INFLUENCES OF PROTEOLYTIC ENZYMES ON PAROTIN VII.
ISOLATION AND SOME PROPERTIES OF PAROTIN-T.B₁
AND PAROTIN-T.B₂
(STUDIES ON THE PHYSIOLOGICAL CHEMISTRY
OF THE SALIVARY GLANDS LVI*)

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In the previous paper (Shinoda, 1961), the author reported the separation of two biologically active fractions, the A- and the B-fraction, from parotin-digest by trypsin. And from the A-fraction a biologically active protein named parotin-T.A was isolated in a homogeneous state. The B-fraction showed only the increasing response in the leukocytes activity and was lacking in the calcium activity. This fraction seemed to be a mixture of some peptides.

This paper deals with the isolation of parotin-T.B₁ and parotin-T.B₂ from the B-fraction, their properties and the comparison of the characteristics between these degradation products of parotin and the intact parotin.

MATERIALS AND METHODS

B-fraction and parotin-T.A were prepared as described before (Ito and Shinoda, 1961; Shinoda, 1961).

Measurement of biological activities The biological potency was assayed by the method reviewed by Ito (1954 and 1960). A half mg of sample per kg of rabbit body weight was injected intravenously.

Paper electrophoresis was done as described before (Shinoda, 1961).

Preparation of 'acid' alumina column and procedure of chromatography The preparation of the 'acid' alumina column using the Merck alumina Brockmann No. 51049 and the procedure of chromatography were the same as previously reported (Shinoda, 1961).

Assessment of proteolytic activity Five mg of sample was dissolved in 5 ml of the physiological saline, and used as sample solution. The tryptic activity was measured by the method of Anson (1938).

Estimation of activity of sensitization to parotin According to the method of Campbell and McCasland (1944), the activity of sensitization of each sample was assayed against parotin as the sensitinogen. To 200 to 250 g body weight of female guinea pig, 1 mg of parotin suspended with Bayol-F and Arlacel-A (2:3) (surface active agents) was subcutaneously injected per day for 3 days (total 3 mg of parotin). At 3 weeks after the injection, contractive movement

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of the dissected uterus was traced kymographically with Magnus's apparatus*.

The amino acids component was determined by the paper chromatography of acid hydrolyzates as described before (Shinoda, 1961).

Determination of N-terminal amino acids Parotin-T.B₁ and parotin-T.B₂ were dinitrophenylated, according to Sanger and Thompson (1953) and then were analyzed by one-dimensional paper chromatography as described before (Shinoda, 1961).

RESULTS

Fractionation of the B-fraction with trichloroacetic acid

As shown in Figure 1, 200 mg of the B-fraction was dissolved in 10 ml of water and adjusted to pH 6.0. To this solution 2 ml of 30%

Fig. 1. Flow sheet of the fractionation of the B-fraction by fractional precipitation with trichloroacetic acid (TCA)

TCA was added slowly with stirring under cooling with ice water, and the mixture was left in refrigerator over one night. After standing, the produced white precipitate and the supernatant solution were separated by centrifugation. Thus separated precipitate was washed twice with 30 ml of acetone and then twice with 30 ml of ether. The precipitate was dried *in vacuo* (B₁-fraction). To the supernatant solution, 10 times amounts of ether-acetone (1:1) mixture was added. The white precipitate produced was separated from the ether-acetone solution by centrifugation, dissolved in a small amount, approximately 2 ml, of water and then lyophilized (B₂-fraction). After evaporating the ether and acetone off

* This measurement was kindly carried out by Dr. S. Aonuma and Mr. T. Mimura, Pharmaceutical Faculty, University of Osaka.

in vacuo, the solution was lyophilized (B₃-fraction). The yields of each fraction were shown in Figure 1.

The biological activities of these fractions were shown in Table 1 and Figure 2. Although the B₁-fraction showed a decreasing response in the calcium activity and only the increasing response in the leukocyte activity with a dose of

Table 1. Yield and calcium activity of sub-fractions of the B-fraction

Fraction	Yield (%)	Decrease of serum calcium * Mean $\pm$ S.E. (%)
B-fraction	—	7.84 $\pm$ 1.78
B ₁ -fraction	26.5	8.45 $\pm$ 0.49
B ₂ -fraction	54.0	4.78 $\pm$ 0.13
B ₃ -fraction	6.5	2.24 $\pm$ 0.90

* Dose: 0.5 mg per kg body weight intravenous injection in rabbits

Fig. 2. Leukocyte activity of sub-fractions of the B-fraction.

Dose: 0.5 mg/kg body weight intravenous injection in rabbits

— B-fraction
 - - - B₁-fraction
 - - - B₂-fraction
 . . . B₃-fraction

0.5 mg per kg body weight. In B₂-fraction it was found to have only the leukocyte increasing response of showing the maximum at 4 to 6 hrs. after injection. The B₃-fraction did not show any biological activities.

On the paper electrophoretical pattern, as shown in Figure 3, the B₁-fraction revealed a single peak in the anodic area, the B₂-fraction showed three peaks just similarly as the original B-fraction and the B₃-fraction was composed of many ingredients.

Fig. 3. Paper electrophoretical patterns of separated fractions by the fractionation of the B-fraction with TCA.

Toyo Roshi filter paper No. 51, 0.05M phosphate buffer of pH 8.0, 1 mA/cm, 3 hrs.

Fig. 4. Chromatography of the B₁-fraction on acid alumina column.
Seventy mg of the B₁-fraction was applied into 1.5×16 cm column.

Purification of the B₁-fraction by acid alumina column chromatography

The B₁-fraction obtained as before mentioned was purified by the column chromatography using the acid alumina as the adsorbent as shown in Figure 4. This procedure was as similar as the isolating method of parotin-T.A previously described (Shinoda, 1961). Seventy mg of the B₁-fraction was dissolved in 2 ml of water, adjusted to pH 6.0 with 0.1N NaOH, and submitted to the acid alumina chromatography (1.5 × 16 cm). The elution diagram was obtained by determining the optical densities of the effluents at 275 mμ of wave length.

The yields and the ultraviolet absorption spectra of each eluted fractions were shown in Table 2 and Figure 5, respectively only the B₁₋₃-fraction showed the maximum absorption at 275 mμ, and other fractions did not show any maximum absorption.

The biological activities of these eluted fractions and the B₁-fraction with a dose of 0.5 mg were shown in Table 2 and Figure 6. Among those only the

Fig. 5. Ultraviolet absorption spectra of eluted fractions by acid alumina column chromatography of the B₁-fraction.

Fig. 6. Leukocyte activity of eluted fractions by acid alumina column chromatography of the B₁-fraction. Dose : 0.5 mg per kg body weight intravenous injection in rabbits.

B₁ : B₁-fraction before elution
 B₁₋₁ : B₁₋₁-fraction eluted with distilled water
 B₁₋₃ : B₁₋₃-fraction eluted with 0.1M Na₂HPO₄

Table 2. Yield and calcium activity of eluted fractions by acid alumina column chromatography of the B₁-fraction

Fraction	Yield (%)	Decrease of serum calcium* Mean ± S.E. (%)
B ₁ -fraction	—	8.45 ± 0.49
B ₁₋₁ -fraction eluted with distilled water	10.1	3.36 ± 1.06
B ₁₋₂ -fraction eluted with 0.1M NaH ₂ PO ₄	4.1	—
B ₁₋₃ -fraction eluted with 0.1M Na ₂ HPO ₄	72.5	8.49 ± 0.67

Dose : 0.5 mg/kg body weight intravenous injection in rabbits

B₁₋₃-fraction was active in both the calcium and leukocyte activities.

Hereafter the B₁₋₃-fraction will be called the "parotin-T.B₁". This seemed to be homogeneous by the paper electrophoretical analysis, as mentioned later (Fig. 11).

Purification of the B₂-fraction by acid alumina column chromatography

Fifty mg of the B₂-fraction was dissolved in 1 ml of water, adjusted to pH 6.0 with 0.1 N NaOH, and submitted to the acid alumina column chromatography (1.5 × 18 cm). Since the B₂-fraction showed the maximum absorption at 275 mμ of wave length, the elution diagram was also made by the ultraviolet absorption method at 275 mμ (Fig. 7). The yield of each eluted fractions was shown in Table 3.

The ultraviolet absorption spectra of these eluted fractions were shown in Figure 8. The B₂₋₁-fraction showed the weak maximum absorption at 270~272 mμ and the B₂₋₃-fraction showed the maximum at 274.5~275 mμ. But the B₂₋₂-fraction did not show any maximum absorption.

Fig. 7. Chromatography of the B₂-fraction on acid alumina column.
Fifty mg of the B₂-fraction on acid alumina column.

Table 3. Yield and calcium activity of eluted fractions by acid alumina column chromatography of the B₂-fraction

Fraction	Yield (%)	Decrease of serum calcium* Mean ± S.E. (%)
B ₂ -fraction	—	4.78 ± 0.13
B ₂₋₁ -fraction eluted with distilled water	23.1	1.24 ± 0.01
B ₂₋₂ -fraction eluted with 0.1M NaH ₂ PO ₄	3.6	—
B ₂₋₃ -fraction eluted with 0.1M Na ₂ HPO ₄	69.3	2.28 ± 0.95

* Dose: 0.5 mg/kg body weight intravenous injection in rabbits

The biological activities of these eluted fractions at the B_2 -fractions were shown in Table 3 and Figure 9. Each fraction lacked the calcium activity and the B_{2-3} -fraction showed only the leukocyte increasing response rather stronger than that of the original B_2 -fraction. The B_{2-1} -fraction showed neither the calcium- nor the leukocyte-activity.

The B_{2-3} -fraction was demonstrated to be homogeneous by the paper electrophoretal analysis as mentioned later (Fig. 11). Hereafter the B_{2-3} -fraction was named as "parotin-T. B_2 ".

Fig. 8. Ultraviolet absorption spectra of eluted fractions by acid alumina column chromatography of the B_2 -fraction

Fig. 9. Leukocyte activity of eluted fractions by acid alumina column chromatography of the B_2 -fraction. Dose: 0.5 mg/kg body weight intravenous injection in rabbits

B_2 : B_2 -fraction before elution
 B_{2-1} : B_{2-1} -fraction eluted with distilled water
 B_{2-3} : B_{2-3} -fraction eluted with 0.1M Na_2HPO_4

Adsorptive affinity of parotin-T. B_1 and parotin-T. B_2 on acid alumina

The equal amount of parotin-T. B_1 and parotin-T. B_2 were adsorbed on the acid alumina column of the same size, and the elution diagrams of both fractions were made under the same condition as shown in Figure 10.

The paper electrophoretal patterns of parotin-T. B_1 and parotin-T. B_2 were compared with those of the B_1 - and the B_2 -fractions, as shown in Figure 11, the B_1 -fraction showed already single peak by the paper electrophoresis, and parotin-T. B_1 was furthermore purified by the column chromatography. On the other hand, although the B_2 -fraction showed three peaks, parotin-T. B_2 showed a single symmetrical pattern. Under this experimental conditions, the mobility of parotin-T. B_1 for anodic side was slightly larger than that of parotin-T. B_2 .

Distribution of tryptic activity in the various fractions

In this experiment, trypsin added for digestion might be distributed in some of these fractions. For that reason, the proteolytic activities of all separated fractions were measured by the viscosity method of Anson (1938) and shown in Figure 12 together with the yields of these fractions. The trypsin content could be assayed until 10 μg per 5 mg of sample by this determination. And the

Fig. 10. Comparison of adsorptive affinities of parotin-T.B₁ and parotin-T.B₂. Twenty mg of each sample was applied into 1.2×15 cm of acid alumina column.

Fig. 11. Paper electrophoretical patterns of parotin-T.B₁ and parotin-T.B₂. Toyo Roshi filter paper No. 51, 0.05M phosphate buffer of pH 8.0, 1 mA/cm, 3 hrs.

Fig. 12. Flow sheet of distribution of trypsin added for digestion in separated fractions from the digested parotin

T : tryptic activity. Alumina : acid alumina column chromatography

amount of trypsin under $10 \mu\text{g}$ per 5 mg of sample was described as 0% in Figure 12. A small part, approximately 15%, of the trypsin added went into the B-fraction, and approximate 5% of the trypsin was estimated in the B₁-fraction. The remaining activity in these fraction was perfectly diminished when passed through the column of the acid alumina. Other greater parts of trypsin was either destroyed or separated during these treatments. Each biologically active peptides, parotin-T.A, -T.B₁ and -T.B₂, showed no proteolytic activity.

Activity of sensitization of the separated fraction against parotin

By the method of Campbell and McCasland (1944) using the uterus of guinea pig sensitized by parotin, the activity of sensitization of these separated fractions was measured as shown in Figure 13. The parotin used as the protein

Fig. 13. Activity of sensitization against parotin by the separated fractions from the parotin-digest.

A : 50 μ g of parotin	B : 30 μ g of parotin-T.A
C : 80 μ g of B-fraction	D : 120 μ g of B-fraction
E : 50 μ g of parotin	F : 1000 μ g of C-fraction
G : 80 μ g of parotin-T.B ₁	H : 500 μ g of parotin-T.B ₂
I : 50 μ g of parotin	J : 60 μ g of parotin

Fig. 14. Ultraviolet absorption spectra of parotin-T.A, parotin-T.B₁ and parotin-T.B₂.

Solvent: 0.05M phosphate buffer of pH 8.0.

———— parotin-T.A
 - - - - - parotin-T.B₁
 - · - · - parotin-T.B₂

sensitogen in this measurement was the same preparation as the material for the tryptic digestion. As the results on these fractions, 30 μ g of parotin-T.A, 120 μ g of the B-fraction and 80 μ g of parotin-T.B₁, respectively, showed the activity of sensitization corresponding with that of the 50 μ g of parotin. But the C-fraction and parotin-T.B₂ showed no activity.

Fig. 15. Paper partition chromatogram of acid hydrolyzate of parotin-T.B₁. Filter paper: Toyo Roshi No. 51.

Fig. 16. Paper partition chromatogram of acid hydrolyzate of parotin-T.B₂. Filter paper: Toyo Roshi No. 51.

Comparison of ultraviolet absorption spectra of parotin-T.A, -T.B₁ and -T.B₂

Though the maximum absorption of parotin-T.A was found at $276\text{ m}\mu$ of wave length, those of parotin-T.B₁ and -T.B₂ were shown at $275\text{ m}\mu$, respectively, as shown in Figure 14. Although parotin-T.B₁ and -T.B₂ showed the similar type of the absorption spectra, the extinction was relatively larger with parotin-T.B₁ than with parotin-T.B₂.

Amino acids components of parotin-T.B₁ and parotin-T.B₂

By the two-dimensional paper chromatography as shown in Figure 15, the acid hydrolyzate of parotin-T.B₁ contained the following 13 or 14 kinds of amino

acids; alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine (and/or isoleucine), lysine, proline, serine, threonine, tyrosine and valine. The presence of arginine, histidine and tyrosine were also revealed by Sakaguchi's and diazo reagents, but methionine and cystine could be estimated by neither sodium azide reagent nor iodine reaction. On the other hand, the paper chromatogram of the alkaline hydrolyzate of parotin-T.B₁ by 10% Ba(OH)₂, showed the presence of tryptophan by the Ehrlich's reagent, 1% *p*-dimethylaminobenzaldehyde in 1 *N* HCl solution. The parotin-T.B₁ *per se* also showed the positive Ehrlich's reaction showing the presence of tryptophan.

It was also demonstrated that the acid hydrolyzate of parotin-T.B₂ contained 13 or 14 kinds of amino acids similarly as the case of the parotin-T.B₁ (Fig. 16). And the presence of tryptophan in parotin-T.B₂ *per se* was also shown by the Ehrlich's reagent.

N-terminal amino acid of parotin-T.B₁ and parotin-T.B₂

As the developing solvent of dinitrophenyl amino acids, 17 kinds of solvents and their combination were examined. All kinds of standard dinitrophenyl amino acids were submitted to one-dimensional paper chromatography with Toyo Roshi filter paper No. 50 or 51. In the case of the solvents containing the buffer, the filter paper was previously washed well with the same buffer before the development and dried at room temperature. Among the solvent combinations studied, (a) *n*-butanol - pH 6 phthalate buffer (3:2), (b) *n*-butanol - ethanol - pH 6 phthalate (9:1:5), (c) *n*-butanol - 0.1% NH₄OH (1:1) and (d) *n*-butanol - 0.1% NH₄OH (5:1) were found to be useful for the detection of the N-terminal amino acids. Consequently (a) and (d) of solvents were used.

Fig. 17. Paper partition chromatograms of acid hydrolyzates (ether-soluble fraction) of DNP-parotin-T.B₁ and DNP-parotin-T.B₂. Filter paper: Toyo Roshi No. 51.

DNA: dinitroaniline, DNP: dinitrophenol B₁: DNP-parotin-T.B₁, B₂: DNP-parotin-T.B₂

(I): solvent, upper layer of *n*-butanol - pH 6 phthalate buffer (3:2)

(II): solvent, upper layer of *n*-butanol - 0.1% NH₄OH (5:1)

By the paper chromatography of the ether-soluble fractions of DNP-parotin-T.B₁ and DNP-parotin-T.B₂, the N-terminal amino acids of both parotin-T.B₁ and parotin-T.B₂ were determined samely as aspartic acid, as shown in Figure 17. The ϵ -lysine was also recognized in either case of parotin-T.B₁ or parotin-T.B₂ in the water-soluble fraction of these DNP-peptides.

DISCUSSION

For the purpose of the purification of B-fraction, the column chromatography using the acid alumina as the adsorbent and the fractional precipitation with TCA were attempted. Although the acid alumina column chromatography failed to purify, it succeeded by the fractional precipitation with TCA. The B₁-fraction precipitated readily at the concentration of 5% of TCA as shown in Figure 1, while no more precipitate was produced by increasing the TCA-concentration of the solution. Li *et al.* (1952) reported that the adrenocorticotrophic peptides prepared by peptic digestion of sheep ACTH protein was purified by fractional precipitation with TCA, and the activity in the digest was found in the precipitate at the concentration from 5 to 25% of TCA. In contrast with the result of ACTH peptide, the precipitate at the concentration from 5 to 25% of TCA was not obtained in author's experiment of tryptic digest of parotin. However, on the other hand, the precipitate (B₂-fraction) could be obtained by the addition of ether-acetone mixture (1:1) to the TCA supernatant.

Table 4. Comparison of the properties of parotin-T.A, parotin-T.B₁, parotin-T.B₂ and parotin

Properties	Parotin	Parotin-T.A	Parotin-T.B ₁	Parotin-T.B ₂
Isoelectric point	pH 5.4	approximate pH 4.5		
Molecular weight	132,000	99,000		
Wave length of maximum absorption (m μ)	277	27.5-276	275	275
Amino acids components	Ala., Arg., Asp., Cys., Glu., Gly., His., Leu. and/or Ileu., Lys., Met., Phe., Pro., Ser., Thr., Try., Tyr. Val.	Ala., Arg., Asp., Cys., Glu., Gly., His., Leu. and/or Ileu., Lys., Phe., Pro., Ser., Thr., Try., Tyr., Val.	Ala., Arg., Asp., Glu., Gly., His., Leu. and/or Ileu., Lys., Pro., Ser., Thr., Try., Tyr., Val.	same kinds as parotin-T.B ₁
N-terminal amino acid	Ala., Asp., Gly.	Asp., Gly.	Asp.	Asp.
Calcium activity	+	+	+	—
Leukocyte decreasing activity	+	+	—	—
Leukocyte increasing activity	+	+	+	+
Activity of sensitization against parotin	+	+	+	—

Both parotin-T.B₁ and parotin-T.B₂ were purified by similar procedure, *i.e.* the acid alumina column chromatography, as the case of parotin-T.A. During elution the pH values of effluents increased step by step and it was able to check the elution of each fraction by measuring the pH value of the effluents. Parotin-T.B₁ and parotin-T.B₂ seemed to have similar adsorptive affinity on the acid alumina column. So it seems to be impossible to separate these 2 substances by the column chromatography without applying the method of fractional precipitation with TCA. It also seems to be that the biological activities of parotin-T.B₁ and parotin-T.B₂ are relatively stable against TCA.

The properties of these 3 peptides and the intact parotin were compared as shown in Table 4. The molecular weight of parotin-T.A was 99,000 (Shinoda, 1961), namely the size is about three quarters of that of parotin, 132,000, measured by Ito *et al.* (1960). The molecular weight of parotin-T.B₁ and parotin-T.B₂ were unable to be estimated by the ultracentrifugal procedure, because these peptides were considerably diffused during the operation. They did not precipitated at their isoelectric points. The ultraviolet absorption maxima of these were at slightly shorter wave length than that of parotin (Fig. 14).

While parotin-T.A showed both the calcium and the leukocyte activities, in the parotin-T.B₁ the calcium activity was very weak and the leukocyte activity showed only the increasing response. Parotin-T.B₂ showed only the leukocyte increasing response and no calcium activity. By the method of Campbell and McCasland (1944) using the uterus of guinea pig, the activity of sensitization against parotin was recognized in parotin-T.A and parotin-T.B₁. But in the parotin-T.B₂, which had no calcium activity, this activity was not observed (Fig. 13). Ito, Aonuma and Mimura (unpublished) had found that S-parotin and Serum-parotin, parotin-like substances in bovine submaxillary gland and serum, showed this activity against parotin. It, therefore, seems in all likelihood that the activity of sensitization might be connected with the calcium activity but not with the leukocyte increasing activity.

Both parotin-T.B₁ and parotin-T.B₂ contained 14 kinds of amino acids as shown in Figures 15 and 16. The parotin-T.A did not contain methionine, contrary to parotin. And parotin-T.B₁ and parotin-T.B₂ did contain neither methionine nor cystine and phenylalanine as compared with parotin-T.A. It seems probably to be that methionine might be dispensable for the calcium- and leukocyte-activities, while cystine might be essential for only the calcium activity. By the method of Sanger and Thompson (1953), *N*-terminal amino acids of both parotin-T.B₁ and parotin-T.B₂ were shown to be aspartic acid as seen in Figure 17. For dinitrophenylation, the method of Sanger and Thompson (1953), using trimethylamine as the solvent containing no inorganic salts was found to be more useful than the method of Sanger (1945), using NaHCO₃. Parotin-T.A and other 2 peptides were shown to have no alanine as the *N*-terminal amino acid, and parotin-T.B₁ and parotin-T.B₂ were found to have the aspartic acid only as *N*-terminal amino acid. It seems likely that the parotin molecule might be splitted at first at the alanyl chain, and later at glycyl chain by tryptic digestion, and the aspartic chain of parotin might be relatively stable against the digestion. It also seems probably that the glycyl

chain is responsible for the biological activities at parotin, and the process of the tryptic degradation of parotin might have the following pathway: Parotin $\rightarrow$ parotin-T.A $\rightarrow$ parotin-T.B₁ $\rightarrow$ parotin-T.B₂.

SUMMARY

The active leukocyte-increasing fractions prepared by tryptic digestion of parotin were separated by fractional precipitation with trichloroacetic acid, and two active peptides, parotin-T.B₁ and parotin-T.B₂ were isolated by the acid alumina column chromatography. Both peptides were estimated as to be homogeneous by the paper electrophoresis. Parotin-T.B₁ showed the calcium activity and the leukocyte increasing response. Parotin-T.B₂ showed only the leukocyte increasing response.

Some properties of parotin-T.B₁ and parotin-T.B₂ were described and also compared with those of parotin and parotin-T.A.

Parotin-T.B₁ and parotin-T.B₂ showed same ultraviolet absorption maximum at 275 m μ of wave length. The amino acids components of both peptides were shown to be the same following 14 kinds; alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, leucine (and/or isoleucine), lysine, proline, serine, threonine, tryptophan, tyrosine and valine. The *N*-terminal amino acid of these peptides were estimated as aspartic acid.

The activity of sensitization against parotin was recognized in parotin-T.A and parotin-T.B₁ by the method using the uterus of guinea pig sensitized by parotin previously. But in parotin-T.B₂ it was lacking.

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