ENZYMATIC HYDROLYSIS OF DENATURED PIG PEPSIN BY TRYPSIN

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The first stage in the investigation of the primary structure of pepsin is the splitting of its molecule into comparatively large polypeptide fragments. This can be effected by various methods; however, as a practical determination of the sequence of aminoacides in proteins has shown, it is most convenient to use trypsin hydrolysis for this purpose. Trypsin, a proteolytic enzyme with a clearly expressed specificity, selectively hydrolyzes peptide bonds formed with the participation of the carboxyl groups of the basic aminoacids arginine and lysine. In infringement of this rule, the non-specific splitting of other peptide bonds by trypsin is sometimes found, most frequently those which are readily split by chymotrypsin. The cause of this may be the presence in trypsin of a small amount of chymotrypsin difficult to separate or else an inerent capacity of trypsin itself for hydrolyzing peptide bonds of aromatic aminoacids [1]. The existence of "cross" specificity with trypsin and chymotrypsin has been shown repeatedly on synthetic substrates.

We have investigated the action of trypsin on pig pepsin with the object of obtaining fragments of the pepsin molecule suitable for further investigation of its primary structure. As a substrate for the action of trypsin, pepsin possesses some special features.

In the first place, it differs from the majority of proteins by an exceptionally low content of arginine and lysine. According to Brand's data [2], the pepsin molecule contains only two lysine residues and two arginine residues. A determination of the aminoacid composition of crystalline pepsin with the aid of an automatic aminoacid analyzer [3] has shown the presence in pepsin of one lysine residue and two arginine residues (out of a total number of 341). Thus, it follows from the literature data that three or four bonds in pig pepsin can be split by trypsin.

In the second place, pepsin readily undergoes denaturation in a weakly alkaline medium, while observations indicate that alkaline denaturation is accompanied by a splitting of the pepsin molecule [4, 5, 6]. Since the range of activity of trypsin is in the alkaline region, it is necessary to prevent non-specific splitting of the peptide chain of pepsin through denaturation. It has been shown in our laboratory that it is possible to use the prior inactivation of the pepsin by means of dissolution in phenol for this purpose [6]. The presence in pepsin of three disulfide bonds might adversely affect trypsin hydrolysis. It might prove that the disulfide bonds couple to one another peptide fragments formed in the splitting of the pepsin and thereby prevent their separation. In order to avoid these complications, we used pepsin reduced by thioglycolate, the sulfhydryl groups of which were blocked by iodoacetic acid [7].

The reduced carboxymethylated pepsin was split by trypsin at pH 8.0 and 38°. In order to select the optimum time of hydrolysis and to evaluate the nature and number of the peptide bonds undergoing hydrolysis, the content of N-terminal aminoacids in the hydrolyzate was determined after the trypsin had acted for 2, 4, 8, and 24 hours. This was achieved by the dinitrophenylation of the reaction mixture with subsequent acid hydrolysis and chromatographic identification of the DNP-aminoacids.

In considering the results obtained, which are given in the Table, it is necessary to bear in mind that on prolonged acid hydrolysis of DNP-peptides, the splitting out of DNP-aminoacids occurs, the extent of this being particularly great for DNP-glycine. The introduction of corrections for the loss of DNP-aminoacids on hydrolysis and chromatography presents considerable difficulties, but in our case corrections were unnecessary since it was not the absolute values of the measured magnitudes but their change with time that was of primary interest. It can be seen from the data in the Table that hydrolysis takes place to a considerable extent even in the first two hours; however, subsequently, a slower increase in the content of N-terminal aminoacids is always found.

During the reaction, a pH of 8.0 was maintained by means of a pH-stat fitted with a recording device. The splitting of peptide bonds at pH 8.0 leads to the appearance of hydrogen ions and a displacement of the pH to the acid region. The latter was automatically compensated by the addition of alkali. Thus, the curve of the consumption of alkali permits an evaluation of the course of the hydrolysis. In our experiments, the consumption of alkali slowed down after 2 hours' hydrolysis, fell sharply after 8 hr and practically ceased after 12 hr. In preliminary experiments on the splitting of pepsin, we increased the time of hydrolysis to 19 hr; in order to compensate for the inactivation of the trypsin during the hydrolysis, small amounts of enzyme were added four and eight hours after the beginning of incubation.

The data in the table make it possible to estimate the N-terminal aminoacids of the peptides formed in the trypsin hydrolysis of the pepsin. In all cases, 20 mg of pepsin or the mixture of peptides obtained by the hydrolysis of 20 mg of pepsin was subjected to dinitrophenylation. As can be seen from the Table, the eluate of the DNP-isoleucine spot obtained from 20 mg of pepsin had an optical density of 0.985. It is known that the molecule of pig pepsin contains one N-terminal isoleucine residue [8]. Thus, under the given conditions an optical density of 0.985 corresponds to approximately one amino group

per mole of pepsin. Comparing this magnitude with the optical densities of the eluates of the DNP-aminoacids obtained by the dinitrophenylation of the 4-24 hour pepsin hydrolyzates, it may be concluded that the trypsin hydrolyzate contains three peptides with N-terminal leucine (or isoleucine, since these DNP-aminoacids are not separated on chromatography), one peptide with N-terminal valine, one with N-terminal alanine, and two with N-terminal threonine. The presence of a peptide the N-terminal aminoacid of which is aspartic or glutamic acid (or either of the corresponding amides) is likely. The appearance of DNP-serine in an amount of approximately 0.5 mole per mole of protein is apparently due to the non-specific hydrolysis of a peptide bond. It is more difficult to explain the presence of DNP-glycine.

Optical densities of eluates of DNP-aminoacids obtained by the dinitrophenylation of a trypsin hydrolyzate of reduced carboxymethylated pig pepsin (at 360 mμ).

DNP-Aminoacids	Time of trypsin hydrolysis, hours				
	0	2	4	8	24
DNP-leucine (isoleucine)	0.985	2.80	3.250	2.82	2.83
DNP-valine	0.015	0.94	0.945	0.94	0.94
DNP-alanine	0.190	0.94	1.150	1.24	1.10
DNP-threonine	0.105	1.50	1.980	2.00	1.92
DNP-glycine	0.0	0.20	0.270	0.54	0.23
DNP-serine	0.025	0.37	0.495	0.56	0.42
DNP-aspartic (DNP-glutamic) acid	0.085	0.84	1.250	1.30	1.27

This DNP-aminoacid is very strongly decomposed on acid hydrolysis [9]; consequently, in spite of the fact that the DNP-glycine is found in small and, moreover, variable, amounts, it is not excluded that the trypsin hydrolyzate contains a peptide with N-terminal glycine.

Thus, it may be assumed that in the trypsin hydrolysis of reduced carboxymethylated pepsin 8 or 9 peptides are formed, i.e. 7 or 8 peptide bonds are split. Unfortunately, the published data on the content of arginine and lysine in pepsin and hence on the probable number of products of trypsin hydrolysis are contradictory and relate to pepsin not subjected to chromatographic purification. If one starts from Brand's data (2 arginine and 2 lysine residues), one should expect the formation of five peptides as a result of the trypsin hydrolysis of pepsin. According to Blumenfeld and Perlmann's data (two arginine residues and one lysine residue), there should be four such peptides. The number of pepsin fragments that we have observed is considerably greater. It is quite probable that some of them owe their appearance to the splitting of bonds formed by aromatic aminoacids. It is not excluded that the low content of bonds corresponding to the specificity of trypsin in pepsin favors the attack by the enzyme of bonds that are not usually hydrolyzed by it. In addition, it must be emphasized that information on the content of arginine and lysine in pepsin requires careful checking on a chromatographically pure preparation.

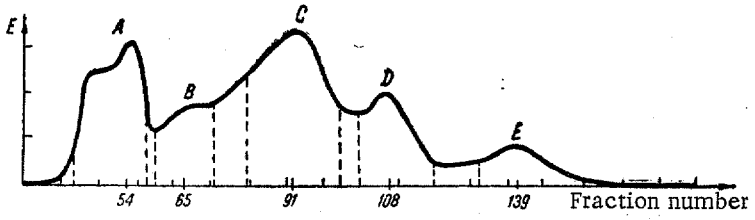
Since the number of bonds capable of being split is small, the trypsin hydrolysis of pepsin must form comparatively large peptide fragments. If seven or eight bonds are ruptured, the mean molecular weight of the hydrolysis products of pepsin will be approximately 4000-4500, which corresponds to 40-45 aminoacid residues. It is obvious that there is no basis for assuming a uniform distribution of the arginine and lysine residues in pepsin; the dimensions of the peptides will differ considerably from the figure given. However, there is no doubt that the hydrolyzate will contain very large peptides, approximating proteins in length.

Considering the difficulty of chromatographing macropeptides on ion-exchange resins, we decided to use fractionation with respect to moleculary weight by means of "Sephadex" molecular sieves as the first step in the separation of the products of tryptic hydrolysis. The best results were obtained with "Sephadex" G-50.

In the chromatography of the macropeptides on "Sephadex", we encountered serious difficulties due to the repeatedly observed tendency of peptides to undergo association. To suppress the association of the peptides, it proved to be necessary to carry out the chromatography in a 4 M solution of urea. Furthermore, it was necessary to take care that the concentration of the peptides was not too high.

The results of the fraction of a tryptic hydrolyzate of pepsin on "Sephadex" G-50 are shown in the Figure. Rechromatography of fraction A on "Sephadex" G-100 permitted its separation into two components. Rechromatography of fraction B on "Sephadex" G-100 gave one well-defined peak. It was possible to purify fraction D by rechromatography on "Sephadex" G-50.

The results obtained show that chromatography on molecular sieves can be used successfully for the initial fractionation of mixtures of high-molecular-weight peptides.



Separation of a trypsin hydrolyzate of reduced carboxymethylated pepsin on "Sephadex" G-50 in 4 M urea. The number of 5-ml fractions of eluate is plotted along the abscissa and the optical density at 253 mμ (in arbitrary units) along the ordinate. The boundaries of the fractions combined are shown by broken lines.

Experimental

Pepsin. The work was carried out with reduced carboxymethylated pig pepsin obtained from pepsin purified by chromatography* on DEAE-cellulose and inactivated by dissolving in aqueous phenol [7].

Trypsin. 10 mg of a crystalline precipitation from the "Spofa" firm (Czechoslovakia) was dissolved in 2 ml of 0.0001 N HCl, and water was added to 10 ml.

Hydrolysis of the carboxymethylated pepsin with trypsin (analytical experiment). With constant stirring, 100 mg of reduced carboxymethylated pepsin was dissolved over 1 hour at 37° in 10 ml of water (pH of the solution 6-7). A 2-ml sample of the solution was taken for dinitrophenylation (control experiment). The remaining solution, containing 80 mg of pepsin, was brought to pH 8.0, and 1.2 mg of trypsin was added to it. Hydrolysis was carried out in a TTT-1 automatic titrator ("Radiometer," Denmark) working under pH-stat conditions, a pH of 8.0 being maintained by the addition 0.5 N caustic soda. The temperature was constant (37°) during the experiment. Samples (2 ml each, containing 20 mg of protein), which were taken after 2, 4, and 8 hours, were subjected to dinitrophenylation. The remaining 20 mg of pepsin was hydrolyzed for 24 hr, after which it was also dinitrophenylated.

Determination of the N-terminal aminoacids in the trypsin hydrolyzate of pepsin. The samples of hydrolyzate obtained in the preceding experiment were dinitrophenylated at pH 9.0 and 40°. The mixture of DNP-peptides was hydrolyzed for 40 hr with 5.7 N HCl at 105°. Ether extraction of the hydrolyzate gave the DNP-aminoacids, after which they were separated by paper chromatography. The spots of the DNP-aminoacids were eluted with sodium bicarbonate and the optical densities of the eluates were determined in an SF-4 spectrophotometer at 360 mμ. The technique of the determination of N-terminal aminoacids used in our laboratory has been described in detail previously [8].

Hydrolysis of carboxymethylated pepsin by trypsin (preparative experiment). At 37°, 600 mg of the pepsin was dissolved in 60 ml of water, and alkali was added to pH 8.0. The solution was incubated at 37° with 4 mg of trypsin, the pH being maintained at 8.0 by means of the automatic titrator. After 4 hr, 2.5 mg of trypsin was added and after 8 hr a further 2.5 mg. After 19 hr incubation, the mixture was freeze-dried.

Separation of the tryptic hydrolyzate of pepsin. A solution of 300 mg of hydrolyzate in 15 ml of 4 M urea was transferred to a 2.5 × 150 cm column filled with "Sephadex" G-50 which had been swollen for several days in a 4 M solution of urea. Elution was carried out at room temperature with a 4 M solution of urea, 5-ml fractions being collected. The appearance of peptides in the eluates was followed by the ultraviolet absorption at 253 mμ (for this purpose, a flow-through UV photometer of the firm LKB, Sweden, was used). The measurement of the optical density of the eluate fractions at 280 mμ in a SF-4 spectrophotometer gives similar results.

Chromatography on "Sephadex" G-100 was carried out under similar conditions. The only difference was the use of a weak borate buffer to maintain a pH of 8 during the separation.

Summary

1. The conditions of the enzymatic hydrolysis of reduced carboxymethylated pepsin by trypsin have been studied.

2. The usefulness of chromatography on molecular sieves ("Sephadex") as a method for the initial fractionation of a mixture of macropeptides has been demonstrated.

*The chromatographic purification of the pepsin was carried out by A. F. Lyubsteva.

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