INFLUENCES OF PROTEOLYTIC ENZYMES ON PAROTIN VII.
ISOLATION AND SOME PROPERTIES OF PAROTIN-T.B_1
AND PAROTIN-T.B_2
(STUDIES ON THE PHYSIOLOGICAL CHEMISTRY
OF THE SALIVARY GLANDS LVIII*)

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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.B_1 and parotin-T.B_2 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; Shi-
noda, 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 chro-
matography 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 Mc-
Casland (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

Received for publication September 26, 1960.

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June 1961

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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 with 0.1 N NaOH. To this solution 2 ml of 30% TCA was added with stirring. The white precipitate was left in refrigerator over a night. Centrifuged.

Next, the supernatant solution was adjusted to pH 6.0 with 0.1 N NaOH. To this solution 2 ml of 30% 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 of water, and then lyophilized (B₂-fraction). After evaporating the ether and acetone off

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

* 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>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (%)</th>
<th>Decrease of serum calcium Mean±S.E. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-fraction</td>
<td>—</td>
<td>7.84±1.78</td>
</tr>
<tr>
<td>B₁-fraction</td>
<td>26.5</td>
<td>8.45±0.49</td>
</tr>
<tr>
<td>B₂-fraction</td>
<td>54.0</td>
<td>4.78±0.13</td>
</tr>
<tr>
<td>B₃-fraction</td>
<td>6.5</td>
<td>2.24±0.90</td>
</tr>
</tbody>
</table>

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

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 electrophoreetical 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 0.1 N 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 m\(\mu\) 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 m\(\mu\), 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.](image)

![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.](image)

<table>
<thead>
<tr>
<th>Table 2. Yield and calcium activity of eluted fractions by acid alumina column chromatography of the B1-fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>B1-fraction</td>
</tr>
<tr>
<td>B1-1-fraction eluted with distilled water</td>
</tr>
<tr>
<td>B1-2-fraction eluted with 0.1M Na(_2)HPO(_4)</td>
</tr>
<tr>
<td>B1-3-fraction eluted with 0.1M Na(_2)HPO(_4)</td>
</tr>
</tbody>
</table>

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.](image)

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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield (%)</th>
<th>Decrease of serum calcium*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean±S.E. 1%</td>
</tr>
<tr>
<td>B2-fraction</td>
<td>—</td>
<td>4.78±0.13</td>
</tr>
<tr>
<td>B2-1-fraction eluted with distilled water</td>
<td>23.1</td>
<td>1.24±0.01</td>
</tr>
<tr>
<td>B2-2-fraction eluted with 0.1M Na2HPO4</td>
<td>3.6</td>
<td>—</td>
</tr>
<tr>
<td>B2-3-fraction eluted with 0.1M Na3HPO4</td>
<td>69.3</td>
<td>2.28±0.95</td>
</tr>
</tbody>
</table>

*Dose: 0.5 mg/kg body weight intravenous injection in rabbits*
The biological activities of these eluted fractions at the B2-fractions were shown in Table 3 and Figure 9. Each fraction lacked the calcium activity and the B2-3-fraction showed only the leukocyte increasing response rather stronger than that of the original B2-fraction. The B2-1-fraction showed neither the calcium nor the leukocyte-activity.

The B2-3-fraction was demonstrated to be homogeneous by the paper electrophoretical analysis as mentioned later (Fig. 11). Hereafter the B2-3-fraction was named as "parotin-T.B2".

**Adsorptive affinity of parotin-T.B1 and parotin-T.B2 on acid alumina**

The equal amount of parotin-T.B1 and parotin-T.B2 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 electrophoretical patterns of parotin-T.B1 and parotin-T.B2 were compared with those of the B1- and the B2-fractions, as shown in Figure 11, the B1-fraction showed already single peak by the paper electrophoresis, and parotin-T.B1 was furthermore purified by the column chromatography. On the other hand, although the B2-fraction showed three peaks, parotin-T.B2 showed a single symmetrical pattern. Under this experimental conditions, the mobility of parotin-T.B1 for anodic side was slightly larger than that of parotin-T.B2.

**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.
amount of trypsin under 10 μg 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 approximately 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 μg of parotin  
B: 30 μg of parotin-T.A  
C: 80 μg of B-fraction  
D: 120 μg of B-fraction  
E: 50 μg of parotin  
F: 1000 μg of C-fraction  
G: 80 μg of parotin-T.B₁  
H: 500 μg of parotin-T.B₂  
I: 50 μg of parotin  
J: 60 μg 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

sensitinogen in this measurement was the same preparation as the material for the tryptic digestion. As the results on these fractions, 30 μg of parotin-T.A, 120 μg of the B-fraction and 80 μg of parotin-T.B₁, respectively, showed the activity of sensitization corresponding with that of the 50 μg of parotin. But the C-fraction and parotin-T.B₂ showed no activity.
Comparison of ultraviolet absorption spectra of \( \text{parotin-T.A, T.B}_1 \) and \( \text{T.B}_2 \)

Though the maximum absorption of parotin-T.A was found at 276 m\( \mu \) of wave length, those of parotin-T.B\(_1\) and T.B\(_2\) were shown at 275 m\( \mu \), respectively, as shown in Figure 14. Although parotin-T.B\(_1\) and T.B\(_2\) showed the similar type of the absorption spectra, the extinction was relatively larger with parotin-T.B\(_1\) than with parotin-T.B\(_2\).

Amino acids components of \( \text{parotin-T.B}_1 \) and \( \text{parotin-T.B}_2 \)

By the two-dimensional paper chromatography as shown in Figure 15, the acid hydrolyzate of parotin-T.B\(_1\) contained the following 13 or 14 kinds of amino
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-dimethyl-aminobenzaldehyde in 1N 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.

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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 similarly 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 adrenocorticotropic 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.B₁ | Parotin-T.B₂ |
| Isoelectric point               | pH 5.4  | approximate pH 4.6 |
| 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 lleu., Lys., Met., Phe., Pro., Ser., Thr., Try., Tyr., Val. |
|                                 | Ala., Arg., Asp., Cys., Glu., Gly., His., Leu. and/or lleu., Lys., Phe., Pro., Ser., Thr., Try., Tyr., Val. |
|                                 | Ala., Arg., Asp., Cys., Glu., Gly., His., Leu. and/or lleu., Lys., Phe., Pro., Ser., Thr., Try., Tyr., Val. |
| 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 trypic degradation of parotin might have the following pathway: Parotin → parotin-T.A → parotin-T.B₁ → parotin-T.B₂.

SUMMARY

The active leukocyte-increasing fractions prepared by trypic 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\(\mu\) 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.

ACKNOWLEDGEMENTS

The author would like to express his deep gratitude to Prof. Y. Ito of this institute for his encouragement and advice to carry out this work. Thanks are also due to Prof. S. Aonuma and Mr. T. Mimura, University of Osaka, for their conducting the experiment on the determination of sensitization and kind advice during this work, and to Dr. N. Ogawa, staff of Research Laboratory, Mochida Pharm. Mfg. Co., for his co-experiment on the determination of trypic activity.

REFERENCES