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VESICLE PENICILLINASE OF *BACILLUS LICHENIFORMIS* 749/C

APPARENT IDENTITY WITH THE PLASMA MEMBRANE ENZYME

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Summary

A substantial fraction of the total membrane penicillinase of *Bacillus licheniformis* 749/C is attached to the vesicles released during conversion of the cells to protoplasts. This enzyme was purified since there was indirect evidence that it differed from the enzyme that remained with the protoplast. The purified vesicle penicillinase has the same molecular weight and general properties as the plasma membrane (protoplast) enzyme and, similarly, contains a covalently linked phosphatidylserine residue. Treatment of the two enzymes with trypsin produced phosphatidylserine-containing peptides which could not be distinguished by gel or paper electrophoresis. The two membrane penicillinases are very similar, if not identical.

Introduction

Bacillus licheniformis 749/C produces a hydrophilic extracellular penicillinase (penicillin amido-β-lactamhydrolase; EC 3.5.2.6) and a hydrophobic cell-bound form(s) [1,2]. About 50% of the cell-bound enzyme is released when cells are treated with lysozyme in osmotically stabilized media [2]; the remaining enzyme is bound to the protoplast membrane but can be solubilized by detergent [3]. The released penicillinase is associated with vesicular structures presumably produced by eversion of mesosomes [3,4]. The plasma membrane penicillinase is a phospholipoprotein [5] that differs from the exoenzyme in carrying an additional 24 amino acid residues with phosphatidylserine as the NH_2 terminus. Earlier studies [3] had indicated that the crude vesicle enzyme

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differed from the plasma membrane enzyme in that it was converted readily to the exo-form; however, we have recently shown [6] that the two enzymes behave identically on gel permeation chromatography in the presence or absence of detergent. The earlier vesicle preparations probably contained a protease that converts the vesicle enzyme to the hydrophilic exoenzyme [7].

Since vesicle penicillinase is the major source of exoenzyme at alkaline pH [8], we purified the vesicle enzyme and have found it to be essentially identical to the plasma membrane penicillinase.

Materials and Methods

Organisms and growth conditions. B. licheniformis 749/C is a magnoconstitutive mutant of the penicillinase-inducible strain 749 [1]. Inocula were prepared as described by Lampen [2]. Cultures were grown at 30° C with vigorous shaking in 500 ml volumes of casein hydrolysate-salts medium containing 0.03 M KH_2PO_4 (pH 6.3). After 3 h, 1 mCi of [2-^{3}H]glycerol was added to each of 10 flasks, and the cultures were allowed to grow to a cell density of 0.8 mg (dry weight equivalent)/ml. Growth was measured as turbidity with a Klett-Summerson colorimeter (No. 54 filter). Cells were harvested by sedimenting at 12 000 × *g* for 20 min at 2° C.

Preparation of vesicles. The cells were suspended at 10 mg/ml in a medium consisting of 0.75 M sucrose, 0.03 M KH_2PO_4 (pH 6.0), 1 mM $MgCl_2$, lysozyme, 100 μg/ml, chloramphenicol, 40 μg/ml and 1 mM quinacrine hydrochloride (to minimize cleavage of the bound penicillinase [9]). Protoplast formation was complete in 30 min at 30° C. The suspension was centrifuged at 12 000 × *g* for 20 min at 2° C, then at 20 000 × *g* for 30 min, and finally at 100 000 × *g* for 2 h to sediment the vesicles.

Purification of vesicle penicillinase. Stage 1. Extraction from vesicles. The vesicle fraction was homogenized with 20 volumes of 0.05 M KH_2PO_4 buffer (pH 6.3) containing 0.1% Triton X-100 and stirred gently overnight at 20° C. The mixture was brought to 80% saturation with $(NH_4)_2SO_4$ (pH maintained at 7.0), stirred for 3 h at 2° C, and then spun at 10 000 × *g* for 15 min. The floating lipid layer, which contained 95% of the penicillinase activity, was dissolved in 10 volumes of 0.05 M Tris buffer (pH 7.5) containing 0.1% Triton X-100 (Buffer A).

Stage 2. DEAE-Sephadex chromatography. The enzyme solution from Stage 1 was directly applied to a column (2.5 × 108 cm) of DEAE-Sephadex A-50 equilibrated in Buffer A; the membrane enzyme was eluted following the addition of 0.15 M NaCl to the buffer. Active fractions were pooled and concentrated by pressure filtration under nitrogen (UM-10 Diaflo ultrafiltration membrane; Amicon Corp., Lexington, Mass.).

Stage 3. Bio-Gel A-5M gel filtration. The enzyme concentrate (Stage 2) was applied to a column (2 × 50 cm) of Bio-Gel A-5M equilibrated in Buffer A. Fractions were collected at a flow rate of 20 ml/h.

Stages 4 and 5. DEAE-Sephadex chromatography. The active fractions from Stage 3 were applied directly to a column (2 × 20 cm) of DEAE-Sephadex A-50 equilibrated in Buffer A. The resin was washed with three column volumes of Buffer A, and the enzyme eluted with a linear gradient of 0—0.15 M NaCl in

Buffer A. Fractions containing penicillinase were pooled, dialyzed for 24 h against Buffer A, applied to a DEAE-Sephadex column as in Stage 2, and gradient elution repeated. The active fractions were pooled, dialyzed against Buffer A for 24 h, and concentrated by pressure filtration.

Stage 6. Gel permeation on Bio-Gel A-5M. The enzyme from Stage 5 was applied to a column (2 × 50 cm) of Bio-Gel A-5M equilibrated in and eluted with Buffer A. Vesicle penicillinase eluted as a single peak with a V_e/V_o ratio of 1.68. This approximates an apparent molecular weight of 100 000, as observed for plasma membrane penicillinase in the presence of Triton X-100 [5].

Stage 7. Affinity chromatography on cephalosphorin C-Sepharose. The pooled enzyme from Stage 6 was dialyzed against 0.05 M sodium acetate buffer (pH 4.0) containing 0.1% taurodeoxycholate. About 100 000 units of enzyme (the approximate binding capacity of the column) were placed on a column (1 × 9 cm) of cephalosporin C-Sepharose 4B equilibrated and washed with the above buffer [10]. The enzyme was eluted with 0.05 M sodium phosphate (pH 6.0) containing 0.1% taurodeoxycholate (Fig. 1). The active fractions [9–13] were pooled and stored at −20° C.

Assays. Penicillinase was measured by the method of Sargent [11]. One unit hydrolyzes 1 μmol of benzylpenicillin per h at 30° C. The assay buffer was 0.01 M KH_2PO_4 (pH 7.0) containing 0.1% taurodeoxycholate. Protein was determined by the alkaline ninhydrin procedure [12].

Thin-layer chromatography was performed on plates of silica gel G (Brinkmann Instruments, Inc., Westbury, N.Y.) with three solvent systems; A, methylisobutylketone/acetic acid/water (40 : 25 : 5, v/v); B, chloroform/methanol/water (70 : 30 : 5, v/v); C, chloroform/methanol/acetic acid/water (25 : 15 : 4 : 2, v/v). The phosphatidylserine standard was detected with 0.2% ninhydrin in acetone/lutidine (9 : 1, v/v), and the lipid from vesicle penicillinase by assay for radioactivity.

Chemicals. Lysozyme was obtained from Sigma Chemical Co., St. Louis, Mo.; pronase and sodium taurodeoxycholate from Calbiochem., La Jolla, Calif.;

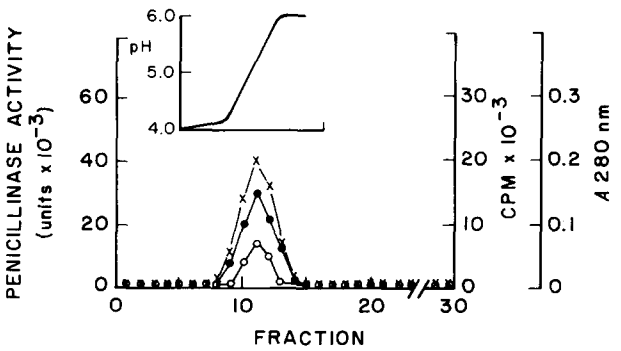

Fig. 1. Purification of vesicle penicillinase by affinity chromatography (Stage 7). The vesicle penicillinase was absorbed onto a column of cephalosporin C-Sepharose 4B equilibrated in 0.05 M sodium acetate buffer (pH 4.0) containing 0.1% taurodeoxycholate. The enzyme was eluted sharply from the column with 0.05 M sodium phosphate (pH 6.0) containing 0.1% taurodeoxycholate. •, penicillinase activity; ×, radioactivity; ○, extinction at 280 nm.

quinacrine hydrochloride from Mann Research Labs., Orangeburg, N.Y.; and [2-^{3}H]glycerol (200 Ci/mol) from New England Nuclear Corp., Boston, Mass. Exopenicillinase was purified by the method of Pollock [1]. Plasma membrane penicillinase was a gift of Dr. S. Yamamoto of this institute.

Results

Purification of vesicle penicillinase. The procedure (Table I) was a modification of the method for preparation of the plasma membrane penicillinase [13]. The purified vesicle enzyme had a specific activity of 360 units per μg of protein, which is similar to the values for the exo and plasma membrane penicillinases [13].

Purity of vesicle penicillinase and incorporation of [2-^{3}H]glycerol. The purified vesicle enzyme eluted from the cephalosporin C-Sepharose column (Stage 7) as a single coincident peak of protein, penicillinase activity, and readioactivity derived from [2-^{3}H]glycerol (Fig. 1). Upon electrophoresis in the presence of 0.1% sodium dodecyl sulfate the enzyme exhibited a single protein band (Fig. 2) and retained tritium activity which was coincident with penicillinase activity. Under the conditions of this experiment the sodium dodecyl sulfate does not interfere with the assay of penicillinase.

Nature of lipid moiety. It was important to determine if vesicle penicillinase contains a phosphatidylserine residue as does the plasma membrane enzyme [5]. Purified ^{3}H-labelled vesicle penicillinase (170 μg) was digested with pronase (10 μg) in 0.01 M sodium borate buffer (pH 8.0) containing 0.01 M calcium acetate for 24 h at 30°C. The lipid was extracted by the method of Bligh and Dyer [14] and the chloroform phase was subjected to thin-layer chromatography on silica gel G. In the three solvent systems tested, the R_F values for the

TABLE I

PURIFICATION OF VESICLE PENICILLINASE FROM *B. LICHENIFORMIS* 749/C

Stage	Enzyme (units × 10^{-5})	Protein (mg)	Specific activity (units/μg protein)	Yield (%)
Vesicle pellet [a]	29.5	—	—	100
1. Triton X-100 extraction and ammonium sulfate fractionation	18.0	—	—	61
2. DEAE-Sephadex A-50, 0.15 M NaCl elution	16.0	—	—	54
3. Bio-Gel A-5M	13.6	12.9	105	46
4. DEAE-Sephadex A-50, 1st NaCl gradient	9.84	4.5	220	33
5. DEAE-Sephadex A-50, 2nd NaCl gradient	5.4	1.8	300	18
6. Bio-Gel A-5M filtration	3.9	1.2	330	13
7. Affinity chromatography cephalosporin C-Sepharose	3.5	0.9	360 [b]	11

[a] From 5 l of bacterial culture (4200 mg dry weight equivalents of cells).
[b] Approx. 200 cpm (as ^{3}H)/μg protein.

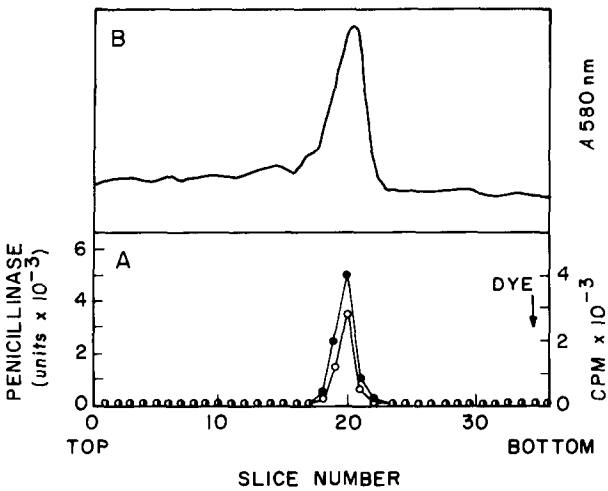

Fig. 2. Gel electrophoresis of purified vesicle penicillinase. (A) Retention of label from [2-^{3}H]glycerol. Approx. 10 000 units (28 μg) of penicillinase were subjected to electrophoresis in 0.05 M phosphate buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate. Slices (2.0 mm) were extracted with 0.05 M Tris/maleate buffer (pH 6.0) containing 0.2% taurodeoxycholate and assayed for penicillinase (•) and radioactivity (○). (B) Approx. 8000 units of enzyme were electrophoresed as in A, followed by staining with Coomassie Blue. A scanning densitometer (Schoeffel Instrument Co., Westwood, N.J.) was used to measure absorbance.

single lipid spot from vesicle penicillinase and for authentic phosphatidylserine were, respectively: A, 0.31, 0.32; B, 0.27, 0.27; and C, 0.59 and 0.58. The lipid fragment from vesicle penicillinase clearly has the properties of phosphatidylserine.

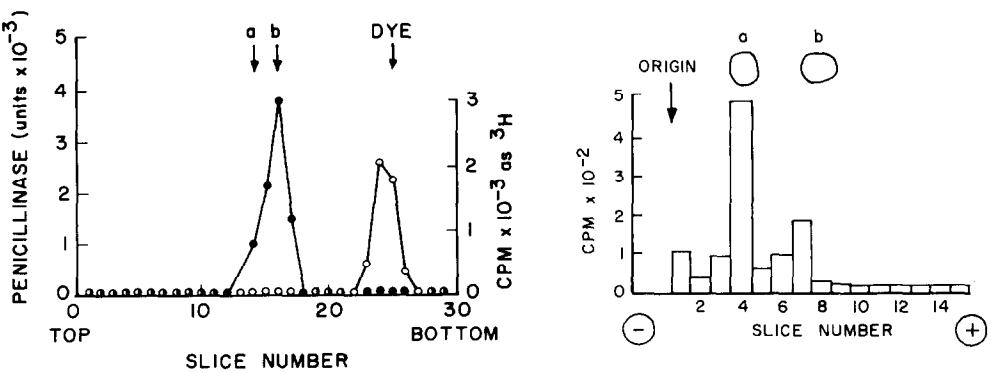

Fig. 3. Gel electrophoresis of trypsin-treated vesicle penicillinase. Approx. 35 μg of enzyme was incubated with 2 μg of trypsin for 1 h and subjected to electrophoresis in 0.05 M Tris buffer (pH 7.2) containing 0.1% sodium dodecyl sulfate. •, penicillinase activity; ○, ^{3}H activity. a, migration of untreated vesicle enzyme; and b, migration of authentic exopenicillinase.

Fig. 4. Comparison of the phospholipopeptides from vesicle and plasma membrane penicillinases. ^{3}H-labelled vesicle enzyme (58 μg) was incubated with 2 μg of trypsin for 2 h at 30°C in 0.05 M barbital buffer (pH 8.5) with 0.1% Triton X-100. Samples equivalent to 1.6 μg (3000 cpm) of vesicle enzyme were electrophoresed on Whatman No. 1 paper strips in 0.05 M barbital buffer (pH 10) at 175 V for 2 h at 30°C. The strips were cut into 0.5-cm segments which were assayed for radioactivity. Exopenicillinase (b) and the phospholipopeptide (a) from plasma membrane enzyme were detected on parallel strips by the chlorine-tolidine method for protein [16].

Cleavage of vesicle penicillinase by trypsin. Incubation of the ^{3}H-labelled vesicle penicillinase with trypsin at pH 8.0 yielded a hydrophilic enzyme with the electrophoretic mobility (in sodium dodecyl sulfate) of exopenicillinase and a radioactive fragment that moved just behind the marker dye (Fig. 3). This fragment has the same mobility as the phospholipopeptide released from the plasma membrane enzyme [15].

The ^{3}H-labelled fragments from the two enzymes were also compared by electrophoresis on Whatman No. 1 paper in the presence of Triton X-100 (Fig. 4). The mobility of the vesicle penicillinase fragment (detected by its radioactivity) was identical with that of the well-characterized phospholipopeptide from the plasma membrane enzyme.

Discussion

The findings presented here demonstrate that the purified vesicle penicillinase of *B. licheniformis* 749/C is similar to the plasma membrane enzyme. (1) Both enzymes elute from Bio-Gel A-5M with apparent molecular weights of about 100 000 in the presence of Triton X-100, 45 000 in the presence of deoxycholate, and 600 000 in the absence of detergent. (2) When the enzymes were purified from cells grown in a medium containing [2-^{3}H]glycerol, both retained radioactivity which was not removed by gel electrophoresis in the presence of 0.1% dodecyl sulfate. (3) The vesicle enzyme, like the plasma membrane enzyme, contains a phosphatydylserine residue and on treatment with trypsin yielded a hydrophilic penicillinase and an ^{3}H-labelled fragment which could not be distinguished from the products obtained from the plasma membrane enzyme. We have not had enough of the pure vesicle enzyme to determine the sequence of the ^{3}H-labelled fragment. Nevertheless, there is no reason to believe that the membrane penicillinases from the two locations in the cell are chemically different.

A prominent feature of penicillinase secretion is the preferential formation of exoenzyme at alkaline pH from the area of the cell membrane which would be expelled during protoplast formation [3,9]. With only a single chemical form of membrane penicillinase available, this preferential release must reflect a greater accessibility of the bound enzyme in the releasable vesicle fraction to the penicillinase-releasing protease of *B. licheniformis* 749 [7].

Acknowledgements

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