INVESTIGATION OF SUBTILISIN DIGEST OF PEPSIN CHAIN BETWEEN HALF-CYSTINES II AND III

Vladimír KOSTKA

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

Received November 8th, 1979

Aminoethylated hog pepsin was subjected to tryptic digestion and the longest fragment, arising from cleavage at S-(β-aminoethyl)-cysteine residue No II and III was isolated from the digest. This fragment was subjected to additional cleavage with subtilisin and the digest resolved into crude fractions by chromatography on Dowex 1. The isolation and final purification of the peptides was carried out by paper electrophoreses and paper chromatography. By these methods 55 peptides were obtained which were subjected to sequential analysis by stepwise degradation. The amino acid sequences of these peptides and their positions in the pepsin chain are given. These sequences provided overlaps for peptides obtained by hydrolysis of this part of the pepsin chain by other enzymes.

Systematic studies on the primary structure of hog pepsin, carried out in this Laboratory, permitted us to propose a tentative structure[1] of this protein in 1973. This structure summarizing the results of our preceding studies[2-5] as well as of studies by then unpublished[1], and also of this investigation, provided information on 324 residues of the molecule; of this number 113 residues formed a continuous N-terminal sequence and 96 were comprised in the C-terminal sequence. The determination of the complete covalent structure[6] of the entire protein required the knowledge of 8 overlaps of which 6 were localized in the region between methionine residues No I and II.

The fragmentation procedures which had been used in this Laboratory afforded this region in the form of high molecular weight fragments only. After cyanogen bromide cleavage[5] of S-sulfo-pepsin this region is obtained in fragment CB3 and partly also in longer fragment CB2, which is a result of incomplete cleavage of the chain at Met I (ref.[1]). If aminoethylated pepsin (RAE-pepsin) is digested with trypsin, then this part of the chain is contained in a 156-residue fragment (RAEP-tA 22, *cf.*[7]), originating from cleavage at S-(β-aminoethyl)-cysteine residues No II and III. In both cases, however, the size of the fragments does not permit their amino acid sequence to be determined directly, *e.g.* by stepwise degradation in the sequencer. For these reasons the fragments obtained by both procedures were subjected to additional enzymatic digestion with chymotrypsin[8-10] and thermolysin[8]. The sequential analysis of the peptides thus obtained provided data on the amino acid sequence

of the predominant part of the region studied, did not permit, however, the complete sequence to be derived. For these reasons other fragmentation procedures were sought, likely to yield the overlaps required.

The aim of this study was the fragmentation of tryptic fragment RAEP-tA 22 of RAE-pepsin[7] by subtilisin and the sequential analysis of the peptides thus obtained, mainly of those yielding overlaps for the chymotryptic and thermolysin peptides studied earlier[8-10].

EXPERIMENTAL

Material

The preparation of fragment RAEP-tA 22 by tryptic hydrolysis of RAE-pepsin and its purification were described in the preceding paper[7]. Subtilisin Novo was a dialyzed and lyophilized preparation of Novo Industri A/S, Copenhagen, Denmark. Carboxypeptidase A (DFP-treated) was purchased from Worthington Biochem. Corp., Freehold, N. J., U.S.A. Dowex 1-X2 (200 to 400 mesh) was from Fluka, A. G., Switzerland. Silica gel thin layer sheets (Silufol) and polyamide layer sheets were purchased from Kavalier, Czechoslovakia and BDH Chemicals Ltd., Poole, England, respectively. All the remaining chemicals used in this study were of analytical purity.

Methods

The peptide maps of the individual fractions were prepared by a combination of paper electrophoresis and chromatography, as described elsewhere[5,7]. The techniques of paper electrophoreses[11,12] and paper chromatography[13], elution of peptides and their identification, and certain special detection techniques were described in detail before[14]. Amino acid analysis was performed on samples of peptides hydrolyzed at 110°C, 20 or 70 h in 6M-HCl. The method of Spackman and coworkers[15] as modified by Benson and Patterson[16] was used and the analyses were carried out in Beckman-Spinco Model 120B Amino Acid Analyzer or Model 6020 Amino Acid Analyzer manufactured by the Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague. Tryptophan was determined by the qualitative test only[17]. The N-terminal amino acids of peptides were examined by dansylation[18]. Dansyl amino acids were identified by thin-layer chromatography[19,20] on silica gel or polyamide layer sheets. The C-terminal amino acids of the peptides were determined from the kinetics of carboxypeptidase A cleavage[7] or by hydrazinolysis[21,22]. The manual sequential degradation of peptides[23] and the identification of its products were carried out by the techniques described in detail earlier[3].

Digestion of fragment RAEP-tA 22 *by subtilisin*: Fragment RAEP-tA 22 (600 mg, 38 μmol) was dissolved in 60 ml of 1·25M pyridine acetate buffer, pH 6·3. This solution was heated at 37°C and subsequently 6 mg of subtilisin dissolved in 6 ml of water was added (enzyme to substrate ratio 1 : 100, w/w). The digestion was allowed to proceed 1 h at 37°C. After this period subtilisin (6 mg in 6 ml of water) was again added and the digestion was allowed to proceed for another hour; it was discontinued by the addition of acetic acid (pH of digest 5·0). The solution of the digest was evaporated almost to dryness at room temperature in a rotary evaporator. The residue was dissolved in 10 ml of water and the solution was rotary evaporated to dryness at a bath temperature of 30°C. This procedure was repeated once more. The resulting dry residue was dissolved in water and lyophilized. The weight of the lyophilized product was 580 mg.

Fractionation of substilisin digest by chromatography on Dowex 1: The lyophilisate of the subtilisin digest was dissolved in 200 ml of 1% *sym.* collidine and the pH of the solution was adjusted to 9·0 by 2M-NaOH. A small quantity of undissolved material was centrifuged off. The clear solution was applied onto a Dowex 1-X2 column (2·4 × 50 cm) equilibrated with 1% *sym.* collidine (pH 8·7) according to Guest and coworkers[24]. The chromatography was effected by an exponential elution gradient of increasing molarity of acetic acid, described by the authors and slightly modified by us. A 1000 ml mixing device, filled up completely with 1% solution of *sym.* collidine, was fed 500 ml of 0·1M acetic acid from a reservoir. The latter was then filled with 0·3, 1, 5, 10M, and glacial acetic acid (always 500 ml) and the elution was continued at a flow rate of 22 ml per 10 min (1 fraction). After the last eluent had been pumped out from the reservoir and mixing device, the column was eluted by glacial acetic acid. The total number of fractions collected was 168. The course of the chromatography was monitored by checking aliquots removed from each fraction and corresponding to 4·5% of its volume. The aliquots were taken to dryness *in vacuo* over potassium hydroxide and chromatographed. The effluent was pooled into 24 fractions according to the results of chromatography.

Isolation of homogeneous peptides and their analyses. The pooled fractions were analyzed by the method of peptide maps. The individual peptides were isolated by paper electrophoreses, or chromatography, or by a combination of several techniques, and subjected to amino acid analysis. The homogeneous peptides were characterized by end group analysis and subjected to stepwise degradation wherever necessary.

RESULTS AND DISCUSSION

The studies on the enzymatic digest of high molecular weight fragments comprising the middle region of the pepsin chain, *i.e.* between the first and second methionine residue, permitted us to unambiguously define[1] the N-terminal part of this region from methionine No 80 to tyrosine No 113. The remaining part of this region, corresponding to 86 residues, was represented by several mutually interchangeable segments of known sequence. As one of the possible lines of approach to obtaining the overlapping peptides which would permit the arrangement of these segments we chose fragmentation of this part of the chain by another enzyme. Fragment RAEP-tA 22, obtained by tryptic digestion of RAE-pepsin[7] and containing this region, was used to start with and subtilisin was employed for its degradation. This enzyme shows an extremely low specificity[25] and is usually used, like papain or pronase, for secondary cleavage of short peptides. If subtilisin is to be used for the degradation of large peptides, the cleavage should be carried out under conditions limiting the multiplicity of the arising products, *i.e.* at a lower enzyme concentration and for a shorter period. In this study we examined the cleavage of fragment RAEP-tA 22 in orienting experiments and evaluated the digest by peptide maps. We carried out the cleavage in pyridine acetate buffer at pH 6·5 in which fragment RAEP-tA 22 was readily soluble. The activity of the enzyme at this pH, which is roughly by 2 units lower than the pH-optimum, is approximately 40% of the maximal activity[26]. The cleavage at room temperature and at an enzyme to substrate ratio of 1 : 100 afforded after 2 h a digest with a very low undigested part and an adequately rich

mixture of smaller peptides. We carried out therefore the preparative scale experiment under these conditions. The enzyme was added in two portions (each corresponding to 1/100 of the quantity of the substrate) at an interval of one hour; the total time of cleavage was 2 h and the final enzyme to substrate ratio was 1 : 50. The cleavage was terminated by acidification of the digest by glacial acetic acid to pH 5 where the activity of subtilisin is practically zero[26]. The acidification was not paralleled by the formation of a precipitate. This indicated a low content of large fragments and therefore their separation by gel filtration was omitted. The lyophilized digest was dissolved to a *c.* 0·15 mM solution in 1% *sym.* collidine, pH 8·7, which after adjustment of pH to 9·0 and removal of a small amount of turbidity by centrifugation was directly applied to the anion exchanger. The elution was effected by an exponential gradient which was used to advantage for the fractionation of low molecular weight peptides derived from hog pepsin before[27]. In this manner we resolved the digest into 168 fractions which were studied by paper chromatography. According to the results of chromatography and of specific tests for the presence of tyrosine and tryptophan the effluent was divided into 24 portions. The latter, save for a few exceptions, contained mixtures of peptides and were therefore subjected to additional separation procedures on paper. Homogeneous peptides were obtained in most cases by the application of one separation procedure only. The amino acid composition of the 55 homogeneous peptides thus obtained is shown in Table I. Of these peptides 6 only were not analyzed further. All the remaining peptides were subjected to end group analysis. Peptides assumed to provide new information (altogether 20) were analyzed sequentially. The sequential data obtained by the analysis of the subtilisin digest of fragment RAEP-tA 22 are summarized in Table II. As demonstrated, the subtilisin digestion of the fragment afforded a rich mixture of peptides ranging in size from dipeptide to a 27-residue peptide. Because of the experimental conditions used (pH, enzyme to substrate ratio, temperature) the cleavage was not excessively deep; this is evidenced by the fact that peptides containing 6 amino acid residues and more represented 50% of the material and that the quantity of free amino acids was minimal. Subtilisin can be used to advantage under appropriate conditions not only for deep, secondary cleavage of peptides of intermediate size but also for primary cleavage of large peptide fragments. The broad specificity of subtilisin often commented[25,28] makes it impossible to define a marked preference of the enzyme for certain bonds; under the conditions used in this study very often (in 24 peptides) bonds were cleaved in which the amino group of glycine was involved. Relatively rare was cleavage at tyrosines and hydrophobic aliphatic residues; this permitted us to obtain overlaps for a number of peptides obtained by digestion of this part of the chain[8-10] or of whole S-sulfo-pepsin[27,29] by chymotrypsin and thermolysin. This was also facilitated by the fact that because of the low specificity of subtilisin most of the peptides obtained (with the exception of peptides from the N- and C-terminal region and with the exception of peptide

50 60 70

Cys-Ser-Asp-His-Asn-Gln-Phe-Asn-Pro-Asp-Asp-Ser-Ser-Thr-Phe-Glu-Ala-Thr-Ser-Gln-Glu-Leu-Ser-Ile-Thr-Tyr-

55

24 22

44

80 90 100

Gly-Thr-Gly-Ser-Met-Thr-Gly-Ile-Leu-Gly-Tyr-Asp-Thr-Val-Gln-Val-Gly-Gly-Ile-Ser-Asp-Thr-Asn-Gln-Ile-

23 25

33 5

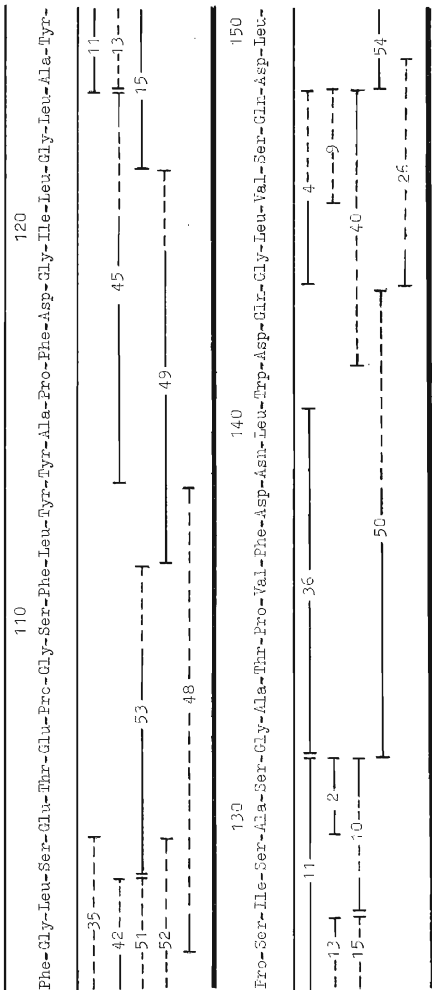
8

3 35

1 37 42

51

52


110
120
Phe-Gly-Leu-Ser-Glu-Thr-Glu-Pro-Gly-Ser-Phe-Leu-Tyr-Tyr-Ala-Pro-Phe-Asp-Gly-Ile-Leu-Gly-Leu-Ala-Tyr-
35
11
42
45
13
51
53
15
52
49
48
130
140
150
Pro-Ser-Ile-Ser-Ala-Ser-Gly-Ala-Thr-Pro-Val-Phe-Asp-Asn-Leu-Trp-Asp-Gln-Gly-Leu-Val-Ser-Gln-Asp-Leu-
11
36
4
13
2
9
15
10
40
50
54
26

160 170
Phe-Ser-Val-Tyr-Leu-Ser-Ser-Asn-Asp-Asp-Ser-Gly-Ser-Val-Val-Leu-Leu-Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr-

20 41 27 19 32 21 40 56 47

180 190 200
Thr-Gly-Ser-Leu-Asn-Trp-Val-Pro-Val-Ser-Val-Glu-Gly-Tyr-Trp-Gln-Ile-Thr-Leu-Asp-Ser-Ile-Thr-Met-Asp-

19 29 18 28 21 30 34 6 36 7 31 39 43

206
Gly-Glu-Thr-Ile-Ala-Cys

34 39

FIG. 1

Cleavage of Tryptic Fragment of RAE-pepsin (RAEP-tA 22) by Subtilisin and Survey of Peptides Isolated

The complete amino acid sequence of hog pepsin[6] between half-cystine residues No II and III is shown in the first line. The sequence of this region was derived from the results of several independent studies (including this study) which are discussed in the adjoining paper[9]. The peptides from the subtilisin digest of fragment RAEP-tA 22 comprising this region are marked by horizontal bars (——— sequences determined in full, ------ undetermind sequences) and the serial number of the peptide (with the omission of the symbol SL, *cf.* Table II). The residue numbers correspond to the numbering of the complete amino acid sequence of hog pepsin[6].

TABLE I

Amino Acid Analyses of Peptides from the Subtilisin Digest of Fragment RAEP-tA 22

The analyses were performed on 20-h hydrolysates by methods described under Experimental. The values of molar amino acid ratios, calculated according to[30], are not corrected unless stated otherwise. None of the peptides described contained either lysine or arginine.

Amino acid	Peptide SL-													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Aminoethylcysteine														
Histidine														
Aspartic acid														
Threonine	2·0		1·0					0·9	0·9					1·0
Serine	1·2	1·0	1·0	0·9	1·0		1·0	0·8	1·0	1·8	2·9		0·9	
Glutamic acid				1·0			1·0							
Proline											1·2		1·1	
Glycine	2·1		2·1	1·0	1·0	1·0		1·0				0·6		
Alanine		1·1								1·0	2·1		1·0	
Valine				1·0					0·9					
Methionine	0·8							0·5						0·8
Isoleucine					0·9					0·9	1·0	0·8		
Leucine				1·0		1·0						1·2		
Tyrosine											1·0		1·0	
Phenylalanine														
Tryptophan														

TABLE I
(*Continued*)

Amino acid	Peptide SL-													
	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Aminoethylcysteine														
Histidine														
Aspartic acid					1·1						1·1	1·1	1·1	1·2
Threonine				1·1	1·1		0·7	0·9		1·0	0·8			
Serine	1·1	1·9			1·1	1·2	0·7	1·0			1·1	1·2		
Glutamic acid				1·1						1·1	1·2	1·2		
Proline	1·0	1·0												
Glycine	1·0		1·1		1·1		0·9	0·6	1·9		1·9	1·1	1·8	
Alanine	1·0	1·0								1·0				
Valine						1·0					1·8	0·9		
Methionine														
Isoleucine		1·0		1·0				0·7	1·0		0·9		0·9	
Leucine	1·0				1·1		0·7	0·9	1·0			1·1		1·0
Tyrosine	0·7	0·8	1·0		1·7	0·8	1·0	1·0	0·9					
Phenylalanine														
Tryptophan														

Amino acid	Peptide SL-													
	29	30	31	32	33	34	35	36	37	38	39	40	41	42
Aminoethylcysteine											0·8			
Histidine														
Aspartic acid		1·0	0·8	1·2	1·1	2·1	2·6	1·8	1·0		1·9	0·9	3·0	2·1
Threonine					1·0	1·7	1·9	0·8	1·1	1·0	1·9			1·0
Serine	1·1	1·0	1·0	1·7		1·1	1·7				1·0	0·8	3·6[a]	
Glutamic acid	1·0	1·0	1·1		1·0	1·2	1·6		1·3	1·1	1·1	1·6		1·0
Proline	1·1	1·0	1·2					1·1						
Glycine				1·8	1·9	1·3	3·9	1·1	2·2	1·2	1·1	1·0	1·3	1·1
Alanine						0·9		1·0			1·0			
Valine	2·9	2·9	3·2		1·8		2·0	1·1	1·6			0·8	1·8[b]	
Methionine						0·4					0·9			
Isoleucine				1·0		1·6	1·5		1·1	1·0	1·9			0·8
Leucine			0·7			1·0	0·8	1·0	0·8		1·0	0·8	2·1	1·1
Tyrosine					0·8		0·8		0·6	0·8				
Phenylalanine							0·6	0·9						0·8
Tryptophan		+	+							+				

TABLE I
(*Continued*)

Amino acid	Peptide SL-												
	43	44	45	46	47	48	49	50	51	52	53	54	55
Aminoethylcysteine													
Histidine													0.7
Aspartic acid	1·2		1·0	2·3	1·3		1·1	3·0	2·0	2·0		0·5	4·9
Threonine		0·9		1·0	1·1	1·1		1·0	1·0	1·1	1·0		2·6
Serine	1·6	1·0		2·8	2·1	2·0			1·3	0·9	1·7	0·7	4·5[a]
Glutamic acid	1·1	0·9				2·1		1·1	1·0	1·4	1·9		3·9
Proline	1·0		1·3			1·0	0·8	0·9			0·9		1·1
Glycine	1·4		1·9	2·7	3·0	1·5	1·4	1·1	2·0	1·3	1·0		0·8
Alanine			1·0				1·0	0·8					1·1
Valine	3·0							1·0				1·0	
Methionine													
Isoleucine		0·7	0·9	0·9	1·0		0·9		1·6	0·7			1·0
Leucine	0·8	0·9	1·5	1·3		1·5	2·1	1·3	1·1	1·0		1·1	1·4
Tyrosine	0·9	0·6	+	1·4	1·9	0·6	1·3					+	0·9
Phenylalanine			1·0			1·0	1·1	0·8	0·8	0·9	1·0	1·0	1·9
Tryptophan	+							+					

+ Qualitative determination; [a] extrapolated value; [b] values from 70-h hydrolysate.

TABLE II

Peptides Obtained from Subtilisin Digest of Fragment RAEP-tA 22 of Hog Aminoethyl-Pepsin

The peptides are marked by the symbol SL and serial numbers which correspond to the order of emergence of the peptides from the Dowex 1 column. The N-terminal end groups were determined by dansylation, the C-terminal groups by hydrazinolysis or from the kinetics of cleavage of the peptides by carboxypeptidase A. In the latter cases the determined C-terminal groups or sequences are marked by arrows (←). The sequences of the peptides given in this Table were determined by stepwise degradation by using the procedures described in detail elsewhere[3]. The presence of amides in the peptides not studied sequentially was judged by their mobility at high voltage electrophoresis (pH 5·6). The position of the peptides in the pepsin chain are shown on the background of its complete structure[6]. Unsequenced peptides or peptides sequenced uncompletely are given in the form corresponding to their complete structure.

Designation of peptide	Structure (composition) of peptide	Residue numbers
SL-1	Gly(Thr,Gly,Ser)Met	76—81
SL-2	Ala-Ser	130—131
SL-3	Gly(Thr,Gly)Ser	76—79
SL-4	Gly-Leu-Val(Ser,Gln)	144—148
SL-5	Gly-Ile-Ser	93—95
SL-6	Gly-Leu	[a]
SL-7	Ser-Gln	[a]
SL-8	Gly(Ser,Met,Thr)	78—81
SL-9	(Val,Ser,Gln)	146—148
SL-10	Ile(Ser,Ala)Ser	128—131
SL-11	Ala-Tyr-Pro-Ser-Ile-Ser-Ala-Ser	124—131
SL-12	Gly-Ile-Leu	[a]
SL-13	(Ala,Tyr,Pro)Ser	124—127
SL-14	(Met,Thr)	[a]
SL-15	Gly-Leu-Ala-Tyr(Pro,Ser)	122—127
SL-16	(Tyr,Pro,Ser,Ile,Ser,Ala)	125—130
SL-17	Gly-Tyr	[a]
SL-18	Gln-Ile-Thr	191—193

TABLE II
(*Continued*)

Designation of peptide	Structure (composition) of peptide	Residue numbers
SL-19	Tyr(Tyr,Thr,Gly,Ser,Leu,Asn)	174—180
SL-20	Ser-Val-Tyr	152—154
SL-21	Tyr(Thr,Gly,Ser,Leu)	175—179
SL-22	Leu(Ser,Ile,Thr,Tyr,Gly)	71—76
SL-23	Gly(Ile,Leu,Gly,Tyr)	82—86
SL-24	(Glu,Ala,Thr)	65—67
SL-25	(Thr,Val,Glx,Val,Gly,Gly,Ile,Ser,Asx)	88—96
SL-26	Gly(Leu,Val,Ser,Glx,Asx)	144—149
SL-27	Gly(Gly,Ile,Asp)	168—171
SL-28	Leu-Asp	194—195
SL-29	Val-Pro-Val-Ser-Val-Glu	182—187
SL-30	(Asx,Trp,Val,Pro,Val,Ser,Val,Glx)	180—187
SL-31	Leu(Asx,Trp,Val,Pro,Val,Ser,Val,Glx,)	179—187
SL-32	Gly(Gly,Ile,Asp,Ser,Ser)	168—173
SL-33	Gly-Tyr-Asp-Thr-Val-Gln(Val,Gly)	85—92
SL-34	Leu-Asp-Ser-Ile(Thr,Met,Asx,Gly,Glx,Thr,Ile,Ala)	194—205
SL-35	Gly-Tyr-Asp-Thr-Val(Glx,Val,Gly,Gly,Ile,Ser,Asx,Thr,Asx,Glx,Ile,Phe,Gly,Leu,Ser)	85—104
SL-36	Gly-Ala-Thr-Pro-Val-Phe-Asn-Asn-Leu	132—140
SL-37	Gly(Ile,Leu,Gly,Tyr,Asx,Thr,Val,Glx,Val)	82—91
SL-38	Gly(Tyr,Trp,Gln,Ile,Thr)	188—193
SL-39	Leu-Asp-Ser-Ile-Thr-Met-Asp-Gly(Glx,Thr,Ile,Ala)Aec[b]	194—206
SL-40	(Asx,Glx,Gly,Leu,Val,Ser,Glx)	142—148
SL-41	Leu-Ser-Ser-Asn-Asp-Asp-Ser-Gly(Ser,Val)Val-Leu ← ←	155—166
SL-42	Asp-Thr-Asn-Gln-Ile-Phe(Gly,Leu)	96—103
SL-43	Ser(Leu,Asx,Trp,Val,Pro,Val,Ser,Val,Glx,Gly,Tyr)	178—189

Designation of peptide	Structure (composition) of peptide	Residue numbers
SL-44	Glu-Leu-Ser-Ile-Thr-Tyr	70—75
SL-45	Tyr-Ala-Pro-Phe-Asp-Gly-Ile(Leu,Gly)Leu	114—123
SL-46	Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr-Thr-Gly-Ser-Leu-Asn	168—180
SL-47	Gly-Gly-Ile-Asp-Ser-Ser-Tyr-Tyr-Thr-Gly	168—177
SL-48	Gly(Leu,Ser,Glx,Thr,Glx,Pro,Gly,Ser,Phe,Leu,Tyr)	102—113
SL-49	Leu-Tyr-Tyr-Ala-Pro-Phe-Asp-Gly(Ile,Leu)	112—121
SL-50	Gly-Ala-Thr-Pro-Val-Phe-Asp(Asx,Leu,Trp,Asx,Glx)	132—143
SL-51	Gly(Ile,Ser,Asx,Thr,Asx,Glx,Ile,Phe,Gly,Leu)	93—103
SL-52	Asx(Thr,Asx,Glx,Ile,Phe,Gly,Leu,Ser)	96—104
SL-53	Ser-Glu-Thr-Glu-Pro-Gly(Ser,Phe)	104—111
SL-54	Asp-Leu-Phe-Ser-Val-Tyr	149—154
SL-55	Ser-Asp-His-Asn(Glx,Phe,Asx,Pro,Asx,Asx,Ser,Ser,Thr,Phe,Glx,Ala,Thr,Ser,Glx,Glx,Leu,Ser,Ile,Thr,Tyr,Gly)	51—76

[a] Not given; the peptide cannot be ascribed unambiguously one position only in the region studied; [b] Aec = S-(β-aminoethyl)cysteine.

SL-41) were overlapping. The localization of the individual subtilisin peptides in the middle part of the peptide chain of hog pepsin is shown on the background of the complete structure of this region in Fig. 1. The determination of this complete structure is also the subject of the adjoining paper describing the role of the subtilisin peptides in this determination.

I am indebted to Mrs E. Bulantová for skillful technical assistance. I thank Mr K. Grüner and Mrs A. Čermáková for the sequential degradation of peptides and to Mr J. Zbrožek, Miss V. Himrová and Mrs E. Dršková for the amino acid analyses of peptides.

REFERENCES

1. Morávek L., Kostka V.: FEBS Lett. *35*, 276 (1973).
2. Keil B., Morávek L., Šorm F.: This Journal *32*, 1968 (1967).
3. Kostka V., Morávek L., Šorm F.: Eur. J. Biochem. *13*, 447 (1970).
4. Morávek L.: FEBS Lett. *23*, 337 (1972).
5. Morávek L., Kostka V.: This Journal *38*, 304 (1973).
6. Morávek L., Kostka V.: FEBS Lett. *43*, 207 (1974).
7. Kostka V.: This Journal *43*, 2942 (1978).
8. Morávek L.: This Journal *39*, 1933 (1974).
9. Morávek L., Kostka V.: This Journal *46*, 655 (1981).
10. Kostka V.: *Thesis*. Czechoslovak Academy of Sciences, Prague 1980.
11. Prusík Z., Štěpánek J.: J. Chromatogr. *87*, 73 (1973).
12. Mikeš O.: This Journal *22*, 831 (1957).
13. Waley S. G., Watson J.: Biochem. J. *55*, 328 (1953).
14. Meloun B., Kostka V., Šorm F.: This Journal *28*, 2749 (1963).
15. Spackman D. H., Stein W. H., Moore S.: Anal. Chem. *30*, 1190 (1958).
16. Benson jr. J. V., Patterson J. A.: Anal. Chem. *37*, 1108 (1965).
17. Smith I.: Nature *171*, 43 (1953).
18. Gray W. R., Hartley B. S.: Biochem. J. *89*, 59P (1963).
19. Novotný J., Franěk F.: Chem. Listy *62*, 995 (1968).
20. Meloun B.: Personal communication.
21. Akabori S., Ohno K., Narita K.: Bull. Chem. Soc. Jap. *25*, 214 (1952).
22. Meloun B., Vaněček J., Šorm F.: This Journal *23*, 1788 (1958).
23. Edman P.: Acta Chem. Scand. *4*, 283 (1950).
24. Guest J. R., Carlton B. C., Yanofsky C.: J. Biol. Chem. *242*, 5397 (1967).
25. Hill R. L.: Adv. Protein Chem. *20*, 37 (1965).
26. Graae J.: Acta Chem. Scand. *8*, 356 (1954).
27. Kostka V., Salvetová A.: This Journal *39*, 2291 (1974).
28. Smyth D. G.: Methods Enzymol. *11*, 214 (1967).
29. Morávek L., Kysilka Č.: This Journal *39*, 2301 (1974).
30. Hirs C. H. W., Moore S., Stein W. H.: J. Biol. Chem. *219*, 623 (1956).

Translated by the author.