PEPTIDES OBTAINED BY CHYMOTRYPTIC DIGESTION OF S-SULFO DERIVATIVE OF HOG PEPSIN

V.KOSTKA and A.SALVETOVÁ

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

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Hog pepsin was subjected to sulfitolysis and the resulting S-sulfo derivative was digested with chymotrypsin. The digest was fractionated by chromatography on Dowex 50. The crude fractions obtained were separated further either by additional chromatography on Dowex 1 or by gel filtration. The isolation and final purification of the peptides was effected by high-voltage paper electrophoresis and by paper chromatography. These methods afforded a total of 43 individual peptides. Selected peptides were subjected to sequential analysis. The amino acid sequences of 82 nonoverlapping chymotryptic peptides are given and their location in the polypeptide chain of hog pepsin is described.

The sequential work on hog pepsin, carried out in this Laboratory, has followed two main lines of approach aimed at the isolation of well-defined high molecular weight fragments of the molecule of the enzyme. These two lines are represented by studies on fragments arising from cyanogen bromide cleavage[1] and by studies on tryptic fragments of reduced and aminoethylated pepsin[2]. In both cases fragments are obtained which are suitable for sequential investigation of well-defined regions of the molecule. On the other hand, the size of these fragments, which sometimes equals the size of small protein molecules, virtually eliminates the possibility of direct determination of their complete amino-acid sequence by stepwise degradation. The sequencing of these fragments must then proceed by way of additional enzymatic or chemical cleavage; this increases the quantity of starting material required. We decided therefore to follow in our sequence studies, in addition to the main line leading *via* high molecular weight fragments, also another route: to cleave the pepsin molecule directly to a great number of low molecular weight peptides. The aim of these experiments was to obtain these peptides by routine procedures from a large quantity of starting material in high yields, to determine their amino acid sequences, to correlate the results obtained with data from other experiments and thus to determine the location of the peptides in the individual regions of the pepsin molecule. We tried to use the sequential information obtained with these peptides especially in cases where the corresponding chymotryptic peptides were isolated from the large fragments in low yields.

In another study, peptides from the thermolysin digest[3] of S-sulfo-pepsin were also isolated.

This paper reports on the chymotryptic digestion of S-sulfo-pepsin, the isolation and characterization of the peptides obtained, and on the sequential analysis of some of them.

EXPERIMENTAL

Material

S-sulfo-pepsin was prepared from twice crystallized and lyophilized hog pepsin (E.C. 3.4.4.1), a product of Worthington Biochem. Corp., Freehold, N.Y., U.S.A. The sulfitolysis was effected by an earlier described modification of the procedure of Pechère and coworkers[4]. The derivative obtained was freed of low molecular weight contaminants by gel filtration through a column of Sephadex G-100 equilibrated with 0·3M ammonium acetate (pH 6·0), 8M in urea[1]. Chymotrypsin (E.C. 3.4.4.5) was obtained by tryptic activation of chymotrypsinogen; the latter was prepared by 5-fold crystallization[5] of a commercial product of Léčiva, Prague. Thermolysin, used for additional cleavage of certain chymotryptic peptides, was a B-grade quality product purchased from Calbiochem, Los Angeles, Calif., U.S.A. Carboxypeptidase A (E.C. 3.4.2.1) was a diisopropyl fluorophosphate-treated preparation from Worthington. Dowex 50-X2 and Dowex 1-X2 (both 200—400 mesh) were products of Fluka, A. G., Switzerland. Sephadex G-25 fine (particle size 20—80 μ) and Sephadex G-100 (particle size 40—120 μ) were from Pharmacia (Uppsala, Sweden). Silufol, a thin silica gel layer with starch binder on aluminum sheet, was a product of Kavalier, Czechoslovakia. Polyamide layer sheets were purchased from BDH Chemicals Ltd. (Poole, England). All solvents for paper chromatography of peptides or for thin-layer chromatography of PTH or DNS* derivatives of amino acids were of analytical purity.

Methods

Paper chromatography of peptides was carried out in the system n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12 v/v) on Whatman No 3 paper. High voltage electrophoresis[6] was likewise carried out on Whatman No 3 perpa, either in the horizontal arrangement[7] (buffer formic acid– acetic acid–water, 50 : 150 : 800, v/v, at pH 1·9 and a potential gradient of 120 v/cm) or in the descending arrangement[8] (buffer pyridine–acetic acid–water, 2 : 0·5 : 997·5, at pH 5·6 and a potential gradient of 35 v/cm). The techniques used for the elution of peptides from paper, for the identification of peptides and amino acids, and certain other, special techniques have been described in detail elsewhere[9].

Amino–acid analysis of peptides was carried out by the method of Spackman, Stein, and Moore[10] as described in detail earlier[11]. The N-terminal amino acids were determined by dinitrophenylation[12] or by the dansyl[13] technique. In the latter case, the DNS-amino acids were analyzed by thin-layer chromatography on Silufol sheets[14] or on polyamide layers two-dimensionally[15]. The C-terminal amino acids were determined after their liberation by carboxypeptidase A (*e.g.*[16]) or by hydrazinolysis[17,18]. The sequential degradation of peptides according to Niall and Edman[19] has been described in detail before[20].

Chymotryptic digestion of S-sulfo-pepsin. S-sulfo-pepsin (10 g) was dissolved in water whose pH was adjusted to 8·2 by concentrated ammonia. This solution (final volume 750 ml) was thermostated at 37°C and 100 mg of chymotrypsin in 100 ml of water was added (enzyme to substrate ratio 1 : 100, w/w). The reaction mixture was thermostated at 37°C and the pH was maintained in the alkaline range according to the indicator (phenol red) color by occasional addition of 0·2M-$(NH_4)_2CO_3$. After 2 h of incubation, a fresh 100 mg portion of chymotrypsin was added (final enzyme to substrate ratio 1 : 50, w/w). The digestion was continued for 2 h. The reaction mixture was then acidified (yellow color of indicator) by glacial acetic acid, concentrated by rotary evaporation *in vacuo* and lyophilized. The weight of the lyophilized product was 9·87 g.

* Abbreviations used: DNS 1-dimethylamino-naphthalene-5-sulfonyl.

Fractionation of chymotryptic digest by chromatography on Dowex 50. The entire quantity of the lyophilized digest was dissolved in 860 ml of H_2O. The slightly turbid solution obtained was applied on a 3·5 . 120 cm column of Dowex 50, equilibrated with 0·05M pyridine formate at pH 2·8. The column was then eluted by the same buffer, at room temperature and a flow rate of 100 ml/h. Fractions of 50 ml were collected. After the emergence of 40 fractions, the column was eluted by a linear gradient of buffers developed from 10 l of 0·05M pyridine formate, pH 2·8, and 10 l of 0·2M pyridine acetate, pH 3·5. After the emergence of fraction No 400, the elution was continued by another gradient, developed from 10 l of 0·2M pyridine acetate, pH 3·5, and 2M pyridine acetate, pH 5·0. The column was then eluted by 2M pyridine. The total number of fractions collected was 664. The course of the ion-exchange chromatography was checked by the analysis of 0·2 ml aliquots (0·4%), taken from each fraction. The dry residues of these aliquots were analyzed by paper chromatography. According to the results obtained, preliminary pools of the effluent fractions were made.

Isolation of pure peptides. The individual preliminary pools were studied by the technique of peptide maps. A combination of horizontal high-voltage electrophoresis with paper chromatography, in certain cases also of descending electrophoresis with chromatography was used. The inspection of the peptide maps obtained showed that many preliminary pools partly contained identical peptides. Therefore additional pools were made; the total number of the basic fractions thus obtained was then 11 (C I through C XI). These basic fractions were then subjected to additional resolution by one of the following methods: *1*) chromatography on a column of Dowex 1 (1·8 . 64 cm), using an elution gradient of volatile buffers according to Guest and coworkers[21], *2*) gel filtration on Sephadex G-25 (column 4·6 . 200 cm or 2·4 . 150 cm), equilibrated in water adjusted to pH 9 by concentrated ammonia or 0·5% NH_4HCO_3, *3*) paper chromarography and paper electrophoreses. The final purification of the peptides was effected as a rule by paper techniques. The processing of the basic fractions is shown below.

Basic fraction No	Effluent fraction No	Separation technique
C I	4—38	Sephadex G-25
C II	39—79	Dowex 1
C III	80—238	Dowex 1
C IV	239—319	paper techniques
C V	320—374	Dowex 1
C VI	375—430	Dowex 1
C VII	431—448	paper techniques
C VIII	449—492	paper techniques
C IX	493—608	Sephadex G-25
C X	609—634	Sephadex G-25
C XI	635—644	paper techniques

RESULTS AND DISCUSSION

The difficulties encountered in sequential studies on the molecule of hog pepsin were discussed in our preceding paper[1]. This enzyme contains only 2 residues of arginine and one residue of lysine[1,22] which moreover are clustered in the small C-terminal region of the chain[16]; for this reason tryptic digestion cannot be used as a fragment-

TABLE I

Amino-Acid Analyses of Peptides from Chymotryptic Digest of S-Sulfo-Pepsin

The analyses were performed on 20 h hydrolysates of the peptides by methods given in the text[9,10]. The molar ratio values given are uncorrected. The tryptophan content was determined only qualitatively.

Designation of peptide	LYS	HIS	ARG	CYS	ASP	THR	SER	GLU	PRO	GLY	ALA	VAL	MET	ILE	LEU	TYR	PHE	TRP
C-I-1														1·1	1·0			
C-I-2						0·9								1·0		0·8		
C-I-3						1·9	1·9	2·8			1·0			1·0	1·1	0·7		
C-II-1							1·0								1·0			
C-II-2					1·0	1·0	1·0						0·7	1·0				
C-II-3					2·1	1·0	2·0			1·1					0·9			
C-II-4					3·2		3·7			1·1		1·6			2·0			
C-II-5					2·1		1·3	1·7		1·1		1·1			1·9		0·8	
C-II-6					5·0	2·0	4·2	3·1	1·3	3·3		1·4	1·0	0·6	2·2		1·3	+
C-III-1						1·0	1·0			1·8			0·8					
C-III-2					1·0					2·0				1·1	2·0			
C-III-3						1·0	1·0	3·2			1·1				1·1			
C-III-4					3·0		3·9			1·3		1·4			2·1			
C-III-5						0·9	1·6	1·0	0·9	1·0		1·0			1·0			+
C-III-6						1·0	2·1	2·0	1·0	2·0					1·1		1·0	
C-III-7					1·1	2·0		1·1	1·0	3·0	1·0			2·0			1·0	
C-III-8		0·8		0·7	4·7	1·0	2·8	1·1	1·1		0·9						1·9	
C-V-1						0·8						1·3					0·9	
C-V-2					1·8	0·9	1·6			0·9					1·0			+
C-V-3					1·0	0·9		1·0	1·0	1.0		1·0		0·9	1·0	0·7		
C-V-4						2·0	1·0			3·8			0·9	1·0	1·0	0·8		

TABLE I
(*Continued*)

Designation of peptide	LYS	HIS	ARG	CYS	ASP	THR	SER	GLU	PRO	GLY	ALA	VAL	MET	ILE	LEU	TYR	PHE	TRP
C-V-5					2·9	1·0	2·8		1·0	1·0				0·8	1·9	1·6		
C-V-6					1·0	1·0	1·8	1·6	1·0	2·0		1·0	0·2		1·0			+
C-V-7					1·0	1·1	1·8	1·0	2·0	1·4	1·0	1·1		1·1	1·0	1·8		
C-VI-1												0·9					1·0	
C-VI-2						1·0	1·7	1·8	1·2	1·9					1·7	1·0	1·0	
C-VI-3					2·9	1·0		2·9	1·0	1·1	1·1			1·0	2·8	0·8	1·0	
C-VI-4					4·8	1·4	4·0	2·5	2·1	2·6	3·0	2·2		1·2	3·4	0·9	2·1	+
C-VII-1															1·0	0·9		
C-VII-2			1·0		2·6						0·9							
C-VII-3	0·9		1·0		2·8				1·1	0·9	2·8	1·9			1·0			
C-VII-4					2·9	1·0		2·7	1·1	1·1				0·9	2·0	1·6	0·9	
C-VIII-1							0·9					1·1				1·0		
C-VIII-2									0·9		0·9					0·9	1·0	
C-VIII-3	0·9								1·1	0·9	1·8	1·9			1·0			
C-VIII-4					1·2		2·1			2·1				1·0	1·0	1·8		
C-IX-1	0·8									1·2		1·1			1·0			
C-IX-2			1·1					1·2						0·8		1·0		
C-IX-3							1·9		1·0		1·1					0·9		
C-IX-4							0·8	1·0		1·2		1·2				0·9		+
C-X-1					1·0		1·5	1·0	1·0	1·8		2·9			0·8	1·0		+
C-XI-1	0·8									1·2		1·1			1·0	2·0		
C-XI-2							0·9		1·0			2·1				1·0		+

TABLE II

Peptides Isolated from Chymotryptic Digest of S-Sulfo-Pepsin

The individual peptides are marked by a symbol, corresponding to the basic fraction from which they were isolated (Roman numeral, in order of emergence from the Dowex 50 column) and a serial number (Arabic numeral). The complete amino acid sequences of the peptides were determined by stepwise degradation by techniques given in the text, individual degradation steps are marked by arrows (→). The N-terminal end groups of unsequenced peptides were established by the Dansyl technique, exceptionally by dinitrophenylation. The C-terminal end groups were determined in kinetic experiments with carboxypeptidase A digestion of the peptides (marked ←), exceptionally by hydrazinolysis. In the remaining cases the peptide was ascribed the C-terminal amino acid with regard to the known specificity of chymotrypsin. The presence of amides in unsequenced peptides was judged by the mobility of the peptides on high-voltage electrophoresis at pH 5·6. Unsequenced peptides are given in a form corresponding to our present knowledge[23] of the pepsin structure in order to facilitate their alignment.

Designation of peptide	Structure or composition
C-I-1	Ile-Leu
C-I-2	Ile-Thr-Tyr
C-I-3	(Glx,Ala,Thr,Ser,Glx,Glx,Leu,Ile,Thr)Tyr
C-II-1	Ser-Leu
C-II-2	Asx(Ser,Ile,Thr,Met)
C-II-3	$\overrightarrow{\text{Asp-Thr-Gly-Ser-Ser-Asn}}$-Leu
C-II-4	$\overrightarrow{\text{Leu-Ser-Ser-Asn-Asp-(Asx,Ser)Gly-Ser-Val-Val}}$-$\overleftarrow{\text{Leu}}$
C-II-5	$\overrightarrow{\text{Asp-Gln-Gly-Leu-Val-Ser-Gln-Asp-Leu-Phe}}$
C-II-6[a]	$\overrightarrow{\text{Ile-Leu-Gln-Asp-Asp-Asp(Cys,Thr,Ser,Gly,Phe,Glx,Gly,Met,Asx,Val,Pro,Thr,Ser,Ser,Gly,Glx,Leu)Trp}}$
C-III-1	$\overrightarrow{\text{Gly-Thr-Gly-Ser-Met}}$
C-III-2	$\overrightarrow{\text{Asp-Gly-Ile-Leu-Gly-Leu}}$
C-III-3	Glx(Ala,Thr,Ser,Glx,Glx)Leu
C-III-4	Leu(Ser,Ser,Asx,Asx,Asx,Ser,Gly,Ser,Val,Val)$\overleftarrow{\text{Leu}}$

TABLE II
(*Continued*)

Designation	Structure or composition
C-III-5	(Val,Pro,Thr,Ser,Ser,Gly,Glx,Leu)Trp
C-III-6	Gly(Leu,Ser,Glx,Thr,Glx,Pro,Gly,Ser)Phe-Leu-Tyr
C-III-7	(Gly,Thr,Ile,Gly,Ile,Gly,Thr,Pro,Ala,Glx,Asx)Phe
C-III-8	Ala(Cys,Ser,Asx,His,Asx,Glx,Phe,Asx,Pro,Asx,Asx,Ser,Ser,Thr)Phe
C-V-1	Thr-Val-Phe
C-V-2	Asx(Thr,Gly,Ser,Ser,Asx,Leu)Trp
C-V-3	(Thr,Ile,Asn,Gly,Val,Gln,Tyr,Pro,Leu)
C-V-4	Gly(Thr,Gly,Ser,Met,Thr)(Ile,Leu,Gly)Tyr
C-V-5	Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr-Leu-Asp-Thr-Glu-Tyr
C-V-6	(Glx,Gly,Met,Asx,Val,Pro,Thr,Ser,Ser,Gly,Glx,Leu)Trp
C-V-7	Thr-Ile-Asn-Gly-Val-Gln-Tyr(Pro,Leu,Ser,Pro,Ser,Ala)Tyr
C-VI-1	Val-Phe
C-VI-2	Gly(Leu,Ser,Glx,Thr,Glx,Pro,Gly,Ser,Phe,Leu,Tyr)
C-VI-3	Ala-Leu-Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr(Leu,Asx,Thr,Glx,Tyr,Phe)
C-VI-4	Ala-Tyr-Pro-Ser-Ile-Ser-Ala(Ser,Gly,Ala,Thr,Pro,Val,Phe,Asx,Asx,Leu,Trp,Asx,Glx,Gly,Leu,Val,Ser,Glx,Asx,Leu,Phe)
C-VII-1	Leu-Tyr
C-VII-2	Asx(Arg,Ala,Asx,Asx)
C-VII-3	(Asx,Arg,Ala,Asx,Asx,Lys,Val,Gly,Leu,Ala,Pro,Val,Ala)

TABLE II
(*continued*)

Designation of peptide	Structure or Composition
C-VII-4	Ile(Gly,Asx,Glx,Pro,Leu,Glx,Asx,Tyr,Leu,Asx,Thr,Glx,Tyr,Phe)
C-VIII-1	Ser-Val-Tyr
C-VIII-2	Tyr-Ala-Pro-Phe
C-VIII-3	Lys(Val,Gly,Leu,Ala,Pro,Val,Ala)
C-VIII-4	Leu-Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr
C-IX-1	Lys(Val,Gly)Leu
C-IX-2	Ile(Arg,Glx)Tyr
C-IX-3	(Ser-Pro,Ser,Ala)Tyr
C-IX-4	Ser-Val-Glu-Gly-Tyr-Trp
C-X-1	(Thr,Gly,Ser,Leu,Asx,Trp,Val,Pro,Val,Ser,Val,Glx,Gly,Tyr,Trp)
C-IX-1[b]	Tyr-Tyr
C-XI-2	(Trp,Val,Pro,Ser,Val,Tyr)

[a] The presence of Cys in the peptide was not determined. The half-cystine residue was asigned to this position on the basis of the knowledge of an analogous peptide isolated from the chymotryptic digest of the tryptic fragment of aminoethylated pepsin. [b] The complete hydrolysate of the peptide contained tyrosine only. The position of the peptide on peptide maps was different from that of free tyrosine.

ation procedure. Therefore in this study, intended to provide low molecular weight fragments which could readily be isolated by routine techniques, we decided to use chymotrypsin for the digestion because it is the relatively most specific enzyme besides trypsin. We used again sulfitolysis to unfold the molecule of the native enzyme because we had good experience with this procedure from our previous studies[1]. The peptide maps of the chymotryptic digest showed that S-sulfo-pepsin had been degraded to a great number of peptides. This result was expected because of the high content of aromatic amino acids and of leucine in pepsin[1,22]. The complexity of the digest was observed also during its first fractionation on the Dowex 50 column. The 11 basic fractions of the effluent from the column required additional fractionation. Fraction C-I, which contained also unadsorbed material, and fractions C-IX and C-X, showing the presence of tryptophan- and tyrosine-containing peptides, were separated to advantage by gel filtration on Sephadex G-25. Fractions containing mostly acidic peptides were separated on Dowex 1. This additional separations afforded peptides which were isolated in homogeneous form by paper techniques. Certain peptides were isolated from more than one fraction; they are, however, listed under one symbol only. The amino–acid composition of 43 peptides selected is given in Table I. The peptides are marked by symbol C (for chymotryptic digestion) and by a Roman numeral indicating the basic fraction from which the peptide was isolated. The Arabic numeral stands for the serial number of the peptide in the fraction. The peptides given in Table I were submitted to additional characterization in most cases. Peptides which had been obtained in parallel studies on the high-molecular weight fragments not at all or in a low yield only, were chosen for sequential analysis. The results of the work on these peptides are summarized in Table II. These data represent 82 nonoverlapping amino–acid sequences. A comparison of these data with the tentative amino–acid sequence of hog pepsin, reported[23] by us earlier, permits us to assign most of these peptides to defined regions of the pepsin molecule. Peptides C-III-5, C-V-1, C-VII-2, C-VII-3, and C-IX-2 are derived from the C-terminal region of pepsin, between Met IV and the C-terminal alanine. This region extend peptides C-II-6 and C-V-6. All the remaining peptides belong to the region of the molecule between Cys II and Cys III, except for peptides C-II-3, C-III-7, C-V-2, C-V-3, C-V-7, C-IX-3, and C-XI-2, derived from other parts of the polypeptide chain[23], and peptides C-V-5, C-VI-3, and C-VII-4, which represent the N-terminus of the pepsin molecule. Of the latter, special attention deserves peptide C-VI-3, Ala-Leu-Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn--Tyr(Leu,Asx,Thr,Glx, Tyr,Phe). The sequence of residues 3 through 11 in this peptide is identical with the N-terminal amino–acid sequence of the pepsin molecule, determined in independent experiments[1,24]. The peptide contains an additional sequence, Ala-Leu, at its N-terminal end. It has been shown a long time ago that pepsin originates in its inactive precursor, pepsinogen, whose polypeptide chain is (from the viewpoint of primary structure) identical with that of pepsin, yet longer by approximately 44

amino acid residues at the N-terminus. These residues are split off in the process of zymogen activation in the form of peptides of varying chain length (according to the conditions of activation) and active pepsin, N-terminated by the sequence Ile-Gly-Asp- ... is obtained. Quite recently, a few papers (summarized in[25]) appeared reporting that "Ile-pepsin" is not the sole product of pepsinogen activation and that pepsin with additional Ala-Leu sequence at the N-terminus ("Ala-pepsin") is another, yet minor product of pepsinogen activation. This "Ala-pepsin" is also the minor component of commercial pepsin preparations. Peptide C-VI-3, found by us, provides additional experimental evidence in favor of this fact.

Our data presented here will be compared with the results of other authors[24] in papers reporting on the complete structures of the high molecular weight fragments.

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