SUCCESSIVE CLEAVAGE OF PEPSIN WITH TRYPSIN AND CYANOGEN BROMIDE ISOLATION OF A PEPTIDE FRAGMENT BELONGING TO THE C-TERMINAL REGION OF PEPSIN

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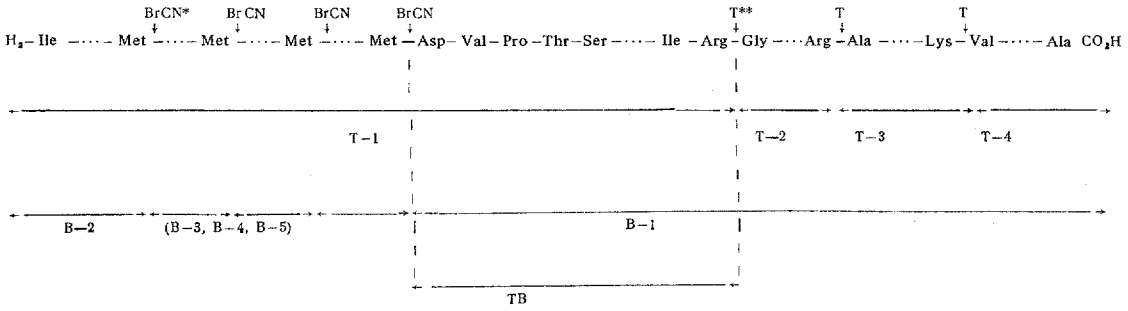
Specific cleavage with cyanogen bromide is the most convenient method for obtaining the peptide fragments necessary to determine the primary structure of pepsin. The action of cyanogen bromide on pepsin gives, in particular, the peptide B-1 consisting of 46 amino acid residues and representing the C-terminal section of the polypeptide chain of pepsin [1] (Scheme). In the C-terminal part of the polypeptide chains are concentrated all the lysine and arginine residues of the pepsin at which the specific hydrolysis with trypsin takes place [2]. This has permitted three groups of investigators, working independently, to explain the sequence of the 27 amino acid residues in the C-terminal section of pepsin [2–4].

Scheme of the Arrangement of the Peptide Fragments of Pepsin Formed Under the Action of Trypsin and Cyanogen Bromide



*BrCN) position of cleavage of a peptide bond by cyanogen bromide.
**T) position of cleavage of the peptide bond by trypsin.

Thus, the C-terminal part of the peptide B-1, consisting of slightly more than half its length, has been deciphered at the present time. The analysis of the N-terminal part of this peptide is considerably more difficult. In view of this, it appears of interest to develop a method of obtaining the corresponding peptide fragment not from the peptide B-1, the isolation of which is comparatively complex, but directly from pepsin. As can be seen from the Scheme, this problem can be solved by a combination of trypsin hydrolysis and cyanogen bromide cleavage. Under the action of trypsin on pepsin, the peptide bonds localized in the immediate vicinity of the carboxyl end of the molecule are cleaved, as a result of which the comparatively short peptides T-2, T-3, and T-4, and the high-molecular-weight peptide T-1 corresponding to almost the whole of the polypeptide chain of the pepsin from the N-terminal isoleucine to the arginine residue furthest from the carboxyl end of the residue one formed [2]. The specific cleavage of this peptide with cyanogen bromide must form— in addition to the peptides B-2, B-3, B-4, and B-5—the peptide TB which corresponds to the fragment of the polypeptide chain of pepsin of the greatest interest to us.

In order to isolate the peptide TB by the proposed scheme, the homogeneity of the macromolecular peptide T-1 formed by the action of trypsin on pepsin is of decisive importance. It has been shown previously that in the hydrolysis of pepsin with trypsin a nonspecific cleavage of the peptide bonds frequently takes place which complicates the isolation of the individual peptides from the hydrolysate. This deviation of the trypsin hydrolysis from the normal process is due to two factors. In the first place, denatured pepsin possesses to some extent a stable spatial structure and this makes the peptide bonds corresponding to the specificity of trypsin difficultly accessible to the attack of the proteinase. It has been found that this difficulty can be circumvented by performing the hydrolysis with trypsin in 40% ethanol which apparently ensures a more complete extension of the globule. In the second place, the result of hydrolysis depends largely on the quality of the trypsin, especially on the presence in it of traces of chymotrypsin. The best results were obtained in our laboratory by using porcine trypsin of the Danish firm "Novo". This is due not only to the absence of appreciable amounts of chymotrypsin in this preparation but also, possibly, to the fact that percine trypsin, in contrast to the bovine trypsin usually used, exhibits a "secondary" specificity of the

chymotrypsin type to a smaller extent. A sample of bovine trypsin produced by the Leningrad factory for medicinal preparations that we had available exhibited an appreciable activity with respect to the specific substrate of chymotrypsin. Its action on pepsin led to an extremely intensive cleavage of peptide bonds not corresponding to the specificity of trypsin. We followed the hydrolysis process by determining the N-terminal groups in the hydrolysate by the dinitrophenyl method.

It is known that in the specific cleavage of pepsin with trypsin, N-terminal residues of valine, glutamic acid, and alanine appear in addition to the N-terminal residue of isoleucine already present in pepsin [2]. Table 1 shows that the hydrolysis of pepsin with the trypsin of the Leningrad factory for medicinal preparations leads to the appearance of threonine, glycine, serine, and aspartic acid, in addition to the N-terminal amino acids mentioned above.

Table 1. Determination of the N-Terminal Amino Acids in Trypsin Hydrolysates of CM-Pepsin Referred to DNP-Isoleucine (The amount of which was taken as 1).

DNP-amino acid	A	B	C
Ile	1	1	1
Glu	1	0.64	1.00
Ala	1	0.79	0.88
Val	1	0.65	0.90
Ser	0	0.55	0.33
Thr	0	1.10	Traces
Gly	0	0.55	0.08
Asp	0	0.35	Traces

Note. A) number of expected residues of the DNP-amino acids; B) number of DNP-amino acid residues obtained when using the trypsin of the Leningrad factory for medicinal preparations; C) number of residues of DNP-amino acids obtained when using the trypsin of the Leningrad factory for medicinal preparations after its incubation with the chymotrypsin inhibitor chloromethyl 1-tosylamido-2-phenyl ketone.

Since the isolation of the peptide T-1 from such a hydrolysate is difficult, to inhibit the chymotrypsin present in the trypsin we used treatment of the latter with chloromethyl 1-tosylamido-2-phenylethyl ketone [7]. This expedient enabled us to obtain considerably better results in the treatment of pepsin. It can be seen from Table 1 that the hydrolysis of pepsin with trypsin treated with the inhibitor leads to the appearance of N-terminal isoleucine, alanine, valine, and glutamic acid in equivalent proportions, as should be the case in the cleavage of the peptide bonds corresponding to the specificity of trypsin. Nonspecific hydrolysis, which is shown by the appearance of small amounts of N-terminal serine, is slight and does not interfere with the subsequent operations (see Table 1).

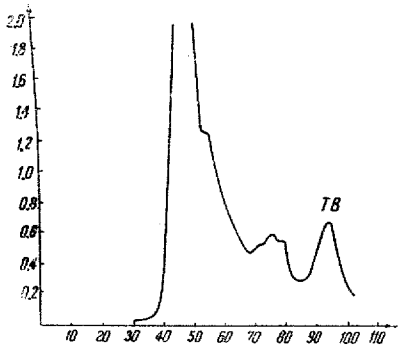
After the performance of the trypsin hydrolysis and the isolation of the high-molecular-weight peptide T-1, this peptide was treated with an excess of cyanogen bromide in formic acid under conditions analogous to the conditions for the cleavage of pepsin with cyanogen bromide.

Separation of the cyanogen bromide hydrolysate of the peptide T-1 by gel filtration on Sephadex G-50 gave us the peptide TB with a yield of 30%. It can be seen from the figure that the peptide TB is the fraction of lowest molecular weight in the cyanogen-bromide hydrolysate of the peptide T-1.

The peptide TB consists of approximately 27 amino acids and contains among these one arginine residue and two tryptophan residues.

The amino acid composition of the C-terminal, B-1, fragment of pepsin can be illustrated as the sum of the peptide TB that we have obtained and the peptides T-2, T-3, and T-4 (Table 2).

As follows from Table 2, the amino acid composition of the peptide B-1 is in fact in good agreement with the sum of the amino acids contained in the peptides TB, T-2, T-3, and T-4. There are appreciable deviations only in the case of alanine, valine, and phenylalanine. In the peptide TB, we detected only traces of alanine. The content of valine in the peptide B-1 is possibly lower because of the stability of the peptides of valine to hydrolysis. In relation to phenylalanine, we can make no final conclusion, and the question of the number of phenylalanine residues in the peptide TB will be decided in the course of the structural work.



Separation of 0.25 g of the cyanogen-bromide hydrolysate of the peptide T-1 on a column (25 × 970 mm) of Sephadex G-50 in 4 M urea and 1% sodium bicarbonate (the number of 4.8-ml fractions is plotted along the axis of abscissas and the optical density at 280 M in arbitrary units along the axis of ordinates).

Table 2. Amino Acid Composition of the Peptides

Amino acid	Number of residues of the given amino acid in the peptide					
	TB	T–2	T–3	T–4	Sum of TB, T-2, T-3, and T-4	B–1
Lys	—	—	1	—	1	1
Arg	1	1	—	—	2	2
Asp	3	1	2	—	6	6
Thr	2	1	—	—	3	3
Ser	3	—	—	—	3	3
Glu	2	1	—	—	3	3
Pro	2	—	—	1	3	3
Gly	3	—	—	1	4	4
Ala	—	—	1	2	3	4
Val	3	1	—	2	6	6
Ile	2	—	—	—	2	2
Leu	3	—	—	1	4	4
Tyr	—	2	—	—	2	2
Phe	1–2	1	—	—	2–3	2
Trp	2	—	—	—	2	2

In the determination of the N-terminal amino acid of the peptide TB, aspartic acid was detected by Sanger's dinitrophenyl method [9] and by Grey and Hartley's dansyl method [10].

The determination of the N-terminal sequence of the peptide TB was carried out by the stepwise splitting off of methylthiohydantoins [11], and gave the following results (the yield of the corresponding methylthiohydantion is given in parentheses): Asp (32%)—Val (90%)—Pro (53%). This sequence coincides with the N-terminal sequence of the peptide B-1 determined previously: Asp—Val—Pro—Thr—Ser [1]. Consequently, the peptide TB has the N-terminal sequence Asp—Val—Pro—Thr—Ser.

For further investigation, the peptide TB was subjected to partial acid hydrolysis. As is well known, hydrolysis in a mixture of acids leads to the cleavage of the peptide bonds formed by aspartic acid, this being isolated in the form of the free amino acid [13]. We have found the optimum conditions for the acid cleavage of the peptide TB.

The course of the hydrolysis was followed by quantitatively determining the free amino acids in samples of the hydrolysate. After hydrolysis for 23 hr the following amino acids were found in a sample containing 0.25 μM of the peptide TB, which corresponds to 0.75 μM of aspartic acid: Asp 0.481; Thr 0.018; Ser 0.036; Gly 0.054. Hydrolysis to aspartic acid took place to the extent of 64%, with the formation of very small amounts of other amino acids. From the hydrolysate was isolated the tetrapepside Val-Ile-Phe-Arg.

The N-terminal sequence of the tetrapeptide was determined by Edman's method in combination with that of Grey and Hartley [12] as Val-Phe-Ile. Thus, the peptide has the structure Val-Phe-Ile-Arg, which agrees with the results obtained previously [3].

Summarizing what has been said above, the following scheme for the peptide TB forming part of the C-terminal fragment of pepsin can be written Asp-Val-Pro-Tre-Ser. . . -(Asp_2, Thr, Ser_2, Pro, Gly_3, Val, Ile, Leu_3, Glu_2, Trp_2)-Val-Phe-Ile-Arg.

EXPERIMENTAL

Pepsin. Porcine pepsin purified by chromatography on DEAE-cellulose [5] and then reduced and carboxymethylated by the method described previously (CM-pepsin).

Trypsin. Trypsin of the Leningrad factory for medicinal preparations was used. It possessed chymotrypsin activity.

To eliminate the chymotrypsin impurity, the trypsin preparation was treated with the chymotrypsin inhibitor chloromethyl 1-tosylamido-2-phenylethyl ketone [7].

To a solution of 0.240 g of trypsin in 80 ml of 0.001 M calcium chloride was added 7 mg of the inhibitor in 1.8 ml of ethanol, the pH being kept at 7.2 (TTT-1 autotitrator, "Radiometer," Denmark) with 0.5 N caustic soda. After 5 hr at room temperature, the mixture could be used for hydrolysis. For its storage, it was acidified with 1 N HCl to pH 3,

frozen, and left in the cold, being used as required without the excess of inhibitor being removed.

Trypsin hydrolysis [2]. A solution of 8 g of CM-pepsin in 245 ml of 2% triethylamine was kept at 37° C for 1 hr, and then the pH of the solution was brought to 8 with 50% acetic acid and 207 ml of ethanol was added so that its final concentration was 40%. To this solution was added 54 ml (0.160 g) of the trypsin solution containing the inhibitor in suspension, the latter dissolving in the 40% ethanol. The concentration of pepsin in the solution was approximately 1.5%. The solution was incubated at 37° C for 1 hr, and then another 27 ml (0.080 g) of trypsin solution was added and incubation was continued for 1 hr. The enzyme/substrate ratio was 1:33.

The solution was acidified with acetic acid to pH 2–3 and the precipitate formed was separated off on the centrifuge, washed with acidified water, and recentrifuged. Then the precipitate was dissolved at pH 8–9, precipitated by acidification, and centrifuged. The moist residue was used for the subsequent cyanogen bromide cleavage.

Cyanogen bromide cleavage [1]. The moist precipitate of the twice-reprecipitated peptide was transferred to a flask with a ground-in stopper and treated with 500 ml of formic acid which had been distilled and kept under argon. The solid dissolved well, forming a slightly turbid solution. To this was added 12 g of cyanogen bromide and the mixture was stirred at room temperature for 12 hr.

To eliminate the formic acid completely, the solution was freeze-dried, water was added, and it was dried again, and this operation was repeated once more.

The dry residue was dissolved in 0.5 N triethylammonium carbonate buffer with pH 8.5, and after 12 hr 1 g of sodium bicarbonate and 48 g of urea were added to the solution. The total volume was 100 ml.

Gel filtration on Sephadex G-50. The gel filtration of 100 ml of the solution obtained after the cyanogen bromide cleavage was carried out on a column (50 × 1100 mm) of Sephadex G-50 equilibrated with a 4 M solution of urea in 1% bicarbonate (pH 9). The rate of elution was 63 ml/hr. The peptide TB issued in a volume of 1280 ml.

The gel filtration on Sephadex G-50 was carried out twice. After each experiment the peptide was desalted on a column (46 × 360 mm) of Sephadex G-25 "medium" equilibrated with water.

Amino acid composition of the peptide TB. The total amino acid hydrolysis of the peptide TB was carried out with 5.7 N HCl at 110° C for 48 hr (Table 3).

Table 3. Amino Acid Composition of the Hydrolysate of Peptide TB

Amino acid	A	B	C	A	B	C
Arg	0.150	1	1	0.165	1	1
Asp	0.493	3.3	3	0.574	3.5	3
Thr	6.260	1.7	2	0.284	1.7	2
Ser	0.505	3.4	3	0.568	3.4	3
Glu	0.259	1.7	2	0.387	2.3	2
Pro	0.220	1.5	2	0.317	1.9	2
Gly	0.492	3.3	3	0.557	3.4	3
Ala	0.034	0.02	—	0.029	0.02	—
Val	0.407	2.7	3	0.439	2.7	3
Ile	0.337	2.3	2	0.373	2.3	2
Leu	0.441	3.0	3	0.445	2.7	3
Tyr	0.051	0.03	—	Traces	—	—
Phe	0.215	1.5	1—2	0.209	1.3	1
Trp	0.291	—	—	—	—	—

Note. A) content in μM; B) content in the residues; C) rounded-off content in the residues. The tryptophan was determined by a colorimetric method [8].

Partial acid hydrolysis of the peptide TB. A solution of 8 mg of the freeze-dried peptide in 1.2 ml of formic acid was treated with 4.8 ml of acetic acid and 12 ml of water and was kept at 110° C. The conditions for the electrophoresis of the mixture of peptides were: pH 5.6, 2 hr, 1000 V, Whatman 3 MM paper, in a chamber for vertical electrophoresis. The zone revealed by the Sakaguchi reagent was eluted with water and hydrolyzed with 5.7 N HCl at 110° C for 23 hr. The peptide had the following composition: Val 0.281, Ile 0.331, Phe 0.256, Arg 0.260.

CONCLUSIONS

1. The peptide TB has been isolated from the C-terminal fragment of pepsin.

2. The amino acid composition of this region of pepsin has been refined.

3. The sequence of nine amino acids in the peptide TB has been established.

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