Purification and Characteristics of Plasma Membrane Penicillinase from Bacillus licheniformis 749/C*

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SUMMARY

The plasma membrane-bound penicillinase of Bacillus licheniformis 749/C has been purified from bacteria grown in a medium containing [2-3H]glycerol and 14C-labeled aminoacids, and the purified enzyme compared with exopenicillinase. The procedure consisted of repeated chromatography on DEAE-Sephadex in the presence of Triton X-100, and gel filtration on Sephadex G-75 in the presence of taurodeoxycholate. The purified membrane enzyme moved as a single band in sodium dodecyl sulfate acrylamide gel electrophoresis, and its specific penicillinase activity was 354 units per mg of enzyme protein (equivalent to that of the exoenzyme). The enzyme has an apparent molecular weight of 48,000 in the presence of taurodeoxycholate, as estimated by gel filtration, and exhibited high susceptibility to inactivation by heating and by iodine. The purified enzyme retained characteristic lipophilic properties and contained 3H activity which could not be removed from the enzyme protein by treatment with 8 M urea, 0.2% sodium dodecyl sulfate at 80° or by extraction with chloroform-methanol. The tritium activity could be separated from the enzyme protein by treatment with trypsin or phospholipase D followed by gel filtration and acrylamide gel electrophoresis. The small component(s) released by trypsin contained both 14C and 3H activity. The resulting enzyme had lost its lipophilic character and showed properties similar to exopenicillinase.

Although the penicillinase (EC 3.5.2.6) of Bacillus licheniformis 749/C is known as an extracellular enzyme, about half of the enzyme produced by the organism is cell-bound and associated with the plasma membrane and with periplasmic vesicles. The penicillinase attached to the periplasmic vesicles is released along with the vesicles during protoplast formation; the plasma membrane penicillinase remains tightly associated with the protoplast and can be solubilized from membrane preparations by detergent (1). In a preliminary communication (2) we reported the purification of a small amount of the membrane enzyme solubilized by taurodeoxycholate, and presented evidence that the purified enzyme is probably a phospholipid-protein, i.e. the phospholipid-like moiety and the enzyme protein appear to be linked by a covalent bond. Although recent studies have suggested that the membrane enzyme is not an obligatory intermediate in the formation of exoenzyme (3), the membrane enzyme is probably an important source of exoenzyme during growth at a pH above neutrality (4, 5). The chemical structure of the membrane enzyme is of special interest since this enzyme has unusual lipophilic properties. It was necessary to develop an improved purification procedure because the method based on detergent acrylamide gel electrophoresis (2) was unsuitable for obtaining native enzyme in quantity and gave a product of low specific activity.

This paper describes the purification of the membrane penicillinase and reports some properties of the purified native enzyme.

MATERIALS AND METHODS

Organisms, Growth Medium and Culture—Bacillus licheniformis 749/C is a penicillinase-magnusconstitutive mutant of the penicillinase-inducible wild type strain 749 (6). Strain 749/C was maintained as spores on slants of Andrade agar (7). Inocula were prepared as described by Lampen (8) and cultures were grown in 150 ml of casein hydrolysate salts (CH/S) medium, pH 6.5, at 30° in 500-ml flasks with vigorous rotatory shaking until the cell concentration reached 0.6 to 0.7 mg dry weight per ml. Growth and cell concentration were followed by measuring turbidity with a Klett-Summerson colorimeter (filter No. 54). For preparation of labeled membrane penicillinase, 150 μCi of 14C-aminoacids mixture and 1.5 mCi of [2-3H]glycerol were added to the culture at the time of inoculation.

Preparation of Plasma Membrane—Cells in exponential phase were spun down and converted to protoplasts with lysozyme; the protoplasts were lysed and the plasma membrane fraction was washed as described previously (1).

Preparation of Exopenicillinase—Exopenicillinase of B. licheniformis 749/C was purified by the method of Pollack (9). It migrated as a single band in sodium dodecyl sulfate acrylamide gel electrophoresis, and its specific penicillinase activity was 350 units per μg of protein.

Radiochemicals—Uniformly labeled 14C-aminoacids mixture and [2-3H]glycerol, 200 mCi per mmole, were products of New England Nuclear Corp. Radiochemical purity of the [2-3H]glycerol was given by the manufacturer as greater than 98%. This was confirmed in our laboratory by the paper chromatographic method of Sargent (10).

Analytical Methods—The method of Sargent (10) was generally used for assay of penicillinase activity, with units expressed as

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micromoles of benzyl penicillin hydrolyzed per hour. Substrate specificity for five penicillins and a cephalosporin was determined by a modification of Perret's method (11). Unless otherwise noted, membrane penicillinase was assayed in 0.1 M phosphate buffer, pH 7.0, containing 0.1% taurodeoxycholate, and exopenicillinase in the same buffer but without detergent.

The protein content of pooled column eluates (from the peaks of enzyme activity) was estimated by the absorbance at 240 nm (approximate minimum in the absorption curve of Triton X-100) against a blank containing the detergent. Bovine serum albumin was the standard protein and at 50 µg per ml gave an E280 of 0.050. The method of Lowry et al. (12) was used for assay of protein in solutions not containing Triton X-100.

SUMMARY AND CONCLUSIONS

The purified enzyme, with a specific activity of 354 units per µg of protein (12) was obtained in 26% over-all yield from the plasma membrane fraction. All procedures were carried out at 0 to 5°.

Stage 1. Extraction from Plasma Membrane—The plasma membrane fraction was washed three times with 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.01 M MgCl2. The washed membranes were suspended in 20 ml of the buffer and mixed with 200 ml of 0.05 M pyrophosphate buffer, pH 6.5, containing 0.1% taurodeoxycholate. The mixture was stirred vigorously by Sorvall homogenizer for 1 min and centrifuged at 14,000 × g for 60 min. More than 90% of the total penicillinase activity was extracted. The supernatant was concentrated to about 50 ml in a dialysis tube with the aid of Aquasol II (Calbiochem) and then dialyzed for 24 hours against 3 liters of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1% Triton X-100.

Stage 2. DEAE-Sephadex Chromatography—A column of DEAE-Sephadex A-50 (1.2 x 15 cm) was equilibrated with 0.05 M Tris-HCl, pH 7.5, containing 0.1% Triton X-100. The enzyme solution from Stage 1 was diluted with an equal volume of the buffer and applied to the column which then was washed with 80 ml of the buffer. Addition of buffer containing 0.5 M NaCl eluted the enzyme activity as a sharp peak. About 70% of the activity applied was detected within 12 ml of eluted solution. The enzyme solution was dialyzed against 2 liters of the buffer for 2 days.

Stage 3. DEAE-Sephadex Chromatography—The dialyzed enzyme solution from Stage 2 was diluted with an equal volume of the Tris-Triton buffer and applied to a column prepared as for Stage 2. The column was washed with about 50 ml of the buffer before the enzyme was eluted by a linear gradient of 0 to 0.3 M NaCl. The gradient is constructed from 100 ml of the buffer and 100 ml of the buffer containing 0.3 M NaCl. The enzyme was eluted at a NaCl concentration of about 0.05 M. The fractions of the main enzyme peak were pooled (25 ml total) and dialyzed against 2 liters of the buffer for 1 day.

Stage 4. DEAE-Sephadex Chromatography—A column of DEAE-Sephadex A-50 was prepared as for Stage 2. The dialyzed solution from Stage 3 was diluted with an equal volume of the buffer and applied to the column which was washed with about 50 ml of the buffer. At this stage, enzyme activity was occasionally detected in the washings; however, the amount was less than 10% of the total applied. The membrane penicillinase shows weaker affinity with the ion exchanger as the preparation becomes purer. Also, Triton X-100 interferes with the binding of highly purified enzyme to the ion exchanger since the enzyme binds more tightly at the lower concentrations of detergent; however, at these concentrations the enzyme peak eluted by a linear gradient of NaCl became wider (see below Fig. 4).

After the column had been washed, the enzyme was eluted with a NaCl gradient as in Stage 3. Fig. 1 shows the eluting patterns. A small peak of enzyme activity was observed between Fractions 84 to 90 (not always present); it was superimposed on a small peak of 14C activity but not accompanied by a peak of 3H activity. Such a minor component may be enzyme...
which has lost the lipophilic component present in the membrane penicillinase molecule. Its eluting position was about the same as that of exopenicillinase. Fractions 68 to 80 from the major peak were pooled and concentrated to about 1 ml by the use of Aquacid II and then freeze-dried.

Stage 5. Gel Filtration on Sephadex G-75—The enzyme from Stage 4 was dissolved in 1 ml of 0.05 M pyrophosphate buffer, pH 6.5, containing 0.1% taurodeoxycholate and loaded onto a Sephadex G-75 column (1.2 x 85 cm) equilibrated with the same buffer, which was also used for elution of the enzyme. The elution pattern is shown in Fig. 2. Fractions 24, 25, and 26, with specific penicillinase activities of 300 to 370 units per µg of protein, were combined, dialyzed against 2 liters of 5 mM Tris-maleate buffer, pH 6.0, containing 0.02% taurodeoxycholate for 2 days, and then freeze-dried. In the experiments with this Sephadex G-75 column, Triton X-100 was eluted just after the enzyme fractions.

Purity of Membrane Penicillinase

Rechromatography of the final preparation of membrane penicillinase on DEAE-Sephadex and Sephadex G-75 columns yielded a single coincident peak of protein, enzyme activity and radioisotopic activities derived from 14C-aminoacids and [2-3H]-glycerol. The purified enzyme exhibited a single protein band upon electrophoresis in 7.5% acrylamide gel containing 0.1% sodium dodecyl sulfate (Fig. 3). As reported with earlier, partially denatured preparations (2), the tritium activity derived from [2-3H]glycerol was not removed from the purified enzyme (also labeled with 14C-aminoacids) by treatment with 8 M urea, 0.2% sodium dodecyl sulfate at 80° for 30 min followed by acrylamide gel electrophoresis in 8 M urea, 0.1% sodium dodecyl sulfate, pH 7.2 (Fig. 4), even though about 75% of enzymatic activity was lost. Also, extraction of the dried enzyme with chloroform and methanol according to the method of Bligh and Dyer (13) did not alter the 3H:14C ratio.

Behavior of Membrane Penicillinase on DEAE-Sephadex

During preliminary experiments for purification of membrane penicillinase on DEAE-Sephadex A-50, we observed characteristics of the enzyme which were very different from those of exopenicillinase. The relationship between affinity of the enzyme to the ion exchanger and concentration of Triton X-100 is illustrated graphically in Fig. 5.

In 0.05 M Tris-HCl buffer, pH 7.5, membrane enzyme bound tightly to DEAE-Sephadex columns and could not be eluted by 0.1 M acetate buffer, pH 4.0, or by 6 M urea. The tightly bound
membrane enzyme could be eluted by high concentrations of 
NaCl, but the elution profile was very broad. In contrast, 
exopenicillinase, in the absence of added detergent, bound to 
DEAE-Sephadex at pH 7.5 and was sharply eluted by a low 
concentration of NaCl, about 0.05 M.

In the presence of Triton X-100, membrane enzyme was 
readily eluted from the DEAE-Sephadex column by NaCl; 
however, the concentration of NaCl required for elution was 
dependent on the concentration of detergent in the eluting buffer. 
The affinity of membrane-enzyme for DEAE-Sephadex decreased 
with increasing concentration of the detergent. To obtain 
a sharp elution peak of the enzyme, 0.1% detergent was 
suitable. When a small amount of the highly purified enzyme 
was applied to the DEAE-Sephadex column, a substantial portion 
of the enzyme activity was sometimes washed from the 
column with buffer containing the detergent but no NaCl. This 
tendency of the enzyme was also seen at the last stage of the 
purification by DEAE-Sephadex (Stage 4).

**Molecular Weight**

The apparent molecular weight of membrane penicillinase 
purified by Sarkosyl NL-97 acrylamide gel electrophoresis was 
estimated to be 48,000 by gel filtration through Sephadex G-75 
in the presence of 0.1% taurodeoxycholate (2). The same value 
purified by Sarkosyl NL-97 acrylamide gel electrophoresis was 
also obtained for the membrane penicillinase purified by the 
method described here.

**Enzymatic Properties of Membrane Penicillinase: pH Optimum**

The pH optimum of membrane enzyme was from pH 6.0 to 
7.0 in buffer containing 0.1% taurodeoxycholate. This range 
and the shape of the pH activity curve are in agreement with 
those of exopenicillinase (detailed results not given). In the 
absence of added detergent, a definite pH activity curve could 
not be obtained because the results were extremely variable. 
When a small amount of the highly purified enzyme 
was applied to the DEAE-Sephadex column, a substantial portion 
of the enzyme activity was sometimes washed from the 
column with buffer containing the detergent but no NaCl. This 
tendency of the enzyme was also seen at the last stage of the 
purification by DEAE-Sephadex (Stage 4).

**Substrate Specificity**

The relative rates of substrate hydrolysis by exo- and membrane 
penicillinas at 60° in 0.1 M phosphate buffer, pH 7.0, in the 
presence or absence of 0.1% taurodeoxycholate (4) or 0.1% 
Triton X-100 (B). The enzyme was treated for indicated times 
at 60° and quickly cooled in ice. The remaining activity was 
assayed at 30° in 0.1 M phosphate buffer, pH 7.0, containing 0.1% 
taurodeoxycholate; incubation time was 8 min. Exopenicillinase, 
in the presence of detergent (O—O) and in its absence 
(●—●). Membrane penicillinase, in the presence of detergent 
(O—O) and in its absence (●—●).

**Effect of Temperature**

At 60° the activity of exopenicillinase was unchanged during a 
5-min incubation in 0.1 M phosphate buffer, pH 7.0, in the 
presence or absence of 0.1% taurodeoxycholate. The maximum 
temperature at which membrane penicillinase was stable for this 
period in buffer containing 0.1% of the detergent was somewhat 
lower and was estimated to be between 50 and 60°.

The stability of exo- and membrane enzymes at 60° with time 
differed sharply as shown in Fig. 6. Membrane enzyme was 
markedly more stable, especially in the absence of detergents. 
Taurodeoxycholate was more effective in protecting the mem-
brane enzyme than was Triton X-100. Neither detergent af-
fected the activity or stability of exopenicillinase. In relation to 
these experiments we examined the possibility that membrane 
enzyme has a tendency to absorb on the surface of glass, but 
this was excluded since similar results were obtained using sil-
icon-coated glassware.

**Effect of Iodine**

Membrane penicillinase was markedly more susceptible to 
iiodine at 0° than was the exoenzyme (Fig. 7). This result, 
together with the observations on thermostability, indicates some 
difference in tertiary structure between exo- and membrane en-
zymes even though they are believed to have nearly the same 
primary amino acid sequence (14).

**Substrate Specificity**

The relative rates of substrate hydrolysis by exo- and mem-
brane enzymes were measured for five penicillins and a cepha-
losporin in 0.1 M phosphate buffer, pH 7.0, containing 0.1% 
taurodeoxycholate. As shown in Table II, no significant differ-
ence was observed between the substrate specificity profiles 
of the two enzymes.

FIG. 6 (left). Rates of heat inactivation of exo- and membrane 
penicillinases at 60° in 0.1 M phosphate buffer, pH 7.0, in the 
presence or absence of 0.1% taurodeoxycholate (A) or 0.1% 
Triton X-100 (B). The enzyme was treated for indicated times 
at 60° and quickly cooled in ice. The remaining activity was 
assayed at 30° in 0.1 M phosphate buffer, pH 7.0, containing 0.1% 
taurodeoxycholate; incubation time was 8 min. Exopenicillinase, 
in the presence of detergent (O—O) and in its absence 
(●—●). Membrane penicillinase, in the presence of detergent 
(O—O) and in its absence (●—●).

FIG. 7 (right). Effect of iodine on the activities of exo- and 
membrane penicillinases. The enzyme was treated at 0° with 
3.8 mM iodine and 0.02 M potassium iodine in 0.02 M phosphate 
buffer, pH 7.0, containing 0.1% taurodeoxycholate. At the 
indicated times, sodium thiosulfate equivalent to the iodine in 
the enzyme solution was added, and the remaining enzyme activity 
was assayed. Exopenicillinase (O—O) and membrane peni-
cillinase (O—O).
TABLE II
Substrate specificity profilea

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Exopenicillinase</th>
<th>Membrane penicillinase</th>
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<tr>
<td>Benzylpenicillin</td>
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<td>100</td>
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<tr>
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<td>76</td>
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<td>129</td>
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<tr>
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<td>2</td>
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<tr>
<td>Cephaloridine</td>
<td>36</td>
<td>32</td>
</tr>
</tbody>
</table>

a Rates of hydrolysis of the six substrates are expressed in percent of rate with benzylpenicillin.

Conversion of Membrane Penicillinase to Exoenzyme Form

In a preliminary communication (2) we reported that membrane penicillinase could be converted to a form closely resembling the exoenzyme by treatment with trypsin or phospholipases C or D. Membrane enzyme lost its lipophilic properties during the conversion process and the thermostability and apparent molecular weight of the converted enzymes were about the same as those of exopenicillinase. However, it was assumed from the higher specific activity of the membrane enzyme purified as described in this paper that samples prepared by the older method contained a large amount of denatured enzyme though they were pure from the standpoint of being a single protein. We therefore confirmed the conversion to exoenzyme by using enzyme labeled by growth in the presence of [2-3H]glycerol and 14C-amino acids and purified by the new method.

Fig. 8A shows the eluting patterns of membrane enzyme which had been treated with trypsin. The peak of enzyme activity was superimposed on a peak of 14C activity, and its eluting position corresponded to that of exoenzyme. The peaks of enzyme activity and tritium were not coincident, the tritium eluting somewhat earlier than the converted enzyme. Following the enzyme peak, two broad peaks of 14C-labeled peptides were observed; these digested peptides were also noted previously (2). In the experiment of Fig. 7A, 21 × 10⁶ units of membrane enzyme were treated with trypsin and 13 × 10⁶ units (82% of the applied activity) were found in the converted enzyme peak. At the same time, about 45% of the applied 14C activity (amino acids) remained with the converted enzyme and the rest was distributed over the digested peptide range.

The phospholipases, especially phospholipase C (2) were much less active than trypsin in converting membrane enzyme to exo form, but this conversion was not accompanied by a degradation of the penicillinase protein. Fig. 8B shows the eluting patterns of membrane enzyme labeled with [2-3H]glycerol and 14C-amino acids which was treated with phospholipase D (EC 3.1.4.4) from cabbages (Sigma Chemical Co.). More than half of the treated enzyme was apparently unchanged and was eluted from the Sephadex G-75 column before the converted enzyme. The peak of converted enzyme (Fractions 30 to 39) was superimposed on a peak of 14C activity but not on a peak of tritium activity. Its eluting position was about the same as that of exopenicillinase. The tritium peak eluted somewhat later and differed in its position from the peak produced by trypsin.

Analysis of Converted Enzymes by Detergent-Acrylamide Gel Electrophoresis

The converted enzymes had not been separated from the [2-3H]glycerol-derived tritium activity by gel filtration on Sephadex G-75, although the peaks of enzyme activity and of tritium were not completely superimposed (Fig. 8). However, acrylamide gel electrophoresis separated them readily.

The fractions containing the converted enzymes (Fractions 30 to 39 in Fig. 8A and B) were pooled, concentrated with Aquacide, and dialyzed against 0.005 M Tris-maleate buffer, pH 6.5, containing 0.02% taurodeoxycholate. Neither tritium nor 14C activity was lost from the cellophane tubes during dialysis. The dialyzed solutions were further concentrated by lyophilization, and the enzymes were solubilized in a small amount (80 to 100 µl) of a solution consisting of 0.1% Sarkosyl NL-97, 25% (v/v) glycerol, and 0.002% bromphenol blue in 0.01 M phosphate buffer, pH 7.2. Samples (30 µl each) were subjected to Sarkosyl NL-97 acrylamide gel electrophoresis. The electrophoretic patterns of the enzymes and the radioisotopic activities in the gels are shown in Fig. 9.

The trypsin-converted enzyme was separated from the tritium activity by the electrophoresis (Fig. 9A). The tritium activity migrated rapidly with a position close to that of the dye marker, and was accompanied by a small peak of 14C activity.

The products of conversion by phospholipase D gave different electrophoretic patterns (Fig. 9B) than did those formed by trypsin. The tritium-labeled compound showed a faster electrophoretic mobility than the compound produced by trypsin treatment and was not accompanied by 14C activity.
enzymes extracted and purified from cell membranes may be hydrophobic because of their high content of nonpolar amino acids (15) or, more often, have a nonrandom distribution of their polar groups so as to produce separated hydrophobic and hydrophilic surface regions (16). Such enzymes usually exhibit strong affinity for phospholipids but are not linked to them by covalent bonds. The membrane penicillinase of Bacillus licheniformis is hydrophobic but is very similar in amino acid composition to the highly hydrophobic exopenicillinase which has, at most, a weak affinity for detergents (2, 4). Our results strongly suggested that the purified membrane penicillinase carries a hydrophobic component that is labeled during growth in the presence of [32P]phosphate (2) and [2-3H]glycerol (possibly a phospholipid) and is covalently bound to the hydrophilic enzyme protein. Thus membrane penicillinase is not a typical membrane-bound enzyme.

Membrane penicillinase purified to homogeneity by the improved procedure described here has the same specific activity as the pure exoenzyme (8). Since there is no change in total penicillinase activity during treatment of membrane preparations (or detergent extracts) with trypsin to convert the membrane enzyme to the protease-resistant exo form (4, 17), we conclude that our purified samples are native in that they retain the specific activity of the membrane-bound enzyme. It is also clear that the catalytic activity and substrate specificity (Table II) of penicillinase are not affected by the presence or absence of the 32P- and 2-3H-labeled glycerol fragment. There is a substantial difference, however, between our purified preparations and the enzyme as it exists in the membrane in that approximately half of the total activity of our current high specific activity preparations, or of the partially inactivated ones tested earlier, was destroyed by trypsin during conversion to exoenzyme. The enzyme may be stabilized in the membrane by its interactions with other proteins and with phospholipids, or the purified enzyme, although fully active, may have a conformation sufficiently different from the membrane-bound protein to be somewhat susceptible to degradation by trypsin.

Treatment of the membrane enzyme with either trypsin or phospholipase D caused the formation of a penicillinase that is very similar to the exoenzyme (2, 4, 14) and of a fragment containing the label incorporated from [2-3H]glycerol. The fragments released by the two enzymes differ in mobility during gel electrophoresis, and 14C-labeled amino acids are found only with the fragment formed by trypsin. We do not have conclusive evidence for direct linkage between the peptide(s) and the tritium-labeled compounds; however, their concurrent elution (apparently as an aggregate) from the Sephadex G-75 column (Fig. 8), the retention of the 14C activity during dialysis, and the agreement in shape between the tritium and 14C peaks during electrophoresis (Fig. 9) indicate such a bond. Characterization of this fragment, which gives a color test (18) for phospholipid, is under way.

Although the purified membrane enzyme closely resembles exopenicillinase in its substrate specificity (Table II), pH activity curve and immunoreactive groupings (4), it differs strikingly in its hydrophobic character, especially as evidenced by its tendency to complex with detergents (1, 2, 4). These differences are illustrated by the behavior of the two enzymes on DEAE-Sephadex columns (Fig. 5). The membrane enzyme seems to be held by two different means. One is the usual ionic interaction between protein and ion exchange resin and is affected by both pH and ionic strength. The other probably is a binding or adsorption dependent on the lipophilic part of the enzyme. This should be relatively insensitive to changes in pH and ionic strength and primarily dependent on the concentration of the detergent (Triton X-100). In this regard it should be noted that the [2-3H]glycerol-containing material released from the purified membrane enzyme by trypsin tended to adsorb on glass surfaces and could be removed from them by chloroform-methanol. The intact membrane enzyme did not show such a tendency.

At low concentrations of detergent, the membrane enzyme would be bound to the DEAE-Sephadex by both ionic interaction and adsorption. At a suitable concentration (0.1%) of Triton X-100 the lipophilic portion of the enzyme may be effectively shielded by the neutral detergent so that the enzyme behaves much like exopenicillinase; in fact, the large quantity of bound detergent may actually interfere sterically with binding between the protein portion of the enzyme and the ion exchanger. Triton X-100 appeared to exist as large micelles under these conditions since it eluted from a Sephadex G-75 column equilibrated with buffer containing 0.1% taurodeoxycholate just after the peak of membrane enzyme (apparent molecular weight 48,000). Also the apparent molecular weight of membrane enzyme in buffer containing 0.1% Triton X-100 was greater than 100,000 as estimated by gel filtration through a Sephadex G-75 column.2

Despite the many similarities between exopenicillinase and the protein portion of the membrane enzyme, the two proteins almost certainly differ in tertiary structure as shown by the studies on heat stability (Fig. 6 and Ref. 4) and sensitivity to iodine (Fig. 7). We also have pointed out earlier that the purified membrane enzyme may in turn have a different tertiary structure than the enzyme bound in the plasma membrane.

FIG. 9. Polyacrylamide gel electrophoresis of converted membrane penicillinases. A, the enzyme produced from membrane penicillinase by trypsin (Fig. 8A; Fractions 30 to 39) was layered on a 7.5% cross-linked gel containing 0.1 m phosphate buffer, pH 7.2, and 0.1% Sarkosyl NL-97. Electrophoresis was for 2.5 hours at 5°. The gel was then cut in 3-mm slices. Each slice was extracted with 1 ml of 0.06 m Tris-maleate buffer, pH 6.0, containing 0.2% taurodeoxycholate and assayed for penicillinase and the radioisotopic activities (methods described in Fig. 1). B, the enzyme produced from membrane penicillinase by phospholipase D (Fig. 8B; Fractions 30 to 39) was analyzed as in A. In both A and B, the bar indicates the position of a dye marker (bromophenol blue) in the gel.

DISCUSSION

Enzymes extracted and purified from cell membranes may be hydrophobic because of their high content of nonpolar amino acids (15) or, more often, have a nonrandom distribution of their polar groups so as to produce separated hydrophobic and hydrophilic surface regions (16). Such enzymes usually exhibit strong affinity for phospholipids but are not linked to them by covalent bonds. The membrane penicillinase of Bacillus licheniformis is hydrophobic but is very similar in amino acid composition to the highly hydrophobic exopenicillinase which has, at most, a weak affinity for detergents (2, 4). Our results strongly suggested that the purified membrane penicillinase carries a hydrophobic component that is labeled during growth in the presence of [32P]phosphate (2) and [2-3H]glycerol (possibly a phospholipid) and is covalently bound to the hydrophilic enzyme protein. Thus membrane penicillinase is not a typical membrane-bound enzyme.

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At low concentrations of detergent, the membrane enzyme would be bound to the DEAE-Sephadex by both ionic interaction and adsorption. At a suitable concentration (0.1%) of Triton X-100 the lipophilic portion of the enzyme may be effectively shielded by the neutral detergent so that the enzyme behaves much like exopenicillinase; in fact, the large quantity of bound detergent may actually interfere sterically with binding between the protein portion of the enzyme and the ion exchanger. Triton X-100 appeared to exist as large micelles under these conditions since it eluted from a Sephadex G-75 column equilibrated with buffer containing 0.1% taurodeoxycholate just after the peak of membrane enzyme (apparent molecular weight 48,000). Also the apparent molecular weight of membrane enzyme in buffer containing 0.1% Triton X-100 was greater than 100,000 as estimated by gel filtration through a Sephadex G-75 column.2

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1 S. Yamamoto, personal communication.
2 Unpublished data.
In addition to the major membrane-bound penicillinase described here, Bettinger and Lampen, using protoplasts of B. licheniformis 749/C preincubated at pH 9.0 to 9.5 to remove much of their membrane-bound enzyme, were able to demonstrate the transient presence during active penicillinase synthesis of a protease-sensitive form presumably in an incompletely folded state (19). The precise biosynthetic relation of this molecule to the membrane-bound and exopenicillinases is as yet uncertain, although it is already clear that the stabilized membrane-bound penicillinase is not an obligatory intermediate in the formation of the exoenzyme (3).

REFERENCES
Purification and Characteristics of Plasma Membrane Penicillinase from *Bacillus licheniformis* 749 / C
Tetsuo Sawai and J. Oliver Lampen


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