Purification and Characterization of a Novel Cholesterol Esterase from *Pseudomonas aeruginosa*, with Its Application to Cleaning Lipid-stained Contact Lenses

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With the aim of developing a new cholesterol esterase for eliminating lipids on used contact lenses, microorganisms were screened for the enzyme activity. A *Pseudomonas aeruginosa* isolated from soil was found to produce a desirable enzyme. The enzyme had an isoelectric point of 3.2, and molecular mass of 58 kDa. The optimal temperature was around 53°C at pH 7.0, and the optimal pH was from 5.5 to 9.5. The enzyme was stable between pH 5 and 10 for 19 h at 25°C, and retained its activity up to 53°C on 30 min of incubation at pH 7.0. The rates of hydrolysis of cholesteryl esters of different fatty acids were in the following order: linoleate > oleate > stearate > palmitate > caprylate > myristate > laurate, caprate > caproate > butyrate, acetate. Addition of (tauro)cholate to a final concentration of 100 mM markedly promoted the hydrolysis of triglycerides of short-, medium-, and long-chain fatty acids. When used with taurocholate, the enzyme acted as an effective cleaner for contact lenses stained with lipids consisting of cholesteryl oleate, tripalmitin, and stearyl stearate.

Key words: cholesterol esterase; cholesteryl ester; *Pseudomonas aeruginosa*; contact lens cleaner

Cholesterol esterase (EC 3.1.1.13) has been extensively examined in a number of mammalian tissues including pancreas, liver, adrenal, placenta, aorta, and brain, primarily because of the involvement of sterol esters in atherosclerosis.1–5) In addition to cholesteryl esters, acylglycerols, retinyl esters, vitamin esters, and phospholipids are also among the physiological substrates of the enzyme.6) On the other hand, in spite of the widespread distribution and variety of sterols and fatty acids in fungi and yeasts, production of microbial cholesterol esterase has been demonstrated only from *Pseudomonas* (P.) *fluorescens*,7) *Staphylococcus aureus*,8) *Fusarium oxysporum*,9,10) and *Sacccharomyces cerevisiae*.11,12) The practical use of these enzymes is limited to the measurement of cholesterol in human blood sera. Hence the commercially available enzymes are highly purified and expensive.

We have turned our attention to the wide substrate specificity of cholesterol esterase, and began studies to develop a new microbial enzyme for cleaning used contact lenses. High-grade contact lenses are reused after being cleaned with a cleaning fluid, to which protease, triglyceride lipase, or both may be added. However, lipids sticking to the used contact lenses are difficult to remove even with the aid of lipase because of the high contents of wax (32–35%) and sterol (mainly cholesterol) esters (27–29%)13) that are not appropriate substrates for the enzyme. We examined cholesterol esterase for its usefulness in eliminating cholesteryl ester, triglyceride, and wax on lipid-stained contact lenses. This paper deals with purification and characterization of a novel cholesterol esterase from a *P. aeruginosa* isolated from soil, and its application to cleaning contact lenses artificially stained with three typical lipids which are secreted with tears.

**Materials and Methods**

**Materials.** Cholesterol oxidase and peroxidase were purchased from Toyobo (Osaka). Cholesteryl acetate, butyrate, caproate, caprylate, myristate, palmitate, and stearate were obtained from Tokyo Kasei (Tokyo). Cholesteryl oleate, cholesteryl linoleate, and Igepal CA-630 were from ICN Pharmaceuticals (Costa Mesa, CA). Simple triglycerides and Quick CBB (Coomassie Brilliant Blue solution) were the products of Wako Pure Chemical Industries.
(Osaka). Stearyl stearate was from Funakoshi (Tokyo). Protein molecular weight markers (MW-GF-200) for gel filtration were obtained from Sigma-Aldrich (St. Louis, MI). Molecular weight standards for SDS-PAGE (LMW Marker Kit), Pharmalyte 3-10, a Superdex 200 column (1 × 30 cm), and a Mono Q column were from Amersham Biosciences (Uppsala, Sweden). Lipase M from Mucor javanicus was obtained from Amano Enzyme (Aichi). Other reagents were of analytical grade.

**Enzyme assay.** Cholesterol esterase activity was measured by the method of Gallo with micellar cholesteryl linolate as the substrate. The assay was at 40°C in 110 mM phosphate buffer, pH 7.0, containing 0.2 mM cholesteryl linolate, 1.5 mM 4-aminoantipyrin, 22 mM phenol, 10 units of Streptomycetes sp. cholesterol oxidase, 5 purpurogallin, and 0.2 mM sodium cholate, and 0.68 M isopropanol. To 920 μl of this reaction mixture kept at 40°C was added 35 μl of enzyme solution, and the increase in absorbance at 500 nm was recorded for 30 sec on a Shimadzu UV-1600 spectrophotometer (Shimadzu, Kyoto). The cholesterol esterase activity was assayed from the initial slope with a molar absorbance of 13,780 for quinoneimine dye. One unit of activity was defined as the amount of enzyme producing 1 μmol of free cholesterol/min under these conditions.

**Protein assay.** Protein was estimated spectrophotometrically with a Bio-Rad protein assay kit with bovine serum albumin as the standard. The chromatographic elution patterns were monitored in terms of the absorbance at 280 nm.

**Purification of enzyme.** P. aeruginosa isolated from soil was cultivated in a 500-ml flask containing 150 ml of a medium composed of 2% peptone, 1% bonito meat extract, and 0.1% NaCl, pH 7.0, with shaking for 4 d at 26°C. All the following operations were at 15°C unless otherwise specified. The culture broth was centrifuged for 20 min at 8000 × g. To the supernatant was added solid ammonium sulfate to 70% saturation. The resulting precipitate was left overnight, and then recovered by filtration through Celite. The crude enzyme precipitate was dissolved in a minimal amount of water, and then purified by gel filtration on a Superdex 200 column. Proteins were eluted with 50 mM phosphate, pH 7.0 containing 150 mM NaCl at a flow rate of 0.5 ml/min. Active fractions were pooled, and concentrated by use of an ultrafiltration membrane with a molecular cutoff of 10 kDa. The enzyme solution was treated with 1% Igepal CA-630 for 1 h at 30°C, and then rechromatographed on the gel filtration column. Active fractions were dialyzed against 1 mM phosphate, pH 7.0, and put on a Mono Q column (0.5 × 5 cm) equilibrated with the same buffer. Proteins were eluted with a linear gradient of 0–600 mM NaCl in 1 mM phosphate, pH 7.0, at a flow rate of 0.5 ml/min. Active fractions were collected, concentrated, and dialyzed against deionized water.

**Isoelectric focusing.** The enzyme solution was treated by sucrose-density gradient isoelectric focusing in a 110 ml column with Pharmalyte 3-10 as the carrier ampholytes by the method of Vesterberg. The electrophoresis was run at 450 V for 48 h at 2°C, and then proteins were collected with a fraction collector at 1 ml/tube.

**Molecular weight estimation.** The molecular mass of the purified enzyme was estimated by gel filtration on a Superdex 200 column equilibrated with 50 mM phosphate, pH 7.0, containing 150 mM NaCl. Alcohol dehydrogenase (150 kDa), bovine serum albumin (67 kDa), carbonic anhydrase (29 kDa), and horse heart cytochrome c (12.4 kDa) were used for calibration. The molecular mass of denatured enzyme was estimated by SDS-PAGE by the method of Laemmli on a 9% polyacrylamide gel.10 The marker proteins used were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa). After the electrophoresis, the gel was stained with Quick CBB.

**Effect of pH on activity.** To 0.90 ml of Britton-Robinson buffer of different pHs containing 0.2 mM cholesteryl linolate, 0.33% Triton X-100, 0.2% sodium cholate, and 0.68% isopropanol was added 10 μl of the enzyme solution (1.5 U), and the mixture was incubated for 2 min at 40°C. After boiling of the mixture for 1 min, 0.5 ml of 500 mM phosphate, pH 7.0, containing 16 units of cholesterol oxidase, 8 purpurogallin U of peroxidase, 2.64 M 4-aminoantipyrin, and 9% phenol was added, and the absorbance at 500 nm was measured. Control experiments were done with water instead of the enzyme solution.

**Inhibitory assay.** The enzyme was incubated with a chemical inhibitor at 1 mM for 1 h at 25°C in 50 mM phosphate, pH 7.0. The remaining enzyme activity was assayed with cholesteryl linolate as the substrate.

**Fatty acid specificity.** Fatty acid specificity of the enzyme was measured at pH 7.0 with a variety of cholesteryl esters, as described under "Enzyme assay".

**Effects of detergents on lipase and cholesterol esterase activities measured in a bulk assay.** The lipase activity of the enzyme on triglycerides was measured by a bulk assay as follows. Simple
triglycerides of fatty acids of 10 carbons or less are liquid at 53°C, and hence activity on them was measured individually with stirring at 500 rpm at the temperature by the method described previously. The assay was started by addition of 100 μl of enzyme solution to a mixture containing 0.3 g of a simple triglyceride, 2.0 ml of 50 mM phosphate, pH 7.0, in the absence and presence of 100 mM sodium cholate or Triton X-100. Activity on triolein also was measured by this method. One activity unit in the bulk assay (U-BA) was defined as the amount of the enzyme releasing 1 μmol of fatty acid/min under these conditions. Activity on triglycerides of fatty acids of 12 carbons or more including linoleic, linolenic, arachidonic, eicosapentaenoic, and docosahexaenoic acids were examined by use of a mixture of randomly interesterified triglycerides as described previously. The bulk assay on cholesteryl olate was done as described above.

**Thin-layer chromatography.** A reaction mixture composed of 0.3 g of triolein, 2.5 ml of phosphate, pH 6.0, containing 100 mM sodium cholate, and 10–20 U (1.5–3.0 U-BA) of the enzyme was incubated at 30°C for 20 min with stirring at 500 rpm. The reaction products were then extracted with 20 ml of an equimolar mixture of hexane and ethyl acetate, and portions of the organic layer were put on a silica gel G 60 plate and a Chromarod SIII silica gel-coated quartz capillary (Iatron Laboratories, Tokyo). The silica gel plate was developed in a mixture of chloroform, acetone, and acetic acid (96/4/1, by volume) as noted previously. After the development, the chromatogram was sprayed with 50% sulfuric acid in ethanol and heated at 120°C for 5 min. The silica gel-coated quartz capillary was developed in a mixture of benzene, chloroform, and acetic acid (50/20/0.5, by volume), and was examined by TLC/FID with Iatroscan TH-10 (Iatron Laboratories, Tokyo).

**Evaluation of lipid-degrading ability of the enzyme.** On the basis of the composition of lipid secreted with tears, cholesteryl oleate, tripalmitin, and stearyl stearate were selected as the model fats to stain contact lenses. A mixture of these fats (25 mg of each) dissolved in 1 ml of chloroform was put to 49 ml of saline, and the whole solution was homogenized by vigorous stirring for 30 min. A new silicone contact lens was immersed in 2 ml of the homogenate for 1 d at room temperature to prepare a fat-stained lens. Cholesterol esterase from *P. aeruginosa* was dissolved in 10 mM Tris-HCl (pH 7.4) containing 0.5% (w/v) sodium cholate (buffer C) to prepare an enzyme solution of 2.1 U/ml (CE solution). To compare the cholesteryl ester-degrading ability of the enzyme with that of trilglyceride lipase Lipase M with no cholesterol esterase activity was dissolved in buffer C (L solution, 20 U-BA/ml), and tested for its lipid-degrading ability. A piece of the fat-stained contact lens was immersed in buffer C, CE, or L solution for 2 h at 30°C. The test piece was then rinsed with deionized water, and dried. The fats remaining on it were extracted with 0.5 ml of chloroform containing 0.011% (w/v) cholesteryl acetate as the internal standard, and were assayed on a UA-1 capillary column (0.25 mm × 15 m, Frontier Laboratories, Fukushima) connected to a Hewlett-Packard HP 6890 gas chromatograph (Hewlett-Packard, Palo Alto, CA). Analysis was done in a split ratio of 5:1 with 2-μl sample injection. The injector and the detector (FID) temperatures were 300 and 360°C, respectively. The column temperature was raised from 180 to 360°C at a rate of 30°C/min, and then kept there for 3 min. Each experiment was done for three test pieces.

**Results**

**Purification of cholesterol esterase**

Cholesterol esterase from *P. aeruginosa* existed as aggregates in the culture filtrate, and was eluted in the void volume on gel filtration. The aggregates with enzyme activity were combined, and treated by different kinds of chromatography after dialysis against 1 mM phosphate, pH 7.0. However, the aggregates failed to adsorb to the support of ion-exchange, hydrophobic interaction, or adsorption chromatography. Detergents were then screened for the ability to dissociate the aggregated enzyme. Igepal CA-630 at a final concentration of 1% was used without causing enzyme inactivation. Figure 1 shows gel filtration on a Superdex 200 column before and after the treatment of the enzyme with the detergent. Succeeding anion-exchange chromatography on a Mono Q column separated the enzyme from contaminant proteins (data not shown). Table 1 summarizes the purification of the cholesterol esterase. The enzyme was purified 116-fold in 19% yield on the basis of the initial activity, and was homogeneous as judged by SDS-PAGE (Fig. 2).

**Properties of enzyme**

Gel filtration of the purified enzyme on the Superdex 200 column gave a value of 53 kDa, and SDS-PAGE showed a single band at 58 kDa (Fig. 2). Iselectric focusing afforded a single active peak at pH 3.2. The enzyme showed its optimum pH from pH 5.5 to 9.5, and was stable between pH 5 and 10 for 19 h at 25°C (Fig. 3). The activity was highest at or near 53°C at pH 7.0, and stayed high up to 53°C on 30 min of incubation at pH 7.0 (Fig. 4). Table 2 lists the effects of some typical inhibitors. Only phenylmethanesulfonyl fluoride caused much inhibition.

**Fatty acid specificity on cholesteryl ester hydrolysis**

Table 3 lists relative rates of hydrolysis of different
Fig. 1. Results of Gel Filtration of Culture Filtrate of *P. aeruginosa* before and after 1% Igepal Treatment.

To the culture filtrate was added ammonium sulfate to 70% saturation, and the crude enzyme precipitate obtained was dissolved in a minimal amount of water, and subjected to gel filtration on a Superdex 200 column (A). Active fractions were collected, treated with 1% Igepal, and chromatographed again on the same column (B). ○, Absorbance at 280 nm; ●, cholesterol esterase activity (U/ml).

Table 1. Purification of *P. aeruginosa* Cholesterol Esterase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture filtrate</td>
<td>123,500</td>
<td>9,000</td>
<td>0.07</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate ppt.</td>
<td>16,760</td>
<td>8,300</td>
<td>0.50</td>
<td>92</td>
<td>7</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>2,250</td>
<td>3,840</td>
<td>1.7</td>
<td>43</td>
<td>23</td>
</tr>
<tr>
<td>Igepal CA-630</td>
<td>1,280</td>
<td>3,540</td>
<td>2.8</td>
<td>39</td>
<td>38</td>
</tr>
<tr>
<td>Superdex 200</td>
<td>430</td>
<td>2,640</td>
<td>6.1</td>
<td>29</td>
<td>84</td>
</tr>
<tr>
<td>Mono Q</td>
<td>210</td>
<td>1,780</td>
<td>8.5</td>
<td>20</td>
<td>116</td>
</tr>
</tbody>
</table>

Fig. 2. SDS-PAGE of *P. aeruginosa* Cholesterol Esterase after Two Steps of Gel Filtration and then Mono Q Anion-exchange Chromatography.

SDS-PAGE of the enzyme after two steps of gel filtration and then anion-exchange chromatography was done on a 9% polyacrylamide gel. The gel was stained with Quick CBB. Lane 1, cholesterol esterase; lane 2, molecular mass marker proteins: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

Effects of detergents on lipase and cholesterol esterase activities in the bulk assay

Figure 5 shows the effects of bile salts and Triton X-100 on the hydrolysis of triolein. The enzyme did not act on the substrate in the absence of the bile salts, but had activity in proportion to the bile salt concentration. On the other hand, with Triton X-100, the enzyme had little activity. We did the same experiments using cholesteryl oleate instead of triolein. Activation was similar, although to a lesser extent. In view of the highly stimulating effect of the bile salts on triolein hydrolysis, we then investigated this effect on a variety of simple triglycerides and randomly interesterified triglycerides (Fig. 6). The concentration of cholate was fixed at 100 mM. As expected, cholate stimulated the hydrolysis of all fatty acid specificities, molecular weights, and isoelectric points indicate that the enzyme from *P. aeruginosa* is a novel cholesterol esterase.
Cholesterol Esterase from *Pseudomonas aeruginosa*

The enzyme activity towards 0.2 mM cholesteryl linoleate was assayed in Britton-Robinson buffers of different pHs at 40°C. The values were expressed as the percentage of that at pH 7.0. Stability of the enzyme was examined by measurements of the residual activities after the incubation in Britton-Robinson buffer of different pHs for 19 h at 25°C. Relative enzyme activity (\(z\)); \(\text{Residual activity (z)}\).

**Fig. 3.** Effects of pH on Activity and Stability of *P. aeruginosa* Cholesterol Esterase.

**Table 2.** Effects of Chemicals on *P. aeruginosa* Cholesterol Esterase

<table>
<thead>
<tr>
<th>Chemical (1 mM)</th>
<th>Activity remaining (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>97</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>104</td>
</tr>
<tr>
<td>BaCl₂</td>
<td>101</td>
</tr>
<tr>
<td>SnCl₂</td>
<td>98</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>95</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>99</td>
</tr>
<tr>
<td>CuCl₂</td>
<td>92</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>103</td>
</tr>
<tr>
<td>PMSF</td>
<td>6</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>92</td>
</tr>
<tr>
<td>DTNB</td>
<td>97</td>
</tr>
<tr>
<td>Tetranitromethane</td>
<td>94</td>
</tr>
</tbody>
</table>

The enzyme was incubated with one of the chemicals at 1 mM for 1 h at 25°C in 50 mM phosphate, pH 7.0. The enzyme activity remaining was assayed with cholesteryl linoleate as the substrate.

PMSF, Phenylmethanesulfonyl fluoride. DTNB, 5,5'-Dithiobis(2-nitrobenzoic acid).

The results of TLC showed that the enzyme cleaved \(sn-2\)-positioned ester bond faster than the \(sn-1(3)\)-positioned bond. TLC/FID afforded a value of approximately 12 as the ratio of \(sn-1,2(2,3)\)- to \(sn-1,3\)-diolein accumulated during the 20 min of hydrolysis.

**Lipid-degrading ability of enzyme**

In order to check the effects of cholesterol esterase activity on lipid degradation, we selected, as a reference enzyme, *Mucor javanicus* lipase devoid of cholesterol esterase activity. Figure 8 shows the abilities of the two enzymes to clean fat-stained contact lenses. *Mucor javanicus* lipase eliminated tripalmitin and stearyl stearate, yet it did not act on cholesteryl olate much. Cholesterol esterase from *P. aeruginosa*, on the other hand, proved to be effective in reducing all these fats, especially cholesteryl olate, two-thirds of which being removed.

**Discussion**

We purified and characterized cholesterol esterase from newly isolated *P. aeruginosa*. The enzyme was secreted extracellularly, and existed as aggregates in the culture filtrate like mammalian and some microbial cholesterol esterases. The use of Igepal CA-630 dissociated the aggregated enzyme without causing enzyme inactivation, and lead to successful purification and characterization. The enzyme had a wide range of optimum pH from 5.5 to 9.5, and was stable over the pH range 5 to 10. Besides the cholesterol esterases listed in Table 3, those from *Staphylococcus aureus* and *Saccharomyces cerevisiae* have been purified and some of their properties examined. The former was reported to have a molecular weight of 175 kDa and a PI of 9.1, but no fatty acid specificity was described. The latter was shown to have the simple triglycerides and randomly interesterified triglycerides.

**Positional specificity on triglyceride hydrolysis**

Figure 7 shows results of TLC of products from triolein hydrolysis in the presence of 100 mM cholate when developed on a silica-gel-coated capillary. The extents of hydrolysis of the two samples were estimated to be 7% and 13% from the amounts of released free fatty acids. Spontaneous acyl group migration was considered negligible in view of the short time and weakly acid pH for the hydrolysis.\(^{19}\)
Table 3. Fatty Acid Specificities of Microbial Cholesterol Esterases

<table>
<thead>
<tr>
<th>Cholesteryl ester</th>
<th>Cholesterol esterase from</th>
<th>P. aeruginosab</th>
<th>P. fluorescensc</th>
<th>COE-311d</th>
<th>CHE-1e</th>
<th>F. oxysporumf</th>
<th>F. oxysporumg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td></td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Butyrate</td>
<td></td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Caproate</td>
<td></td>
<td>22</td>
<td>3</td>
<td>17</td>
<td>—</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Caprate</td>
<td></td>
<td>62</td>
<td>—</td>
<td>59</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Laurate</td>
<td></td>
<td>41</td>
<td>12</td>
<td>40</td>
<td>28</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Myristate</td>
<td></td>
<td>58</td>
<td>—</td>
<td>49</td>
<td>84</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Palmitate</td>
<td></td>
<td>75</td>
<td>64</td>
<td>34</td>
<td>94</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Stearate</td>
<td></td>
<td>80</td>
<td>21</td>
<td>8</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Oleate</td>
<td></td>
<td>92</td>
<td>53</td>
<td>105</td>
<td>90</td>
<td>27</td>
<td>40</td>
</tr>
<tr>
<td>Linoate</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Molecular weight (kDa) | 58 | 129 | 300 | — | — | 15, 60, 75 |
pI | 3.2 | 3.8, 4.9 | 6.0 | — | — | — |

a Activities are relative to the activity on cholesteryl linoleate.
b This study.
c Reference 7.
f Reference 9.
g Reference 10.

Fig. 5. Effects of Detergent on the Hydrolysis of Triglyceride and Cholesteryl Oleate by P. aeruginosa Cholesterol Esterase.
Assays were at 53°C for 30 min with triglyceride or cholesteryl oleate as the substrate, and the oleic acids released were titrated with 50 mM KOH. ○, Effects of cholate on triolein hydrolysis; ●, effects of Triton X-100 on triolein hydrolysis; △, effects of taurocholate on triolein hydrolysis; ▲, effects of cholate on cholesteryl oleate; ■, effects of Triton X-100 on cholesteryl oleate.

Fig. 6. Fatty Acid Specificity of P. aeruginosa Cholesterol Esterase on Triglyceride Hydrolysis.
The enzyme activity on different simple triglycerides was assayed in the presence (■) of 100 mM sodium cholate or its absence (□). The values are expressed as the percentage of the value toward trioctanoin in the presence of 100 mM cholate.

triglyceride lipase activity, and cleaves the sn-1(3)-positioned bond approximately 6 times as fast as the sn-2-positioned bond of triolein. That the ratio of sn-2- to sn-1(3)-monoolein was 1.7 supports this interpretation.

Because not only cholesterol esters but also triglycerides are important components of edible fats and oils, meat, fish, and dairy products, and are emulsified with bile salts such as cholate and taurocholate in the mammalian duodenum, the effects of these bile salts on the hydrolysis of these
Fig. 7. TLC Showing the Positional Specificity of *P. aeruginosa* Cholesterol Esterase on Triolein Hydrolysis.

A reaction mixture containing 300 mg of triolein, 2.0 ml of phosphate buffer, pH 7.0, containing 100 mM sodium cholate, and 10–20 U of the enzyme was incubated at 50°C for 20 or 30 min with stirring at 500 rpm. The reaction products were extracted with 20 ml of an equimolar mixture of hexane and ethyl acetate, and portions of the organic solvent layer were treated by TLC on a Kieselgel G 60 plate (A) and on an Iatroscan TH-10 (B).

Fig. 8. Ability of *P. aeruginosa* Cholesterol Esterase to Remove Three Typical Skin Fats from a Contact Lens.

A piece of contact lens stained with a mixture of cholesteryl oleate, tripalmitin, and stearyl stearate was immersed in 10 mM Tris-HCl buffer containing 0.5% taurocholate and *P. aeruginosa* cholesterol esterase or *Mucor javanicus* lipase for 2 h at 30°C. The test piece was then rinsed with deionized water, and the fats remaining on it were assayed by GLC. C, Treated with neither sodium taurocholate nor enzyme; ST, treated with sodium taurocholate; ST + L: treated with sodium taurocholate containing lipase M; ST + CE: treated with sodium taurocholate containing *P. aeruginosa* cholesterol esterase. ■, Cholesteryl oleate; □, tripalmitin; △, stearyl stearate. Values are results of triplicate measurements.

substrates were examined. Mammalian cholesterol esterase is strictly dependent on bile salts for hydrolytic activity on insoluble fats and oils, kinetic and biochemical studies have been focused to analyze the activation mechanism.23–28) Wang and Lee have done experiments to probe the accessibility of the active site of the human milk cholesterol esterase, and have indicated that taurocholate was a nonessential activator for the enzyme with water-soluble substrates, but an essential activator with water-insoluble long chain triglycerides.27) They suggested one of the probable reasons for the difference toward these substrates could be the inaccessibility of the active site of the enzyme to the long chain triglyceride molecule caused by a steric hindrance effect. On the basis of this reasoning, they did assays by incubating simple triglycerides (0.2 mM) of different fatty acid chain lengths (carbon numbers 6 to 12) individually with the enzyme (1 mg/ml) for probing the size of the active site of the enzyme. They found that trihexanoyl- and triheptanoylglycerol were degraded completely during the 10 min of the incubation, during which time trioctanoylglycerol of the same concentration was partially degraded. On the other hand, trinonanoyl-, tridecanoyl-, and tridodecanoylglycerol were not degraded even with prolonged (60 min) incubation. They also did kinetic analyses with triolein at 10 mM while varying the taurocholate concentration from 0 to 50 mM. They observed that the enzyme showed a sigmoidal increase in enzyme activity starting from null with increasing taurocholate concentration from 0 to 50 mM. They observed that the enzyme showed a sigmoidal increase in enzyme activity starting from null with increasing taurocholate concentration. A similar sigmoidal response was reported previously by Hernell and Olivecrona.28) Recently three-dimensional structures of bovine pancreatic cholesterol esterase and its complex with taurocholate have been elucidated by X-ray analysis.29–31) Two taurocholate binding sites were found in the complex structure. One of these sites was close to the cholesterol esterase-specific hairpin loop near the active site. In the absence of taurocholate, the loop partly blocks the entrance of the active site, and does not allow access of bulky substrates to the active site. On the capture of taurocholate, this loop has a drag-
tic conformational change, and opens the passage to the active site. This structural change allows a bulky substrate to enter the active site without difficulty. The taurocholate molecule is located such that its 3α hydroxyl group is hydrogen-bonded to the backbone amide group of Leu 124 in the hairpin loop, and its 7α-hydroxyl group is hydrogen-bonded to Oγ1 atom of Thr 449. Thus binding of taurocholate presumably stabilizes the open conformation of the loop. The other taurocholate binding site is remote from the active site. Though no obvious conformational change is associated with the second binding site, it is not known if the site has a biological function.

So far no reports have been published on bile salt activation of microbial cholesterol esterase, partly because the environment for bile salt activation is questioned in microorganisms. However, the abovementioned activation mechanism for mammalian cholesterol esterase prompted us to investigate the effects of bile salt on the microbial enzyme. We found that bile salt raised triolein hydrolysis by \textit{P. aeruginosa} cholesterol esterase, and also cholesterol ester hydrolysis though to a lesser extent. In contrast, reduction of cholesteryl oleate was not achieved by triglyceride lipases such as one from \textit{Mucor javanicus}, suggesting the potential use of the cholesterol esterase as a contact lens cleaner.

**References**


22) Durham, L. A., and Grogan, W. M., Characteriza-


