Primary Structure of Porcine Pepsin

I. PURIFICATION AND PLACEMENT OF CYANOGEN BROMIDE FRAGMENTS AND THE AMINO ACID SEQUENCE OF FRAGMENT CB5*

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Fragments resulting from the cyanogen bromide cleavage of reduced and aminoethylated porcine pepsin were purified. Only four of the five fragments theoretically present could be accounted for in major yield when the cyanogen bromide reaction was carried out at room temperature. The NH₂-terminal fragment, CB2, contained an internal homoserine which was not cleaved to any significant extent. The amino acid sequence around this internal homoserine was determined by isolating and partially determining the sequence of an α-chymotryptic peptide. Cleavage at this methionine was increased by 50% when the cyanogen bromide reaction was carried out at 37°. The NH₂- and COOH-terminal sequences of five major fragments were determined. The placement of these fragments in the native pepsin molecule was demonstrated.

The amino acid sequence of one of the fragments, CB5, was determined. This fragment contains 44 residues with an internal disulfide bridge. The COOH-terminal methionine of this fragment was connected to another 37-residue cyanogen bromide fragment of known sequence. Together these two fragments formed the COOH-terminal 81 residues of porcine pepsin.

Although porcine pepsin was one of the first enzymes to be discovered and purified in crystalline form, structure-function relationships are only superficially understood. The question of the structure and function of pepsin is interesting, because this enzyme catalyzes proteolysis in highly acidic solutions, in the range of pH 1 to 4. Thus, the enzymic mechanism and the catalytic groups of pepsin, as well as other acidic proteases, must differ strikingly from those of alkaline proteases. The latter groups are much better understood, largely due to the available information on their primary and tertiary structures. Therefore, before significant progress can be made in the understanding of the acidic proteases, a systematic elucidation of their primary structure appears necessary.

We selected porcine pepsin as a model for the sequence study of an acid protease mainly for two reasons. First, the enzymic properties of porcine pepsin have been studied more extensively than other acid proteases. Not only the specificity and kinetic parameters have been well documented (2), but also the catalytically essential residues have been identified as 2 aspartyl residues (3–6). Second, the tertiary structure of porcine pepsin is being studied (7). In addition, it is known that many acid proteases are homologous in amino acid sequence (8–10). The knowledge of the amino acid sequence of pepsin hopefully will simplify the elucidation of the structure of other acid proteases.

Porcine pepsin contains 4 methionine and 6 half-cystine residues in a single polypeptide chain of about 321 amino acid residues (11). For the initial cleavage method in our sequencing strategy, we decided to use cyanogen bromide. This approach seemed particularly promising because the overlapping methionyl sequences had already been studied by the methionine diagonal technique (12). Whenever feasible, we planned further cleavage at the half-cystinyl residues by means of reduction, aminoethylation, and tryptic digestion. This approach appeared advantageous because the only 2 arginyl residues and the single lysyl residue in pepsin are located in the last 20 residues near the COOH terminus of pepsin (13). The fact that the sequences around the cystinyl residues had been studied (12) would aid in disclosing some of the needed overlaps.

Although cyanogen bromide cleavage has previously been used to obtain fragments for structural studies of pepsin (14, 15), all the fragments have not yet been isolated and their
overlaps have not been demonstrated. Therefore, to elucidate the complete primary structure of pepsin, a thorough investigation of the purification and characteristics of all cyanogen bromide fragments appeared necessary.

In the experiments reported below, we purified fragments from the cyanogen bromide cleavage of reduced and aminoethyalted pepsin. The NH₂- and COOH-terminal sequences of the fragments were studied. More evidence on the sequence of a methionine overlap was obtained. From these results, we were able to construct a complete scheme of the pepsin molecule. In addition, we also determined the amino acid sequence of a 44-residue fragment, CB5.

EXPERIMENTAL PROCEDURE

Materials

Crystalline porcine pepsin, diisopropylphosphorofluoridate-treated carboxypeptidase A, and α-chymotrypsin were obtained from Worthington Biochemical Corp. Thermolysin was obtained from Calbiochem. Trypsin (once crystalized, diphenyl carbamyl chloride-treated, type XI) was purchased from Sigma. Subtilisin was a product of Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Aminopeptidase M, manufactured by Röhm GmbH Chemische Fabrik was obtained from Henley Co., Long Island. Reagents used in the Edman degradation procedure were purchased from commercial sources and redistilled in our laboratory. Ethylenimine, cyanogen bromide, and mercaptoethanol were purchased from Eastman Kodak Co. Ethylenimine was redistilled over barium oxide. Dithiothreitol was obtained from Calbiochem. Polyamide sheets were obtained from Gallard-Schlesinger Chemical Manufacturing Corp. (a product of Cheng Chin Co., Taipei, Taiwan). Other chemicals used were of the highest purity commercially available and were used without further purification.

Methods

Reduction and Aminoethylation of Porcine Pepsin—Pepsin was reduced and aminoethylated using a modified procedure of Slobin and Singer (16). A 2 % solution of porcine pepsin (1 g/50 ml) was prepared in 6 M guanidine hydrochloride, 2 M Tris-chloride, and 0.01 M EDTA, pH 8.2. Dithiothreitol was then added to a concentration of 0.1 M. After flushing with nitrogen gas, the flask was closed and the contents stirred for 3 hours at 37°C. One milliliter of ethylenimine was then added and the flask was again flushed with nitrogen. This process was repeated after 30 min. After 30 min more of stirring, the contents of the flask were dialyzed in the cold against several changes of distilled water and lyophilized. Amino acid analysis of the modified pepsin gave 5.8 residues of aminoethylcysteine and 4 residues of methionine.

Amino Acid Analysis—Amino acids were determined with a Spino model 125B amino acid analyzer. The recorder permitted quantitative analysis in the range from 1 to 10 nmol of amino acid. Analysis was performed according to the accelerated procedure of Spackman (18). The samples were hydrolyzed in 5 N HCl in sealed evacuated tubes for 24 hours at 110 ± 2°C. After removal of the HCl under reduced pressure, the residues were dissolved in 0.2 N sodium citrate buffer, pH 2.2.

For the analysis of homoserine, the dried HCl hydrolysates were incubated in 0.2 M ammonium acetate buffer, pH 9.3. The fragments in the eluents were detected by determination of absorbance at both 280 nm and 215 nm. The eluted material was recovered by pooling of the fractions and lyophilization. For peptide separation, a column (3 × 150 cm) of Sephadex G-25 was eluted with 0.1 M ammonium acetate buffer, pH 9. The eluents were detected by determination of absorbance at both 280 nm and 215 nm. The eluted material was recovered by pooling of the fractions and lyophilization. For peptide separation, a column (3 × 150 cm) of Sephadex G-25 was eluted with 0.1 M NH₄OH. The effluent was collected in 10 ml fractions. Aliquots of 0.2 ml were taken from every other tube, dried in a desiccator, and subjected to paper electrophoresis at pH 6.0 to determine the peptides present.

Nomenclature of Peptides—In this paper, as well as in the second and third papers in this series, all of the prefixes in the peptide (or fragment) numbers are designated according to the cleavage or hydrolysis methods. They are described as follows: CB, cyanogen bromide fragment; C, chymotrypsin digest; H, partial acid hydrolysis; AP, alkaline hydrolysis; B, papain digest; T, trypsin digest; Th, thermolysin digest; P, papain digest; C, chymotrypsin digest; H, partial acid hydrolysis; AP,
proteolytic digests of the fragments. These peptides were sequenced in the sequence studies of each individual fragment in this paper and two other papers of this series (27, 28), the detailed procedures for the isolation and sequence determination of homoserine peptides shown in Table II are described only in the repository data.2

CB1 contained 37 residues. The composition and terminal sequences revealed that this fragment was derived from the COOH terminus of porcine pepsin. The sequence of this fragment is known (13, 29). Fragments CB4 and CB5, containing 47 and 41 residues, respectively, were obviously unique. Fragments CB2 and CB3 had NH₂-terminal sequences identical with each other and also to pepsin (30). However, CB2 contained about 200 residues, whereas CB3 contained only about 80 residues. Both fragments released free homoserine from their COOH termini after carboxypeptidase A digestion. Therefore, we concluded that CB2 must have contained an internal methionine (or its derivative) which cleaved only partially to produce small quantities of CB3 and a missing 120-residue fragment (CB4 and CB5 were too small). Indeed, this was confirmed by two-dimensional peptide maps of CB2 and CB3 (Fig. 3). The results clearly showed that the maps of CB2 and CB3 contained large numbers of peptides appearing at the same positions. Additionally, CB2 produced about 40% more peptide spots than were found in the peptide map of CB3. We concluded that a unique fragment of about 120 residues had not been recovered. Various conditions were tried for the cyanogen bromide cleavage reaction. Only at higher temperature was it possible to cleave well the homoserine-containing peptides on the peptide maps of the fragments. These peptides were isolated and their sequence determined. Because these sequences are confirmed in the sequence studies of each individual fragment in this paper and two other papers of this series (27, 28), the detailed procedures for the isolation and sequence determination of homoserine peptides shown in Table II are described only in the repository data.2

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were corrected for 10% and 7% losses, respectively. The values for isoleucine and valine were those of 48-hour hydrolyses (except CBl).

Comparison with Fig. 1 makes it clear that the size of the CB3 cleaved, produced fragments CB3 and CBSA. To confirm this, the chromatographic pattern of fragments derived from cyanogen bromide cleavage at 37°C was shown in Fig. 4. These results were below.

Cyanogen Bromide Cleavage at 37°C and Isolation of Fragment CB2A—The chromatographic pattern of fragments derived from cyanogen bromide cleavage at 37°C is shown in Fig. 4. Comparison with Fig. 1 makes it clear that the size of the CB3 peak increased as the size of the CB2 peak decreased. In addition, a new peak, CB2A, appeared between CB2 and CB3. The fractions contained under the main portion of the peak were pooled and lyophilized, and chromatography on Sephadex G-75 was repeated (Fig. 4B) to separate contaminants resulting from the partial overlap of the other peaks. Only fractions under the center of the peak (area indicated in Fig. 4B) were recovered by pooling and were lyophilized. The amino acid composition indicated that fragment CB2A contained 118 residues (Table I). The combined amino acid compositions of CB3 and CB2A agreed reasonably well with the composition of CB2 (Table I), indicating that CB3 and CB2A were products derived from the cleavage of an internal methionine in CB2. This point was further substantiated by the isolation of the internal homoserine peptide, described in the next section. A peptide map of CB2A accounted for the differences in the peptide maps of CB2 and CB3 (Fig. 3). The NH2-terminal sequence of CB2A was determined to be Thr-Gly-Ile-Leu- and the COOH-terminus was homoserine. We, therefore, concluded that CB2A represented about 120 residues at the COOH-terminal end of CB2.

The five cyanogen bromide fragments, CBl, CB2A, CB3, CB4, and CB5, contained an aggregate of 321 residues, which is the number of residues in pepsin calculated by Rajagopalan et al. (11) from amino acid analysis.

Isolation and Sequence of Internal Homoserine Peptide from Fragment CB2—As described in the preceding section, CB2 appeared to contain an internal homoserine, which, when cleaved, produced fragments CB3 and CB2A. To confirm this
Fig. 3. Two-dimensional high voltage electrophoretic patterns of peptides derived from α-chymotryptic digestion of the fragments, from the top, CB2A, CB2, and CB3. The first dimension was obtained at pH 6.0 at about 60 volts/cm for 50 min. The second dimension was obtained at pH 2.0 at the same voltage for 40 min. The shaded peptide spots from CB2 (middle) are those found in CB3 (bottom). The unshaded peptide spots in CB2 are those found in CB2A (top). The peptide spots indicated in dotted circles are those that appeared yellow after treatment with cadmium-ninhydrin reagent. The peptide numbers are those used in the sequence determinations of the fragments (27, 28).

possibility, we attempted to isolate the homoserine-containing peptides in fragment CB2. Two chymotryptic peptides, (CB2)C1 and (CB2)C2, which contained homoserine residues were isolated from the neutral bands of pH 6 high voltage electrophoresis after two additional electrophoretic separations in pH 3.5 and pH 2.0. The sequences of the peptides were determined using Edman degradation and thermolytic cleavages. The sequences of these peptides are: Peptide (CB2)C1, Gly-Thr-Gly-Ser-Hse-(Thr,Gly)-Ile-Leu-Gly-Tyr; Peptide (CB2)C2, Gly-Thr-Gly-Ser-Hse-(Thr,Gly)-Ile-Leu. The sequences shown above apparently consist of the COOH-terminal portion of CB3, Gly-Thr-Gly-Ser-Hse, and the NH2-terminal portion of CB2A, Thr-Gly-Ile-Leu (see Table II).

Amino Acid Sequence of Fragment CB5—Cyanogen bromide fragment CB5 was digested separately with trypsin, thermolysin, and papain. The resulting peptides were separated on columns of Sephadex G-25. Further purifications were carried out using high voltage electrophoresis at pH 6.0, pH 2.0, and pH 3.5. Five tryptic peptides (T1 to T5), eight thermolytic peptides (Th1 to Th8), and nine papain peptides (P1 to P9) were obtained. The sequence determinations of these peptides were carried out using the Edman procedure, carboxypeptidase A digestion, hydrazinolysis, partial acid hydrolysis, and enzymic cleavages with subtilisin and thermolysin. The determined sequences are shown in Table III for all of the tryptic and thermolytic peptides. Only one papain peptide, P6, is shown in Table III, because it provides a unique overlap for the chymotryptic and thermolytic peptides. The sequence determination of other eight papain peptides is described in detail in the repository data.3

Fig. 4. A, chromatographic pattern of fragments of aminomethyl pepsin which resulted from the cyanogen bromide reaction at 37°C. A column (4 x 200 cm) of Sephadex G-75 was eluted with 0.1 M ammonium acetate, pH 9.5. B, chromatographic pattern of the material collected under peak CB2A in A. The conditions are the same as that in A. The horizontal bar under the peak indicates the fractions collected as the final material of fragment CB2A.

DISCUSSION

The information on the NH2- and COOH-terminal sequences of the individual cyanogen bromide fragments derived from these experiments gives us a composite picture of how these fragments are assembled in the original pepsin molecule. Fig. 6 depicts the relative positions of the cyanogen bromide fragments. The overlap connecting fragments CB3 and CB2A (at methionine I, Fig. 6) becomes apparent from the amino acid sequence of the internal homoserine in CB2. The evidence for the overlap at methionine II (Fig. 6) is provided by previous experiments (12) which elucidated a methionine partial sequence: Asp-Ser-Ile-Thr-Met-(Asx,Gly,Glx). This overlap sequence, however, has been confirmed in an α-chymotryptic peptide, peptide (OP)C2,2 which provided a sequence of Asp-Ser-Ile-Thr-Met-Asp-Gly-Glu-Thr-Be------. The evidence for the overlap at methionine III was provided in the previous study (12) of a partial methionine sequence: Ala-Ser-Glu-Ahx-Ser-Ahx-Glu-Glu-Met-(Ile,Val,Tyr). Admittedly, this sequence agrees only moderately well with the sequence at methionine III, shown in Fig. 6. However, because the other three methionine overlaps are firmly established, we feel that the evidence is sufficient to confirm the only remaining alternative. The overlap at methionine IV was provided in our previous work (5), which demonstrated a peptide sequence of Phe-Glu-Gly-Met-Asp-Val-Pro-Thr-Ser-Ser-Gly-Glu-Leu.

The poor cyanogen bromide cleavage at methionine I (Fig. 6) produced a large yield of fragment CB2, which contained an
TABLE III

Summary of electrophoretic mobility, amino acid composition, and sequence of peptides from CB5

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Mobility a</th>
<th>pH 2.0</th>
<th>pH 6.0</th>
<th>No. of Residues</th>
<th>Amino Acid Composition b</th>
<th>Sequence</th>
<th>Sequencing Procedures c</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>1.20</td>
<td>-0.98</td>
<td>red</td>
<td>4</td>
<td>Ser-3, Gly-1, Glu-3, Cys(Ae)-5</td>
<td>Thr-Ile-Asp-Gly-Thr-Pro-Leu-Ser-Ala-Val-Tyr</td>
<td>DE, Hx</td>
</tr>
<tr>
<td>T2</td>
<td>0.38</td>
<td>-0.44</td>
<td>brown</td>
<td>11</td>
<td>Asp-3, Ser-3, Pro-4, Val-4, Leu-1, Thr-1, Cys-1</td>
<td>Ser-Ser-Ile-Asp-Ser-Leu-Asp-Ile-Val-Phe</td>
<td>DE, Co</td>
</tr>
<tr>
<td>T3</td>
<td>0.32</td>
<td>-0.22</td>
<td>yellow</td>
<td>14</td>
<td>Asp-3, Gly-2, Ser-2, Glu-1, Thr-1, Gly-1, Ala-1, Cys(Ae)-5</td>
<td>Thr-Ile-Asp-Gly-Thr-Pro-Leu-Ser-Pro-Ser-Ala-Val-Tyr</td>
<td>DE, Co, Th, 5</td>
</tr>
<tr>
<td>T4</td>
<td>0.75</td>
<td>-0.48</td>
<td>red</td>
<td>8</td>
<td>Asp-3, Ser-3, Gly-1, Glu-1, Cys(Ae)-5</td>
<td>Ile-Leu-Gln-Asp-Asp-Ser-Cys(Ae)</td>
<td>DE, Hx, HCl</td>
</tr>
<tr>
<td>T5</td>
<td>0.52</td>
<td>-0.31</td>
<td>yellow</td>
<td>7</td>
<td>Thr-1, Ser-1, Gly-1, Thr-1, Gly-1, Thr-1, Cys(Ae)-5</td>
<td>Thr-Ile-Asp-Gly-Thr-Pro-Leu-Ser-Pro-Ser-Ala-Val-Tyr</td>
<td>DE, Co</td>
</tr>
<tr>
<td>T6</td>
<td>0.55</td>
<td>-0.23</td>
<td>red</td>
<td>6</td>
<td>Ser-3, Gly-1, Glu-1, Cys(Ae)-5</td>
<td>Val-Ile-Ser-(Cys-Ae, Ser)-Ser</td>
<td>DE, Co</td>
</tr>
<tr>
<td>T7</td>
<td>0.54</td>
<td>-0.60</td>
<td>red</td>
<td>6</td>
<td>Asp-3, Ser-3, Pro-4, Val-1, Leu-1, Cys(Ae)-5</td>
<td>Ile-Asp-Ser-Leu-Pro-Asp</td>
<td>DE</td>
</tr>
<tr>
<td>T8</td>
<td>0.64</td>
<td>0</td>
<td>red</td>
<td>4</td>
<td>Thr-1, Val-1, Gly-1, Cys(Ae)-5</td>
<td>Ile-Leu-Pro-Thr</td>
<td>DE</td>
</tr>
<tr>
<td>T9</td>
<td>0.48</td>
<td>-0.30</td>
<td>red</td>
<td>7</td>
<td>Asp-3, Gly-1, Pro-4, Gly-1, Val-1, Thr-1, Cys(Ae)-5</td>
<td>Asp-Ser-Gly-Thr-Pro-Leu-Ser-Pro-Ser</td>
<td>DE</td>
</tr>
<tr>
<td>T10</td>
<td>0.97</td>
<td>0</td>
<td>red</td>
<td>4</td>
<td>Ser-3, Gly-1, Leu-1, Cys(Ae)-5</td>
<td>Leu-Pro-Ser</td>
<td>DE</td>
</tr>
<tr>
<td>T11</td>
<td>0.77</td>
<td>-0.43</td>
<td>red</td>
<td>11</td>
<td>Asp-3, Gly-1, Ser-1, Gly-1, Glu-1, Val-1, Leu-1, Cys(Ae)-5</td>
<td>Ile-Leu-Gln-Asp-Ser-Cys(Ae)-Thr-Ser-Gly</td>
<td>DE, Co</td>
</tr>
<tr>
<td>T12</td>
<td>0.66</td>
<td>-0.43</td>
<td>red</td>
<td>4</td>
<td>Gly-1, Gly-1, Thr-1, Cys(Ae)-5</td>
<td>Thr-Leu-Val-Gly</td>
<td>DE</td>
</tr>
<tr>
<td>T13</td>
<td>0.61</td>
<td>0</td>
<td>red</td>
<td>4</td>
<td>Glu-1, Gly-1, Thr-1, Cys(Ae)-5</td>
<td>Thr-Ile-Leu-Gln</td>
<td>DE, Co</td>
</tr>
</tbody>
</table>

a. Electrophoretic mobilities are relative to serine (=1.0) at pH 2.0 and to aspartic acid at pH 6.0. -0- indicates the peptides which are considered neutral in electrophoresis at pH 6.0.

b. The amino acid analyses are molar ratios. Serine was corrected for 5% of loss during the hydrolysis. Hse and Cys(Ae) are the abbreviations for homoserine and aminoethylcysteine, respectively.

c. The symbols indicate the methods used in the sequencing of the peptides. DE = dansyl-Edman degradation, Hz = hydrazinolysis, Cp = carboxypeptidase A digestion, HCI = partial acid hydrolysis, Th = thermolysin digestion, and S = subtilisin digestion.

FIG. 5. Amino acid sequence of cyanogen bromide fragment CB5 from aminomethyl pepsin. The horizontal arrows under the sequence indicate the positions of the peptides. Hse, homoserine.

Internal homoserine residue. This poor cleavage was obviously due to the methionyl-threonine bond which is known to resist cyanogen bromide cleavage (31-32). Although we were able to increase the extent of cyanogen bromide cleavage at this site by raising the reaction temperature to 37°C, this may be unfeasible as a general method to increase the cleavage of methionyl-threonine (or methionyl-serine) bonds. For example, Schroeder et al. (33) obtained negative results with the high temperature reaction. In spite of the poor cleavage at one of the methionyl bonds, the results of this study indicate that the use of cyanogen bromide cleavage as the first step in the sequence study of pepsin is feasible.

Ostoslavskaya et al. (15) purified and partially characterized several cyanogen bromide fragments from reduced and carboxymethylated pepsin, and our data agree in general with theirs. The purified fragments which we labeled CB1, CB2, CB4, and CB5 in our work were called fragments R1, R9, R5, and B4, respectively, in the report of Ostoslavskaya et al.
The sum of the amino acid compositions of five fragments (Table I) gives a total of 321 residues, which agrees with the number of residues obtained from analysis of the whole molecule (11). The amino acid composition also shows that fragments CB3, CB4, and CB5 each contain 2 half-cystine residues. The single histidyl residue of pepsin is located in a previously reported sequence, Cys-Ser-Ser-Leu-Ala-Cys-Ser-Asp-His-Asn-Gln-Phe. Because CB2 and CB3 contain a histidyl residue, they must contain this sequence. The 2 half-cystine residues contained in CB5 are those previously shown to be linked in a disulfide bond (12). Therefore, fragment CB4 must contain the third disulfide bridge with the amino acid sequence of Cys-Ser-Gly-Gly-Cys-Glu (12).

In constructing the amino acid sequence of CB5, only two 1-residue overlaps are present, at Thr-16 and Tyr-29 (see Fig. 5). However, because fragment CB5 contains only 2 residues each of threonine and tyrosine (Table I), the proposed structure is the only possible arrangement.

Some discrepancies have emerged in the sequences near 2 half-cystine residues. Originally, a sequence of Cys-Ser-Ile-Asp-Gln was obtained (12). In this work, serine, instead of glutamine, was identified as the last residue. The reason for this difference is not clear. The evidence in both cases appears to be incontrovertible. We suggest that the discrepancy may be due to a genetic variant in one of the two pepsin samples used. The evidence for the other half-cystine sequence in the previous work (12) suggests a sequence of Glu-Asx-Asx-Ser-Cys-Thr-Ser. We now believe that the correct sequence is as shown in this work, that is, Gln-Asp-Asp-Asp-Ser-Cys-Thr-Ser.

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K C Chen, N Tao and J Tang


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