Lipase from *Candida paraliopolytica*

Part III. Further Studies on the Activation of the Enzyme Systems with Bile- or Calcium Salts

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The extracellular lipase from *Candida paraliopolytica* required essential activators* (usually bile- or calcium salts) for the in vitro hydrolysis of triglycerides. The reaction systems emulsified with gum arabic, gelatin, lecithin, methyl cellulose, pectin, polyvinyl alcohol, sodium cholate, or without emulsifier were compared concerning requirement for essential activator, inhibition with sodium chloride and maximum reaction rate, and the following findings have been obtained. (1) The emulsions used can be classified into five groups by the essential activator requirement. (2) The inhibition with sodium chloride depended on reaction system. (3) Each reaction system gave a similar reaction rate at pH 8.2. (4) Long-chain fatty acid dissolved in substrate was necessary to the activation with calcium salts.

INTRODUCTION

It is well known that bile salts have activating effects on lipase, and a large number of studies on the activation were reviewed by Wills.¹⁾ His conclusion was that the exact alignment of enzyme molecules in the interfacial layer was important and that bile salts might promote this alignment.

Calcium salts also have activating effects on lipase. This was first described by Willstätter et al.,²⁾ and Schönheyder and Volqvartz³⁾ sug-

gested that the role of calcium ions was to remove fatty acids formed during the hydrolysis as insoluble calcium soaps. Wills⁴⁾ showed that calcium ions had an additional function in maintaining lipase stability.

Contrary to *Candida cylindracea* lipase, the extracellular lipase from *Candida paraliopolytica* requires essential activators, at least in vitro, for the hydrolysis of triglycerides. As shown in the previous reports,^{5,6)} it required alkaline-earth metal ions including calcium ions in the non-emulsified reaction systems and bile salt or anionic surfactant in the polyvinyl alcohol-emulsified systems. However, the activation mechanism by bile- or calcium salts was not elucidated sufficiently. In the present paper a further study is described using emulsions

* Essential activator means substances necessary to activate enzyme or enzyme systems. In the present paper it includes metal ions previously⁶⁾ termed cofactor.

Abbreviations: B, Tributyrin; CaAc₂, Calcium acetate; GA, Gum arabic; Gel, Gelatin; Lec, Lecithin; MC, Methyl cellulose; Non, Non-emulsified; O, Olive oil; OA, Oleic acid; Pec, Pectin; PVA, Polyvinyl alcohol; SC, Sodium cholate; STC, Sodium taurocholate; PVA (O; STC 0.2%), for example, shows the polyvinyl alcohol-emulsified reaction system activated with 0.2% of sodium taurocholate, using olive oil as the substrate.

1) E. D. Wills, *Advan. Lipid Research*, **3**, 197 (1965).

2) R. Willstätter, E. Waldschmidt-Leitz und F. Memmen, *Z. Physiol. Chem.*, **125**, 93 (1923).

3) F. Schönheyder and K. Volqvartz, *Acta Physiol. Scand.*, **10**, 62 (1945).

4) E. D. Wills, *Biochim. Biophys. Acta*, **40**, 481 (1960).

5) Y. Ota and K. Yamada, *This Journal*, **30**, 351 (1966).

6) Y. Ota and K. Yamada, *This Journal*, **30**, 1030 (1966).

changed in emulsifier.

On the other hand, the difference of requirement for essential activator existing between the two kinds of reaction systems suggested that substrate particles prepared by the two methods had different surface natures probably due to emulsifier. Up to date, many kinds of emulsifier have been employed in the studies on lipase, but the specific effect on lipase action has scarcely been discussed. Tomizuka et al.⁷⁾ found that the optimum pH of *C. cylindracea* lipase changed according to the kind of substrate preparations. Therefore, it is an interesting problem to classify emulsions prepared with various macromolecular emulsifiers by the essential activator requirement. The present paper deals with the action of *C. parapolytica* lipase in eight kinds of reaction systems.

MATERIALS AND METHODS

Enzyme. Crude lipase powder obtained from the culture fluid of *Candida parapolytica* was purified according to the method described in the previous paper.⁵⁾

Preparation of emulsions. An aqueous solution of emulsifier was added to 20 ml of olive oil or 10 ml of tributyrin, adjusting the total volume to 100 ml, and the mixture was emulsified for ten minutes by a homogenizer cooled with ice below 10°C. The olive oil (acid value 0.42*) and tributyrin used were obtained from Iwaki Seiyaku Co. and Tokyo Kaseikogyo Co., respectively.

Polyvinyl alcohol (PVA, Kurashiki Poval 117) was dissolved in distilled water by gradual heating and the solution (2%) was filtered after cooling. The PVA emulsion was stable for several days. PVA emulsions were employed by Fiore and Nord,⁸⁾ Hágony et al.,⁹⁾ Yamada et al.¹⁰⁾ and Arima et al.¹¹⁾

A 10% solution of Japanese pharmacopoeial gum arabic (GA) was neutralized with sodium hydroxide, dialyzed in a cellophane tube for six days to eliminate substances of low molecular weight and finally filtered. The GA emulsion was as stable as PVA emulsion. GA emulsions were used by Foder,¹²⁾ Hofstee,¹³⁾ Desnuelle et al.,¹⁴⁾ and Chandan and Shahani.¹⁵⁾

Methyl cellulose (MC) obtained from Kanto Kagaku Co. was suspended in a hot water and washed several times with the hot water on a Büchner's funnel to remove soluble impurities. The solution (1%) was prepared by cooling with ice. Before use MC emulsion was stored overnight in a refrigerator to make it free from fine bubbles. The MC emulsion was stable, but very viscous.

Gelatin (Gel) was washed with streaming cold water for a few days to remove soluble substances and dissolved in distilled water to give a 3% solution. The solution was filtered and the pH was adjusted to a desired value. The Gel emulsion of olive oil was as stable as PVA emulsion, but the emulsion of tributyrin was less stable. Before use the emulsions were warmed in a bath at 37°C, because they were coagulated in the refrigerator. Gelatin was introduced by Wills⁴⁾ into the study on pancreatic lipase.

A 1% solution of pectin (Pec) from citrus was neutralized with sodium hydroxide and dialyzed for six days to eliminate substances of low molecular weight. The Pec emulsion was not so stable and was prepared just before use.

Sodium cholate (SC) was dissolved in distilled water to give a 0.15% solution. Emulsification will be prevented by intense foaming, if the cholate concentration is increased to 0.3%. The SC emulsion of olive oil was prepared every day, but the emulsion of tributyrin was stable. Recently Benzonana and Desnuelle¹⁶⁾ employed bile salts themselves as emulsifiers to avoid the complexity owing to macromolecular emulsifiers.

Lecithin (Lec) emulsion was made as follows. One gram of lecithin from soya bean was mixed with the substrate and the total volume was adjusted to 100 ml

7) N. Tomizuka, Y. Ota and K. Yamada, This Journal, **30**, 576 (1966).

* The acid value of the olive oil used in Part II was 0.36 (KOH mg/g).

8) J. V. Fiore and F. F. Nord, *Arch. Biochem.*, **23**, 473 (1949).

9) P. L. Hágony, L. Vekerdi and T. Keleti, *Enzymologia*, **23**, 321 (1961).

10) K. Yamada, Y. Ota and H. Machida, *J. Agr. Chem. Soc. Japan*, **36**, 860 (1962).

11) K. Arima, T. Narasaki, Y. Nakamura and G. Tamura, This Journal, **30**, 515 (1966).

12) P. J. Foder, *Arch. Biochem.*, **35**, 311 (1952).

13) B. H. J. Hofstee, *J. Biol. Chem.*, **199**, 357 (1952).

14) P. Desnuelle, M. J. Constantin et J. Baldy, *Bull. Soc. Chim. Biol. France*, **37**, 285 (1955).

15) R. C. Chandan and K. M. Shahani, *J. Dairy Sci.*, **46**, 275 (1963).

16) G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, **105**, 121 (1965).

by the addition of distilled water. The mixture was emulsified by the method described above. The Lec emulsion was very stable. The nature of the emulsion did not change apparently, when lecithin was decreased to 0.5 g.

Determination of Lipase Activity

1) Activity in non-emulsified (shaken) systems.

Into an L-shaped flask were introduced 1 ml of substrate (olive oil or tributyrin), 2 ml of 0.2 M tris acid maleate-0.2 M sodium hydroxide buffer (pH 8.2), 1 ml of essential activator solution (0.1 M calcium acetate) and 5 ml of distilled water. The mixture was preheated at 37°C with constant shaking (66 oscillations/min.) for ten minutes and 1 ml of enzyme solution was added (total volume 10 ml). The incubation was carried out at 37°C for one or two hours with constant shaking, using a Monod-type shaking machine, and the reaction was stopped with 20 ml of a 1:1 mixture of acetone and ethanol. The mixture was titrated with 0.05 N aqueous sodium hydroxide with phenolphthalein as an indicator. A blank was shaken without enzyme, which was added immediately before titration.

The addition of free fatty acid to substrate was done as follows. Weighed fatty acid was dissolved in a certain quantity of chloroform and the solution was pipetted into each L-shaped flask. After evaporating chloroform, substrate was added and mixed with remaining fatty acid.

The concentration of essential activator or other water-soluble additives is expressed in molar concentration or in per cent (weight/volume) in the reaction mixture, the volume of which is 10 ml, including insoluble substrate, and this is the same in the following determination method.

2) Activity in emulsified systems. In a test tube (2×13 cm) were mixed 2 ml of the same buffer, 1 ml of essential activator (sodium taurocholate or calcium acetate) and 1 ml of distilled water. Then 5 ml of emulsion (containing 1 ml of olive oil or 0.5 ml of tributyrin) was added and mixed sufficiently. After preheating at 37°C for ten minutes, 1 ml of enzyme solution was poured as quickly as possible and again mixed sufficiently (total volume 10 ml). The mixture was incubated at 37°C without shaking. The procedure of determination followed was similar to the former method.

Lipase activity of these systems was calculated from the difference between ten and twenty minutes of reaction time.

FIG. 1. Relation between Reaction Time and Titer Values.

- —● Gel (O; STC 0.2%)
- —○ MC (O; STC 0.2%)
- ▲ —▲ GA (O; STC 0.2%)
- △ —△ GA (O; none)
- × —× SC (O; NaCl 0.075M)
- —● Pec (O; STC 0.1%)
- —○ Pec (O; none)
- —■ Gel (B; STC 0.1%)
- —□ PVA (B; STC 0.1%)

Abbreviations are described in the footnote of the first page. All reaction mixtures were at pH 8.2.

Fig. 1 shows the relation between reaction time and titer values in various emulsified systems. The zero-order reaction is observed till a titer value reaches to about 2 ml, changing emulsifier, substrate or essential activator. Similar results have been obtained in PVA-emulsified¹⁰⁾ and non-emulsified⁶⁾ systems.

Materials. Sodium taurocholate was obtained from Tokyo Kaseikogyo Co. and fatty acids were commercial products.

RESULTS

Fatty Acid Requirement for the Calcium Activation

As previously reported,⁶⁾ tributyrin was an unsuitable substrate for the lipase especially in the non-emulsified system, and it was supposed that the substrate specificity might change owing to reaction system. However, it has been found that fatty acid dissolved in tributyrin is necessary to its hydrolysis in addition to calcium ions dissolved in the aqueous phase.

Fig. 2 shows the activation with fatty acids. Of the fatty acids tested, linoleic acid was

FIG. 2. Fatty Acid Requirement for Calcium Activation (1).

Lipase activity was determined at pH 8.2 in the non-emulsified system activated with 10^{-2} M CaAc_2 , using tributyrin as the substrate. Relative activity is based on the maximum reaction rate observed for linoleic acid.

- ▲—▲ Caproic acid
- Lauric acid
- △—△ Stearic acid
- Oleic acid
- Linoleic acid
- ×—× Ricinoleic acid
- Na-oleate

FIG. 3. Fatty Acid Requirement for Calcium Activation (2).

Lipase assay was carried out at pH 8.2 in the non-emulsified system activated with 10^{-2} M CaAc_2 , using purified olive oil as the substrate. Olive oil was purified by chromatography on an Amberlite-400 column.¹⁷⁾ Relative activity is based on values for 2.5 mg/ml of oleic acid.

the most effective activator, and oleic and ricinoleic acids followed. Stearic acid was not able to activate the enzyme system and this is probably due to its low solubility in tributyrin. Beside solubility, length of fatty acid chain seems to be important to determine the ability of activation, since lauric acid was more effective than caproic acid. Sodium oleate dissolved in the aqueous phase was not comparable with free oleic acid mixed in the substrate, though the appearance of reaction mixture did not change.

On the other hand, olive oil was hydrolyzed easily in the non-emulsified system only by the addition of calcium salt.⁶⁾ As shown in Fig. 3 and Table I, this is due to long-chain

TABLE I. RECOVERY OF LIPASE ACTIVITY WITH OLEIC ACID

	Acid value (KOH mg/g)	Ratio of hydrolysis
Original olive oil	0.29	1.00
Purified olive oil +oleic acid 1.37 mg/ml	ca. 0.3	0.92

The assay was carried out at pH 8.2 in the non-emulsified system activated with 10^{-2} M CaAc_2 .

fatty acids naturally mixed in olive oil. Fig. 3 shows the activation of purified olive oil with oleic acid. Lipase activity increased to the maximum and declined as further oleic acid was added. The concentration of oleic acid in olive oil producing the maximum reaction rate was about 2.5 mg/ml (converting into acid value, ca. 0.55 KOH mg/g). With respect to acid value, the natural content of fatty acids in the olive oil used in our studies was enough to give the lipase activity of more than 90% according to Fig. 3. Table I shows that the natural activation was able to be recovered approximately by the addition of oleic acid after purification.

Comparison of Reaction Systems Concerning Essential Activator Requirement

The non-emulsified and the polyvinyl alcohol

17) J. Cason, G. Sumrell and R. S. Mitchell, *J. Org. Chem.*, **15**, 854 (1950).

FIG. 4. Reaction Systems Activated with either Sodium Taurocholate or Calcium Acetate.

The left half shows the results in the methyl cellulose-emulsified system and the right half those in the gelatin-emulsified system. The acid value of olive oil used was 0.42 KOH mg/g and all reaction mixtures were at pH 8.2. Relative activity is based on values for 0.1% STC.

(PVA)-emulsified systems were studied earlier.^{5,6} Fig. 4 indicates that both of sodium taurocholate and calcium acetate can act as the essential activator in the methyl cellulose (MC)-emulsified and the gelatin (Gel)-emulsified systems. Moreover, it is found that the two kinds of activators did not differ in their activating abilities in the MC system. In the Gel system lipase activity did not increase when adding both 0.1% of taurocholate and 10^{-2} M calcium acetate. A slight activity in the absence of activator is probably due to calcium ions contaminating the reaction mixture, since it was reduced nearly to zero with 10^{-3} M EDTA.

FIG. 5. Reaction Systems Requiring no Essential Activator.

The left half shows the results in the pectin-emulsified system and the right half those in the gum arabic-emulsified system. The acid value of olive oil used was 0.42 KOH mg/g and all reaction mixtures were at pH 8.2. Relative activity is based on values for 0.1% STC (Pec system) or 0.2% STC (GA system).

In Fig. 5 are shown the reaction systems which did not require essential activators. Lipase activities appearing without activator were hardly affected with 10^{-3} M EDTA. However, these activities were not the respective maximum reaction rates of the pectin (Pec)-emulsified and the gum arabic (GA)-emulsified systems, and both systems demanded non-essential activator. As the non-essential activator, sodium taurocholate was much more effective than calcium acetate. The activation with taurocholate was 1.6 and 2.1 times under the conditions employed in the Pec and the GA systems, respectively. Similar activations were observed in the studies on lipases

TABLE II. ACTIVATION OF GUM ARABIC SYSTEM (TRIBUTYRIN).

Na-taurocholate (w/v %)	Relative activity (%) ^{*1}
0	0 ^{*2}
0.1	100
0.2	87
0.3	72

^{*1} Relative activity is based on values for 0.1% STC.

^{*2} Lipase activity was not observed similarly, using tributyrin emulsion emulsified with pectin.

from pancreas^{10,18~20)} and *Candida cylindracea*,⁷⁾ using various reaction systems.

In contrast with the results mentioned above, the GA-emulsified system using tributyrin required sodium taurocholate essentially, as shown in Table II. From this finding, it is suggested that gum arabic and pectin were not contaminated with activators such as taurocholate, and that "emulsifier activation" may depend on fatty acids mixed in the substrate as well as the calcium activation.

Sodium cholate (SC) is an emulsifier of low molecular weight and acts as an activator in

the reaction systems emulsified with macro-molecular emulsifier. Therefore, the SC-emulsified systems are expected to show unknown properties. In Fig. 6 is graphed the activation of the systems with sodium chloride. The hydrolysis of olive oil occurring without addition of sodium chloride may be due to sodium ions (0.024 M) derived from the buffer used. Changing the substrate to tributyrin, again the enzyme system was not activated by the same method. However, the problem was partly solved by dissolving oleic acid in tributyrin.

Comparison of Reaction Systems Concerning Sodium Chloride Inhibition

The inhibition of lipolytic activity with sodium chloride was used to separate lipoprotein lipase from other lipases such as pancreatic lipase.^{11,21)} However, it has been found that the inhibition also depends on reaction system.

As shown in Fig. 7, the GA system, which was mentioned in many papers on pancreatic

FIG. 6. Activation of Cholate-emulsified Systems.

Reaction pH was 8.2. Relative activity is based on conditions, olive oil and 0.075 M NaCl

○—○ Olive oil as substrate.
●—● Tributyrin as substrate.

FIG. 7. Effect of Sodium Chloride on Some Reaction Systems.

All reaction mixtures were at pH 8.2. Abbreviations are shown in the footnote.

●—● GA (O; none)
□—□ Lec (O; none)
×—× Non (O; CaAc_2 10^{-2} M)
○—○ PVA (O; STC 0.2%)

18) P. Desnuelle, *Advan. Enzymol.*, **23**, 129 (1961).
19) P. J. Fritz and P. Melius, *Can. J. Biochem. Physiol.*, **41**, 719 (1963).

20) B. Baskys, E. Klein and W. F. Lever, *Arch. Biochem. Biophys.*, **102**, 201 (1963).

21) E. D. Korn, *J. Biol. Chem.*, **215**, 1 (1955).

lipase, was not inhibited but activated a little with 0.2M sodium chloride. On the other hand, the complete inhibition was observed with the same concentration in both the lecithin (Lec)-emulsified system and the PVA system activated with sodium taurocholate. These results may be due to the difference in activation mechanisms by bile salts, calcium salts and some macromolecular emulsifiers.

Comparison of Reaction Systems Concerning Maximum Reaction Rate

It was found that the non-emulsified and the PVA-emulsified systems gave approximately equal reaction rates at pH 8.2, using different essential activators.⁶¹ Fig. 8 shows the further comparison of all reaction systems employed in our studies. Various olive oil emulsions except Lec emulsion were hydro-

FIG. 8. Comparison of Reaction System Concerning Maximum Reaction Rate.

All reaction mixtures were at pH 8.2. Relative activity is based on Non (O). Abbreviations are shown in the footnote.

lyzed at similar speeds at pH 8.2 ($100 \pm 10\%$) and analogous results were obtained using tributyrin emulsions. The activities of pectin esterase and phospholipases were not observed under these conditions.

The data in the present paper indicate that the change of substrate specificity according to reaction system is not so large, when suitable essential or non-essential activators are added. The hydrolysis ratio of tributyrin to olive oil seems to be near to 0.6.

DISCUSSION

Emulsions tested in our studies can be classified by the requirement for essential activators as follows: (1) Non, only calcium ions (in combination with fatty acids in the substrate), (2) PVA, only sodium taurocholate (STC), (3) Gel and MC, either STC or Ca⁺⁺, (4) GA and Pec, apparently no requirement when using olive oil, (5) SC, sodium chloride (a special case). Such distinction of emulsions is certainly due to a specific enzyme-emulsifier or substrate-emulsifier interaction.

The comparison of emulsions concerning reaction rate (Fig. 8) suggests that macromolecular emulsifiers inhibit neither the lipase itself nor its action, after essential activators are added properly and enough. However, the inhibition before the addition cannot be discussed at the present time. Shahani and Chandan²²⁾ investigated the inhibitory milk lipase- β -casein interaction, though the substrate was emulsified with gum arabic.

Most of the experimental results obtained on the bile salt activation in our studies support Wills' consideration. At first, the lipase essentially required bile salt in many reaction systems, and this denies that its primary role is to assist diffusion processes of reaction products. Then bile salts were able to be replaced by synthetic anionic surfactants.⁵¹ The wide range of bile salt-type activators

22) K. M. Shahani and R. C. Chandan, *Arch. Biochem. Biophys.*, **111**, 257 (1965).

indicates that bile salts do not act as extremely specific activators (e.g. coenzymes) of the lipase, differing from the case of pancreatic cholesterol esterase.²³⁾ The requirement for a surface-active property shows that the activation mechanism may have a relation with the oil-water interface and the requirement for a negative charge is probably due to the structure of the enzyme. Furthermore, the bile salt activation can be replaced completely by the combination of calcium ions and long-chain fatty acids, as shown in Figs. 4 and 8.

The role of calcium ions investigated by us clearly differs from the role proposed by Shønheyder and Volqvartz,³⁾ since long-chain fatty acid dissolved in the substrate was required essentially. The requirement for fatty acid also suggests that phenomena at the oil-water interface are important to elucidate the activation mechanism by calcium ions. As reported previously,⁶⁾ the sigmoidal activation with calcium ions was first order and a cationic surfactant was a competitive inhibitor against calcium ions. These findings indicate that the enzyme requires one calcium ion per active center, which may be connect-

ed with unknown negatively charged groups.

In summary, it seems likely that the roles of bile- and calcium salts during lipolysis by *Candida parailipolytica* lipase are to promote the alignment of enzyme or substrate molecules or/and the transconformation of enzyme²⁴⁾ at the interface.

Other activating effects of fatty acid mixed in the substrate were described by Yamada et al.¹⁰⁾ and Hofmann and Borgström.²⁵⁾ Its action in the cholate-emulsified system using tributyrin can be explained by the mechanism elucidated by Hofmann.

It was reported that sodium chloride inhibited lipoprotein lipases in a specific manner. However, it has been found that the inhibition depends on reaction system, i.e., kind of emulsion, in addition to kind of lipase, using *C. parailipolytica* lipase. Therefore, for the purpose of lipase classification, it is necessary to control reaction conditions exactly.

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23) G. V. Vahouny, S. Weersing and C. R. Treadwell, *Biochim. Biophys. Acta*, **98**, 607 (1965).

24) P. Desnuelle, L. Sarda and G. Ailhaud, *Biochim. Biophys. Acta*, **37**, 570 (1960).

25) A. F. Hofmann and B. Borgström, *ibid.*, **70**, 317 (1963).