EVIDENCE FOR PHOSPHOLIPID IN PLASMA MEMBRANE PENICILLINASE OF BACILLUS LICHENIFORMIS 749/C

TETSUO SAWAI, LAURA J. CRANE And J. O. LAMPEN

Institute of Microbiology, Rutgers University, The State University of New Jersey, New Brunswick, New Jersey 08903

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SUMMARY: The plasma membrane-bound penicillinase of Bacillus licheniformis 749/C has been purified. Amino acid analysis showed no significant differences in composition between the enzyme and exopenicillinase. Enzyme purified from cultures containing $H_3{}^{33}PO_4$ or [^{3}H]-glycerol contained ^{33}P or [^{3}H]-glycerol activity and treatment with 8 M urea, 0.2% sodium dodecyl sulfate at 80° C did not remove the ^{3}H-activity from the enzyme protein. Trypsin readily cleaved the glycerol-containing moiety from the enzyme protein, forming enzyme with molecular weight and heat stability like that of the exoenzyme. Phospholipase D and C also produced enzyme resembling the exo-form.

INTRODUCTION: B. licheniformis 749/C secretes approximately half its penicillinase (EC 3.5.2.6) into the medium in a very stable hydrophilic form that is not adsorbed to the cell membrane and shows no tendency to aggregate. The remaining penicillinase is cell-bound, associated with the plasma membrane and with periplasmic vesicles that are released into the medium upon protoplasting (1). There is only one penicillinase structural gene (2), and, indeed, cell-bound enzyme released by trypsin differs in amino-acid sequence from the exoenzyme only by the absence of the N-terminal lysine residue (3). The cell-bound enzyme, however, particularly the plasma membrane form, is very tightly associated with the membrane and requires solubilization by a combination of detergent and chelating agent. The solubilized enzymes readily aggregate, and in general have the characteristics of hydrophobic proteins (4, 5).

Recent studies, confirming earlier work, have shown that a portion of the cell-bound enzyme is involved in the secretion process, though the membrane-bound enzyme is not an obligatory intermediate in the formation of exoenzyme(6). In order to clarify its relation to the secretion process and to understand the basis for its association with the membrane, we have purified and partially characterized the plasma membrane form of the enzyme.

B. licheniformis 749/C, a mutant which produces penicillinase constitutively, was grown in CH/S medium at pH 6.5 to exponential growth phase and converted to protoplasts with lysozyme, as described previously (4). Protoplasts were lysed by suspension in 0.05 M Tris-HCl buffer, pH 7.5, 0.01 M $MgCl_2$ and the membrane fraction prepared and the enzyme extracted as described by Sargent and Lampen (4) except that taurodeoxycholate was used instead of deoxycholate.

The extracted enzyme was first purified on a Biogel A5M column (2 x 45 cm) equilibrated with 0.05 M pyrophosphate buffer, pH 6.5, 0.1% taurodeoxycholate. The main active fractions from the column were pooled and subjected to further purification by electrophoresis in the presence of 0.1% Sarkosyl NL-97 (sodium N-lauroyl sarcosinate) on 7.5% acrylamide gels (0.8 x 6.5 cm). After electrophoresis in 0.1 M sodium phosphate, 0.1% Sarkosyl, pH 7.2 at 20 mA/tube for 5 hours, the gels were cut in three mm slices and the enzyme was extracted from the slices by 0.05 M Tris-maleate buffer, pH 6.0 containing 0.2% taurodeoxycholate. Two cycles of Sarkosyl-acrylamide gel electrophoresis resulted in a preparation which showed a single band in Sarkosyl and in sodium dodecyl sulfate-acrylamide gel electrophoresis.

The amino acid composition of the purified enzyme was determined

and compared with that of exoenzyme purified according to Pollock (7). The exoenzyme migrated as a single band in sodium dodecyl sulfate-acrylamide gel electrophoresis and its specific penicillinase activity (350 units/μg protein) agreed with the value reported by Pollock. Both preparations were dialyzed against deionized water, freeze-dried and then hydrolyzed at 110° C in 6 N HCl under vacuum in sealed tubes for 24 hours. Amino acid analyses were performed by Dr. David Strumeyer (Biochemical Data Corporation, Highland Park, New Jersey) on a Durrum-500 amino acid analyzer. There was no significant difference in amino acid composition between the membrane enzyme and the exoenzyme.

This finding ruled out the possibility that the hydrophobic properties of the membrane enzyme were due to a hydrophobic "tail" of amino acids linked to the N-terminal lysine as in the cytochrome b_5 from liver microsomes (9,10). Such a "tail" of amino acids would explain the trypsin-catalyzed release of enzyme indistinguishable from the exo-form.

Although the purified enzyme showed no difference in amino acid composition from the exo-form, it retained the characteristic hydrophobic properties of the crude plasma membrane-bound enzyme. Sargent and Lampen (4) observed that the crude enzyme showed an apparent molecular weight on Biogel A5M in the presence of deoxycholate of 45,000. In the absence of deoxycholate, crude enzyme aggregated to an apparent molecular weight of over 600,000. A more accurate determination of the gel filtration behavior of the purified enzyme in the presence and absence of taurodeoxycholate was made using a longer Biogel A5M column (1.2 x 82 cm). With alpha-chymotrypsinogen A, ovalbumin, bovine albumin, and bovine gamma-globulin as internal standards, the molecular weight of the membrane enzyme was found to be 48,000 in the presence of 0.1% taurodeoxycholate and 102,000 and 170,000 in

its absence (two peaks of enzyme activity). In both buffers, the exoenzyme shows a molecular weight of 29,000 in agreement with the amino acid sequence reported by Ambler and Meadway (3). Sargent and Lampen suggested that the apparent molecular weight in the presence of bile salts was the result of complex formation between hydrophobic portions of the membrane enzyme and bile salt molecules. The molecular weight of 48,000 corresponds to a complex of 1 enzyme molecule and 38 molecules of taurodeoxycholate. Recent observations indicate that in 0.1% [^{14}C]-taurodeoxycholate, 37 molecules of taurodeoxycholate are bound per molecule of enzyme.

In order to examine the possibility that the hydrophobic properties of the membrane enzyme are due to association with phospholipid, the enzyme was purified from plasma membrane prepared from cells grown in CH/S medium containing $H_3{}^{33}PO_4$ or [2-^{3}H]-glycerol. Cultures in 500 ml of CH/S medium contained 0.5 mCi $H_3{}^{33}PO_4$ and 1 mCi ^{3}H-labeled amino acid mixture, or 5 mCi [^{3}H]-glycerol and 0.5 mCi ^{14}C-labeled amino acid mixture. Purified enzyme preparations were, indeed, found to contain ^{33}P (data not shown) or [^{3}H]-glycerol activity (Fig. 1). Exoenzyme partially purified from the same cultures by cellulose phosphate column chromatography (7) contained no radioisotopic activities other than that of the labeled amino acids. Assuming that the protein moiety of the enzyme has the same molecular weight as the exoenzyme, the [^{3}H, ^{33}P]-enzyme was estimated to contain the enzyme: phosphorus in molecular ratio of 1:1.3. The [^{3}H]-glycerol, [^{14}C]-amino acid labeled membrane enzyme was heated at 80° C for 30 min in 8 M urea and 0.2% sodium dodecyl sulfate, then subjected to acrylamide gel electrophoresis in 8 M urea, 0.1% sodium dodecyl sulfate, pH 7.2. There was no separation of [^{3}H]-glycerol from the ^{14}C-labeled protein or change in the ^{3}H/^{14}C ratio. Similar results were obtained with the ^{33}P and [^{3}H]-

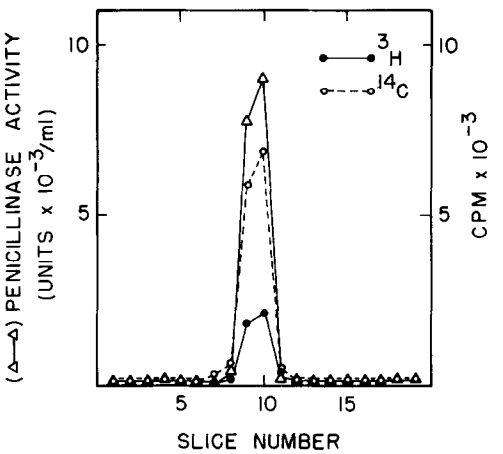

Fig. 1. Acrylamide gel electrophoresis of membrane penicillinase isolated from a culture containing [^{3}H]-glycerol and [^{14}C]-amino acids. Electrophoresis was carried out in a 7.5% cross-linked gel containing 0.1 M phosphate buffer, pH 7.2, 0.1% Sarkosyl for 5 hours. The gel was then cut in 3 mm slices and the enzyme extracted from each slice by 0.7 ml of 0.05 M Tris-maleate buffer pH 6.0 containing 0.2% taurodeoxycholate. Penicillinase activity was assayed by the method of Sargent (11). The radioactivity was counted in Aquasol (New England Nuclear Corp.) with a Packard Tri-Carb liquid scintillation counter.

amino acid labeled enzyme preparation. The [^{14}C, ^{3}H]-enzyme was also treated with chloroform and methanol according to the method of Bligh and Dyer (8). After the extraction, the ^{3}H/^{14}C ratio had not changed significantly.

The differing mobilities of the two enzyme forms on Biogel A5M in the presence of taurodeoxycholate were used to test the conversion of the [^{3}H]-glycerol, [^{14}C]-amino acid labeled enzyme to the exo-form. As shown in Fig. 2A, brief treatment with trypsin changed the eluting position of the membrane enzyme to that of exoenzyme. About half of the enzyme activity was lost, and a peak of ^{14}C-labeled peptides was observed. The presence of more ^{14}C-activity in the lower molecular weight peptides peak than expected from the decrease in enzyme activity is presumably a result of preferential digestion of the denatured molecules in the enzyme preparation. This loss of activity does not usually occur with less rigorously purified preparations.

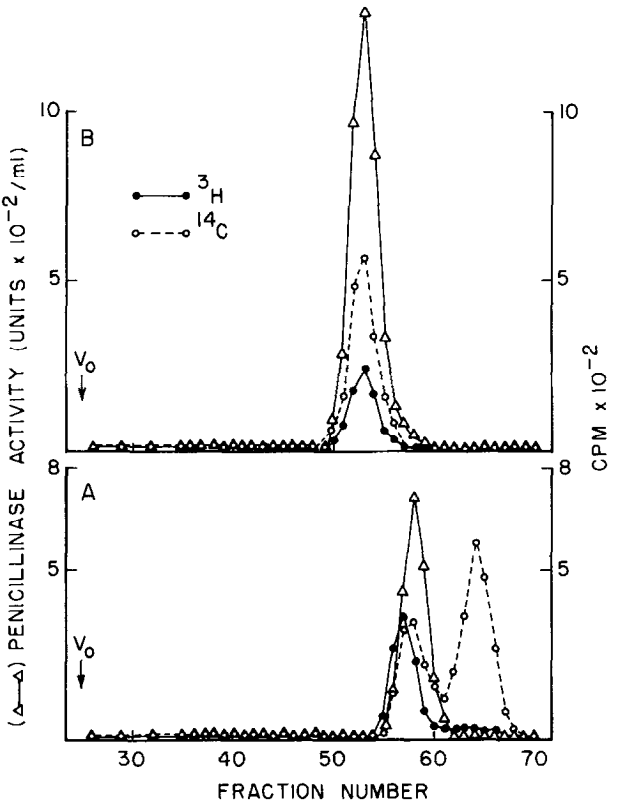

Fig. 2. Gel filtration on Biogel A5M of trypsin-treated membrane enzyme. (A) 10^4 units of enzyme labeled with [^{3}H]-glycerol and [^{14}C]-amino acids were incubated in 0.4 ml of 0.01 M Tris-HCl buffer, pH 7.9, containing 0.02 M $CaCl_2$ and 50 µg of trypsin at 30° C. After 1 hour, 50 µl of 0.16 M EDTA and 50 µg of soybean trypsin inhibitor in 50 µl of water were added. The sample was chromatographed on a Biogel A5M column (1.2 x 82 cm) equilibrated and eluted with 0.05 M pyrophosphate buffer, pH 6.5, 0.1% taurodeoxycholate. Fraction size was 1.5 ml. Penicillinase activity and radioactivity were assayed as described in Fig. 1. (B) As A, except that the trypsin was omitted.

When the enzyme was treated under identical conditions except that trypsin was omitted, no degradation or change to exoenzyme was observed (Fig. 2B). ^{3}H-activity (presumably as [^{3}H]-glycerol) was not separated from the enzyme peak in the Biogel A5M column, although the peaks of enzyme activity and tritium were not superimposible (Fig. 2A). However, when the trypsin-treated enzyme was subjected to Sarkosyl-acrylamide gel electrophoresis, ^{3}H-activity was completely separated from the enzyme protein and moved out of the gel.

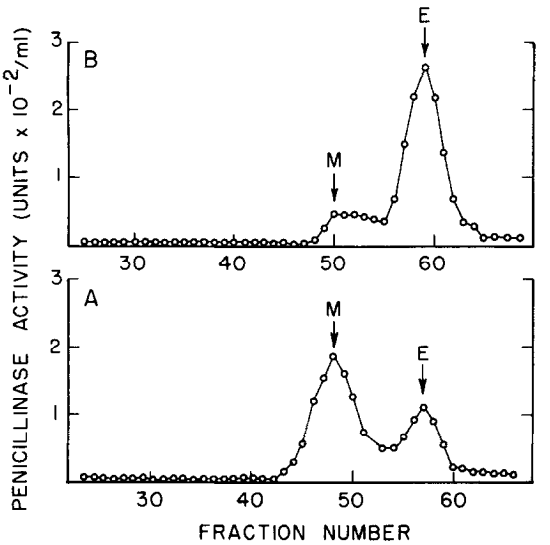

Fig. 3. Gel filtration on Biogel A5M of phospholipase D-treated enzyme. 3 x 10^3 units of membrane enzyme was incubated in 0.4 ml of 0.04 M acetate buffer, pH 5.6, containing 0.05 M $CaCl_2$, 0.1% taurodeoxycholate and phospholipase D (A: 0.8 units; B, 31 units) at 26° C for 1 hour. The reaction mixture was then mixed with 100 µl of 0.16 M EDTA, cooled in an ice-bath, and chromatographed on a Biogel A5M column under the conditions described in Fig. 2. With the crude taurodeoxycholate preparation used for this experiment the membrane enzyme had an apparent molecular weight of approximately 100,000. M and E show the eluting positions of membrane enzyme and exoenzyme, respectively.

A distinguishing characteristic of the two enzyme forms is the much greater stability of the exo-form than of the membrane form at 55° C. The converted enzyme showed heat stability similar to that of exoenzyme. These results provide strong evidence that trypsin cleaves the membrane enzyme into the enzyme protein and a compound(s) that contains phosphate and glycerol and can be separated from the enzyme by gel electrophoresis.

Because it seemed probable that the purified membrane enzyme is a phospholipid-protein, we tested the effect of phospholipase D (E. C. 3.1.4.4) from cabbage (Sigma Chemical Co., St. Louis, Mo.) and phospholipase C (E. C. 3.1.4.3) from Clostridium perfringens (Worthington Biochemical Co., Freehold, N. J.) on the membrane enzyme. As shown in Fig. 3, the enzyme

(unlabelled) was converted to a form with the apparent molecular weight of the exoenzyme by phospholipase D without substantial loss of activity and the amount of enzyme converted was proportional to the amount of phospholipase D added. The phospholipase D was a partially purified preparation, but proteinase activity could not be detected. Phospholipase C was tested in a similar experiment. When 10^4 units of the membrane enzyme were treated at 37° C for 1 hour with 1 unit of phospholipase C in 0.5 ml of 0.04 M Tris-HCl buffer, pH 7.3, containing 2 mM $CaCl_2$, about 20% of the enzyme was converted to the exo-form. The activity of phospholipase C on the membrane enzyme is thus much weaker than phospholipase D. These results provide strong evidence for the presence of phospholipid in the plasma membrane-bound penicillinase of B. licheniformis 749/C.

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