EVIDENCE FOR THE EXTRUSION OF AN INCOMPLETELY FOLDED FORM OF PENICILLINASE DURING SECRETION BY PROTOPLASTS OF BACILLUS LICHENIFORMIS 749/C [1]

George E. Bettinger and J. O. Lampen

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

Received February 24, 1971

Summary. The cell-bound and exocellular penicillinases of B. licheniformis 749/C are resistant to trypsin and chymotrypsin. The production of penicillinase sensitive to these proteases has been detected utilizing protoplasts stripped of part of their bound enzyme. The proteases most likely act at the outer surface of the membrane, degrading the emerging polypeptide before it can assume a resistant conformation. In the absence of protease, these protoplasts incorporate nascent penicillinase in amounts approximating that previously removed.

Bacillus licheniformis 749/C produces a hydrophilic exoenzyme and membrane bound, lipophilic forms (8). The latter are convertible to the exo-form without loss of activity by trypsin (1, 2), while all three are equally resistant to degradation by chymotrypsin (2). Penicillinase secretion is relatively tightly coupled to its synthesis (7), and we have suggested that the nascent protein is extruded into the membrane from the polysome in an incompletely folded form (9).

We have examined penicillinase secretion by protoplasts under the hypothesis that if the enzyme being secreted is only partially folded, then it may be susceptible to degradation by the two proteases as it emerges.

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[1] This research was supported by Public Health Service grant AI-04572 from the National Institute of Allergy and Infectious Diseases. G. Bettinger is a predoctoral trainee under Public Health Service training grant GM-507 from the National Institute of General Medical Sciences.

We find that there does exist a protease sensitive form of penicillinase, and additionally, are able to show that protoplasts can incorporate nascent penicillinase into a bound form.

MATERIALS AND METHODS

Protoplasts of B. licheniformis 749/C, penicillinase constitutive and induced for alpha-glucosidase (3), were prepared as previously reported (6). All protoplast weights are expressed as the dry weight equivalent of initial cells. Incubations were performed at 30 C in a gyratory shaker at 250 rpm, and all media, buffers and glassware pre-warmed to 30 C. The protoplasts were sedimented at room temperature for 5 min at 12,000 x g, and incubated for 30 min at 4 mg/ml in a 0.75 M sucrose medium (7), pH 6.5, 7.5, 8.5 or 9.5, without amino acids and containing 40 μg/ml chloramphenicol (CAM). Depending on the pH of this medium, different amounts of penicillinase were removed from the protoplasts during the incubation (see Fig. 1A legend). Protoplasts stripped in this manner were washed once with a pH 7.5 buffer of 0.4 M Na-succinate, 0.001 M $MgCl_2$, 0.02 M KH_2PO_4 and resuspended in 0.4 M succinate growth medium, pH 7.5 (7). The resuspended protoplasts always showed a 30 min lag before penicillinase synthesis began, therefore zero time in all experiments was chosen as 30 min after the addition of the protoplasts to the medium.

Free and cell bound enzyme was followed as previously described (7). Samples were diluted 1:5 into ice cold 0.1 M KH_2PO_4, pH 7.0, containing per ml: 40 μg CAM and 50 μg soybean trypsin inhibitor. Alpha-glucosidase and penicillinase were assayed as previously reported (3, 5). Total protein synthesis was measured by the

incorporation of a ^{14}C-amino acid mixture (1.0 μCi/ml protoplast medium) into hot TCA precipitable material.

RESULTS

When stripped protoplasts are incubated in growth medium containing trypsin and chymotrypsin (25 μg each/ml), the net increase in penicillinase activity is strikingly reduced (Fig. 1A, 1B). Either protease alone is equally as effective in preventing the increase in penicillinase as when combined (unpublished results), and no significant

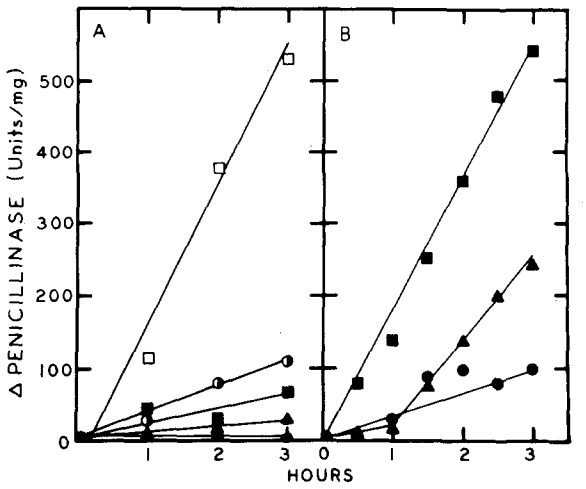

Fig. 1A. The effect of trypsin and chymotrypsin (T/C) on net penicillinase formation at different initial levels of bound enzyme. Stripped protoplasts were incubated in the 0.4 M succinate growth medium at pH 7.5 (7), using T/C added at 25 μg each/ml as noted. The pH of the stripping medium and the % penicillinase removed are indicated. □——□ 50% penicillinase removed at pH 9.5, no T/C; ◐——◐ 17% removed at pH 6.5, + T/C; ■——■ 21% removed at pH 7.5, + T/C; ▲——▲ 42% removed at pH 8.5, + T/C; and ●——● 50% removed at pH 9.5, + T/C.

Fig. 1B. Kinetics of recovery of net penicillinase formation from trypsin and chymotrypsin inhibition following the addition of soybean trypsin inhibitor (STI). Protoplasts, previously stripped of 50% of their bound penicillinase, were incubated as in Fig. 1A. ■——■ no T/C; ●——● + T/C; ▲——▲ + T/C, at 1 hr STI (275 μg/ml) added.

loss of cellular alpha-glucosidase was observed with protease incubation. The ability of the proteases to limit penicillinase increase is inversely related to the amount of penicillinase remaining on the stripped protoplast (Fig. 1A). Inhibition is between 80 - 100% on protoplasts having half the normal level of bound penicillinase.

The possibility exists that the proteases are not degrading nascent penicillinase, but rather damaging the enzyme forming system. If the penicillinase synthetic system were being altered, some recovery period might be expected upon removal of the restriction. When sufficient soybean trypsin inhibitor to bind all protease present is added to protoplasts, the net penicillinase formation of which is restricted by trypsin and chymotrypsin (Fig. 1B), penicillinase increase begins without a noticeable lag. Removal of the proteases by washing the protoplasts and resuspending them in fresh medium also removes the restriction on the penicillinase forming system after a 15 - 30 min lag (unpublished results), characteristic of protoplasts transferred to fresh growth medium (see Materials and Methods).

The effect of the proteases on total cellular protein synthesis and, more specifically, on alpha-glucosidase formation is shown in Table I. Trypsin and chymotrypsin cause a slight decrease (12 - 15%) in the rates of formation of alpha-glucosidase and total protein while causing an 85% drop in the appearance of new penicillinase activity. The two proteases do not decrease the level of internal alpha-glucosidase beyond that expected due to minimal lysis (7).

If the distribution of the nascent penicillinase formed by protoplasts stripped of 50% of their bound enzyme is followed (Table II), it can be seen that they accumulate, as bound enzyme, approximately the amount

Table I. Synthetic capacity of stripped B. licheniformis 749/C protoplasts during incubation with trypsin and chymotrypsin (T/C)[a]

	Synthesis/mg/hr	
	+T/C	-T/C
penicillinase	20 units	175 units
alpha-glucosidase	4.6 units	5.3 units
total cellular protein[b]	14,000 dpm	17,000 dpm

[a]50% of the bound penicillinase had been removed by treatment at pH 9.5. The protoplasts were incubated in 0.4 M succinate growth medium (7) pH 7.5, supplemented with 2 mg/ml maltose to maintain alpha-glucosidase formation.

[b]Measured by the incorporation of ^{14}C-amino acids into hot TCA precipitable material.

Table II. Incorporation of nascent penicillinase by stripped and by untreated protoplasts of B. licheniformis 749/C

Time (hrs)	Stripped protoplasts		Untreated protoplasts
	Exp. 1	Exp. 2	Exp. 3
	% original bound penicillinase		
0	50	56	100
1	103	75	90
2	122	90	97
3	130	95	97

Stripped and untreated protoplasts were incubated in 0.4 M succinate growth medium. The original level of bound penicillinase was approximately 400 units/mg, and both types of protoplasts formed about 500 units during the 3 hr. incubation.

of enzyme previously removed, after which there is no net retention of penicillinase. Untreated protoplasts do not incorporate any additional penicillinase (Table II, 7).

DISCUSSION

Trypsin and chymotrypsin prevent a net increase in penicillinase

activity under conditions which support near normal synthesis of cytoplasmic protein (Table I), and this constraint is inversely related to the amount of penicillinase present on the protoplast (Fig. 1A). It is unlikely that the proteases directly damage the penicillinase-forming apparatus, since there is no evidence that they are able to penetrate the membrane of intact protoplasts (Table I, 1). There is the possibility that the proteases indirectly prevent penicillinase synthesis by acting at the outer surface of the membrane, consequently altering internal relationships, and stopping penicillinase synthesis. However, the absence of a recovery period upon removal of the protease (Fig. 1B) indicates that the penicillinase forming system is operational during the incubation, and that the nascent penicillinase is degraded by the proteases.

We suggest that penicillinase is formed by membrane bound polysomes (9) and that the growing chain is extruded into the membrane in an extended form as proposed by Redman (4). It seems likely that the proteases act on the emerging penicillinase before it can assume a resistant conformation (9). An analogous partially folded protein form has been implicated in the secretion of an extracellular protease in a Sarcina sp. (Bissell, M. J., R. Tosi and L. Gorini, J. Bacteriol. 1971, in press).

It is particularly intriguing that protoplasts previously stripped of half their bound enzyme could subsequently re-incorporate approximately that amount of penicillinase which had been removed (Table II). Apparently protoplasts can retain only a fixed number of penicillinase molecules, and when these sites are saturated no net accumulation occurs. This explains why incorporation of penicillinase by protoplasts was not previously observed (7).

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