Amino Acid Sequence of Bovine Carboxypeptidase A. Isolation and Characterization of the Thermolytic Peptides of the Cyanogen Bromide Fragment F_I*

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ABSTRACT: Following the isolation of the tryptic and chymotryptic peptides, the thermolytic peptides of the largest fragment of bovine carboxypeptidase A, F_I, obtained by cleavage with cyanogen bromide, were isolated and characterized. In contrast to the tryptic and chymotryptic digests, cleavage by thermolysin resulted in the solubilization of 96% of the protein with 189 of the 198 amino acid residues present in the soluble fraction.

Analysis of the cleavage pattern indicated that about one-quarter of the peptide bonds in F_I were hydrolyzed, the majority of which (69%) were composed of the X-Leu, X-Ile, X-Phe, and X-Val type.

In the previous report, the soluble tryptic and chymotryptic peptides derived from the largest fragment (F_I) of bovine carboxypeptidase A, obtained by cleavage with cyanogen bromide, were described (Bradshaw *et al.*, 1971a). However, in view of the fact that each of these digests produced appreciable amounts of insoluble material, the use of thermolysin as the cleavage agent was deemed a probable route to produce peptides from the portions of the fragment that were difficult to solubilize. The choice of this enzyme was prompted by its specificity (Matsubara *et al.*, 1966; Ohta and Ogura, 1965; Matsubara *et al.*, 1969) and by its successful use in other sequence problems (Matsubara and Sasaki, 1968; Ambler and Meadway, 1968; Blow *et al.*, 1969; Bradshaw, 1969). The efficiency of this enzyme is documented by the fact that 190 of the 198 residues (96%) were recovered in the soluble thermolytic peptides. Their isolation and characterization are documented below.

Experimental Procedure

The method of preparation of *S*-CM F_I[1] has been reported previously (Nomoto *et al.*, 1969). The conditions for digestion with thermolysin (obtained from Daiwa Kasei K.K., Osaka, Japan) are the same as those used for the hydrolysis of fragment F_{III} (Bradshaw, 1969). The methods for the isolation and characterization of the peptides are the same as those described previously (Bradshaw *et al.*, 1969a).

Results

Thermolytic Digest. The digest of *S*-CM F_I with thermolysin was prepared at 37°, pH 8.0, in the pH-Stat. The enzyme was added to a 1–2% solution of F_I to give a final concentration of 0.5% weight of substrate. After 2 hr of digestion, a second equal aliquot was added and the reaction was allowed to proceed for an additional hour. The reaction was terminated by adjustment to pH 2.0 with 6 N HCl.

Isolation of the Thermolytic Peptides. The thermolytic digestion mixture, which contained only small amounts of insoluble material after acidification to pH 2.0, was fractionated on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine–acetic acid buffers which yielded a linear gradient with regard to pH (Bradshaw *et al.*, 1969b). The elution profile is shown in Figure 1. The fractions were pooled according to the solid bars and examined for purity by high-voltage electrophoresis. Impure peptides were fractionated on columns of Dowex 1-X2, Dowex 50-X2, and by paper electrophoresis. The amino acid composition of each of the purified peptides obtained is given in Table I. The number of residues, the per cent yield, the subsequent purification procedure used, and the location of the peptide in the completed sequence are also given at the bottom of Table I (Bradshaw *et al.*, 1971b). In keeping with the format employed in the analysis of tryptic and chymotryptic digests, the peptides were numbered on the basis of their sequence of elution from the Dowex 50-X8 column. The characterization of the peptides is described below.

Characterization of the Thermolytic Peptides. FRACTION I. Fraction I was subfractionated on a 0.9 × 150 cm column of Dowex 1-X2, as shown in Figure 2. Nine pools were obtained. Fraction Th I-7 and -9 contained peptides in low yield derived from contaminating amounts of F_{III} in the preparation of F_I, whereas fractions Th I-3 and -8 contained insufficient material for further analysis. Fraction Th I-1 was further purified on Dowex 50-X2 (Figure 3) and yielded two peptides, described in Table II. The first was positioned from composition while the second was completely sequenced by Edman degradation. The latter was judged by high-voltage electrophoresis to contain a residue of glutamine. Fraction Th I-2 contained a single peptide which was subjected to two rounds of Edman degradation before the degradation termi-

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[1] Abbreviations used are: Th-, thermolytic peptide; *S*-CM, *S*-carboxymethylcysteine.

TABLE I: Amino Acid Composition of the Thermolytic Peptides of Fragment F_1.[a]

Amino Acid	Th-I-1-1	Th-I-1-2	Th-I-2	Th-I-4-1	Th-I-4-2	Th-I-5-1	Th-I-5-2	Th-I-6-1	Th-II-1-1	Th-II-1-2 PC-1	Th-II-1-2 PC-2	Th-II-4
Lysine			0.98 (1)									
Histidine												
Arginine												
S-Carboxymethyl-cysteine				0.80 (1)								
Aspartic acid			2.00 (2)		2.15 (2)	1.89 (2)		1.92 (2)				1.03 (1)
Threonine	1.80 (2)		2.67 (3)	1.07 (1)	1.00 (1)	1.13 (1)	1.61 (2)		1.34 (1)	1.00 (1)	1.78 (2)	
Serine	0.83 (1)	1.01 (1)	1.32 (1)	3.08 (3)				2.73 (3)	3.00 (3)			
Glutamic acid		0.99 (1)	3.01 (3)			1.23 (1)	2.01 (2)	1.20 (1)				0.99 (1)
Proline		1.01 (1)	1.23 (1)		1.00 (1)	1.13 (1)	1.01 (1)					
Glycine	1.00 (1)			1.07 (1)	1.01 (1)	1.01 (1)		2.91 (3)		1.10 (1)		
Alanine		0.98 (1)			1.02 (1)	1.03 (1)	0.99 (1)	1.11 (1)				
Valine				2.02 (2)	0.95 (1)[b]	0.99 (1)[b]			1.34 (1)	1.06 (1)		
Isoleucine	1.92 (2)[b]		1.10 (1)		0.96 (1)[b]	1.02 (1)[b]	1.77 (2)[b]	0.91 (1)			1.97 (2)[b]	1.02 (1)
Leucine		1.94 (2)	1.21 (1)	1.07 (1)		0.89 (1)				2.00 (2)		1.02 (1)
Tyrosine								0.65 (1)				
Phenylalanine					0.97 (1)	0.73 (1)						0.94 (1)
Tryptophan							0.40 (1)	0.35 (1)				
Homoserine and Lactone												
Total	6	6	13	9	9	11	9	13	5	5	4	5
% yield	18	84	22	7	16	10	15	4	33	27	25	10
Purification procedure[d]	D X1; D X50	D X1; D X50	D X1	D X1; D X50	D X1; D X50	D X1; D X50	D X1; D X50	D X1; D X50	D X1; D X50	D X1; D X50, PC	D X1; D X50, PC	D X1
Residue No.	241–246	280–285	209–221	132–140	109–117	107–117	286–294	250–262	132–136	295–299	243–246	104–108

Amino Acid	Th-III-1	Th-III-2-1	Th-III-2-2	Th-III-3	Th-III-4-1	Th-III-5	Th-IV-1	Th-IV-2	Th-IV-3-1	Th-IV-4-1	Th-IV-5	Th-IV-6
Lysine				0.99 (1)				1.15 (1)				
Histidine												
Arginine												
S-Carboxymethyl-cysteine										1.04 (1)		
Aspartic acid			1.14 (1)	1.26 (1)				1.07 (1)	1.02 (1)			1.73 (1)
Threonine				2.68 (3)			0.98 (1)				1.82 (2)	
Serine		1.84 (2)		1.07 (1)				1.87 (2)				0.96 (1)
Glutamic acid			0.90 (1)	2.15 (2)	1.08 (1)			2.00 (2)			2.00 (2)	1.07 (1)
Proline				1.00 (1)								
Glycine		2.00 (2)				1.06 (1)						1.08 (1)
Alanine	3.20 (3)	1.02 (1)			1.88 (2)			1.00 (1)			1.00 (1)	
Valine	0.90 (1)				1.00 (1)	0.83 (1)	1.00 (1)	1.78 (2)	1.44 (1.5)[b]			
Isoleucine				1.00 (1)					0.53 (0.5)[b]			0.97 (1)
Leucine			1.00 (1)			1.11 (1)	1.02 (1)			1.00 (1)		
Tyrosine				0.57 (1)								0.97 (1)
Phenylalanine												
Tryptophan											0.61 (1)	0.51 (1)
Homoserine and Lactone												
Total	4	5	3	11	4	3	3	9	3	2	6	8
% yield	39	13	23	19	31	17	28	77	31	20	5	12
Purification procedure[d]	D X1	D X1; D X50	D X1; D X50	D X1	D X1; D X50	D X1	D X1	D X1	D X1; D X50	D X1; D X50	D X1	D X1
Residue No.	226–229	250–254	219–221	208–218	226–229	295–297	298–299	170–178	179–181	137–138	289–294	255–262

Amino Acid	Th-V-1	Th-V-3	Th-V-4	Th-V-6	Th-VI-2-1	Th-VI-2-2	Th-VI-3	Th-VI-4	Th-VII-5	Th-VII-6	Th-VII-7	Th-VII-8
Lysine			1.00 (1)									
Histidine												
Arginine										1.28 (1)		
S-Carboxymethyl-cysteine												
Aspartic acid			1.01 (1)	1.02 (1)						4.20 (4)		
Threonine								0.93 (1)	1.04 (1)			
Serine	0.98 (1)	1.01 (1)	1.90 (2)		1.81 (2)							
Glutamic acid		1.05 (1)	2.05 (2)		1.00 (1)		1.05 (1)	1.08 (1)			0.99 (1)	
Proline												0.94 (1)
Glycine	1.02 (1)									2.03 (2)		1.07 (1)
Alanine			1.01 (1)							1.98 (1)		
Valine			1.95 (2)							2.01 (2)		
Isoleucine				1.00 (1)								
Leucine		0.94 (1)				0.92 (1)	0.95 (1)					
Tyrosine		0.73 (1)	0.56 (1)		0.60 (1)							1.99 (2)
Phenylalanine				0.97 (1)		1.09 (1)		1.88 (2)	0.95 (1)		1.01 (1)	
Tryptophan										0.40 (1)		
Homoserine and Lactone												
Total	2	4	10	3	4	2	2	4	2	11	2	4
% yield	25	9	4	49	6	5	41	12	6	5	11	4
Purification procedure[d]	D X1	D X1	D X1	D X1	D X1; D X50	D X1; D X50	D X1	D X1	D X1	D X1	D X1	D X1
Residue No.	241–242 (253–254)	198–201	169–178	104–106	197–200	279–280 (106–107) (192–193)	107–108	267–270	267–268	139–150	269–270	204–207

TABLE I (*Continued*)

Amino Acid	Th-VIII-1	Th-VIII-2	Th-IX-2-3	Th-IX-2-4	Th-IX-4	Th-IX-5	Th-XI-1	Th-XI-2	Th-XI-4	Th-XI-5	Th-XII-1	Th-XII-2
Lysine				1.12 (1)			1.02 (1)		1.03 (1)	1.07 (1)	1.24 (1)	2.17 (2)
Histidine			1.04 (1)	1.00 (1)				1.00 (1)	1.01 (1)	1.01 (1)		1.07 (1)
Arginine						1.05 (1)						
S-Carboxymethylcysteine										0.62 (1)		0.81 (1)
Aspartic acid				1.15 (1)		3.60 (4)			2.71 (3)			
Threonine			1.00 (1)	0.98 (1)						0.82 (1)		0.98 (1)
Serine				3.60 (4)			1.00 (1)	1.90 (2)		2.84 (3)	1.14 (1)	3.58 (4)
Glutamic acid			1.00 (1)	1.10 (1)						0.95 (1)		0.99 (1)
Proline				1.02 (1)						0.99 (1)		0.99 (1)
Glycine				1.12 (1)	0.98 (1)	0.99 (1)			1.06 (1)	0.85 (1)		2.75 (3)
Alanine		0.98 (1)		1.00 (1)		1.95 (2)	2.24 (2)				1.00 (1)	1.86 (2)
Valine						1.01 (1)	0.85 (1)		1.72 (2.5)[c]		1.00 (1)	
Isoleucine	1.00 (1)		0.92 (1)					0.86 (1)	0.28 (0.5)[c]			
Leucine					0.90 (1)			1.04 (1)				
Tyrosine				1.70 (2)	1.03 (1)					0.67 (1)		1.42 (2)
Phenylalanine		1.00 (1)							0.92 (1)			0.88 (1)
Tryptophan						0.30 (1)						
Homoserine and Lactone	0.95 (1)		0.88 (1)									
Total	2	2	5	14	3	10	5	5	10	11	4	19
% yield	7	5	8	17	4	33	20	3	5	5	53	6
Purification procedure[d]	D X1	D X1	D X1; D X50	D X1; D X50	D X1	D X1	D X1	D X1	D X1	D X1	D X1	D X1
Residue No.	300–301	117–118 (191–192)	300–304	156–169	233–235	141–150	222–226	193–197	179–188	158–168	222–225	151–169

Amino Acid	Th-XIII-1	Th-XIV-1-1	Th-XIV-1-2	Th-XIV-1-3	Th-XIV-3	Th-XV-1	Th-XVI-1	Th-XVI-2	Th-XVI-3	Th-XVII	Th-XIX-1	Th-XX-1	Th-XXI
Lysine		1.14 (1)	1.00 (1)	1.08 (1)	0.90 (1)	1.18 (1)		1.34 (1)	1.06 (1)		1.88 (2)		
Histidine	1.07 (1)				1.07 (1)					0.96 (1)	0.98 (1)		
Arginine							1.04 (1)			0.98 (1)		2.00 (2)	2.05 (2)
S-Carboxymethylcysteine													
Aspartic acid					1.81 (2)					1.06 (1)	1.89 (2)	1.29 (1)	1.03 (1)
Threonine				0.84 (1)			0.99 (1)			1.04 (1)		0.97 (1)	0.96 (1)
Serine	2.50 (2)	1.01 (1)		0.97 (1)		0.99 (1)	0.97 (1)			1.00 (1)			
Glutamic acid	1.44 (1)									1.03 (1)			
Proline													
Glycine			2.03 (2)	0.88 (1)	1.04 (1)						1.16 (1)	2.31 (2)	1.08 (1)
Alanine			1.00 (1)					0.76 (1)			1.00 (1)		
Valine					0.62 (1)						0.88 (1)		
Isoleucine	0.86 (1)					0.86 (1)							
Leucine		0.99 (1)		0.66 (1)								1.05 (1)	0.89 (1)
Tyrosine	0.74 (1)			1.54 (2)		0.98 (1)						0.85 (1)	0.99 (1)
Phenylalanine			0.77 (1)					1.12 (1)	0.94 (1)	0.91 (1)	1.60 (2)		
Tryptophan													
Homoserine and Lactone													
Total	6	3	5	7	6	4	3	3	2	7	10	8	7
% yield	5	73	25	20	15	23	27	19	12	75	4	6	24
Purification procedure[d]	D X1	D X1; D X50	D X1; D X50	D X1; D X50	D X1	D X1	D X50	D X50	D X50		D X50	D X50	
Residue No.	195–200	230–232	151–155	233–239	183–188	263–266	129–131	189–191	189–190	118–124	182–191	271–278	271–277

[a] Values are given in residues/mole. The assumed integral values are given in parentheses. [b] Values obtained from 72-hr hydrolysates. [c] See discussion in the text. [d] Abbreviations used are: D X1, Dowex 1-X2; D X50, Dowex 50-X2.

nated, presumably by pyrrolidonecarboxylic acid formation from glutamine in the third position of the peptide. Fractions Th I-4 and -5 were purified on Dowex 50-X2 columns (Figures 4 and 5) and each yielded two pure peptides, as described in Table II. Fraction Th I-5-3 was devoid of material. Of the two peptides from pool Th I-4, the first was identified directly by the presence of *S*-carboxymethylcysteine and was not structured further. Six of the nine residues of the second peptide were sequenced by Edman degradations. Six successful Edman degradations were performed on each of the peptides from Th I-5. The subfractionation of Th I-6 on Dowex 50-X2 yielded a single peptide, Th I-6-1, which is described in Table II.

FRACTION II. The separation of fraction II on Dowex 1-X2 is shown in Figure 6. The major pool, Th II-1, was further purified on Dowex 50-X2 to yield two major fractions (Figure 7). Fraction Th II-1-1 contained a single peptide, as described in Table III, while fraction Th II-1-2 had to be further purified by paper chromatography. The two peptides recovered are listed in Table III. Fractions Th II-2 and -3 were devoid of sufficient material to warrant further consideration. The final pool, Th II-4, contained a single pentapeptide whose sequence was fully established by Edman degradation. (See Table III.) Its acidic nature on high-voltage electrophoresis indicated at least one acid residue.

FRACTION III. Fraction III was subfractionated into five pools as shown in Figure 8. Pools Th III-2 and -4 were further purified on Dowex 50-X2. Two peptides, Th III-2-1 and Th III-2-2, were obtained in the first case (Figure 9), while only one major peptide was obtained from Th III-4.

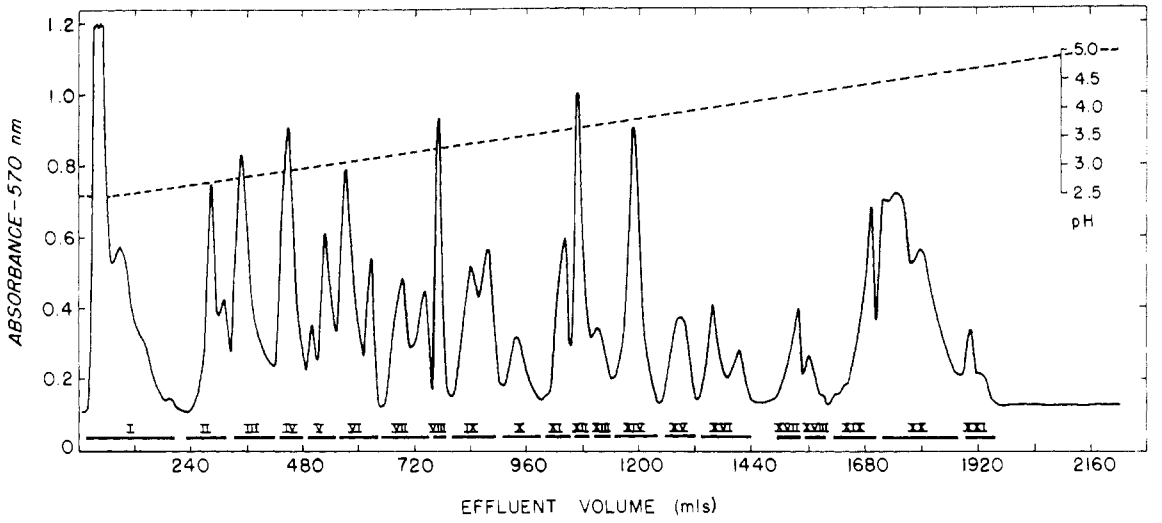

FIGURE 1: Elution profile of the soluble thermolytic peptides from fragment F_I of bovine carboxypeptidase A on a 2.0 × 25 cm column of Dowex 50-X8 at 55°. The column was developed at 80 ml/hr with a double linear gradient of pyridine–acetic acid, as described in the text. Fractions of 6.0 ml were collected and monitored by ninhydrin analysis at 570 nm after alkaline hydrolysis. Fractions were pooled according to the solid bars. The pH gradient is indicated by the dashed line.

Each of the remaining fractions in this pool contained a single peptide, as detailed in Table III. It should be noted that Th III-1 and Th III-4-1 represent the same segment of F_I since they include the two forms comprising one of the allotypic replacement sites of bovine carboxypeptidase A (Pétra *et al.*, 1969).

FRACTION IV. After purification of this fraction on Dowex 1-X2, as shown in Figure 10, six pools were obtained. Fractions Th IV-3 and -4 were further purified on Dowex 50-X2 but yielded only one major peptide in each case. One of these, peptide Th IV-3-1, contained a mixture of two peptides comprising the second allotypic replacement site found in

TABLE II: Characterization of the Thermolytic Peptides of Fraction I.[a]

Fraction I:
- Peptide Th I-1-1:
  - Sequence: (Gly,Ser,Ile,Ile,Thr,Thr)
- Peptide Th I-1-2:
  - Sequence: Leu-Leu-Pro-Ala-Ser-Gln
  - HVE, pH 6.5: Neutral
- Peptide Th I-2:
  - Sequence: Thr-Thr-(Glx,Ser,Ile,Pro,Asx,Lys,Thr,Glx,-Leu,Asx,Glx)
- Peptide Th I-4-1:
  - Sequence: (Val,Thr,Ser,Ser,Ser,Leu,CMCys,Val,Gly)
- Peptide Th I-4-2:
  - Sequence: Ile-Val,Thr-Asx-Pro-Asx(Gly,Phe,Ala)
- Peptide Th I-5-1:
  - Sequence: Leu-Glx-Ile-Val-Thr-Asx-(Pro,Asx,Gly,Phe,-Ala)
- Peptide Th I-5-2:
  - Sequence: Ile-Ile-Pro-Thr-Ala-Glx-(Glx,Thr,Trp)
- Peptide Th I-6-1:
  - Sequence: Ala-Ser-Gly-Gly-Ser(Ile,Asx,Trp,Ser,Tyr,-Asx,Glx,Gly)

[a] Edman degradations are indicated by (→), whereas leucineaminopeptidase and carboxypeptidase A and B hydrolyses are indicated by (→) and (←), respectively.

TABLE III: Characterization of the Thermolytic Peptides of Fractions II and III.

Fraction II:
- Peptide Th II-1-1:
  - Sequence: Val-Thr-Ser-Ser-Ser
- Peptide Th II-1-2 PC-1:
  - Sequence: (Leu,Gly,Val,Leu,Thr)
- Peptide Th II-1-2 PC-2:
  - Sequence: (Ile,Ile,Thr,Thr)
- Peptide Th II-4:
  - Sequence: Asx-Ile-Phe-Leu-Glx
  - HVE, pH 6.5: Acidic

Fraction III:
- Peptide Th III-1:
  - Sequence: Ala-Val-Ala-Ala
- Peptide Th III-2-1:
  - Sequence: Ala-Ser-Gly-Gly-Ser
- Peptide Th III-2-2:
  - Sequence: Leu-Asn-Gln
  - HVE, pH 6.5: Neutral
- Peptide Th III-3:
  - Sequence: Tyr-Thr-Thr-Glx-Ser-Ile(Pro,Asx,Lys,Thr,Glx)
  - HVE, pH 6.5: Acidic
- Peptide Th III-4-1:
  - Sequence: Ala-Val-Glu-Ala
  - HVE, pH 6.5: Acidic
- Peptide Th III-5:
  - Sequence: Leu-Gly-Val
  - Carboxypeptidase A: 1 hr; Val, 1.00

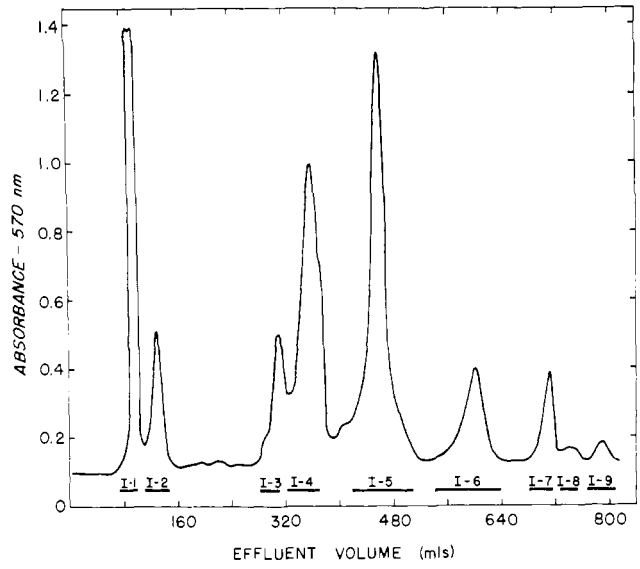

FIGURE 2: Elution profile of Fraction Th-I separated on a 0.9 × 150 cm column of Dowex 1-X2 at 35°. The column was developed at 30 ml/hr with a gradient of pyridine–acetic acid, as described in the text. Fractions of 2.0 ml were collected and monitored by ninhydrin analysis at 570 nm. Fractions were pooled as indicated by the solid bars.

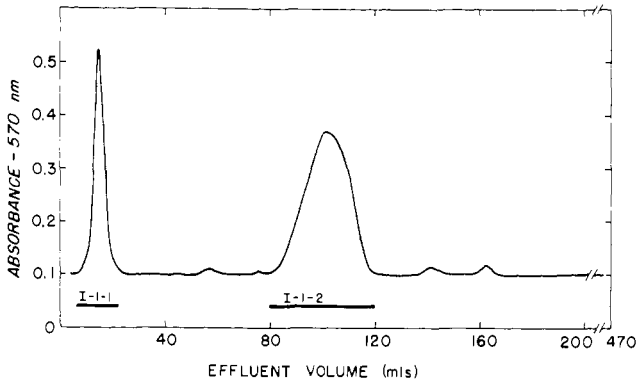

FIGURE 3: Elution profile of the separation of Fraction Th-I-1 on a 0.9 × 50 cm column of Dowex 50-X2 at 55°. The column was developed at 30 ml/hr and 2.0-ml fractions were collected. The gradient was composed of 250 ml of 0.05 N pyridine-acetic acid, pH 2.4, and 250 ml of 0.2 N pyridine–acetic acid, pH 3.1.

F_I (Pétra *et al.*, 1969). Each of the other pools contained single peptides which are described in Table IV. A complete structure for each peptide was obtained by Edman degradations and enzymic hydrolyses with the exception of peptide Th IV-4-1. This dipeptide was readily identified as part of the half-cystinyl segment (Bradshaw *et al.*, 1971a) already constructed from the tryptic and chymotryptic peptides and hence was not examined further. Peptide Th IV-6, which was completely sequenced by Edman degradations, was subjected to chymotryptic digestion and yielded two peptides, one of which was electrophoretically neutral. Based on the acidic nature of the intact peptide, the amide assignments of the three acidic residues can be made.

FRACTION V. This fraction was subfractionated on Dowex 1-X2 and gave the elution profile shown in Figure 11. Fraction Th V-5 contained a peptide derived from F_{III}, whereas Th V-2, after purification on Dowex 50-X2, gave several minor yield peptides. The remaining fractions each contained a

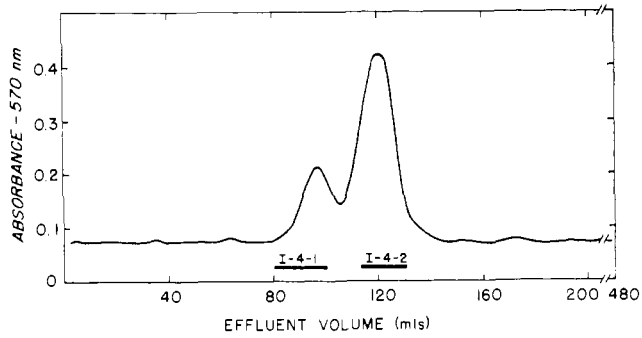

FIGURE 4: Elution profile of the separation of Fraction Th-I-4 on a 0.9 × 50 cm column of Dowex 50-X2. Details as in Figure 3.

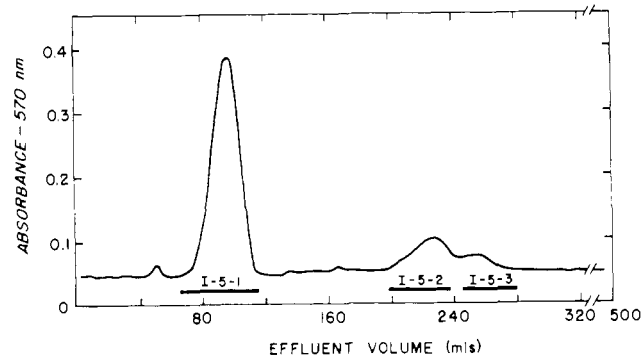

FIGURE 5: Elution profile of the separation of Fraction Th-I-5 on a 0.9 × 50 cm column of Dowex 50-X2. Details as in Figure 3.

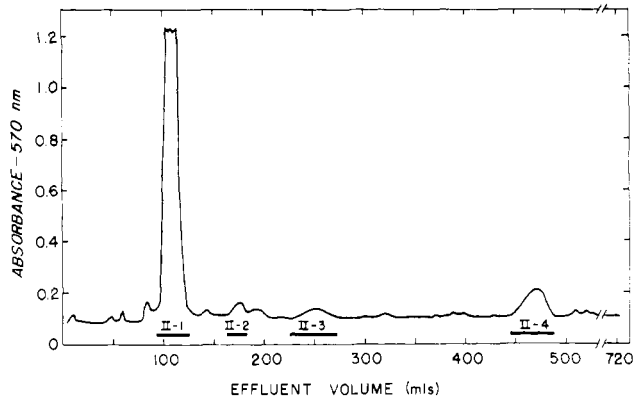

FIGURE 6: Elution profile of the separation of Fraction Th-II on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

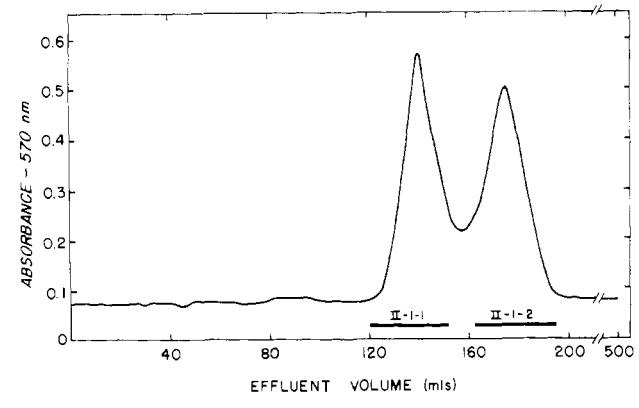

FIGURE 7: Elution profile of the separation of Fraction Th-II-1 on a 0.9 × 50 cm column of Dowex 50-X2. Details as in Figure 3.

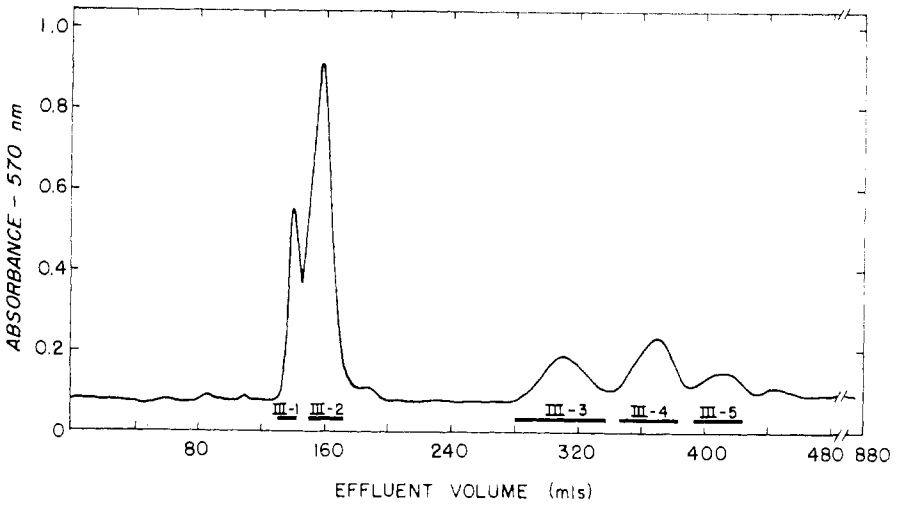

FIGURE 8: Elution profile of the separation of Fraction Th-III on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

TABLE IV: Characterization of the Thermolytic Peptides of Fraction IV.

Fraction IV:
- Peptide Th IV-1:
  - Sequence: Val-Leu-Thr
- Peptide Th IV-2:
  - Sequence: Ala-Asn-Ser-Glu-Val-Glu-Val-Lys-Ser
  - Carboxypeptidase A: 3 hr; Ser, 1.00
  - Leucineaminopeptidase: 3 hr; Ala, 0.39; Asn, 0.22
  - HVE, pH 6.5: Acidic
- Peptide Th IV-3-1:
  - Sequence: Ile/Val-Val-Asp
  - HVE, pH 6.5: Acidic
- Peptide Th IV-4-1:
  - Sequence: (Leu,CMCys)
- Peptide Th IV-5:
  - Sequence: Thr-Ala-Glx-Glx-Thr-Trp
  - HVE, pH 6.5: Acidic
- Peptide Th IV-6:
  - Sequence: Ile-Asp-Trp-Ser-Tyr-Asn-Gln-Gly
  - HVE, pH 6.5: Acidic
  - Chymotryptic subdigest:
    - Peptide Th IV-6 C-1:
      - Sequence: (Ile,Asx,Trp)
    - Peptide Th IV-6 C-2:
      - Sequence: (Ser,Tyr,Asx,Gln,Gly)
      - HVE, pH 6.5: Neutral

TABLE V: Characterization of the Thermolytic Peptides of Fractions V and VI.

Fraction V:
- Peptide Th V-1:
  - Sequence: Gly-Ser
- Peptide Th V-3:
  - Sequence: Tyr-Ser-Gln-Leu
  - HVE, pH 6.5: Neutral
- Peptide Th V-4:
  - Sequence: Tyr-Ala-Asx-(Ser,Glx,Val,Glx,Val,Lys,Ser)
- Peptide Th V-5:
  - Sequence: Asp-Ile-Phe
  - HVE, pH 6.5: Acidic

Fraction VI:
- Peptide Th VI-2-1:
  - Sequence: (Ser,Tyr,Ser,Glx)
- Peptide Th VI-2-2:
  - Sequence: (Phe-Leu)
- Peptide Th VI-3:
  - Sequence: Leu-Glu
  - HVE, pH 6.5: Acidic
- Peptide Th VI-4:
  - Sequence: Phe-Thr-Phe-Glu
  - HVE, pH 6.5: Acidic

single peptide, as described in Table V. Three of the peptides were completely structured and partial sequence data were obtained on the fourth, Th V-4.

FRACTION VI. After fractionation on Dowex 1-X2 (Figure 12), four pools were obtained. Fraction Th VI-1 contained free lysine, while fraction Th VI-2 had to be separated on Dowex 50-X2. Two peptides were obtained in low yield with the compositions listed in Table I. Fractions Th VI-3 and -4 each contained a single peptide, which was completely sequenced, described in Table V.

FRACTION VII. Fractionation of this pool on Dowex 1-X2 gave eight fractions (Figure 13). However, fractions Th VII-1 and -2 contained only peptides derived from F_{III}, and fraction Th VII-3 was devoid of sufficient material for further characterization. Fraction Th VII-4 contained only leucine after hydrolysis and might correspond to Leu-Leu derived from residues 201 and 202. The other fractions contained single peptides, as shown in Table VI. The dipeptides found in fractions Th VII-5 and -7 were structured by one round of Edman degradation.

FRACTION VIII. This fraction was found to contain only two dipeptides which were separated on Dowex 1-X2. Their com-

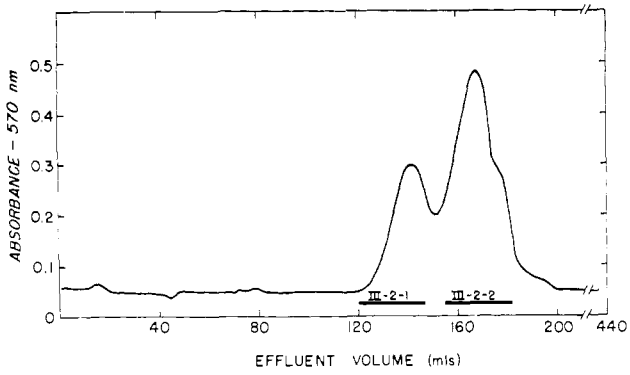

FIGURE 9: Elution profile of the separation of Fraction Th-III-2 on a 0.9 × 50 cm column of Dowex 50-X2. Details as in Figure 3.

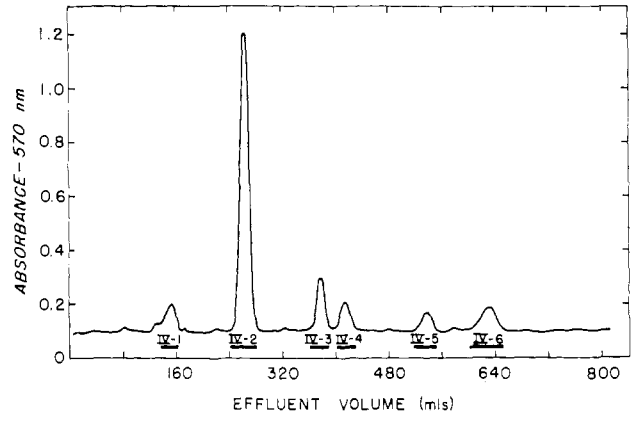

FIGURE 10: Elution profile of the separation of Fraction Th-IV on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

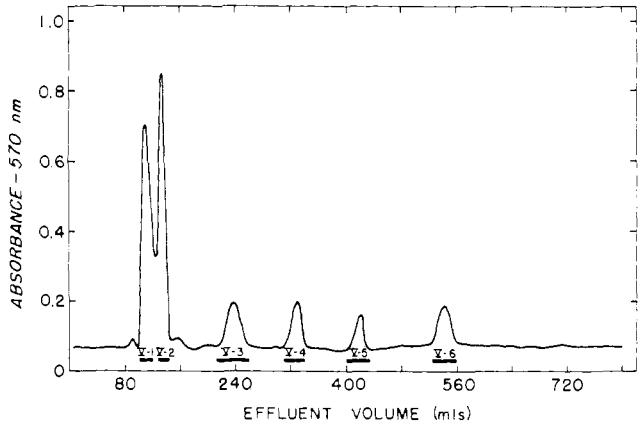

FIGURE 11: Elution profile of the separation of Fraction Th-V on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

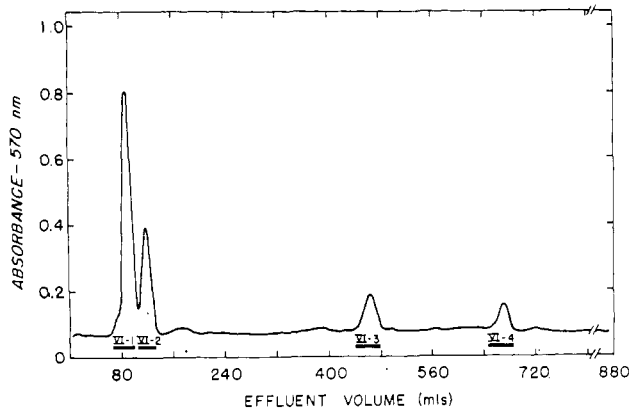

FIGURE 12: Elution profile of the separation of Fraction Th-VI on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

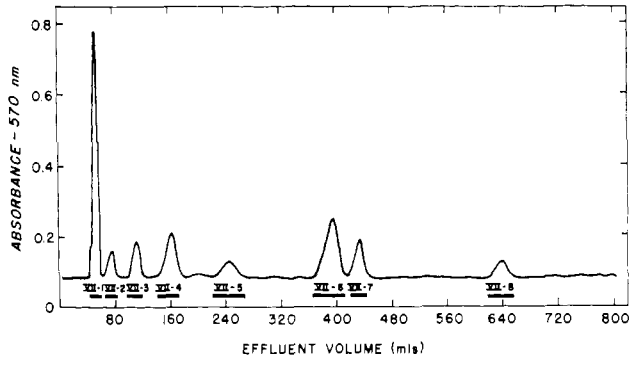

FIGURE 13: Elution profile of the separation of Fraction Th-VII on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

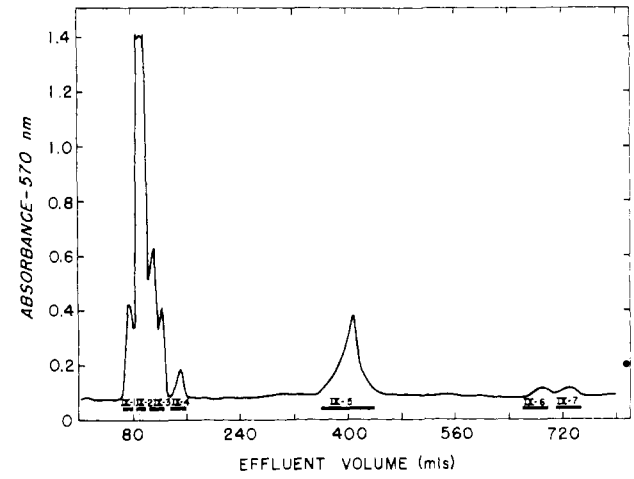

FIGURE 14: Elution profile of the separation of Fraction Th-IX on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

positions are listed in Table I. The sequence of the first, Th VIII-1, is assumed from the presence of homoserine which is derived from methionine during cyanogen bromide cleavage. The sequence of the second dipeptide was determined by Edman degradations and is given in Table VI.

FRACTION IX. Fractionation of this pool on Dowex 1-X2 gave the pattern shown in Figure 14. Fraction Th IX-1 contained negligible amounts of peptidic material. Fraction Th IX-2 was further purified on Dowex 50-X2, as shown in Figure 15. Of the four peaks that were obtained, the first two to be eluted, peptides Th IX-2-1 and Th IX-2-2, both contained the dipeptide Ile-HSer seen in Fraction Th VIII-1. The reason for their separation undoubtedly lies in the homoserine–homoserine lactone equilibrium. Peptide Th IX-2-3 was of particular interest in that it represented a bridge peptide between fragments F_I and F_C caused by the reaction os cyanogen bromide with methionine, followed by hydrolysif

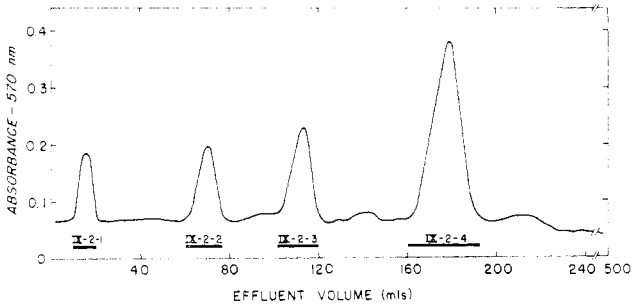

FIGURE 15: Elution profile of the separation of Fraction Th-IX-2 on a 0.9 × 50 cm column of Dowex 50-X2. The gradient was composed of 250 ml of 0.2 N pyridine acetic acid, pH 3.1, and 250 ml of 2.0 N pyridineacetic acid, pH 5.0. Other details as in Figure 3.

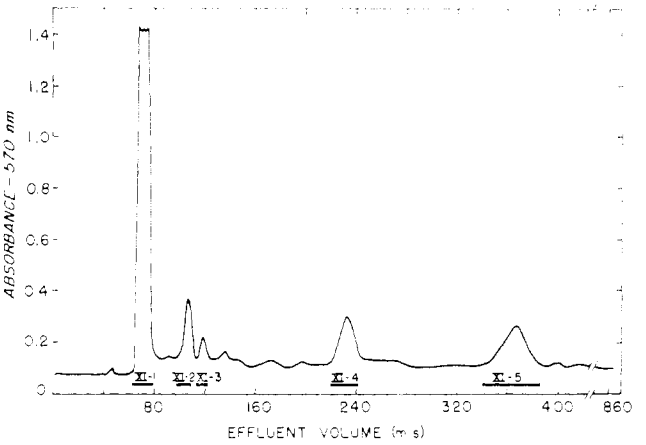

FIGURE 16: Elution profile of the separation of Fraction Th-XI on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

of the sulfonium salt by the solvent, resulting in noncleavage of the peptide chain. This peptide serves to further confirm the order of the cyanogen bromide fragments deduced from the peptic peptides of native carboxypeptidase A (Nomoto *et al.*, 1969). Peptide Th IX-2-4 was readily located in the final structure because of the presence of *S*-carboxymethylcysteine. The carboxyl-terminal residue was identified as tyrosine by carboxypeptidase A digestion. Peptides Th IX-4 and -5 were isolated in a pure state and their characterization is described in Table VI. Fractions Th IX-6 and -7 were devoid of material.

TABLE VI: Characterization of the Thermolytic Peptides of Fractions VII, VIII, and IX.

Fraction VII:
- Peptide Th VII-5:
  - Sequence: Phe-Thr
- Peptide Th VII-6:
  - Sequence: Val-Gly-Val-Asx-Ala(Asx,Arg,Asx,Trp,Asx,-Ala,Gly)
- Peptide Th VII-7:
  - Sequence: Phe-Glx
- Peptide Th VII-8:
  - Sequence: Tyr-Pro-(Tyr,Gly)

Fraction VIII:
- Peptide Th VIII-1:
  - Sequence: Ile-HSer
- Peptide Th VIII-2:
  - Sequence: Ala-Phe

Fraction IX:
- Peptide Th IX-2-3:
  - Sequence: Ile-(HSer,Glx,His,Thr)
- Peptide Th IX-2-4:
  - Sequence: (Ala,Ser,Ser,Ser,Pro,CMCys,Ser,Glx,Thr,-Tyr,His,Gly,Lys)-Tyr
  - Carboxypeptidase A: 1 hr; Tyr, 0.72
- Peptide Th IX-4:
  - Sequence: Leu-Tyr-Gly
- Peptide Th IX-5:
  - Sequence: Val-Asx-Ala-Asx-Arg-Asx-Trp(Asx,Ala,Gly)
  - HVE, pH 6.5: Acidic

FRACTION X. This pool was further purified on Dowex 1-X2, yielding only a single major peptide which was identical with Th IX-2-4. It was not examined further.

FRACTION XI. This pool was fractionated on Dowex 1-X2 and yielded five fractions, as shown in Figure 16. Each pool, except Th XI-3, contained a single peptide, described in Table VII. Pool Th XI-3 was devoid of sufficient material to allow further characterization. It is interesting to note that peptide Th XI-4 also corresponds to the region surrounding the second allotypic replacement site in fragment F_I. The fractional yields of Ile and Val (see Table I) in this peptide are a result of the mixture and the fact that the Ile–Val and Val–Val bonds are only partially hydrolyzed in 24 hr. If, in fact, the allotypic peptides of this pool are present in equal amounts, then each peptide would give one residue of valine plus one-half residue each of the two valine residues in consecutive sequence and one-half residue of isoleucine and valine in consecutive sequence in the second peptide (assuming 50% hydrolysis in

TABLE VII: Characterization of the Thermolytic Peptides of Fractions XI and XII.

Fraction XI:
- Peptide Th XI-1:
  - Sequence: Val-Ala-Lys-Ser-Ala
- Peptide Th XI-2:
  - Sequence: Leu-Ser-(Ile,His,Ser)
- Peptide Th XI-4:
  - Sequence: Ile/Val-Val-Asx-Phe-Val-Lys(Asx,His,Gly,-Asx)
- Peptide Th XI-5:
  - Sequence: Ser-Ser-Pro-CMCys(Ser,Glx,Thr,Tyr,His,Gly,Lys)

Fraction XII:
- Peptide Th XII-1:
  - Sequence: Val-Ala-Lys-Ser
  - Carboxypeptidase A: 3 hr; Ser, 0.91
- Peptide Th XII-2:
  - Sequence: (Phe,Gly,Lys,Ala,Gly,Ala,Ser,Ser,Ser,Pro,-CMCys,Ser,Glx,Thr,Tyr,His,Gly,Lys,Tyr)

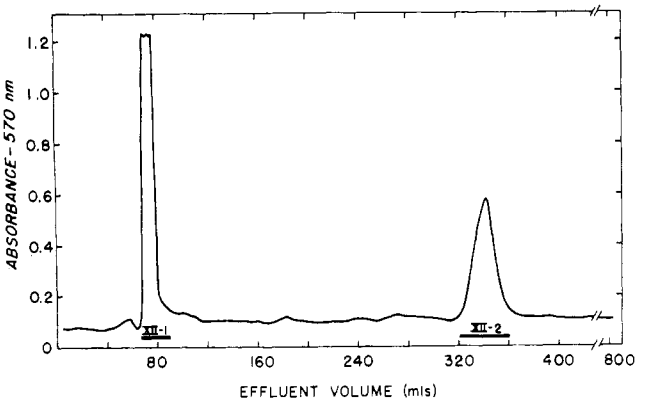

FIGURE 17: Elution profile of the separation of Fraction Th-XII on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

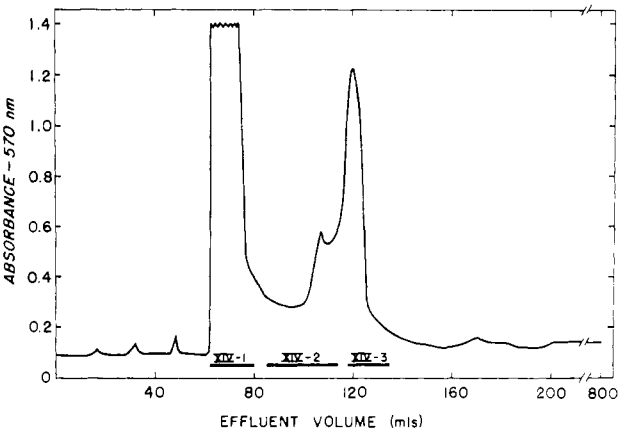

FIGURE 18: Elution profile of the separation of Fraction Th-XIV on a 0.9 × 150 cm column of Dowex 1-X2. Details as in Figure 2.

24 hr). By summation, the mixture would give 1.75 residues of valine and 0.25 residue of isoleucine, in excellent agreement with the observed values of 1.72 and 0.28.

FRACTION XII. Separation of Fraction XII on Dowex 1-X2 yielded two major peptides (Figure 17). Peptide Th XII-1 was completely structured while peptide Th XII-2 was placed by composition only (Table VII). The latter assignment was easily made due to the presence of *S*-carboxymethylcysteine in the peptide.

FRACTION XIII. Fractionation of pool XIII on Dowex 1-X2 yielded a single hexapeptide which is described in Table VIII. One round of Edman degradation gave amino-terminal isoleucine.

FRACTION XIV. After fractionation of pool XIV on Dowex 1-X2 (Figure 18), Fraction Th XIV-1 was further purified on Dowex 50-X2 (Figure 19). Fraction Th XIV-2 contained no usable peptides, while Fraction Th XIV-3 contained a single peptide, shown in Table VIII. The three peptides found in pool Th XIV-1 are also described in Table VIII. The four peptides finally isolated from this fraction were structured to a large degree by Edman analyses.

TABLE VIII: Characterization of the Thermolytic Peptides of Fractions XIII, XIV, and XV.

Fraction XIII:
- Peptide Th XIII-1:
  - Sequence: Ile-(His,Ser,Tyr,Ser,Glx)

Fraction XIV:
- Peptide Th-XIV-1-1:
  - Sequence: Leu-Lys-Ser
  - Carboxypeptidase A: 3 hr; Ser, 1.00
- Peptide Th XIV-1-2:
  - Sequence: Phe-Gly-Lys-Ala-Gly
- Peptide Th XIV-1-3:
  - Sequence: Leu-Tyr-Gly-Thr-(Ser,Tyr,Lys)
- Peptide Th XIV-3:
  - Sequence: Val-Lys-Asx(His,Gly,Asx)

Fraction XV:
- Peptide Th XV-1:
  - Sequence: Ile-Lys-Tyr-Ser

FRACTION XV. Fractionation on Dowex 1-X2 gave two peptides from this pool. The second, Th XV-2, was identical with Th XIV-3 and was not studied further. Peptide Th XV-1 was completely sequenced by Edman degradation, as described in Table VIII.

FRACTION XVI. This pool was purified on Dowex 50-X2 and yielded four pools (Figure 20). Peptide Th XVI-4 was identical with peptide Th XVII (*vide infra*) and was not treated further. Peptides Th XVI-1 and Th XVI-2, described in Table IX, were sequenced by Edman degradation and carboxypeptidase A digestion. Fraction Th XVI-3 contained a single dipeptide which was not sequenced.

FRACTION XVII. This pool contained a single peptide, with a net positive charge at pH 6.5, which was structured by Edman degradation and carboxypeptidase A and B hydrolyses, as described in Table IX. In view of the identification of the penultimate residue as asparagine, the remaining Glx residue was assigned as glutamic acid.

FRACTION XVIII. This pool contained the same peptide as Fraction XVII and was not examined further.

FRACTION XIX. After purification on Dowex 50-X2, only a single decapeptide was obtained, Th XIX-1. The first three residues were assigned by Edman degradation.

FRACTION XX. Purification of this fraction on Dowex 50-X2 yielded only a single peptide along with a large amount of ninhydrin-positive nonpeptidic material. The structure of

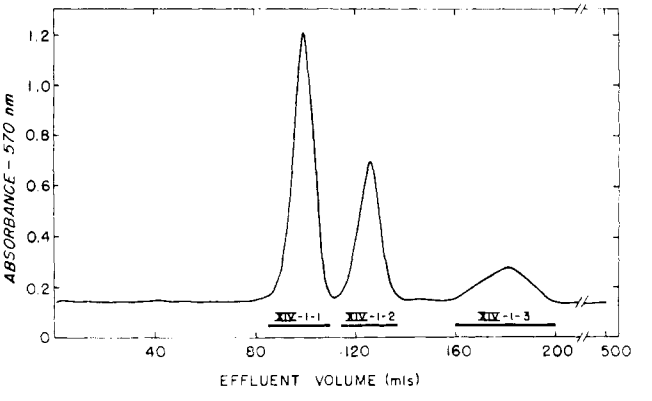

FIGURE 19: Elution profile of the separation of Fraction Th-XIV-1 on a 0.9 × 50 cm column of Dowex 50-X2. The gradient was composed of 250 ml of 0.5 N pyridine–acetic acid, pH 3.75, and 250 ml of 2.0 N pyridine–acetic acid, pH 5.0. Other details as in Figure 3.

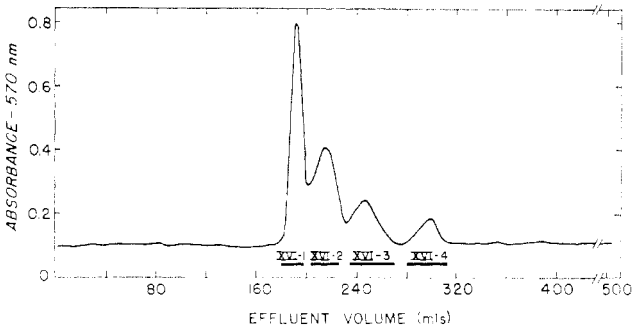

FIGURE 20: Elution profile of the separation of Fraction Th-XVI on a 0.9 × 50 cm column of Dowex 50-X2. Details as in Figure 15.

peptide Th XX-1 is given in Table IX. Only the amino-terminal leucine was identified because of its obvious relationship to peptide Th XII (*vide infra*).

FRACTION XXI. This pool contained a single peptide of seven residues. It was completely structured by Edman degradation, as described in Table IX. In view of the obvious similarity of this peptide to peptide Th XX, it was tentatively concluded that Th XX terminates in carboxyl-terminal glycine. This conclusion was substantiated by other peptides (Bradshaw *et al.*, 1971b).

Discussion

Alignment of the purified peptides (summarized in Table I) in the complete sequence of bovine carboxypeptidase A (Bradshaw *et al.*, 1971b) indicates that 189 of 198 residues found in the F_I fragment were recovered as soluble thermolytic peptides. Accordingly, the recovery of peptides from the thermolytic digest was nearly complete. Not all of these were included in the final proof of structure, however. Only five peptides, isolated in less than 10% yield, were used, *i.e.*, Th-I-6-1, Th-IV-5, Th-V-3, Th-XI-4, and Th-XIX-1. In each case, the structure of these peptides was substantiated by the analysis of peptides from other digests (Bradshaw *et al.*, 1971a,b). Nevertheless, the complete characterization of all thermolytic peptides was deemed important, first, to define further the specificity of thermolysin by a complete set of data, and second, as additional support of the final structure of fragment F_I.

The thermolytic digest failed to produce several of the overlaps necessary for the complete elucidation of the amino acid sequence and the present data were therefore combined with those obtained from the peptic and Nagarse digests. The complete compilation of the data that support the final structure is given in the last paper of this series (Bradshaw *et al.*, 1971b).

It is of interest to compare the specificity of thermolysin in the digestion of F_I with that observed in the digestion of fragment F_{III} (Bradshaw, 1969). These data are summarized in Table X, in which the number of cleavages observed in fragment F_I are listed in terms of the type of susceptible bonds. (For purposes of comparison, the data obtained with fragment F_{III} are also listed.) Clearly, the same profile is observed in both cases. Of the 50 bonds of the X-Leu, X-Ile, X-Phe, and X-Val type 37 (74%) were cleaved in F_I compared to 17 of 22 cleavages (77%) of the same variety in F_{III}. In fact, hydrolysis of these types of bonds amounted to 69% (37 of 54) of all of the cleavages observed. Although this number was somewhat higher (81%) in F_{III}, there were fewer total cleavages (21) in the smaller F_{III} fragment. Interestingly, thermolysin cleaved about one-quarter of the total available bonds in each fragment. Thus the specificity of the enzyme observed in fragment F_{III} is paralleled very closely by the F_I fragment,

TABLE IX: Characterization of the Thermolytic Peptides of Fractions XVI, XVII, XIX, XX, and XXI.

Fraction XVI:
- Peptide Th XVI-1:
  - Sequence: Thr-Arg-Ser
- Peptide Th XVI-2:
  - Sequence: Phe-Lys-Ala
  - Carboxypeptidase A: 30 min; Ala, 0.55
- Peptide Th XVI-3:
  - Sequence: (Phe,Lys)

Fraction XVII:
- Peptide Th XVII:
  - Sequence: Phe-Thr-His-Ser-Glx-Asn-Arg
  - Carboxypeptidase B: 30 min; Arg, 0.75
  - Carboxypeptidases A and B: 3 hr; Asn,1.00; Arg, 0.94
  - HVE, pH 6.5: Basic

Fraction XIX:
- Peptide Th XIX:
  - Sequence: Phe-Val-Lys(Asx,His,Gly,Asx,Phe,Lys,Ala)

Fraction XX:
- Peptide Th XX-1:
  - Sequence: Leu-(Arg,Asx,Thr,Gly,Arg,Tyr,Gly)

Fraction XXI:
- Peptide Th XXI:
  - Sequence: Leu-Arg-Asx-Thr-Gly-Arg-Tyr

TABLE X: Comparison of the Thermolytic Cleavage Sites in Fragments F_I and F_{III}.

	F_I			F_{III}		
Bond Type	Total Present	Number of Cleavage Sites	%	Total Present	Number of Cleavage Sites	%
X-Leu	15	13	87	7	5	71
X-Ile	13	10	77	7	6	86
X-Phe	10	8	80	4	4	100
X-Val	12	7	58	4	2	50
X-Tyr	13	5	38	3	0	0
X-Trp	4	0	0	3	1	33
X-Ala	14	4	29	5	1	20
Other	117	8	7	47	2	4
Total	198[a]	55	28	80	21	26

[a] The total in this column, 198, includes one more bond than is present in fragment F_I, since the allotypic replacement site at position 179, containing either isoleucine or valine, was treated as two sites.

further substantiating the conclusion that this enzyme has great utility in sequence studies.

Acknowledgments

The authors would like to thank Mrs. Brita Moody and Mrs. Lynne Maston for their assistance with the sequence analyses and Mr. C. V. Shih for preparation of the F_I fragment. The amino acid analyses were carried out by Mr. R. R. Granberg and Mr. Thomas A. Oas.

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Amino Acid Sequence of Bovine Carboxypeptidase A. Isolation and Characterization of Selected Peptic and Nagarse Peptides and the Complete Sequence of Fragment F_I*

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ABSTRACT: The isolation and characterization of selected peptides derived from peptic and Nagarse digests of the cyanogen bromide fragment F_I of bovine carboxypeptidase A are described. These data were combined with those obtained from the tryptic, chymotryptic, and thermolytic peptides of this fragment to produce the complete amino acid sequence of this 198-residue fragment. In addition, each of the side chains of Glx and Asx residues was identified as to its acid or amide character.

Attention was directed toward those residues whose identification differed from the assignments made on the basis of X-ray diffraction studies.

In the previous reports (Bradshaw *et al.*, 1971a,b), the isolation and characterization of the soluble tryptic, chymotryptic, and thermolytic peptides of fragment F_I of bovine carboxypeptidase A were described. These data were insufficient to provide an unambiguous sequence for this fragment. Digestion of the whole fragment by pepsin and of the insoluble core remaining after tryptic hydrolysis by Nagarse yielded additional peptides which enabled the completion of the structure. In order to simplify the information that led to the final structure, only those peptides which are necessary for the proof are described. The final assembly indicates that fragment F_I is composed of 198 amino acid residues and that the composition derived from the sequence is in exact agreement with that obtained from acid hydrolysates of fragment F_I.

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Experimental Procedure

Materials. After CNBr treatment of bovine carboxypeptidase A, as described previously (Nomoto *et al.*, 1969), fragment F_I was isolated on Sephadex G-75 which had been equilibrated with 0.1 M propionic acid. Pepsin was purchased from Worthington Biochemical Corporation, and Nagarse from Teikoku Chemical Industry Co., Ltd., Osaka, Japan. Other materials were the same as described previously (Bradshaw *et al.*, 1969a).

Methods. Peptic digestion of F_I was carried out by the addition of pepsin, dissolved in 0.5 M NaCl, to a final concentration