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Sequence Determination of Amino- and Carboxyl-Terminal Residues of Hypocalcemic Protein TP_1 extracted from Bovine Thymus Gland[1]

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End group amino acid sequence was determined on a hypocalcemic protein TP_1 purified from bovine thymus gland. Amino-terminal amino acid sequence was deduced to be His-Asx-Ala-Glx- by the dansyl-Edman degradation method. Carboxyl-terminal amino acid sequence was analyzed by digestion with carboxypeptidase A and Y, and it was deduced to be -Thr-Ala-Lys. Furthermore, the carboxyl-terminal amino acid was confirmed to be lysine alone by the hydrazinolysis method. According to these results and from previous data, it became apparent that TP_1 did not bear the subunit structure, and it was clearly different from other hypocalcemic proteins obtained from bovine thymus and parotid gland.

Keywords——bovine thymus gland; TP_1; hypocalcemic protein; dansyl-Edman degradation; amino-terminal sequence; carboxypeptidase A; carboxypeptidase Y; hydrazinolysis; carboxyl-terminal sequence

Recently, we have isolated two hypocalcemic proteins, TP_1 and TP_2, from the saline extract of bovine thymus gland using fractionation with ammonium sulfate, diethylaminoethyl (DEAE)-cellulose chromatography, gel filtration, and preparative disc electrophoresis.[3] Both TP_1 and TP_2 showed a single band by analytical disc electrophoresis, and their electrophoretic mobilities were 0.68 and 0.48, respectively. In addition to the hypocalcemic effect, we previously reported that these proteins had stimulated lymphocytes and increased plaque-forming cells in mice.[4] The molecular weight of TP_1 was determined to be about 68000 by sodium dodesyl sulfate (SDS)-polyacrylamide gel electrophoresis and the protein consisted of about 620 amino acid residues.[3c] Examinations by SDS polyacrylamide gel electrophoresis after reduction with β-mercaptoethanol, sedimentation equilibrium, and gel filtration suggested that TP_1 did not bear a subunit structure.[5] The present paper reports the determination of its terminal amino acid sequence, and comparison of TP_1 with other hypocalcemic proteins purified from bovine parotid gland.

Materials and Methods

Materials——The hypocalcemic protein TP_1 was purified from bovine thymus gland according to the method previously reported,[3b,c] with a little modification, and the procedure is outlined as follows: Thymus gland from cattle of 12—18 months of age was extracted with saline at pH 8.0, and $(CH_3)_2CO$-dried powder was prepared from the extract by the addition of $(CH_3)_2CO$ to give 60% (w/w) concentration of $(CH_3)_2CO$ at pH 5.4. This powder was extracted with saline at pH 8.0, followed by fractionation with $(NH_4)_2SO_4$, and the fraction precipitated at 0—15% (w/w) concentration of $(NH_4)_2SO_4$ was obtained in a yield of about 0.7% from the fresh gland. This product was chromatographed over DEAE-cellulose, and fraction DE-III was eluted with 0.067 M phosphate buffer containing 0.1 M NaCl (pH 6.50, $\mu=0.23$) in about 7.2% yield from the $(NH_4)_2SO_4$ precipitate. The fraction DE-III was chromatographed over Ultrogel AcA-22 (LKB Products,

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Sweden) and fraction G-2 was eluted at Kav value of 0.5—0.8 in about 38.5% yield from DE-III. Fraction G-2 was further purified by preparative disc electrophoresis, using 7.5% polyacrylamide gel (column size, 7.5×7.0 cm). On the anode side of gel column, 50 mg of the fraction dissolved in 5 ml of 40% sucrose solution was applied and run at 600 V for about 8 hr. After electrophoresis, the gel column was sliced and the slices containing the protein band with electrophoretic mobility of 0.68 against Bromophenol Blue (BPB) as a marker were collected. After extraction of these gel slices with distilled water, contaminated gel particles were removed by DEAE-cellulose chromatography and lyophilized TP_1 fraction was obtained. The fraction TP_1 showed a single band in analytical disc electrophoresis and its yield was about 12% of the fraction G-2.

Analysis of Amino-terminal Sequence——Amino-terminal sequence was analyzed by the method of Weiner, *et al.*,[6] and dansyl-Edman degradation involving the procedure of Gray and Hartly.[7] Standard dansylated amino acids were prepared according to Weiner, *et al.*[6] After dansylation of 3 mg of TP_1 fraction, the reaction product was hydrolyzed, and the resulting dansylated amino acid was spotted on a polyamide layer plate (Cheng Chin Trading, Hong Kong) and the known dansylated amino acids were spotted on the opposite side of the plate. The plates were chromatographed with the solvent systems of (a) 1.5% formic acid in H_2O, (b) C_6H_6–CH_3COOH (9:1, v/v), or (c) $CH_3COOC_2H_5$–CH_3COOH–MeOH (20:1:1, v/v/v). Solvents (b) and (c) were run in the same direction perpendicular to solvent (a). The spots of dansylated amino acids were detected under ultraviolet ray (254 nm).

Analysis of Carboxyl-terminal Sequence by Carboxypeptidase——Carboxyl-terminal sequence was first analyzed using carboxypeptidase A according to the procedure of Frankel-Conrat, *et al.*[8] Carboxypeptidase A [EC 3.4.2.1] (CPase A-DFP, 20 mg/ml suspension) was purchased from Sigma (U.S.A.). A suspension of 0.005 μM of the enzyme (20 μl) was mixed with 13.984 mg (0.21 μM) of the sample dissolved in 5 ml of H_2O at pH 8.0 and the final volume was made up to 6 ml. After incubation of this solution at 25° for 0, 0.5, 1, 2, or 4 hr, the digestion was successively stopped by adding 0.1 ml of 1 M citric acid, and the amino acid fraction was obtained by gel filtration on Sephadex G-10, followed by analysis of the amino acids with Hitachi KLA-3B automatic amino acid analyzer.

Later, carboxypeptidase Y [EC 3.4.12] (Oriental Yeast Co., Osaka) was employed for the digestion.[9] The enzyme (0.20 mg, 0.003 μM) was added to 5 ml of the sample solution (12.823 mg, 0.18 μM) at pH 5.5 and the final volume was made up to 6 ml. This solution was incubated at 25° for 0, 1, 2, 4, or 8 hr in the presence of 6 M urea. The digestion was terminated by the addition of Dowex 50W-12X (H^+ form) to the solution with shaking until pH of the supernatant was 2.5. The absorbed amino acids were eluted with 5 M NH_4OH according to the procedure of Ambler,[10] and subjected to amino acid analysis.

Analysis of Carboxyl-terminal Amino Acid by Hydrazinolysis.[11] The fraction TP_1 (1.2 mg) was hydrazinolyzed with 0.5 ml of anhydrous N_2H_4 at 65° for 9 hr in the presence of 65 mg of $N_2H_4 \cdot H_2SO_4$ as a catalyst. The liberated carboxyl-terminal amino acid was determined as in the case of dansylated amino acid according to the procedure of Seiler, *et al.*[12] after the amino acid hydrazide was removed.

Assay for Hypocalcemic Activity——The assay was the same as in our previous report.[3a] Six male rabbits of more than 2.0 kg in body weight were used as one group. The rabbits were fasted for 24 hr before the experiment and with the sample dissolved in saline was injected into their aural vein. The control rabbits were injected with saline. Blood was drawn from the aural vein before the injection, and 4, 5, and 6 hr after the injection. The amount of calcium in 0.1 ml of serum was determined with Shimadzu AA-610 atomic absorption spectrophotometer at 422.7 nm.

The rate of fall in serum calcium after the injection was calculated and the average of the maximum values of the 3 rates of fall was taken as the hypocalcemic rate. The difference in the hypocalcemic rate between the experimental and control groups was examined by the *t*-test and the value was considered effective when the difference was significant at below 5% level of significance.

Analytical Polyacrylamide Gel Electrophoresis——The purity of proteins was examined by polyacrylamide gel (7.5%) disc electrophoresis according to Davis' method,[13] at a current of 3 mA/gel column in the tris(hydroxymethyl)aminomethane glycine buffer (pH 8.3, $\mu=0.05$). After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250 (Sigma, U.S.A.), and were scanned at 610 nm with a autodensitometer Model FDA-A-IV (Fuji Riken, Tokyo).

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Determination of Proteins——Concentration of proteins was determined by measuring its absorption at 280 nm or by the method of Lowry, *et al.*,[14] with bovine serum albumin as a standard.

Results and Discussion

Hypocalcemic activity of the fraction at each step of the purification procedure is shown in Table I. The fraction TP_1 was homogeneous as judged by analytical disc electrophoresis (Fig. 1) and by electrofocusing, and its isoelectric point was estimated to be pH 5.5. The hypocalcemic activity of the purified TP_1 was similar to that of the product previously reported.[5]

Table I. Hypocalcemic Activity of the Fractions obtained from the Each Step of Purification

Sample	Dose (mg/kg)	Decrease (%) (mean ± standard error)
Acetone-dried powder	10.0	8.90±0.90[a]
15% $(NH_4)_2SO_4$ fraction	1.0	14.80±2.20[a]
DE-III	0.5	9.90±1.85[a]
G-2	0.1	8.59±1.49[a]
TP_1	0.02	6.10±0.56[b]

Significantly different from control, (*a*) $p<0.01$, (*b*) $p<0.05$).

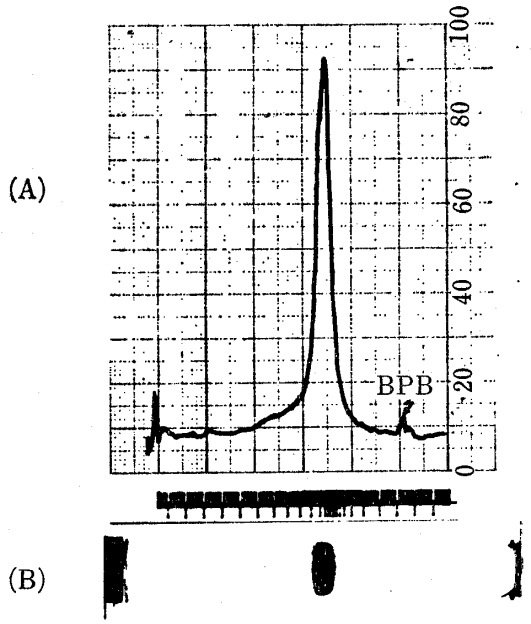

Fig. 1. (A) Densitometric Tracing of Disc Electropherogram of purified TP_1 (λ=610 nm)
(B) Polyacrylamide Gel Electrophoretic Pattern of TP_1 purified from Bovine Thymus Gland

Amino-terminal amino acid of TP_1 fraction was analyzed as dansyl-histidine. Fig. 2 shows polyamide layer chromatogram obtained after dansyl-Edman degradation. In the first Dansyl-Edman degradation, dansyl-aspartic acid and dansyl-alanine were detected on the chromatogram and the second degradation resulted in the dansyl-alanine and dansyl-glutamic acid, and thirdly dansyl-glutamic acid. On the fourth degradation, however, dansylated amino acid was not detected. For the reason of this failure, it is considered that the amount of TP_1 fraction decreased gradually with progress of the degradation cycle and that removal of diphenylthiourea of reaction by-product was not complete. According to Blombäck, *et al.*,[15] amino terminal histidine was sometimes cloven into histidyl-dipeptide by the Edmän degradation, hence TP_1 was broken into two peptides with different terminal amino acid. Therefore, the third amino acid from amino-terminal was detected in addition to the second amino acid on the second chromatogram, and the same result was occurred on the third chromatography. Dansyl-Edman degradation cannot distinguish aspartic acid and glutamic acid from asparagine and glutamine, respectively. For the distinction of whether these amino acids are amino type or free type, it must be identified as the amino acid phenylthiohydantoins (PTH-amino acid) cleaved, thus we tentatively represented those as Asx and Glx, respectively. Consequently, amino terminal sequence of TP_1 was deduced to be His-Asx-Ala-Glx-. In our preliminary experiment, sodium carbonate buffer was used as the reaction solvent according to Weiner, *et al.*,[6] and they determined the amino terminal sequence

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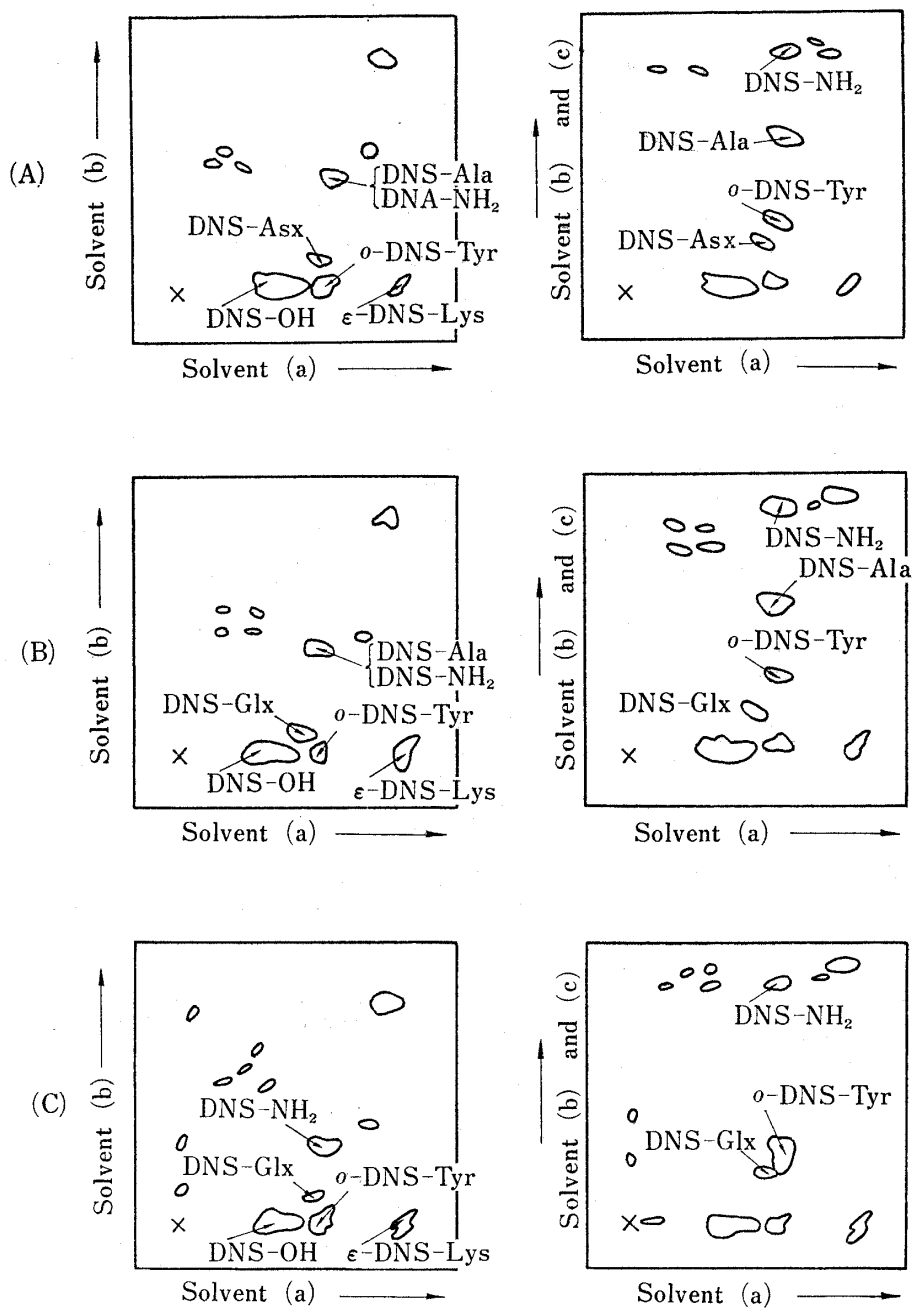

Fig. 2. Chromatography of the Dansylated (DNS) Amino Acid obtained from Edman Degradation of TP_1

(A) Chromatography after 1st Edman Degradation.
(B) Chromatography after 2nd Edman Degradation.
(C) Chromatography after 3rd Edman Degradation.

of 5 to 19 residues for any proteins, but for TP_1 only one amino acid of amino terminous was determined because TP_1 was hardly soluble in this buffer. Later, we employed N-ethylmorpholine-pyridine buffer for the reaction according to Gray and Hartly.[7a] This solvent enabled high molecular protein, such as TP_1, to dissolve.

Fig. 3 shows the time course of amino acids liberated from TP_1 by digestion with carboxypeptidase A. Lysine was liberated as the first amino acid and alanine as the second from the carboxyl-terminus, but these profiles show unsatisfactory digestion, and the results might be due to short digestion time and by substrate specificity of this enzyme. On the other hand, Fig. 4 shows the time course of TP_1 digested with carboxypeptidase Y, which has a limited substrate specificity. From these graphs, lysine was found to be the first amino acid liberated,

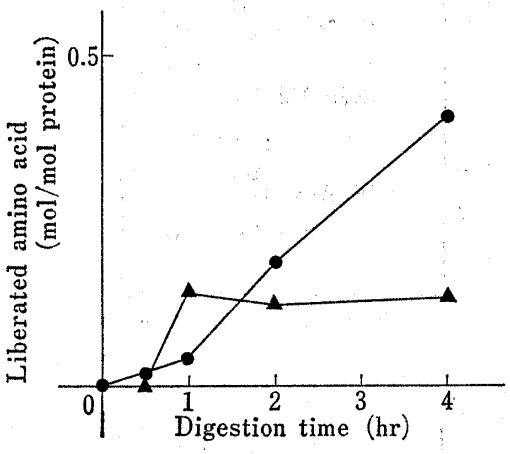

Fig. 3. Time Course of Amino Acids liberated from TP_1 digested with Carboxypeptidase A

● lysine, ▲ alanine.

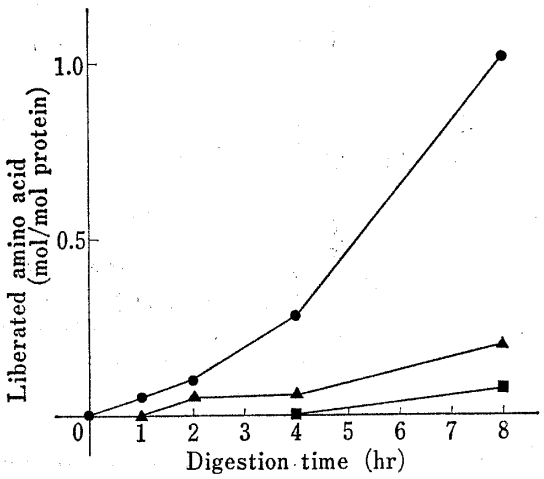

Fig. 4. Time Course of Amino Acids liberated from TP_1 digested with Carboxypeptidase Y

● lysine, ▲ alanine, ■ threonine.

and alanine and threonine might follow as the second and third, respectively. The carboxyl-terminal dansylated amino acid obtained from hydrazinolysis was detected as dansyl-lysine only. Thus carboxyl-terminal sequence of TP_1 was deduced to be -Thr-Ala-Lys. On the basis of these results and that previously reported,[3c,5] it was revealed TP_1 did not bear subunit structure.

Table II. Comparison of Hypocalcemic Proteins purified from Bovine Thymus Gland or Bovine Parotid Gland

Protein	Molecular weight[a]	Relative electrophoretic mobility[b]	Amino acid residue	N-Terminal sequence	C-Terminal sequence	Hypocalcemic activity dose (mg/kg) Mean±S.E.
TP_1	68000	0.68	620	His–Asx–Ala–Lys–	–Ala–Lys	0.02 6.10±0.56[d]
TP_2	57000	0.48	450	Unknown	Unknown	0.05 6.29±1.41[d]
Hypocalcemic protein from parotid gland[c]	48000	0.28	395	Lys–Leu–	–Val–Leu	0.03 6.50±0.62[d]

a) Determined by SDS-polyacrylamide gel electrophoresis.
b) Calculated against that of BPB.
c) Cited from Mizutani, *et al.*[16a]
d) Significantly different from control, ($p<0.05$).

Table II shows the comparison of TP_1, TP_2, and the hypocalcemic protein which was purified from bovine parotid gland.[16] These data indicate that TP_1 is clearly different from other products.

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