase preparation was hydrolyzed with $1\text{ N H}_2\text{SO}_4$ at 100°C for 3 hr. The hydrolyzate was neutralized to pH 5.3 with BaCO_3 . After removal of the precipitate by filtration, the filtrate was concentrated to 0.1 ml under reduced pressure. Sugar components of the hydrolyzate were analyzed by paper chromatography using a solvent system of *n*-butanol: pyridine: water (6: 4: 3) with double ascending development. Localization of reducing sugar on the filter paper was detected by spraying aniline hydrogen phtharate.

Analysis of amino acid. Each 2.57 mg of the crystallized lipase preparation was hydrolyzed with 6 N HCl at 110°C for 24, 48, or 72 hr, respectively. Amino acids in the hydrolyzate were analyzed by a Hitachi amino acid analyzer KLA-5.

Analysis of lipid. One hundred milligrams of the crystallized lipase preparation were dissolved in chloroform methanol mixture (2: 1, v/v) and oscillated by ultrasonic wave apparatus. Resulting solution was evaporated in vacuum and the solid matter was dissolved in ethyl ether and the insoluble residue was removed. The soluble fraction was saponified with alcoholic 0.5 N KOH and acidified with HCl to pH 2.0. After removing alcohol, ethyl ether was added to the solution. Methylation of the ether soluble fraction was then carried out by the addition of diazomethane.⁸⁾ Then methylated material was subjected to gas liquid chromatography (Column: Diethylene glycol succinate 5%, 165°C).

Other reagents and materials. DEAE-Sephadex A-50, Sephadex G-100 and Sephadex G-200 were the products of Pharmacia Co., Ltd. Carrier Ampholites for electrofocusing were the products of the LKB-Productor. All the other materials used were special grade reagents purchased from Wako Pure Chemicals Ltd.

RESULTS AND DISCUSSION

1. Purification procedures of lipase

Ammonium sulfate fractionation. Solid ammonium sulfate was added to 23.3 liters of the culture filtrate of *G. candidum* Link up to the concentration of 0.35 saturation and the precipitate formed was filtered off. To the filtrate, solid ammonium sulfate was further added to give 0.75 saturation. The resulting precipitate was collected by filtration and dissolved in a minimum amount of deionized water. The solution was applied to a column of Sephadex G-25 (coarse) to remove the ammonium sulfate.

DEAE-Sephadex column chromatography. To the desalted solution, a given amount of 1 M acetate buffer, pH 5.0, was added up to 0.01 M in final concentration. The solution was then dialyzed against 0.01 M acetate buffer, pH 5.0, for 2 days. After removal of the precipitate, the filtrate (2780 ml) was applied to a column of DEAE-Sephadex A-50 ($6.2 \times 60\text{ cm}$) which was equilibrated with the 0.01 M acetate buffer, pH 5.0. The column was washed thoroughly with the same buffer, and protein adsorbed was eluted by a linear gradient concentration of NaCl at a flow rate of 42 ml per hr. The effluent was fractionated into 12 ml portions. The main fractions (tube No. 200-220) were combined and concentrated to 50 ml by dialysis against polyethylene glycol (Fig. 1).

Gel filtration on Sephadex G-100. The concentrated solution was chromatographed on a column of Sephadex G-100 ($3.8 \times 120\text{ cm}$) conditioning with 0.01 M acetate buffer (pH 5.0). Elution was made with the same buffer at a flow rate of 15 ml per hr and the effluent was fractionated into 12 ml portions. A

FIG. 1. Column Chromatography of *G. candidum* Lipase on DEAE-Sephadex A-50.

The experimental details are described in the text.

○—○ Lipase activity
 ----- Protein (O.D. at 280 mμ)
 NaCl

FIG. 2. Column Chromatography of *G. candidum* Lipase on Sephadex G-100.

The experimental details are described in the text.

○—○ Lipase activity
 ----- Protein (O.D. at 280 mμ)

typical chromatographic pattern obtained in gel filtration was presented in Fig. 2. The active fractions (No. 53 to 62) were combined and dialyzed against deionized water for 2 days and lyophilized. About 1.5 g of lyophilized powder was obtained.

Gel filtration on Sephadex G-200. The lyophilized powder (1.5 g) was dissolved in 60 ml of 0.01 M acetate buffer, pH 5.0. Twenty ml each of the enzyme solution was loaded three times (total, 60 ml) on a column of Sephadex G-200 (2.8 × 104 cm) equilibrated with 0.01 M acetate buffer, pH 5.0. Elution was carried out with the same buffer at a flow rate of 15.2 ml per hr. Effluent was frac-

tionated into 6.4 ml portions. As shown in Fig. 3, a symmetric peak of protein with a constant specific activity of lipase was obtained. The fractions having more than 1000 units per ml were combined and dialyzed against deionized water for 3 days.

FIG. 3. Column Chromatography of *G. candidum* Lipase on Sephadex G-200.

The experimental details are described in the text.

○—○ Lipase activity
 ----- Protein (O.D. at 280 mμ)

Crystallization. The dialyzed solution was concentrated in a collodion bag under reduced pressure. The crystals slightly appeared when the concentration of protein solution reached to about 10%. After standing the concentrated solution in a cold room (8°C) for 3 days under reduced pressure, the crystallization was almost completed. The enzyme crystallized from an aqueous solution was separated from mother liquor by centrifugation. The crystals were washed several times with cold deionized water and resuspended in a small amount of cold deionized water. Then, 0.01 N NaOH was gently added to the crystal suspension in an ice bath. When pH of the solution reached to 8.8, the crystals dissolved completely. After adjusting pH of the solution to 6.0 with diluted acetic acid, insoluble matter was removed by centrifugation. The supernatant was dialyzed against deionized water. Recrystallization was carried out in the same procedure as the first

FIG. 4. Crystals of Lipase from *Geotrichum candidum* Link.

crystallization. The microphotograph of crystals of lipase is shown in Fig. 4. The crystals formed were washed three times with cold deionized water and lyophilized.

The overall procedures of the purification and results are summarized in Tables I and II. The purification results in a 40 fold increase in specific activity with a recovery of 20% of the original activity.

2. Physical properties of the crystalline lipase

The homogeneity of the crystallized preparation of lipase from *G. candidum* Link was examined by analytical electrophoresis, disc electrophoresis on polyacryl amide gels and sedimentation. From the results shown in Figs. 5, 6 and 7, it is revealed that the preparation is homogeneous.

The sedimentation constant, $s_{20,w}$ was determined to be 4.0, when the experiment

TABLE I. PURIFICATION PROCEDURE OF THE LIPASE FROM *Geotrichum candidum* LINK

Culture filtrate	fractionated with ammonium sulfate (0.25~0.75 saturation)
Precipitate	dissolved in deionized water and desalted with a column of Sephadex G-25
Desalted solution	chromatographed on DEAE-Sephadex column equilibrated with 0.01 M acetate buffer, pH 5.0, and eluted by NaCl
Eluted solution	chromatographed on Sephadex G-100 column equilibrated with 0.01 M acetate buffer, pH 5.0, and lyophilized
Lyophilized powder	dissolved in 0.01 M acetate buffer, pH 5.0, and chromatographed on Sephadex G-200 column equilibrated with 0.01 M acetate buffer, pH 5.0.
Eluted solution	dialyzed against deionized water and concentrated by means of a collodion bag under reduced pressure
First crystals	recrystallized
Second crystals	

was made from 0.856% to 0.428% in protein concentration. Figure 8 shows a plot of sedimentation constants in various protein concentrations.

The molecular weight of the lipase was estimated to be 53000-55000 by the method of gel filtration using Sephadex G-200 column (1.6×93 cm). Chymotrypsinogen A (M.W. 25000), ovalbumin (M.W. 45000), bovine

TABLE II. LIPASE ACTIVITIES IN THE COURSE OF PURIFICATION

Fraction and step	Total volume (ml)	Total activity (U)	Specific activity (U/mg protein)	Recovery of activity (%)
Culture filtrate	23300	1.53×10^6	11	100
Solution of precipitate	1060	1.29×10^6	37	84
Desalted solution	2780	1.16×10^6	50	76
DEAE-Sephadex Chromatography	252	1.00×10^6	259	65
Sephadex G-100 Chromatography	120	7.42×10^5	385	49
Solution of lyophilized powder	60	5.73×10^5	377	37
Sephadex G-200 Chromatography	258	4.57×10^5	447	30
Solution of first crystals	100	3.83×10^5	447	25
Recrystals	—	3.07×10^5	447	20

FIG. 5. Electrophoretic Patterns of Lipase from *G. candidum* Link.

The experiment was carried out at a concentration of 1% lipase in 0.1 ionic strength phosphate buffer at pH 7.9 under the condition of temperature 15°C, Voltage 100 volt, Ampere 15 mA. The photographs were taken at 0, 15, 30, 45 and 60 min.

FIG. 6. Disc Electrophoretic Pattern of Lipase from *G. candidum* Link.

The enzyme, 200 μ g was applied to the stacking gel and was subjected to electrophoresis in 0.1 M glycine-NaOH buffer, pH 9.4, at a current of 5 mA for 35 min (A) and 70 min (B).

FIG. 7. Sedimentation Patterns of Recrystallized Lipase.

The experiment was carried out at a concentration of 0.856% lipase in deionized water at 21°C. The photographs were taken every 10 min after reaching the final speed (55700 rpm).

serum albumin (M.W. 67000) and γ -globulin (M.W. 160000) were used as standard proteins for molecular weight determination (Fig. 9). From the result of amino acid analysis as described later, it is considered that the value of M.W. 53000 is reasonable.

The isoelectric point of the lipase was deter-

mined by isoelectric-focusing method. It was estimated to be pH 4.33 from the effluent pattern shown in Fig. 10.

The ultraviolet absorption spectrum is shown in Fig. 11, from which $E_{280\text{nm}}^{1\%}$ was calculated to be 10.8.

FIG. 8. Concentration Dependence of the Sedimentation Constant of Lipase from *G. candidum* Link.

The crystals were dissolved into deionized water up to the concentrations as indicated in the text. The sedimentation measurement was carried out at 21°C. The rotor speed was 55700 rpm.

FIG. 9. Estimation of Molecular Weight of the Lipase by Gel Filtration.

Sephadex G-200 column (1.6 × 93 cm) equilibrated with 0.01 M acetate buffer, pH 5.0, containing 0.1 M KCl was used. The elution point of blue dextran was taken as void volume (V_0) and V_e/V_0 (elution volume) was plotted against logarithm of molecular weight.

A: Chymotrypsinogen A, B: Ovalbumin
C: Bovine serum albumin D: γ -globulin
S: Lipase

3. Chemical composition of the crystalline lipase

Carbohydrate content of the crystalline lipase preparation was estimated to be 7.5% as mannose. In the hydrolyzate of the preparation, xylose, arabinose, galactose and mannose were detected by paper chromatography. The paperchromatogram suggested that mannose is the main component among the sugars detected.

FIG. 10. Electro-focusing Pattern of *G. candidum* Lipase.

The experimental details are described in the text.

○—○ Lipase activity
----- pH

FIG. 11. Ultraviolet Absorption Spectrum of Lipase from *Geotrichum candidum* Link.

The crystallized preparation (0.56 mg/ml) was dissolved in 0.01 M acetate buffer (pH 5.0).

Table III is summarized the results of amino acid analysis of the crystallized lipase preparation. Sulfur-containing amino acids, such as cysteine and methionine were not detected in the hydrolyzate. The lipase is characterized as an enzyme without sulfur-containing amino acid. Accordingly, it seems that the lipase has no S-S bond in the molecule structure.

By extraction with chloroform methanol mixture, about 1.1 mg of oily substance was obtained from 101 mg of the crystallized lipase preparation. According to the gas chromatography

TABLE III. AMINO ACID COMPOSITION OF THE LIPASE FROM *Geotrichum candidum* LINK

Amino acid	Amino acid ^{a)} determined after hydrolysis for 24 hr	Numbers of ^{b)} residue per mole of the enzyme
Tryptophan ^{c)}	0.0389	8
Lysine	0.0825	16
Histidine	0.0530	10
Ammonia	0.2440	46
Arginine	0.080	20
Aspartic acid	0.283	54
Threonine	0.113	22
Serine	0.147	28
Glutamic acid	0.160	30
Proline	0.141	26
Glycine	0.212	40
Alanine	0.177	34
Half-cystine	0	0
Valine	0.130	24
Methionine	0	0
Isoleucine	0.093	18
Leucine	0.215	40
Tyrosine	0.0963	18
Phenylalanine	0.143	28

^{a)} Expressed as micro mol of amino acid residue in 321 micro gram of the enzyme.

^{b)} Calculated on the basis of the value obtained by subtracting the carbohydrate content (7%) from molecular weight, 53,000.

^{c)} By the method of Goodwin and Movton.⁹⁾

graphic pattern, it appears that the oily substance is composed of several kinds of fatty acids. Although, the identifications of all of them are expected to further study, palmitic and oleic acids have been identified so far.

In the literature,¹⁰⁾ it has been reported that partial purified preparation of ricinus lipase contains a small amount of lipid which plays an essential role on the enzyme action. In the case of the lipase from *G. candidum* Link, however, it is not still clear whether the lipid is necessary for the enzyme activity. The fact that a small amount of lipid exists in the crystalline lipase might only suggest that the enzyme is happened to be contaminated.

4. Enzyme property

Figures 12 and 13 show the effect of pH on the activity and on the stability of *G. candidum* lipase, respectively. The enzyme was the most

FIG. 12. pH Activity Curve of Lipase from *G. candidum* Link.

The enzyme was incubated with olive oil at 30°C for 60 min at various pH values adjusted by McIlvain buffer (from pH 4 to pH 7) or glycine-NaOH buffer (from pH 6 to pH 10).

FIG. 13. pH Stability Curve of Lipase from *G. candidum* Link.

Lipase was dissolved in a buffer solution having a pH-value indicated in the figure and incubated at 30°C for 24 hr. The buffer solution used was 0.05 M McIlvain buffer (from pH 4 to pH 8) or Sorensen's glycine-NaOH buffer (from pH 7 to pH 11). Each solution was then diluted with 0.1 M acetate buffer, pH 5.6. The remaining activity was determined by the standard assay method.

active at pH values between 5.6 and 7.0, and was stable in the range of pH 4.6 to 9.8 during 24 hr incubation at 30°C.

The effect of temperature on the enzyme activity was examined in the range of 20°C to

FIG. 14. Effect of Temperature on Activity.

Enzyme reaction was carried out by the standard assay method for 60 min except temperature was varied.

50°C. The optimal temperature was estimated to be at about 40°C as shown in Fig. 14.

As shown in Fig. 15, the enzyme was stable at the temperatures below 55°C at pH 5.6 for 15 min.

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FIG. 15. Effect of Temperature on the Stability.

Each enzyme solution, dissolved in 0.02 M acetate buffer, pH 5.6, was incubated in a test tube at each temperature for 15 min. Then the test tube was kept at 0°C for 30 min and remaining activity was determined by the standard assay method.

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