CHARACTERIZATION AND ARRANGEMENT OF TRYPTIC FRAGMENTS FROM N-TERMINAL REGION OF HOG PEPSIN

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1. Introduction

The cyanogen bromide hydrolysis of hog pepsin at the methionine residues [1] gives rise to a large fragment which involves the N-terminal half of its chain, lacking arginine and lysine residues [2,3]. For additional specific cleavage it is convenient to aminoethylate the half-cystine residues [4, 5] as has been described for the whole pepsin molecule [6]. Trufanov et al. [7] aminoethylated the N-terminal cyanogen bromide fragment of pepsin and isolated from its tryptic digest the N-terminal peptide which they assumed to contain 105 amino acid residues.

In this study the tryptic fragments derived from the aminoethylated N-terminal cyanogen bromide fragment [2] were isolated and their sequence in the chain determined. The phosphoserine residue contained in pepsin [8] was localized and material was obtained for sequence studies on the N-terminal half of the molecule of this enzyme.

2. Experimental

The N-terminal fragment CB2 was isolated in an earlier study [2] from the cyanogen bromide hydrolysate of *S*-sulfo-pepsin by discontinuous recycling chromatography [9]. For the aminoethylation of 3 g of fragment CB2, the procedure described for pepsin [6] was employed and the same excess of reagents per one half-cystine residue was retained. Ethylene imine and 2-amino-2-methyl-1,3-propanediol were preparations of Koch and Light (UK), 2-mercaptoethanol was purchased from Fluka (Switzerland). The aminoethylated fragment (CB2-RAE) was isolated from the reaction mixture by gel filtration on Sephadex G-25 in dilute ammonium hydroxide (pH 9). A 1% solution of this material (2.7 g) was made alkaline (phenol red) by ammonium bicarbonate and digested with TPCK-trypsin [10] (Worthington, U.S.A.) at a weight ratio 1:50, 4 hr, at 37°. The digest was fractionated on an 80 × 8 cm column of Sephadex G-25 in dilute ammonium hydroxide (pH 9). Fractions (100 ml/15 min) were assayed by absorbance measurement at 280 nm and by paper chromatography of aliquots (1 ml). Only one peak, involving the high molecular weight fraction CB2-RAE-T1 was detected by absorbance measurement. The retarded fraction, CB2-RAE-T2, detected on paper chromatograms, was fractionated further by paper chromatography and electrophoresis. Fraction CB2-RAE-T1 was fractionated by discontinuous recycling chromatography (fig. 1) on a 260 × 10 cm column of Sephadex G-100 in 0.3 M ammonium acetate containing 8 M urea (pH 5.0), using the equipment and procedure described elsewhere [9]. The material contained in peaks CB2-RAE-T11b and CB2-RAE-T12 was desalted on Sephadex G-25 and lyophilized.

The phosphopeptide was isolated from 80 mg of CB2-RAE-T11b after digestion with chymotrypsin (4 hr at 37° and a molar ratio 1:50). The purified phosphopeptide CB2-RAE-T11b-C21 (1.25 μmole) was then digested with thermolysin (4 hr at 37° and a molar ratio 1:50). The thermolysin peptides were isolated by paper chromatography and electrophoresis. These techniques and also the methods of characterization of peptides have been described elsewhere [2] as well as the technique of Edman degradation used [11].

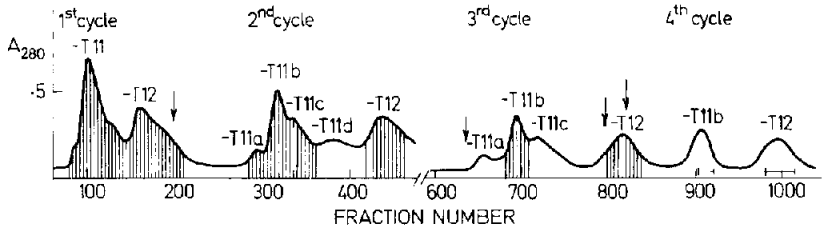

Fig. 1. Discontinuous recycling chromatography of the tryptic digest of fragment CB2-RAE. The sample (2.5 g) was dissolved in 500 ml of 0.3 M ammonium acetate, containing 8 M urea, pH 5.0. The Sephadex G-100 column (260 × 10 cm, equilibrated and eluted with the same solution) and the recycling technique have been described elsewhere [9]. Fractions of 100 ml/hr were collected. The areas selected for recycling are marked by hatching. The sites of reapplication of fractions to be recycled are marked by arrows, except for the site of application of regions -T11a to -T11c in the third cycle, which is at fraction no. 560, situated in the interrupted part of the graph. The arrow at fraction no. 637 designates the beginning of reapplication of region -T12. After 4 cycles the effective column height was 10.4 m.

The phosphate ester was detected by the Hanes–Isherwood reagent [12].

3. Results and discussion

Three tryptic fragments were isolated from the digest. Fragment CB2-RAE-T12 behaved as a homogeneous material when subjected to Edman degradation and its N-terminal sequence Ile–Gly–Asp–Glu–Pro–Leu–Glu–Asn–Tyr––X––Asp was determined by this technique. The sequence of the first 9 residues is identical with the reported N-terminal sequence of 9 residues of pepsin [13,2]. The product in the 10th step "X" was not determined unambiguously. Fragment CB2-RAE-T12 thus involves the proper N-terminal region of pepsin to the first aminoethyl-cysteine residue (fig. 2). The lower analytical value for aminoethyl-cysteine (table 1) was explained in subsequent sequential studies [14] on this region by partial cleavage of the C-terminal bond ...Tyr-AEC during tryptic hydrolysis (AEC stands for aminoethyl-cysteine).

Only one peptide, CB2-RAE-T2121 (table 1) was isolated from fraction CB2-RAE-T2. Its amino acid sequence Ser–Ser–Leu–Ala-AEC was determined by Edman degradation. In accordance with the reported data on the sequence around the first disulfide [15–17] it may be assumed that this peptide has been formed by cleavage at the AEC residues and that it represents the inner part of the disulfide loop [15] ...Cys–Ser–Ser–Leu–Ala–Cys–Ser–Asp–His... extending the N-terminal fragment CB2-RAE-T12.

As fragment CB2-RAE-T11b has the lowest elution volume on gel filtration, it is the largest of all components isolated. Its N-terminal sequence Ser–Asp was determined by Edman degradation and the presence of histidine was revealed by amino acid analysis. The homoserine content indicates that this tryptic fragment represents the C-terminal part and completes the sequence of the three tryptic fragments obtained (fig. 2). The presence of a small quantity of aminoethyl-cysteine (table 1) indicates traces of contaminants. Final conclusions in its exact amino acid composition will emerge from the sequence studies on this region.

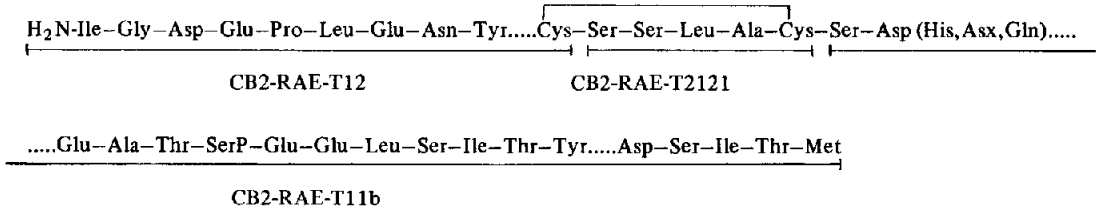

Fig. 2. Arrangement of tryptic fragments in N-terminal half of pepsin molecule.

Table 1
Amino acid composition of peptides.

	T11b	T12	T2121	T11b-C21 -	T11b-C21 -TH1	T11b-C21 H
His	0.6	–	–	–	–	–
Asp	13.0	6.2	–	–	–	–
Thr	8.1	5.0	–	2.0	1.0	–
Ser	14.0	4.5	1.8	1.8	1.0	1.1
Glu	8.1	3.4	–	3.0	2.8	–
Pro	4.7	2.8	–	–	–	–
Gly	11.7	5.2	–	–	–	–
Ala	4.0	1.2	1.0	1.0	1.0	–
Val	6.4	3.0	–	–	–	–
Ile	6.1	3.3	–	0.8	–	–
Leu	10.1	3.7	1.0	1.0	–	0.9
Tyr	5.9	3.0	–	0.4	–	–
Phe	4.7	2.6	–	–	–	–
HSE[a]	1.0	0.1	–	–	–	–
AEC[a]	0.1	0.5	0.8	–	–	–
Trp[b]	+	+	–	–	–	–

The peptides are marked by symbols shorter by the initial designation CB2-RAE-.
The peptides were analyzed after 20-hr hydrolysis; none of the samples contained arginine, lysine, or methionine. a) The symbols HSE and AEC are those for homoserine and aminoethyl-cysteine, respectively. b) Tryptophan was determined only qualitatively on chymotryptic maps.

From the chymotryptic digest of fragment CB2-RAE-T11b a characteristic acidic peptide, -C21 (CB2-RAE-T11b-C21), was isolated. Its amino acid composition (table 1) is identical with that of the phosphopeptide, localized in the N-terminal half of pepsin and sequenced by Vakhitova et al. [18]. Peptide -C21 gave a positive test with the Hanes–Isherwood reagent for phosphate esters, present in pepsin as a phosphoserine residue [8]. Its partial amino acid sequence was determined by Edman degradation. The product from the fourth step "Y" was not characterized. The C-terminal position of tyrosine can be deduced from the specificity of chymotrypsin. From the thermolysin digest of peptide -C21 peptide -TH1, reacting with the reagent for phosphate esters, as well as peptide -TH2 were isolated.
(SerP stands for the phosphoserine residue)

The remaining C-terminal tripeptide was not obtained, however, due to the specificity of thermolysin, isoleucine should be its N-terminal amino acid.

From a summary of these results the sequence Glu–Ala–Thr–SerP–Glu–Glu–Leu–Ser–Ile–Thr–Tyr is obtained in agreement with the reported data [18] and partly with [15]. Its localization in fragment CB2-RAE-T11b, however, permits the phosphopeptide to be assigned to a narrower region between the second half-cystine and the first methionine residue (fig. 2) of pepsin.

The additional components -T11a,-T11c, and -T11d, whose chymotryptic peptide maps resemble that of the homoserine fragment CB2-RAE-T11b, have not been characterized in detail. Trufanov et al. have determined the size of the N-terminal tryptic fragment as being 105 of the total 158 amino acid residues of original fragment CB2 [7]. This finding is not in agreement with its highest elution volume on gel filtration. In subsequent sequential studies [14] the size of fragment CB2-RAE-T12 has been determined as being 45 amino acid residues; hence, the half cystines occupy positions no. 45 and 50 with respect to the N-terminus of pepsin. The results of this study and previous data from the Prague Laboratory [2, 16] are summarized in fig. 2. The region involved in fragment CB2-RAE-T11b is being studied at present.

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CB2-RAE-T11b-C21: Glu–Ala–Thr –Y– Glu–Glu–Leu(Ser, Ile, Thr)Tyr
-TH1: (Glu, Ala, Thr, SerP, Glx, Glx)
-TH2: Leu–Ser

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