THE N-TERMINAL AMINO ACID SEQUENCE OF BOVINE PEPSIN* (EC 3.4.4.1) AND THE SEQUENCE OVERLAPPING THE ACTIVATION FRAGMENT

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1. Introduction

In our current research project on the structure and function of acidic proteases we have investigated the N-terminal amino acid sequence of bovine pepsin. From a tryptic digest of bovine pepsinogen we have isolated and analysed a peptide which includes the C-terminal part of the peptide fragment cleaved off during the activation of the zymogen, and the N-terminal part of pepsin.

Preliminary sequence studies on the activation fragment [1, 2] and on thermolytic peptides of pepsin [3] were important for the design of the experiment from which the overlapping sequence was obtained. The first mentioned experiments indicated that an arginine residue was present near the C-terminus of the activation fragment, in the latter an arginine containing peptide was found which was very homologous to the sequence around arginine no. 57 in chymosin (rennin EC 3.4.4.3) [2]. As the two other arginine groups in pepsin are located near the C-terminus [4] a tryptic digest of maleylated pepsinogen should give the wanted overlap in a peptide of ca. 60 residues. This turned out to be the case. The results substantiate the homology in amino acid sequences of the gastric proteases found in previous investigations e.g. [2, 4, 5 and 6].

* Where nothing else is stated the terms pepsin and pepsinogen in this letter refer to the bovine zymogen and enzyme.

2. Materials

2.1. *Pepsinogen*

Pepsinogen was prepared from the fourth stomach of the cow. The mucosa was homogenized and extracted with 0.5 M NaCl. The extract was clarified by forming a gel of aluminum phosphate in situ as previously described for the preparation of prochymosin (prorennin) [7]. The zymogen was purified by batchwise absorption on DEAE-cellulose followed by ion exchange chromatography and gel filtration as described by Chow and Kassell [8].

2.2. *Pepsin*

Pepsin was prepared from the pepsinogen by activation at pH 2 for 8 min in an ice bath. Separation of activation peptides and pepsin was performed by ion exchange chromatography on DEAE-cellulose using 0.02 M phosphate buffer pH 5.3 and a linear salt gradient to 0.5 M NaCl dissolved in the starting buffer. In order to avoid autolysis the pepsin was denatured immediately after chromatography. The eluate was diluted with an equal volume of acetone; and ammonia was added to a reading at the pH-meter of 9. The preparation was concentrated on a rotary vacuum evaporator, dialyzed against 0.05 M NH_3 and freeze-dried [9].

2.3. *Auxiliary preparations and chemicals*

Trypsin TPCK-treated (Worthington), pancreatic trypsin inhibitor "Trasylol"® (Bayer), thermolysin (Daiwa Kasei), carboxypeptidase A (Sigma), maleic anhydride (Fluka), DEAE-cellulose (Whatman DE 32); all other chemicals were of reagent grade.

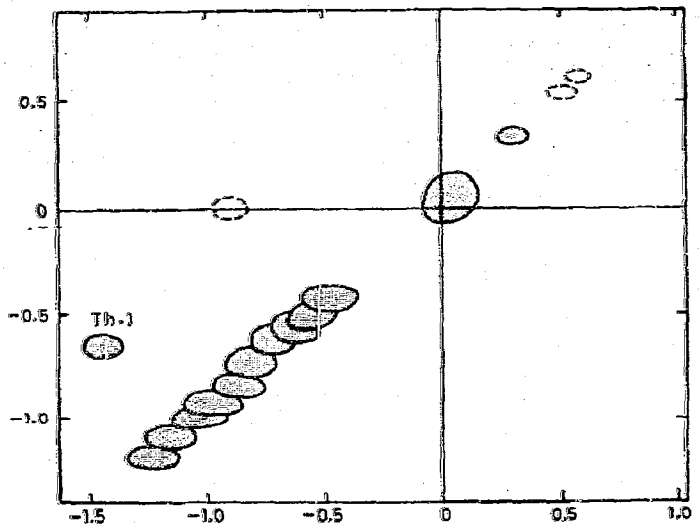


Fig. 1. Demaleylated pH 6.5 diagonal electrophoretic map of a thermolytic digest of maleylated pepsin. Conditions are described in the text. The mobilities are expressed relative to the mobility of an internal marker of 1-dimethylamino naphthalene-5-sulphonic acid (−1.0).

3. Experimental

175 mg (approx. 5.3 μmole) of the denatured pepsin was maleylated according to Butler et al. [10]. After dialysis the preparation was digested with 6 mg of thermolysin for 17 hr at 37°C (*N*-ethyl morpholine acetate buffer pH 8, 0.02 M). One third of the digest was directly subjected to high voltage paper electrophoresis in pyridine acetate buffer pH 6.5. After demaleylation a guide strip of the electropherogram was examined by the diagonal electrophoretic technique [10]. Only one predominant off diagonal spot showed up after staining with ninhydrin (fig. 1). The corresponding band of the main sheet was demaleylated and subjected to electrophoresis under the same conditions as the guide strip. The peptide (Th. 1) was further purified by electrophoresis at pH 3.5 before it was eluted, analysed and sequenced by sequential Edman degradation and dansylation [11].

From the remaining part of the digest several other peptides were purified after gel filtration on Sephadex G-25 (fine) followed by paper electrophoresis and paper chromatography. The sequence of one peptide (Th. 2) will be considered here since it appears to be adjacent to Th. 1. The other results will be reported in a subsequent paper.

The freeze-dried pepsinogen was denatured by dissolution in 50% acetone and addition of ammonia. After dialysis and lyophilization it was redissolved in 5 M guanidinium-HCl and maleylated. The preparation

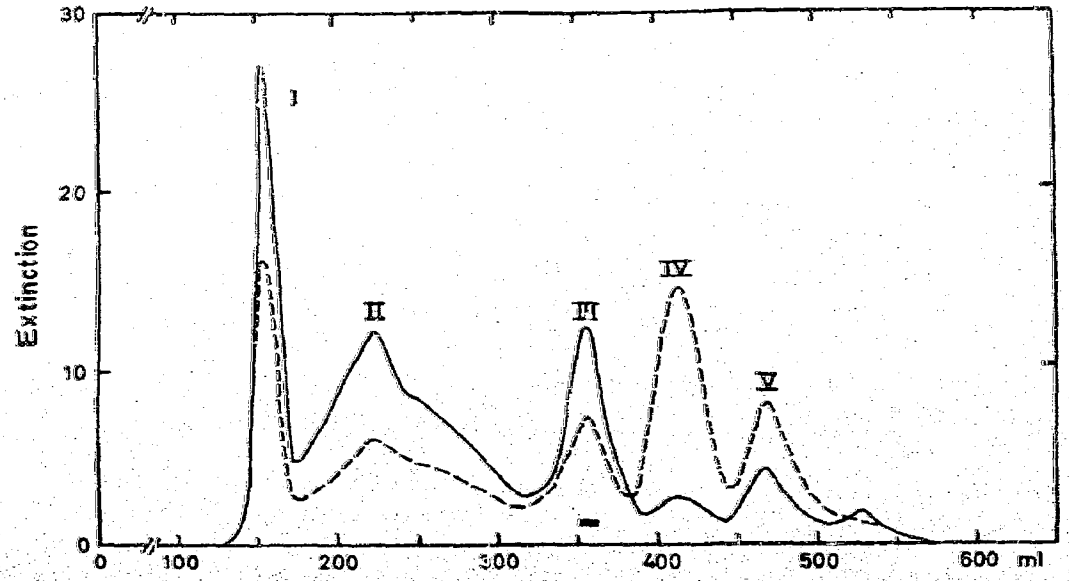
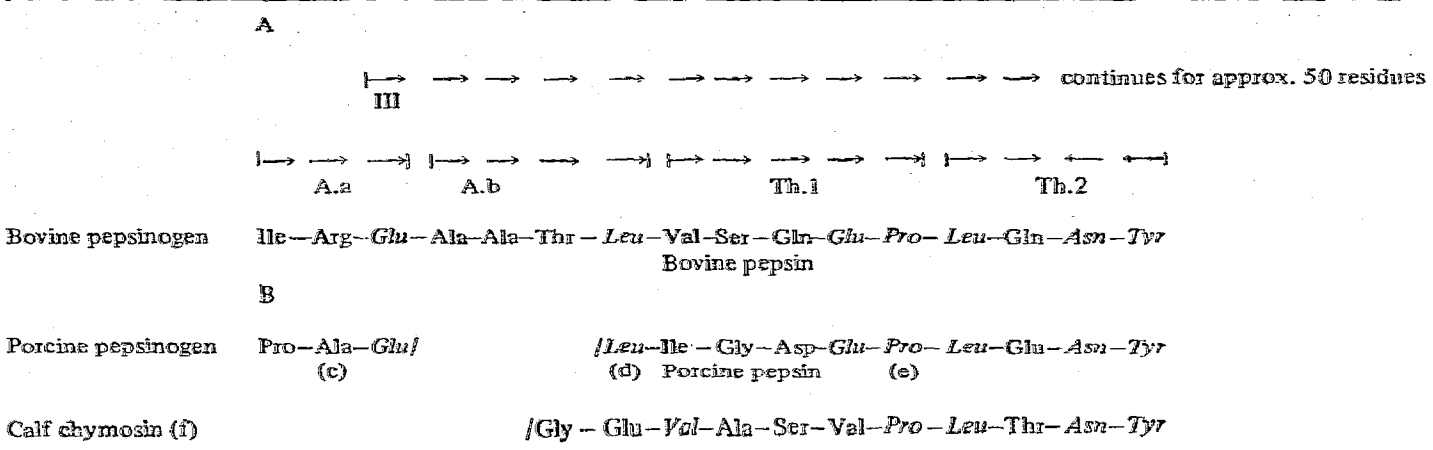


Fig. 2. Gel filtration of a tryptic digest of maleylated pepsinogen. Conditions: Sephadex G-100, 2.5 × 100 cm. Eluant: 0.05 M NH_4HCO_3. Flow rate: 25 ml/hr. Solid line: E_{280}, dashed line: E_{255}.

Table 1

The amino acid sequence overlapping the activation fragment and the pepsin moiety of bovine pepsinogen compared with known fragments from porcine pepsinogen, pepsin (EC 3.4.4.1) and calf chymosin (rennin) (EC 3.4.4.3).



→ Indicates that the position of the amino acid residue was located by sequential Edman degradation/dansylation. ← Shows liberation of amino acids with carboxypeptidase A. Bold face amino acid residues mark the N-termini of the enzyme formed during normal acid activation of the zymogens. Italics indicate that the same amino acid is present in two or more of the enzymes. Part A: Th.1 and Th.2 represent the N-terminal amino acid sequence of bovine pepsin. III shows the N-terminus of the tryptic peptide from peak III (fig. 2). A.a and A.b are two peptides isolated from an activation mixture of bovine pepsinogen (see [1] and [2]). Part B: c) is the C-terminus of the activation fragment from porcine pepsinogen according to Ong and Perlmann [16]. d) Leu preceeding the N-terminus of porcine pepsin according to Stepanov et al. [17], e) N-terminus of porcine pepsin (Tang [6]), f) N-terminus of calf chymosin [5], (the sequence of the C-terminal section of the activation fragment from this zymogen is not yet completed).

was again dialyzed and lyophilyzed. 140 mg (approx. 3.6 μmole) was then redissolved in 10 ml of distilled water and digested with 2 mg of trypsin for 10 minutes at 10°C, pH was kept constant at 7.8 by addition of 0.05 NaOH [12]. The reaction was terminated by addition of 0.5 ml "Trasylol"® solution. The digest was fractionated by gel filtration on Sephadex G-100 using 0.05 M NH_4HCO_3 as eluant (fig. 2). The central part of peak III (marked with the solid bar) appeared better than 95% homogeneous by disc electrophoresis in 12.5% polyacrylamide [13]. The peptide was freeze-dried and subjected to sequential Edman degradation [11] and dansylation [14].

4. Results

The amino acid sequences of peptide Th.1 and Th.2 are illustrated in table 1. The absence of lysine in Th.1 and the fact that the peptide did not stain with ninhydrin before demaleylation clearly identify Th.1 as the N-terminal amino acid sequence in pepsin which has had the N-terminal NH_2-group blocked by the maleylation. Calculation of the electrophoretic mobilities relative to the molecular weight [15] indicates that the peptide carried 3 negative charges in the first electrophoretic dimension and 1 in the second. After the third Edman degradation the remaining peptide behaved acidic indicating that residue no. 4 is Glu. Since Th.2 is a neutral peptide Asn and Gln must be present here. In fig. 2 the front peak is an aggregation product and peak II contains most of the pepsin part of the peptide chain. The peptides included in peak IV and V of the tryptic pepsinogen digest comprise the major part of the activation fragment. The sequence of these will be published in a joint paper together with Beatrice Kassell and coworkers. The peptide from peak III was sequenced through 12 steps of Edman degradation as shown in table 1. It is obvious that this peptide includes the N-terminal part of pepsin and further that it provides an overlap for Th.2. In addition to the peptides analysed in this letter,

table 1 includes homologous sequences from other gastric proteases and some peptides from the activation fragment reported in [1] and [2]. The neutral activation peptide Ile—Arg—Glu includes the arginine residue which presumably preceeds peptide III and it indicates that the first residue in peptide III is Glu.

5. Discussion

With reference to the experimental conditions it is worthy to note that we found proper denaturation of pepsinogen was required before maleylation. In a preliminary experiment a considerable amount of activation peptides were liberated during the maleylation, indicating that local environments of low pH were able to produce short term peptic activity. Another series of preliminary experiments have shown that pepsinogen and pepsin are rather prone to unspecific tryptic hydrolysis. This is the reason for the very restricted conditions of tryptic digestion used here. Previous comparison between the N-termini of porcine pepsin and calf chymosin [6] has shown that the ultimate residues have little resemblance, but from Pro (no. 5 in porcine pepsin) the homology appears. The results from bovine pepsin fit into this pattern.

For the preliminary arrangement of peptides obtained from the activation fragment of bovine pepsinogen both Kassell and coworkers [1] and we [2] have relied on an expected homology with the N-terminal amino acid sequence of porcine pepsinogen reported by Ong and Perlmann [16]. However both groups found a tetrapeptide Ala—Ala—Thr—Leu which did not correspond to any of the sequences reported by Ong and Perlmann. The present results show that this peptide preceeds the pepsin moiety of the zymogen, this means that this segment either is missing in porcine pepsinogen or it has not yet been observed. The data of Stepanov et al. [17] favour the latter possibility. These authors found that a limited proteolysis of porcine pepsinogen with an *Aspergillus* proteinase produced a pepsin with an additional N-terminal leucine. The enzymatic activity of the leucine-pepsin described by Stepanov et al. makes it difficult to state which bond is essential to hydrolyse in order to activate the zymogens of the gastric proteases. Considering the minor degree of homology in these parts of the primary structures we are in favour of the hypothesis that unlike the pancreatic serine proteinases the N-terminus formed during the activation does not directly participate in the formation of the active center. Activation takes place by hydrolysis of an exposed peptide bond; thereby liberating a peptide which carries positive charges, which at pH above 5 have kept the zymogen in inactive conformation [2, 7 and 16].

Acknowledgements

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