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Penicillinase-Releasing Protease of *Bacillus licheniformis*: Purification and General Properties

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The membrane penicillinase of *Bacillus licheniformis* 749/C is a phospholipoprotein which differs from the exoenzyme in that it has an additional sequence of 24 amino acid residues and a phosphatidylserine at the NH_2 terminus. In exponential-phase cultures, the conversion of membrane penicillinase to exoenzyme occurs at neutral and alkaline pH. An enzyme that will cleave the membrane penicillinase to yield the exoenzyme is present (in small amounts) in exponential-phase cells and is released during their conversion to protoplasts. The enzyme is found in the filtrate of a stationary-phase culture of the uninduced penicillinase-inducible strain 749 and has been purified to apparent homogeneity from this source. The protease has an approximate molecular weight of 21,500 and requires Ca^{2+} ions for stabilization. It has a pH optimum of 7.0 to 9.5 for hydrolysis of casein and for the cleavage of membrane penicillinase. Both activities are inhibited by diisopropylfluorophosphate; hence, the enzyme is a serine protease. This enzyme may be entirely responsible for the formation of exopenicillinase by this organism, since the other neutral and alkaline proteases of strain 749 have little, if any, activity in releasing exopenicillinase. The enzyme has been termed penicillinase-releasing protease.

It has been documented that the hydrophobic membrane-bound penicillinase of *Bacillus licheniformis* 749/C is an intermediate in the formation and release of the hydrophilic exopenicillinase, even though the membrane-bound enzyme is readily converted to the exoform only at pH values of 7 and above (3, 4, 7, 11-13). The membrane penicillinase differs from exopenicillinase in that it has 24 additional amino acid residues (only glycine, serine, aspartic acid or asparagine, and glutamic acid or glutamine) and a covalently linked phosphatidylserine at the NH_2 terminus (16). The sequence of this phospholipopeptide fragment has been determined (17).

In the preceding communication (14) it was shown that certain periplasmic macromolecules released upon protoplasting exponential-phase cells of *B. licheniformis* 749/C have the capacity to convert the membrane-bound penicillinase to a hydrophilic form. The enzyme thus released from the vesicle preparation has lysine or glutamic acid at its NH_2 terminus, as does the exopenicillinase released by growing cells at pH 6.5 or by washed cells or protoplasts at pH 9.0 (1, 14). Thus the formation of exopenicillinase in the various systems appears to involve a common proteolytic processing of the membrane enzyme.

We here describe the isolation, purification, and some properties of the enzyme (penicillinase-releasing protease [PR protease]) that appears to be responsible for the conversion of membrane penicillinase to exopenicillinase. Large amounts of this enzyme are present in the culture fluid of stationary-phase cultures of the penicillinase-constitutive mutant *B. licheniformis* 749/C and of the inducible parent strain 749 grown without inducer. Strain 749 was used to avoid interference in the assay system by the penicillinase produced by the constitutive mutant. We have also attempted to relate some of the properties of this enzyme to the characteristics of the various secretion systems (3, 4, 7, 12, 14).

MATERIALS AND METHODS

Materials. Diethylaminoethyl (DEAE)-Sephadex A-50, carboxymethyl (CM)-Sephadex C-50, and Sephadex G-50 were purchased from Pharmacia Fine Chemicals, Piscataway, N.J. Bovine serum albumin, alpha-casein, phenylmethylsulfonyl fluoride, tosylphenylalanylchloromethylketone, tosyllylsylchloromethylketone, *p*-aminobenzidine, and ethyleneglycoltetraacetic acid were products of Sigma Chemical Co., St. Louis, Mo. Diisopropyl-

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fluorophosphate and taurodeoxycholate were the products of Calbiochem, San Diego, Calif. Soybean trypsin inhibitor and casein were purchased from Nutritional Biochemical Co., Cleveland, Ohio. Membrane penicillinase of *B. licheniformis* 749/C was purified in our laboratory (18). Ovalbumin was purchased from Worthington Biochemicals Corp., Freehold, N.J.

Growth conditions. *B. licheniformis* 749 was maintained as spores on Wickerham agar slants. Inocula were prepared by growing cells overnight at 30°C in casein hydrolysate-salts medium (6). A 1.5-liter culture was added to 30 liters of casein hydrolysate-salts medium, and growth was continued for 20 h at 32°C with aeration at 20 liters/min and with 5 ml of polyglycol 2000 added as anti-foaming agent. The cells were harvested with a Sharples supercentrifuge no. 16 at 13,200 × *g*, and the supernatant fluid was concentrated to 1.78 liters in an Amicon Diaflo thin-layer TC3E ultrafiltration system with UM-10 filters (Amicon Corp., Lexington, Mass.). The concentrate was centrifuged at 10,000 × *g* for 15 min and frozen until used.

Protease assay. Proteolytic activity in the culture supernatant and in the various fractions during purification was assayed by a slight modification of the method of Belew and Porath (2) with casein as the substrate. Two milliliters of 0.5% casein in 0.2 M Tris-hydrochloride buffer (pH 7.5) with 2 mM $CaCl_2$ was equilibrated at 37°C. The enzyme preparation (5 to 20 μl) was added, and the mixture was incubated for 1 h. Two milliliters of 10% trichloroacetic acid was added to stop the reaction. The sample was held for 15 min at 4°C before centrifuging at 16,000 × *g* for 15 min. The absorbance of the supernatant at 280 nm (A_{280}) was measured. Control mixtures were similarly prepared except that the trichloroacetic acid was added before the enzyme. Proteolytic activity was measured as the difference in absorbance between the assay sample and the control. One unit of protease activity produces an increase in A_{280} of 0.1 under the assay conditions.

For the purified PR protease, the proteolytic activity and the kinetic properties were determined by the method of Lin et al. (8) with *N,N*-dimethyl casein as the substrate. Methylated casein was prepared as described (8). Activity is expressed as moles of peptide bond cleaved per minute per milliliter during a 10-min period.

Penicillinase-releasing activity. Conversion of the vesicle penicillinase to exopenicillinase through cleavage by the protease and release from the vesicle was assayed as described in the preceding communication (14). One unit of penicillinase-releasing activity converts (cleaves) 10 units of vesicle penicillinase to exopenicillinase in 10 min under the assay conditions. Penicillinase was assayed as described by Sargent (10).

Protein determination. Protein was estimated by the method of Lowry et al. (9).

Purification of the PR protease. All procedures were carried out at 4°C.

Step 1. The concentrated culture supernatant (1.78 liters; see growth conditions) was brought to 20% saturation with powdered ammonium sulfate and held for 3 h, and the precipitate was collected by centrifugation at 18,000 × *g* for 30 min. The supernatant fluid was then brought to 90% saturation with ammonium sulfate and held for 18 h, and the precipitate was collected as before. The precipitates were dissolved in a minimum volume of 0.05 M sodium phosphate buffer (pH 6.0) and dialyzed for 30 h against 60 liters of the same buffer with four buffer changes (4 × 15 liters). Protease and penicillinase-releasing activities were associated with the precipitate between 20 to 90% ammonium sulfate saturation.

Step 2. DEAE-Sephadex A-50 anion exchange chromatography. The active fraction (420 ml) from step 1 was passed through a column (4.5 by 60 cm) of DEAE-Sephadex A-50 equilibrated with 0.05 M sodium phosphate buffer (pH 7.0). The column was washed with 0.05 M sodium phosphate buffer (pH 6.5) until no more A_{280}-absorbing material was eluted. Most of the proteolytic and penicillinase-releasing activities were washed from the column. The material subsequently eluted with a gradient of 0.1 to 1 M NaCl did not have either activity. The active fractions were pooled and concentrated to 70 ml by ultrafiltration (Amicon ultrafiltration system with a UM-10 filter). The concentrate was centrifuged at 18,000 × *g* for 10 min and further concentrated to 15 ml by ultrafiltration.

Step 3. First Sephadex G-50 gel filtration chromatography. The fraction from step 2 (15 ml) was applied to a column (2 by 140 cm) of Sephadex G-50 equilibrated with 0.05 M sodium phosphate buffer (pH 6.0). The column eluted with the same buffer at a flow rate of 10 ml/h, and 5-ml fractions were collected. The protein content of the fractions was estimated by their A_{280} values. Protease and penicillinase-releasing activities were measured, and the fractions containing penicillinase-releasing activity (fractions 53 to 82, Fig. 1) were pooled and concentrated to 15 ml as in step 2.

Step 4. CM-Sephadex C-50 cation exchange chromatography. The fraction from step 3 was adjusted to pH 6.5 and loaded on a column (4 by 20 cm) of CM-Sephadex C-50 equilibrated with 0.05 M sodium phosphate buffer (pH 6.5). The column was washed with 5 column volumes of the same buffer until the elution of A_{280}-absorbing material had reached a low level. Elution was then carried out with a 400-ml linear gradient of 0.1 to 1.0 M NaCl in 0.05 M sodium phosphate buffer (pH 6.5) at a flow rate of 50 ml/h, and 5-ml fractions were collected. The fractions from the major peak of penicillinase-releasing activity (fractions 125 to 155, Fig. 2) were pooled and concentrated to 6 ml as in step 2.

Step 5. Second Sephadex G-50 gel filtration chromatography. The active material from step 4 was applied to a column (2 by 140 cm) of Sephadex G-50 equilibrated with 0.05 M sodium phosphate buffer (pH 6.0). The column was developed as described in step 3 and with a flow rate of 6 ml/h. Fractions with penicillinase-releasing activity were pooled (fractions 62 to 69, Fig. 3) and stored at −80°C.

SDS-polyacrylamide disc-gel electrophoresis. Electrophoretic separation of the proteins was carried out in 7% polyacrylamide gels with 0.2% sodium

dodecyl sulfate (SDS) according to the method of Weber and Osborn (15). Samples containing 10 to 50 μg of protein in 10 to 50 μl of 0.05 M phosphate buffer (pH 7.0) were heated for 5 min with 1% SDS, 2 M urea, and 5% mercaptoethanol. The electrode buffer was 0.1 M sodium phosphate buffer (pH 7.2) with 0.1% SDS, and bromphenol blue was the tracking dye. A constant current of 8 mA/tube was applied for 4 h, by which time the tracking dye had reached the lower end of the gel. The gels were soaked overnight in 15% trichloroacetic acid containing 0.5% Coomassie brilliant blue and then destained electrophoretically.

RESULTS

Purification of the PR protease. Table 1 summarizes the purification of PR protease from the supernatant fluid of a 30-liter culture of *B. licheniformis* 749. The precipitate formed between 20 and 90% saturation of the concentrated culture fluid with ammonium sulfate contained most of the protease and penicillinase-releasing activities. On DEAE-Sephadex A-50 anion exchange chromatography, the protease and penicillinase-releasing activities were eluted by washing the DEAE-Sephadex A-50 with 0.05 M sodium phosphate buffer (pH 6.5), whereas most of the proteins that lack protease activity were tightly bound to the resin. Elution of the enzyme was rather slow, however, and a considerable amount of activity was lost during this step (Table 1). Gel filtration on Sephadex G-50 eliminated the smaller polypeptides, but the protease and penicillinase-releasing activities were eluted together (Fig. 1).

Figure 2 illustrates the separation of the PR protease from the bulk of the other proteases by CM-Sephadex C-50 cation exchange chromatography. Most of the protease activity was eluted with the 0.05 M sodium phosphate (pH 6.5) buffer wash, but about 0.4 M NaCl was required for elution of the penicillinase-releasing activity. In a final gel filtration on Sephadex G-50 (Fig. 3), one major peak that had both activities was obtained. Gel electrophoresis of this fraction in 0.2% SDS also gave one major protein band. Since this protein releases penicillinase from vesicles and hydrolyzes casein, we have named it the penicillinase-releasing protease (PR protease) of *B. licheniformis* 749.

Determination of the molecular weight. The molecular weight of the PR protease was determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn (15). Bovine serum albumin, ovalbumin, membrane penicillinase of *B. licheniformis* 749/C, and alpha-casein were used as reference proteins. An approximate molecular weight of 21,500 was estimated for the PR protease (Fig. 4).

Kinetic parameters. The activity of the protease in releasing penicillinase from the vesicle fraction appeared to be linear with respect to time and enzyme concentration (Fig. 5). The

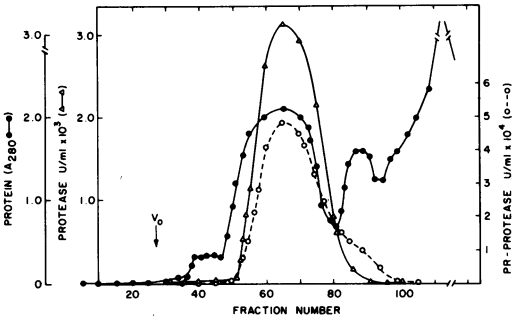

FIG. 1. *Gel filtration of the PR protease on Sephadex G-50. The active fraction (15 ml) from step 2 was applied to a column (2 by 140 cm) which was developed with 0.05 M sodium phosphate buffer (pH 6.0) at a flow rate of 10 ml/h. Protein, protease, and penicillinase-releasing activities of the 5-ml fractions were determined as described in the text.*

TABLE 1. *Purification of the PR protease*

Step	Vol (ml)	Protein (mg)	Protease		PR protease			
			Units (× 10^5)	Units/mg of protein	Units (× 10^8)	Units/mg of protein (× 10^5)	Fold purification	Yield (%)
Culture fluid UM-10 concentrate	1,780	12,015	9.3	80	5.7	0.48	1.0	100
Ammonium sulfate precipitate (20 to 90%)	420	9,240	7.4	80	3.7	0.4	0.84	64
DEAE-Sephadex A-50	70	2,940	2.2	74	1.6	0.54	1.12	28
Sephadex G-50 (I)	83	581	2.5	426	1.6	2.7	5.6	27
CM-Sephadex C-50	55	27	0.15	540	0.83	30	63	14.4
Sephadex G-50 (II)	40	16	0.12	725	0.7	43	90	12.2

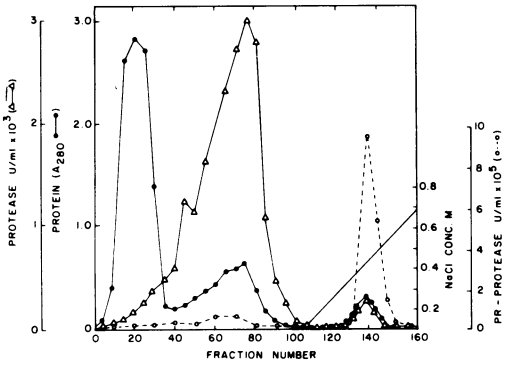

FIG. 2. *CM-Sephadex C-50 cation exchange chromatography of the PR protease. The active fraction (15 ml) from step 3 (Fig. 1) was applied to a column (4 by 20 cm) in 0.05 M sodium phosphate buffer (pH 6.5), and the column was washed with 5 column volumes of the buffer. The retained proteins were eluted with 400 ml of a linear gradient of 0.1 to 1.0 M NaCl in the same buffer. Protein and protease and penicillinase-releasing activities were determined as described in the text.*

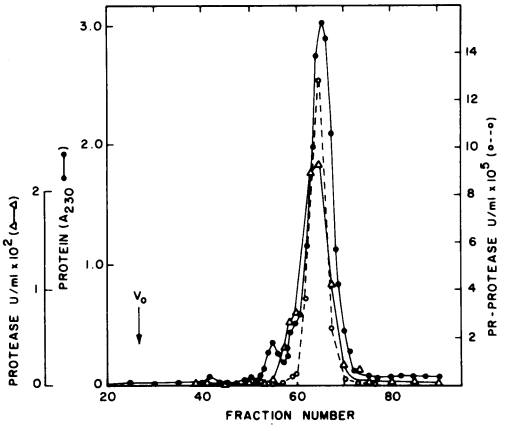

FIG. 3. *Second Sephadex G-50 gel filtration of the PR protease. The active fraction (6 ml) from step 4 (Fig. 2) was subjected to gel filtration on Sephadex G-50 as described in Fig. 1, with a flow rate of 6 ml/h. Protein and protease and penicillinase-releasing activities of 5-ml fractions were measured as described in the text.*

hydrolysis of *N,N*-dimethyl casein by the PR protease deviated from linearity after 5 min and halted after 20 to 30 min (Fig. 6). This suggests that the PR protease is relatively specific and cleaves only a limited class of peptide bonds (15 to 20% of the available bonds as determined by a parallel incubation with Pronase). Double-reciprocal plots of the initial rate of reaction against the concentration of substrate (*N,N*-dimethyl casein) allowed the calculation of V_{max} and K_m. Values of 1.6×10^{-3} mol/mg per min and 3.1×10^{-4} mol/liter were obtained for the V_{max} and K_m, respectively.

Effect of protease inhibitors. Table 2 shows the effect of various inhibitors on the protease and penicillinase-releasing activities of the enzyme. Both activities were nearly completely inhibited by 5×10^{-3} M diisopropylfluorophosphate, although 10^{-3} M phenylmethylsulfonyl fluoride, the maximum soluble level, had no effect. Ethylenediaminetetraacetic acid had no appreciable inhibitory action on the enzyme, nor did the thiol reagent *p*-chloromercuribenzoate at 4×10^{-3} M. In other tests, soybean trypsin inhibitor (200 μg/ml), 10^{-2} M tosylphenylalanylchloromethylketone, tosyllysylchloromethylketone, or *p*-aminobenzidine did not inhibit either activity (P. S. Aiyappa and J. O. Lampen, unpublished data).

Effect of pH. The effect of pH on the protease and penicillinase-releasing activities is illus-

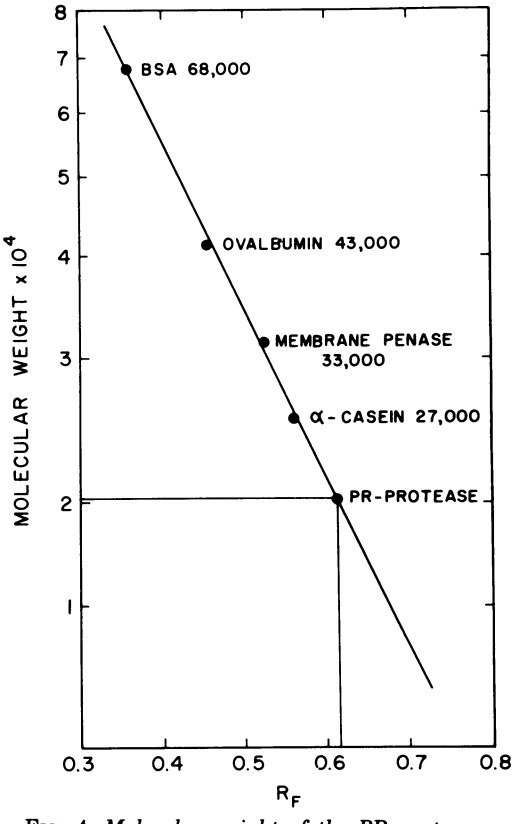

FIG. 4. *Molecular weight of the PR protease as determined by SDS-polyacrylamide gel electrophoresis. The migration of reference proteins relative to the tracking dye was plotted against molecular weight. Abbreviations: BSA, Bovine serum albumin; membrane penase, membrane penicillinase of B. licheniformis 749/C.*

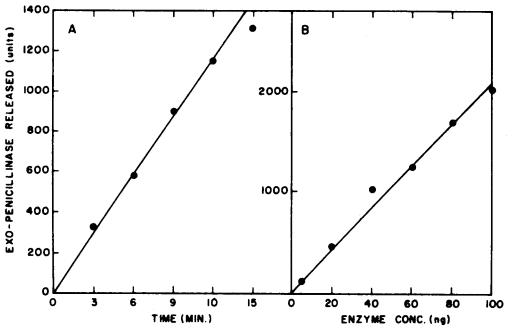

FIG. 5. *(A) Penicillinase release by the PR protease as a function of time. The assay was carried out at 37°C with 2,600 units of vesicle penicillinase and 10 ng of enzyme in 0.45 ml of 0.05 M Tris-hydrochloride buffer (pH 7.5) for the indicated times. (B) Penicillinase release as a function of enzyme concentration. The assay was carried out as in (A) for 10 min.*

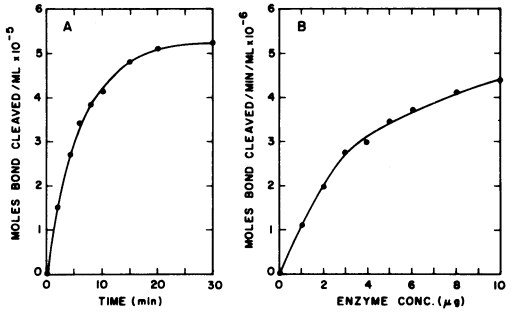

FIG. 6. *(A) Cleavage of N,N-dimethyl casein by PR protease as a function of time. One-milliliter volumes of 0.05 M sodium phosphate buffer (pH 7.5) containing 1 mg of substrate and 2 μg of enzyme were incubated at 37°C for the indicated times. The proteolytic activity was determined by the reaction of trinitrobenzene sulfonic acid with the newly formed NH_2 groups (9). (B) Cleavage of N,N-dimethyl casein by PR protease as a function of enzyme concentration. Incubation time, 10 min; other conditions as in (A).*

trated in Fig. 7. The PR protease has a broad optimum of pH 7 to 9.5 for the hydrolysis of methylated casein. The release of penicillinase from vesicles and the conversion of purified membrane penicillinase to the exoenzyme also showed an optimum from pH 7 to 9; however, at higher pH the penicillinase-releasing activity declined much more abruptly than the activity on methylated casein. A similar activity profile over the pH range 6 to 9 was observed for the release of penicillinase from protoplasts by the PR protease (P. S. Aiyappa and J. O. Lampen, unpublished data).

Stability and general properties of the PR protease. The protease appeared to be very unstable in that its specific activity decreased 50% when the enzyme was frozen and thawed twice. Attempts were made to stabilize the enzyme by the addition of Ca^{2+} and Mg^{2+} ions (Table 3). When these ions were added to the reaction mixture prior to the addition of the PR protease (to start the reaction), $CaCl_2$ at 1 mM appeared to stabilize the enzyme as indicated by a 30% increase in the penicillinase-releasing activity over the 10-min assay period. Higher concentrations of $CaCl_2$ or $MgCl_2$ were inhibitory, and

TABLE 2. *Effect of inhibitors on the PR protease*[a]

Inhibitor	Concn	Protease activity (% of control)	Penicillinase-releasing activity (% of control)
None		100	100
Diisopropyl-fluorophosphate	5×10^{-3} M	0	13
	2.5×10^{-3} M	10	43
Phenylmethylsulfonyl fluoride	1×10^{-3} M	100	100
p-Chloromercuribenzoate	4×10^{-3} M	80	82
Ethylenediaminetetraacetic acid	4×10^{-3} M	85	85
Ethyleneglycoltetraacetic acid	1×10^{-3} M	73	73
	1×10^{-2} M	31	33
Taurodeoxycholate	0.01%	120	35
	0.05%	119	40
	0.1%	125	72

[a] Penicillinase-releasing and protease activities were measured as described in Fig. 5 and 6. The inhibitors were added to the reaction mixture before the addition of the enzyme. Incubation was for 10 min at 37°C.

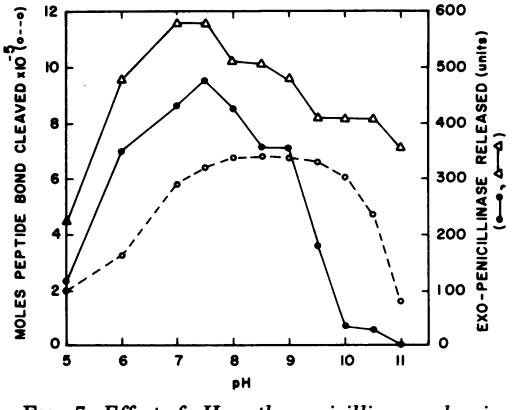

FIG. 7. *Effect of pH on the penicillinase-releasing and proteolytic activities of the PR protease (determined as in Fig. 5 and 6). Substrates:* (○) *N,N-dimethyl casein (1 mg);* (●) *purified membrane penicillinase (2,600 units);* (△) *vesicle preparation (2,600 units).*

TABLE 3. *Effect of Ca^{2+} and Mg^{2+} ions on the activity of PR protease*[a]

Addition	Concn (M)	Penicillinase-releasing activity (units)
None		175
$CaCl_2$	1×10^{-3}	225
	2×10^{-3}	208
	4×10^{-3}	151
	1×10^{-2}	108
$MgCl_2$	1×10^{-3}	192
	2×10^{-3}	170
	4×10^{-3}	66
	1×10^{-2}	66
$CaCl_2$ + $MgCl_2$ (each)	1×10^{-3}	182
	2×10^{-3}	132

[a] Penicillinase-releasing activity was measured as described in Fig. 5. The metal ions were present before the addition of the enzyme. The reactions were carried out for 10 min at 37°C.

the activity could not be recovered by subsequent dilution to reduce the $CaCl_2$ concentration from 20 to 2 mM. Addition of a specific chelator for Ca^{2+} ions, ethyleneglycoltetraacetic acid, decreased both the penicillinase-releasing and the protease activities by a factor of about two-thirds (Table 2).

The anionic detergent taurodeoxycholate prevented the release of penicillinase from vesicles but not the hydrolysis of *N,N*-dimethyl casein (Table 2). (A high concentration of taurodeoxycholate solubilizes the membrane and presumably increases the accessibility of membrane penicillinase to PR protease. Hence, an increase in activity in the presence of 0.1% taurodeoxycholate was noted.) Addition of bovine serum albumin (10 to 20 μg/ml) appeared to stabilize the PR protease and increased the penicillinase-releasing activity by 45% (P. S. Aiyappa and J. O. Lampen, unpublished data).

DISCUSSION

Cultures of *B. licheniformis* 749 or 749/C produce an enzyme(s) that releases exopenicillinase from the membrane vesicles formed during protoplasting (14). This activity is concentrated in the periplasm during the exponential growth phase and accumulates in the culture filtrate in stationary phase. Its formation is not dependent upon the concurrent production of penicillinase.

Since the exopenicillinase released from *B. licheniformis* 749 or 749/C under a variety of conditions always has lysine or glutamic acid as its NH_2-terminal residue (1, 14), it seemed likely that there is a single general process for the formation of the exoenzyme. These organisms produce large amounts of several proteases that are active in the pH range of 7 to 10, but only one minor component had substantial activity in releasing exopenicillinase from the vesicle fraction (Fig. 2 and 3). After a 90-fold purification from the supernatant fluid of a stationary-phase culture of the uninduced strain 749 (Table 1), the releasing enzyme migrated as a single band (molecular weight, 21,500) on SDS-polyacrylamide gel electrophoresis. This protein hydrolyzed casein, released penicillinase from the membrane vesicles, and cleaved the purified membrane penicillinase to yield exoenzyme. We have therefore named the enzyme the "penicillinase-releasing protease" (PR protease).

Both penicillinase release and the hydrolysis of *N,N*-dimethyl casein were inhibited by diisopropylfluorophosphate (Table 2), an inhibitor of proteases and esterases which have serine at their active center (5). The enzyme was not substantially inhibited by the chelating agent ethylenediaminetetraacetic acid, by the thiol-reactive agent *p*-chloromercuribenzoate, or by several other protease inhibitors. Hence, PR protease should be classed as a serine protease.

Taurodeoxycholate prevented penicillinase release from vesicles but not the hydrolysis of casein. Thus it probably does not inhibit the PR protease directly, and its effect on penicillinase release may be due to the interaction of such detergents with the membrane penicillinase (14) or with the membrane itself.

The PR protease appears to be relatively unstable. It shows a tendency to hydrolyze itself, and the specific activity of the enzyme was reduced 50% by two cycles of freezing and thawing. However, Ca^{2+} and Mg^{2+} ions appear to stabilize the enzyme, as indicated by an increase in activity following the addition of 1 to 2 mM $CaCl_2$ or $MgCl_2$ to the assay mixture (Table 3). Penicillinase release could also be enhanced by the addition of bovine serum albumin, which probably serves as a protective agent for the enzyme.

The inhibition of both proteolysis and penicillinase release after the addition of the Ca^{2+}-chelating agent ethyleneglycoltetraacetic acid is additional evidence that these ions stabilize the enzyme. High concentrations of $CaCl_2$ or $MgCl_2$ inhibited the penicillinase-releasing activity, and activity could not be restored by diluting the reaction mixture to reduce the $CaCl_2$ concentration from 20 to 2 mM.

The pH optimum for the release of penicillinase from vesicles by the PR protease (pH 7 to 9;

Fig. 7) is consistent in a general way with the characteristics of in vivo penicillinase release, which increases rapidly above pH 7 (11, 12, 14). There is a discrepancy, however, in that the isolated enzyme is still relatively active at pH 6 either in releasing penicillinase from the vesicle fraction or from protoplasts or in cleaving the purified membrane enzyme (Fig. 7), whereas the release of penicillinase from intact cells or protoplasts (presumably by the endogenous PR protease) is extremely slow (11, 12). The explanation of this differential accessibility at pH 6 remains obscure.

Since the formation of PR protease is not dependent upon the concurrent synthesis of penicillinase, its function may not be restricted to that of releasing exopenicillinase. The membrane of uninduced 749 cells contains several other proteins which appear to have covalently linked phosphatidylserine (P. S. Aiyappa and J. O. Lampen, Biochem. Biophys. Acta, in press), and it is conceivable that the PR protease plays a comparable role in the processing of these proteins. Nevertheless, the precise physiological function of the PR protease will probably be clear only when the properties of a mutant that lacks the enzyme can be examined.

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