Studies on the Effects of Surfactants on Lipase Activity

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In a series of studies on the application of lipases to detergency, the influence of pH and surfactants on the activities of various lipases was examined by assaying residual lipase activity. Five microbial lipases of Candida cylindracea (Can), Mucor sp. (Mu), Rhizopus chinensis (Rhi), Chromobacterium viscosum (Chr), and Pseudomonas (Pse), and an animal lipase of porcine pancreas (Pan) were used. Two methods A) and B), the prior contact of surfactant with substrate or with lipase, respectively, were used.

The following results were obtained:
1) The effects of change in ionic groups on lipase activity were briefly examined by varying the pH of the system. These effects were small for Rhi and Chr but large for Can, Mu, Pse, and Pan.
2) In method A, all lipase activity was reduced almost linearly with rise in surfactant concentration. Measurements of the kinetic parameters of lipase hydrolyses indicated the inhibition to be quite likely due to change in the surface properties of substrate as a result of surfactant adsorption.
3) In method B, the nonionic surfactant activated each lipase, and the activity was maximum at the characteristic concentration.
4) The effects of anionic surfactants in method B varied according to the lipase. Can, Mu, Pse, and Pan were strongly inhibited, while the activity of Rhi and Chr increased once and remained in a wide range of concentration. Maximum Can, Rhi, Chr, and Pse activity in the presence of anionic surfactants may possibly be due to the formation of a new and more active surface lipase-surfactant complex.
5) The effects of surfactants at pH 10 were similar to those at pH 7.
6) Classification of lipases on the basis of inhibition mode by anionic surfactants was consisted with that by pH effect.
7) The inhibition of lipase activity by anionic surfactants could be avoided by mixing nonionic surfactants.

1 Introduction

Enzymes in laundry detergents, such as alkaline protease, have been developed in the last decade owing to the trends to lower phosphate builder and/or lower washing temperature. In the previous paper the authors reported that lipase from Candida cylindracea improved the removal of olive oil from cotton fabric by 15-20% at the optimum conditions. Moreover, as it is widely accepted that free fatty acids are easily removed by laundering with alkaline solution of surfactant in preference to other glycerides owing to the formation of water soluble free fatty acid soap, it can be expected that lipolytic enzyme, that is lipase, will be applied for detergency to promote the removal of triglycerides by lipolytic hydrolysis to di-, monoglycerides and free fatty acids in the future.

It has also been reported that surfactants, the main components of laundry detergents, strongly inhibit enzyme reactions. As for lipases, Wills reported that anionic detergents almost completely inhibited the pancreatic lipase hydrolysis at the concentration of more than 0.05%. However, there have been reported few studies about the effects of surfactants on other lipases originated from microorganisms though they are very important in industry. Moreover, though the effect of surfactants on lipase hydrolysis in the case of the prior contact of
surfactants with lipase would be more important in considering the practical laundry, such effects have scarcely been studied\(^{13}\).

Here, as a series of studies on application of lipases to detergency, we wish to report the influence of pH and surfactants on the activities of various lipases and also the protective effect by mixing nonionic surfactant against the large decrease in lipase activity by anionic detergents alone.

2 Experimental

2.1 Materials

2.1.1 Lipases

Five kinds of lipases from various microorganisms (yeast, mold, and bacterial lipase) and a pancreatic lipase shown in Table-1 were used in this study. Lipase activities (unit/g) were measured according to Yamada et al.\(^{14}\), where one lipase unit was defined as the amount of lipase which liberates 1 \(\mu\)mol of fatty acid from olive oil per one minute at 37°C\(^{15}\). The original activities of lipases were also shown in Table-1.

2.1.2 Surfactants

One nonionic and four anionic surfactants, as shown in Table-2, were used without any purification to investigate the effect on activity of lipase.

2.2 Methods

For the determination of lipase activity was employed the following two methods according to Yamada et al.'s\(^{14}\) with slight modifications.

2.2.1 Preparation of Olive Oil Emulsion

As a substrate of lipase hydrolysis, olive oil (Japan pharmacopeia grade, Yamakei Sangyo Co.) was used as received. The olive oil emulsion was prepared by homogenizing twice a mixture of 25 ml of olive oil and 75 ml of poly(vinyl alcohol) solution consisting of 1.85 \% PVA 117 (polymerization degree 1725±25, saponification value 98.5±0.5 mol\%, Kuraray Co. Ltd.) and 0.15 \% PVA 205 (550±50, 88.0±1.5 mol\%, respectively) with Emulsion (Te=raoka Co.) at 11,000 r.p.m. for each 5 min at 5~10°C.

2.2.2 Buffer

Buffer solution (pH 7) was prepared by mixing 0.1 M KH\(_2\)PO\(_4\) and 0.1 M Na\(_2\)HPO\(_4\). To investigate the effect of pH, other buffer solutions (pH 7.5~10) were prepared by addition of 0.1 N NaOH to the above buffer of pH 7.

2.2.3 Preparation of Lipase Solution

Lipase solution was prepared by dissolving each lipase in distilled water just prior to use. Preliminary experiments revealed that the amounts of produced free fatty acids were proportional to the concentration of lipase up to 4 unit/ml for all six lipases employed in this study. Though detergent tolerance of lipase would depend on the concentration of lipase,

<table>
<thead>
<tr>
<th>Table-1</th>
<th>Lipases used for experiments.</th>
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<tbody>
<tr>
<td>Yeast lipase</td>
<td>Candida cylindracea</td>
</tr>
<tr>
<td>Mold lipase</td>
<td>Mucor sp.</td>
</tr>
<tr>
<td>Rhizopus chinensis</td>
<td>800</td>
</tr>
<tr>
<td>Bacterial lipase</td>
<td>Chromobacterium viscosum</td>
</tr>
<tr>
<td>Pseudomonas</td>
<td>22900</td>
</tr>
<tr>
<td>Pancreatic lipase</td>
<td>Porcine pancrea</td>
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<table>
<thead>
<tr>
<th>Table-2</th>
<th>Surfactants used for experiments.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RO(CH(_2)CH(_2))(_n)H</td>
<td>Nikko Chemicals</td>
</tr>
<tr>
<td>CH(_2)(_3)OSO(_4)Na</td>
<td>Wako (for biochemical)</td>
</tr>
<tr>
<td>C(_12)H(_25)-C(_8)H(_3)-SO(_4)Na</td>
<td>Wako (for chemical)</td>
</tr>
<tr>
<td>Sodium (\alpha)-olefinsulfonate</td>
<td>Lion</td>
</tr>
<tr>
<td>Sodium N-stearoylglutamate</td>
<td>Ajinomoto</td>
</tr>
</tbody>
</table>
the concentration was held constant of 2.5 unit/ml for all lipases, which unit value was taken from original activity, in order to compare them under the same condition.

2.2.4 Experimental Method

Method A: A mixture of 5 ml olive oil emulsion, 3 ml phosphate buffer and 1 ml distilled water or surfactant solution was preheated at 37°C for 10 min before the reaction was started. The hydrolysis was started by adding 1 ml lipase solution. After 20 min, 20 ml of a mixture of acetone/ethanol (1:1, vol/vol) was added to the reaction mixture to stop lipase hydrolysis. As control, the above procedure was repeated except that lipase was added after the addition of a mixture of acetone/ethanol. The amount of free fatty acids in the reaction mixture and control mixture was determined by titration with 0.05 N NaOH using phenolphthalein as indicator. The activity of lipase was calculated according to the following equation,

\[
\text{Activity of lipase (unit/g)} = \frac{(T_r - T_c) \times f \times (1/w)}{2.5},
\]

where \(T_r\) and \(T_c\) are titration values (ml) for reaction mixture and control mixture, respectively, \(f\) is a factor of 0.05 N NaOH, and \(w\) is the weight of lipase dissolved in 1 ml of lipase solution.

Method B: A mixture of 3 ml phosphate buffer, 1 ml lipase solution and 1 ml distilled water or surfactant solution was preheated at 37°C for 10 min. The hydrolysis was started by adding 5 ml olive oil emulsion. After 20 min, the lipase reaction was stopped, the free fatty acids were titrated and the activity of lipase was calculated in a similar manner as method A.

2.3 Effect of pH

The effect of pH on lipase activity was studied by changing pH of buffer from 7 to 10 by 0.5 in both method A and B.

2.4 Effect of Surfactant

The effects of surfactants on the activities of lipases were studied by replacing 1 ml distilled water to 1 ml surfactant solution of a given concentration (0.05~1.0%) in both method A and B.

In method A the surfactant was allowed to contact first with substrate emulsion, while in method B the surfactant contacted first with lipase, which are illustrated in Scheme-1.

2.5 Effect of Mixed Surfactant

With respect to the system of surfactant mixture, the experiments were done only for method B. In this experiment the concentration of buffer (pH 7) was slightly modified to 0.15 M and the total concentration of surfactants was held constant at 0.05%.

As shown in Scheme-2, to examine the influence of the order of contact of two surfactants with lipase on the degree of protection against inhibition, the residual lipase activity was measured by the following three methods:

Method Ba: After the prior contact of anionic surfactant with lipase for 5 min, nonionic one was added and, after 10 min in total, the lipase hydrolysis was started by adding emulsion.

Method Bn: After the prior contact of non-ionic surfactant with lipase for 5 min, anionic
one was added and operated similarly.

Method Bm: From the beginning, two surfactants were mixed and allowed to contact with lipase for 10 min, and operated similarly.

2.6 Expression of Results

The results were expressed in terms of relative activity (residual lipase activity experimentally obtained to the original one listed in Table-1). With respect to the concentration of surfactants, it was expressed using the value of the system at the beginning of the lipase hydrolysis, not using that of original surfactant solution.

3 Results

Though there have been many studies on the effects of pH and/or surfactants on lipase hydrolysis\(^9\)\(^{-}13\), in those studies such effects were investigated under several different conditions (pH, buffer, substrate and surfactants) for each lipase, and hence those results cannot be compared simultaneously. Therefore, in this paper the effects of pH and surfactants on lipase activity were studied under same conditions for various lipases.

The procedures consisted of following two methods: (1) Similar to conventional method, the lipase hydrolysis was started by the addition of lipase solution to the mixture of substrate and surfactant (Method A). (2) The lipase hydrolysis was started by the addition of substrate to the mixture of lipase and surfactant, because it is reasonably more practical under consideration of detergency that lipase contacts prior with detergents and acts on substrate (Method B).

3.1 Effect of pH

In the point of view of application of lipase under the practical laundry conditions, the effect of pH on the lipase activity was examined by changing the pH of the hydrolysis system in the range of 7 to 10. The results are shown in Fig.-1.

As can be seen in Fig.-1, lipases were grouped into three classes in method A. The optimum pH for Mu, Rhi, Chr, Pse, which are said alkaline-stable, and Can lies at 7, while that for Pan, alkaline lipase, lies at 8–9. The lipase activities of Mu, Rhi, and Chr did not decrease so much with a rise in pH, while those of Can and Pse decreased significantly and became half of their original activity at pH 9 and 10, respectively. On the other hand, that of Pan increased with a rise of pH and became 1.5 times larger at about pH 9.

In method B, even at pH 7, Can, Mu, Chr, and Pse have smaller relative activities by the values of 0.75, 0.9, 0.9, and 0.6, respectively. It can be interpreted in terms of thermal stability because such small values would be caused by the preheating for 10 min at 37°C before starting lipase hydrolysis. In method B, lipases were grouped into two classes. With a rise in pH, the relative values of Rhi, Chr, and Pan did not change or decrease so much, while those of Can, Mu, and Pse changed remarkably.

3.2 Effect of Surfactants

Effects of various surfactants on lipase activity were examined. Each surfactant was used at the concentrations of 0.005 to 0.1%.

3.2.1 APE-10

As shown in Fig.-2, in method A, the relative activity of lipase decreased almost linearly with rise in concentration of APE-10 regardless of the kinds of lipases. Wills, though he employed shaking method, reported similar result for triolein hydrolysis of Pan when nonionic detergents were used in high concentration (more than 0.02%)\(^12\). To the contrary, different from method A, with rise in concentration in method B the lipase activities once increased. Kokusho et al., though they studied only at one concentration (0.05%), also reported the activation of Pan by nonionic surfactants\(^13\). After passing the maximum at
the characteristic concentration for each lipase, lipase activities again decreased. However, the degree of decrease after activation was not so large as that in method A. Especially, the relative activities of Pan and Mu were kept larger than unity in a wide range of concentration, and it is noteworthy that the relative activity of Pan reached up to 2.3 at 0.03% of APE-10.

3.2.2 SDS

As can be seen in Fig.-3, similar to the result mentioned above for nonionic surfactant, the relative activities of lipases decreased linearly in method A with rise in the concentration of SDS. Further, complete inhibition was observed for Can and Pan at 0.07% of SDS. Except for the value of concentration, this is in accord with the previous reports by Wills that complete inhibition of lipase action (Pan) occurred at the concentration of 0.15%\(^{12}\). On the other hand, the inhibition of lipase action was not so strong for mold lipases, Mu and Rhi.

In method B, however, it proved that lipases can be roughly divided into two groups. The lipase hydrolyses of Can, Mu, Pse, and Pan were strongly inhibited and even at the low concentration (less than 0.02%) the inhibition was complete. Such inhibition of lipase action by SDS in method B was also reported for Can and Pan despite of different reaction conditions\(^{13}\). On the other hand, the presence of low concentration of SDS caused the increase in activities of Rhi and Chr and their lipase activities were remaining in a wide range of concentration. More detail elucidation allowed to find that the maximum of lipase activities can be observed for Can, Rhi, Chr, and Pse, but not for Mu and Pan.

3.2.3 DBS and AOS

The effects of DBS and AOS in method B are shown in Fig. 4, where the effects in method A were omitted as there was no great difference from that in the case of SDS.

In these cases, lipases can also be divided into two groups. The lipase activities of Can, Mu, Pse, and Pan were strongly inhibited and the complete inhibition occurred at the lower concentration in comparison with the case of SDS. On the other hand, those of Rhi and Chr were remaining even at the concentration of 0.1%. Also, though not clear for Rhi, there observed the maximization of lipase activities of Can, Rhi, Chr, and Pse. Therefore, it can be reasonably concluded that the effect
of SDS represent the typical ones of anionic surfactants on lipase activity.

3.2.4 NAG

Sodium N-acylglutamate, though classified into anionic surfactants, gave different results from other anionic surfactants mentioned above.

<table>
<thead>
<tr>
<th>Method A</th>
<th>Method B</th>
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<tbody>
<tr>
<td>Can</td>
<td>0.07%</td>
</tr>
<tr>
<td>Mu</td>
<td></td>
</tr>
<tr>
<td>Rhi</td>
<td></td>
</tr>
<tr>
<td>Chr</td>
<td></td>
</tr>
<tr>
<td>Pse</td>
<td></td>
</tr>
<tr>
<td>Pan</td>
<td></td>
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</tbody>
</table>

In method A, though SDS completely inhibited the lipase action of Pan at 0.07%, NAG did not inhibit Pan so much, but significantly inhibited Can and Pse. In method B, indeed the maximization was clearly observed for Can, Rhi, Chr, and Pse, but the inhibition was smaller than the case of SDS. Especially, Pan retained its relative activity by the value of 0.5 even at the concentration of 0.1%.

3.3 Effect of Surfactant at pH 10

As in the practical laundry the pH of the system reaches at pH 9~10 owing to the alkaline builders, similar experiments were done at pH 10 in order to elucidate whether the results obtained at pH 7 can be applied for more practical high alkaline system. In these experiments, the lipase Can was neglected because of its poor pH stability. Fig.-6 shows the representative results of APE-10 and SDS in method B.

With rise in concentration of APE-10, there observed the maximization of relative activities, for example Pan stimulated its relative activity by twofold. But, different from the case of pH 7, there found the critical concentration of APE-10 to produce the increase in activity.

In the case of SDS, lipase action of Mu, Pse, and Pan was completely inhibited at lower concentration than the case of pH 7. Though there could not be observed the maximization for Rhi and Chr, their lipase activities were remaining even at the high concentration. Likewise, in the cases of DBS and AOS, similar tendencies were observed.

According to the results that there appeared the maximization of lipase activities for nonionic surfactant and lipases could be divided into two groups for anionic surfactants at pH 10 as well as pH 7, the results at pH 7 can be concluded qualitatively applicable for the cases of high pH.

3.4 Protective Effect of Surfactant Mixture

When lipases are formulated in the practical laundry detergents, it is quite necessary to protect the large decrease in lipase activity at high concentration of surfactants in order to utilize lipase hydrolysis more effectively. For such purpose, referring to the above results, the effects of mixture of nonionic and anionic surfactants on lipase activity were examined as one method to protect large decrease in lipase activity by anionic surfactants. In these experiments, lipase Rhi was used and the concentration of surfactant mixture was held at 0.05% with varying the mixing ratios. Experiments were done by three methods (method Ba, Bn and Bm, see experimental section in detail) to discuss the order of contact of lipase with two surfactants. The results are shown in Fig.-7 a (APE-10/DBS) and Fig.-7 b (APE-10/SDS). The values of the symbol ⊪ were the products of each relative activity at the given concentration of surfactants by assuming their indepen-
dent action to lipase.

The relative activities of three methods were aligned in the order of method Bn > method Bm > method Ba. In method Ba, the subsequent addition of nonionic surfactant no longer reactivated the lipase activity. On the other hand, in method Bn and Bm the protective effect by mixing of nonionic surfactant was clearly observed, and at the low mixing ratio of nonionic surfactant the protective effect in method Bn was slightly larger than that in method Bm.

Such protective effect (method Bn and Bm) is well compatible with the previous result that the inhibition of Can and Mu by SDS and DBS was efficiently protected by the addition of nonionic surfactants.

4 Discussion

It is wellknown that lipases act at oil/water interface and the properties of the interface exert an influence on lipase hydrolysis. Wills concluded that the change in surface properties of substrate caused by the adsorption of surfactants affects lipase hydrolysis and it would be the reason for the large inhibition by anionic surfactants. In this study, we adopted two methods, method A and B, where the orders of contact of surfactant, lipase and substrate were varied. Such concept introduced by Wills would

hold in the case of prior contact of surfactant with substrate (method A), but another explanation is necessary to be considered in method B.

In general, several factors can be supposed for the change in lipase activity; that is, (1) the change in ionic groups of lipase protein, (2) the change in conformation of lipase, and (3) subsequent change in surface activities of lipase.

First, the effect of change in ionic groups was briefly examined by varying the pH of the system. In the comparison of method A with method B (Fig.-1), as there will be little difference in the effect of pH on the rate of lipase hydrolysis between two methods, the influence of the change in ionic groups would appear more remarkably in method B. Therefore, as a measure of the influence of the change in ionic groups, the ratios of the relative activity in method B to that in method A were estimated. The results are shown in Fig.-8. The followings were obtained: (1) For Rhi and Chr, there was little change in a range of pH 7-10. The effect of change in ionic groups would be small. (2) For Mu and Pan, steep decrease was observed up to pH 8.5. The effect would be large. (3) For Can and Pse, there was little change up to about pH 8, but remarkable change was observed in higher pH region. The effect would be large at high pH.
As the ionic groups are important also in the adsorption of surfactants on lipase, this result suggests that the smaller the change in ionic groups due to pH variation is, the smaller the effect of surfactants on lipase hydrolysis would be.

With respect to the effect of surfactant, every lipase activity was reduced almost linearly with rise in surfactant concentration independently on the kinds of surfactants in method A. As the surface properties of emulsion would be changed in method A first of all, this result is reasonably explained according to the Wills’ concept. Further, from the measurement of kinetic parameters, maximum reaction rate \( V_{\text{max}} \) and Michaelis constant \( K_m \), at the concentration of 0.005% of APE-10 or SDS in method A, it turned out that the change in surface property (charge) caused by the adsorption of surfactants exactly resulted in inhibition of lipase hydrolysis. As seen in Table-3, in the case of SDS \( V_{\text{max}} \) was almost unchanged and \( K_m \) increased, while in the case of APE-10 both \( V_{\text{max}} \) and \( K_m \) increased. In the case of SDS, this result represents the typical mode of competitive inhibition\(^{17}\). Namely, the \( SO_3^- \) groups of SDS adsorbed on emulsion surface influence the active point of lipase. In the case of APE-10, however, the result is complex and the mode of inhibition is not clear at the present.

Table-3 Kinetic parameters of lipase hydrolysis reaction in the presence of surfactants (Method A).

<table>
<thead>
<tr>
<th>Surfactant</th>
<th>( V_{\text{max}} ) (pmol/\text{lit} \cdot \text{min})</th>
<th>( K_m ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>260.4</td>
<td>3.62</td>
</tr>
<tr>
<td>APE-10</td>
<td>345.1</td>
<td>19.54</td>
</tr>
<tr>
<td>SDS</td>
<td>249.4</td>
<td>6.67</td>
</tr>
</tbody>
</table>

Lipase : Can (2.5 unit/system)
Surfactant concentration : 0.005% |

On the other hand, in method B where the surface activity of lipase would be changed by the addition of surfactant, the mode of change in lipase activity depended on the kinds of surfactants. Nonionic surfactant activated every lipase with rise in concentration and gave the maximum of activity, but the effect of anionic surfactants depended on the kinds of lipases. The classification of lipases according to the mode of inhibition by anionic surfactants is well consisted with that according to pH effect.

The appearance of maximum in Can, Rhi, Chr, and Pse in method B is very interesting because there have been no reports about such phenomenon. In these cases, similar to the interactions of various surfactants with proteins, anionic surfactant would interact with lipase protein and probably produce a new lipase-surfactant complex which should be more surface active than original lipase\(^{19}\). It can be considered that such complex would act on substrate and apparently stimulate the lipase hydrolysis, but at higher concentration excess surfactant would attack the active point of lipase and produce inhibition. No detection of maximization for Mu and Pan could be explained by the rapid conformational change of them similar to the large change by pH variation.

To the contrary, in the case of nonionic surfactant, such conformational change would hardly occur till high concentration because of week electrostatic interaction of surfactant with protein and active point of lipase. The fact that the tendency of the effect of surfactant at pH 10 was similar to that at pH 7 is also consistent with this explanation.

At last, as a result of examination of mixing effect (nonionic/anionic surfactants) as one method to protect the decrease of lipase activity, considerable protective effect was obtained. Taking the fact into consideration that alkaline protease formulated in the present laundry detergents has been pretreated with nonionic surfactant during granulation\(^{15}\), this method would be very valuable.

Further detail studies on the interaction of lipase with various surfactants from the view point of surface chemistry of lipase are now under investigation.

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