Purification and Characterization of Canine Pepsinogen*

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SUMMARY

Pepsinogen has been purified from the fundic mucosa of the dog. The preparation is homogeneous by chromatography, amino terminal analysis, ultracentrifugation, electrophoresis on cellulose acetate strips, and disc electrophoresis.

Analysis indicates the presence of 352 amino acid residues, at least 12 moles of carbohydrate including some amino sugars, and 1 mole of organic phosphate per mole of protein. The amino terminus is Ala-Ile-. Canine, porcine, and bovine pepsinogens are similar in size, in the large number of acidic and small number of basic residues, and in the number of aromatic residues and cysteine. The main differences are in the number of hydroxy amino acids and methionines; canine pepsinogen with 8 methionine residues is unlike the other mammalian zymogens, but resembles dogfish and chicken pepsinogens.

The molecular weight of canine pepsinogen is 39,900 by amino acid and carbohydrate content and 41,667 by ultracentrifugation; the sedimentation coefficient, s_{20,w}, is 3.38 S and the diffusion coefficient, D_{20,w}, is 7.37 \times 10^{-7} \text{ cm}^2/\text{sec}.

The pepsins are unusual enzymes, characterized by the extremely low pH of their isoelectric points and of their enzymatic activity. The present paper describes the purification to homogeneity and some properties of one of the canine pepsinogens.

EXPERIMENTAL PROCEDURE

Materials—Porcine pepsin (twice crystallized, Lot PM695) and porcine pepsinogen (crystalline, Lot PG 0HA) were obtained from Worthington. Dovine hemoglobin (twice crystallized) was purchased from Pentex. Sephadex G-100 was a product of Pharmacia. DEAE-cellulose was No. 71, type 20, from Schleicher and Schuell, Keene, New Hampshire. Deionized distilled water was used throughout. Chemicals were reagent grade.

Pepsin Assay—Potential pepsic activity of the pepsinogen was routinely determined by a modification (5) of the hemoglobin method of Anson (10). Potential specific activity of the canine pepsinogen was expressed as microgram equivalents of porcine pepsin per A_{280} unit of the canine pepsinogen solution. Milk-clotting activity (5, 11) was used to test for active pepsin in the pepsinogen solution. A solution was considered to be free of active pepsin if it did not clot in 2 min when it contained at least 25 \mu g of pepsinogen.

Analytical Methods—Amino acid analysis was carried out on a Beckman-Spinco model 120B analyzer, essentially as described by Moore and Stein (12); the details were presented in a previous paper (5). Cystine was determined as cysteic acid (13) and tryptophan was measured spectrophotometrically in guanidine hydrochloride (14). The Edman procedure (15) was used for sequential degradation of the zymogen. The amino terminal group at each step was measured as the PTI derivative (15) and identified by thin layer chromatography on silica gel plates (16, 17). The dansylation procedure as described by Gray and Hartley (18) was also used for amino terminal analysis of the zymogen. The amino terminal group was identified by thin layer chromatography in Systems I and II of Gros and Labouesse (19). Total carbohydrate was determined by the phenol-sulfuric acid method (20) after deamination as described by Lee and Montgomery (21); the standard was an equimolar mixture of glucose and galactosamine. Carbohydrate content, exclusive of amino sugars, was determined directly by the phenol-sulfuric acid method (20) with a glucose standard. The presence of amino sugars was established by amino acid analysis (22). Moisture content was determined as described by Chow and Kassell (5). The ashing of the

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2 We are grateful to Aldridge Associates and Company, Inc., Washington, D. C., for a gift of highly purified guanidine hydrochloride.

3 The following abbreviations have been used: PTH-, phenylthiohydantoain, DNS-, or dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.
protein for total phosphate determination was carried out as described by Meitner and Kassell (23); the method of Ames (24) was used for the colorimetric evaluation. Cellulose acetate strip electrophoresis of the purified protein was carried out in pyridine-acetic acid buffer, pH 6.4, with a Shandon electrophoresis apparatus, model U77, Shandon Scientific Company, Sewickley, Pennsylvania. Standard procedures were followed. Disc electrophoresis of the purified protein was carried out with a Canaleo instrument, Canal Industrial Corporation, Rockville, Maryland. The sample was applied as a 2-mm sucrose stack, pH 6.9 (imidazole-HCl buffer), on a separating gel, pH 8.3 (imidazole-HCl buffer). Stacking was effected by the difference in pH. Molecular weight was determined with a model E analytical ultracentrifuge (Beckman Instruments, Spinco Division, Palo Alto, California) by sedimentation and diffusion (25). Conductivity of the chromatographic effluents was measured on a conductivity bridge, model 31, from Yellow Springs Instrument Company, Yellow Springs, Ohio.

RESULTS

Purification of Fundic Pepsinogen

Immediately after the death of the mongrel dogs, their stomachs were removed and washed thoroughly to prevent activation of the zymogen by the acidic gastric juice. The fundic and pyloric portions were separated and frozen until used. The fundic portions were partially thawed; the mucosae were separated, trimmed, and ground. All subsequent operations were carried out at 5°C.

Step 1: Extraction—The ground mucosae (540 g per nine stomachs) were extracted with 1100 ml of 0.1 M sodium phosphate buffer, pH 7.0, for 30 min. Celite 545, 150 g, was added, and the mixture was centrifuged for 1 hour at 13,000 × g in a Sorvall refrigerated centrifuge. The sediment was resuspended in 1100 ml of buffer and centrifuged again. The combined supernatant solutions were filtered through cloth on a Buchner funnel.

Step 2: Ammonium Sulfate Fractionation—Solid (NH₄)₂SO₄ was added to 20% saturation, with NaOH introduced as required to keep the pH close to 7.0. Celite 545, 150 g, was added, and the mixture was centrifuged for 1 hour at 13,000 × g in a Sorvall refrigerated centrifuge. The sediment was resuspended in 1100 ml of buffer and centrifuged again. The combined supernatant solutions were filtered through cloth on a Buchner funnel.

Step 3: First Batch Absorption on DEAE-cellulose—The (NH₄)₂SO₄ cake was suspended in 350 ml of 0.01 M sodium phosphate buffer, pH 7.0, and dialyzed against two changes of the same buffer. The suspension was centrifuged for 3 hours at 13,000 × g in a Sorvall refrigerated centrifuge. The Celite that sedimented was resuspended in another 350 ml of buffer and centrifuged again. The combined supernatant solutions were centrifuged at 36,000 × g for 3 hours in a Spinco L-2 ultracentrifuge. The sediment was washed with 1100 ml of buffer and centrifuged again. The combined supernatant solutions were filtered through Schleicher and Schuell No. 588 folded paper.

The crude pepsinogen solution, equivalent to 16,100 A₂₅₀ units and containing 1.9 g of pepsinogen, was diluted to 5.5 liters with 0.05 M Tris-phosphate buffer, pH 7.0, and mixed with 1.5 liters of settled DEAE-cellulose, previously regenerated and then equilibrated with the same buffer. The mixture was stirred gently for 1 hour and filtered through cloth on a Buchner funnel. The cake was washed with 2 liters of the same buffer. The moist cake was resuspended in fresh 0.05 M buffer and transferred to a chromatographic column (7 × 60 cm). The column was washed with the same buffer (about 1 liter) until the absorbance of the effluent at 280 nm fell to about 0.1. The pepsinogen was then eluted in a single peak with 0.6 M NaCl in 0.05 M Tris-phosphate buffer, pH 7.0.

The fractions containing potential peptic activity greater than 100 µg per A₂₅₀ unit were pooled (700 ml) and concentrated to a final volume of 250 ml in a Dia-Flo model 400 ultrafiltration cell, Amicon Corporation, Cambridge, Massachusetts. The solution was desalted in the ultrafilter by repeated concentration and dilution of the sample. The solution was stirred very slowly during ultrafiltration to avoid denaturation.

Step 4: Second Batch Absorption on DEAE-cellulose, with Gradient Elution—The solution, containing 8170 A₂₅₀ units and 1150 mg of pepsinogen, was diluted to 3 liters with 0.05 M Tris-phosphate buffer, pH 7.0, mixed with 1100 ml of settled DEAE-cellulose, and stirred for 1 hour. The total volume was made up to 5 liters. The suspension was filtered through cloth on a Buchner funnel. The cake was resuspended in 1200 ml of the same buffer and transferred to a column (5 × 60 cm) containing a 10-cm packed bed of fresh DEAE-cellulose. The column was washed with 4 liters of the same buffer; the absorbance of the effluent at 280 nm fell to about 0.02. The pepsinogen was eluted by a gradient of NaCl up to 0.5 M. The elution profile (Fig. 1) shows that potential activity against hemoglobin is present in more than one peak. Fractions from Peak C with potential peptic activity greater than 175 µg per A₂₅₀ unit were pooled (Table 1). The pooled fractions were desalted by ultrafiltration and concentrated to 55 ml. Other peaks with potential activity were stored for future investigation.

Step 5: Gel Filtration on Sephadex G-100—The sample, containing 1600 A₂₅₀ units and 370 mg of pepsinogen, was introduced, in
Table I

Purification steps and yield of canine pepsinogen

<table>
<thead>
<tr>
<th>Step</th>
<th>Total pepsinogen recovered at each step</th>
<th>Specific potential activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>1. Extraction</td>
<td>2700</td>
<td></td>
</tr>
<tr>
<td>2. Ammonium sulfate fractionation:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 20% filtrate</td>
<td>2420</td>
<td>87</td>
</tr>
<tr>
<td>(b) 45% cake</td>
<td>1030</td>
<td>70</td>
</tr>
<tr>
<td>3. First batch absorption on DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1150</td>
<td>41</td>
</tr>
<tr>
<td>4. Second batch absorption on DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>370</td>
<td>13</td>
</tr>
<tr>
<td>5. Gel filtration on Sephadex G-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>324</td>
<td>12</td>
</tr>
<tr>
<td>6. Chromatography on DEAE-cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>236</td>
<td>8.5</td>
</tr>
<tr>
<td>7. Gel filtration on Sephadex G-100</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>193</td>
<td>7</td>
</tr>
</tbody>
</table>

* Recovery at each step is based only on the center portion of the peak actually pooled. The yield could be improved by combining and rechromatographing the edges of the peaks from several runs.

Fig. 2. First gel filtration on Sephadex G-100 (see the text, Step 5). The column was 5 x 105 cm, the sample was 370 mg in 61 ml, and the buffer was 0.05 M Tris-phosphate, pH 7.0. The upward flow rate was 22 ml per hour at the start; then it was increased and maintained at 36 ml per hour. ---, A280; •—•—•-••, specific activity; the fractions pooled for further purification are indicated above the curve.

Fig. 3. Chromatography on DEAE-cellulose (see the text, Step 6). The column was 2.8 x 52 cm; the sample was 524 mg. The first open bottle contained 1600 ml of equilibrating buffer, pH 7.0. The gradient solution bottle contained 600 ml of 0.6 M NaCl in the same buffer. The diameter of the first open bottle was 2.3 times that of the gradient solution bottle. The flow rate was 40 ml per hour. ---, A280; •—•—•, specific activity; ----, conductivity.

Fig. 4. Second gel filtration on Sephadex G-100 (see the text, Step 7). The sample was 230 mg in 50 ml. The conditions were the same as in Fig. 2.

The pooled fractions (139 ml) were placed on a column (2.8 x 52 cm) of DEAE-cellulose equilibrated with 0.05 M Tris-phosphate buffer, pH 7.0. A concave gradient from 0 to 0.6 M NaCl in the buffer was used to elute the pepsinogen. Gradient bottles of two different sizes were used to develop the gradient, as suggested by Bock and Ling (26). The elution profile is shown in Fig. 3. Fractions with potential peptic activity greater than 480 µg per A280 unit were pooled; the solution was desalted by ultrafiltration and concentrated to 50 ml.

Step 7: Gel Filtration on Sephadex G-100—A sample of 50 ml, containing 450 A280 units and 236 mg of pepsinogen, was passed through a Sephadex G-100 column as in Step 5. A single peak was obtained (Fig. 4). Fractions with potential peptic activity greater than 480 µg per A280 unit were pooled.

The pooled fractions were dialyzed against two 6-liter changes of 0.01 M sodium acetate, pH 7.0, and finally against two 6-liter changes of water adjusted to pH 7.0. After dialysis, the sample was lyophilized. No active pepsin was detectable.

Yield—Table I summarizes the yields and specific potential activities during purification.

Properties of Canine Pepsinogen

Amino Acid Composition—The complete amino acid composition of the canine zymogen is shown in Table II. For the readily hydrolyzed, stable amino acids there was very good agreement...
among the 24-, 48-, and 72-hour hydrolysates. On the chromatogram of the amino acid analysis, it was difficult to determine the base line for lysine because of the appearance of galactosamine immediately before the lysine peak. It is quite possible that the lysine peak included a trace of material other than the amino acid. This explains the 5% deviation of the lysine value from a whole number (6.3 instead of 6.0).

The absorbance of the purified pepsinogen at 280 nm is 1.279 for 1 mg of protein per ml.

**Carbohydrate Content of Zymogen**—The total carbohydrate determined by the phenol-sulfuric acid method (20) after deamination (21) accounts for 12 carbohydrate eq per mole of protein (Table II). Of these 12 residues, approximately 4 residues are non-amino sugars. This estimation of the carbohydrate is only an approximation, since the nature of much of the carbohydrate is not known. An attempt was made to decrease the carbohydrate content by precipitation of the zymogen five times by 70% saturation with ammonium sulfate, pH 7, as described by Portmann and Freseol (27). No decrease was observed, suggesting that the sugar is covalently bound. Pepsinogens from the pig (28), chicken (29), and cow (30) also contain covalently bound carbohydrate. Further studies on the carbohydrate will be the subject of a future investigation.

**Organic Phosphate Content**—After extensive dialysis (Step 7) the protein was ashed to determine total phosphate. Samples were assayed for the presence of any residual inorganic phosphate in the same manner as for total phosphate except for the ashing step, and appropriate small corrections (0.15 μmole per μmole of protein) were made. The recovery of organic phosphate was determined in the presence of protein; when serine-O-phosphate was added to phosphate-free trypsin, the recovery of the phosphate was 82% (6). This correction factor was used in the calculation of organic phosphate. The corrected organic phosphate content of the canine pepsinogen is 0.89 mole per mole of protein. Porcine pepsin, analyzed as a control and also corrected for the recovery of a future investigation, yielded PTH-alanine, identified by thin layer chromatography in the molecular weight calculated by amino acid and carbohydrate calculated by these methods is 41,667, which is in agreement with 0.94% and 0.25%. The sedimentation pattern (Fig. 5) yielded a peak in the position of glycine, thus explaining this result. We can conclude from these combined results that the pepsinogen has amino terminal Ala–Ile–.

**Homogeneity**—Five criteria of purity were used. (a) At the final step of purification (Fig. 4), the potential peptic activity was uniformly distributed across the peak (see "Stability," below). (b) Only one amino terminal residue was detected by both the dansyl and the Edman methods. (c) There was a single symmetrical peak in the ultracentrifuge (Fig. 5). (d) Cellulose acetate strip electrophoresis at initial concentrations of 0.05 M Tris-phosphate buffer, pH 7.0, lost some of their potential activity when stored for 24 hours at 0°. The loss is concentration-dependent; at 170 μg per ml there was a 3% loss in potential activity, while at 17 μg per ml a 15% loss occurred in 24 hours. The poor stability of the dilute solutions accounts for the low activity on the edges of the peak in Fig. 4.

<table>
<thead>
<tr>
<th>Residue</th>
<th>Amino acid residues</th>
<th>Residues per molecule to nearest integer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>6.33 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.91 ± 0.07</td>
<td>7</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.00 ± 0.05</td>
<td>6</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>38.72 ± 0.26</td>
<td>39</td>
</tr>
<tr>
<td>Threonine</td>
<td>19.82 ± 0.11</td>
<td>20</td>
</tr>
<tr>
<td>Serine</td>
<td>38.19 ± 0.11</td>
<td>38</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>33.35 ± 0.25</td>
<td>33</td>
</tr>
<tr>
<td>Proline</td>
<td>21.59 ± 0.38</td>
<td>22</td>
</tr>
<tr>
<td>Glycine</td>
<td>35.22 ± 0.48</td>
<td>35</td>
</tr>
<tr>
<td>Alanine</td>
<td>23.32 ± 0.02</td>
<td>23</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>5.95 ± 0.02</td>
<td>6</td>
</tr>
<tr>
<td>Valine</td>
<td>25.62 ± 0.02</td>
<td>25</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.70 ± 0.04</td>
<td>8</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>24.5 ± 0.0</td>
<td>25</td>
</tr>
<tr>
<td>Leucine</td>
<td>25.11 ± 0.24</td>
<td>25</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>17.62 ± 0.14</td>
<td>18</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>15.22 ± 0.05</td>
<td>15</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>4.65 ± 0.25</td>
<td>5</td>
</tr>
<tr>
<td>NH3</td>
<td>15.17 ± 0.53</td>
<td>(21)</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>38.72 ± 0.26</td>
<td>39</td>
</tr>
<tr>
<td>Histidine</td>
<td>7.91 ± 0.07</td>
<td>7</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.00 ± 0.05</td>
<td>6</td>
</tr>
</tbody>
</table>

* Arginine was taken as 6 residues to calculate the residues of amino acids per mole. The short column values are based on single 24-, 48-, and 72-hour hydrolysates. The long column values are based on duplicate hydrolysates for each time.

**Stability**—Pepsinogen solutions in 0.05 M Tris-phosphate buffer, pH 7.0, lose some of their potential activity when stored for 24 hours at 0°. The loss is concentration-dependent; at 170 μg per ml there was a 3% loss in potential activity, while at 17 μg per ml a 15% loss occurred in 24 hours. The poor stability of the dilute solutions accounts for the low activity on the edges of the peak in Fig. 4.
4 (bovine and porcine) methionine residues, 14 instead of 16 (bovine) or 17 (porcine) basic residues, and 58 instead of 77 (bovine) instead of 15 (bovine) or 19 (porcine) proline residues, 8 instead of number of hydroxy amino acids. Canine pepsinogen has 22 in and methionine, in the number of basic amino acids, and in the number of glycine, alanine, valine, isoleucine, and leucine residues in all three zymogens; the canine zymogen has 134, bovine has 133, and porcine has 135. Notable differences do occur in proline and methionine, in the number of basic amino acids, and in the number of hydroxy amino acids. Canine pepsinogen with 8 residues is unlike the other mammalian zymogens, but resembles dogfish pepsinogen with 6 to 7 residues (34) and chicken pepsinogen with 9 to 10 residues (29, 35).

In agreement with the occurrence of several pepsinogens in the porcine (36), human (11), dogfish (34), and chicken (35) gastric mucosa, the synthesis of several pepsinogens in the canine mucosa appears likely. Figs. 1 and 6 show the clear separation of the canine pepsinogens during DEAE-cellulose chromatography. This is in contrast to the bovine pepsinogens, which separated only partially on DEAE-cellulose and which proved to be the same protein with different amounts of organic phosphate (23).

The variations in the relative amounts of the potentially active peaks, which is of physiological interest, requires further work, since pooled stomachs from several mongrel dogs were used. There is, however, enough pepsinogen in a single stomach, so that isolations could be made under controlled conditions of nutrition, hormone injection, and so forth. Further investigations of these variations is of interest. Taylor (37) has demonstrated that gastric pepsin I (the fastest moving electrophoretic fraction) is increased in patients with gastric ulcers, whereas Seijffers, Segel, and Miller (38) detected only their pepsinogen I (showing the least binding to DEAE-cellulose), instead of the usual three pepsinogens, in the gastric juice of a patient with pernicious anemia.

It is our intention to study the structure and kinetic properties of the pepsin(s) obtained by activation of the canine pepsinogen. Investigations going on in several laboratories should make data available in the near future for several species of pepsins, thus making it possible to make some correlations of structure with enzymatic activity.

Acknowledgments—We are grateful to the Pharmacology and Physiology Departments of this institution for supplying the canine stomachs, to Dr. Albert Girotti of this department for the molecular weight determination, and to Dr. William Whish for the disc electrophoresis. We wish to express appreciation to Dr. Patricia A. Meitner and to Dr. John Kay for helpful suggestions and fruitful discussions.

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