Large Exopenicillinase, Initial Extracellular Form Detected in Cultures of *Bacillus licheniformis*†

K. Izui,‡ J. B. K. Nielsen, M. P. Caulfield, and J. O. Lampen*

ABSTRACT: *Bacillus licheniformis* 749 and 749/C (constitutive for penicillinase production) produce two related hydrophilic extracellular penicillinases: exo-large, 30 500 daltons, and exo-small, 29 500 daltons. The presence of exo-large was recognized only when discontinuous sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis and immunoprecipitation of the exoenzyme from the culture supernatant began to be used. Exo-large is the sole form detectable in early to midexponential phase cultures of mutant 749/C. Exo-small appears only as the cultures approach the stationary phase. Conversion of exo-large to exo-small can be accomplished by the sterile supernatant of late exponential or early stationary phase cultures. The enzyme(s) is more active at an alkaline pH and is absent in early to midexponential phase culture supernatants. Both the large and small exoenzymes were purified and their N-terminal amino acid sequences determined. That of exo-small penicillinase agreed with the previously published sequence [Meadway, R. J. (1969) *Biochem. J. 115*, 12]. The exo-large enzyme contained an additional eight residues: Ser-Gln-Pro-Ala-Glu-Lys-Asn-Glu-exo-small. A minor form of exo-large had the same sequence except the penultimate amino acid was Glu. This agrees with the sequence obtained by Simons and co-workers [Simons, K., Sarvas, M., Garoff, H., & Helenius, A. (1978) *J. Mol. Biol. 126*, 673], who isolated a large hydrophilic enzyme as a breakdown product of the hydrophobic membrane penicillinase. The N-terminal sequence of the exo-large enzyme is not compatible with the N-terminal sequence of membrane penicillinase reported by S. Yamamoto and J. O. Lampen [(1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 1457]. The membrane enzyme currently isolated in this laboratory appears to be identical with that of Simons and co-workers. The closely related organism *Bacillus licheniformis* 6346/C also produces an exopenicillinase, different from that of *B. licheniformis* 749/C, and this too is secreted primarily as a larger form.

Bacillus licheniformis produces highly soluble exopenicillinase which is released into the medium and a hydrophobic membrane-bound form which can be converted to exopenicillinase by proteolytic cleavage (Yamamoto & Lampen, 1976a,b). Ambler & Meadway (1969) noted heterogeneity of their exopenicillinase preparations which arose from charge variations of a single form of 29 500 daltons. This species was sequenced by Meadway (1969). Now, however, the use of highly resolving discontinuous sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis and of precipitating antibody capable of rapid removal of penicillinase from the culture medium has led us to the conclusion that penicillinase first accumulates in the medium as a species eight residues longer than the penicillinase previously characterized. A similar form has recently been reported by Simons et al. (1978) as a cleavage product of membrane penicillinase and designated exo-slow on the basis of its mobility in $NaDodSO_4$ gels. We will refer to this species as exo-large and the previously reported 29 500-dalton species (Simons' exo-fast) as exo-small. The previously published sequence for membrane penicillinase (Yamamoto & Lampen, 1976c) is not consistent with the structure of the exo-large enzyme.

Materials and Methods

Exopenicillinase (β-lactamase; EC 3.5.2.6) was isolated from *B. licheniformis* 749/C (ATCC 25972) and assayed as described by Sargent (1968). Cells were grown in 30-L batches of casamino acids, 10 g/L, KH_2PO_4, 2.8 g/L, 10 mM $MgCl_2$, and 0.1% Pollock's salts (Pollock, 1965), pH 6.5, to a cell density of 1.2 mg/mL. (All cell weights are given as dry weight equivalents.) Growth was monitored with a Klett–Summerson colorimeter using a green filter (540 nm); at a cell density of 1.2 mg/mL, the absorbance was 270 Klett units. The culture, now pH 6.8, was centrifuged directly without prior adjustment to pH 9.0 as had been done by Yamamoto & Lampen (1976a) to obtain maximum release of exopenicillinase. With the exception of these changes and the addition of a final chromatographic step on DEAE-cellulose (DE-52, Whatman), the purification of exo-large was performed as described by Yamamoto & Lampen (1976a). The several penicillinase forms were separated on the final DEAE-cellulose column with a shallow gradient of 0.05–0.2 M Tris-HCl buffer, pH 7.5.

Antibodies to purified exo-small and exo-large were raised separately in male albino New Zealand rabbits injected intradermally at multiple sites with an emulsion made with Freund's complete adjuvant according to the following schedule: 1.0 mg on day 1 and 0.5 mg on days 8, 15, and 29. Blood was withdrawn on day 36. The immunoglobulin (IgG) fraction from each rabbit was purified by ammonium sulfate precipitation followed by chromatography on DEAE-cellulose and CM-cellulose as described by Palacios et al. (1972). The two antibody preparations were equally active in precipitating exo-small and exo-large; on Ouchterlony gel diffusion plates each gave lines of identity with the two enzyme forms.

Antibody precipitation was performed in 1.0% Triton X-100, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 150 mM NaCl. After 1 h at room temperature, the precipitated pellet was collected by centrifugation and washed three times in the above buffer and once in 10 mM Tris-HCl buffer, pH 8.0. Samples for gel electrophoresis were prepared by dissolving the washed pellet in the appropriate sample buffer. For $NaDodSO_4$ gels the sample was boiled 5 min.

† From the Waksman Institute of Microbiology, Rutgers University, Piscataway, New Jersey 08854. *Received July 13, 1979.* This investigation was supported by U.S. Public Health Service Grant AI-04572 and by grants from Miles Laboratories, Inc., and the Charles and Johanna Busch Memorial Fund.

‡ Present address: Department of Chemistry, Faculty of Science, Kyoto University, Kyoto, Japan. K. Izui held fellowships from the Japanese Society for the Promotion of Science and the Charles and Johanna Busch Memorial Fund.

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Three polyacrylamide gel systems used were: the nondenaturing system of Davis (1964), the continuous $NaDodSO_4$ system of Weber & Osborn (1969), and the discontinuous $NaDodSO_4$ system of Laemmli (1970). Davis and Laemmli gels were 10% polyacrylamide; Weber and Osborn gels were 7.5%. Gels were fixed 1 h in 10% trichloroacetic acid, stained 4 h in 0.1% Coomassie blue R250, destained, and dried. Penicillinase activity was eluted from unfixed gels sliced into 0.95-mm sections. Each slice was incubated in 150 μL of 50 mM Tris-HCl buffer, pH 7.5, and 0.1% Triton for 1 h at 37 °C, and enzymatic activity was then measured by the iodometric assay.

Samples (5 mg) to be sequenced were dialyzed against four changes of 0.6% acetic acid at 4 °C, lyophilized, and processed in a Beckman sequenator 890C with a 1 M Quadrol program. Phenylthiohydantoin derivatives were identified by high-pressure liquid chromatography as described by Bhown et al. (1978).

Hydrophobicity Measurement. Hydrophobicity was determined by the behavior of the enzyme on octyl-Sepharose (Pharmacia). About 6000 units of penicillinase was applied to an octyl-Sepharose column (0.5 × 5 cm) equilibrated with 0.05 M Tris-HCl buffer, pH 7.5. The column was eluted with 5 mL of the starting buffer and then with 5 mL of 0.05 M Tris and 0.3% taurodeoxycholic acid, pH 7.5. Exo-large and exo-small were not absorbed and were eluted in the starting buffer. Membrane penicillinase was eluted exclusively in the buffer containing detergent.

Results

Until about 2 years ago in our laboratory exopenicillinase was examined for purity in $NaDodSO_4$ gels according to the method of Weber & Osborn (1969). In this system protein species differing in size by a small amount (±3%) were not resolved due to spreading of the bands. When exopenicillinase samples dating from our previous investigations were first examined in the system of Laemmli (1970), we noted considerable heterogeneity where none was expected; three sharp bands ranged in apparent molecular weight from about 30000 to 32000. While not all the heterogeneity in this discontinuous system can be attributed to size alone (de Jong et al., 1978), a closer examination of the mobilities in the continuous gel system indicated that there were two major species of exopenicillinase, one of apparent molecular weight 30000, close to that expected for the known exopenicillinase, and a larger one of 31 500. A sample typical of our older preparations is shown in Figure 2, slot F. Since these species were detected in purified preparations of exopenicillinase, we examined exopenicillinase secreted in growing cultures to determine how the heterogeneity was arising.

Appearance of Exo-Large and Exo-Small in the Culture Medium. The production of fast (exo-small) and slow (exo-large) electrophoretic forms of soluble penicillinase was examined as a function of the age of the cells and of the pH of the culture medium. pH values of 7.5 and higher enhance the release of membrane-bound penicillinase and are known to favor the activity of the penicillinase-releasing protease (PR-protease) (Aiyappa et al., 1977) which is synthesized and secreted by *B. licheniformis* toward the end of exponential growth. The use of precipitating antibody allowed rapid detection and separation of the forms present at any stage of the growth curve.

Strain 749/C was grown in casamino acid medium adjusted to pH 6.5 or to pH 7.5 to a final cell density of about 1.6 mg/mL (Figure 1). At pH 6.5 only exo-large was detectable in early to midexponential phase cultures (up to 5.5 h).

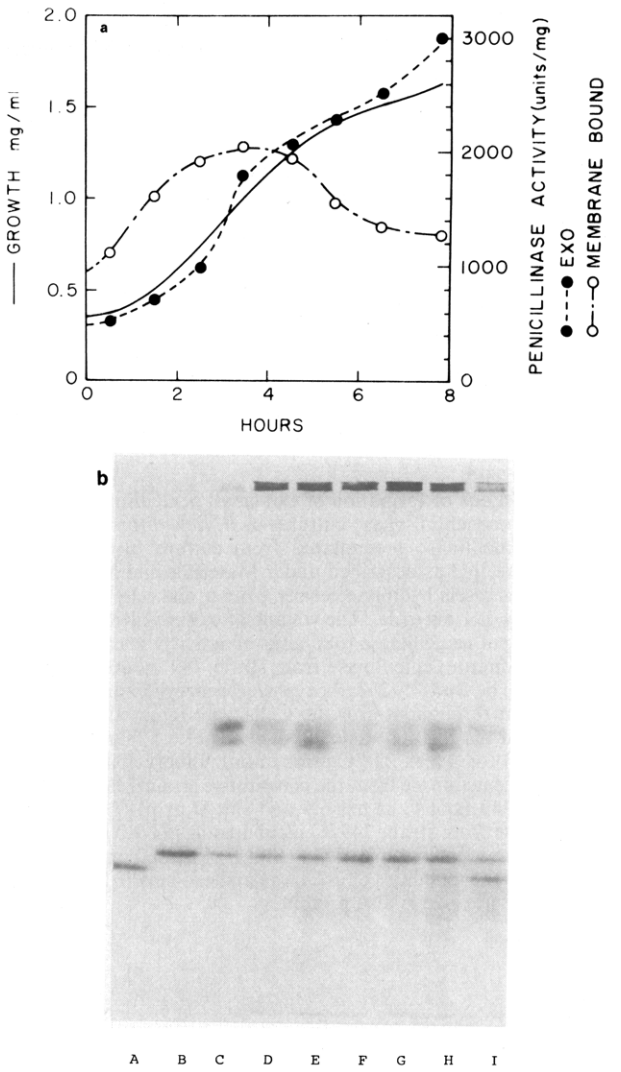

FIGURE 1: Appearance of exo-large and exo-small penicillinases as a function of culture age. Strain 749/C was grown in casein hydrolysate–salts medium (pH 6.5) as described under Materials and Methods. Figure 1a shows growth and the appearance of penicillinase in the medium and as the membrane-bound form. Figure 1b presents a Laemmli gel of exopenicillinase immunoprecipitated from the medium at various times during growth. Slot A is purified exo-small, slot B is purified exo-large, and slots C–I show exopenicillinase immunoprecipitated at t = 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, and 7.75 h, respectively.

Exo-small was first seen after 6.5 h of growth, and the relative amount increased as growth entered the stationary phase. Even in the early stationary phase (7.75 h) only about one-half of the total exopenicillinase was exo-small. In a culture buffered at pH 7.5, in which penicillinase is produced and released at a faster rate, exo-small was detected slightly earlier and represented more of the total at all times than at the lower pH (data not shown). Thus exo-large is the first major form of penicillinase to be detected when exposure to proteases is minimized by rapid removal of the secreted enzyme from the medium. Only late in the growth cycle is exo-small being secreted or produced by processing of exo-large in the medium by secreted proteases. In early log phase cultures (less than 3 h of growth), a small amount of a larger molecule precipitated by antipenicillinase IgG was sometimes detected (not seen in Figure 1). However, its extremely transient nature made it very difficult to purify, and it was not characterized any further.

Conversion of Exo-Large to Exo-Small Penicillinase. That the production of exo-small late in the growth cycle was the

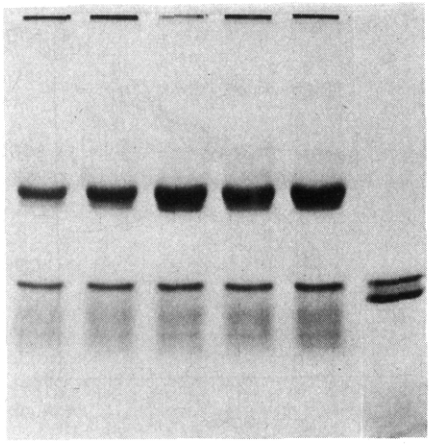

FIGURE 2: Lack of formation of exo-small penicillinase in filtrates from midexponential phase cultures of *B. licheniformis*. Laemmli gels of penicillinase precipitated from culture filtrates by anti-penicillinase IgG as described under Materials and Methods. The medium was casein hydrolysate–salts, pH 6.5, and cells were harvested at a density of 1 mg/mL. The volume of culture filtrate was varied to give 2 μg of penicillinase (680 units of activity) in each slot. Slots A and B contain penicillinase from strain 749 induced to produce penicillinase by 5 μM 2-(2′-carboxyphenyl)benzoyl-6-aminopenicillanic acid present throughout growth. Cells were removed by sterile filtration, and the filtrate was incubated at pH 6.5 (slot A) or at pH 9.0 (slot B) for 2 h at 22 °C prior to immunoprecipitation. (Slots C and D) Penicillinase from the constitutive strain 749/C treated as for strain 749 (slot C at pH 6.5 and slot D at pH 9.0). (Slot E) Penicillinase from strain 749/C incubated at pH 9.0 for 16 h at 22 °C before immunoprecipitation. (Slot F) Purified exopenicillinase to illustrate the mobilities of exo-large and exo-small. Some enzyme of intermediate size is also present.

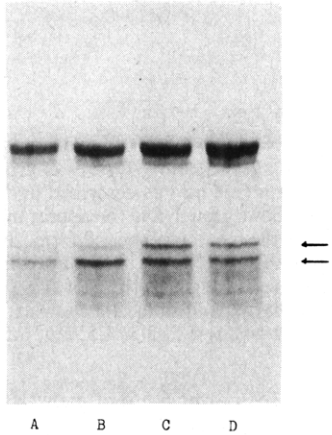

FIGURE 3: Conversion of exo-large to exo-small penicillinase in supernatants of early stationary phase cultures of strain 749/C. Laemmli gels of penicillinase forms present in supernatants incubated for 16 h at 4 °C, pH 6.5 (slot A) or pH 9.0 (slot B), or for 2 h at 22 °C, pH 6.5 (slot C) or pH 9.0 (slot D). The original early stationary phase culture supernatants contained a barely detectable level of exo-small (see slot H, Figure 1). The arrows indicate the mobilities of purified exo-large and exo-small. The immunoprecipitation at pH 6.5 is not as efficient or as reproducible as at higher pH. Thus the relative proportion of the forms and not the absolute densities should be compared between lanes.

result of proteolytic cleavage in the medium rather than secretion in that form from the cell was suggested by the experiments shown in Figures 2 and 3. Filtrates from cultures in midexponential growth (samples tested in Figure 2, slots C–E) caused no conversion of the exo-large present to smaller forms even when incubated overnight at room temperature. However, filtrates from cultures approaching the stationary phase (Figure 3) were active in causing this conversion.

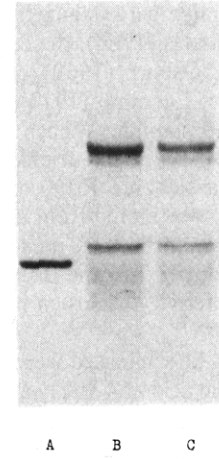

FIGURE 4: Nature of penicillinases released by protoplasts of strain 749/C. Laemmli gel of penicillinases released by protoplasts at pH 6.5 and pH 9.0 in the absence of protein synthesis. Cells from a midexponential phase culture were converted to protoplasts in 0.75 M sucrose, 0.02 M sodium phosphate, pH 6.5, and 0.001 M $MgCl_2$ medium containing 100 μg of lysozyme per mL. Protoplasts were harvested and suspended in the same medium with 40 μg of chloramphenicol per mL but no lysozyme. One portion was adjusted to pH 9.0 (slot C) and the other maintained at pH 6.5 (slot B) during incubation for 40 min at 30 °C. The protoplasts were removed and the penicillinases in the supernatants immunoprecipitated for electrophoresis. Slot A is purified exo-small.

Exo-small, barely detectable at the time of sterile filtration, constituted about 50% of the total exoenzyme after a subsequent 2-h incubation at room temperature and about 90% after storage overnight at 4 °C. The latter conditions approximate those in earlier purifications (Ambler & Meadway, 1969; Yamamoto & Lampen, 1976a), where 24 h can be required for the steps before adsorption and elution from phosphocellulose. The medium from such cultures is sufficiently active in causing this conversion to explain why the secretion of a larger species was not noted before. In the earlier work, the purity of exopenicillinase was examined only in the final stages of purification from cells harvested in high pH and high density. Moreover, mobilities were examined in Weber–Osborn and $NaDodSO_4$–urea gels which resolve proteins less sharply than Laemmli gels.

Simons et al. (1978) noted that until several years ago the principal secreted form of exopenicillinase was exo-large but that recently a change took place and exo-small became the dominant form. We have checked our lyophilized stock cultures of strain 749/C dating back as far as 1967 and find no differences among them in the pattern of secretion of exo-small and exo-large. The primary secretion product was always exo-large. This was true also in strain 749 induced to produce penicillinase by the addition of 2-(2′-carboxyphenyl)benzoyl-6-aminopenicillanic acid (Bettinger & Lampen, 1970) as can be seen in Figure 2 (slots A and B). The cultures behaved the same way in all media tested, both enriched and minimal. Furthermore, the penicillinase released from protoplasts (Figure 4, slots B and C) incubated in an osmotically supported medium in the presence of chloramphenicol was exclusively large in size. About 50% of the penicillinase released under these conditions was hydrophilic (G. E. Bettinger and J. O. Lampen, unpublished experiments) and 50% was hydrophobic, probably sloughed from membranes. Thus it is clear that the exopenicillinase released from protoplasts is exclusively exo-large even at pH 9.0.

Resolution of Exopenicillinase into Multiple Forms. Exopenicillinase was purified from the medium of cultures in midexponential growth. Conversion of exo-large to smaller

Table I: Apparent Molecular Weight of Penicillinases of *B. licheniformis* 749/C (in Daltons)[a]

	Laemmli gel	Weber–Osborn gel	from sequence
exo-small	30 500	30 000	29 503
exo-large	32 000	31 500	30 459

[a] Mobilities in Laemmli and Weber–Osborn gels. The standards used were bovine serum albumin (67 000 daltons), hen egg ovalbumin (45 000 daltons), rabbit muscle aldolase (39 500 daltons), bovine erythrocyte carbonic anhydrase (30 000 daltons), and bovine pancreatic chymotrypsinogen (25 000 daltons). The values from three runs were averaged.

forms is extremely slow at this stage of growth as was shown in Figures 1 and 2. Exopenicillinase isolated and purified by chromatography on phosphocellulose and Sephadex G-100, as described by Yamamoto & Lampen (1976a), can be resolved by DEAE-cellulose into several forms. Enzyme activity is eluted in a broad five-peaked region centered around 0.1 M Tris-HCl buffer, pH 7.5 (Figure 5). The first peak is exo-small: 30 500 daltons on Laemmli gels and 30 000 daltons on Weber–Osborn gels (Table I). This is the slowest moving exopenicillinase band migrating toward the anode on a nondenaturing (Davis) gel, a mobility consistent with its being the least negatively charged exopenicillinase species. The second peak is a mixture of three large species as characterized on Laemmli gels. The third is exo-large: 32 000 daltons on Laemmli gels and 31 500 daltons on Weber–Osborn gels. The later peaks probably represent charge variants of exo-large. Their mobility in $NaDodSO_4$ gels is the same as that of exo-large, but they migrate faster than exo-large in non-$NaDodSO_4$ gels, apparently having greater negative charge. The fifth peak appears to be the exo-large of Simons et al. (1978), i.e., X-Glu-Pro (see below) where an amide group has been lost from the Gln residue next to the N-terminal residue in the exo-large described here, the third peak in the DEAE-cellulose profile. The relative proportions of the five peaks varied from one preparation to another, but the third, exo-large, usually predominated. The longer the time taken in purifying any batch of penicillinase the more exo-small there was. All species have the same specific activity (340 units/μg of protein) and were equally reactive with antibodies made either to exo-small or exo-large and thus could not be distinguished on the basis of catalytic activity or immune reactivity.

The three peaks in this profile (Figure 5) which showed single bands on gels run under both denaturing and nondenaturing conditions were sequenced by automated Edman degradation. The first peak, exo-small on the basis of its mobility in $NaDodSO_4$ gels, had the N-terminal sequence Lys-Thr-Glu-Met–, in agreement with authentic penicillinase sequenced by Meadway (1969). The major peak, the third, had the N-terminal sequence Ser-Gln-Pro-Ala-Glu-Lys-Asn-Glu-Lys-Thr-Glu-Met-Lys-Asp–. The yields for all residues except the first, serine, were at least 90% of the expected value. Even when identification of the phenylthiohydantoin derivative by high-pressure and thin-layer chromatography was done immediately after derivatization, the yield for serine was low. As this residue is known to be labile, the complete absence of any other phenylthiohydantoin derivative in both aqueous and organic phases and the small but definite peak of serine phenylthiohydantoin identified the N-terminal residue as serine. Sequencing was carried out to 14 residues, the last 6 of which agreed completely with the N-terminal sequence of exo-small. The fifth peak has the sequence Ser-Glu-Pro-Ala–, differing from that of the major peak only by the presence of Glu in the penultimate position instead of Gln. The N-terminal residue behaved identically with that of exo-large as described above (third peak) and was identified as serine.

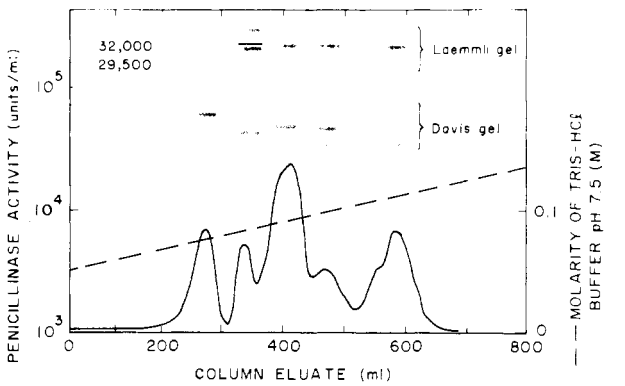

FIGURE 5: Resolution of exopenicillinase forms on DEAE-cellulose. Exopenicillinase (3.7×10^6 units) purified as under Materials and Methods was applied to a column (1.2×100 cm) of DE-52 previously equilibrated in 0.05 M Tris-HCl buffer, pH 7.5, and was eluted with a 2.0-L gradient of 0.05–0.2 M Tris-HCl buffer, pH 7.5. Mobilities of peak fractions in Laemmli and Davis gels are indicated. Migration toward the anode is downward.

Discussion

Until a few years ago *B. licheniformis* 749 and 749/C were thought to secrete a single form of penicillinase with a molecular weight of approximately 29 500 and a known sequence (Meadway, 1969). When discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis became available, a number of purified exoenzyme preparations were shown to contain larger forms (slot F, Figure 2). If the antibody to purified exopenicillinase was used to harvest the secreted enzyme from early to midexponential phase cultures, only one larger form (exo-large, apparent molecular weight 32 000) was detected; exo-small (29 500 daltons) did not appear until the cultures approached the stationary phase. Supernatants of these late exponential or early stationary phase cultures were capable of converting exo-large to exo-small, and this process was more rapid at pH 9.0 than at pH 6.5.

To facilitate isolation and characterization of the exo-large penicillinase, we attempted to minimize its conversion to exo-small. Cultures were harvested in the early to midexponential phase when the pH was still below 7.0, and the incubation at pH 9.0 before harvesting employed by Yamamoto & Lampen (1976a) was omitted. The subsequent procedures for purification of the enzyme from the culture supernatants were those of Yamamoto & Lampen (1976a). Finally, several forms present in the purified exopenicillinase preparation were separated by ion-exchange chromatography on DEAE-cellulose. Two peaks of exo-large enzyme were obtained; one (peak 3, Figure 5) was the major component and the other, more negatively charged, was a minor form (peak 5).

The purified exo-large penicillinase was sequenced, and eight residues were identified prior to the known sequence of exo-small. The N-terminal sequence was Ser-Gln-Pro-Ala-Glu-Lys-Asn-Glu-exo-small. Exo-large and exo-small appear to be equally hydrophilic as measured by chromatography on octyl-Sepharose; the preponderance of hydrophilic amino acids in the N-terminal extension is consistent with this. Furthermore, the three charged residues in the extension sequence may well explain why the apparent molecular weight of exo-large is so high in Laemmli gels (de Jong et al., 1978; Wyckoff et al., 1977).

Simons et al. (1978) have recently characterized a large hydrophilic penicillinase obtained as a breakdown product of the membrane penicillinase of strain 749/C. This hydrophilic

form, which they called exo-slow for its behavior on NaDodSO$_4$ gel electrophoresis, was reported to have eight amino acid residues attached at the N terminus of the usual exoenzyme. The sequence was X-Glu-Pro-Ala-Glu-Lys-Asn-Glu-exo-small. This sequence is identical with that of the minor form of our exo-large enzyme except that we identified Ser as the N-terminal group. Our major form of exo-large has Gln as the second residue, and we infer that the minor form arises by deamidation of the initial biosynthetic product.

In contrast to our results on the timing of the appearance of exo-large and exo-small penicillinases, Simons et al. (1978) reported that prior to 1976 exo-large was the predominant secreted form in their cultures, but now the predominant form is exo-small. It is possible that their newer cultures produced a protease that cleaved exo-large to exo-small at an earlier stage in growth than did their older cultures.

Various *B. licheniformis* isolates form one or the other of two types of penicillinase usually identified with the high-producing strains 749 and 6346. These enzymes are approximately the same size, are antigenetically cross-reactive (Pollock, 1964), and show a high degree of sequence homology (Ambler & Meadway, 1969); however, they differ in electrophoretic mobility and in substrate specificity (Pollock, 1965). The pattern of exoenzyme production by strain 6346/C was very similar to that of strain 749/C. The apparent molecular weight of the exoenzyme harvested from young cultures was 30 000 and from older cultures was 28 000 (J. B. K. Nielsen, unpublished experiments). It seems likely that there is a common mechanism for secretion of these two closely related enzymes.

We conclude from the data presented here that the initial penicillinase accumulating in the supernatant of *B. licheniformis* 749/C is a species eight residues longer than the previously described 29 500-dalton exoenzyme. One cannot necessarily conclude, however, that this is the initial species secreted. We have occasionally detected a yet larger form in very young cultures. This species might well be secreted and cleaved very rapidly to the more stable exo-large enzyme.

Several years ago Yamamoto & Lampen (1976a–c) characterized a form of the membrane penicillinase of strain 749/C which differed from the 29 500-dalton exoenzyme in that it carried an additional 24 amino acid residues that resembled a repeated tetrapeptide and had a phosphatidylserine residue as its N terminus. That structure is clearly incompatible with the eight N-terminal residues of the exo-large enzyme described here and with the analysis and properties of the membrane penicillinase recently described by Simons et al. (1978). The type of material sequenced by Yamamoto and Lampen cannot now be detected in similar cultures whether they are derived from current stock cultures or from stocks lyophilized as long ago as 1967. This is true for membrane enzyme isolated from the inducible strain 749 and from the penicillinase-constitutive mutant 749/C. The material now isolated in our laboratory appears to be essentially identical with that described by Simons' group.

We have no specific basis for doubting the original results, but we cannot identify or reproduce the type of material sequenced before 1976. Unfortunately, no samples remain for comparison with current materials. One possibility is that a tightly bound phosphopeptide was present (in nearly stoichiometric amounts) in our earlier preparations; this would be analogous to the situation with staphylococcal penicillinase (Ambler, 1979). In that instance earlier samples contained a tightly bound peptide that is not detected in current preparations. This problem remains under investigation.

Acknowledgments

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