CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 26, No. 7 July 1978

Regular Articles

[Chem. Pharm. Bull. 26(7)1957—1965(1978)]

UDC 547.963.02.05.09 : 615.272.2.011.5.076.9

Purification and Characterization of Bovine Parotid Hypocalcemic Factor obtained *via* Extraction with Glacial Acetic Acid[1]

Akira Mizutani, Ikukatsu Suzuki, and Hajimu Yamamoto

Faculty of Pharmaceutical Sciences, Nagoya City University[2]

(Received August 6, 1977)

The acetone-dried powder (PA), obtained from supernatant left after separation of the pH 5.4-precipitating fraction (crude Parotin) from the aqueous extract of bovine parotid gland, was extracted with glacial acetic acid, and the extract had a hypocalcemic activity in rabbits, but its activity fluctuated. On the other hand, the glacial acetic acid extract of the acetone-dried powder (PAI) obtained by incubation of a suspension of the sample PA in saline, had nearly constant activity. From the purification of PAI by DEAE-cellulose chromatography and gel chromatography on Sephadex G-100, a hypocalcemic protein (P-MSY) was obtained in a yield of 37 mg from 10 kg of the fresh gland. The sample P-MSY was homogeneous in disc electrophoresis and electrofocusing, and it was effective in a dose of 0.01 mg/kg at 1% level of significance against control rabbits given saline, and it has almost the same activity as purified Parotin, which was effective in a dose of 0.01 mg/kg as reported previously. P-MSY had a molecular weight of 66000 by SDS-polyacrylamide gel electrophoresis, isoelectric point of pH 5.60, and sugar content of 2.53%. These properties and the results of amino acid analysis suggest that P-MSY is a hypocalcemic protein differing from purified Parotin. Further, P-MSY was also proved to be different from β-Parotin or calcitonin in its character.

Keywords——parotid gland; glacial acetic acid extraction; hypocalcemic protein; gel chromatography; electrofocusing; amino acid analysis; SDS polyacrylamide gel electrophoresis; dose-response relationship

Previously, we reported the physicochemical properties[3] and the immune competence promotion[4] of the hypocalcemic protein (M.W. 48000) obtained through the ammonium sulfate fractionation of the product precipitated at pH 5.4 (pH 5.4-precipitate, crude Parotin) from the aqueous extract of bovine parotid gland.[5] On the other hand, a hypocalcemic fraction, PA, was obtained from the mother liquor left after separation of the pH 5.4-precipitate, and a hypocalcemic protein was later isolated from PA *via* extraction with glacial acetic acid.[6] In the present paper, we describe purification of the hypocalcemic substance and its properties.

1) Presented at the 49 th Annual Meeting of Biochemical Society of Japan, Hokkaido, September 1976.
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya 467, Japan*
3) A. Mizutani, T. Mizutani, and P-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2733 (1976).
4) A. Mizutani, T. Mizutani, I. Suzuki, and P-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **25**, 1601 (1977).
5) A. Mizutani, T. Kitamura, N. Yamada, H. Ianaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).
6) I. Suzuki, H. Yamamoto, and A. Mizutani, *Seikagaku*, **48**, 466 (1976).

Materials and Methods

Materials——The material used was a fresh parotid gland obtained from cattle. Extraction of the gland and preparation of the glacial acetic acid extract are outlined in Fig. 1. According to the method re-

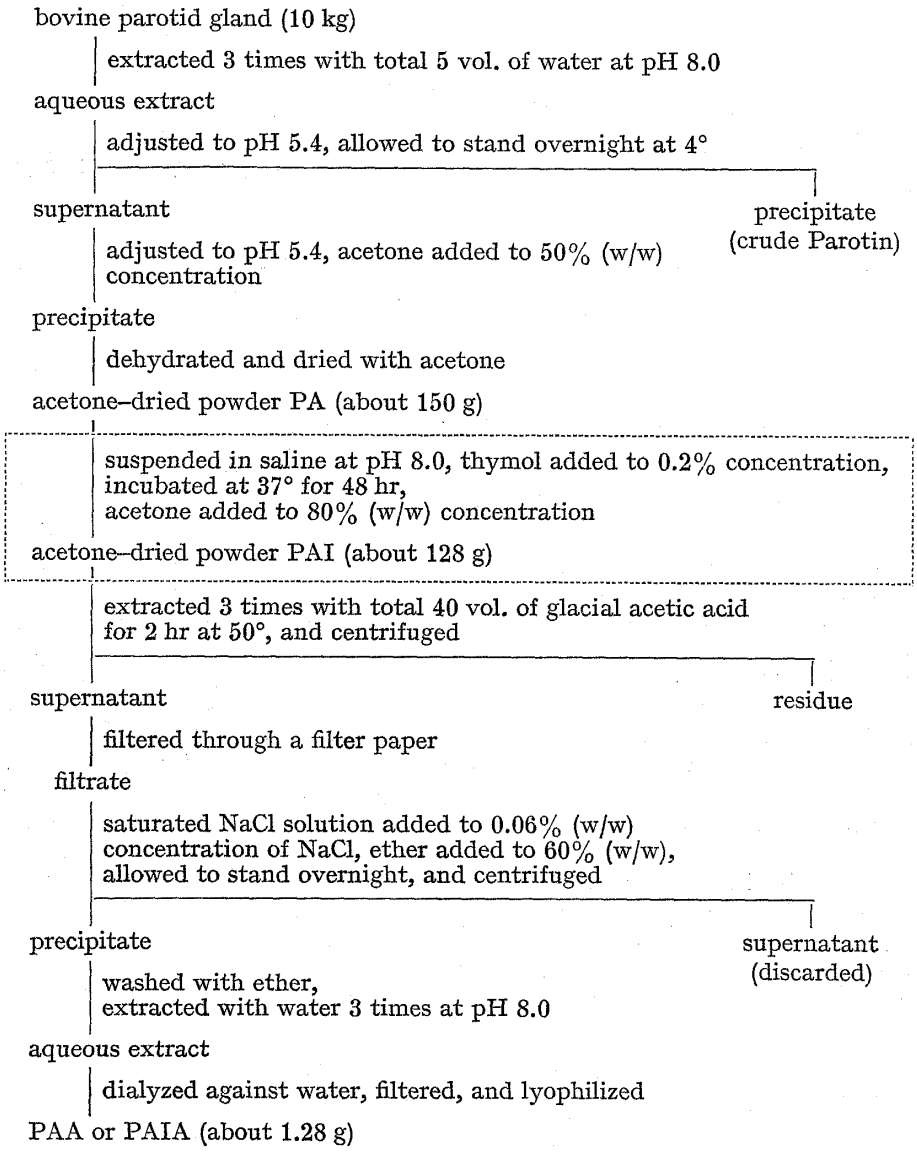

Fig. 1. Flow Sheet for the Preparation of Glacial Acetic Acid Extract from Bovine Parotid Gland

ported previously,[7] the fresh gland was extracted with water at pH 8.0, the pH 5.4-precipitate was removed, then acetone was added to the supernatant to give 50% (w/w) concentration, and the resulting precipitate was dried with acetone to obtain the acetone-dried powder, PA. In our earlier experiment, fraction PA was extracted with glacial acetic acid for 2 hr at 50°, except the course enclosed with a broken line in Fig. 1. After filtration of the glacial acetic acid extract, saturated solution of NaCl was added to the filtrate to give 0.06% (w/w) concentration of NaCl, and then ether was added up to 60% (w/w) concentration. The resulting precipitate was extracted with water at pH 8.0, followed by dialysis of the aqueous extract and lyophilization to obtain the glacial acetic acid extract, PAA. However, we could not constantly obtain an active product. Therefore, the additional method of Ito and Yamamoto[8] for Uroparotin, enclosed with a broken line in Fig. 1, was employed. The suspension of PA (5%) in saline was adjusted to pH 8.0 with 1 N NaOH and thymol was added to give 0.2% concentration as an antiseptic. After incubation of the suspension at 37°

7) Y. Ito and A. Mizutani, *Yakugaku Zasshi*, **72**, 237 (1973).
8) Y. Ito and M. Yamamoto, *Endocrinol. Jpn.*, **5**, 250 (1958).

for 48 hr, acetone was added to 80% (w/w) concentration, and the resulting precipitate was made into the acetone-dried powder, PAI. Fraction PAI was extracted with glacial acetic acid and the lypohilized product, PAIA, was obtained in the same way as described above.

Fractionation of PAIA by DEAE-cellulose Chromatography——DEAE-cellulose (exchange capacity, 0.97 meq/g, Tohoku Pulp product) was pretreated by the conventional method and packed into a column (5.0×105 cm). A solution of PAIA (8.08 g/400 ml) was applied to the column. The adsorbed materials were eluted with the following buffers at a flow rate of 200 ml/hr: (1) Tris-HCl buffer (μ=0.005, pH 8.32), 2) Tris-maleate buffer (μ=0.02, pH 6.40), 3) Tris-maleate buffer +0.1 M NaCl (μ=0.12, pH 5.40). The fractions of eluate involved in each peak were combined, dialyzed against deionized water, and then lyophilized, resulting in five fractions; PAID-I, PAID-II, PAID-III, PAID-IV, and PAID-V (Fig. 2). Fraction PAID-IV had the most potent hypocalcemic activity and showed the highest yield of these five fractions. It was then subjected to further purification by gel chromatography.

Gel Chromatography on Sephadex G-100——Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala) was equilibrated with 0.025 M phosphate buffer (μ=0.06, pH 6.98) and packed into a column (3.7×102 cm). Fraction PAID-IV (600 mg) was dissolved in the same buffer to make a concentration of 20 mg/ml, and loaded on the column. The proteins were eluted with the same buffer by the descending method, at a hydraulic pressure of 30 cm and a flow rate of 20 ml/hr (Fig. 3a). The resulting fraction PAID-IV.GII (100 mg) was dissolved in 5 ml of the phosphate buffer, loaded on a smaller column (2.8×70 cm), rechromatographed with the same buffer, and the final purified fraction P-MSY was obtained (Fig. 4a).

Determination of Protein——Protein was determined by the method of Lowry *et al.*[9]

Examination of the Purity of Proteins——The purity of proteins was examined by the disc electrophoresis of Davis[10] (7.5% gel, pH 8.9) at a constant current (3 mA/tube) for 2 hr. The gel was stained with Coomassie Brilliant Blue R-250 by the method of Fazekas *et al.*[11]

Hypocalcemic Activity——Hypocalcemic activity was assayed by the method reported previously.[12] Groups of six normal mature male rabbits, deprived of food for about 24 hr, were used. A saline solution of the sample was intravenously injected into rabbits and percentage decrease of serum calcium was calculated against the value before the injection, then averaged for the six animals (mean rate). The difference between the mean rates of the test group and the control group injected with saline was examined by the *t*-test. The sample injected was considered to be effective when the difference was significant at 5% level of significance.

Time Course of Hypocalcemia Caused by the Injection of the Fraction P-MSY——Fraction P-MSY was intravenously injected into rabbits in a dose of 0.005 mg/kg. Blood was drawn before and after (2, 4, 5, 6, 8, and 24 hr) the injection, and the hypocalcemic activity was plotted against the time after the injection.

Dose-Response Relationship——The hypocalcemic activity was determined in doses of 2.5, 5.0, and 10.0 μg/kg of P-MSY and plotted against the logarithms of the dose. The linearity of dose-response curve was statistically examined.

Molecular Weight Determination by Sodium Dodecyl Sulfate (SDS)-polyacrylamide Gel Electrophoresis——Molecular weight of P-MSY was determined by the split gel method of Dunker and Rueckert,[13] using 10% polyacrylamide gel in the presence of 0.1% SDS. After electrophoresis, the gel was stained and traced with an autodensitometer. The mobilities of P-MSY and the reference proteins (Schwarz-Mann product) were calculated from the top of the peaks in the profile obtained by tracing. The molecular weight of P-MSY was estimated by comparing its mobility with those of the reference proteins.

Electrofocusing——Isoelectric point of P-MSY was measured with an LKB Model Multiphor electrofocusing apparatus.[14] Two kinds of ampholine-carrier ampholytes were mixed (LKB 1809-116 pH 4—6 and LKB 1809-121 pH 5—7; 2: 1). The filter paper moistend with a solution of P-MSY was placed on the polyacrylamide gel plate (Multiphor Complate, 125×260×1 mm). During the operation, the voltage was gradually elevated to 1080 V in 2 hr. After electrophoresis, the pH gradient on the gel was measured at 1 cm intervals and plotted against the mobility, and the isoelectric point of P-MSY was estimated.

Determination of Sugar——The solution of P-MSY (1.778 mg/ml) was colored by the phenol–H_2SO_4 method of Dubois *et al.*[15] and its absorbance was measured at 490 nm. The amount of sugar was estimated from the calibration curve for glucose.

Amino Acid Analysis——A sample of P-MSY (3.716 mg) was dissolved in 5 ml of 6 N HCl, and 3 portions of 1 ml of the solution were placed in 3 glass tubes. They were hydrolyzed for 24, 36, and 72 hr at 110°±2°.[16]

9) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
10) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
11) S. Fazekas, S.T. de Groth, R.G. Webster, and A. Datyner, *Biochim. Biophys. Acta*, **71**, 377 (1963).
12) A. Mizutani, K. Yamamoto, H. Sato, Y. Saito, H. Kitsugawa, and H. Yamamoto, *Yakugaku Zasshi*, **91**, 297 (1971).
13) A.K Dunker and R.R. Rueckert, *J. Biol. Chem.*, **244**, 5074 (1969).
14) L.S. Bates and C.W. Deyoe, *J. Chromatogr.*, **73**, 296 (1972).
15) M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
16) D.H. Spackman, W.E. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

Amino acids were analyzed with a Hitachi Model KLA-3B amino acid analyzer. The amount of tryptophan was determined by Spies and Chambers' method.[17]

Results and Discussion

By the earlier method of extraction without the process of incubation, three of eight samples of PA showed the hypocalcemic activity in a dose of 10 mg/kg. The yield (dry weight) of fraction PA was about 1.5% of the fresh gland (average of 8 samples). In the case of PAA, only two samples were effective among sixteen samples, in a dose of 0.1 mg/kg. The yield of PAA was about 1.4% of PA (average of 16 samples). In the improved method including the incubation of PA, four of five samples of PAI were effective in a dose of 10 mg/kg. The yield of PAI was about 1.3% of the fresh gland (average of 5 samples). Nine of eleven samples of PAIA were effective in a dose of 0.1 mg/kg. The specific activity of PAIA increased by about 100-fold over that of PAI. The yield of PAIA was about 1.0% of PAI (average of 11 samples). Thus the frequency of obtaining active products (PAI, PAIA) increased by the incubation of PA, and fraction PAI was used hereafter as a material for extraction with glacial acetic acid. It is considered that by incubation, hydrolytic action of

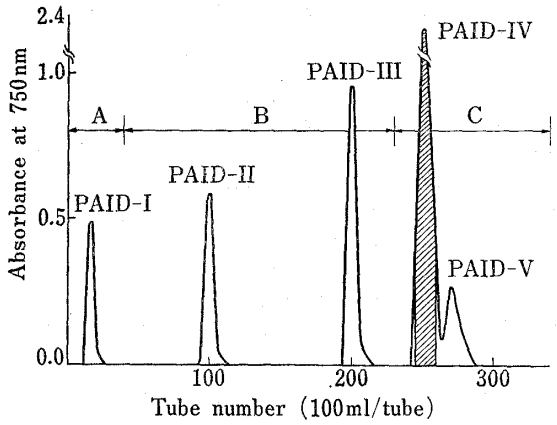

Fig. 2. Elution Pattern of PAIA Obtained by Chromatography on DEAE-cellulose

Buffer system A: Tris-HCl buffer (μ=0.005, pH 8.32), B: Tris-maleate buffer (μ=0.12, pH 6.40), C: Tris-maleate buffer+0.1M NaCl (μ=0.12, pH 5.40). Column size: 5.0×105 cm, sample: PAIA 8.08 g, flow rate: 200 ml/hr. The shaded peak (PAID-IV) showed the highest hypocalcemic activity.

TABLE I. Hypocalcemic Activity and Yield of the Fractions Obtained from the Fresh Parotid Gland

Fraction	Dose (mg/kg)	Decrease in serum Ca (%) Mean±S.E. (n=6)	Relative specific[a] activity	Yield[b] (g)
PA	10	6.98±1.22		150
PAI	10	11.33±1.49[c]	1	128
PAIA	0.1	12.18±1.32[c]	100	1.28
PAID-I	0.05	5.61±1.61		0.033
PAID-II	0.05	7.46±1.57		0.099
PAID-III	0.05	7.58±1.02		0.127
PAID-IV	0.05	13.43±1.14[c]	200	0.361
PAID-V	0.05	9.13±0.72[d]		0.072
PAID-IV.GI	0.02	6.15±1.95		0.019
PAID-IV.GII	0.02	9.43±0.92[c]	500	0.098
PAID-IV.GIII	0.02	7.02±1.08		0.051
PAID-IV.GIV	0.02	5.81±1.14		0.006
P-MSY	0.01	10.67±1.65[c]	1000	0.037
	0.005	9.41±1.93[d]	2000	—
	0.0025	5.85±1.25		—

a) Specific activity of each fraction against the activity of PAI.
b) Results from 10 kg of bovine parotid gland.
c) Significantly different from control $p<0.01$.
d) Significantly different from control $p<0.05$.

17) J.R. Spies and D.C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

some proteases (*e.g.*, cathepsin) contained in the products freed the protein from blocking its active site and this led to appearance of the activity. When incubation was not made, the active site might be exposed by inadvertently developed enzymic action during operation. The hypocalcemic activities and yields of the five fractions obtained from DEAE-cellulose chromatography of PAIA (Fig. 2) are given in Table I. Both fractions PAID–I and PAID–II were not effective in a dose of 0.05 mg/kg, while fraction PAID–IV was effective at 1% level of significance and was obtained in the highest yield. Although fraction PAID–V was active, it was not purified because of its low yield. From the gel chromatography of PAID–IV on

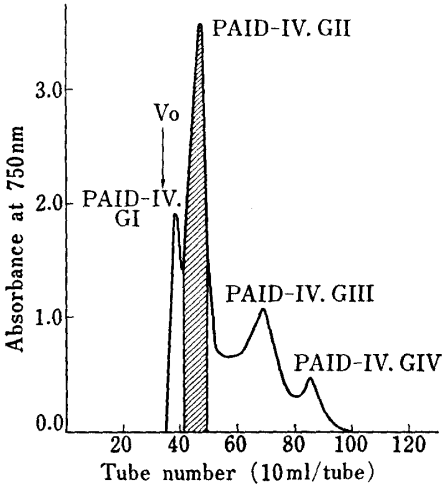

Fig. 3a. Gel Chromatography on Sephadex G-100

Sample: PAID–IV 600 mg, column size: 3.7×102 cm, buffer: 0.025 M, pH 6.98 phosphate buffer, flow rate: 20 ml/hr. The shaded area (PAID–IV. GII) showed the highest hypocalcemic activity.

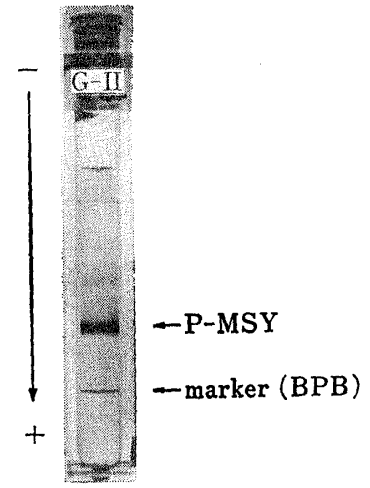

Fig. 3b. Disc Electrophoresis of Fraction PAID–IV.GII 7.5 % gel, pH 8.9

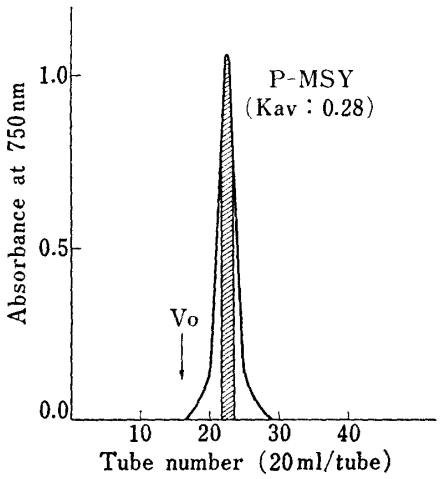

Fig. 4a. Second Gel Chromatography on Sephadex G-100

Sample: PAID–IV.GII 100 mg, buffer: 0.025 M, pH 6.98 phosphate buffer, flow rate: 20 ml/hr.

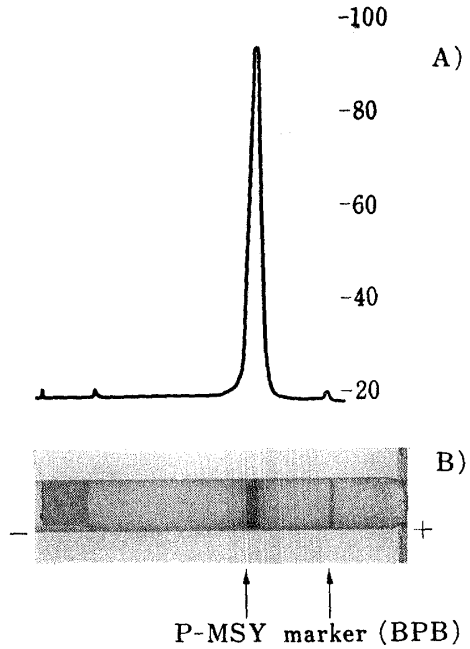

Fig. 4b. A) Densitometric Tracing of Disc Electrophorogram of Fraction P–MSY, B) Disc Electrophoresis of Fraction P–MSY

Sephadex G-100, four fractions (PAID-IV.GI, PAID-IV.GII, PAID-IV.GIII, and PAID-IV.GIV) were separated (Fig. 3a). The hypocalcemic activities and yields of these fractions are given in Table I. Fraction PAID-IV.GII (Kav: 0.28) was effective in a dose of 0.02 mg/kg at 1% level of significance and its yield was comparatively high, but not homogeneous in disc electrophoresis (Fig. 3b). In the second gel chromatography, the fractions eluted at around 0.28 of Kav were collected to obtain fraction P-MSY (Fig. 4a), giving a single band on disc electrophoresis and good symmetry in its profile of densitometric tracing (Fig. 4b). Fraction P-MSY gave the mean hypocalcemic rate of 9.41±1.93% in a dose of 0.005 mg/kg. Its activity was significant at 5% level of significance and increased by about 2000-fold over that of fraction PAI (Table I).

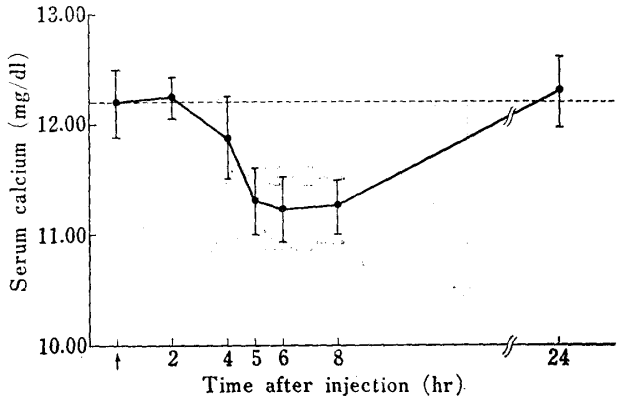

Fig. 5. Time Course of the Hypocalcemic Effect of P-MSY after Intravenous Injection of 0.005 mg/kg into Rabbits

↑ : time of injection of material.

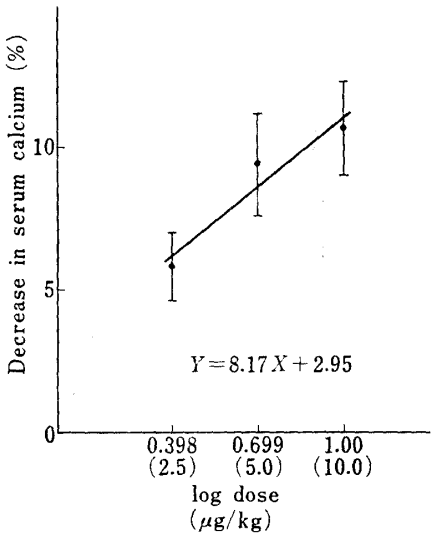

Fig. 6. Dose-Response Relationship of P-MSY

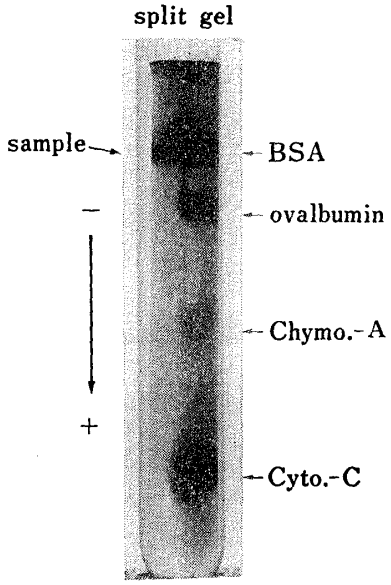

Fig. 7a. Electrophoresis on 10% Polyacrylamide Gel in the Presence of 0.1% SDS by the Split Gel Technique

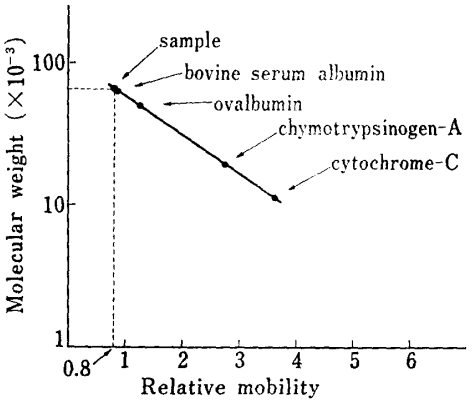

Fig. 7b. Molecular Weight Calibration Curve Obtained from SDS Polyacrylamide Gel Electrophoresis

Sample: P-MSY.

The time course study of the hypocalcemic effect of fraction P–MSY (Fig. 5) showed that the serum calcium level was lowered at 4, 5, and 6 hr after the injection, then tended to rise, and returned to the level before the injection in 24 hr. This pattern was almost similar to that of purified Parotin (unpublished).

The dose-response relationship of fraction P–MSY is shown in Fig. 6. The linearity of dose-response curve was revealed by statistical examination (analysis of variance) at 5% level of significance, and the regression line was given by an equation $Y=8.17X+2.95$. Consequently, the dose-response relationship may be linear over a moderate range of doses.

The pattern of P–MSY obtained by SDS polyacrylamide gel electrophoresis is shown in Fig. 7a. Molecular weight of P–MSY was estimated to be 66000 from the calibration curve for the reference proteins (Fig. 7b).

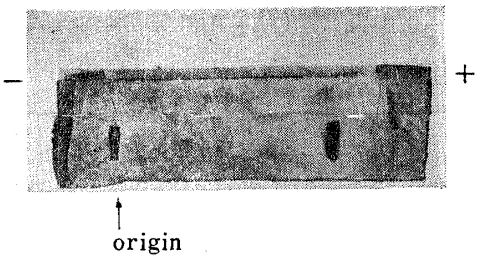

Fig. 8a. Electrofocusing of a Sample of P–MSY on Polyacrylamide Gel Plate

pH ranges: pH 4—7.

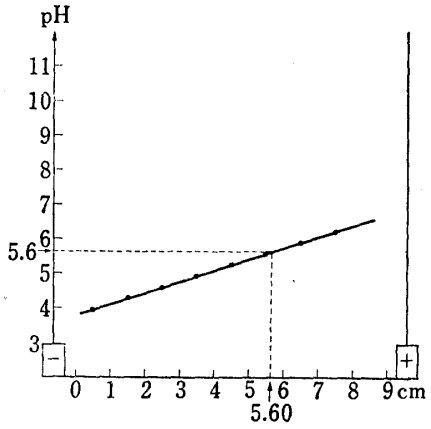

Fig. 8b. Plot of pH Gradient on Polyacrylamide Gel Plate in the Electrofocusing

Table II. Amino Acid Composition of P–MSY and Purified Parotin

Amino acid	Mol/mol protein	
	P–MSY	Purified parotin[a)]
Lysine	46.0	25.6
Histidine	9.4	3.9
Arginine	12.0	25.1
Aspartic acid	45.2	48.8
Threonine	24.6	19.2
Glycine	23.8	24.2
Serine	31.9	20.8
Proline	25.4	3.4
Glutamic acid	53.9	66.2
Leucine	36.7	48.1
Valine	22.6	19.6
Tyrosine	52.8	8.4
Isoleucine	89.3	22.0
Methionine	2.8	7.3
Alanine	30.1	37.9
Phenylalanine	18.7	8.1
Cystine/2	5.7	1.4
Tryptophan	4.4	5.1
Total	544.6	395.1
Molecular weight	66000	48000

a) Purified Parotin obtained from pH-5.4 precipitate.[5)]

In the electrofocusing, this sample gave a single band (Fig. 8a) and its isoelectric point was pH 5.60 (Fig. 8b).

The sugar content of P–MSY was estimated to be 2.53%. The results of amino acid analysis and the molar content of each amino acid are shown in Table II, providing a molecular weight of P–MSY as 66000. This table shows that P–MSY contains a comparatively large amount of glutamic acid, tyrosine, and isoleucine. In comparison with purified Parotin, P–MSY contains more proline, tyrosine, isoleucine, and half cystine, and less arginine and methionine than purified Parotin, and hence a marked difference can be seen between these two substances.

Table III. Comparison of Properties of Various Hypocalcemic Substances from Bovine Parotid Gland

		P–MSY	Purified Parotin[a]	β-Parotin[b]	Calcitonin[c]
Hypocalcemic activity	Dose (mg/kg)	0.01	0.01	0.5	0.03
	Decrease in serum Ca (%) Mean±S.E.	10.67±1.65[d]	10.24±1.06[d]	12.06±0.71	—
Kav in gel chromatography		0.28[e]	0,[f] 0.3[g]	—	0.60[h]
Relative mobility[i]		0.69	0.28	—	0.31
Molecular weight[j]		66000	48000	—	4000
Isolectric point		5.60	5.30	4.20	—
Sugar (%)		2.53	0.50	2.40	—

a) The results of Mizutani *et al.*[5]
b) Taken from the data of Ito *et al.*[18]
c) The results of Brewer *et al.*[19]
d) Significantly different from control $p<0.01$.
e) Sephadex G-100.
f) Sephadex G-200.
g) Sepharose 6B.
h) Sephadex G-25.
i) Relative mobility to BPB in disc electrophoresis.
j) SDS polyacrylamide gel electrophoresis.

The properties of P–MSY, purified Parotin,[5] β-Parotin,[18] and calcitonin[19] are shown in Table III. The hypocalcemic activity of P–MSY was almost the same as that of purified Parotin, which is effective in a dose of 0.01 mg/kg. Purified Parotin showed molecular weight of 48000, isolectric point of pH 5.30, relative mobility[20] of 0.28 against the marker (Bromophenol Blue) in disc electrophoresis, and sugar content[5] of 0.5%, and these values are lower than those of P–MSY. β-Parotin, which was homogeneous in Tiselius' electrophoresis,[18] was less active than P–MSY, because the former is effective in a dose of 0.5 mg/kg. The sugar content of P–MSY was similar to that of β-Parotin (2.40%). In comparison of P–MSY with calcitonin, the latter showed relative mobility of 0.31 against the marker (BPB) in disc electrophoresis and molecular weight of 4000, and these values are smaller than those of P–MSY. The time course of hypocalcemic activity of P–MSY almost the same as those of purified Parotin and β-Parotin. However, the lag in the appearance of hypocalcemic activity of P–MSY was larger (5—6 hr) and the duration of hypocalcemic activity was longer than those that of calcitonin (1 hr, in rabbits). From these facts, it is clear that P–MSY is a hypocalcemic protein different from the above three active proteins.

18) Y. Ito, S. Okabe, and S. Namba, *Endrocrinol. Jpn.*, **12**, 79 (1965).
19) H.B. Brewer, R.J. Schlueter, and J.P. Aldred, *J. Biol. Chem.*, **245**, 4232 (1970).
20) A. Mizutani, A. Okano, Y. Isobe, T. Mizutani, Y. Matsushita, and H. Goto, *Chem. Pharm. Bull.* (Tokyo), **24**, 2391 (1976).

As noted above, various hypocalcemic proteins having different physical and chemical characteristics were obtained from the parotid gland. Thus it is assumed that in these protein molecules there is a similar amino acid sequence or peptide conformation bearing hypocalcemic activity, and this assumption is left for future study.

Acknowledgment The authors are grateful to Mr. Akira Murayama for his cooperation in preparing the crude material used for this experiment.