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Purification and Characterization of Hypocalcemic Protein from Porcine Parotid Gland

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A hypocalcemic protein was isolated for the first time from porcine parotid gland by the procedures of aqueous extraction, precipitation at pH 4.8, ammonium sulfate fractionation, chromatography on DEAE-cellulose, and repeated gel filtration on Sephadex G-200. The purified protein lowered the serum-calcium concentration in albino rabbits by 12.5±1.3% at a dose of 20 μg/kg body weight 4—6 hr after the injection. The purified protein was found to be homogeneous, being a single band by polyacrylamide gel electrophoresis and by analytical ultracentrifugation. The molecular weight of the porcine hypocalcemic protein was determined to be 52000 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Amino acid analysis revealed that the protein was rich in aspartic and glutamic acids and in leucine, and that the ratio of acidic/basic amino acid residues was 2.13, and the ratio of histidine/lysine was 3.07 in this protein. The latter ratio is characteristic, since the ratio is below 1.00 in many proteins. The chemical properties of the porcine parotid hypocalcemic protein were slightly different from that of the bovine parotid hypocalcemic protein, although they were similar in biological effect.

Parotin, which was obtained previously from bovine parotid gland by Ito and Mizutani,[2] exerts a serum-calcium lowering effect in rabbits, with other several physiological effects.[3] Furthermore, several parotin-like substances had been obtained from horse parotid gland,[4] from the submaxillary glands of bovine,[5] of horse,[6] and of porcine,[7] from bovine serum,[8] and from human saliva[9] and urine.[10]

Recently, we found that the parotin-preparation described above was still heterogeneous by polyacrylamide gel electrophoresis, and presented a method of complete purification and characterization of a hypocalcemic protein (HC protein) from bovine parotid gland,[11] which was homogeneous by polyacrylamide gel electrophoresis and approximately 100 times more potent than the parotin-preparation.

The present investigation was undertaken to isolate an HC protein from porcine parotid gland in order to obtain more information concerning parotid HC proteins from comparative biochemical interest. This report describes the extraction, fractionation, further purification, and some properties of the porcine parotid HC protein.

Materials and Methods

Materials——The parotid glands were collected from pigs (the white F_1 between *Mid-yorkshire* and *Landrace* species), 7—10 months after birth.

1) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
2) Y. Ito and A. Mizutani, *Yakugaku Zasshi*, **72**, 244 (1952).
3) *a*) Y. Ito, *Endocrinol. Japon*, **1**, 1 (1954); *b*) *Idem*, *Ann. N. Y. Acad. Sci.*, **85**, 228 (1960).
4) Y. Ito and A. Mizutani, *Yakugaku Zasshi*, **72**, 1462 (1952).
5) Y. Ito, S. Aonuma, H. Takikawa, and M. Shinoda, *Yakugaku Zasshi*, **76**, 83 (1956).
6) S. Aonuma, *Yakugaku Zasshi*, **76**, 199 (1956).
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8) Y. Ito, S. Aonuma, and T. Mimura, *Yakugaku Zasshi*, **82**, 425 (1962).
9) Y. Ito and S. Okabe, *Endocrinol. Japon*, **6**, 166 (1959).
10) Y. Ito and M. Yamamoto, *Endocrinol. Japon*, **5**, 280 (1958).
11) A. Mizutani, T. Kitamura N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974).

Bovine serum albumin, ovalbumin, chymotrypsinogen A, and horse heart cytochrome c were purchased as a set of calibration proteins (Boehringer Mannheim products). All other materials were reagent grade.

Bioassay——Hypocalcemic activity was biologically assayed by measuring the maximum decreasing rate of the serum calcium level with six albino male rabbits over 2 kg body weight according to the method previously reported.[12] The significance of the difference between the data of the test and saline control groups was statistically estimated with the aid of the *t*-table. When the difference was significant at the level of 0.01 or 0.05 probability, the preparation tested was evaluated to be active at the dose injected.

Protein Determination——Protein was determined by ultraviolet absorption at 280 nm or by the method of Lowry, *et al.*[13] Bovine serum albumin was employed as a protein standard.

Disc Electrophoresis——Disc electrophoresis of the samples was performed by the method of Davis[14] on 7.5% polyacrylamide gel. After electrophoresis, the gels were stained with Amido Black 10-B in 7% acetic acid followed by destaining in 7% acetic acid. The resulting gels were submitted to recording with a Fuji-Riken model FD-A4 autodensitometer.

Molecular Weight Determination——Molecular weight estimation was performed by the split gel techniques of Dunker and Rueckert[15] on 10% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. In the plot of relative mobility against logarithm of molecular weight, four kinds of the proteins of known molecular weight were employed as calibration standards.

Amino Acid Analysis——Amino acid analyses were performed on a Hitachi model KLA-3B amino acid analyzer with a single column (0.9×55 cm) by the use of buffer 1 (0.2 M Na^+, pH 3.25), buffer 2 (0.2 M Na^+, pH 4.25),[16] and buffer 3 (0.8 M Na^+, pH 5.9, prepared from 234.9 g sodium citrate $2H_2O$, 3 ml phenol, 50 ml 35% HCl, 3 g BRIJ-35 detergent, and water to make 3 liters). Hydrolyses of the sample were conducted in a nitrogen atmosphere in 6 N HCl at 110° for 24 or 72 hr in a sealed tube.

Carbohydrate Determination and the Instruments——Carbohydrate content was determined with anthrone–sulfuric acid method.[17]

The instruments used were a Hitachi model UCA-1 ultracentrifuge for analytical ultracentrifugation, a JASCO model UV/ORD-5 spectrophotometer for optical rotatory dispersion (ORD) spectrum, and a Shimadzu model QV-50 spectrophotometer for ultraviolet (UV) spectrum.

Purification Procedure——Unless otherwise stated all steps were carried out in a cold room at about 5° or under ice cooling, and toluene was added to solutions as a preservative.

Step 1. Extraction and Isoelectric Precipitation——The parotid glands were cautiously freed from lymph nodes, connective tissues, and adipose tissues. The fresh glands were minced, and then extracted with 2 vols. of water at pH 8.0 for 2 hr. After the suspension had been sieved, the remainder was again extracted with 2 vols. of water as above. The extracts were combined, and frozen in blocks. The frozen blocks were thawed on gauze, the bulk of residue was discarded, and the filtrate was adjusted to pH 4.8, and allowed to stand overnight. The suspension was centrifuged at 2500 rpm for 10 min, the supernatant was discarded. The precipitate was washed with acetone and dried over $CaCl_2$ in the vacuum desiccator, yielding acetone powder of the pH 4.8 fraction.

Step 2. Ammonium Sulfate Fractionation——The acetone powder (50 g) was suspended in 0.15 M NaCl (2 liters) and solubilized at pH 8.0 with slow stirring overnight. The suspension was brought gradually to 7% (w/w) ammonium sulfate concentration by the addition of saturated $(NH_4)_2SO_4$ solution with stirring at pH 8.0, and allowed to stand overnight. The insoluble materials were filtered off through fluted Toyo No. 2 paper. To the clear filtrate, saturated $(NH_4)_2SO_4$ solution was added dropwise up to 22.5% (w/w) ammonium sulfate concentration at pH 6.8, and allowed to stand overnight. The precipitate formed was collected by centrifugation at 2500 rpm for 20 min. The pellet resulted was again dissolved in a minimal amount of water, and dialyzed against water. Saturated $(NH_4)_2SO_4$ solution was added dropwise to the dialyzed inner solution with stirring up to 15% (w/w) ammonium sulfate concentration at pH 6.0. After the mixture had stood overnight, the precipitate formed was collected by centrifugation as above. The resulting pellet (7—15% $(NH_4)_2SO_4$ fraction) was dissolved in water, dialyzed exhaustively against water, and lyophilized.

Step 3. DEAE-cellulose Column Chromatography——DEAE-cellulose D-1 (Tohoku Pulp Co. product, exchange capacity 0.95 meq/g) (30 g) was pre-treated according to the manufacturer's instructions. The exchanger was then bufferized with 67 mM phosphate buffer (pH 8.16, $\mu=0.116$) and packed in a column (5.2×90 cm), followed by equilibration with the same buffer. The 7—15% $(NH_4)_2SO_4$ fraction obtained in step 2 (4.28 g) was dissolved in the same buffer (200 ml), and loaded to the column, and then eluted as de-

12) A. Mizutani, K. Yamamoto, H. Sato, Y. Saito, H. Kitsugawa, and H. Yamamoto, *Yakugaku Zasshi*, **91**, 297 (1971).
13) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
14) B.J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
15) A.K. Dunker and R.R. Rueckert, *J. Biol. Chem.*, **244**, 5074 (1969).
16) S. Moore, D.H. Spackman, and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).
17) F.A. Loewvs, *Anal. Chem.*, **24**, 219 (1952).

scribed in the legend to Fig. 2. Appropriate fractions were pooled, concentrated with a Diaflo ultrafiltration apparatus (UM-10 membrane), dialyzed exhaustively against water, and then lyophilized.

Step 4. Sephadex G-200 Gel Filtration——Sephadex G-200 (Pharmacia) was swollen in 67 mM phosphate buffer (pH 7.16, $\mu=0.146$) and packed in column (2.6×110 cm) equipped with an upward flow adaptor. The DEAE-cellulose fraction D obtained in step 3 (60 mg) was dissolved in the same buffer (4 ml) and loaded to the column, and then developed with the same buffer at a flow rate of 10 ml/hr. Ten ml fractions were collected. Appropriate fractions were pooled, dialyzed exhaustively against water, and lyophilized.

For further purification by repeating the gel filtration, a sample of the active fraction was applied to column (1.45×75 cm) and developed upward with the same buffer at a flow rate of 2.4 ml/hr. Five ml fractions were collected. Appropriate fractions were pooled, dialyzed against water, and lyophilized.

Results

Purification

Figure 1 shows the flow chart of the purification of the HC protein from porcine parotid gland. The yield of the pH 4.8 fraction from the fresh glands (32 kg) was 2.03% (650 g). Ammonium sulfate fractionation of the pH 4.8 fraction yielded three fractions: 7—15%, 15—22.5%, and 22.5—42% $(NH_4)_2SO_4$ fractions. The yields and hypocalcemic activities of each fractions are shown in Table I. Most of the activity was found in the 7—15% $(NH_4)_2SO_4$ fraction and the fraction lowered the serum calcium concentration by 15.79±2.68% in a dose of 2 mg/kg in rabbits.

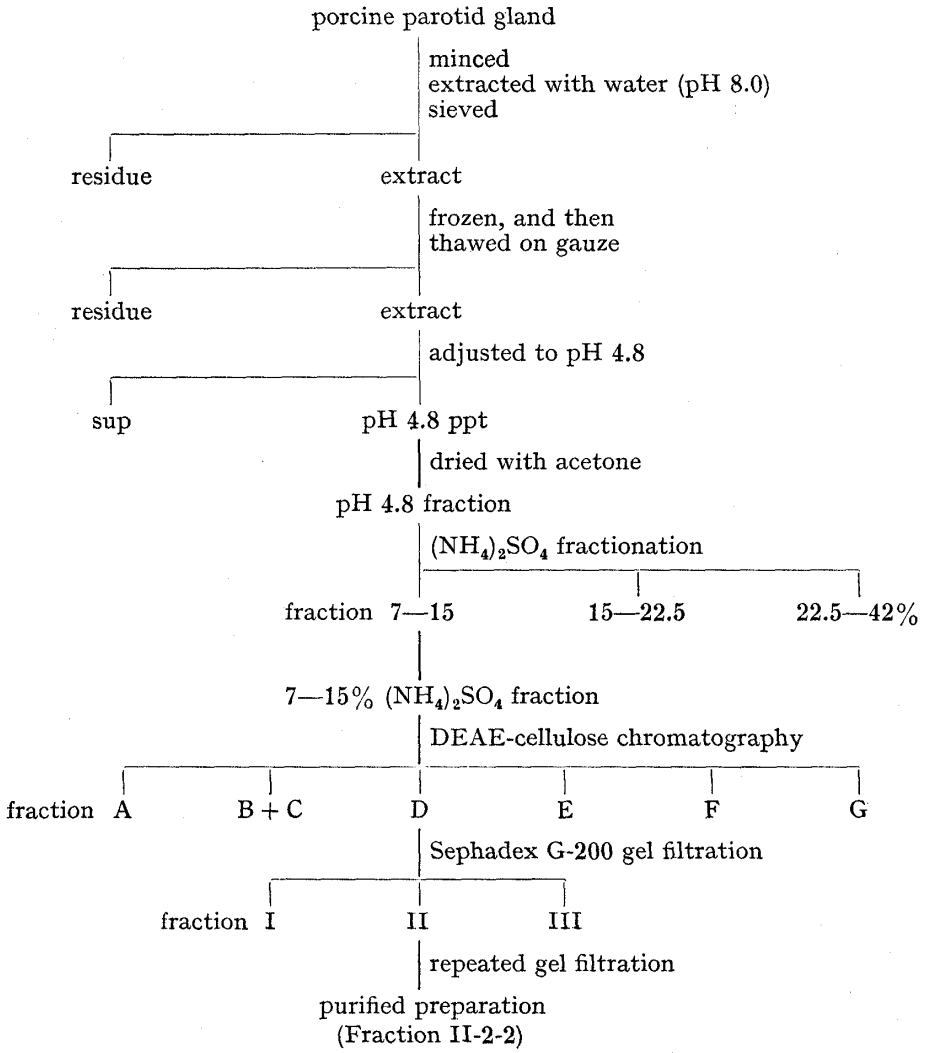

Fig. 1. Flow Chart of the Purification of the Hypocalcemic Protein from Porcine Parotid Gland

Table I. Yields and Hypocalcemic Activities of the $(NH_4)_2SO_4$ Fractions

Fractions	Yield (%)	Hypocalcemic activity	
		Dose (mg/kg)	Percent decrease of serum calcium mean ± SE(n=6)
Acetone powder	100	10	8.73±1.54[a]
7—15% $(NH_4)_2SO_4$	1.8	2	15.79±2.68[a]
15—22.5% $(NH_4)_2SO_4$	1.2	2	0.93±3.58[b]
22.5—42% $(NH_4)_2SO_4$	2.1	2	5.07±2.19[b]

a) significantly different from saline control, $p<0.01$
b) $p>0.05$

The 7—15% $(NH_4)_2SO_4$ fraction was fractionated into seven fractions by column chromatography on DEAE-cellulose. The elution profile is shown in Fig. 2. The yields and activities of the fractions are summarized in Table II. The fraction D possessed the highest activity (10.4±2.0% in a dose of 0.5 mg/kg). The fraction A and E were less active and the others were inactive.

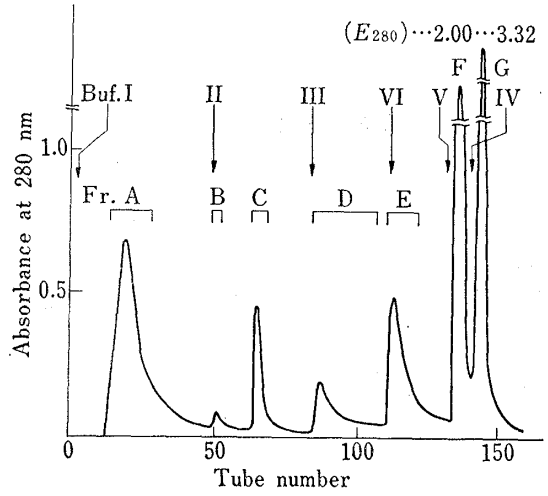

Fig. 2. DEAE-cellulose Chromatography of the 7—15% $(NH_4)_2SO_4$ Fraction obtained in Step 2

The 7—15% $(NH_4)_2SO_4$ fraction (4.28 g) was dissolved in the equilibration buffer, 67 mM phosphate buffer (pH 8.16, μ=0.116), and applied to a column (5.2×90 cm), and then eluted with the following buffers: I, 67 mM phosphate buffer (pH 8.16, μ=0.116); II, 67 mM phosphate buffer (pH 7.16, μ=0.146); III, the buffer II plus 0.08 M NaCl; IV, the buffer II plus 0.16 M NaCl; V, the buffer II plus 0.36 M NaCl; VI, 0.5 M NaOH. The flow rate was maintained at 260 ml/hr. 114 ml fractions were collected.

The DEAE-cellulose fraction D was further fractionated into three fractions by gel filtration on Sephadex G-200 as shown in Fig. 3-A. The yields and activities of the fractions in Fig. 3-A are shown in Table III. The fraction II possessed a hypocalcemic activity. No activity was found in fractions I and III. For further purification, the active fraction II was subjected to the second gel filtration (Fig. 3-B). The fraction II-2 in Fig. 3-B shows an increase in activity. The third gel filtration of the fraction II-2 yielded a single peak (as shown in Fig. 3-C), which appeared to be homogeneous (fraction II-2-2) as judged by disc electrophoresis, (as shown in Fig. 4), SDS gel electrophoresis, and analytical ultracentrifugation. The final purified product (fraction II-2-2) reduced the calcium level by 12.50±1.30% in a dose of 0.02 mg/kg.

Activity

Activities and yields at each step of the purification procedures are summarized in Table IV. The final preparation was purified more than 250 times over the pH 4.8 fraction.

Figure 5 shows the time course of the hypocalcemic response after the administration of the purified preparation to rabbits.

Molecular Weight

Figure 6 shows the plot of mobility *versus* logarithm of molecular weight for the porcine HC protein and standard marker proteins. The molecular weight of the porcine HC protein was estimated to be 52000.

TABLE II. Yields and Hypocalcemic Activities of the DEAE-cellulose Fractions

Fractions	Yield (mg)	Yield (%)	Hypocalcemic activity: Dose (mg/kg)	Hypocalcemic activity: Percent decrease of serum calcium mean±SE[a]	
Starting material[b]	4280	100	2	15.8±2.7[c]	active
			1	10.1±1.9[c]	
A	610	14.2	0.5	6.8±2.2[d]	active
B+C	124	2.9	0.5	4.3±0.8[e]	—
D	278	6.5	0.5	10.4±2.0[c]	active
			0.2	6.0±1.0[c]	
E	328	7.7	0.5	8.0±1.6[c]	active
F	659	15.4	0.5	4.0±0.9[e]	—
G	695	16.2	—	—	—

a) mean ± standard error; six rabbits in each group
b) the 7—15% $(NH_4)_2SO_4$ fraction obtained in step 2
c) significantly different from saline control, $p<0.01$
d) significantly different from saline control, $p<0.05$
e) $p>0.05$.

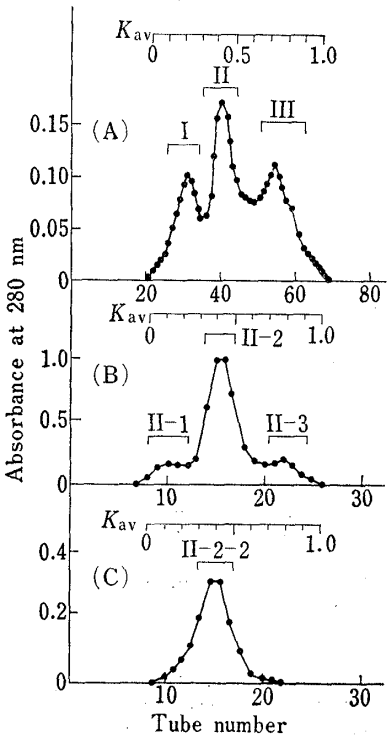

Fig. 3. Sephadex G-200 Gel Filtration of the DEAE-cellulose Fraction D obtained in Step 3

A) The first gel filtration. The DEAE-cellulose fraction D (60 mg) was dialyzed against the equilibration buffer, 67 mM phosphate buffer (pH 7.16, $\mu=0.146$), and applied to a column (2.6×110 cm), and developed upward with the same buffer at a flow rate of 10 ml/hr. Ten ml fractions were collected.
B) The second gel filtration. The fraction II in Figure 3-A (40 mg) was dialyzed against the same buffer as above and applied to a column (1.45×75 cm), and then developed upward at a flow rate of 2.4 ml/hr. Five ml fractions were collected.
C) The third gel filtration. The fraction II-2 in Figure 3-B (12.9 mg) was dialyzed against the same buffer as above, and applied to a column (1.45×75 cm), and then developed upward at a flow rate of 2.4 ml/hr. Five ml fractions were collected.

TABLE III. Yields and Hypocalcemic Activities of the Fractions obtained from Gel Filtration

Fractions	Yield (%)	Hypocalcemic activity: Dose (mg/kg)	Hypocalcemic activity: Percent decrease of serum calcium mean±SE ($n=6$)
The DEAE-cellulose fraction D	100	0.5	10.4±2.0[a]
I	21	0.1	5.6±2.0[b]
II	40	0.1	9.9±1.9[a]
III	33	0.1	3.7±1.2[b]

a) significantly different from saline control, $p<0.01$
b) $p>0.05$

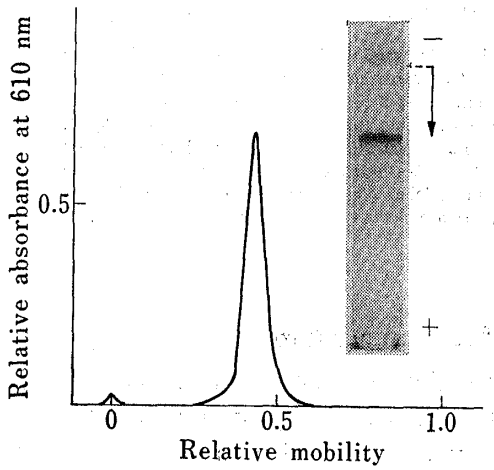

Fig. 4. Polyacrylamide Gel Electrophoresis of the Porcine Hypocalcemic Protein obtained in Step 4

Electrophoresis was carried out on a 7.5% gel at pH 8.6, and then the stained gel was scanned densitometrically at 610 nm.

Table IV. Purification of the Hypocalcemic Protein from 10 kg of Porcine Parotid Gland

Purification steps	Yield (mg)	Yield (%)	Hypocalcemic activity: Dose (mg/kg)	Hypocalcemic activity: Percent decrease of serum calcium mean ± SE[a]	Purification (×)
Step 1. acetone powder	203000	100	5	9.0±1.6[b]	1
Step 2. the 7—15% $(NH_4)_2SO_4$ fraction	3654	1.80	1	10.1±1.9[c]	>5
Step 3. the DEAE-cellulose fraction D	238	1.117	0.5	10.4±2.0[c]	>10
			0.2	6.0±1.0[c]	
Step 4. Sephadex G-200 gel filtration					
The 1st run (fraction II)	50.4	0.025	0.1	9.9±1.9[c]	>50
The 2nd run (fraction II-2)	14.9	0.007	0.05	16.1±3.1[c]	>100
The 3rd run (fraction II-2-2)	7.1	0.003	0.02	12.5±1.3[c]	>250

a) mean ± standard error; six rabbits in each group
b) significantly different from saline control, $p < 0.05$
c) significantly different from saline control, $p < 0.01$

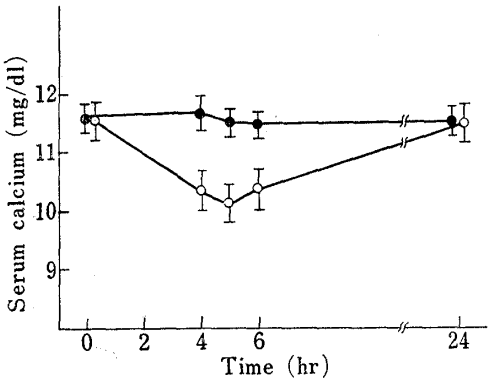

Fig. 5. Time Course of the Porcine Hypocalcemic Protein on Serum Calcium Level in Rabbits

Rabbits were subjected to 24-hr fasting and injected intraveneously with the protein of the fraction II-2-2 (0.02 mg/kg body weight) (—○—) or with saline (0.5 ml/kg body weight) (—●—). After administration, the treated rabbits were bleed at the times indicated in the figure and then the serum calcium concentration was determined. The values are means ± SE determined in six rabbits.

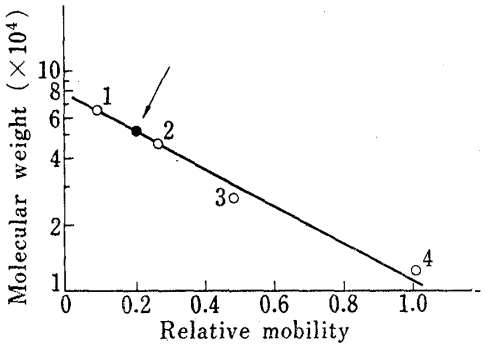

Fig. 6. Determination of the Molecular Weight of the Porcine Hypocalcemic Protein

Standards are: 1, bovine serum albumin (66000); 2, ovalbumin (46000); 3, chymotrypsinogen A (25000); 4, horse heart cytochrome *c* (12400). Arrow (↙) indicates the porcine hypocalcemic protein.

Amino Acid Composition

Table V shows the result of amino acid analysis of the porcine HC protein, including the result of the bovine HC protein recently reported by Mizutani, *et al.*[11] for comparison. Aspartic and glutamic acids, leucine, threonine, and valine were the predominant amino acids in the porcine HC protein.

Carbohydrate Content and Other Properties

The carbohydrate content of the porcine HC protein was quantified to be 1.78%. The UV absorption spectrum of the fraction II-2-2 has an absorption maximum at 278 nm and the ORD spectrum revealed a negative Cotton effect at 233 nm.

Table V. Amino Acid Composition of the Hypocalcemic Proteins from Porcine and Bovine Parotid Glands

Amino acid	Porcine[a] moles/52000 g protein			Bovine[b] moles/48000 g protein
	Hydrolysis		Maximum or extrapolated value	
	24 hr	72 hr		
Aspartic acid	44.4	43.9	44.4	48.8
Threonine	31.9	30.1	32.8[c]	19.2
Serine	22.2	18.5	24.6[c]	20.8
Glutamic acid	43.5	42.6	43.5	66.2
Proline	18.0	16.9	18.8	3.4
Glycine	23.9	23.6	23.9	24.2
Alanine	27.7	28.9	28.9	37.9
Half cystine	10.2	8.8	10.2	2.8
Valine	31.7	34.4	34.4	19.6
Methionine	1.3	0.7	1.3	7.3
Isoleucine	23.7	26.3	26.3	22.0
Leucine	31.4	31.6	31.6	48.1
Tyrosine	11.1	9.9	11.1	8.4
Phenylalanine	18.0	17.4	18.0	8.1
Lysine	5.4	5.3	5.4	25.6
Histidine	16.6	15.9	16.6	3.9
Arginine	19.2	18.0	19.2	25.1

a) this report
b) the result of Mizutani, *et al.*[11]
c) the recovery extrapolated to zero hydrolysis time

Discussion

From porcine parotid gland, an HC protein was isolated here for the first time. The porcine HC protein possesses a biological activity nearly equal to that from bovine. However,

Table VI. Comparison between the Porcine and Bovine Hypocalcemic Proteins

	Porcine[a]	Bovine[b]
Hypocalcemic activity	12.5±1.3% at a dose of 20 μg/kg (0.38 nmoles/kg)	10.8±1.0% at a dose of 8 μg/kg (0.17 nmoles/kg)
Isoelectric point	pH 4.1—4.4[c]	pH 5.3
(K_{av}) in gel filtration	Sephadex G-200 ($K_{av}=0.4$)	Sephadex G-200 ($K_{av}=0$) Sepharose 6B ($K_{av}=0.3$)
Molecular weight	52000	48000
Electrophoretic mobility	0.43	0.28
Amino acid composition	rich in:	rich in:
	Asp, Glu, Leu	Asp, Glu, Leu
	Pro	—
	Cys	Met
	Thr, Val	Ala
	His/Lys=3.07	His/Lys=0.15
	acidic/basic=2.13	acidic/basic=2.11
Carbohydrate content	1.78%	0.50%

a) this report
b) the result of Mizutani, *et al.*[11]
c) tentatively measured by zone electrophoresis with cellulose-acetate membrane

some differences in properties were observed between the porcine and bovine HC proteins, as shown in Table VI.

In step 1 of purification, the porcine active protein was successfully precipitated to obtain a crude material at an acidic pH of 4.8 from the aqueous extract of the parotid gland. At the same pH (5.4) as employed for the preparation of bovine crude material, only a small amount of precipitate had been formed.

In step 2, the 7—15% $(NH_4)_2SO_4$ fraction was obtained by fractionation under the condition of a lower pH 6.0 than that of the bovine preparation (pH 6.8). These observations in step 1 and 2 may reflect the fact that the porcine and bovine active proteins have different isoelectric points from each other. The isoelectric point of the porcine HC protein was tentatively measured by zone electrophoretic techniques with cellulose-acetate membrane to be pH 4.1—4.4 (unpublished data from our laboratory), which is lower than that of the bovine HC protein (pH 5.3).

In step 3, the partially purified preparation of porcine (the 7—15% $(NH_4)_2SO_4$ fraction) was further fractionated into seven fractions by column chromatography on DEAE-cellulose. Hypocalcemic activity was confined in three fractions: the fraction D was most potent, the fractions A and E were less so. The others were not potent. The porcine fraction D possessed a hypocalcemic activity nearly equal to the corresponding bovine fraction D-III. The fractions A and E will be reported elsewhere.

In step 4, the porcine HC protein from the fraction D was successfully purified by gel filtration on Sephadex G-200 (K_{av}=0.4) to a homogeneous state as judged by disc electrophoresis. On the other hand, the bovine HC protein had passed through Sephadex G-200 column (K_{av}=0), although its molecular weight (48000) is rather smaller than that of the porcine HC protein (52000). Consequently, the bovine HC protein had been purified by gel filtration on Sepharose 6B (K_{av}=0.3). These observations suggest that the bovine HC protein may have some molecular shapes with an unusually large dissymmetry.

On disc electrophoresis (7.5% gel, pH 8.6), the porcine HC protein moved as a single band with a mobility of 0.43 faster than the bovine HC protein (0.28). This result indicates that molecular weight is not inversely proportional to electrophoretic mobility in the cases of the porcine and bovine HC proteins. This discrepancy may reasonably be due to both an unusual shape of the bovine HC protein molecule as above and more negatively charged groups on the porcine HC protein as expected from its lower isoelectric point.

The time course of the porcine HC protein on serum calcium level in rabbits is analogous to that of the bovine HC protein: that is, both the porcine and bovine HC proteins cause the maximum hypocalcemic effect 4—6 hr after the administration, and then the effect disappears by 24 hr. On the other hand, these time courses and molecular weight of the porcine and bovine HC proteins are distinctly different from that of the porcine calcitonins.[18]

A comparison of the amino acid composition between the porcine and bovine HC proteins indicates the following: (*a*) a similarity in that they are rich in aspartic and glutamic acids, and in leucine; (*b*) some differences in that the porcine HC protein has a relatively higher content of proline, and higher contents of histidine and cystine with supplementally lower contents of lysine and methionine, respectively, as compared with the bovine HC protein. A characteristic feature of the porcine HC protein in amino acid composition is a histidine/lysine ratio of 3.07, since the ratio is below 1.00 in many kinds of proteins with a few exceptions like porcine calcitonin, collagenase, γ-crystalline, and cytochrome b_5 and *c*, *etc.*[19] The porcine

18) *a*) D.H. Copp, C.E. Brooks, B.S. Low, F. Newsome, R.K. O'dor, C.O. Parkes, V. Walker, and E.G. Watts, "Calcitonin 1969 (Proceedings of the Second International Symposium, London, 1969)," William Heinemann Medical Books Ltd., London, 1970, pp. 281—294; *b*) R. Maier, R. Neher, W. Rittel, and M. Staehelin, "Calcitonin 1969 (Proceedings of the Second International Symposium, London, 1969)," William Heinemann Medical Books Ltd., London, 1970, pp. 381—385.

19) D.M. Kirschenbaum, *Anal. Biochem.*, **44**, 159 (1971); *idem*, *ibid.*, **49**, 248 (1972); *idem*, *ibid.*, **52**, 234 (1973); *idem*, *ibid.*, **61**, 567 (1974).

calcitonin molecule contains one mole of histidine, but lysine is absent.[20] The ratio of acidic/basic amino acid residues in the porcine HC protein (2.13) was slightly larger than that of the bovine HC protein (2.11). However, acidic amino acids may be partially in the amide form in the protein molecule. Using the data presented in Table V, we have calculated the Metzger Different Index[21] to be 22.1. This value suggests, according to the view of Metzger, the possible presence of a sequence homology of 30—40% between the porcine and bovine parotid HC proteins.

20) H.B. Brewer, H.T. Keutmann, J.T. Potts, R.A. Reisfeld, R. Schlueter, and P.L. Munson, *J. Biol. Chem.*, **243**, 5739 (1968).
21) H. Metzger, M.B. Shapiro, J.E. Mosimann, and J.E. Vinton, *Nature*, **219**, 1166 (1968).