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Molecular Weight Determination and the Secondary Structure of the Hypocalcemic Protein purified from Bovine Parotid Gland

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Molecular weight determinations were carried out on a hypocalcemic protein purified from bovine parotid gland by sedimentation equilibrium, gel chromatography on Sepharose 6B in 6 M guanidine hydrochloride (Gun HCl), and viscometry in 6 M Gun HCl 0.1 M mercaptoethanol, and the values obtained were 45000, 47000, and 45700, respectively. These results agreed well with the result (48000) from sodium dodecyl sulfate polyacrylamide gel electrophoresis. The optical rotatory dispersion and circular dichroism spectra of the purified hypocalcemic protein showed that the contents of α-helix, β-structure, and random coil were 54, 26, and 20%, respectively. It was found that this protein contains large amounts of α-helix and thereby has a rigid structure.

Ogata found a biologically active factor in bovine parotid gland which lowered serum calcium levels in rabbits.[2,3] Mizutani, *et al.* have recently purified a hypocalcemic protein from the gland by isoelectric precipitation at pH 5.4, fractional precipitation with ammonium sulfate, column chromatography on diethylaminoethyl (DEAE)-cellulose, gel chromatography on Sepharose 6B, preparative polyacrylamide gel electrophoresis, and second gel chromatography on Sepharose 6B, resulting in a single band by analytical disc electrophoresis.[4] In addition to the hypocalcemic action in rabbits, this substance was found to have a lymphocyte-increasing action in mice.[5] In an amino acid analysis, this protein showed large content of aspartic acid, glutamic acid, and leucine, small content of cystein, histidine, and tryptophan as in other proteins. The characteristic feature was a comparatively small amounts of proline. It was also reported that its isoelectric point was pH 5.3 and its molecular weight was 48000 from sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis.

The value of molecular weight, 48000, differed considerably from 132000 calculated previously from the sedimentation constant and the diffusion constant by Ito, *et al.*[6] Investigation was, therefore, undertaken to clarify the cause of this discrepancy. For that, molecular weight of the hypocalcemic protein was determined by sedimentation equilibrium, gel chromatography on Sepharose 6B in 6M guanidine hydrochloride (Gun HCl) and measurements of the viscosity in 6M Gun HCl. We also report in this paper the content of α-helix, β-structure and random coil calculated from the optical rotatory dispersion (ORD) and circular dichroism (CD) spectra.

Materials and Methods

Materials——The hypocalcemic protein was purified from bovine parotid gland according to the method of Mizutani, *et al.*[4] All the fractionation procedures were carried out in a cold room at about 5° or under ice cooling, and toluene was added to the buffer solutions as a preservative. Following the extraction of

1) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
2) A. Ogata, Y. Ito, and S. Okabe, *Yakugaku Zasshi*, **64**, 79 (1944).
3) Y. Ito, *Ann. N. Y. Acad. Sci.*, **85**, 228 (1960).
4) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).
5) I. Suzuki, H. Inaba, Y. Naito, T. Mizutani, ann A. Mizutani, *J. Biochem.* (Tokyo), **78**, 697 (1975).
6) Y. Ito, Y. Kubota, and Y. Shibuya, *J. Biochem.* (Tokyo), **47**, 422 (1960).

bovine parotid gland with water (pH 8.0), an acetone dried powder was prepared from the aqueous extract through the precipitation at pH 5.4. The powder was extracted with saline, and the extract was fractionated with ammonium sulfate. The fraction precipitated at 7—15% of ammonium sulfate concentration (w/w) was obtained in a yield of about 5% from the acetone-dried powder. The 7—15% ammonium sulfate fraction was chromatographed on DEAE-cellulose and the fraction eluted with the solution of 0.27 M NaCl in 0.05 M phosphate buffer (pH 7.38 $\mu=0.13$) was obtained. This fraction was chromatographed on Sepharose 6B-(Pharmacia) and the fraction eluted at $K_{av}=0.3$ was obtained. The fraction ($K_{av}=0.3$) was further purified by preparative polyacrylamide gel electrophoresis. Fractions giving a single band and having an identical relative mobility were pooled, and loaded on a column of Sepharose 6B to remove the contaminants of fine gel-particles. The protein purified by this method was used for the following experiments and significantly lowered the serum calcium concentration by 6.50 ± 0.62% in a dose of 0.03 mg/kg in rabbits on bioassay of hypocalcemic activity which is described below.

Molecular Weight Determination by Sedimentation Equilibrium——Molecular weight determination was carried out by the Yphantis sedimentation equilibrium method[7] with a Hitachi model UCA analytical ultracentrifuge. The purified material was dissolved at a concentration of 0.49 mg/ml in 0.15 M NaCl and 0.05 M phosphate buffer. After the centrifugation at 20°, 28700 rpm for 16 hr, the pattern was recorded by Rayleigh interference.

Molecular Weight Determination by Gel Chromatography on Sepharose 6B in 6 M Gun HCl——Gel chromatography was performed on Sepharose 6B in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 8.1) containing 6 M Gun HCl and 0.02 M mercaptoethanol at room temperature by the method of Fish. *et al.*[8,9] A distribution coefficient K_d defined as $K_d=(V_e-V_o)/(V_i-V_o)$ was calculated. Void volume (Vo) of the column was measured by use of Blue dextran 2000 (Pharmacia). Inosine-5′-phosphate was used as a marker for the internal volume (V_i) of the column. V_e was the elution volume of the protein. The proteins used for standards were bovine serum albumin (M.W. 66000), ovalbumin (M.W. 46000), chymotrypsinogen A(M.W. 25741), and myoglobin (M.W. 17600). The hypocalcemic protein and proteins for standards were dissolved at a concentration of 20 mg/ml in 0.1 M Tris-HCl buffer (pH 8.1) containing 6 M Gun HCl and 1 M mercaptoethanol and treated at 30° for 48 hr in the dark. The solution (0.2 ml) treated as above was loaded on a column (0.95×55 cm) and fractions of 0.45 ml were collected. The elutions of blue dextran and proteins were followed by the measurement of absorbance at 630 nm and 280 nm, respectively. The molecular weight of the sample treated in the absence of mercaptoethanol, was also estimated on the same column in the buffer without mercaptoethanol.

Molecular Weight Determination by Measurements of the Viscosity——Molecular weight determination by viscometry was carried out by the method of Tanford, *et al.*[10,11] with Ostwald microviscometer, flow time was 157 second for water, at 20.0 ±0.1° in the solution containing 6 M Gun HCl, 0.1 M mercaptoethanol, and 0.1 M Tris–HCl buffer(pH 7.2).

The concentrations of sample were 1.632 mg/ml, 1.224 mg/ml, 0.816 mg/ml and 0.408 mg/ml of the buffer. The concentrations of bovine serum albumin used as a standard were 2.775 mg/ml, 2.044 mg/ml, and 1.363 mg/ml.

The intrinsic viscosities (η) were determined by linear extrapolation of reduced viscosity obtained from these protein concentrations to zero concentration. (η) was expressed in the following equation[10] $(\eta)=0.716\,n^{0.66}$ and the number of average residues (n) was calculated. The molecular weight of this sample was estimated from the n value and the mean residue weight which was obtained by amino acid analysis.

Optical Rotatory Dispersion and Circular Dichroism——The sample was dissolved at a concentration of 0.204 mg/ml in 0.2 M NaCl and 0.01 M phosphate buffer (pH 7.3) and was measured with a JASCO J-20 automatic recording spectropolarimeter at 20°. For CD spectra, polyglutamic acid (0.286 mg/ml) and lysozyme (0.15 mg/ml) was used as standard.

Bioassay——This was performed as reported previously.[12] Six male rabbits of more than 2 kg body weight were used as one group. The rabbits were deprived of diet for 1 day prior to the experiments and were injected into their aural vein with the sample dissolved in physiological saline in a dose of 0.5 ml/kg of the body weight. The control rabbits received an injection of physiological saline. Blood was drawn from the aural vein before the injection and 4, 5, and 6 hr after the injection. After 10 ml of 1000 ppm strontium solution was added to 0.1 ml of serum, the amounts of calcium in the solution was determined with a Shimazu AA-610 atomic absorption spectrophotometer at 422.7 nm.

The rate of fall of serum calcium after the injection compared with that before the injection was calculated and the average of the maximum values of the 3 rates of fall was taken as a hypocalcemic rate.

7) D.A. Yphantis, *Biochemistry*, **3**, 297 (1964).
8) W.W. Fish, K.G. Mann, and C. Tanford, *J. Biol. Chem.*, **244**, 4989 (1969).
9) C. Courte and J. Willemot, *J. Biol. Chem.*, **247**, 4429 (1972).
10) C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.*, **89**, 729 (1967).
11) J.C. Lee and R.P. Frigon, *J. Biol. Chem.*, **248**, 7253 (1973).
12) A. Mizutani, K. Yamamoto, H. Sato, Y. Saito, H. Kitsugawa, and H. Yamamoto, *Yakugaku Zasshi*, **91**, 297 (1971).

The difference in the hypocalcemic rate between the experimental group and the control group was examined by the t-test and the value was considered effective when the difference was significant at below 5% level of significance.

Determination of Protein——Protein concentration in the sample solutions was estimated by the method of Lowry, *et al.*[13] and bovine serum albumin was used as a standard. Folin phenol reagent was added to the sample solution and allowed to stand for 1 hr and then optical density of the solutions was measured at 750 nm against a blank solution.

Results

Molecular Weight Determination by Sedimentation Equilibrium

Figure 1 shows the results of the sedimentation equilibrium studies. The protein concentrations with a logarithmic scale and the square of the radius were plotted on ordinates and abscissae, respectively. A linear relationship of In(c) *versus* r^2 was obtained and indicated a monodisperse system. The slope (dIn/dr^2) was calculated to be 1.94 from the figure, and molecular weight was evaluated by the equation $Mw=2RT/(1-\bar{V}\rho)\omega^2 \times dInc/dr^2$, resulting in 45000. In this equation, R is the gas constant, T is the absolute temperature, ω is the angular velocity, $\bar{V}$ is the partial specific volume of the protein and ρ is the density of the solvent. The value employed for $\bar{V}$ was 0.753 which was measured previously by Ito, *et al.*[6] with 7--12% ammonium sulfate fraction.

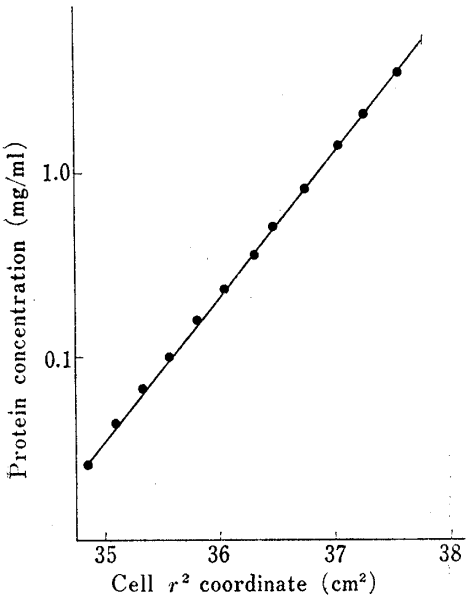

Fig. 1. Sedimentation Equilibrium of Sample (0.49 mg/ml) in 0.05M Phosphate Buffer (pH 7.3) and 0.15M NaCl at 20°, 28700 rpm for 16 hr

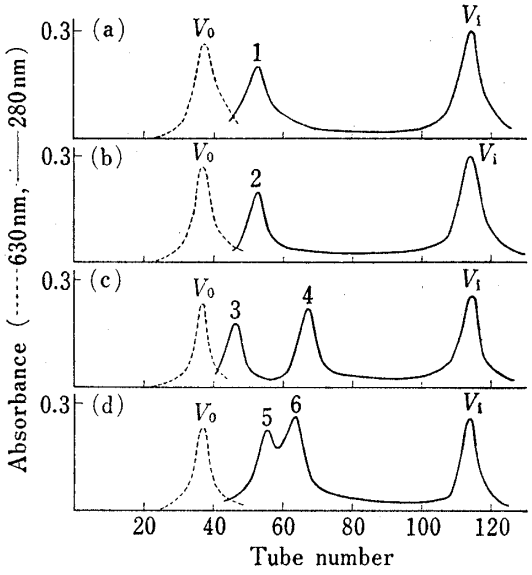

Fig. 2. Elution Patterns by Gel Chromatography on Sepharose 6B in 6M Gun HCl

The same column (0.95×55 cm) was used and fractions (0.45 ml) were collected throughout. (a), the hypocalcemic protein (1); (b), the hypocalcemic protein (2)+mercaptoethanol; (c), bovine serum albumin (3)+myoglobin (4); (d), ovalbumin (5)+chymotrypsinogen A (6).

Molecular Weight Determination by Gel Chromatography on Sepharose 6B in 6M Gun HCl

Figure 2 shows the elution patterns by gel chromatography on Sepharose 6B in 6M Gun HCl. Four experiments were performed on the same column. Fig. 2a and 2b show in the absence and the presence of mercaptoethanol in the solution and the both patterns are identical. In Fig, 2c and 2d, each of peaks shows standard proteins.

13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

Figure 3 shows the calibration curve of molecular weight of the protein in the terms of semi-logarithmic plot of molecular weight *versus* K_d. The plots of standard proteins show a good straight line. The K_d value of sample is near to that of ovalbumin and its molecular weight was 47000 in both the cases of intact and reducing state.

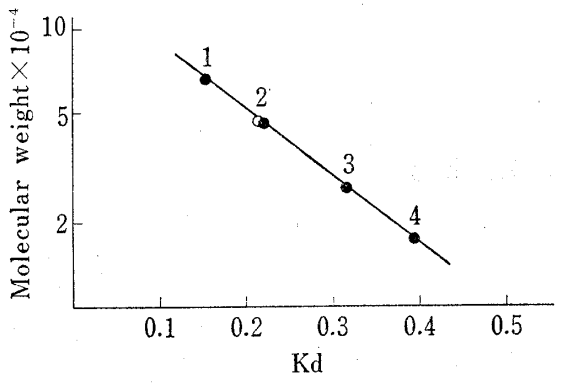

Fig. 3. Molecular Weight Determination by Gel Chromatography on Sepharose 6B in 6M Gun HCl

Calibration curve in terms of semilogarithmic plots of molecular weight *versus* Kd was obtained for the column operated in the presence of mercaptoethanol. The hypocalcemic protein is represented by ○. The protein standards are ●, and their molecular weights are: 1, bovine serum albumin (66000); 2, ovalbumin (46000); 3, chymotrypsinogen A (25700); 4, Myoglobin (17600).

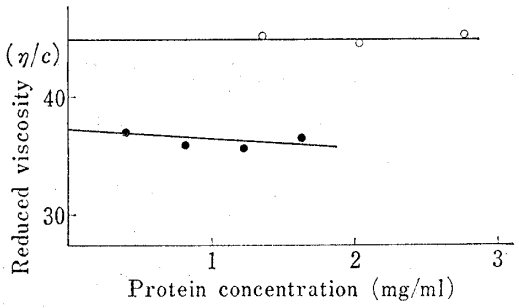

Fig. 4. Reduced Viscosity *versus* Concentration Curve for Protein in 6M Gun HCl (pH 7.2)

The symbols for the samples are: ●—●, the hypocalcemic protein; ○—○, bovine serum albumin.

Molecular Weight Determination by Measurements of the Viscosity

The reduced viscosity was plotted against the protein concentrations as shown in Fig. 4. Intrinsic viscosity (η) of this protein was found on the Fig. 4 to be 37.0. The number of average residues (n) was calculated by the equation $(\eta)=0.716n^{0.66}$ to give 394. Combining this value 394 with the known mean residue weight of 116, which was obtained from amino acid analysis,[4] resulted in a molecular weight of 45700. The molecular weight of bovine serum albumin from the result of $n=529$ and the mean residue weight 124 was 65000.

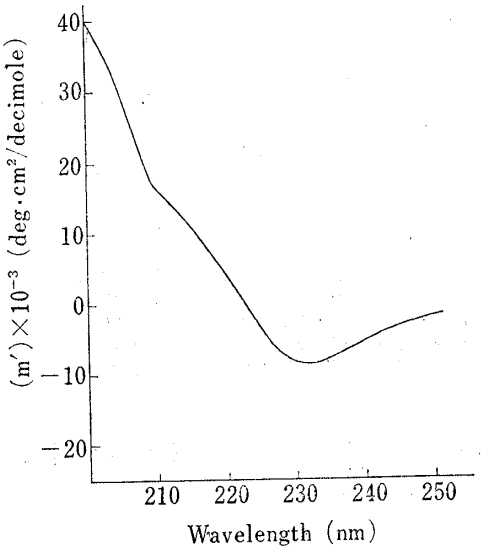

Fig. 5. ORD Spectrum of the Hypocalcemic Protein

The hypocalcemic protein was dissolved in 0.2M NaCl and 0.01M phosphate buffer (pH 7.3) and measured at 20°.

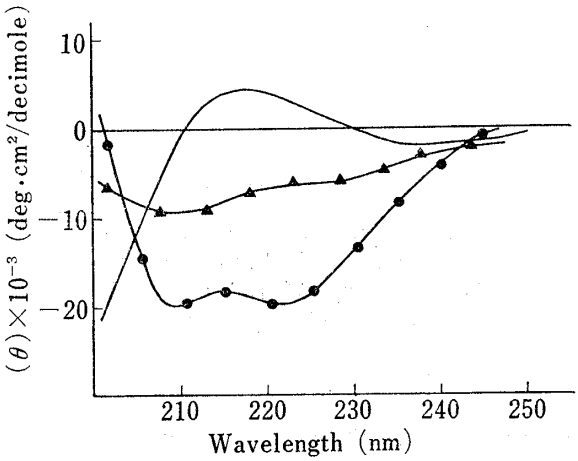

Fig. 6. CD Spectra of the Hypocalcemic Protein

The proteins were dissolved in 0.2M NaCl and 0.01M phosphate buffer (pH 7.3) and measured at 20°. The symbols for the sample are: ●—● the hypocalcemic protein; ▲—▲, lysozyme; —— poly-glutamic acid.

Optical Rotatory Dispersion and Circular Dichroism

Figures 5 and 6 show ORD and CD spectra, respectively.

Using Moffit equation, α-helix content was calculated to be 56% from ORD spectrum. In CD spectrum of the sample, the value of $(\theta)_{208}$ (−19600 deg. cm²/decimole) was applied to the following equation,[14] α-helix content $=(\theta)_{208}-(-4000)/-33000-(-4000)$ and it was found to be 54%. When the value of $(\theta)_{222}$ was also used,[15] its α-helix content was similar to above results. For estimation of β-structure and random coil, CD spectra of synthetic poly (L-lysine) by Greenfield, *et al.*[14] was used and the contents were about 26% and 20%, respectively.

Discussion

The results of the molecular weight measurements are summarized in Table I. Molecular weight of the hypocalcemic protein, which was isolated from bovine parotid gland, was 45000

Table I. Summary of Molecular Weight Determinations

Method	Molecular weight
SDS polyacrylamide gel electrophoresis in 1.0 % SDS	47000[a]
+1% mercaptoethanol	48000[a]
Sedimentation equilibrium	45000
Gel chromatography on Sepharose 6B in 6M Gun HCl	47000
+0.02M mercaptoethanol	47000
Viscometry in 6M Gun HCl	45700

a) The results obtained by Mizutani, *et al.*[4]

by sedimentation equilibrium, 47000 by gel chromatography on Sepharose 6B in 6M Gun HCl. Since these results agreed with our previous result 48000 by SDS polyacrylamide gel electrophoresis,[4] it can be seen that the molecular weight of the hypocalcemic protein did not change with the methods used and on the state of the di-sulfide bridges in the protein. The average value of the molecular weight was 46500±1500. The molecular weight of the protein sample precipitated at 7—12% of ammonium sulfate concentration was determined previously to be 132000[6] by the centrifugal sedimentation velocity as described above. One of the causes may be due to that their preparation was inferior to our preparation in purity, although theirs showed a single peak by Tiselius electrophoresis. Furthermore, it was considered that the error might become large when both the curves of sedimentation and diffusion were extrapolated to zero concentration owing to the rapid rise in them at low concentration of the protein. The K_{av} value of purified sample was 0.3 by gel chromatography on Sepharose 6B, and apparent molecular weight from that value was about 500000 using a standard curve of globular proteins. However, the average molecular weight obtained as above was 46500± 1500, and this value was lower than the value obtained from $K_{av}=0.3$, and the α-helix content of the protein was fairly large (54%). Accordingly, it seems that this protein has a long, slender, and rigid structure. In the experiments of SDS polyacrylamide gel electrophoresis, gel chromatography on Sepharose 6B in 6M Gun HCl, and viscosity measurements in 6M Gun HCl, we might be able to obtain the molecular weight of the dissociated protein (subunit). The subunit, on the contrary, may tend to associate in aqueous solution. Neverthless, the molecular weight obtained by sedimentation equilibrium in native state of the protein in aqueous solution was agreed well with the values from other experiments mentioned above.

14) N. Greenfield and G.D. Fasman, *Biochemistry*, **8**, 4108 (1969).
15) Y.H. Chen and J.T. Yang, *Biochem. Biophys. Res. Commun.*, **44**, 1285 (1971).

Further, Fig. 1 shows good linearity, hence it suggests that the protein in the solution was homogeneous and association did not occur. Consequently, we could not prove in our experiments that the protein associates to a larger molecule by –s–s– bond or hydrogen bond and dissociates to the subunit by the treatment with various agents. Aonuma, *et al.* have isolated one of the hypocalcemic components from MP-parotin.[16] They showed that the purified protein was constituted from two subunits, whose molecular weight was 45000. The isolectric point of the protein was pH 4.5 and the K_{av} value was about 0.1 (estimated by us from their figure) by gel chromatography on Sephadex G-100. Our hypocalcemic protein had an isoelectric point of pH 5.3[4] and the K_{av} value was 0.3 by gel chromatography on Sepharose 6B and these results were different from the results obtained by Aonuma, *et al.*[16] Furthermore, two kinds of hypocalcemic proteins clearly differed from one another in amino acid composition. We consider, therefore, that the hypocalcemic protein dealt here would be unlike that obtained by Aonuma, *et al.*[16]

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16) S. Aonuma, Y. Kohama, S. Nakajin, S. Yashiki, and H. Egawa, *Nippon Naibumpi Gakkai Zasshi*, **50** 1468 (1974).