[Chem. Pharm. Bull. 25(12)3306—3311(1977)]

UDC 547.963.02 : 543.47.06

Some Properties of Bovine Thymic Hypocalcemic Factor TP1[1)]

Akira Mizutani, Yoshihiro Matsushita, and Takaharu Mizutani

Faculty of Pharmaceutical Sciences, Nagoya City University[2)]

(Received May 4, 1977)

One of two hypocalcemic substances from the bovine thymus (TP1) was investigated. As a material for purification, it was confirmed by chromatography on diethylaminoethyl-cellulose that the fraction precipitated at 0—15% ammonium sulfate concentration was better than the 0—20% precipitate. Molecular weight of TP1 was 66000 by sedimentation equilibrium and this result agreed with that obtained by sodium dodecyl sulfate polyacrylamide gel electrophoresis (68000) and suggested that TP1 was not composed of subunit. The circular dichroism spectrum of TP1 showed that the contents of α-helix, β-structure, and random form were 7, 39 and 54%, respectively. TP1 contained 2.07 mol of the sulfhydryl residues by the mercury orange method. By the treatment at 56° for 30 min, hypocalcemic activity disappeared and the activity also disappeared by the treatment at 37° for 1 hr in acidic (pH 1.4) or alkaline (pH 12.6) solutions.

Keywords——thymus; hypocalcemic factor; TP1; sedimentation equilibrium; CD; ORD; analysis of sulfhydryl residues; stability of activity

In the course of our investigation on the function of the thymus, two hypocalcemic factors (TP1 and TP2) were purified from bovine thymus extracts.[3)] TP1 was a protein of molecular weight 68000 by sodium dodecyl sulfate (SDS) gel electrophoresis, contained 2.43% of carbohydrate, and its isoelectric point was 5.65.[4)] The amino acid composition of TP1 was also reported. Miller[5)] had demonstrated that the thymus played a decisive role in immune function, and immunoactive substances such as thymosin and lymphocyte-stimulating hormone (LSH) were later isolated from the thymus extracts.[6)] Meanwhile, we reported that TP1 had lymphocyte stimulating and antibody-forming cell increasing activities.[7)]

In this paper, we describe the improved purification method for TP1, some properties of purified TP1, such as molecular weight by sedimentation equilibrium, the secondary structure by the circular dichroism (CD) measurements, the contents of the sulfhydryl and disulfide residues, and stability of activity.

Materials and Methods

Materials——Acetone-dried powder was prepared from bovine thymus gland by the method reported previously.[3b)] The saline extract from the powder was fractionated with $(NH_4)_2SO_4$, and the fraction precipitated at 15% (w/w) concentration of $(NH_4)_2SO_4$ (0—15% precipitate) was obtained. Saturated solution of $(NH_4)_2SO_4$ was added to the supernatant of the 0—15% precipitate up to 20% concentration of $(NH_4)_2SO_4$ and the resulting precipitate was collected (15—20% precipitate).

Purification——Diethylaminoethyl (DEAE)-cellulose which was activated by the conventional method and fully equilibrated with 7 mM phosphate buffer (pH 7.90, 0.02 μ) was packed in a column (5×122 cm).

1) Presented at the 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1976.
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) *a*) A. Mizutani, M. Terada, Y. Toda, and K. Yamamoto, *Ann. Rept. Pharm. Nagoya City Univ.*, **17**, 16 (1969); *b*) A. Mizutani, Y. Saito, H. Sato, and M. Saito, *Yakugaku Zasshi*, **90**, 445 (1970); *c*) A. Mizutani, K. Yamamoto, H. Sato, Y. Saito, H. Kitsugawa, and H. Yamamoto, *Yakugaku Zasshi*, **91**, 297 (1971).
4) A. Mizutani, M. Shimizu, I. Suzuki, T. Mizutani, and S. Hayase, *Ann. N.Y. Acad. Sci.*, **249**, 220 (1975).
5) J.F.A.P. Miller, *Lancet*, **2**, 748 (1961).
6) *a*) T. Hand, P. Caster, and T.D. Luckey, *Biochem. Biophys. Res. Commun.*, **26**, 18 (1967); *b*) A.L. Goldstein, A. Guha, M.M. Zatz, M. Hardy, and A. White, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1800 (1972).
7) A. Mizutani, I. Suzuki, Y. Matsushita, and K. Hayakawa, *Chem. Pharm. Bull.* (Tokyo), **25**, 2156 (1977).

A solution of the 0—15% precipitate (9.09 g) in 400 ml of the above buffer was charged on the column, and developed with following four buffers at the flow rate of 180 ml/hr; I, 7 mM phosphate buffer (pH 7.90, 0.02 μ); II, 0.067 M phosphate buffer (pH 6.80, 0.13 μ); III, II+0.1 M NaCl (pH 6.50, 0.23 μ); IV, II+0.2 M NaCl (pH 6.30, 0.33 μ). The 15—20% precipitate (1.56 g) was also chromatographed on a column (3×60 cm) with the above buffer system. The fraction eluted with the buffer III by chromatography of the 0—15% precipitate was gel-chromatographed and the fraction eluted at $K_{av}=0.53$ was obtained.[4] This fraction was subjected to preparative polyacrylamide gel electrophoresis and purified TP1 was obtained.

Determination of Partial Specific Volume——The partial specific volume ($\bar{V}$) of TP1 was determined by the usual method[8] using a pycnometer (1.993237 ml). Three parts of the TP1 sample were dissolved in 0.1 M NaCl–0.067 M phosphate buffer (pH 6.47) to the concentrations of 2.0, 2.7 and 4.5 mg/ml and the pycnometer containing the solution was weighed by a micro auto balance. Density of the protein solution at each concentration was calculated from the mean value of three times measurements at 25.00±0.05°. The mean values at three concentrations were plotted against the protein concentrations, and the value of $\bar{V}$, namely partial specific volume, was obtained by extrapolation of the apparent values to zero protein concentration. The partial specific volume of TP1 was also calculated from the amino acid composition in the manner described by McMeekin and Marshall.[9]

Molecular Weight Determination——Molecular weight of TP1 was examined with the aid of a Beckman Spinco model E analytical ultracentrifuge having shlieren optical system by the sedimentation equilibrium method of Yphantis.[10] The purified TP1 was dissolved at a concentration of 0.63 mg/ml in 0.1 M NaCl–0.067 M phosphate buffer (pH 6.47) and centrifuged at 20.36° and 12590 rpm for 24 hr.

Circular Dichroism and Optical Rotatory Dispersion Spectra——The sample was dissolved at a concentration of 0.176 mg/ml in 0.2 M NaCl and 0.01 M phosphate buffer (pH 7.38) and was measured with a JASCO model J-20 automatic recording spectropolarimeter at 21°. The mean residue molecular weight was 109 from amino acid analysis.[4]

Analysis of Sulfhydryl Residues——The sulfhydryl residues in the protein were quantitatively analyzed by the mercury orange method.[11] The solution (1 ml) containing 1.5 mg of TP1 was mixed with 9 ml of acid acetone and the mixture was centrifuged at 2000 rpm for 1 min. The precipitate was suspended in 5 ml of a mixture of mercury orange dissolved in acetone and 0.1 M phosphate buffer at pH 7 (1: 1), and the suspension was allowed to stand at room temperature for 15 min. After centrifugation, the precipitate was washed four times with 7 ml aliquots of acetone and dried, then mixed with 5 ml of acid acetone. The mixture was centrifuged for 5 min at 3000 rpm, and the absorbance of the supernatant was measured at 470 nm and the amount of the sulfhydryl residues in TP1 was calculated from a standard curve. The content of the disulfide residues was estimated from the difference between the sulfhydryl residues and the half-cystine residues from amino acid analysis.

Stability of Activity——Heat stability was studied in the same way as the experiment with LSH.[12] TP1 (0.6 mg) was dissolved in 15 ml of physiological saline and incubated at 56° for 30 min. After standing at room temperature, the solution was used for bioassay. The stability in acidic or alkaline solutions was examined in the same manner as Ito.[13] The sample (0.6 mg) was dissolved in 0.5 ml of HCl (pH 1.4) or NaOH (pH 12.6), and the solution was incubated at 37° for 1 hr. After neutralized, the solution was mixed with 14.5 ml of saline and was used for assay.

Bioassay for Hypocalcemic Activity——Hypocalcemic activity was measured according to the method previously reported.[14] Serum calcium was determined by atomic absorption spectrophotometry. The dose-response relationship for purified TP1 was studied by the method previously reported using the partially purified sample.[4]

Determination of Protein——Protein concentrations in the sample solution were determined by the method of Lowry, *et al.*[15] Analytical disc electrophoresis was done according to the method of Davis.[16]

Results

The results of chromatography of the 0—15% precipitate and the 15—20% precipitate on DEAE-cellulose are shown in Fig. 1a and 1b, respectively. The yield of the active fraction

8) T. Katurai, "Colloid No Riron," Kawaide Shobo, Tokyo, 1951, p. 57.
9) T.L. McMeekin and K. Marshall, *Science*, **116**, 142 (1952).
10) D.A. Yphantis, *Biochemistry*, **3**, 297 (1964).
11) H. Sakai, *Anal. Biochem.*, **26**, 269 (1968).
12) T.D. Luckey (ed.), "Thymic Hormones," University Park Press, Baltimore, 1973, p. 167.
13) Y. Ito, *Ann. N.Y. Acad. Sci.*, **85**, 228 (1960).
14) A. Mizutani, T. Mizutani, and P.-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2733 (1976).
15) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
16) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 409 (1964).

D-3a containing TP1 in Fig. 1a was 6.4% of the total proteins loaded on the column although the fraction was 19% of the total proteins in the effluent. The yield was relatively low, because the 0—15% precipitate was not well dissolved in buffer, but the mean yields were 8—11% in the experiments with other lots. The hypocalcemic rate of the fraction D-3a was 8.80±1.44% in a dose of 0.5 mg/kg and was effective at 5% level of significance. The yield of the active fraction D-3b in Fig. 1b was low and that of the inactive fraction D-2b was high. The ratio of the fraction D-3b to the total proteins in the effluent was 14% though the ratio of the fraction D-3a was 19%. These results showed that the

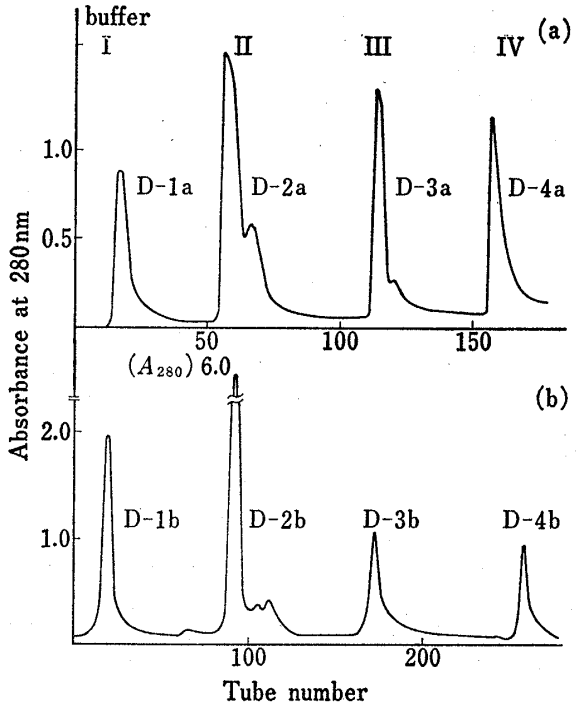

Fig. 1. Elution Patterns ot the Ammonium Sulfate Fractions in Chromatography on DEAE-cellulose

(a) sample, the 0—15% precipitate (9.09 g); column size, 5×122 cm; fraction volume, 110 ml/tube; flow rate, 180 ml/hr. (b) sample, the 15—20% precipitate (1.56 g); column size, 3×60 cm; fraction volume, 20 ml/tube; flow rate, 90 ml/hr. Elution system; buffer I, 7 mM phosphate buffer (pH 7.90, 0.02 μ); II, 0.067 M phosphate buffer (pH 6.80, 0.13 μ); III, II+0.1 M NaCl (pH 6.5, 0.23 μ); IV, II+0.2 M NaCl (pH 6.3, 0.33 μ).

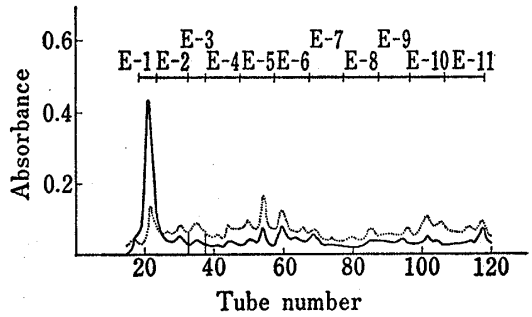

Fig. 2. Preparative Disc Electrophoresis of the Sample Purified by Gel Chromatography

For separation 7.5% polyacrylamide gel was used. Solution of 79.4 mg of the sample in 5 ml of buffer was loaded. Buffer, Tris-glycine (pH 8.6); electrophoretic condition, 30 mA, 450—600 V; apparatus, Toyo Model CD-50; fraction volume, 10 ml/tube; flow rate, 50 ml/hr; solid line, absorbance at 280 nm; dashed line, absorbance at 750 nm by the Lowry method.

Table I. Mobility, Yield, and Hypocalcemic Activity of the Fractions Obtained from Preparative Disc Electrophoresis

Fraction	Relative mobility to BPB	Yield (%)	Percent decrease of serum calcium Mean±S.E. (n=6)[a]
E-1	1.0	1.25	2.25±0.51[b]
E-2	0.85	1.76	2.92±2.05[b]
E-3	0.7	1.25	6.77±1.69[c]
E-4	0.63	2.39	2.00±1.57[b]
E-5	0.55	3.52	3.69±1.79[b]
E-6	0.48	2.89	1.94±1.25[b]
E-7	0.30	1.25	3.58±1.29[b]
E-8	0.20	1.25	2.21±1.26[b]
E-9	0.19	1.51	3.11±1.37[b]
E-10	0.18	1.65	1.66±0.77[b]
E-11	0.17	1.26	1.71±1.61[b]

a) Dose: 0.02 mg/kg of body weight of rabbits.
b) $p>0.05$.
c) Significantly different from control ($p<0.05$).

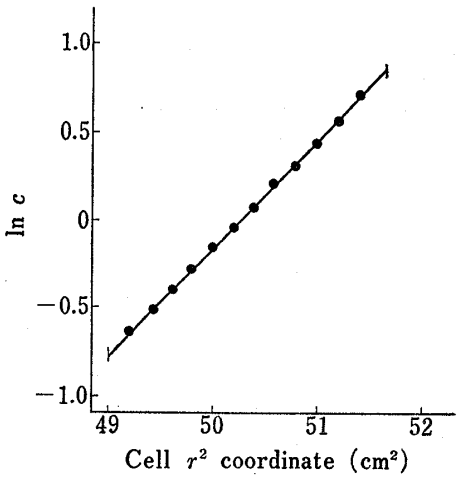

Fig. 3. Sedimentation Equilibrium of Sample

(0.63 mg/ml) in 0.067 M phosphate buffer (pH 6.47) and 0.10 M NaCl at 20.36°, 12590 rpm for 24 hr.

content of the active component in the 0—15% precipitate was higher than that in the 15—20% precipitate. The elution pattern of preparative disc electrophoresis is shown in Fig. 2. Yield, hypocalcemic activity, and relative mobility of the proteins to bromphenol blue (BPB) in Fig. 2 are shown in Table I. Only the fraction E-3, from which TP1 was prepared, was active.

The apparent partial specific volumes at the protein concentrations of 0.2%, 0.27%, and 0.45% were 0.741, 0.732, and 0.735, respectively, and the true value obtained by extrapolation to zero protein concentration was 0.736 which was used in the calculation of molecular weight from the sedimetation equilibrium data. The value calculated from the amino acid composition was 0.729 and this value was coincident with the observed value.

Figure 3 shows the results of the sedimentation equilibrium studies. Protein concentrations in logarithmic scale and the square of the radious were plotted on the ordinate and the abscissa, respectively. Molecular weight of TP1 was calculated to be 66000 from the slope by use of the following equation $Mw=2\boldsymbol{R}T/(1-\bar{V}\rho)\omega^2\times$ dln c/dr^2. Where $\boldsymbol{R}$ is the gas constant, T is the absolute temperature, ω is the angular velocity, V is the partial specific volume (0.736 in the present work), and ρ is the density of the solvent. A straight line in Fig. 3 also indicates a monodisperse system and suggests that the purified TP1 is homogeneous.

The optical rotatory dispersion (ORD) and CD spectra are shown in Figs. 4 and 5, respectively. The α-helix content, calculated from the value of $[\theta]_{208}$ by the equation of Greenfield, was 7%.[17] Using the standard curves of poly (L-lysine) for α-helix, β-structure, and random form, the contents of β-structure and random form were estimated to be 39% and 54%, respectively, and the curve estimated from those contents is also shown in Fig. 5. From these results, the α-helix content of TP1 was relatively low, and hence it was supposed that TP1 had the loosened structure.

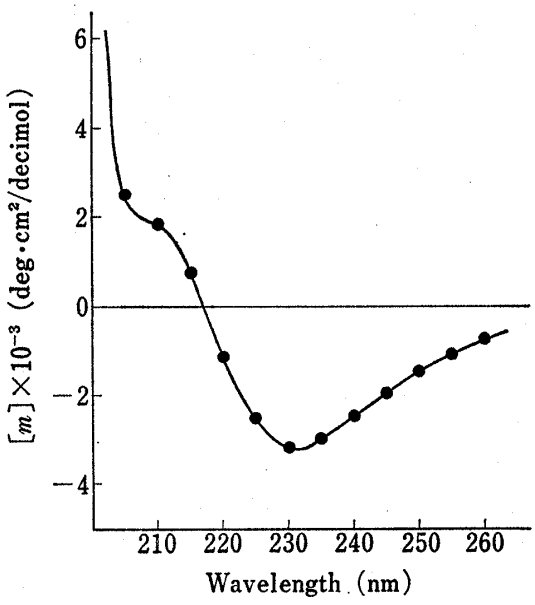

Fig. 4. ORD Spectrum of the Hypocalcemic Factor TP1

The factor was dissolved in 0.2 M NaCl and 0.01 M phosphate buffer (pH 7.38) and measured.

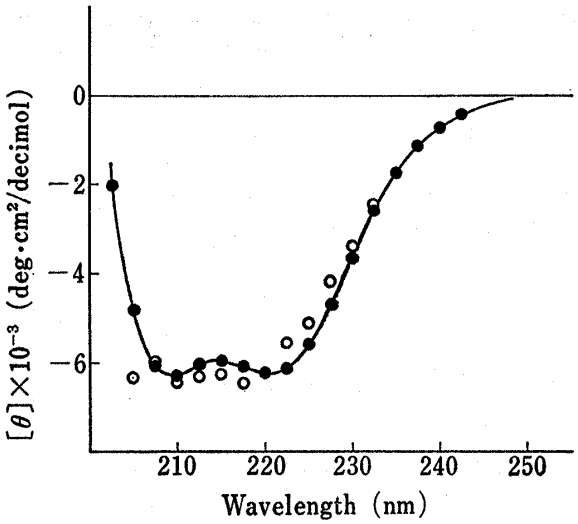

Fig. 5. CD Spectrum of the Hypocalcemic Factor TP1

The factor was dissolved in 0.2 M NaCl and 0.01 M phosphate buffer (pH 7.38) and measured at 21°. Filled circles, the found values; open circles, the computed values based on α-helix=7%, β-structure=39%, and random form=54%.

The sulfhydryl residues in the TP1 protein were determined to be 2.07 mol/mol of protein. The half-cystine residues found in the protein by amino acid analysis were 13.5 mol. Therefore, the disulfide residues in TP1 were estimated to be 5.75 mol. Bovine serum albumin

17) N. Greenfield and G.D. Fasman, *Biochemistry*, **8**, 4108 (1969).

Table II. Stability of Hypocalcemic Activity of TP1

Conditions			Percent decrease of serum calcium Mean±S.E. ($n=6$)[a]
Temperature	pH	Hours	
56°	6	0.5	3.51±1.47[b]
37°	1.4	1	2.28±1.14[b]
37°	12.6	1	1.04±1.21[b]
Untreated	6	—	7.62±1.17[c]

a) Dose: 0.02 mg/kg.
b) $p>0.05$.
c) Significantly different from control ($p<0.01$).

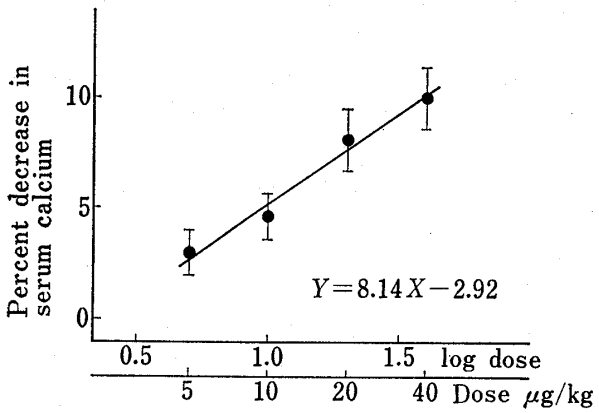

Fig. 6. Dose-response Curve of the Hypocalcemic Factor TP1

Lineality of regression was found from the analysis of variance. Regression coefficient ($b=8.14$) differed from 0 at a significant level of 0.05 in t-table.
⊥●⊥=mean±S.E.
$n=6$.

used for a control experiment contained 0.7 mol sulfhydryl group per mol of protein and this value was coincident with the results of Sakai.[11]

The results of the stability experiments in the various conditions are shown in Table II. By the treatment at 56° for 30 min, the activity was destroyed, and thereby TP1 appears to be a heat labile substance. By treatment of TP1 in acidic or alkaline solution at 37° for 1 hr, the activity was also destroyed.

The dose-response relationship of purified TP1 is shown in Fig. 6. In the previous paper, the relationship was examined using the partially purified TP1, which was prepared by gel filtration. The curve shows that the regression of hypocalcemic response on log dose may be effectively linear over a range of 5—40 μg/kg.

Discussion

We purified TP1 from the 0—15% precipitate or the 0—20% precipitate as reported previously.[4] The apparent yield of the 0—20% precipitate was higher than that of the 0—15% precipitate; hence it was considered that the active component may be obtained in better yield from the 0—20% precipitate than from the 0—15% precipitate. However, the conclusive proof for the superiority of the 0—20% precipitate as a material for purification of TP1 was lacking. In this paper, we compared the merits of two precipitates as the raw materials for further purification. In chromatography of the 15—20% precipitate on DEAE-cellulose, the yield of the active fraction was low and that of the other inactive fractions was high. Meanwhile, the yield of the active fraction in the 0—15% precipitate was higher than that in the 15—20% precipitate. Therefore, in order to exclude the inactive proteins contained in the raw materials for chromatography on DEAE-cellulose, the 0—15% precipitate was better for purification than the 0—20% precipitate, even if the apparent yield of the 0—15% precipitate was low. In preparative disc electrophoresis, the hypocalcemic active fraction did not disperse but moved as a single protein.

Molecular weight of TP1 purified from thymus was 68000 by SDS polyacrylamide gel electrophoresis, the sample gave single band in SDS electrophoresis and in disc electrophoresis,

and its mobility did not change after incubation in 8M urea.[4] The behavior of TP1 by gel filtration on Sepharose 6B (Kav=0.53) suggested that the apparent molecular weight was extremely larger than 68000. However, we confirmed by the sedimentation experiments that TP1 was not composed of subunit and TP1 in solution of the native condition existed as a protein of the molecular weight 66000. From these results, TP1 may have a fairly large dissymmetric shape and has the relatively loose structure by the CD spectrum. The α-helix content of the bovine parotid hypocalcemic protein was 54% and higher than that of TP1.[14]

In the previous work, the time course of TP1 was investigated in a dose of 5 μg/kg,[4] and in this work it was demonstrated that the dose was minimum effective from the dose-response curve of TP1. By the experiments of heat stability, TP1 was more labile than LSHr and thymosin, and the stability was similar to that of LSHh although LSH and thymosin were assayed for lymphocytopoietic activity and TP1 was for hypocalcemic activity. Meanwhile, in comparison with the bovine parotid hypocalcemic protein, the hypocalcemic protein was heat stable in contrast to TP1.[13] TP1 was inactivated by incubation in acidic or alkaline solution, whereas the parotid hypocalcemic protein was stable at pH 12.6 and inactivated at pH 1.4. From these results, it was concluded that TP1 was labile on heating and treatment in acidic or alkaline solution.

During preservation of the preparations of TP1 for long time in a vacuum desiccator at room temperature, the samples showed a tendency of decrease in solubility in a buffer solution. It is considered that air oxidation of the sulfhydryl residues in TP1 may play a role in this phenomenon. Furthermore, the loose structure of this protein might be an additional reason.

Acknowledgement The authors are grateful to Dr. T. Totsuka for CD measurement, to Mrs. K. Hayashi for ultracentrifugation and to Mr. I. Suzuki for his continuing interest and encouragement.