The Activation of Bovine Pepsinogen

SEQUENCE OF THE PEPTIDES RELEASED, IDENTIFICATION OF A PEPsin INHIBITOR*

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MARIANNE HARBOE, PER MAACK ANDERSEN, AND BENT FOLTLMANN
From the Institute of Biochemical Genetics, University of Copenhagen, Copenhagen K, Denmark

JOHN KAY† AND BEATRICE KASSELL
From the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53283

SUMMARY

Bovine pepsinogen is converted into pepsin by removal of 45 amino acid residues from the NH2-terminal of the single polypeptide chain. The complete sequence of the activation peptides has been deduced from two overlapping sets of peptides, one set obtained by autoactivation of the zymogen at pH 2 and the second from a trypsin digest of maleylated pepsinogen. A peptide derived from the 17 residues at the NH2 terminus of bovine pepsinogen has been identified as an inhibitor of the milk-clotting action of pepsin.

Extensive sequence homologies exist among the activation peptides of bovine and porcine pepsinogens and bovine prochymosin. In the peptides from the two pepsinogens, 30 of the 44 or 45 residues are identical, and most of the substitutions in the other residues are conservative. The preservation of positive charges in the NH2-terminal portion of the zymogen molecules, lost during activation, agrees with current ideas on the involvement of this segment in stabilizing the physical structure of the zymogens.

Vertebrate enzymes involved in the digestion of dietary proteins are synthesized in precursor form and are secreted as such into the gastrointestinal tract. Recent studies (reviewed in (1)) have shown that these precursor molecules, or zymogens, have inherent activity by which those that undergo autoactivation can convert themselves into the more active form of the respective enzymes. In the case of the zymogens of the gastric proteases, this activity is developed by a reversible change into the active conformation at low pH (2-4).

The irreversible transformation into active enzymes takes place by limited proteolysis during which a peptide segment is cleaved from the NH2 terminus in each case (cf. Ref. 5). The primary structures of the NH2-terminal portions of the zymogens are of considerable interest in order to understand, first, how the zymogens are able to maintain their less active conformation, and second, how the irreversible activation occurs under the proper conditions.

In the conversion of porcine pepsinogen to pepsin, 44 amino acid residues are cleaved from the NH2 terminus of the molecule. Ong and Perlmann (6) determined the sequence of the first 41 residues, while Pedersen and Foltmann (7) and Stepanov et al. (8) found an additional sequence of 3 residues which connects the first 41 residues with the pepsin moiety of the zymogen.

Preliminary studies on the sequence of the activation peptides of bovine pepsinogen have been reported by Kassell et al. (9), Kay (10), and Foltmann et al. (11). The major portion of the sequence, determined independently in both laboratories, was in agreement, although there were some errors in both sequences. The discrepancies have now been resolved by re-examination of the earlier results and by further experiments. The total sequence is presented here.

A pepsin-inhibiting substance formed during activation of porcine pepsinogen has been known for many years (12). This inhibitor was partially characterized by Van Vunakis and Herriott (13). A recent analysis by Anderson and Harthill (14) showed that it corresponds to the NH2-terminal 16 amino acids in the sequence of Ong and Perlmann (6), except for 1 extra lysine residue. We report here the identification of an inhibitory peptide containing the first 17 amino acids of bovine pepsinogen.

MATERIALS AND METHODS

Bovine Pepsinogen-The pepsinogen was extracted from the mucosa of fourth-stomachs (abomasum), which had been stored frozen. Stomachs from both Danish and American cattle were used, so that two different preparations of pepsinogen were obtained, designated [D] and [A]. For the [D] preparation, the

1 Where nothing else is stated, the terms pepsinogen and pepsin refer to the bovine proteins.
2 The letters [D] and [A] are used to designate Danish and American preparations, procedures, etc. Other abbreviations used are: TPCK, 1-tosylamido-2-phenylethyl chloromethyl ketone derivative of; DNS- or dansyl-, 5-dimethylaminonaphthalene-1-sulfonyl-; PTH-, phenylthiohydantoin of; BAWP, 1-butanol-acetic acid-water-pyridine, 15:8:12:10; TLC, thin layer chromatography; --, Edman degradation with identification as the PTH derivative

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procedure of Chow and Kassell (15) was modified as previously described (16). For the [A] preparation, purified pepsinogen (15) was subjected to chromatography on polylysine-Sepharose 4B at pH 6.5 (29) as the final step of purification (10). Pepsinogen [A] was resolved into three potentially active peaks differing only in their content of covalently bound phosphate (0.4, 1.5, and 2.8 moles per mole of protein). During activation of bovine pepsinogen, all the phosphate groups remain attached to the pepsin moiety (18), indicating that no phosphate is present in the activation peptides. The three components were therefore pooled and treated as one. Pepsinogens [D] and [A] were identical in composition within the limits of analysis.

Other Enzymes and Inhibitors—TPCK-trypsin was obtained from Worthington Biochemicals, Inc., Freehold, N. J. Carboxypeptidase A and B were either Worthington or Sigma preparations (Sigma Chemical Co., St. Louis, Mo.). Portions of the carboxypeptidase A suspension were prepared for use as described by Ambler (19). Pancreatic trypsin inhibitor (Trasylol®) was purchased from Bayer, Germany.

Reagents—Reagents for manual sequencing were redistilled according to the method of Edman and Begg (20) and were stored under N₂ at -15°C. The reagents for the Sequencer, phenylisothiocyanate, Quadrol buffer, heptanfluorobutyric acid, heptane, benzene, ethyl acetate, and 1-chlorobutane were all Sequencer Grade from Beckman Instruments. Imidazole-butane thiol (obtained from Fluka) was added (0.005%) to the chlorobutane in order to obtain higher yields of serine. Other reagents were the best grade available.

Chromatography and Electrophoresis—Sephadex and Sepharose were obtained from Pharmacia. DEAE-cellulose was either from Whatman Biochemicals, Ltd., Maidstone, U. K., or from Schleicher and Schuell, Keene, N. H. The anion exchange AG 1-X2 was purchased from Bio-Rad, Richmond, Calif. Polylysine (mol. wt 50,000 to 100,000) was purchased from Pierce Chemical Co., Rockford, Ill. Polylysine-Sepharose 4B was prepared in two batches of 75 g each for a column (2.5 X 45 cm) by the method of Nevardine and Kassell (17). Whatman chromatography papers were used for electrophoresis and chromatography. Polyamide sheets were obtained from Cheng Chin Trading Co., Taipei, Taiwan.

High voltage paper electrophoresis at about 50 volts per cm was conducted (11) in liquid-cooled tanks (coolants and buffers as described by Ambler (21)) or [A] with the use of a flat-plate apparatus (Savant Instruments, Inc., Hicksville, N. Y.). Either a visible marker containing methyl green and crystal violet was used or asparagine-16, glutamic acid-19, and asparagine-20 were identified by thin layer chromatography of the PTH derivatives also. After 20 cycles, the reaction was terminated by the addition of 0.5 ml of Trasylol solution.

Maleylation of Peptides [A]—Modification of lysine residues was carried out at pH 9 (32), the maleic anhydride being added in dioxane. The excess of reagent was removed by passage through a Sephadex G-15 column, equilibrated with 0.1 M NH₄OH, followed by lyophilization of the peptide fractions. Deprotection was achieved by dissolving the material in 5% acetic acid-1% pyridine and heating at 60°C for 1 hour.

RESULTS


The average repetitive yield during the operation was approximately 90%. As far as was possible, all the residues were identified both as the PTH derivatives and as the free amino acids after conversion with HI. However, arginine-14, glutamine-15, asparagine-16, glutamic acid-19, and asparagine-20 were identified with certainty only after conversion to the free amino acids. Amide assignment was made by comparison to the corresponding activation peptides, although the amides were seen in low yields as the PTH derivatives also. After 20 cycles, the background due to unspecific cleavage reached a level prohibitive of further identification.

Activation of Bovine Pepsinogen

To avoid degradation of activation peptides by the newly formed pepsin, optimized conditions of activation were first determined. Fig. 1 shows the course of activation at 0°C between pH 1.5 and 5.0; the optimum pH for activation is 2.0.

Activation experiments were carried out in both laboratories under the same conditions except for the time of activation and the method of separation of the peptides. In the first experiment, 370 mg of pepsinogen [A] was activated at pH 2 and 0°C for 4 min at a protein concentration of 15 mg per ml. The pH was raised to 3.5, and the solution was immediately applied to the column of polylysine-Sepharose at pH 3.5 (Fig. 2). The pure peaks, the A and B fractions were collected after washing with 0.1 M NH₄OH.

Separation on polylysine-Sepharose depends on both affinity chromatography and ion exchange (17). With pepsinogen at pH 6.5, ion exchange appears to predominate, resulting in the separation of 3 peaks differing in bound phosphate. At pH 3.5, the pepsin appeared in a single peak, indicating the predominance of the affinity effect.
The rate of activation of bovine pepsinogen at 0° under varying conditions of pH. A solution of 2 mg of pepsinogen in 3 ml of water, cooled to 0°, was rapidly adjusted to the desired pH with a predetermined amount of 2.5 N HCl. At the designated times, 0.1-ml samples were added to 0.4 ml of 0.1 M Tris-acetate buffer, pH 6.5, also at 0°. The final pH was 5.5. The amount of active pepsin was determined by the milk-clotting method.

The amino acid compositions of the peptide mixtures from the two activations were nearly identical (Table I) and were in reasonable agreement with the composition estimated from the difference in composition between pepsinogen (15) and pepsin (33). The composition determined by the sequence studies below is included in Table I.

**Composition and Sequence of Activation Peptides**

The two peptide mixtures were fractionated by chromatography on Bio-Gel P-2 (2 X 90 cm [A]) or on Sephadex G-25 (1.5 X 90 cm [D]). Each fraction was further purified by high voltage paper electrophoresis and occasionally by chromatography on BAWP (36). Details of the composition, electrophoretic mobility, and yield of each activation peptide are given in Table II. The sequence, determined as described in this section, is summarized in Fig. 3.

**Act-1 (Residues 1 to 17) Ser-Val (Val-Lys,Ile,Pro,Leu)-Val-Lys-Lys-Lys-Ser-Met-Arg-Gln:Asm-Leu—Act-1 was purified further by paper electrophoresis at pH 6.5. Edman degradation gave Ser-Val, corresponding to the NH₂ terminus of pepsinogen. A portion of the peptide was maleylated (32) and digested with TPCK-trypsin (peptide concentration 0.75 μM, 4 hours, 37°, substrate to enzyme ratio 85:1). After deblocking, the peptide

* B. Kassell, unpublished.
mixture was resolved into a neutral fraction (Act-1A) and a larger basic peptide (Act-1B) by electrophoresis at pH 6.5. The basic peptide had NH₂-terminal Ser-Val-, and the sequence at its COOH terminus was established by using carboxypeptidases A and B alternately, determining the amino acids released on the analyzer. Treatment with carboxypeptidase B released only arginine (quantitatively). The mixture was boiled before the addition of carboxypeptidase A, which released leucine (1.0 mole per mole) almost immediately and serine quite slowly. After 28 hours, 1.0 mole each of arginine, leucine, and serine had been released, and introduction of carboxypeptidase B rapidly released 3.0 moles of lysine with a slower release of valine (up to 0.8 mole per mole). These data gave only a partial determination of the sequence of this peptide (see Fig. 3). Peptide Act-1A was easily sequenced by three steps of Edman degradation-dansylation (Fig. 3). The amide assignments at positions 15 and 16 were made on the basis of the mobilities of this tripeptide and of Act-2. Because Act-1 was the NH₂-terminal peptide of pepsinogen, it was possible to confirm and complete the sequence from the sequenator analysis described above.

**Act-2 (Residues 13 to 17)** Leu-Arg-Glu-Asn-Leu—Act-2 was further purified, following DEAE-chromatography on Sephadex G-25, then by paper electrophoresis at pH 6.5, paper chromatography in BAWP, and finally paper electrophoresis at pH 2. The sequence was determined by five steps of Edman degradation, dansylation.

**Act-3 (Residues 18 to 25)** Ile-Glu-Asn-Gly-Lys-Leu-Lys-Glu—The peptide was purified by gel filtration followed by paper electrophoresis at pH 6.5 and pH 2. After paper electrophoresis at pH 6.5, this peptide was isolated both deamidated [D] and with intact asparagine [A]. This is consistent with the observation (28) that the amide in an Asn-Gly linkage is labile. In both peptides, all of the residues were identified as their dansyl derivative following Edman degradation. In addition, for Peptide [A], PTH-glutamic acid-19 was directly identified by TLC (31) in CHCl₃:MeOH, 9:1, v/v, and in CHCl₃:HOCH₃, 95:10, v/v, and PTH-asparagine was identified by TLC in CHCl₃-MeOH.

**Act-4 (Residues 26 to 34)** Thr-His-Lys-Tyr-Asn-Leu—The peptide was further purified by paper electrophoresis at pH 6.5, descending paper chromatography in BAWP, and finally paper electrophoresis at pH 2. The peptide was sequenced by sequential Edman degradation-dansylation. The electrophoretic mobility indicated the presence of asparagine. This was later confirmed by the isolation of a basic peptide (Tyr-32 to Lys-37) after tryptic digestion of demaleylated TM-2 (below).

**Act-5 (Residues 35 to 38)** Gly-Ser-Lys-Tyr—The peptide was further purified by paper electrophoresis at pH 6.5 and pH 2. In addition to the dansyl method for all the residues, PTH-serine was identified by TLC in CHCl₃-MeOH.

**Act-6 (Residues 39 to 41)** Ile-Arg-Glu—The peptide was further purified by paper electrophoresis at pH 6.5 and pH 3.5. PTH-isoleucine was identified by TLC in CHCl₃-MeOH. PTH-arginine was identified in the second step in the aqueous layer. The peptide was neutral during electrophoresis at pH 6.5 and acidic after two cycles of Edman degradation-dansylation. Amino acid analysis of the unhydrolyzed aqueous layer gave free glutamic acid.

**Act-7 (Residues 42 to 45)** Ala-Ala-Thr-Leu—The peptide was
Further purified by paper electrophoresis at pH 6.5 and 3.5. In addition to identification of each residue as the dansyl derivative during Edman degradation, at the second cycle alanine was directly identified as the PTH derivative by TLC in CHCl3-MeOH. At this stage, a portion of the residue was hydrolyzed and yielded only threonine to leucine, 1:1 on the analyzer. After the third cycle, the analysis of the unhydrolyzed residue gave only leucine.

**Tryptic Peptides from Maleyl Pepsinogen**

Pepsinogen contains 6 arginine residues, three in the peptides released upon activation and three in the pepsin moiety of the molecule. Of the latter, two are located within 20 residues of the COOH terminus (37), and the third is 55 residues from the NH2 terminus of the enzyme. Thus, tryptic digestion of maleylated pepsinogen should produce one large fragment, one medium-sized fragment, and five small pieces (three from the NH2 terminus and two from the COOH terminus).

The digest (see under “Materials and Methods”) was fractionated on Sephadex G-100 (Fig. 4, taken from (16)). Peaks I, II, and III were described previously (16). Peak I was an aggregation product; Peak II contained the expected large fragment from the pepsin moiety. Peak III was almost pure Peptide TM-IV, the overlapping sequence between the activation peptides and pepsin (10); part of its sequence is included in Fig. 3.

Peaks IV and V are of present interest. The detailed descriptions of the peptides isolated from these two components are given below and are summarized in Table III and Fig. 3. In addition to the peptides to be described, 2 peptides from the COOH-terminal portion of the pepsinogen were also isolated. The sequences of these have been published (37).

**TM-1 (Residues 1 to 14)** Ser-Val-Val-Lys-Ile-Pro-Leu-Val-Lys-Lys-Lys-Ser-Leu-Arg—After gel filtration on Sephadex G-100 (Fig. 4), the peptides in Peak I were maleylated; TM-1 was purified by paper electrophoresis at pH 2 (mobility 1.2 relative to DNS-arginine) and was sequenced unambiguously by Edman degradation and identification of the free amino acids after conversion with HI.

The low yield (Table III) resulted from unsuccessful attempts to separate TM-1 and TM-2 on DEAE-cellulose and paper electrophoresis without demaleylation before it was found that the deblocked peptides separated well at pH 2.

**TM-2 (Residues 15 to 28)** (Gln, Asn, Leu, Ile, Glu, Asn, Gly, Lys, Leu, Lys, Glu)-Phe-Met-Arg—This peptide was isolated from Peak IV in the same operations as TM-1 (electrophoretic mobility 0.7 at pH 2 relative to DNS-arginine). After elution and drying, the NH2-terminal glutamine was apparently blocked by cyclization to pyrrolidone carboxylic acid, since it was not susceptible to Edman degradation. By digestion with a mixture of carboxypeptidase A and B, phenylalanine, methionine, and arginine were liberated in the ratios 0.5:0.85:1.0. After performic acid oxidation (to protect methionine by conversion to the sulfone), the peptide was digested with trypsin at pH 8 and room temperature overnight at an enzyme to substrate ratio of 1:75. Electrophoresis at pH 3.5 resolved 3 peptides.

**TM-2 (T-1) (Residues 15 to 22)** (Gln, Asn, Leu, Ile, Glu, Asn, Gly, Lys)—This peptide had a blocked NH2 terminus, but its amino acid composition (Table III) corresponded well to the NH2-terminal part of TM-2.

**TM-2 (T-2) (Residues 23 to 24)** Leu-Lys—Leucine was the NH2 terminus. After one Edman degradation, only lysine was left.

**TM-2 (T-3) (Residues 25 to 28)** Glu-Phe-Met-Arg—The sequence was determined by Edman degradation and conversion with HI to free amino acids. Methionine was determined as the sulfone. This peptide represents the COOH-terminal part of TM-2.

**TM-3 (Residues 29 to 40)** Thr-His-Lys-Tyr-Asn-Leu-Gly-Ser-Lys-Tyr-Ile-Arg—This peptide was purified from Peak V of Fig. 4 by gel filtration on Sephadex G-25, followed by paper electrophoresis at pH 3.5. The sequence was obtained by Edman degradation and identification of each amino acid after conversion with HI. Digestion with trypsin was conducted as described for TM-2. The digest yielded 3 basic peptides, resolved by paper electrophoresis at pH 6.5.

**TM-3 (T-2) (Residues 32 to 37)** Tyr-Asn-Glu-Leu-Ser-Lys—TM-3 (T-2), the peptide of lowest mobility (+0.5), was further purified by paper electrophoresis at pH 2. Edman degradation-dansylation established the sequence. The presence of asparagine was determined by the basicity of the peptide.

**TM-4**—This was a large fragment that included the NH2-terminal part of pepsin. The sequence of the first 12 residues has been published (16).

**Deduction of Structures and Order of Activation Peptides**

The results, summarized in Fig. 3, show that every residue has been sequenced, although not in every peptide. The activation peptides plus the tryptic peptides allow an unequivocal sequence to be deduced. Overlaps are complete except for the bond between residues 28 and 29, but this missing overlap does not cause any problem. The confirmation of the NH2-terminal portion of the sequence by the sequenator and of the COOH terminal portion by the continuation of peptide TM-4 into pepsin (16) makes this the only possible arrangement.

**Pepsin-inhibiting Activity of Activation Peptides**

When pepsinogen was activated, the milk-clotting activity of the reaction product almost doubled after the activation peptides were separated by gel filtration [D] or by chromatography on DEAE-cellulose [D] or polylysine-Sepharose [A].

Preliminary experiments have been carried out to investigate the inhibiting properties of the activation peptides. In one series of experiments [D] the activation peptides were first separated from pepsin by chromatography. A concentrated mixture...
The activation peptides reported in this paper are derived from the main pepsinogen of bovine gastric mucosa (15). A second pepsinogen, having NH₄-terminal leucine instead of serine, was isolated by Antonini and Ribadeau-Dumas (38). This minor zymogen separates from the main pepsinogen by chromatography on DEAE-cellulose (15, 38) and was removed in this manner from both pepsinogens [A] and [D] during the preparation.

It is remarkable that no investigation of the activation of gastric zymogens has yielded a single large activation peptide. When bovine pepsinogen was activated at pH 2 for the minimum time to achieve full conversion to pepsin, the events following activation were so rapid that more than 7 peptides were formed (Fig. 3). When the activation time was doubled, at least one additional point of hydrolysis appeared between serine-12 and leucine-13 to produce peptide Act-2 (Fig. 3). Most of the cleavage points were those expected from the rather broad specificity of a pepsin, with the exception of those between methionine-arginine and arginine-threonine. To the best of our knowledge, cleavages between these residues have not been reported for any of the acidic proteases. The activation of porcine pepsinogen (6, 39) and of prochymosin (2, 40, 41) similarly produced a multiplicity of peptides.

It is thus not possible yet to determine the position of the initial split causing irreversible activation. It is not certain that hydrolysis of the bond between leucine-45 of the peptides and valine-1 of pepsin is the first point of attack. Stepanov et al. have found a pepsin with one extra amino acid (leucine) at the NH₄ terminus when porcine pepsinogen was activated by a proteinase complex from Aspergillus oryzae (42) or by thermolysin (43). Tang et al. (44) have shown that a minor component of commercial porcine pepsin contains two extra NH₄-terminal residues, Ala-Leu-.. Thus, elucidation of the succession of reactions in the activation of pepsinogens remains for future work. With the sequence of the activation peptides of bovine pepsinogen now completed, it is possible to compare the NH₄-terminal portions of the bovine zymogen with porcine pepsinogen and with bovine prochymosin (prorennin), the precursor of the enzyme of calf stomach. The comparison is made in Fig. 5. Considerable homology is present among all three proteins. The pepsinogens from the different species are more alike than the two zymogens from young and adult cows.

**DISCUSSION**

For deduction of the sequence presented, two independent series of results have been combined. Different approaches and methods were used by the two groups, but once some preliminary errors were reinvestigated and corrected, the two sets of results were either identical or supplementary to each other.

The activation of pepsinogen at pH 2 for the minimum time to achieve full conversion to pepsin, the events following activation were so rapid that more than 7 peptides were formed (Fig. 3). When the activation time was doubled, at least one additional point of hydrolysis appeared between serine-12 and leucine-13 to produce peptide Act-2 (Fig. 3). Most of the cleavage points were those expected from the rather broad specificity of a pepsin, with the exception of those between methionine-arginine and arginine-threonine. To the best of our knowledge, cleavages between these residues have not been reported for any of the acidic proteases. The activation of porcine pepsinogen (6, 39) and of prochymosin (2, 40, 41) similarly produced a multiplicity of peptides.

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For the two pepsinogens, 30 of the 44 or 45 residues are identical. Most of the substitutions are conservative, e.g., one basic amino acid for another or one hydrophobic residue for another. Other substitutions, glutamic acid for lysine in position 19 and proline for either leucine or arginine in positions 34 and 40, respectively, may result from single base changes. In all other cases the positive charges are conserved. This substantiates the idea previously proposed (2, 45-47) that the positive charges in the NH2-terminal portions of thezymogens are necessary to maintain a zymogen conformation different from that of the active enzyme.

The striking similarity in structure in positions 1 to 17, corresponding to pepsin-inhibiting peptides identified for both species, may indicate an important physiological role for these inhibitors. The inhibiting activity of the bovine activation peptides against porcine pepsin complements the action of porcine inhibitor against bovine pepsin reported by Herriott (12). These cross-reactions are not surprising in view of the homology in primary structure.

Several interesting aspects of inhibition by the activation peptides require additional investigation. At the present time, we are not able to explain why the inhibiting effect is greater in the original activation mixture than in a reconstituted mixture; a tentative suggestion is that a minor conformational change occurs in bovine pepsin once the activation peptides are removed.

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REFERENCES

Fig. 5. Comparison of the NH2-terminal portions of bovine pepsinogen (Bpg), porcine pepsinogen (Ppg), and prochymosin (prorennin, Pch). Amino acids in homologous positions in any two of the peptide chains are written in capitals. Homologous sequences of the two pepsinogens are in boxes.
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