Physical and Chemical Properties of the Lipase of Thermophilic Fungus *Humicola lanuginosa* S-38[†]

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Received April 18, 1973

Some physical and chemical properties of the extracellular lipase from the thermophilic fungus, *Humicola lanuginosa* S-38, were investigated. The results were as follows: Sedimentation coefficient was 2.4×10^{-13} (cm·g/sec·dyne); diffusion coefficient was 8.8×10^{-7} (cm²/sec); and frictional coefficient was 1.22. Molecular weight was $27,500 \pm 500$ and α -helix content was 18.9%. The number of amino acid residues contained in 1 mole of protein of *Humicola* lipase was 224. Sugar and lipid were not detected. The effect of calcium ion and denaturing reagents, such as urea, sodium dodecyl sulfate and dithiothreitol, on the thermostability of *Humicola* lipase was examined. It was concluded that the thermostability of *Humicola* lipase was not influenced by protective cofactors but was attributable to the enzyme itself. Some properties of enzyme structure which were concerned with the thermostability of *Humicola* lipase are also discussed.

The thermostable mechanism of the thermophilic enzyme has evoked the considerable interest of many investigators during the past two decades. Manning, Campbell and Foster²⁾ reported that the thermostability of α -amylase from *Bacillus stearothermophilus* was due to the characteristic less-ordered structure of the enzyme itself. Using α -amylases from other strains of *Bacillus stearothermophilus* other reported^{3,4)} that these enzymes existed as a globular structure as those of many mesophilic α -amylase. They pointed out that a slight excess of amino acids with hydrophobic side chains and the presence of calcium ion played an important role in the thermostability of thermophilic α -amylase molecules. Thermostable protease from *Bacillus thermoproteolyticus* has been reported to be a compact globular protein by Ohta *et al.*⁵⁾ It was concluded that the thermostable enzyme from thermophiles was not always related to the random structure of the enzyme as reported by Manning *et al.*

It is well known that lipase has the unusual property of being functional only at an oil/water interface. It has been postulated that the ability of lipase to act on the triglyceride at an oil/water interface resulted from the relative stability of its characteristic protein structure at an oil/water interface. Unfortunately, owing to the unstable property of lipase, homogeneous lipase was obtained only in a few case and the information on the characteristic structure of lipase is still very limited.

In the previous report,⁶⁾ the lipase produced by a thermophilic fungus, *Humicola lanuginosa* S-38, was found to be much more thermostable than those of mesophiles. The thermostability of this enzyme provides a great advantages in investigating the structure of lipase. The present report is concerned with some physical and chemical properties of this thermostable lipase.

MATERIALS AND METHODS

Preparation of enzyme. The enzyme used in this study was prepared as described previously⁶⁾ and confirmed to be homogeneous with disc electrophoresis and ultracentrifugal analyses.

[†] Studies on the Lipase of Thermophilic Fungus *Humicola lanuginosa*. Part VI. For Part V see Reference 1).

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Estimation of lipase activity. Lipase activity was estimated by the olive oil emulsion method as described previously.⁷⁾ One unit of lipase activity was defined as the amount which liberated 1 μ mole of free fatty acid per minute at 45°C and pH 8.0.

Ultracentrifugal sedimentation rate and equilibrium analyses. Analytical ultracentrifugation was carried out with a Hitachi analytical ultracentrifuge UCA-1 A. Synthetic boundary cell was used in the sedimentation rate analysis. The sedimentation equilibrium analysis was performed by Archibald's method⁸⁾ using a standard cell and a synthetic boundary cell with the Schlieren optical system.

Molecular weight determination (Andrews' method).⁹⁾ Sephadex G-100 was equilibrated with 0.05 M Tris-HCl buffer (pH 8.0) and packed into a column of dimension 2.2 cm $\times$ 48 cm. The blue dextran was used as a void volume marker and bovine albumin (M.W. 67,000), egg albumin (M.W. 45,000), α -chymotrypsinogen (M.W. 25,000) and myoglobin (M.W. 15,000) were also used as standards of molecular weight. The *Humicola* lipase was applied to the column with these markers and eluted with the equilibrating buffer. The amount of blue dextran and other markers were measured spectrophotometrically at 600 and 280 m μ respectively.

Circular dichroism analysis. The measurement of the circular dichroism at 210~250 m μ was performed using a Jasco ORD/UV-5 modified by a circular dichroism attachment (Japan Spectroscopic Co., Ltd.). Molecular ellipticity $[\theta]$ was obtained by the equation, $[\theta] = 3,300 (E_l - E_r)$, where $(E_l - E_r)$ is the difference between the molar extinction coefficients for left and right circularly polarized light. In the calculation of $(E_l - E_r)$, the mean residue weight was used instead of molecular weight. An apparent content of right-handed α -helix was calculated as follows: α -helix content = $-[\theta]_{222}/400,000 \times 100$.³⁾

Amino acid analysis. Amino acid composition of the enzyme was determined with a JLC-5 AH type automatic amino acid analyzer (Japan Electric Co., Ltd.). Hydrolysis was performed with 6 M HCl at 110°C for 24, 48 and 72 hr, respectively. The tryptophan content of the enzyme was determined by the spectrophotometric method.¹⁰⁾ Cystine and cysteine were determined as cysteic acid after performic acid oxidation and hydrolysis of the enzymes.¹¹⁾

Partial specific volume. The partial specific volume of the enzyme was computed from the specific volume of constituent amino acid residues.¹²⁾

Elementary analysis. The enzyme was dialyzed against distilled water, then dried until the weight

became constant. Hydrogen, carbon and nitrogen content of the enzyme were determined by micro-analysis.

Carbohydrate analysis. The hexose and hexosamine content in the enzyme were determined respectively by the phenol-sulfuric acid method¹³⁾ and the Elson-Morgan method.¹⁴⁾

Lipid analysis. The enzyme solution containing 0.1% HCl was extracted at 65°C for 30 min with a chloroform:methanol mixture (1:1, v/v). The upper extracted layer was dried up using a rotary evaporator. The quantitative analysis of the dried sample was performed by thin-layer chromatography. The development solvent used to detect the lipid was a petroleum ether:ethyl ether:acetic acid mixture (80:30:1, v/v) and 10.0% of phosphorus molybdate in 10.0% ethyl alcohol.

Chemicals. Chemicals with a reagent grade were obtained from Tokyo Kasei, Kogyo Co., Ltd. and Kokusan Chemical Work, Ltd.

RESULTS

Sedimentation coefficient

The enzyme was dissolved in 0.05 M Tris-HCl buffer (pH 8.0) and adjusted to three different concentrations (1.2%, 0.75% and 0.35%). The enzyme solutions were employed for the sedimentation rate analysis using a synthetic boundary cell. The sedimentation patterns are shown in Fig. 1. The sedimentation coefficients at various concentrations are plotted in Fig. 2. The straight line obtained was extrapolated to zero concentration. Thus, $S_{20,w}^0$ was estimated to be 2.4×10^{-13} (cm $\cdot$ g/sec $\cdot$ dyne).

Diffusion coefficient

An 0.8% enzyme solution was used for the sedimentation equilibrium analysis using a standard cell and a synthetic boundary cell. The sedimentation equilibrium patterns are shown in Fig. 3. The value of the diffusion coefficient at 20°C was calculated at 8.8×10^{-7} (cm²/sec) and the molecular weight was estimated to be 27,300 by Archibald's method. The frictional coefficient was 1.22. During these estimations, the partial specific volume of the *Humicola* lipase was 0.76 ml/g which

FIG. 1. Ultracentrifuge Patterns of *Humicola* Lipase. Enzyme concentration. A, 1.2%; B, 0.75%; C, 0.35%. The 0.05 M Tris-HCl buffer (pH 8.0) contained 0.1 M KCl. Synthetic boundary cell was used, 60,000 rpm, 25°C.

FIG. 2. Determination of Sedimentation Coefficient of *Humicola* Lipase.

was computed from the specific volume of constituent amino acid residues.

Estimation of the molecular weight by Sephadex G-100 column chromatography

The detailed procedures are described in MATERIALS AND METHODS. As shown

FIG. 3. Sedimentation Equilibrium Patterns of *Humicola* Lipase.

Enzyme concentration = 0.8%, 0.05 M Tris-HCl buffer (pH 8.0) contained 0.1 M KCl; 13,000 rpm, 25°C. Photographed at 10 min intervals.

in Fig. 4, the molecular weight of the *Humicola* lipase was estimated to be around 27,500.

FIG. 4. Estimation of the Molecular Weight of *Humicola* Lipase by Sephadex G-100 Column Chromatography.

A, myoglobin; B, α -chymotrypsinogen; C, egg albumin; D, bovine albumin; V₀, void volume; V_t, total volume of Sephadex column; V_e, elution volume.

Circular dichroism analysis

The circular dichroism (CD) spectra in the

210~250 $m\mu$ region for the enzyme in the native and denatured states were measured at pH 8.0 and 25°C. As shown in Fig. 5, the

FIG. 5. Effect of Urea, SDS and Heat-treatment on the Circular Dichroism Spectrum of *Humicola* Lipase. A, control; B, added 1×10^{-2} M sodium dodecyl sulfate; C, added 8 M urea; D, heated at 80°C for 20 min.

CD spectrum of the native lipase showed a trough at 220~222 $m\mu$. This trough position is characteristic of proteins having a α -helix structure. The molecular ellipticity at 222 $m\mu$, $[\theta]_{222}$ for the *Humicola* lipase was estimated to be -7.56×10^3 (deg·cm²/decimole). The content of α -helix in the enzyme was calculated as 18.9. In the presence of 10^{-2} M sodium dodecyl sulfate at pH 8.0, the CD spectrum of enzyme at room temperature showed a similar pattern to that of the native one. On the contrary, on heating at 80°C for 20 min or in the presence of 8 M urea, the CD spectra of the enzyme showed a different patterns from the native one. In the presence of 8 M urea, the negative value of $[\theta]_{222}$ decreased from -7.56×10^3 to -4.8×10^3 . On heating at 80°C for 20 min, the negative value of $[\theta]_{222}$ decreased further to -4.0×10^3 . From these results, it was apparent that the α -helix structure of enzyme was changed by the 8 M urea or the heat treatment.

Amino acid composition

Three different time periods for hydrolysis of the enzyme were employed. The amino acid composition obtained from the average of three hydrolysates is summarized in Table I. The content of hydrophobic amino acids was higher than the content of hydrophilic amino acids. The abnormally high contents of tyrosine and proline which had been emphasized as a characteristic of thermophilic protease and α -amylase were not found in the *Humicola* lipase.

TABLE I. AMINO ACID COMPOSITION OF THE *Humicola* LIPASE

The values of the amino acid were obtained from the average of 24, 48 and 72 hr hydrolysates. Values for threonine, serine and NH₃ represent the value after extrapolation of each to zero time of hydrolysis. 1/2-Cystine was determined as cysteic acid after performic acid oxidation and hydrolysis of the enzyme. The tryptophan content of the enzyme was determined by spectrophotometric method.

Amino acid	Amino acid (g) per 100 g of protein	Amino acid residues per molecule
Arg	7.27	11
Lys	3.55	7
His	3.47	6
Asp	11.27	23
Glu	7.39	12
Ser	6.60	17
Thr	7.50	19
Tyr	3.68	6
Gly	6.71	25
Ala	6.02	19
Val	6.70	16
Ile	6.03	13
Leu	7.85	17
Pro	4.33	10
Phe	7.62	13
Trp	1.83	3
Met	0.24	1
1/2-Cys	2.80	6
NH ₃	1.40	
Total		224

Elementary analysis and absence of carbohydrate and lipid

Elementary analysis of the purified lipase showed 13.7% of nitrogen, 48.7% of carbon and 6.5% of hydrogen. Hexose and hexos-

amine were not detected in the purified lipase by the phenol-sulfuric acid method or the Elson-Morgan method. Lipid was not detected according to the quantitative analysis by thin-layer chromatography after extraction of the purified lipase with a chloroform and methanol mixture at 65°C for 30 min.

Effect of denaturing reagents on the thermostability

As shown in Fig. 6, on heating at 80°C for 20 min not only was the protein structure

FIG. 6. Effect of Calcium Ion and Denaturing Reagents on the Thermostability of *Humicola* Lipase.

○, control; △, added 1×10^{-2} M calcium ion; ●, added 8 M urea; △, added 1×10^{-2} M sodium dodecyl sulfate. Incubation condition: pH 8.0, 20 min.

changed but the lipase activity was also completely inactivated. However, after the enzyme was treated with 8 M urea at room temperature for 20 min, the full lipase activity returned providing that the urea concentration was reduced by dilution. This indicated that the denaturing action of 8 M urea on the lipase at room temperature was a reversible reaction. But an interesting feature was that the simultaneous action of temperature and urea caused a marked denaturation while the native lipase was stable on heating alone. Therefore, it was clear that the effect of urea on the lipase was to lower the denaturation temperature. To lower the denaturation temperature, sodium dodecyl sulfate was more effective

than 8 M urea. On the other hand, calcium ion did not enhance the thermostability of native lipase.

The activity of *Humicola* lipase was not affected by treatment with a sulfhydryl reducing agent, dithiothreitol, at room temperature but the thermostability of *Humicola* lipase was lowered. In the presence of 8 M urea and sulfhydryl reducing agent, the heat inactivation of the enzyme was accelerated (Fig. 7).

FIG. 7. Effect of Sulfhydryl Reducing and Denaturing Reagents on the Thermostability of *Humicola* Lipase.

○, control; ●, added 8 M urea; ×, added 1×10^{-2} M dithiothreitol; △, added 8 M urea and 1×10^{-2} M dithiothreitol. Incubation condition: pH 8.0, 20 min.

DISCUSSION

Since Manning *et al.*²⁾ reported that the thermostability of the thermophilic α -amylase was due to the characteristic structure of the protein itself, two explanations have been offered about the structure of thermostable protein. The first was that the enzyme existed as a randomly coiled structure and the second explanation was that enzyme existed as a rigid structure. It was pointed out that the randomly coiled structure of thermophilic α -amylase was due to the high proline content¹⁵⁾ and that the rigid structure of thermophilic protease resulted from the abundance of amino acids with hydrophobic side chains and the high tyrosine content.⁵⁾ Although the ratio of the content of hydrophobic amino acids in

the *Humicola* lipase was similar to those of thermophilic α -amylase and protease, an abnormally high content of proline or tyrosine was not found in the *Humicola* lipase (Table I). Meanwhile, the α -helix content of *Humicola* lipase was found to be 18.9% which was similar to that of mesophilic α -amylase. Thus, the explanation of thermostability by Campbell^{3,16)} and Ohta^{5,16)} does not appear satisfactory in the case of *Humicola* lipase.

It was reported^{3,4,17)} that calcium ion played an important role in the thermostability of both thermophilic α -amylase and protease. As shown in Fig. 6, the thermostability of *Humicola* lipase was not promoted by the addition of calcium ion. On the other hand, it was confirmed that the lipase activity was not affected by dialysis or EDTA treatment. The thermostability of *Humicola* lipase was not obviously related to protective cofactors and was attributable only to the enzyme itself.

It was pointed out¹⁸⁾ that the content of hydrophobic amino acids in lipase protein was higher than that of other hydrolase and those proteins which were not related to lipid. On general principles, such a high content of hydrophobic amino acids in the enzyme molecule could provide a compact structure which is not easily denatured by a change in the external environment. However, the composition of hydrophobic amino acids in thermophilic enzymes was diverse, and lipases so far examined were always thermo-unstable. These facts indicate that the higher content of hydrophobic amino acids in the lipolytic enzyme was not sufficient to make them thermostable. It is obvious that the composition or arrangement of hydrophobic amino acids in the enzyme molecule was more important than the content alone.

As shown in Fig. 5, at room temperature, the addition of 8 M urea induced a distinct change of the CD spectrum of *Humicola* lipase indicating a considerable alteration in the enzyme structure. However, after the 8 M urea treatment at 50°C for 20 min, the lipase activity was not affected providing that the urea concentration was reduced by dilution

(Fig. 6). Such extreme stability along with reversibility of the enzyme against conformational change might be due to the strong attractive forces of the hydrophobic structure in the enzyme molecule. Therefore, it is suggested that such a hydrophobic structure might be concerned with the stability of *Humicola* lipase against changes in the external environment such as denaturing reagents and temperature.

The activity of *Humicola* lipase was not affected by treatment with sulfhydryl reducing agent, dithiothreitol, at room temperature but it rendered the enzyme more susceptible to heat inactivation. Furthermore, the heat inactivation of the enzyme was accelerated by the addition of 8 M urea (Fig. 7). According to the results of amino acid analysis, *Humicola* lipase contained six half-cystine residues per enzyme molecule (Table I). However, no free sulfhydryl group was detected in a spectrophotometric study with *p*-chloromercuribenzoate in the presence or absence of 8 M urea,¹⁹⁾ which suggested that these half-cystine residues might exist as disulfide linkages in the enzyme molecule. In addition to hydrophobic structure, these disulfide linkages might play an important role in maintaining the enzyme structure against thermal denaturation.

Acknowledgement. Authors are deeply grateful to Drs. Makari Yamasaki, Takeshi Uozumi and Takashi Saiki, Department of Agricultural Chemistry, the University of Tokyo, for many valuable discussions. The skilled technical assistance of Dr. Tairo Oshima, Mitsubishi Kasei Institute of Life Science, Tokyo; Miss Keiko Tomita, the Institute of Applied Microbiology, the University of Tokyo; and Mr. Hidemasa Hidaka, Meiji Seika Co., Ltd., are also gratefully acknowledged. They performed respectively the circular dichroism analysis; the ultracentrifugal analysis; and the amino acid analysis.

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