AMINO ACID SEQUENCE AROUND THE REACTIVE CYSTEINYL RESIDUE OF CHICKEN PEPSIN

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The amino acid sequence of the cysteine-containing peptide was determined in two different manners. *1*) Chicken pepsin was covalently coupled *via* its SH-group to Thiopropyl-Sepharose 6B, subjected to peptic digestion and cysteinyl peptides, generated by multiplicity of peptic attack on the sequence ..↓Tyr-Tyr-Cys-Asn↓Phe↓.. (marked by arrows), were eluted by 2-mercaptoethanol. *2*) Systematic structure analysis of the tryptic-chymotryptic digest of chicken pepsinogen afforded peptide Tyr-Tyr-Cys-Asn-Phe-Asp-Gly-Ile-Leu-Gly-Leu, whose C-terminal part is highly homologous with other pepsins. These data permitted the cysteinyl residue to be assigned to position 115 (numbered according to hog pepsin A) in a relatively variable region. The same position is occupied by an alanyl residue in hog pepsin A and calf chymosin; this replacement can be considered conservative from the viewpoint of molecular volume.

Pepsin isolated from chicken forestomachs is unique among gastric carboxyl proteinases in containing a reactive cysteinyl residue in its molecule[1,2]. This residue is not situated either in the N- or C-terminal part of the pepsin(ogen) molecule or in the neighborhood of disulfide bonds[3–5]. Even though it does not play a role in the catalytic mechanism of the enzyme and probably is not involved in its binding site either, its covalent modification with bulky reagents markedly affects the kinetic parameters of peptic hydrolysis[6,7]. In this study the primary structure is presented of the peptide containing this reactive cysteinyl residue which was isolated both specifically by thiol-disulfide exchange on Thiopropyl-Sepharose and also by systematic sequential analysis of the tryptic-chymotryptic digest of chicken pepsinogen. The amino acid sequence of the peptide is compared with the known primary structures of the corresponding sites of other carboxyl proteinase molecules.

EXPERIMENTAL

Materials and Methods

Pepsin prepared from chicken pepsinogen[3] contained 0·9—1·0 mol of cysteine per mol of protein, as determined by titration with 2,2'-dithiopyridine[8]. The preparation of the S-sulfo derivative of chicken pepsinogen (SSCPG) has been described elsewhere[3]. Thiopropyl-Sepharose-6B was supplied by Pharmacia Fine Chemicals AB, Uppsala, Sweden.

The bonding of pepsin (2·8 μmol), dissolved in 0·1M Tris-HCl buffer, pH 7·5, containing 1 mM EDTA (12 ml), to Thiopropyl-Sepharose (12 ml) was effected under nitrogen (18 h) as described in[9]. After denaturation in 6M guanidine hydrochloride the immobilized protein and the support were equilibrated with 0·1M formic acid (12 ml) and digestion with hog pepsin (enzyme to substrate ratio 1 : 25, w/w) was allowed to proceed 6 h at 22°C. The column was subsequently washed with 0·1M formic acid, 6M guanidine hydrochloride and water, and the thiol-containing material was eluted by 60 mM 2-mercaptoethanol in 0·1M-NH_4HCO_3, pH 8·5, and lyophilized. The lyophilisate was fractionated on a column of Sephadex G-25 (Fig. 1). The peptides were purified by high voltage electrophoresis and paper chromatography[4].

The trypsinolysis of SSCPG and the fractionation of the digest on Sephadex G-25 fine have been described before[5]. The chymotryptic digestion (enzyme to substrate ratio 1 : 100, w/w) of the high molecular weight fraction of the tryptic peptides was carried out in 15 ml of 0·5% NH_4HCO_3, pH 8·25, for 2 h at 37°C. The digest was resolved on Sephadex G-25 fine (Fig. 2). Aliquots of the individual peaks were oxidized and analyzed for the presence of cysteic acid. In positive cases the SH-peptide was sought by fingerprinting and subsequent amino acid analysis of the eluted zones. The peptides were purified by high voltage electrophoresis and paper chromatography.

Amino acid analyses[10] were performed on Durrum D-500 amino acid analyzer. Cysteine and S-sulfo-cysteine were determined as cysteic acid after oxidation by performic acid[11]. The manual Edman degradation and the identification of amino acid phenylthiohydantoins by thin-layer chromatography on silica gel (Silufol) were carried out as described in[3,5]. The phenylthiohydantoin of cysteic acid was identified by paper electrophoresis[12].

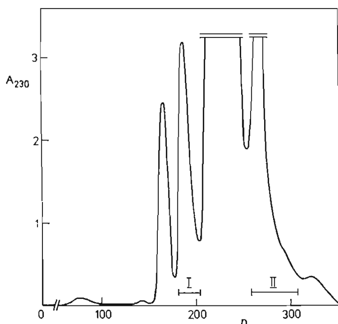

FIG. 1

Chromatography of SH-peptides from Thiopropyl-Sepharose 6B on column (1·7 × 140 cm) of Sephadex G-25 fine in 2 mM formic acid. The fractions were collected at a rate of 2 ml per 10 min; *n*, fraction number

RESULTS AND DISCUSSION

Peptides Isolated by Covalent Chromatography

About 60% (by weight) of the enzyme was bonded to Thiopropyl-Sepharose under the conditions described. The denaturation of the immobilized protein in 6M guanidine hydrochloride was a necessary prerequisite of the subsequent profound peptic hydrolysis. The material eluted by 2-mercaptoethanol was resolved by gel filtration into several fractions (Fig. 1), yet fractions I and II only contained peptide material. The latter contained tyrosine as the single N-terminal amino acid in both cases. Two peptides, Tyr-Cys-Asn-Phe and Tyr-Cys-Asn were isolated from fraction I by paper electrophoresis. Similarly, peptides Tyr-Tyr-Cys-Asn-Phe and Tyr-Tyr-Cys--Asn were found in fraction II. The total yield of the purified peptides based on cysteic acid was about 20% of the starting quantity of immobilized protein. No other peptides were found. The remaining fractions showing absorbance at 230 nm contained material of nonpeptidic character, most likely arising from the thiopyridine structures of the support. This material was not removed by pre-washing the column with 4 mM dithiothreitol at pH 4 as recommended in similar cases[13].

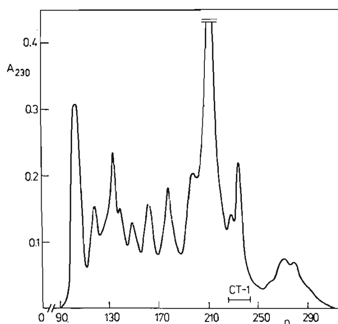

FIG. 2

Chromatography of chymotryptic digest of large tryptic peptides from SSCPG on column (4·4 × 200 cm) of Sephadex G-25 fine in dilute acetic acid (pH 3·5). Flow rate 60 ml/h, fraction volume 10 ml; *n*, fraction number

Tryptic-chymotryptic Digest

When the individual fractions obtained by chromatography of the digest on Sephadex G-25 (Fig. 2) were screened, the half-cystine residues were found in several fractions. The individual peptides were isolated by peptide maps and found, save for one exception, to be derived from the individual disulfide bridges. The fraction containing the peptide which did not correspond to any bridge was considerably retained in the column (Fig. 2, marked by a bar). The primary structure of the peptide (CT-1) obtained from this fraction is shown in Fig. 3.

Assignment to the Structure of Carboxyl Proteinases

Peptide CT-1 is by 6 residues longer at the C-terminus than the peptides isolated on Thiopropyl-Sepharose because of limited chymotryptic attack. These 6 residues fall into the region of the structure of carboxyl proteinases which is highly homologous (Fig. 3) and represents one of the β-strands of the molecule (residues 118 – 126 in the system of numbering according to hog pepsin) (ref.[14,15]). This permits peptide CT-1 to be assigned to positions 113 – 124. The reactive cysteinyl residue occupies position 115 in the relatively variable region. Hog pepsin and calf chymosin bear an alanine residue in the same position. Unlike in intracellular SH-proteinases the sulfhydryl group of chicken pepsin seems to be lacking any more important physiological role; it is obviously localized in the core of the molecule. Viewed from the point of molecular volume the replacement of alanine by cysteine may be regarded as „conservative". The preferential reactivity of the SH-group to reagents with aromatic groups[7] can be caused by effects of cumulated adjacent apolar groups in the sequence of CT-1, as suggested by Schechter and coworkers (ref.[7]). The chicken pepsin modi-

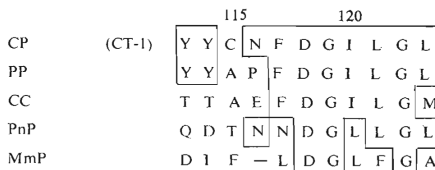

FIG. 3

Amino acid sequences around residue 115 in chicken pepsin (CP), hog pepsin A (PP), calf chymosin (CC), penicillopepsin (PbP), and *Mucor miehei* pepsin (MmP) (ref.[17 – 20]). The system of numbering is according to PP. Identical residues in two or more enzymes are boxed in

fied by these authors displayed a "catalytic approach" to hog pepsin; from this phenomenon the authors concluded that hog pepsin has an aromatic residue in the same position. The elucidation of the complete amino acid sequence of hog pepsin did not confirm this assumption. Another recent finding[16] deserving interest is that still another carboxyl proteinase, rat renin, contains one free, partly buried sulfhydryl group which is inessential for the activity of the enzyme; in this respect rat renin resembles chicken pepsin.

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