Production and Properties of Alkaline Lipase from *Alcaligenes* sp. Strain No. 679[†]

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For the production of extracellular lipase by *Alcaligenes* species No. 679, NaNO_3 , polyoxyethylene alkyl ether, Fe^{++} , sodium citrate and fructose were found to be effective. The enzyme was prepared by acetone precipitation from the filtrate of the culture broth of this strain. The enzyme was most active at pH 9.0 and 50°C, while 35% of its activity was lost on heat treatment at 60°C for 10 min. Sodium salts of bile acids stimulated the enzyme activity. This lipase could hydrolyse natural fats and oils as well as olive oil. During the hydrolysis of olive oil, monoglyceride was found to accumulate up to 70 mol percent. This lipase possesses special properties similar to those of pancreatic lipase as shown in the comparative experiments.

With the recent increase in research on microbial lipases, many lipases of various characteristics have been found. For instance, Watanabe¹⁾ reported on alkaline lipase, and Kosugi²⁾ and Arima³⁾ reported on thermostable lipase. Sugiura⁴⁾ and Tomoda⁵⁾ found that the lipases from *Mucor* and *Phycomyces* were stimulated by bile salts.

As reported in the previous paper,⁶⁾ we found two *Alcaligenes* strains which produced lipases similar to pancreatic lipase. This paper deals with some cultural conditions for lipase production by *Alcaligenes* sp. No. 679 and the enzymatic properties of the acetone precipitated fraction of the culture filtrate compared with those of pancreatic lipase.

MATERIALS AND METHODS

Microorganism. The microorganism used in this study was *Alcaligenes* sp. No. 679.

Materials. Pancreatic lipase preparation (Wilzyme-400) was purchased from Wilson Laboratories. Olive oil and other natural fats were purchased from Yuro Yakuhin

Co., Ltd. SC, SDC, and SGC were obtained from Sigma Chemical Co. and STC was from Difco Laboratories. Other chemicals were obtained from commercial sources.

Cultivation of microorganism. Fifty ml of culture medium in a 500 ml shaking flask was inoculated with a loopful of the microorganism from a slant culture and incubated at 25°C for 2 days with shaking. The basal culture medium consisted of 3% kinako (roasted soybean meal), 1% wheat starch, 1% NaNO_3 and 0.2% K_2HPO_4 .

Preparation of the enzyme sample. The microorganism was cultured in a medium which was composed of 3% kinako, 0.75% NaNO_3 , 0.028% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.6% sodium citrate, 1.25% fructose, 0.5% polyoxyethylene stearyl ether and 0.4% silicon KM-72 (antifoamer), adjusted to pH 7.2. The culture broth was centrifuged to remove cells and other solids. Acetone was added to the supernatant fluid to 80% (v/v). The enzyme precipitate was collected by filtration, washed twice with acetone and dried *in vacuo*. This powder was used in this study. This preparation was assayed and found to contain 1.2×10^5 units/g according to assay method II described later.

Determination of lipase activity. The assay of lipase activity was carried out according to the following two methods. Assay method I was as described in the previous paper.⁶⁾ The activity in the culture fluid was measured according to this method. Assay method II was described

[†] Studies on the Alkaline Lipase. Part II. For Part I, see ref. 6.

Abbreviations: SC, sodium cholate; SDC, sodium deoxycholate; SGC, sodium glycocholate; STC, sodium taurocholate; lipase-Panc, pancreatic lipase preparation; lipase-PL, lipase preparation from *Alcaligenes* sp. No. 679; TLC, thin layer chromatography; HLB, hydrophilic-lipophilic balance; AV, acid value; SV, saponification value.

in the report of the *Federation Internationale Pharmaceutique*.⁷⁾ One ml of enzyme solution was mixed with 10 ml of olive oil emulsion, 17 ml of 5 mM Tris-aminomethane-NaCl buffer and 2 ml of 8% STC solution and kept at 37°C. The olive oil emulsion was prepared according to the method described in the previous paper.⁶⁾ Fatty acid liberated by the lipase reaction was automatically titrated in a pH-stat (TOA Electric Co., HS-2A), to keep the reaction mixture constantly at pH 9.0. One unit is defined as the enzyme amount which liberates one micro-equivalent of fatty acid per minute under the assay conditions. The enzyme properties of the lipase were studied according to this method. One lipase unit for method I is equivalent to about 2.7 lipase units for method II.

Hydrolysis of fats and oils. The reaction mixture containing 2 g of fats or oils, 4 ml of artificial intestinal juice,* 1 ml of 8% STC solution and 1 ml of lipase solution containing 200 units, was shaken at 60 oscillation per min for 60 min at 37°C. Free fatty acid in the reaction mixture was determined according to assay method I.

Hydrolysis of olive oil. The reaction mixture composed of 50 g of olive oil, 1.2 g of STC, 98 ml of 1 M glycine-NaOH buffer (pH 9.1), and 1 g of lipase preparation was kept at 37°C in a 300 ml flask with stirring. During hydrolysis, the pH of the mixture was adjusted to 8~9 with 10 N NaOH solution and an aliquot was taken periodically. The oily fraction of the sample was extracted on the acidic side with ethyl ether. The upper layer was washed with saturated NaCl solution, dehydrated with anhydrous Na₂SO₄, and then ethyl ether was distilled off.

Analysis of hydrolyzate. Determination of AV and SV of the oily fraction were carried out according to the "Standard of the Analytical Method" edited by The Japan Oil Chemical Institute. The rate of hydrolysis was calculated on the basis of AV/SV. Glycerol in the water layer was measured according to the method of Hanahan and Olley.⁸⁾ Mono-, di- and triglycerides in the oily fraction were separated by TLC. The solvent system was petroleum ether-ethyl ether-acetic acid (80:30:1) and the fractions were visualized with iodine. Each zone of mono-, di- and triglyceride on the TLC plate was scraped off separately and saponified with 0.75 N KOH alcohol solution. Glycerol in the saponified solution was measured as above. The molar ratio of mono-, di- and triglyceride was calculated from the quantity of glycerol.

RESULTS

Cultural conditions for lipase production

(a) *Effect of nitrogen sources.* The effects of organic nitrogen sources on the lipase pro-

duction were investigated by adding 3% of each chemical to the basal medium instead of kinako. Corn steep liquor, yeast extract, pepton and casamino acid were less effective than kinako. Inorganic nitrogen sources were investigated in the same way as above and the results are shown in Table I. (NH₄)₂HPO₄ and (NH₂)₂CO were slightly effective but the best result was obtained with NaNO₃.

(b) *Effect of carbon sources.* Various carbohydrates were tested by putting them into basal medium instead of wheat starch. As shown in Table II, effective carbon sources were Na-

TABLE I. EFFECT OF INORGANIC NITROGEN SOURCES ON THE LIPASE PRODUCTION

One percent of each nitrogen source was added to the basal culture medium in place of NaNO₃. Cultural conditions were described in MATERIALS AND METHODS.

Nitrogen sources	Final pH	Lipase activity in supernatant fluid (u/ml)
NH ₄ H ₂ PO ₄	6.4	145
(NH ₄) ₂ HPO ₄	6.7	200
NH ₄ Cl	5.6	111
(NH ₄) ₂ SO ₄	5.2	50
NH ₄ NO ₃	5.2	50
NaNO ₃	8.0	547
(NH ₂) ₂ CO	8.8	300
None	8.1	118

TABLE II. EFFECT OF CARBON SOURCES ON THE LIPASE PRODUCTION

One percent of each carbon source was added to the basal medium in place of wheat starch. Cultural conditions were described in MATERIALS AND METHODS.

Carbon sources	Final pH	Lipase activity in supernatant fluid (u/ml)
Fructose	8.4	950
Glucose	8.0	600
Lactose	8.2	670
Glycerol	8.6	10
Na-Tartarate	8.0	730
Na-Acetate	8.0	800
Na-Citrate	8.0	820
Na-Citrate + Fructose	8.4	1300
Na-Acetate + Fructose	8.4	1180
Wheat starch	8.1	630
None	7.9	660

* "Japanese Pharmacopeia," 9th edition, 1976, p. 639.

TABLE III. EFFECT OF NONIONIC SURFACTANTS ON THE LIPASE PRODUCTION

To the basal culture medium, 0.5 percent of each surfactant was added. Cultural conditions were described in MATERIALS AND METHODS.

Surfactants	<i>n</i> ^c	HLB	Final pH	Lipase activity in supernatant fluid (u/ml)
S ^a monostearate	—	5.1	8.5	143
POE ^b oleyl ether	5	8.8	7.4	95
POE stearyl ether	6	9.4	7.7	306
POE lauryl ether	6	10.5	7.5	105
POE S trioleate	20	11.0	8.0	244
POE lauryl ether	8	12.1	7.6	95
POE oleyl ether	13	13.6	8.1	422
POE lauryl ester	12	13.7	8.3	220
POE stearyl ether	13	13.9	8.4	550
POE cetyl ether	13	14.2	7.9	450
POE S monooleate	20	15.3	8.1	197
None			8.2	155

^a Sorbitan.

^b Polyoxyethylene H(OCH₂CH₂)_{*n*}—.

^c “*n*” means the number of polymerization of oxyethylene.

acetate, Na-citrate and fructose. The combination of fructose and citrate was the most favourable for the enzyme production. Glycerol was effective for bacterial growth but remarkably inhibited enzyme production. A favourable ratio of fructose and NaNO₃ was found to be 1.25% of fructose and 0.75% of NaNO₃.

(c) *Effect of nonionic surfactants.* To the basal medium 0.5% of nonionic surfactants was added and their effects on the enzyme production were investigated. Polyoxyethylene alkyl ethers whose HLB were in the range of 13.6~14.2 were favourable for the enzyme production as shown in Table III. Polyoxyethylene stearyl ether was the most effective.

(d) *Effect of minerals.* As trace elements, 1 mM mineral salts were added to the basal medium and their effects on the enzyme production were investigated. As shown in Table IV, FeSO₄ and FeCl₃ were effective but CoCl₂ inhibited the lipase production remarkably.

(e) *Time course of lipase production.* Time courses of the bacterial growth and enzyme production were investigated with a jar fermentor using the most favourable medium. The cultivation was carried out at 27°C with agitation of 400 rpm and aeration of 0.75

TABLE IV. EFFECT OF MINERALS ON THE LIPASE PRODUCTION

One mM each mineral salt was added to the basal culture medium. Cultural conditions were described in MATERIALS AND METHODS.

Minerals	Final pH	Lipase activity in supernatant fluid (u/ml)
MnCl ₂	8.9	528
CuSO ₄	8.3	577
CaCl ₂	8.7	470
FeSO ₄	8.7	700
MgSO ₄	8.7	450
ZnCl ₂	8.7	518
FeCl ₃	8.7	680
SnCl ₃	8.7	470
CoCl ₃	8.3	96
None	8.7	472

V.V.M. As shown in Fig. 1, the enzyme activity reached its peak after the maximum growth of bacteria and decreased moderately thereafter.

Comparison of the enzyme with pancreatic lipase

(a) *Optimum pH for enzyme activity.* The activities of lipase-PL and lipase-Panc at various pHs were measured according to assay

FIG. 1. Time Courses of Bacterial Growth and Enzyme Production.

The culture medium consisted of 3% kinako, 0.5% citrate, 1.25% fructose, 0.75% NaNO_3 , 0.2% K_2HPO_4 , 0.5% polyoxyethylene stearyl ether (HLB=13.9), and 0.028% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Cultural conditions were as follows: volume of the medium was 15 liters in a 30-liter jar, temperature was 27°C, aeration was 0.5 V.V.M., and agitation was 400rpm. The lipase activity in the culture broth was determined according to assay method II.

○, lipase activity; ■, bacterial growth; ▲, pH.

FIG. 2. Optimum pH for Lipase Activity.

The enzyme activities of lipase-PL (○) and lipase-Panc (●) were determined according to assay method I. They were expressed as percentage relative to that at pH 9.0, which was taken as 100%. Buffer systems; pH 5.0~8.0, McIlvaine buffer; pH 8.0~11.0, Atkins and Pantin buffer; pH 11.0~12.0, Na_2HPO_4 -NaOH buffer.

method I, except that 0.5 ml of 8% STC solution was used in place of distilled water. As shown in Fig. 2, both lipases have optimum pHs for activity around 9.0, however, lipase-PL was active in a wider pH range than lipase-

FIG. 3. Optimum Temperature for Lipase Activity.

The enzyme activity at each temperature was determined according to assay method II. The activity was expressed as percentage relative to that at 50°C with lipase-PL (○) and at 40°C with lipase-Panc (●), which were taken as 100%, respectively.

FIG. 4. Effect of pH on Lipase Stability.

After treatment of lipase preparations in each buffer for 24 hr at 8°C, the residual activity was measured according to assay method II. The activity remaining was expressed as percentage relative to that at pH 9.0 (lipase-PL, ○) and at pH 6.0 (lipase-Panc, ●), respectively, which were taken as 100%. Buffer systems; pH 2.7~8.0, McIlvaine buffer; pH 8.5~12.0, glycine buffer.

Panc.

(b) *Optimum temperature for lipase activity.*

Optimum temperatures for the lipase activities are shown in Fig. 3; they were 50°C for lipase-PL and 40° for lipase-Panc.

(c) *Effect of pH on lipase stability.* The stability of the lipase solutions of various pHs is shown in Fig. 4. While lipase-Panc was

FIG. 5. Effect of Temperature on Lipase Stability.

An aliquot of 1% lipase solution was incubated at various temperatures for 10 min at pH 9.0 (lipase-PL, ○) and at pH 6.0 (lipase-Panc, ●). The enzyme activity remaining was determined according to assay method II and expressed as percentage relative to that at 30°C, which was taken as 100%.

FIG. 6. Effect of Bile Salts on the Lipase Activity.

The lipase activity was measured according to assay method II in the presence of various concentrations of bile salts in the reaction mixture and expressed as percentage relative to that of 0.8% of each bile salt, which was taken as 100%.

○, lipase-PL; ●, lipase-Panc.

stable between pH 5 and 7, lipase-PL was stable in a wider range of pH from 6 to 11.

(d) *Effect of temperature on lipase stability.* Thermal stability of both lipases was investigated at the most stable pH for each lipase: pH

FIG. 7. Hydrolysis of Natural Fats and Oils.

Experimental conditions were described in MATERIALS AND METHODS. Degree of hydrolysis was expressed as percentage relative to that in the case of olive oil, which was taken as 100%.

□, lipase-PL; ■, lipase-Panc.

TABLE V. HYDROLYSIS OF SYNTHETIC ESTERS

According to assay method II, each synthetic ester was hydrolysed. The enzyme activity for the esters was expressed as percentage relative to that for olive oil, which was taken as 100%.

Esters	Lipase-PL (%)	Lipase-Panc (%)
Methyl butyrate	11	13
Methyl caproate	8	4
Methyl caprylate	22	7
Tween 80	7	1
Tween 85	27	1
Span 80	30	8
Tributyrin	180	500
Tri- <i>n</i> -caproin	66	44
Tri- <i>n</i> -caprin	136	133
Triolein	183	167

9.0 for lipase-PL and pH 6.0 for lipase-Panc, and the results are shown in Fig. 5. Lipase-PL was more stable than lipase-Panc towards heat treatment above 40°C.

(e) *Effect of bile salts.* Effects of sodium salts of four bile acids on lipase activities were investigated using assay method II. The results are shown in Fig. 6a, b, c and d. The activities of both lipases were stimulated remarkably in

FIG. 8. Hydrolysis of Olive Oil by Lipase-Panc (A) and Lipase-PL (B).

Experimental conditions and method of analysis of hydrolyzate were described in MATERIALS AND METHODS.

○, triglyceride; △, diglyceride; □, monoglyceride; ●, glycerine.

the presence of the bile salts. The activity of lipase-PL was much more stimulated than that of lipase-Panc with a lower concentration of bile salts. Particularly, the stimulation was remarkable in the case of sodium cholate and sodium deoxycholate.

(f) *Hydrolysis of natural fat or oil.* The hydrolysis of natural fats and oils by both lipases is shown in Fig. 7: Both lipases hydrolysed the fats or oils tested to the same degree as olive oil. Among them, coconut oil was hydrolysed most rapidly.

(g) *Hydrolysis of synthetic esters.* The hydrolysis of various esters of fatty acid by both lipases are shown in Table V. Both lipases were more active on triglycerides than on sorbitan esters and methyl esters of fatty acids. Lipase-PL, however, was more active on sorbitan esters of fatty acids than lipase-Panc.

(h) *Hydrolysis of olive oil.* Both lipases similarly hydrolysed olive oil and the rate of hydrolysis reached above 90%. Hydrolysis products of olive oil with the lipases were analyzed by TLC in order to compare the amounts of mono- and diglycerides accumulated. The results are shown in Fig. 8. When the hydrolysis rate of olive oil with both lipases reached about 70%, triglyceride was not detected and the maximal accumulation of monoglyceride was observed. The results coincided with each other in both cases.

FIG. 9. Effect of Components of Food on the Lipase Activity.

The lipase activity was determined according to assay method II in the presence of various concentrations of components of food in the reaction mixture and expressed as percentage relative to those in the absence of the components of food, which were taken as 100%.

○, lipase-PL; ●, lipase-Panc.

(i) *Effect of components of food on lipase activity.* Each component of food such as milk casein, egg albumin, starch or ethanol was added to the assay mixture and the lipase activities were determined. As shown in Fig. 9, the activity of lipase-PL was stimulated by ethanol and was not affected by starch but it

was suppressed slightly by milk casein and egg albumin. On the other hand, the activity of lipase-Panc was slightly suppressed by the components of food except egg albumin.

DISCUSSION

The main objective of this research was to obtain a large amount of such lipases as pancreatin *via* fermentation with a micro-organism. In the previous report, the authors pointed out three important properties of pancreatic lipase as a digestive aid. The first of these properties is that the lipase is active on the alkaline side. Several researchers have lately found lipases whose optimum pHs were in the alkaline region. For instance, lipases from *Candida parapolitytica*,⁹⁾ *Mucor lipolyticum*,¹⁰⁾ *Sclerotinia libertiana*,¹¹⁾ *Humicola lanuginosa*,³⁾ *Pseudomonas nitroreducens* var. *thermotolerans*¹⁾ and *Achromobactor* sp.¹²⁾ were reported to belong to this type. As far as the optimum pH of the enzyme is concerned, a number of microbial lipases similar to pancreatic lipase have already been found. However, the pH activity curve of lipase-PL is more similar to that of pancreatic lipase than those mentioned above.

The second desirable property is that the enzyme activity should be stimulated by bile salts. A group of microorganisms which produces lipases stimulated by bile salts includes *Phycomyces* sp.,⁵⁾ *Mucor japonicus*,⁴⁾ *Sclerotinia libertiana*¹¹⁾ and *Candida parapolitytica*.¹³⁾ However, the optimum pH for the enzyme activity of the lipases from *Mucor*, *Pseudomonas* and *Phycomyces* were reported to be neutral or slightly acidic, therefore, they are thought to be different from pancreatic lipase. The lipase of *Sclerotinia libertiana* was reported to be an alkaline enzyme and stimulated by bile salts. It was stimulated 2.4 times at pH 6.0 but only 1.3 times at pH 8.5 in the presence of 0.4% STC. Furthermore, the enzyme activity of *Candida parapolitytica* was observed only in the presence of anionic surfactants such as STC and sodium lauryl sulfate.^{13,14)} Lipase-PL was stimulated by bile

salts as shown in Fig. 6 and the stimulation curves against the concentration of STC and SGC were very similar to those of pancreatic lipase, and the optimum pH of this enzyme also agreed with that of pancreatic lipase.

The third property is the accumulation of monoglyceride during enzymatic hydrolysis of fats and oils. Frazer¹⁵⁾ demonstrated that hydrolysis of olive oil by pancreatic lipase brought about a monoglyceride intermediate which he thought played an important role in rapid digestion and absorption of fats and oils by emulsifying oils and fatty acids together with bile salts. Constantin¹⁶⁾ reported that during the course of hydrolysis of olive oil by pancreatic lipase, 80 mol% of monoglyceride and 40 mol% of diglyceride were accumulated. With respect to microbial lipase, the lipase from *Aspergillus niger*¹⁷⁾ accumulated 24 mol% of monoglyceride and the enzyme of *Rhizopus delemar*¹⁷⁾ accumulated 55 mol% during hydrolysis of triolein. The lipase from *Phycomyces* sp. and *Candida cylindracea*¹⁸⁾ accumulated only 8 and 5 mol% of monoglyceride, respectively. As mentioned above, the accumulation of monoglyceride during hydrolysis of fats and oils by microbial lipases was less than that by pancreatic lipase. In contrast to these observations, lipase-PL accumulated 70 and 28 mol% of mono- and diglyceride, respectively, and this seemed to agree well with the case of pancreatic lipase. In addition, both lipases similarly hydrolyzed natural fats and oils as well as olive oil.

There were, however, some differences between the two lipases in thermal stability, pH stability and optimum temperature. Lipase-PL was always more stable than pancreatic lipase.

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