C-TERMINAL AMINO ACID SEQUENCE OF CHICKEN PEPSINOGEN AND ITS HOMOLOGY WITH SEQUENCES OF OTHER ACID PROTEASES

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To determine the primary structure of the C-terminal part of the molecule of chicken pepsinogen the tryptic, chymotryptic and thermolytic digest of the protein were investigated and peptides derived from this region were sought. These peptides permitted the following 21-residue C-terminal sequence to be determined: ...-Ile-Arg-Glu-Tyr-Tyr-Val-Ile-Phe-Asp-Arg-Ala-Asn-Asn-Lys-Val-Gly-Leu-Ser-Pro-Leu-Ser.COOH. A comparison of this structure with the C-terminal sequential regions of the other acid proteases shows a high degree of homology between chicken pepsinogen and these proteases (*e.g.*, the degree of homology with respect to hog pepsinogen and calf prochymosin is about 66%). Additional tryptic peptides, derived from the N-terminal part of the zymogen molecule whose amino acid sequence has been reported before, were also obtained in this study. This sequence was extended by two residues using an overlapping peptide. An ancillary result of this study was the isolation of tryptic peptides derived from other regions of the zymogen molecule.

Chicken pepsinogen, like other pepsinogens, falls into the group of acid protease zymogens. Even though this group of proteins has been studied for a very long time, it was not until recently that the amino acid sequences of its first three representatives[1-5] were completely elucidated and their high degree of homology shown. A hypothesis has been voiced[6] that both the N- and the C-terminal parts of the pepsinogen molecules greatly participate on maintaining the inactive conformation of the zymogens. A key role in this process play cumulated basic amino acid residues which engage in electrostatic interactions with the negatively charged enzyme part of the molecule. It has been[7,8] shown that the N-terminal 26-residue amino acid sequence of chicken pepsinogen contains a great number of basic residues and is highly homologous with the corresponding structures of other pepsinogens.

The aim of the present study was to determine the C-terminal amino acid sequence of chicken pepsinogen and to show to what degree this sequence complies with the general structural features of acid proteases.

EXPERIMENTAL

Material

Chicken pepsinogen, pepsin, and S-sulfo-pepsinogen were prepared by procedures described earlier[7]. TPCK-trypsin and α-chymotrypsin were from Worthington Biochemical Corporation, Freehold, N. J., U.S.A. Thermolysin and soy bean trypsin inhibitor were 3-times crystallized preparations of Calbiochem, San Diego, Cal. U.S.A. Sephadex G-25 fine was a product of Pharmacia, Fine Chemicals Uppsala, Sweden. The chemicals used for sequential degradation were supplied by the sequencer manufacturer, *i.e.* Beckman Instr., Spinco Div., Palo Alto, Cal., U.S.A. Silufol, silicagel layers, were from Kavalier, Czechoslovakia. Polyamide layer sheets were from BDH Chemicals Ltd., Poole, England. All the remaining chemicals used were of analytical purity.

Methods

Small peptides were purified on Whatman No 3MM paper by high voltage electrophoresis at pH 5·6 in the buffer water–pyridine–acetic acid (994 : 5 : 1, v/v) at a potential gradient of 30 V/cm (ref.[9]) or at pH 1·9 in the system water–acetic acid–formic acid (16 : 3 : 1, v/v) at a potential gradient of 70 V/cm (ref.[10]), and by chromatography in the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v, ref.[11]). Quantitative amino acid analysis was carried out by the method of Spackman and coworkers[12] as modified by Benson and Patterson[13]. S-Sulfo-cysteine was determined after oxidation by performic acid according to Moore[14] as cysteic acid. The N-terminal amino acids of the individual peptides were identified after dansylation according to Gray[15] by thin-layer chromatography on polyamide layer sheets[16]. Hydrolysis by 1M-HCl in methanol[17] was used when the presence of N-terminal pyrrolidone carboxylic acid was suspected. The peptide (5 nmol) was hydrolyzed 16 h in 20 µl of acidic methanol at 37°C. The N-terminal glutamic acid γ-methyl ester formed was identified as the dansyl derivative of glutamic acid. The stepwise degradation of peptides was carried out by the phenylisothiocyanate method according to Edman[18]. The structure of the two longest peptides was determined in Beckman-Spinco Model 890 C Amino Acid Sequenator. The samples were fixed by the Braunitzer reagent[19] and the degradation itself was carried out according to Beckman program No 102974. The phenylthiohydantoins obtained after the conversion of the thiazolinones were identified by thin-layer chromatography on silica gel (Silufol)[20] or by gas chromatography in Beckman Model GC-65 Gas Chromatograph either without or after silylation[21].

Tryptic digestion of chicken S-sulfo-pepsinogen and fractionation of the digest. The protein (300 mg) was dissolved in 20 ml of 1% $(NH_4)_2CO_3$ and the pH of the solution was adjusted to 8·55 by glacial acetic acid. Subsequently 3 mg of TPCK-trypsin dissolved in 2·5 ml of water was added. After 2 h incubation at 37°C additional 3 mg of TPCK-trypsin in 1·5 ml of water was added to the mixture and the incubation was continued for 2 more hours (final enzyme to substrate ratio 1 : 50, w/w). The mixture of peptides was separated on a column of Sephadex G-25 fine (4·4 × 200 cm) equilibrated with 0·5% NH_4HCO_3. The fractionation of the peptides was monitored by absorbance measurement of the individual fractions at 230 and 280 nm and by paper chromatography of aliquots (8%) taken from the individual fractions. Peptides deserving interest from the viewpoint of the final goal of this study were purified by paper techniques.

Thermolysin digest of chicken pepsin and its separation. Chicken pepsin (700 mg) was dissolved in 30 ml of 0·1M-Tris-HCl buffer, pH 8·0. The digestion was allowed to proceed 18 h at 38°C. Thermolysin was added in two portions (at 0 and 6 h, final enzyme to substrate ratio 1 : 30, w/w). A slight turbidity was removed by centrifugation. The fractionation of the thermolytic peptides was carried out by the same procedure as the fractionation of the tryptic fragments.

Chymotryptic digest of chicken pepsin and its fractionation. Chicken pepsin (70 mg) was dissolved in 6 ml of water and the pH of the solution was adjusted to 10·7 by 0·1M-NaOH. This pH-value was decreased after 30 min of pepsin inactivation to pH 8·5 by the addition of 0·3M-HCl. Subsequently 1·3 mg of α-chymotrypsin and 1·3 mg of soy bean trypsin inhibitor were added and the mixture was incubated 18 h at 38°C (final enzyme to substrate ratio 1 : 54, w/w). The peptides were isolated by paper techniques.

Chymotryptic digestion of certain tryptic peptides. Peptide TP-2* (50 nmol) was dissolved in 40 µl of water and the solution was made alkaline by 1% $(NH_4)_2CO_3$ (phenol red was used as an indicator). The cleavage was effected by α-chymotrypsin (1 nmol) in the presence of soy bean trypsin inhibitor (2 nmol) at 37°C. After 2 h the same quantity of α-chymotrypsin and the inhibitor was added and the mixture was incubated for 2 more hours. Peptide TP-13* (µmol) was digested under identical conditions yet with the omission of the inhibitor.

Thermolytic digestion of peptide TP-14.* The peptide (100 nmol) was dissolved in 100 µl of 0·1M-NH_4HCO_3, pH 8·0, and 2·5 nmol of thermolysin in 4 µl of buffer was added. After 4 h incubation at 37°C the same quantity of enzyme was added and the incubation was continued for 4 h.

RESULTS

Tryptic peptides: The tryptic digest was cut into 12 portions according to the elution profile and paper chromatography of aliquots; the individual fractions contained in these cuts were pooled and lyophilized (Fig. 1). The pooled fractions were analyzed by the technique of peptide maps which served as a guide for preparative separation by paper techniques. Fractions I and II containing according to their peptide maps large peptides only, have not been treated further since they are not

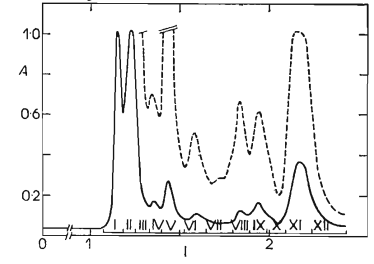

FIG. 1

Chromatography of Tryptic Digest of Chicken S-Sulfo-Pepsinogen on Column of Sephadex G-25 Fine

The quantity of digest applied to the 4·4 × 200 cm column was 300 mg. Flow rate 60 ml/h, fractions 10 ml/10 min. Eluted by 0·5% NH_4HCO_3. The individual pooled fractions are marked by Roman numerals. *A* absorbance at 280 nm ——— and 230 nm – – –, *l* volume in liters.

* See Table I.

TABLE I

Amino Acid Composition of Peptides Isolated

The values are not corrected. The analyses were performed on 20 h hydrolysates. None of the peptides contained methionine or tryptophan. Tryptic, thermolytic, and chymotryptic peptides are marked by the symbols TP, TLP, and CP, respectively.

Designation of peptide	Number of residues															
	Lys	His	Arg	Cys	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe
TP-1		0·7	1·0				1·1						0·8			
TP-2	1·9								0·9			0·9		1·0		
TP-3	1·0									1·0						
TP-4			0·8				0·8							1·0		
TP-5	1·0		1·0				1·0							1·1		
TP-6	1·7							1·0						1·0		
TP-7	0·9							1·0						1·0		
TP-8	0·9	0·8			1·8			1·0		1·0				2·7		1·1
TP-9	1·7				1·0		1·0		1·7		1·0				0·7	
TP-10	1·0	0·9		0·8[a]	1·1		2·7				1·0					
TP-11	1·1		0·8		1·0		0·8		1·1							0·8
TP-12	0·9				1·0		1·0		1·0							0·8
TP-13	0·9				3·0	2·8	1·1	1·2	0·8	3·8		0·9	1·0	1·1	0·8	1·9
TP-14	1·0			0·8[a]	3·8		2·9	1·0		1·8		1·0	1·8	1·0		
TP-15			0·9		1·1			1·0				0·7	0·7		1·6	1·0
TP-16	1·0				2·1						1·0					
TP-17							1·9		1·0	1·2		1·0		2·0		
TLP-1	1·1		1·0		2·8					1·1	1·0	1·0				
TLP-2	1·0						0·9						1·0			
CP-1			0·9					1·2	[illegible]				1·0		0·9	
CP-2					2·0				1·0	2·0			1·0		0·8	
CP-3	1·0					1·7										

[a] Determined as cysteic acid after oxidation by performic acid.

derived either from the N- or the C-terminal part of the molecule. All the remaining pooled fractions were separated by a combination of paper chromatography and electrophoresis. Seventeen tryptic peptides were thus obtained whose homogeneity was examined by amino acid analysis and dansylation. Except for one exception all the peptides obtained were suitable for subsequent sequential work. The amino acid composition of the individual peptides is given in Table I, the primary structures determined are shown in Figs 2 and 4. In four instances the amino acid sequence was studied by other methods. Peptide TP-2 yielded in the 4th and 5th degradation step a considerable quantity of leucine; the structure determined by sequential degradation was therefore checked by the analysis of the chymotryptic digest of the peptide. The peptide map of the digest contained after ninhydrin staining two peptides only whose positions correspond to peptides Val-Pro-Leu and Lys-Lys. Tripeptide TP-7 yielded in the first degradation step traces of glutamine only and it was assumed that pyrrolidone carboxylic acid had formed during the preparation. The structure of this peptide was confirmed by methanolysis and subsequent dansylation and also by a comparison with the structure of peptide TP-6 involving the amino acid sequence of the preceding peptide. Peptide TP-13 was degraded in the sequencer and thus the sequence of the first 16 residues was determined. To elucidate the structure of the remaining C-terminal portion of the peptide its chymotryptic digest was studied. Peptides CP-2 and CP-3 (Table I) were isolated by electrophoresis at pH 5·6; the amino acid sequence of these peptides and of the whole fragment TP-13 is shown in Fig. 4. The total number of amino acid residues contained in both chymotryptic peptides plus the first 10 amino acid residues of the parent fragment corresponded to the amino acid composition of the uncleaved peptide. The sequence of the first 15 residues of peptide TP-14 was also solved in the sequencer. It was not determined beyond doubt though whether the peptide contains 3 or 4 serine residues. Since the peptide contained isoleucine in its terminal portion it was cleaved by thermolysin and the C-terminal tripeptide (TLP-2, Table I) Ile-Ser-Lys was isolated by electrophoresis at pH 5·6. The problem of the number of serine residues in the whole peptide as well as its C-terminal amino acid sequence were thus solved.

Thermolytic and chymotryptic peptides: Only peptides representing overlaps for tryptic peptides derived from the C-terminal part of the molecule were sought; therefore peptides which were basic or neutral at pH 5·6 were isolated. The part of the thermolysin digest which emerged in the hold up volume from the Sephadex G-25 column (4·4 × 200 cm) was subjected to electrophoresis and peptide TLP-1 (Table I) was isolated and purified by paper chromatography. The amino acid sequence of the peptide is shown in Fig. 2. Peptide CP-1 (Table I and Fig. 2) was obtained from the chymotryptic digest by electrophoresis.

C-Terminal amino acid sequence of chicken pepsinogen: The C-terminal amino acid sequence (Fig. 2) was derived from the information provided by tryptic peptides

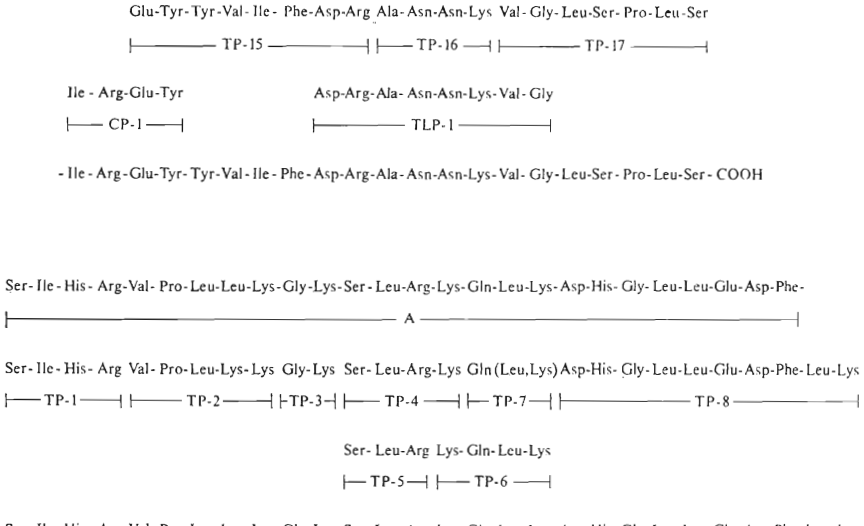

FIG. 2

Amino Acid Sequences of Terminal Regions of Molecule of Chicken Pepsinogen

Top part: C-terminal sequence derived from overlapping peptides isolated from the tryptic, chymotryptic and thermolytic digest of the protein. Bottom part: N-terminal aminoacid sequence of chicken pepsinogen obtained by sequencing of the activation peptide[8] (marked A) and extended from data obtained by the analysis of tryptic fragments. The designation of the peptides is described in the legend to Table I.

		360		370
Pepsinogen				
porcine A	- Ile - Arg - Gln - Tyr - Tyr - Thr - Val -	Phe - Asp	- Arg - Ala - Asn - Asn - Lys - Val -	Gly	- Leu - Ala - Pro - Val - Ala
bovine A	- Ile - Arg - Gln - Tyr - Phe - Thr - Val -	Phe - Asp	- Arg - Gly - Asn - Asn - Gln - Ile -	Gly	- Leu - Ala - Pro - Val - Ala
human A	- Ile - Arg - Gln - Tyr - Phe - Thr - Val -	Phe - Asp	- Arg - Ala - Asn - Asn - Gln - Val -	Gly	- Leu - Ala - Pro - Val - Ala
human C	- Gln - Phe - Tyr - Thr - Val -	Phe - Asp	- Arg - Ala - Asn - Asn - Lys - Glu -	Gly	- Leu - Ala - Pro - Val - Ala
chicken	- Ile - Arg - Glu - Tyr - Tyr - Val - Ile -	Phe - Asp	- Arg - Ala - Asn - Asn - Lys - Val -	Gly	- Leu - Ser - Pro - Leu - Ser
Prochymosin calf	- Ile - Arg - Glu - Tyr - Tyr - Ser - Val -	Phe - Asp	- Arg - Ala - Asn - Asn - Leu - Val -	Gly	- Leu - Ala - Lys - Ala - Ile
Penicillopepsin	- Leu - Lys - Ser - Gln - Tyr - Val - Val -	Phe - Asp	- Ser - Asp - Gly - Pro - Gln - Leu -	Gly	- Phe - Ala - Pro - Gln - Ala

FIG. 3

Homologies in C-terminal Amino Acid Sequences of Various Acid Proteases

The numbering of the chain is according to Foltmann and Pedersen[24] summarizing recorded[1–5,25–29] and unpublished data. Constant amino acid positions are boxed in.

TP-15, TP-16 and TP-17, chymotryptic peptide CP-1, and thermolytic peptide TLP-1. The fact that this sequence represents the C-terminus of the molecule is evidenced also by experiments with carboxypeptidase digestion[7,22,23] of the entire molecule splitting off the C-terminal sequence ...-Leu-Ser.COOH. This sequence is identical with the C-terminal sequence of the only tryptic peptide found in this study which lacks both lysine and arginine (peptide TP-17).

N-*Terminal amino acid sequence of chicken pepsinogen.* The analysis of tryptic fragments isolated in this study confirmed the correctness of the 26-residue N-terminal amino acid sequence established earlier[7,8] with the exception of the eighth residue; this position is occupied by lysine and not by leucine as originally reported. The alignment of the corresponding tryptic peptides with the known N-terminal amino acid sequence is shown in Fig. 2. The overlap of peptide TP-8 and this structure permitted the latter to be extended by two additional amino acid residues.

DISCUSSION

The N-terminal regions of the molecules of acid protease zymogens studied so far as well as their C-terminal regions (identical for both the zymogens and the corresponding active enzymes) show a cumulation of basic amino acid residues as a characteristic feature[24]. We used therefore the tryptic digest as the main source of information to derive the C-terminal amino acid sequence of chicken pepsinogen. Compared with other acid proteases the C-terminal as well as the N-terminal[7] region of chicken pepsinogen display a high conservativeness (Fig. 3). The distribution of basic amino acid residues is exactly the same as in hog pepsinogen. In gastric pepsins the positions of 9 residues including both arginines out of the total number of 21 C-terminal residues are constant, numerous other positions show conservative replacements. If, however, the structure of penicillopepsin is also included in the comparison three positions only are occupied by identical amino acids. The C-terminal region of the molecule of chicken pepsin resembles more the gastric pepsins than penicillopepsin. This is in accordance with the hypothesis that the formation of the precursor of active pepsin, *i.e.* pepsinogen, in higher organisms discontinued the evolutionary process in certain regions of the enzyme in order that the inactive conformation of the zymogen might be retained[24]. The investigation of the tryptic digest permitted us to pick up also the peptides derived from the N-terminus of the molecule. Their sequences confirmed, with one exception, the 26-residue sequence reported earlier; the analysis of peptide TP-2 showed that the eighth amino acid from the N-terminus is lysine and not leucine. The latter was assigned to position 8 in the earlier study for these reasons: when pepsinogen and its derivatives and also the activation peptide[8] were subjected to automatic degradation leucine appeared together with lysine in the 8th degradation step; its yield was considerably higher than the yield of lysine. The amino acid analysis of the activation peptide did not permit an unambiguous conclusion

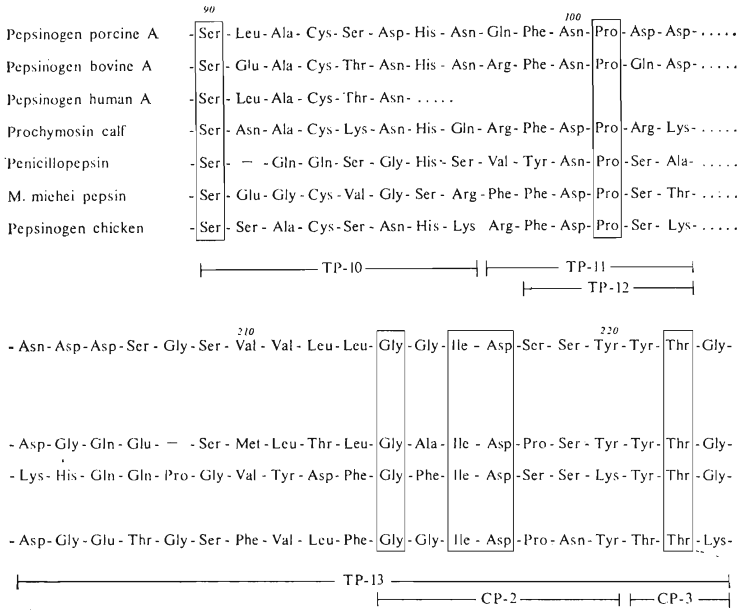

FIG. 4

Homologies between Sequences of Certain Tryptic Peptides from the Middle Part of Chain of Chicken Pepsinogen and Linear Structures of Other Acid Proteases

The numbering, references, and designation of constant positions are described in the legend to Fig. 3. Abbreviations used: M. miehei = Mucor miehei. The amino acid residues in brackets are those aligned with respect to amino acid analysis only.

to be made as to the actual number of leucine and lysine residues contained in the peptide. In view of the sequential analysis of peptide TP-2 isolated in this study the eighth amino acid residue is unambiguously lysine. Since peptide TP-8 overlaps the N-terminal 26-residue sequence the latter can be extended by two residues (Fig. 2). Phenylalanine is followed by the sequence Leu-Lys as in hog pepsinogen. A continuation of the N-terminal amino acid sequence most likely represents peptide TP-9, Lys-Ser-Pro-Tyr-Asn-Pro-Ala-Lys. This assumption is mainly based on the identity of the middle part of its sequence ...-Asn-Pro-Ala-... with the sequence of the corresponding sites of hog pepsinogen A and bovine pepsinogen B (ref.[28]).

An ancillary result of this study represent other tryptic peptides isolated yet not belonging to the N- and C-terminal region of the molecule. Their probable positions which they were aassigned with respect to sequential homologies existing between chicken pepsinogen and other pepsinogens are shown in Fig. 4. Peptides TP-10,TP-11, and TP-12 are derived from the neighborhood of the first -S-S-bridge[30]. This region of the linear structure markedly resembles chymosin (10 positions out of 14 are identical). As in chymosin in chicken pepsin too a characteristic feature of this region is a relatively high cumulation of basic amino acid residues (4 : 14). Altogether 6 positions are constant in animal pepsins. If, however, penicillopepsin and the acid protease from Mucor miehei are also taken into account, only two constant positions remain. Peptide TP-13 is derived roughly from the middle part of the molecule. The region of linear structure covered by this peptide does not show any marked homology in its N-terminal part. Contrarily, the C-terminal part of this region of the chain is homologous in all the sequences known so far. If this region is compared with the corresponding sequence of chicken pepsin four positions only remain constant: Gly (214), Ile-Asp (216–217), and Thr (222). This is most marked with the C-terminal tripeptide where one position only remains constant out of the original three. The region of the linear structure between Tyr (221) and Pro (229) (Fig. 4) should participate according to Foltmann and Pedersen[24] on gene multiplication of acid proteases[24]. However, our data show that chicken pepsin – at least as regards the N-terminal part of the linear structure of this region – is only little homologous with the pepsins sequenced so far. It seems, moreover from other our data (not yet published), that Ser (224) is replaced by a glycine in chicken pepsin. Peptide TP-14 is derived from the neighborhood of half-cystine residue No V which participates on the third -S-S-bridge. The tentative alignment of this peptide is based on the existence of minor homologies in the closest vicinity of the half-cystine residue.

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