THERMOLYTIC DIGEST OF CHICKEN PEPSIN

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Chicken pepsin prepared by the activation of pepsinogen was digested with thermolysin. The thermolytic digest was fractionated by chromatography on Sephadex G-25 fine. Certain fractions were subsequently subjected to ion exchange chromatography on Dowex 50-X2. The final purification was effected by paper chromatography and high voltage electrophoresis. By these procedures a series of homogeneous peptides was obtained; of the latter 54 nonoverlapping (save for a few exceptions) peptides are described in this paper. These peptides in addition to the thermolytic peptides reported before represent 80% of the linear structure of the whole molecule. The N-terminal amino acid sequence of chicken pepsin is discussed from the viewpoint of the recent data obtained by the analysis of the thermolytic digest.

The aim of our studies on the thermolytic digest was two-fold. First, peptides were sought enabling the determination of the S–S-bonds and of the primary structure of the C-terminal part of the molecule. The results of these experiments have been already published[1,2]. The determination of the complete primary structure of the enzyme requires, however, the fragmentation of its molecule to several large fragments and their subsequent subfractionation. Trypsin because of its high specificity is the enzyme of choice most often used for additional cleavage of polypeptide fragments. The use of trypsin for a deeper fragmentation in the case of chicken pepsin is not advantageous because of the very low number of lysine and arginine residues; this feature is a general characteristic of all pepsins. For these reasons, two other proteinases of a relatively high specificity, namely thermolysin and chymotrypsin have to be considered. We have therefore studied in addition to peptides containing S–S-bonds and derived from the C-terminus of the molecule also the remaining thermolytic peptides. The knowledge of their amino acid sequence is necessary if thermolytic peptides are isolated from the high molecular weight fragments in low yield only and their primary structure cannot be determined. Another aim of this study was to obtain methionine and arginine overlaps. We have also made an attempt in this study to cast more light on the N-terminal amino acid sequence of chicken pepsin where automatic sequential degradation has failed[3].

This paper describes the isolation and characterization of the individual peptides of the thermolytic digest and their sequential analysis.

EXPERIMENTAL

Material

Chicken pepsin was prepared by the method described elsewhere[3]. Thermolysin was from Calbiochem, San Diego, Calif., USA. Sephadex G-25 fine was a product of Pharmacia Fine Chemicals, Uppsala, Sweden. Dowex 50-X2 (200–400 mesh) was from Fluka, Switzerland. The chemicals used for sequencing were supplied by Beckman Instr., Spinco Div., Palo Alto, Calif., USA. Silufol, silica gel thin layer sheets, and polyamide layer sheets were purchased from Kavalier, Czechoslovakia and BDH Chemicals Ltd., Poole, England, respectively. The remaining chemicals used were of analytical purity.

Methods

The peptides were purified on Whatman No 3 MM paper by high voltage electrophoresis in the horizontal arrangement in the system water–acetic acid–formic acid (16 : 3 : 1, v/v) at pH 1·9 and a potential gradient of 70 V/cm (ref.[4]) or in the vertical arrangement in the system water–pyridine–acetic acid (994 : 5 : 1, v/v) at pH 5·6 and a potential gradient of 30 V/cm (ref.[5]). Paper chromatography of the peptides was effected on Whatman No 3 MM paper in the system 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12, v/v, ref.[6]).

Amino acid analysis: The samples were hydrolyzed 20 or 70 h in 6M-HCl at 110°C in evacuated sealed tubes and the analysis was performed by the method of Moore and Stein[7] as modified by Benson and Patterson[8] in Spinco Model 120B Amino Acid Analyzer or in an analyzer of Czechoslovak make (Model 6020, Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague). The quantitative analysis of tryptophan was carried out by the method of Matsubara[9]. The N-terminal amino acids of the individual peptides were determined by dansylation according to Gray[10] using thin-layer chromatography on polyamide layers[11]. The stepwise degradation of peptides was effected by the phenyl isothiocyanate method[12] and the phenylthiohydantoins resulting from the conversion of the thiazolinones were identified by thin-layer chromatography on silica gel[13] or by gas chromatography in Beckman GC-65 Gas Chromatograph either without or after silylation[14].

Thermolytic digest of chicken pepsin: The hydrolysis by thermolysin and the fractionation of the digest on a column of Sephadex G-25 fine have been described in detail elsewhere[1]. The effluent was divided into 13 fractions designated TL I-TL XIII and their composition was examined by peptide maps (a combination of descending electrophoresis and paper chromatography). The peptides contained in fractions I–III and IX–XIII were purified by paper techniques only. Fraction IV was rechromatographed on a column of Sephadex G-25 fine (2·6 × 90 cm), equilibrated with 0·1% NH_4HCO_3 and the effluent was divided according to its paper chromatographic pattern into 2 fractions which were subsequently separated by high voltage paper electrophoresis and paper chromatography. Fractions V–VIII were pooled and subjected to ion exchange chromatography on Dowex 50-X2 (column dimensions 1·8 × 35 cm), equilibrated with 50 mM pyridine formate buffer at pH 3·0. The peptides were eluted by a linear gradient developed with the following buffers: 50 mM pyridine formate (pH 3·0)–0·2M pyridine acetate (pH 4·0) (500 + 500 ml) and 0·2M pyridine acetate (pH 4·0)–2·0M pyridine (500 + 500 ml). The flow rate was 30 ml/h and 5 ml fractions were collected. The composition of the effluent was examined by paper chromatography of aliquots (2%) taken from the individual fractions. Altogether 27 fractions were collected and subjected to final purification by paper techniques.

TABLE I

Amino acid composition of peptides isolated from thermolytic digest of chicken pepsin. The values are not corrected. None of the peptides contained half-cystine. Unless specified otherwise the analyses were performed on 20 h hydrolysates

Designation of peptide	Number of residues																
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp
TL-I-1					0·9	1·8			2·0			1·0					
TL-I-2					1·0	0·9									0·8		
TL-I-3						1·0		1·0		2·0				1·0	1·0		
TL-I-4						1·0										1·0	
TL-I-5					0·9		1·0		2·0	0·8			0·8		1·0		
TL-I-6					0·9											1·0	
TL-1-7											1·0				0·8		
TL-I-8						0·9		1·0			1·0			1·0			+[b]
TL-I-9				1·0	0·9	0·9	1·8	0·9	1·0				1·8				
TL-I-10				1·1		1·0				1·0		0·9					
TL-I-11				1·0	1·8	0·9	1·0				1·0						
TL-II-1				2·1	1·9	0·8			2·7				1·0		0·9	0·9	
TL-II-2				1·1	2·6	0·9	2·0	1·0		1·0		0·9			1·6		
TL-II-3					2·1		3·1	0·9	2·0					1·0			0·7
TL-III-1[a]	1·0		0·9	3·0					1·1	1·0	1·6		0·7			1·0	
TL-III-2				1·0	0·9	2·5		0·9							0·8	1·0	
TL-III-3						1·8		1·0						2·0			
TL-III-4[a]				2·0	1·0	1·8			1·0		0·7		0·7			1·0	
TL-III-5[a]				1·1	1·7	0·9			1·0		0·7		0·7	1·1			
TL-III-6				1·1			1·0			0·9	0·9			1·8			
TL-IV-1				2·0			2·1				1·0		1·0				
TL-IV-2					1·0	1·7	2·0	0·9	1·1					0·9		1·0	
TL-A-VI-1					1·0	1·0	1·0			1·1							
TL-A-VII-1					1·0			0·8		1·0							

TABLE I
(*Continued*)

Designation of peptide	Number of residues																
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp
TL-A-VIII-1						1·9							1·0				
TL-A-IX-1					0·9						1·0						
TL-A-X-1									1·0	1·0							
TL-A-X-2						0·9	1·0			1·0				1·1			
TL-A-XI-1				1·0	1·0							1·0					
TL-A-XI-2							1·0	0·9	1·2		0·9	0·9					
TL-A-XI-3									1·0					1·8			
TL-A-XII-1				1·0					1·0				1·0				
TL-A-XII-2					0·9								1·0				
TL-A-XII-3				1·1					1·0				0·9	0·9			
TL-A-XIII-1						1·0			1·0			0·9					
TL-A-XIV-1						1·0		1·0		1·0						1·0	
TL-A-XIV-2					0·8									1·0			
TL-A-XV-1						0·9	2·0	0·9							1·0		
TL-A-XV-2										1·0			0·9	0·9			
TL-A-XVI-1				1·0			1·0									1·0	
TL-A-XVII-1				1·0	1·0										0·9		
TL-A-XVIII-1					1·0	0·9	1·1			1·0			1·0		0·8		
TL-A-XIX-1				2·1							0·9	0·9				1·0	
TL-A-XXI-1	1·0			1·0		0·9		1·0					1·0	0·9			
TL-A-XXII-1	0·9			1·0									1·1				
TL-A-XXIII-1	1·0			1·0					1·0		0·8						
TL-A-XXIV-1			1·0	1·0													

TABLE I
(*Continued*)

Designation of peptide	Number of residues																
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp
TL-A-XXVII-1			0·9				1·0						1·0				
TL-IX-1									1·0				0·8			0·8	
TL-X-1													1·0		0·9		
TL-XI-1		1·0														1·0	
TL-XI-2						0·9		1·0			1·0						
TL-XIII-1															+[c]		

[a] The hydrophobic amino acids were determined after 70 h hydrolysis; [b] the tryptophan content was determined by the qualitative test only; [c] the peptide contained tyrosine only.

RESULTS AND DISCUSSION

Trypsin is the enzyme of choice most often used because of its high specificity for the fragmentation of protein molecules to small peptides which can readily be isolated by routine techniques. The general characteristic of all pepsins, however, is the low content of basic amino acids and trypsin cannot therefore be used as the cleaving enzyme. Chicken pepsin, however, is relatively abundant in hydrophobic amino acids which are the necessary condition of thermolytic cleavage. We used therefore thermolysin for the fragmentation of the pepsin molecule and expected a great number of small and medium-size peptides to be formed. We were able to isolate in fact a great number of peptides of which 54 were selected; the latter are not derived, save for a few exceptions, from the same sites of the chain. The amino acid composition of the peptides is shown in Table I and their amino acid sequences in Table II. Free amino acids isolated from the digest as well as certain dipeptides which could have been formed by cleavage of larger peptides are not listed. The peptides isolated are marked by the symbol TL (for thermolytic digest) and by a Roman numeral designating the basic fraction. An exception represent fractions V – VIII which were pooled (marked fraction A) and resolved by ion exchange chromatography on Dowex 50-X2. (These fractions were marked I – XXVII by Roman numerals). The Arabic numerals designate the serial number of the peptide in each individual fraction. The peptides isolated from more than one fraction are listed once only.

Peptide TL-I-1 was isolated in a low yield and therefore could be degraded to the fourth step only. In the light of the amino acid sequence of peptide TL-A-XIII-1 Met-Ser-Gly and of the existing homologies with other pepsins[15] it can be postulated that the primary structure of peptide TL-I-1 extended by one amino acid residue is the following: Gly-Thr-Gly-Ser-Met-Ser-Gly. Likewise can be postulated the C-terminal primary structures of peptides TL-III-2 and TL-I-8 with regard to peptides TL-I-2 and TL-XI-2 and to the existing homologies with other carboxyl proteinases, even though we have not been able to detect tryptophan in peptide TL-I-8 in the second degradation step. The most probable structure of peptide TL-III-2 is Phe-Asp-Pro--Ser-Lys-Ser-Ser-Thr-Tyr and of peptide TL-I-8 Leu-Trp-Val-Pro-Ser. Peptide TL-I-11 yielded an unidentifiable product in the fourth degradation step. A comparison of the sequence determined with the results of amino acid analysis shows that this site should be occupied by aspartic acid. The amino acid analysis of the peptide pointed moreover to the presence of a carbohydrate component (about 6 glucosamine residues per molecule of peptide). It is therefore obvious that the fourth amino acid from the N-terminus of the peptide is most likely asparagine to which the carbohydrate moiety of chicken pepsin is bound. The amino acid analysis of peptide TL-A-XXII-1 showed even after 70 h hydrolysis the presence of one isoleucine residue only. The results obtained by the dansylation technique, however, indicated the N-terminal sequence Ile-Ile of the peptide. The same result was obtained

by sequential analysis of the peptide. Peptide TL-XIII-1 contained tyrosine only and represented the main component of the fraction retarded most on Sephadex. The R_F-value of this peptide differed from the R_F of free tyrosine and was identical with the R_F of dipeptide Val-Tyr; we concluded from these results that peptide TL-XIII-1 is identical with Tyr-Tyr. The sequence of the first six amino acids of peptide TL-II-2 was identical with the N-terminal amino acid sequence of chicken pepsin determined before. This peptide moreover yielded information on the amino acid composition of the first 12 residues of chicken pepsin. Later we obtained 3 other peptides which cast more light on the N-terminal sequence of chicken pepsin (Fig. 1). Peptide TL-A-XV-1 was homogeneous both according to amino acid analysis and N-terminal end group analysis, yet yielded comparable amounts of glutamic acid and serine in the second and fourth degradation step; the results obtained, however, excluded the possibility that the seventh position from the N-terminus is occupied by aspartic acid as indicated by the automatic sequential degradation of pepsin and its inactive

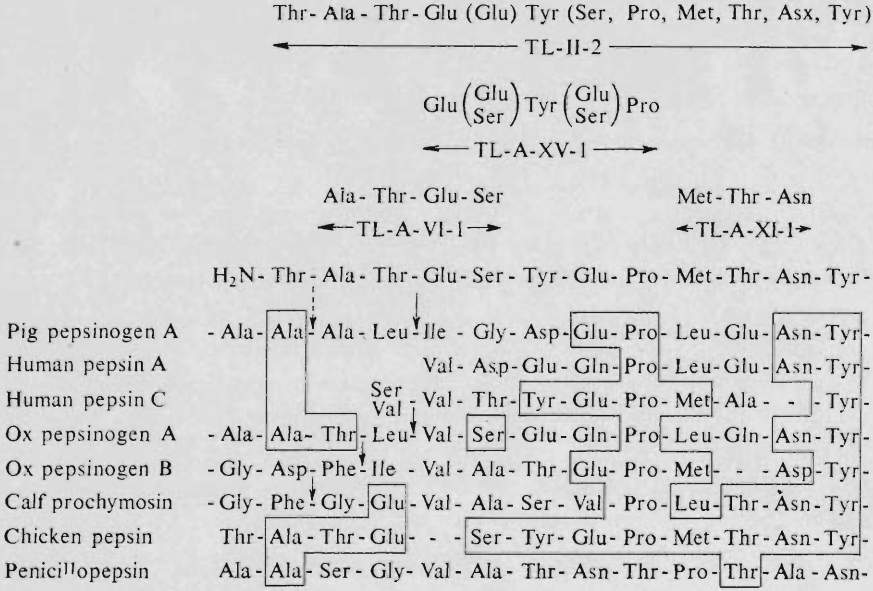

FIG. 1

Top part: probable N-terminal primary structure of chicken pepsin. Bottom part: comparison of the N-terminal primary structure of chicken pepsin with the corresponding regions of primary structures of certain carboxyl proteinases or their zymogens[15–23]. The sequences are arranged according to Foltmann and Pedersen[15]. The sites of formation of pepsins from pepsinogens are marked by arrows. Residues identical with chicken pepsin are boxed in.

derivatives. The problem of the localization of serine and glutamic acid in positions 5 and 7 was solved by the analysis of tetrapeptide TL-A-VI-1 whose sequence of the first three amino acid residues is identical with a part of the N-terminal structure of peptide TL-II-2. The fifth position from the N-terminus is therefore probably occupied by serine, the seventh by glutamic acid which is followed by proline as obvious from the sequence of peptide TL-A-XV-1. It follows from the amino acid analysis of peptide TL-II-2 that 4 residues, Met, Thr, Asx, and Tyr are left. According to the homologies with the N-terminal primary structure of chymosin and other carboxyl proteinases (Fig. 1) tripeptide TL-A-XI-1, Met-Thr-Asn, fits into this site. The most probable N-terminal amino acid sequence of chicken pepsin is therefore Thr-Ala-Thr-Glu-Ser-Tyr-Glu-Pro-Met-Thr-Asn-Tyr. As follows from the comparison of the structures of various carboxyl proteinases the N-terminus of chicken pepsin of all the pepsin sequences extends to the greatest distance into the region involved in the pepsinogen to pepsin conversion. The reason for this is obviously the fact that the part of the chain of chicken pepsinogen where in other pepsinogens the cleavage of the chain accompanied by the liberation of the final activation peptide takes place does not contain a bond susceptible to peptic cleavage (Fig. 1). The primary structures of the N-terminal regions of various pepsins are only little homologous. Chicken pepsin is not an exception in this respect. The positions of proline and tyrosine, however, are retained in all the sequences known.

This study was also expected to contribute to the knowledge of peptides overlapping methionine and arginine residues. Since, however, native pepsin was used to start with, the hydrolysis had to be prolonged. This resulted in partial cleavage before the methionines and 4 peptides only containing this residue in the remaining part of the chain were isolated. One of them, peptide TL-A-XIX-1, had C-terminal methionine, however, and therefore the neighborhood of three out of nine methionines was determined by the analysis of the thermolytic digest. It follows from the known N- and C-terminal structure of chicken pepsinogen that 3 arginine residues only are situated in the middle part of the molecule. One of them is localized close to the first S–S-bridge[2]. We were interested in the structures around the two remaining arginine residues. Two arginine containing peptides, TL-III-1 and TL-A-XXVII-1, derived from the C-terminal portion of the molecule, and dipeptide Asn-Arg were isolated from the digest. It is possible that the sequence Asn-Arg is repeated in the molecule and that the corresponding dipeptide is derived from two different sites of the chain. The overal recovery of the arginines would then be in agreement with the analytical data on arginine residues in chicken pepsin.

The amino acid sequences of the peptides given in Table II represent, together with the peptides derived from the neighborhood of the S–S-bonds[1], 80% of the linear structure of the whole molecule. There may be various reasons why the remaining 20% have not been picked up. First, the thermolytic digestion was relatively long; it is known that thermolysin can cleave nonspecifically during prolonged periods.

TABLE II

Primary structures of peptides isolated from thermolytic digest. The designation of the peptides, the techniques used in the sequencing experiments, and the remaining details are described in the text. The amino acids whose recovery was low in the given degradation step are given in brackets. The C-terminal parts of the peptides with undetermined primary structure are given in brackets, the individual amino acids are separated by commas and ordered according to the probable sequence

Designation of peptide	Sequence
TL-I-1	Gly-Thr-Gly-Ser(Met,Ser)
TL-I-2	Ser-Thr-Tyr
TL-I-3	Leu-Pro-Ala-Ser-Ala-Tyr
TL-I-4	Phe-Ser
TL-I-5	Ile-Ala-Tyr-Gly-Thr
TL-I-6	Phe-Thr
TL-I-7	Val-Tyr
TL-I-8	Leu-X-Val(Pro,Ser)
TL-I-9	Ile-Ser-Ile-Gly-Thr-Pro-Gln-Gln-Asp
TL-I-10	Met-Asp-Ala-Ser
TL-I-11	Val-Ser-Thr-X-Glu-Thr
TL-II-1	Phe-Gly-Gly-Ile-Asp-Pro-Asn-Tyr-Thr-Thr-Lys-Gly
TL-II-2	Thr-Ala-Thr-Glu(Glu)Tyr(Ser,Pro,Met,Thr,Asx,Tyr)
TL-II-3	Gly-Thr-Pro-Thr-Glu-Leu-Gly-Glu-Gln-Trp
TL-IlI-1	Val-Ile-Phe-Asp-Arg-Ala-Asn-Asn-Lys-Val-Gly
TL-III-2	Phe-Asp-Pro-Ser-Lys-Ser(Ser)(Thr,Tyr)
TL-III-3	Leu-Ser-Pro-Leu-Ser
TL-III-4	Val-Ile-Phe-Asp-Thr-Gly-Ser-Ser-Asn
TL-III-5	Ile-Val-Asp-Thr-Gly-Thr-Ser-Leu
TL-III-6	Leu-Val-Ala-Gln-Asp-Leu
TL-IV-1	Ile-Asp-Val-Gln-Asn-Glu
TL-IV-2	Leu-Ser-Glu-Thr-Glu-Pro-Gly-Ser-Phe
TL-A-VI-1	Ala-Thr-Glu-Ser
TL-A-VII-1	Ala-Thr-Pro
TL-A-VIII-1	Ile-Ser-Ser
TL-A-IX-1	Val-Thr
TL-A-X-1	Gly-Ala
TL-A-X-2	Leu-Glu-Ala-Ser
TL-A-XI-1	Met-Thr-Asn
TL-A-XI-2	Val-Met-Pro-Gln-Gly
TL-A-XI-3	Leu-Gly-Leu
TL-A-XII-1	Ile-Asn-Gly
TL-A-XII-2	Ile-Thr
TL-A-XII-3	Ile-Leu-Gly-Asp
TL-A-XIII-1	Met-Ser-Gly
TL-A-XIV-1	Ala-Phe-Pro-Ser

TABLE II
(*Continued*)

Designation of peptide	Sequence
TL-A-XIV-2	Leu-Thr
TL-A-XV-1	Glu(Glu/Ser)Tyr(Glu/Ser)Pro
TL-A-XV-2	Ile-Leu-Ala
TL-A-XVI-1	Phe-Glu-Asn
TL-A-XVII-1	Tyr-Asp-Thr
TL-A-XVIII-1	Ile-Tyr-Ser-Ala-Glu-Thr
TL-A-XIX-1	Val-Phe-Asp-Asn-Met
TL-A-XXI-1	Ile-Ser-Lys-Leu-Pro-Asp
TL-A-XXII-1	Ile-Ile-Lys-Asp
TL-A-XXIII-1	Val-Gly-Asn-Lys
TL-A-XXIV-1	Asn-Arg
TL-A-XXVII-1	Ile-Arg-Glu
TL-IX-1	Ile-Phe-Gly
TL-X-1	Ile-Tyr
TL-XI-1	Phe-His
TL-XI-2	Val-Pro-Ser
TL-XIII-1	Tyr-Tyr

This could explain why almost all free amino acids were found in the digest. It could have happened that some region of the molecule was cleaved down to free amino acids and the peptide belonging to this region was present in a negligible amount only in the digest. The first S–S-bridge, which we were not able to find, can be adduced by way of example. The Table does not list most of the dipeptides since they could have been formed by cleavage of larger peptides. It is most likely though that the sequences of certain di- and tripeptides recur in the molecule and that the dipeptides isolated are derived from other sites of the molecule and therefore do not correspondent to identical dipeptides contained in fragments. This could account for the observed difference of 20%. In cases where we tried to allocate the peptides isolated in the molecule of chicken pepsin with respect to the existing homologies with other carboxyl proteinases we were able to cover 74% of its linear structure.

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