Properties of Penicillinase from *Bacillus cereus* 569*

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**SUMMARY**

Penicillinase from *Bacillus cereus* 569 was purified and examined for homogeneity by sedimentation analysis, aminoterminal analysis, and vertical acrylamide gel electrophoresis. The enzyme preparation was shown to contain three distinct species of extracellular penicillinase which could be separated easily by gel electrophoresis. Kinetic studies failed to reveal any significant differences in the three species of enzyme. The evidence provided suggests that all three species are composed of a single polypeptide chain of approximately 31,000 molecular weight.

The amino acid composition of a mixture of the three species was determined. These data, plus additional experiments, confirmed a lack of cysteine in penicillinase isolated from *B. cereus* 569.

Optical rotatory dispersion data obtained with penicillinase indicate that possibly 30% of the amino acid residues exist in the α-helical configuration. Similar data obtained with penicillinase reveal that the majority of this α-helix is destroyed in the presence of 7.5 M urea or 5.8 M guanidine hydrochloride. These experiments, in conjunction with measurements which show at least a 500-fold reduction in enzyme activity in the presence of the denaturants, indicate that penicillinase from *B. cereus* 569 is denatured when treated with high molar concentrations of urea or guanidine hydrochloride. Removal of the denaturants results in full restoration of activity and rotatory dispersion very similar to those obtained for the native enzyme. These data, collectively, prove that penicillinase from *B. cereus* 569 can readily undergo reversible denaturation.

A wide spectrum of bacteria that are normally sensitive to penicillin can become resistant to the antibiotic (1). Careful examination has shown that this acquired resistance to penicillin is due to the synthesis of penicillinase, an enzyme that hydrolyzes the β-lactam bond of penicillin. Penicillinases from a wide variety of bacterial species have been examined physiochemi-
required to hydrolyze 1 μmole of benzylpenicillin per hour. Protein was determined by the Biuret (12), Folin (13), or absorption method (A's = 10.5 established by comparing absorption measurements with Biuret determinations). Purified phosphocelulose, suspended in 5 X 10^{-3} M Tris-HCl, pH 8.1, was used for the purification columns (0.5 X 5 cm). Sedimentation analysis of penicillinase (0.8%) was made in 0.05 M Tris, pH 7.3, which contained 0.1 NaCl.

Vertical acrylamide gel electrophoresis was performed in a standard Tris-ethylenediaminetetraacetic acid-borate buffer, pH 9.2, at a current of 150 ma for 3 to 4 hours at approximately 10^3. The gel was prepared from 14 g of Cyanogum, 0.28 ml of tetramethylethylenediamine, 0.28 g of ammonium persulfate, and 200 ml of the pH 9.2 buffer described above. For analytical work, the gels were stained with buffalo black for 40 min and destained with 0.5 ml of the pH 9.2 buffer described above. For isolation from the gel, the two outer strips of the gel were excised electrophoretically with an E-C Corporation destainer. Segments of gel between the unstained portion of the gel. These "guide strips" were then used for excision of enzyme from the unstained portion of the gel. Segments of gel between the penicillinase bands were discarded. Gel slices were homogenized in a Potter-Elvehjem tissue homogenizer and the enzyme was extracted with approximately 4 ml of 0.5 M NaCl buffered with 0.01 M phosphate, pH 7.0. For subsequent use, these extracts were dialyzed and lyophilized.

Enzyme preparations used for amino acid composition studies were purified and the protein content was determined as described above. Hydrolysis under reduced pressure for 20, 28, and 38 hours was carried out according to the method of Moore and Stein (14). Actual analysis employed a Spinco automatic amino acid analyzer. Tryptophan content of the enzyme was estimated spectrophotometrically (15).

Tryptic digestion and subsequent fingerprinting were carried out according to the methods of Helsinki and Yanofsky (16). The chromatography solvent used was sec-butyl alcohol-formic acid-water and the electrophoresis buffer system, used in the second dimension, was pyridine-acetic acid-water (pH 6.0).

The free amino-terminal residue of penicillinase was determined by the Folin degradation procedure, as modified by Koningsberg and Hill (17). Unreacted reagents were removed by repeated extraction with benzene. The phenylthiohydantoin was extracted with ethylacetate and the solvent was removed under reduced pressure. The phenylthiohydanatoin residue was taken up in absolute ethanol and again dried under reduced pressure. The mixture of the protein-terminal residue was obtained by electrophoretic analysis of the hydrolyzed phenylthiohydantoin. Hydrolysis was carried out in 5.7 N HCl at 150° for 24 hours (18) and electrophoretic separation was conducted at 3 Kvr for 100 min in a pyridine-acetate buffer, pH 3.5.

Optical rotatory dispersion measurements were made at 27° using a Cary model 60 spectropolarimeter and parallel-faced 1-mm or 1-em quartz cells. All solutions were passed through a filter upon transfer to the cell. The mean residue molecular weight, calculated from the known amino acid composition of penicillinase, is 111. Reduced mean residue rotations were calculated from experimentally observed rotation values and the dispersion constants of the solvents employed (aqueous buffer, urea, and guanidine hydrochloride) according to the following equation:

\[ [\theta]_M = \frac{aM\rho W}{I \times c} \times \frac{3}{n^2 + 2} \]

The helix content was determined by the Moffitt-Yang equation:

\[ [M]_h = \frac{aM\rho W}{I \times c} \frac{3}{n^2 + 2} \left( \lambda^2 - \lambda_0^2 \right) \]

or by the depth of the trough at 233 nm:

\[ \% \text{ helix} = \left( \frac{[M]_h + 1900}{138} \right) \]

RESULTS

Homogeneity of Penicillinase—A crystalline preparation of penicillinase from B. cereus 569 is reported to hydrolyze approximately 0.32 moles of penicillin per mg of protein per hour (20). The specific activity of the dialyzed commercial enzyme preparations used in the studies reported here, as determined by the indicator dye assay (10), was 0.12 mole of benzylpenicillin hydrolyzed per mg of protein per hour. To determine whether the lower specific activity was due to impurities or partial inactivation of the commercial enzyme, the purity of the preparation was examined by ion-exchange chromatography on phosphocelulose. The elution profiles of protein and penicillinase activity, shown in Fig. 1, indicate that although two protein peaks appear, only one peak contains penicillinase activity. The purity of the active peak was further examined by (a) sedimentation analysis, (b) vertical gel electrophoresis, and (c) amino-terminal residue analysis.

Sedimentation Analysis—Sedimentation velocity experiments employing the analytical ultracentrifuge resulted in the appearance of a single symmetrical sedimentation boundary corresponding to an s_{20,w} value of 2.78. The molecular weight of penicillinase estimated from these sedimentation data is approximately 31,500, which agrees well with previously published data (8).

Polyacrylamide Gel Electrophoresis—Homogeneity of penicillinase was also examined by vertical gel electrophoresis. As
FIG. 2. Polyacrylamide gel electrophoresis of penicillinase. Samples of penicillinase (30 μg) were added at various times to Wells 2 through 8 after the current had been started. Running times in minutes were: Well 2, 80 and 300; Well 3, 80; Well 4, 120; Well 5, 160; Well 6, 200; Well 7, 240; Well 8, 300.

Fig. 3. Re-electrophoresis of the three species of penicillinase. Time of electrophoresis was 2½ hours. Approximately 15 μg of the individual bands or 30 μg of the unfractionated enzyme were added as follows. Well 1, unfractionated penicillinase; Well 2, Band 2 plus Band 3; Well 3, Band 1 plus Band 3; Well 4, Band 1 plus Band 2; Well 5, Band 3; Well 6, Band 2; Well 7, Band 1; Well 8, unfractionated penicillinase.

### TABLE I
Amino acid composition of penicillinase

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>20 hours</th>
<th>28 hours</th>
<th>38 hours</th>
<th>Average of extrapolated values</th>
<th>Amino acid residues per nine prolines</th>
<th>Nearest whole integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.342</td>
<td>0.328</td>
<td>0.328</td>
<td>0.333</td>
<td>20.79</td>
<td>21</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.070</td>
<td>0.066</td>
<td>0.067</td>
<td>0.068</td>
<td>4.25</td>
<td>4</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.183</td>
<td>0.178</td>
<td>0.174</td>
<td>0.178</td>
<td>11.16</td>
<td>11</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.560</td>
<td>0.530</td>
<td>0.524</td>
<td>0.528</td>
<td>33.66</td>
<td>34</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.324</td>
<td>0.309</td>
<td>0.272</td>
<td>0.388</td>
<td>24.21</td>
<td>24</td>
</tr>
<tr>
<td>Serine</td>
<td>0.015</td>
<td>0.135</td>
<td>0.165</td>
<td>0.200</td>
<td>12.51</td>
<td>13</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.380</td>
<td>0.307</td>
<td>0.375</td>
<td>0.330</td>
<td>24.30</td>
<td>24</td>
</tr>
<tr>
<td>Proline</td>
<td>0.145</td>
<td>0.144</td>
<td>0.144</td>
<td>0.144</td>
<td>9.00</td>
<td>9</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.317</td>
<td>0.312</td>
<td>0.289</td>
<td>0.315</td>
<td>19.71</td>
<td>20</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.525</td>
<td>0.520</td>
<td>0.484</td>
<td>0.520</td>
<td>32.67</td>
<td>33</td>
</tr>
<tr>
<td>Valine</td>
<td>0.203</td>
<td>0.301</td>
<td>0.279</td>
<td>0.290</td>
<td>18.18</td>
<td>18</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.061</td>
<td>0.063</td>
<td>0.057</td>
<td>0.060</td>
<td>3.75</td>
<td>4</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.269</td>
<td>0.272</td>
<td>0.256</td>
<td>0.266</td>
<td>16.65</td>
<td>17</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.318</td>
<td>0.320</td>
<td>0.301</td>
<td>0.310</td>
<td>19.98</td>
<td>20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.155</td>
<td>0.152</td>
<td>0.143</td>
<td>0.170</td>
<td>10.62</td>
<td>11</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.121</td>
<td>0.124</td>
<td>0.113</td>
<td>0.122</td>
<td>7.62</td>
<td>8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.145</td>
<td>0.144</td>
<td>0.144</td>
<td>0.144</td>
<td>9.00</td>
<td>9</td>
</tr>
</tbody>
</table>

* Calculated number of amino acid residues is based on a molecular weight of 31,500 (see text). Since the frequency of proline is relatively low and the determinations of it were very reproducible, it was used as a frequency standard.

* Samples of 2 ml and 1 ml were added to the long and short columns, respectively.

* Extrapolated to zero time.

* Averages of 20 and 28 hours.

* Estimated from the extinction of the protein (see "Methods").

* Calculated molecular weight = 30,600.

shown in Fig. 2, enzyme preparations purified by the phosphocellulose column procedure can be separated readily into three protein bands by gel electrophoresis. In order to compare the intensity of the stained protein bands with penicillinase activity, the protein was eluted from the individual bands of a nonstained segment of gel and assayed for penicillinase activity. The amount of enzyme activity associated with each band was approximately proportional to the intensity of the corresponding band on the slice of stained gel. No significant enzyme activity was detected between the major bands. Furthermore, when the individual bands were eluted and subjected to electrophoresis again, only the original band appeared in each case (Fig. 3). These data indicate that the phosphocellulose-purified preparations of penicillinase are free of foreign protein and that these preparations contain more than one molecular species of penicillinase.

To determine if the origin of the multiplicity of bands could be the result of artifact production during purification procedures, the strain of *B. cereus* 569 employed for the commercial production of penicillinase was obtained and allowed to form clones. A large culture was grown from a clone and induced with 2 units per ml of penicillin G. The penicillinase from the culture was isolated by the pulverized glass procedure (9) and further purified by the phosphocellulose column procedure described under...
FIG. 4. Relative activities of the three purified species of penicillinase as a function of pH with benzylpenicillin as substrate. The buffers, composed of equal parts of citric acid, phosphate, and Tris were adjusted to the desired pH with KOH. The buffer concentration was 0.1 M for all incubations. ▲, Band 1; ○, Band 2; ●, Band 3.

FIG. 5. Peptide fingerprint of native penicillinase. Electrophoresis was in the horizontal direction and chromatography in the vertical direction. Tryptic digests (Tp) 1, 2, and 3 were observed to be absent in a similar fingerprint of pure fast band penicillinase. Tryptic digests 1 and 3 were also absent from a similar fingerprint of purified middle band.

### Table II

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative rates of hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Band 1</td>
</tr>
<tr>
<td>Benzylpenicillin</td>
<td>100</td>
</tr>
<tr>
<td>Phenoxymethyl penicillin</td>
<td>145</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>8.9</td>
</tr>
<tr>
<td>Methicillin</td>
<td>3.7</td>
</tr>
<tr>
<td>Cephalosporin C</td>
<td>0.015</td>
</tr>
</tbody>
</table>

“Methods.” The number of bands, their relative intensities, and the electrophoretic mobilities were found to be indistinguishable from the commercial preparation of the enzyme.

Amino Acid Composition—Table I shows the amino acid composition data for penicillinase isolated from *B. cereus* 569. To confirm the amino acid composition data that indicated the absence of cysteine in penicillinase, titrations with p-chloromercuribenzoate were carried out in the presence of 8 M urea (21). Addition of the compound to mercaptoethanol-treated enzyme failed to produce a net increase in the optical density of the enzyme solution at 255 mp, indicating a lack of cysteine.

Amino-terminal Residue—Since the enzyme activity studies cited below suggest that the three species of penicillinase are very similar, this analysis was performed on unfractionated enzyme. The amino-terminal residue of unfractionated penicillinase (0.125 pmole) was converted to a phenylthiohydantoin derivative by the modified Edman degradation procedure referred to under “Methods.” The ultraviolet absorption spectrum, (i.e. the ratio of absorbance 245/259 mp) and the apparent molar extinction coefficient of the isolated derivative were in close agreement with the values reported for ε-phenylisothiocarbamyl lysine phenylthiodyantoin (22). The molecular weight of penicillinase calculated from these data (26,100) is in fair agreement with that obtained from analytical ultracentrifuge data and provides evidence that the amino-terminal residue of the three species is identical. Acid hydrolysis of the phenylthiodyantoin derivative and subsequent paper electrophoresis of the hydrolysate revealed a ninhydrin-positive spot which migrated with authentic lysine (0.115 pmole treated in a similar fashion). The ultraviolet spectrum and the extinction coefficient of the authentic lysine derivative agreed well with the accepted values (22) and those obtained for the amino-terminal residue. It is concluded, therefore, that lysine is the N-terminal residue in all three species of penicillinase.

Properties of Three Species of Penicillinase—In an attempt to characterize each of the three species of enzyme and determine their relative concentrations, differences in their enzymatic activities were investigated. As shown in Fig. 4, the catalytic properties of each of the species as a function of pH differ slightly; however, these differences are not sufficient for an accurate determination of the proportionality of the three species of enzyme in a mixture.

Since most penicillinases catalyze the hydrolysis of a number of penicillin-like substrates, differences in substrate specificity among the three species of enzyme were examined. These data, presented in Table II, show that the substrate specificity of Species (Bands) 1 and 2 are very similar to each other and differ only slightly from that of Band 3. These measurements permit a comparison of the maximal velocities for the various substrates but do not reflect the affinity of an enzyme for a substrate. It is concluded, therefore, that the Michaelis constants of the three species of enzyme were determined (23). The values obtained, using benzylpenicillin as the substrate, were $4.6 \times 10^{-5}$, $4.8 \times 10^{-5}$, and $6.4 \times 10^{-5}$ M for Bands 1, 2, and 3, respectively. Thus, the relative proportionality of the three species of enzyme cannot be determined easily from these kinetic measurements.

Additional differences between the three species of penicillinase were noted when a tryptic digest of unfraccionated enzyme was compared with tryptic digests of two of the three bands. Fig. 5 shows a typical fingerprint obtained with unfracionated enzyme. Those peptides which are missing, in the case of the individual species, are noted. Multiple spot differences, in the case of a...
FIG. 6 (left). Effect of treatment with 7.5 M urea or 5.8 M guanidine hydrochloride on the electrophoretic mobility of Band 2.
The type of enzyme added to each well is as follows. Upper gel (A): Well 1, unfractionated penicillinase, 15 µg; Well 2, Band 2, untreated, 30 µg; Well 3, Band 2, urea treated, 10 µg; plus untreated, 15 µg; Well 4, Band 2, urea treated, 20 µg; Well 5, Band 2, urea treated, 10 µg; Well 6, Band 2, untreated, 15 µg; Well 7, unfractinated penicillinase, 30 µg; Well 8, unfractionated penicillinase, 50 µg. Lower gel (B): Well 1, unfractionated penicillinase, 30 µg; Well 2, unfractionated penicillinase, 30 µg; Well 3, Band 2, guanidine hydrochloride treated, 60 µg; Well 4, Band 2, untreated, 25 µg; Well 5, Band 2, guanidine hydrochloride treated, 40 µg; Well 6, Band 2, untreated, 45 µg; Well 7, Band 2, guanidine hydrochloride treated, 20 µg; Well 8, unfractionated penicillinase, 40 µg.

FIG. 7 (right). Effect of treatment with 7.5 M urea or 5.8 M guanidine hydrochloride on the electrophoretic mobility of Bands 1 and 3. The type of enzyme added to each well is as follows. Upper gel (A): Well 1, unfractionated penicillinase, 30 µg; Well 2, Band 3, untreated, 40 µg; Well 3, Band 3, urea treated, 60 µg; Well 4, Band 3, repurified, 30 µg; Well 5, Band 3, repurified, 10 µg; Well 6, Band 3, urea treated, 30 µg; Well 7, Band 3, untreated, 20 µg; Well 8, unfractionated penicillinase, 40 µg. Lower gel (B): Well 1, unfractionated penicillinase, 30 µg; Well 2, Band 1, guanidine hydrochloride treated, 25 µg; Well 3, Band 1, untreated, 25 µg; Well 4, Band 1, treated, 25 µg; Well 5, Band 3, guanidine hydrochloride treated, 30 µg; Well 6, Band 3, untreated, 30 µg; Well 7, unfractionated penicillinase, 40 µg.

single species, do not necessarily indicate multiple amino acid differences, since some spots may arise from incomplete tryptic hydrolysis. Because of the small amount of material involved, no attempt was made to investigate possible amino acid substitutions.

Reversible Denaturation—In order to determine further how the three species differed, an attempt was made to interconvert them. As shown in Figs. 6 and 7, treatment of each of the three species of enzyme with 7.5 M urea or 5.8 M guanidine hydrochloride failed to produce interconversion of the bands since each individual species regained its original electrophoretic mobility after removal of the denaturant.

To determine the effectiveness of each denaturant, enzyme activity was measured prior to, during, and after the treatment. These data, listed in Table III, show that from 80 to 100% of the original enzyme activity is regained spontaneously following
TABLE III

Restoration of enzyme activity upon removal of denaturant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of initial enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfractionated</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Plus 7.5 M urea</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Renatured from 7.5 M urea</td>
<td>80</td>
</tr>
<tr>
<td>Plus 5.8 M guanidine hydrochloride</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Renatured from 5.8 M guanidine hydrochloride</td>
<td>50</td>
</tr>
<tr>
<td>Renatured from 0.1 M NaOH</td>
<td>10</td>
</tr>
</tbody>
</table>

FIG. 8. Optical rotatory dispersion behavior of native, denatured, and renatured penicillinase. Curves are shown for native penicillinase (○); penicillinase in 5.8 M guanidine hydrochloride (△); penicillinase renatured from 5.8 M guanidine hydrochloride (□) or 7.5 M urea (■). Inset shows rotational differences between native and middle band.

TABLE IV

Percentage α helix in penicillinase

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% α helix according to procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillinase</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>31</td>
</tr>
<tr>
<td>Denatured in 7.5 M urea</td>
<td>~5</td>
</tr>
<tr>
<td>Denatured in 5.8 M guanidine hydrochloride</td>
<td>~5</td>
</tr>
<tr>
<td>Renatured from 7.5 M urea</td>
<td>31</td>
</tr>
<tr>
<td>Renatured from 5.8 M guanidine hydrochloride</td>
<td>35</td>
</tr>
</tbody>
</table>

注: 1. h<sub>α</sub><sup>100</sup> = -630, h<sub>α</sub> = 0
2. h<sub>α</sub><sup>100</sup> = -700, h<sub>α</sub> = +100

DISCUSSION

Many highly purified enzyme preparations, when subjected to gel electrophoresis, can be separated into two or more distinct components or bands. The appearance of more than one electrophoretic band may result from any of the following possibilities: (a) the enzyme preparation is contaminated by foreign enzymes or proteins; (b) the extra bands result from aggregation; (c) the composition or structure of some of the enzyme molecules has been altered during the purification procedure and thus the extra bands are artifacts; (d) the enzyme is composed of more than one type of subunit, i.e. the enzyme is a dimer, trimer, etc.; (e) the enzyme can exist in more than one stable conformation; (f) a heterogeneous population of cells is producing more than one type of enzyme; (g) a homogeneous population of cells is producing more than one type of enzyme. This may result from presence of more than one gene or from the removal of the denaturing agent. To establish that the loss of enzyme activity is due to denaturation, evidence of conformational changes was sought.

Optical rotatory dispersion measurements offer insight to gross changes in protein conformation. Thus, such measurements of (a) native penicillinase, (b) penicillinase in 7.5 M urea, (c) penicillinase in 5.8 M guanidine hydrochloride, and (d) penicillinase renatured from a solution of urea or guanidine hydrochloride were made and compared. These results are shown in Fig. 8. It will be noted that the traces obtained for enzyme renatured from urea or guanidine hydrochloride are in good agreement with that of native enzyme. However, traces obtained in the presence of either denaturant are totally different from those of the native enzyme, but are in themselves very similar. These data show that both denaturing agents alter the conformation of the enzyme greatly and to a similar extent.

Table IV lists the approximate percentage α helix present in penicillinase under the different treatments employed. The values obtained, using the Moffitt-Yang equation (24) and a λ<sub>α</sub> value of 212, indicate approximately 30% helix content for both native and renatured. On the other hand, Moffitt-Yang plots of penicillinase in the presence of either denaturant indicates almost total destruction of the helical structure (Table IV). It can also be seen (Fig. 8) that, in the case of guanidine hydrochloride-denatured enzyme, the trough at 233 μm is completely destroyed. Taken collectively, these data show that the enzyme can be reversibly denatured at least to the extent that the 30% of the amino acid residues involved in α helical structure undergo a reversible change in conformation.

During the course of this work, it was possible to compare, using the optical rotatory dispersion technique, differences in rotation between unfractionated penicillinase and enzyme isolated as the middle band from gel electrophoresis. No striking differences were noted in the wavelength region from 240 to 410 μm. It appeared that the rotational properties of the middle band, in the 240 to 320 μm region, are not quite as intense as the unfractionated material (see inset of Fig. 8).
an alteration in size of a certain percentage of the enzyme molecules.

Since purified preparations of penicillinase from \textit{B. cereus} 569 can be separated by acrylamide gel electrophoresis into three components (see Fig. 2), an explanation for the multiplicity of bands was sought. The protein eluted from each of the three bands contained penicillinase of a very high specific activity whereas only a trace of penicillinase activity was detected between the bands. Therefore, the multiplicity of bands is not the result of foreign proteins. Re-electrophoresis of the penicillinase eluted from each of the three bands revealed that the relative rates of electrophoretic migration of the three species of enzyme are properties of the individual species of penicillinase and not a result of aggregation (Fig. 3). Furthermore, repurification of the individual bands failed to produce band interconversion. Indeed, enzyme produced by a clone and purified by absorption onto and elution from pulverized glass also revealed a multiplicity of bands. Thus, the origin of the multiplicity of bands could not be traced to an artifact produced by the purification procedure.

If the enzyme were a dimer composed of two distinct and readily dissociable subunits, one might expect acrylamide gel electrophoresis to yield three bands at approximately the relative intensities observed. This possibility was excluded by isolating each individual band, recombining two of the three bands in all possible combinations, and subjecting the various mixtures to electrophoresis (Fig. 3). Only two bands were obtained for each of the three combinations and each band ran true to its original rate of migration. More convincing, the amino-terminal analysis coupled with the molecular weight determination obtained by sedimentation velocity analysis and from the literature (8) strongly suggest that penicillinase is composed of a single polypeptide chain of approximately 31,000 molecular weight. Therefore, the three bands cannot be explained by the dimer hypothesis.

The interpretation of the gel electrophoresis behavior of penicillinase is that the enzyme is composed of three distinct species. Fingerprint analysis of two of the three forms, and from the literature (8) strongly suggest that penicillinase is composed of a single polypeptide chain of approximately 31,000 molecular weight. Therefore, the three bands cannot be explained by the dimer hypothesis.

The interpretation of the gel electrophoresis behavior of penicillinase is that the enzyme is composed of three distinct species. Fingerprint analysis of two of the three forms tends to confirm this and indicates that the differences are in the primary structure of the enzyme. Moreover, the data represented in Figs. 6 and 7 and in Table III, show that penicillinase is composed of three distinct species of enzyme and that each species can be reversibly denatured. This conclusion is based on the observation that the activity of each species of enzyme is reduced 500-fold in the presence of denaturing compounds and that upon removal of the denaturant, the majority, if not all, of the enzyme activity is restored. In addition, each species of enzyme regains its original electrophoretic mobility upon renaturation. Such experiments rule out the possibility that the three electrophoretic species observed are conformational isozymes since the denaturing treatment would be expected to eliminate any activation energy barriers between the three forms (25).

Optical rotatory dispersion experiments tend to confirm the above conclusions. As seen in Fig. 8, native and renatured enzymes have very similar rotatory properties but exhibit very different properties from enzyme in the presence of denaturant. Destruction of the trough at 233 m\(\mu\), in the case of guanidine hydrochloride-treated penicillinase, further substantiates the ease for reversibility of denaturation.

Further analysis of the data from Fig. 8, using the Moffitt-Yang equation, indicated that penicillinase contains about 30\% of its amino acid residues in the \(\alpha\) helical configuration. Furthermore, in the case of native and renatured enzyme at short wave lengths (below 280 m\(\mu\)), there were serious deviations from the typical linear Moffitt-Yang relationship seen for helical proteins. Fig. 8 shows no unusual behavior which might account for such deviations. It should also be pointed out that the depth of trough at 233 m\(\mu\) is not pronounced enough to represent 30\% \(\alpha\) helix. Therefore, no positive conclusions can be reached concerning this aspect of the secondary structure of penicillinase. The fact that middle band and unfractonated enzymes have very similar optical rotatory properties tends to indicate that any structural differences between the three species do not drastically alter the secondary structure.

Recently, it has been observed (26) that in the case of penicillinase from \textit{B. licheniformis} 749/C, the enzyme can exist as four forms, two of which cannot be separated on the basis of electrophoresis. Sequence analyses of the four different forms have shown that the \(N\)-termini are either Lys-Thr-Glu- or simply Glu- while the C-termini are either --Asn--Gly--Lys-- or --Asp--Gly--Lys (27). The combination of the above variations generates the four possible forms. Similarity between the penicillinase from \textit{B. cereus} 569 and \textit{B. licheniformis} 749/C is suggested by the homology predicted on the basis of similarity of amino acid composition data (28) and by the homology found for penicillinase from \textit{S. aureus} and \textit{B. licheniformis} (26). Thus, it is likely that a similar mechanism is responsible for the three bands observed in the case of \textit{B. cereus} 569. However, contrary to the results for \textit{B. licheniformis}, we have no evidence for a "ragged" \(N\)-terminal end. Our data strongly suggest that all three forms are terminated with lysine.

REFERENCES


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