INFLUENCES OF PROTEOLYTIC ENZYMES ON PAROTIN IV.
SEPARATION OF HYDROLYZATES IN DIGESTED
PAROTIN BY TRYPSIN
(STUDIES ON THE PHYSIOLOGICAL CHEMISTRY
OF THE SALIVARY GLANDS LII*)

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By the tryptic digestion, the serum calcium decreasing activity of parotin slowly diminished, the decreasing activity of circulating leukocytes rapidly diminished, and, on the other hand, the increasing activity of leukocytes was relatively resistant (Ito and Shinoda, 1958a). The free amino-nitrogen content in parotin rapidly increased within 1 hr., and 2 or more hydrolyzates were recognized in non-protein fraction of the digested parotin by paper partition chromatography (Ito and Shinoda, 1958b). The ultraviolet absorption of the perchloric acid-soluble fraction in the digested parotin also increased within half an hr. (Ito and Shinoda, 1961). In view of the results above-mentioned, it can be concluded that the diminution of the biological activities of parotin by the digestion appears to take place later than the alteration in the chemical properties. Therefore it seems likely that the molecular fragments having the calcium- and the leukocyte-activity might be obtained separately from the digested parotin.

This paper deals with the fractionation of the substance obtained by digestion of parotin with trypsin, and also with the biological activities and some properties of the separated fractions. Hereafter the biological activities of parotin are respectively to be abbreviated as calcium activity and leukocyte activity.

MATERIALS AND METHODS

Materials Parotin used in this experiment was prepared as described in the preceding paper (Ito and Shinoda, 1960).

Trypsin** used in this experiment was a crystalline preparation purified by the method of Kunitz and Northrop (1936), having the activity as 1,600 haemoglobin U per mg by the method of Anson (1938).

Paper partition chromatography A small amount of sample was dissolved in some drops of water and submitted to paper partition chromatography by one-dimensional ascending method with No. 51 Toyo Roshi filter paper, using the mixture of *n*-butanol:acetic acid:water (4:1:5) as the developing solvent and ninhydrin as the coloring reagent. In the case of 2-dimensional method, the 1st phenol:0.1% ammonia (4:1), and the 2nd *n*-butanol:acetic acid:water (4:1:5) were used as solvents.

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Paper electrophoresis A small amount of sample was dissolved in some drops of 0.05 *M* phosphate buffer of pH 8.0, and submitted to paper electrophoresis on No. 51 Toyo Roshi filter paper, using the same buffer and the coloring reagent. The electrophoretical pattern thus obtained was fixed with paraffin transparently. The optical density on the filter paper was measured by a densitometer equipped with a green filter.

Determination of ultraviolet absorption spectra Three mg of sample was dissolved in 6 ml of water, adjusted to pH 7.0 with 0.05 *N* NaOH, and used as the sample solution for the measurement of the ultraviolet absorption. The spectral curve was determined by a Beckman spectrophotometer at room temperature.

Measurement of biological activities Ten mg of the sample was dissolved in 5 ml of water and adjusted to pH 8.0. This sample solution was injected intravenously into the male rabbits weighing 2~2.5 kg after fasting for 24 hrs., with the dose of 1 mg of sample per kg of body weight. The biological activities were assayed by the method of Ito (1954 and 1960).

RESULTS

Separation of hydrolyzates in the digested parotin with trypsin

As shown in Figure 1, 50 mg of parotin was dissolved in 10 ml of water, adjusted to pH 8.0 with 0.1 *N* NaOH, and warmed at 37°C in a water bath. To this parotin solution, 1 mg of trypsin was added, and the mixture was incubated at 37°C for 3 hrs. After incubation, the solution was cooled in ice water and then adjusted to pH 4.5 with 0.1 *N* HCl. The white precipitate formed was separated from the supernatant solution by centrifugation. To this precipitate was added about 5 ml of cold water adjusted to pH 4.5 with 0.1 *N* HCl and mixed completely.

Fig. 1. Flow sheet of fractionation of hydrolyzates in parotin digested by trypsin

The mixture was centrifuged and the separated precipitate was taken, dissolved in a small amount of water and lyophilized (A-fraction). To the supernatant was added acetone slowly to make 80% in concentration under cooling in ice water, whereupon a second white precipitate was formed. After standing overnight in a refrigerator, the precipitate was taken by centrifugation and dissolved in a small amount of water, then lyophilized (B-fraction). After evaporating off the acetone in the supernatant solution *in vacuo*, the solution was lyophilized (C-fraction). The yield of each fraction was as shown in Table 1. The parotin

Table 1. Yield of separated fraction as shown in Figure 1.

Fraction*	Yield (mg)			
	3 hrs. incubation		24 hrs. incubation	
	With trypsin	Without trypsin	With trypsin	Without trypsin
A (pH 4.5- or pH 5.4- precipitate)**	23	47	7	47
B (80% acetone-insoluble)	20	2	11	1
C (80% acetone-soluble)	5	—	29	—

* Starting material of each group : 50 mg

** With trypsin : pH 4.5-precipitate, without trypsin : pH 5.4-precipitate

Fig. 2. Paper partition chromatograms of hydrolyzates in parotin digested for 3 hrs. by trypsin

P : Parotin before treatment
 A : A-fraction with trypsin
 B : B-fraction with trypsin
 C : C-fraction with trypsin
 A' : A-fraction without trypsin
 B' : B-fraction without trypsin
 C' : C-fraction without trypsin

Fig. 3. Paper partition chromatograms of hydrolyzates in parotin digested for 24 hrs. by trypsin

P : Parotin before treatment
 A : A-fraction with trypsin
 B : B-fraction with trypsin
 C : C-fraction with trypsin
 A' : A-fraction without trypsin
 B' : B-fraction without trypsin
 C' : C-fraction without trypsin

solution without enzyme was treated similarly as the digested parotin and used as control. The parotin digested for 24 hrs. was also fractionated similarly as that digested for 3 hrs., and the yields of these 3 fractions were compared with those of 3 hrs.-digestion as in Table 1.

Compared with the 3 hrs.-digestion, the A- and the B-fractions in the 24 hrs.-digestion were small and the C-fraction was large in amount. The control parotin without enzyme remained unchanged during the incubation both for 3 hrs. and 24 hrs. and precipitated perfectly at the acidity of pH 5.4 as same as untreated native parotin.

Paper partition chromatography of each fraction separated from digested parotin

The hydrolyzates of each fraction of 3 hrs.-digestion and 24 hrs.-digestion revealed the paper partition chromatogram as shown in Figures 2 and 3. In both the digested and the control parotin (A and A'), the A-fractions remained completely at the original line as same as the parotin before treatment (P). Although the B-fractions of the above 2 digested parotin (B) contained some running ingredients, most part of them remained to stay on the original line. The B-fractions of both controls (B') stayed on the original line similarly as the A-fraction. Every C-fraction of both the digested and the control parotin (C) runned thoroughly, remaining not any part of them at the original line.

From the 2-dimensional paper partition chromatography of the C-fraction of the 24 hrs.-digestion, as shown in Figure 4, 5 or more ingredients seem to be contained in the C-fraction.

Fig. 4. Two dimensional paper partition chromatogram of the C-fraction of digested parotin for 24 hrs.

Fig. 5. Paper electrophoretical patterns of hydrolyzates in parotin digested by trypsin

Paper electrophoresis of separated fractions

As shown in Figure 5, the electrophoretical patterns of the separated fractions from 3 hrs.- digested parotin were revealed on the filter paper. The A-fraction showed the same pattern as that before digestion. But the mobility of the A-fraction under these experimental conditions was larger than that of the parotin itself. In the case of the B-fraction, 3 or more peaks, and in the C-fraction, a lot of ingredients were revealed.

Ultraviolet absorption spectra of separated fractions

The ultraviolet absorption of the A- and B-fraction and the parotin before treatment were shown in Figure 6. The absorption of C-fraction was not measured since it was found to be the mixture of many hydrolyzates by the above mentioned paper electrophoretic analysis. The absorption peaks both of the A- and B-fraction were seen at 276 m μ of wave length. Extinctions of A- and B-fraction were relatively lower than that of the parotin before treatment.

Biological activities of separated fractions

Among these A-, B- and C-fraction, only the A-fraction showed the calcium activity as shown in Table 2. In the leukocyte activity (Fig. 7), the A-fraction showed not only the 1st decrease but also the successive increase. The B-fraction,

Fig. 6. Ultraviolet absorption spectra of hydrolyzates in digested parotin by trypsin

— Parotin before treatment
 - - - A-fraction for 3 hrs. digestion
 B-fraction for 3 hrs. digestion

Fig. 7. Leukocyte activity of hydrolyzates in digested parotin by trypsin.

Dose: 1 mg/kg intravenous injection

— A-fraction
 - - - B-fraction
 C-fraction

Table 2. Calcium decreasing and protein decreasing activities of each separated fraction in Figure 1.

Fraction	Percentage of serum calcium decreasing activity* Mean $\pm$ S.E.	Percentage of serum protein decreasing activity* Mean $\pm$ S.E.
A (pH 4.5-precipitate)	9.40 $\pm$ 0.47	15.06 $\pm$ 4.13
B (80% acetone-insoluble)	6.85 $\pm$ 0.47	15.13 $\pm$ 1.07
C (80% acetone-soluble)	5.82 $\pm$ 0.61	13.46 $\pm$ 0.89
Parotin**	13.63 $\pm$ 1.22	13.10 $\pm$ 1.53

* Dose : 1 mg/kg, intravenous injection. Mean value of 3 rabbits

** Before treatment

however, showed only the increasing action alone lacking the 1st decreasing action. The C-fraction was completely inactive. In contrast with the calcium or the leukocyte activity, every fraction showed equal and similar serum protein level decreasing activity (protein activity) as that of the parotin before treatment (Table 2).

DISCUSSION

Although the biological activities of parotin was completely destroyed after the 24 hrs. of tryptic digestion at 37°C, the calcium activity and the leukocyte increasing activity of parotin yet remained. On the other hand, the free amino-nitrogen content of parotin increased about 4 times and 2 or more hydrolyzates, perhaps peptides, were shown to be formed by paper partition chromatography (Ito and Shinoda, 1958 a and b). From these results it seems that the biologically active fragment(s) could be obtained from the digested parotin with trypsin for 3 hrs. Therefore the fractionation of the tryptic digested parotin was attempted as shown in Figure 1 and Table 1. Although the isoelectric point of parotin is about pH 5.4 (Ito, 1960) and the A-fraction of the control without enzyme has precipitated as the same pH value, the A-fraction of the digested parotin has not precipitated at this pH and precipitated at pH 4.5. According to the comparison in the yield of each fraction after the 3 hrs. digestion and the 24 hrs. digestion, the increase of digesting period cause the increase in the yield of the C-fraction. In the case of the control without enzyme, there is no difference in the yields of the A-fraction between 3 hrs. and 24 hrs. digestion.

By the paper partition chromatography the A-fraction of digested parotin did not move at all with the solvent of *n*-butanol and acetic acid system (Figs. 2 and 3). In consequence, it seems that the A-fraction is composed of relatively high molecule as the parotin itself. Perhaps the developed hydrolyzates in the B-fraction seems to be the intermixture contaminated with a part of the C-fraction. The purity of the A-fraction is considered to be very high from the paper electrophoretic analysis.

The A-fraction has the same biological activities as that of the parotin before digestion and the B-fraction has only the leukocyte increasing activity, but the

C-fraction has none. The protein activity of parotin remains unchanged after the complete degradation of parotin. Thus it seems that the proteolysis of parotin by the action of trypsin progresses to give A-, B- and C-fraction successively.

SUMMARY

The hydrolyzates in the digested parotin by trypsin were separated into the 3 fractions by the isoelectric precipitation and acetone fractionation: the pH 4.5-precipitate, the insoluble fraction in the acetone of 80% concentration, and the soluble fraction in the same solvent. The first pH 4.5-precipitate was relatively pure in view of the paper electrophoresis and the paper partition chromatography, and showed the same biological activities as the parotin before digestion. The 80% acetone-insoluble fraction was the mixture of some hydrolyzates and showed only the leukocyte increasing activity. The 80% acetone-soluble fraction contained many hydrolyzates and was negative in the biological activities.

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