Characterization of Pesticin

SEPARATION OF ANTIBACTERIAL ACTIVITIES*

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SUMMARY

Pesticin was prepared from cell-free extracts of Yersinia pestis strain A1122 by a procedure involving fractionation with (NH₄)₂SO₄ and chromatography with Sephadex G-200, diethylaminoethylcellulose, and calcium hydroxyapatite. A single antibacterial fraction (α-pesticin) was recovered from extracts of uninduced yersiniae, whereas extracts of mitomycin C-induced cells yielded an additional active fraction (β-pesticin) at the final step of purification. Antibacterial specificity and specific activity of the two fractions were identical; their absorption spectra were typical of simple proteins and neither contained significant carbohydrate (measured as hexose or hexosamine). Both pesticides were monomers with a s₂₀,₅₀ of 4.4 and molecular weight of about 65,000 as determined by equilibrium sedimentation and gel filtration in guanidine hydrochloride. In addition, both preparations exhibited similar ratios of amino acids (both lacked detectable cysteine) and possessed common primary structure as judged by peptide analysis of tryptic digests. Isoelectric points of 5.49 and 5.87 were determined for α- and β-pesticin, respectively. Although α- and β-pesticin were interconvertible in vitro, with equilibrium favoring formation of α-pesticin, both forms became rapidly altered to a third form, termed γ-pesticin. The latter was eluted on calcium hydroxyapatite after β-pesticin and was without significant biological activity. The three forms of pesticin were antigenically identical as judged by immunodiffusion. The results suggested that pesticin, like certain cysteine-deficient colicins, can exist in conformer states.

Pesticin is a bacteriocin produced by wild type cells of Yersinia pestis which is active against serogroup I strains of Yersinia pseudotuberculosis and certain isolates of Yersinia enterocolitica and Escherichia coli (1, 2). Although the concentration of pesticin in cell-free extracts of normal yersiniae is sufficient to permit their use as a source of material for purification (3), the specific activity can be increased by induction with mitomycin C or ultraviolet light (1, 3).

Upon attempting to purify large quantities of pesticin by use of extracts of mitomycin C-induced cells, we noted the presence of a new fraction with activity, termed β-pesticin, at the final step of purification with calcium hydroxyapatite. This fraction was eluted after the activity common to uninduced and induced cells, termed α-pesticin, had been collected. Both fractions exhibited identical specific activities when tested against a variety of indicator bacteria suggesting that the two activities were not products of distinct genes. The purpose of this study was to test this hypothesis and to determine the nature of the difference between α- and β-pesticin.

EXPERIMENTAL PROCEDURE

Bacteria—Pesticin was prepared from cells of Y. pestis strain A1122 and assayed with indicator cells of Y. pseudotuberculosis strain PB7+/+ by the procedure of Hu et al. (3).

Cultivation and Induction—The medium and cultural conditions employed previously (3) were used to prepare uninduced bacteria, although in some cases the cells were grown to stationary phase at 26° in fermenter vessels (model SF 305, New Brunswick Scientific Co., New Brunswick, N. J.). Induced cells were prepared by aeration at 26° in 200 ml of medium per 2-liter flask on a shaker (model R26, New Brunswick Scientific Co.). When the population reached a density of 5 × 10⁸ cells per ml, the organisms were collected by centrifugation at 27,000 X g for 15 min and resuspended in fresh medium. After further aeration for 15 min, the cultures received mitomycin C (0.2 μg per ml) and a mixture of amino acids equivalent to 50% of that present in the synthetic medium of Yang and Brubaker (4). This amino acid component was replaced by 200 μCi of ¹⁴C-labeled algal hydrolysate (Calarome, Los Angeles, Calif.) per 200 ml of medium when radioactive ¹⁴C-labeled pesticin was induced. Aeration was continued for an additional 6 hours and the cells were harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.8), resuspended in the same buffer, and subjected to disruption as previously (3) described.

Purification of Pesticin—The process of purification was slightly modified from that used previously (6) by including a step of fractionation on Sephadex G-200 (Pharmacia). The procedure entailed dialyzing that fraction of crude extract which precipitated between 33 and 66% saturated (NH₄)₂SO₄ with 0.05 M Tris-HCl (pH 7.8) containing 1.0 M NaCl. This preparation was then applied to a column (2.0 × 40 cm) of Sephadex G-200 and eluted with the same buffer. The fraction containing pesticin was

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‡ P. C. Hu, unpublished experiments.
concentrated by precipitation with 70% saturated (NH₄)₂SO₄ and dialyzed against 0.05 M Tris-HCl (pH 7.8); the sample was then chromatographed over DEAE-cellulose and calcium hydroxylapatite as previously described.

**Ultracentrifugal Analysis**—Preparations were dialyzed for 24 hours against two changes of 500 ml of potassium phosphate at pH 7.0 containing 0.1 M KCl. The final dialysate was used for dilutions and in reference cells. Analytical sedimentations were performed in a Spinco model E ultracentrifuge equipped with a RTIC temperature control unit and either Rayleigh interference optics with 12-mm double-sectored cells or schlieren optics with 30-mm double-sectored cells. Sedimentation velocity analysis was conducted at 19.2° for undiluted and at 6.5° for induced preparations at a rotor speed of 56,100 rpm; the sedimentation coefficient was corrected to 86° (5). High speed sedimentation equilibrium analysis was performed at 10.9° at rotor speeds of 25,975 rpm and 25,000 rpm for undiluted and induced preparations, respectively. Calculations of molecular weight, according to the meniscus depletion technique (6), were based on the fringe displacement following achievement of equilibrium.

**Analytical Gel Filtration in Guanidine Hydrochloride**—Analytical gel filtration was performed in Chromaflex glass columns (1.2 X 100 cm) (Kontes Glass Co., Vineland, N. J.) containing 6% agarose equilibrated with 6 M guanidine hydrochloride and 0.05 M sodium acetate, pH 8.5 (equilibration mixture) (7). The freshly prepared columns were examined for proper packing by chromatography of 0.2% blue dextran (Pharmacia) in equilibration mixture plus 10% sucrose. Upon demonstration of suitable gel beds, the columns were treated with equilibration mixture containing 0.01 M dithiothreitol prior to application of samples.

**Polyacrylamide Disc Gel Electrophoresis**—This procedure was performed as previously described (3).

**Amino Acid Analysis**—Samples were dialyzed exhaustively against glass-distilled water for 24 hours and, following lyophilization, 0.5 mg of protein was dissolved in 1 ml of 6 N HCl in glass tubes. The tubes were sealed and placed in a drying oven at 110° for 24 hours. Following hydrolysis, portions of the sample equivalent to 24 μg of protein were analyzed for amino acids by the procedure and apparatus described by Robertson et al. (12). Corrections for deamination of aspartic and threonine were made as described by Moore and Stein (13). Trypsinogen was determined by the method of Blevin and Holdway (14).

**Peptide Analysis**—Lyophilized samples of pesticin (8.0 mg) in tubes were dissolved in 2 ml of deionized water. After denaturation at 90° in a water bath for 4 min, the preparations received 2 ml of 0.2 M NH₄HCO₃ (pH 8.0). After mixing, 15 μl of a 1:10-sulfoxamido-2-phenylethyl chloromethyl ketone-treated solution of trypsin were added (5 mg per ml of 1 M HCl) as described by Carpenter (15) plus a small drop of toluene. After incubation for 12 hours at 37°, 5 μl of trypsin solution were added and incubation was continued for another 8 hours. The preparations were centrifuged at 27,000 X g for 15 min to remove insoluble material and the supernatant fluids were transferred to 250-ml round bottom flasks. Each preparation received 2 ml of distilled water prior to shell freezing and lyophilization (which was extended for 4 hours after the samples had reached apparent dryness in order to assure removal of NH₄HCO₃). The samples were dissolved in 1 ml of deionized water, transferred to lyophilization tubes (1.3 X 10 cm), and again lyophilized; this procedure was repeated twice. The residues were dissolved in deionized water to yield a concentration of approx. 25 mg/ml.

**Carbohydrate Analysis**—Hexose was determined with anthrone reagent (16) and amino sugars by the Elson-Morgan reaction (17). A 40-μl sample of each tryptic digest was carefully applied to thin layer plates (Silica Gel 60 F-254, E. Merck, Darmstadt, Germany) and electrophoresis was performed with pyridine-acetic acid-water buffer, pH 6.5 (24:1:225), for 100 min at 900 volts. Following electrophoresis, the plates were air-dried and chromatographed in the second dimension with butanol-acetic acid-water (15:3:10:12) for 8.5 hours. After air-drying followed by exposure to 80° in an oven for 20 min, the cooled plates were sprayed with 0.4% ninhydrin in acetone and heated at 110° for 15 min.

**Immunodiffusion**—Samples of pesticin were diffused in gel against a whole serum to Y. pseudotuberculosis as previously described (5).

**RESULTS**

**Purification**—Pesticin from uninduced and induced cells was purified as described under "Experimental Procedure." Preparations of uninduced cells yielded a single antibacterial fraction during the final step of chromatography on calcium hydroxylapatite. However, a second fraction of activity was observed when preparations of induced cells were carried through the same process of purification (Fig. 1). The first fraction, termed α-pesticin, was evidently common to uninduced and induced organisms, whereas only induced cells expressed significant concentrations of the second fraction, termed β-pesticin. The specific activities of both preparations against indicator cells of Y. pseudotuberculosis were 18,000 units per mg of protein.
acrylamide gels. Similarly, single symmetrical schlieren patterns were obtained during high speed velocity sedimentation. Plots of fringe displacement versus the square of the radius of rotation during high speed equilibrium centrifugation were linear except for slight upward curvature with \( \beta \)-pesticin due, presumably, to the presence of a minor contaminant of low molecular weight (Fig. 2). The preparations exhibited single bands of precipitate when diffused in gels against a complete antiserum (see below).

**Stability**—Preparations of this degree of purity were stable for no longer than a month in 0.15 M sodium phosphate, pH 7.0, at \(-20^\circ\). Longer storage resulted in appearance of an additional band in polyacrylamide gels which was probably identical with the \( \gamma \) form (see below).

**Spectra and Composition**—Absorption spectra of \( \alpha \)- and \( \beta \)-pesticin were typical of simple proteins. At pH 7.0, maximum absorption for both preparations occurred at 278 nm and the absorption ratio (280:260 nm) was 1.92 and 1.94 for the \( \alpha \) and \( \beta \) forms, respectively. The preparations contained approximately 1% carbohydrate (as hexose) and lacked detectable hexosamine.

**Sedimentation Coefficients**—Using an averaged partial specific volume of 0.74 cm\(^3\)/per g calculated from the amino acid composition (see below), an \( s_{20,\text{w}} \) of 4.32, 4.36, and 4.37 was calculated for \( \alpha \)-pesticin (uninduced), \( \alpha \)-pesticin (induced), and \( \beta \)-pesticin (induced).

**Molecular Weights**—Molecular weights determined from sedimentation coefficients approximated 62,500. Fringe displacements obtained during sedimentation to equilibrium were plotted against the square of the distance of rotation (Fig. 2). Weight-average values calculated from these determinations were 66,000, 65,000, and 66,000 for \( \alpha \)-pesticin (uninduced), \( \alpha \)-pesticin (induced), and \( \beta \)-pesticin (induced), respectively. A molecular weight of 63,000 was determined for both \( \alpha \) and \( \beta \)-pesticin by gel filtration in guanidine hydrochloride.

**Subunit Composition**—\( \alpha \)-Pesticin migrated in sodium dodecyl sulfate polyacrylamide gel as a single band at a rate indistinguishable from that of the \( \alpha \) subunit of \( P. \) putida RNA polymerase (mol wt 44,000). In a separate experiment, both \( \alpha \)- and \( \beta \)-pesticin migrated as a single band at rates identical with that of ovalbumin (mol wt 45,000). The parameters described above are summarized in Table 1.

**Amino Acid Analysis**—Results obtained from analyses of \( \alpha \) and \( \beta \)-pesticin are shown in Table II. The preparations ex-

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**Table I**

<table>
<thead>
<tr>
<th>Molecular weight of pesticin</th>
<th>Pesticin</th>
<th>Uninduced pesticin</th>
<th>Induced pesticin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated by equilibrium sedimentation</td>
<td>66,000</td>
<td>65,000</td>
<td>66,000</td>
</tr>
<tr>
<td>Dissociated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated by gel filtration in 6 M guanidine hydrochloride</td>
<td>—(a) 63,000</td>
<td>63,000</td>
<td></td>
</tr>
<tr>
<td>Estimated by sodium dodecyl sulfate acrylamide gel filtration</td>
<td>44,000</td>
<td>44,000</td>
<td>44,000</td>
</tr>
</tbody>
</table>

* Not determined.

**Table II**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Uninduced pesticin</th>
<th>Induced by miytomycin C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>125.6</td>
<td>136.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>47.6</td>
<td>43.2</td>
</tr>
<tr>
<td>Serine</td>
<td>69.2</td>
<td>69.2</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>62.4</td>
<td>80.8</td>
</tr>
<tr>
<td>Proline</td>
<td>33.0</td>
<td>21.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>55.6</td>
<td>70.0</td>
</tr>
<tr>
<td>Alanine</td>
<td>30.2</td>
<td>40.8</td>
</tr>
<tr>
<td>Valine</td>
<td>51.2</td>
<td>55.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>14.8</td>
<td>18.6</td>
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<tr>
<td>Isoleucine</td>
<td>50.0</td>
<td>55.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>64.4</td>
<td>71.6</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>29.6</td>
<td>33.1</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>42.0</td>
<td>43.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>51.0</td>
<td>52.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>13.2</td>
<td>13.8</td>
</tr>
<tr>
<td>Arginine</td>
<td>40.0</td>
<td>48.8</td>
</tr>
<tr>
<td>Tryptophan(a)</td>
<td>3.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Determined by alkaline spectrophotometry (14).
Peptide Analysis—No distinction in peptide composition was detected between preparations of α- and β-pesticin (Figs. 3 and 4). At least 38 common peptides were present in tryptic digests.

Isoelectric Focusing—The isoelectric points of α- and β-pesticin were 5.49 and 5.87, respectively, as judged from results obtained from individual determinations. The activities were clearly separated when a mixture of the two pesticins was examined.

Interconversion in Vitro. A mixture of α- and β-pesticin was mixed, as carrier, with homogeneous 14C-labeled α-pesticin or 14C-labeled β-pesticin. As shown by rechromatography after incubation at 26° for 1, 3, and 7 days, the conversion of β-pesticin to the α form occurred more rapidly than did the reverse (Table III). However, the majority of radioactivity was converted to a new form, termed γ-pesticin, which appeared as a distinct fraction on calcium hydroxyapatite eluting immediately after β-pesticin. This third fraction arose with greater facility from β-pesticin than from α-pesticin and was devoid of antibacterial activity.

Immunodiffusion—Homogenous preparations of α-, β-, and γ-pesticin exhibited a reaction of identity when diffused against antiserum in gel (Fig. 5).

**DISCUSSION**

We previously reported a simple procedure which permitted recovery of high yields of homogenous pesticin from extracts of normal yersiniae (3). Use of a minor modification of this method

with extracts of mitomycin C-induced cells resulted in detection of a new fraction of activity (β-pesticin) which remained associated with the apparent constitutive fraction (α-pesticin) until the final step of purification on calcium hydroxyapatite. These two fractions differed in isoelectric point but exhibited similar or identical antibacterial spectra, specific activities, sedimentation coefficients, subunit structure, molecular weight, amino acid ratios, peptide composition, and antigenic properties. In addition, the two molecules were interconvertible in vitro upon prolonged incubation at 26° although considerable activity was lost in the form of a third form which was biologically inactive (γ-pesticin).

These observations suggest that the difference between α- and β-pesticin does not involve differences in primary structure, subunit aggregation, or epigenetic modification. The two activities possibly reflect alternative configurations or "conformer" states (18) of the same protein. A similar phenomenon has been described for other proteins (18-20) including colicin E₆ (21) and E₅ (22). The latter, like pesticin, are deficient in cysteine and thus unable to form disulfide bonds which can stabilize tertiary structure. This amino acid is present in colicin D (23), but not colicins Ia or Ib (24); none of these colicins have been reported to exist as conformers.

**TABLE III**

**Interconversion of pesticins**

<table>
<thead>
<tr>
<th>Time of incubation at 26°</th>
<th>Recovered radioactivity from 14C-labeled α-pesticin</th>
<th>Ratio of α:β:γ</th>
<th>Recovered radioactivity from 14C-labeled β-pesticin</th>
<th>Ratio of α:β:γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>days</td>
<td>cpm</td>
<td>α-pesticin</td>
<td>β-pesticin</td>
<td>γ-pesticin</td>
</tr>
<tr>
<td>0</td>
<td>15,000</td>
<td>0</td>
<td>100:0:0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12,300</td>
<td>100</td>
<td>99:1:0</td>
<td>350</td>
</tr>
<tr>
<td>3</td>
<td>11,700</td>
<td>350</td>
<td>4,000</td>
<td>74:2:24</td>
</tr>
<tr>
<td>7</td>
<td>7,000</td>
<td>450</td>
<td>4,350</td>
<td>60:4:36</td>
</tr>
</tbody>
</table>

* Not detectable by ultraviolet monitoring.
Although results obtained by guanidine hydrochloride gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis clearly showed that pesticin consists of a single polypeptide chain, the molecular weight determined by the latter method was significantly lower than that obtained by other procedures. Electrophoresis in sodium dodecyl sulfate gels may yield erroneous results for proteins of low molecular weight (25). For example, the molecular weight of the subunit of staphylococcal lactose-specific phosphocarrier protein was 11,600 when determined by gel filtration in 6 M guanidine hydrochloride and 9,200 when assayed by gel electrophoresis in 1% sodium dodecyl sulfate (26). The behavior of pesticin (mol wt 65,000) during electrophoresis in sodium dodecyl sulfate is unusual and suggests that portions of the molecule remain stabilized in this detergent.

Alteration of ionic strength and pH can influence the equilibrium of conformers (18, 19) and further study of these variables may result in defining conditions which prevent accumulation of γ-pesticin or favor maintenance of β-pesticin. It is now recognized that the minor fraction of activity detected following chromatography of crude extracts on calcium hydroxyapatite was β-pesticin (3). This fraction is lost, possibly due to conversion to γ- and α-pesticin, when such extracts are subjected to the complete process of purification.

REFERENCES

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