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An Active Core in a Limited Chymotryptic Digest of Bovine Parotid Hypocalcemic Substance[1]

Takaharu Mizutani, Kazuko Watanabe, Reiko Shimizu, Po-Feng Kuo, and Akira Mizutani

Faculty of Pharmaceutical Sciences, Nagoya City University[2]

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An active core in a limited chymotryptic digest of a bovine parotid hypocalcemic substance was purified and some properties of that core were investigated. After digestion of hypocalcemic protein (400 mg) for 2 hr with chymotrypsin (8 mg) at 37°, the mixture (20 ml) was chromatographed at 4° on a column of Sephadex G-100 (4×70 cm) and the fraction eluted at Kav 0.2 was obtained. The fraction was active on hypocalcemic activity and further rechromatographed on Sepharose 6B in 6 M guanidine hydrochloride (Gdn-HCl)–0.1 M Tris-HCl at pH 8.0. Major fraction on rechromatography (Kd 0.49) was purified by preparative disc electrophoresis and the purified protein (CPD-P) was active in a dose of 0.06 mg/kg (Ca lowering rate 6.5±1.1%). Molecular weight of CPD-P was 11000 by sodium dodecyl sulfate gel (20%) electrophoresis and 12500 by gel chromatography in 6 M Gdn-HCl. The amino-terminal sequence of CPD-P was Lys-Leu- identical to that of native hypocalcemic protein and the carboxyl-terminal amino acid was phenylalanine. Amino acid composition of CPD-P was shown and secondary structure of CPD-P was composed of α-helix (90%) by the measurement of CD spectrum.

Keywords——limited chymotryptic digest; active core; parotin; hypocalcemic protein; bovine parotid gland

We have reported some biochemical properties of a hypocalcemic substance purified from a precipitate at pH 5.4 of the extract of bovine parotid gland, such as amino acid composition, amino-, carboxyl-terminal sequences, and the secondary structure by the circular dichroism studies.[3] Recently, we also suggested by the study of chemical modification of the hypocalcemic protein that histidyl, tryptophanyl, and tyrosyl residues might play a role for appearance of hypocalcemic activity of the protein but methionyl and lysyl residues were not.[4] Meanwhile, the hypocalcemic protein has a lymphocyte-increasing action and an action increasing antibody-producing cells by stimulation of the mesenchymal system in mice.[5] Ito reported that a limited chymotryptic digest of partially purified protein had the activity.[6] Li, *et al.* showed that limited digestion of human growth hormone or ovine prolactine with chymotrypsin caused only a slight loss of the biological activity.[7]

In this paper we report isolation and characterization of an active core in a limited chymotryptic digest of a parotid hypocalcemic substance.

1) T. Mizutani, P.-F. Kuo, and A. Mizutani, *Seikagaku* (in Japanese), **48**, 466 (1976).
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya 467, Japan.*
3) *a*) A. Mizutani, T. Kitamura, N. Yamada, H. Inaba, Y. Naito, and T. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **22**, 1955 (1974); *b*) A. Mizutani, T. Mizutani, and P.-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **24**, 2733 (1976); *c*) A. Mizutani, T. Mizutani, and P.-F. Kuo, *Chem. Pharm. Bull.* (Tokyo), **25**, 1285 (1977); *d*) T. Mizutani, P.-F. Kuo, and A. Mizutani, *Chem. Pharm. Bull.* (Tokyo), **25**, 2821 (1977).
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7) *a*) C.H. Li, *Federation Proc.*, **16**, 775 (1957); *b*) A. Trenkle, C.H. Li, and N.R. Moudgal, *Arch. Biochem. Biophys.*, **100**, 255 (1963); *c*) M. Sluyser and C.H. Li, *Biochim. Biophys. Acta*, **82**, 595 (1964).

Materials and Methods

Materials——The sample used was prepared according to the method described previously.[3a] Chymotrypsin A_4 was obtained from Boehringer Mannheim Co.

Digestion——Hypocalcemic protein was dissolved in a concentration of 20 mg/ml in 0.05 M phosphate buffer (pH 7.4). Chymotrypsin was added in a concentration of 0.4 mg/ml and the solution was incubated at 37°.

Preparation and Purification of Limited Digests——After limited digestion of the hypocalcemic protein with chymotrypsin, the solution (20 ml) containing limited digests was cooled and applied on a column (4×70 cm) of Sephadex G-100, and was eluted with 0.05 M phosphate buffer (pH 7.4). Gel chromatography was operated at 4° and the flow-rate of 0.1 ml/min·cm². The fractions of 10 ml were collected and a protein fraction eluted at Kav 0.2 was dialyzed against deionized water and followed by lyophilization. The fraction (30 mg) was dissolved in 1 ml of 6 M guanidine hydrochloride (Gdn-HCl)–0.1 M Tris-HCl pH 8.0 and chromatographed on a column (1.1×61 cm) of Sepharose 6B in 6 M Gdn-HCl–0.1 M Tris-HCl pH 8.0 at the flow-rate of 0.05 ml/min·cm² at room temperature. The main fraction eluted at Kd 0.49 was obtained and this fraction was further purified by preparative polyacrylamide gel disc electrophoresis and gel chromatography on Sepharose 6B according to the method of purification of native hypocalcemic substance.[3a]

General Methods——Bioassay and the measurements of time course of hypocalcemic activity were done by the methods described previously.[8] Serum calcium was determined with a Shimadzu atomic absorption spectrophotometer at 422.7 nm.[3b] After hydrolyzation of sample with 6 N HCl for 24 and 72 hr at 110±0.5°, the resulting amino acids were analyzed according to the earlier method.[3a] Molecular weight was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel (20%) electrophoresis[9] and by gel chromatography on Sepharose 6B in 6 M Gdn-HCl–0.1 M Tris-HCl.[10] Reference proteins used were bovine serum albumin (BSA), chymotrypsinogen A, cytochrome c, and insulin. Circular dichroism (CD) spectrum was measured at 21° using a JASCO model J-20 automatic recording spectro-polarimeter. The content of α-helix in the protein molecule was determined by using a mean residue molecular weight of 119 by the method described by Chen and Yang.[11] The amino-terminal sequence was determined by the dansyl (DNS)-Edman method in the presence of SDS and the carboxylterminus was analyzed by the micro hydrazinolysis-dansylation method.[3c] Analytical polyacrylamide gel electrophoresis using 7.5% gel was carried out by the method of Davis.[12] Protein concentration in the solution was determined by ultraviolet absorption at 280 nm or by the method of Lowry, *et al.*[13]

Results

Preparation and Purification of a Limited Digest

Disc electrophoretic patterns of the products obtained by the digestion with chymotrypsin for 10 min, 2 hr, and 7 hr are shown in Fig. 1B, C, and D, respectively. The pattern of products in the digestion for 10 min shows that the digest contained the proteins showing relative mobility of 0.7 to BPB even though the mobility of native hypocalcemic protein was 0.3 as shown in Fig. 1A. Major products in the digestion for 7 hr moved at the identical point with BPB marker. The products in the digestion for 2 hr contained proteins having the relative mobility of 0.8 on a gel of disc electrophoresis. Similar results were obtained from the patterns of gel chromatography. The elution patterns of the hydrolyzates (100 mg) in the digestion of hypocalcemic protein for 10 min, 7 hr, and 45 hr on a column (4×70 cm) of Sephadex G-100 are shown in Fig. 2A, B, and C, respectively. Major fragments obtained by the digestion for 10 min were eluted at void volume of the column of Sephadex G-100 (native protein was eluted at void volume) and the products in the digestion for 7 hr or 45 hr were also mainly composed of the substance eluted at total volume of the column. The

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9) A.K. Dunker and R.R. Rueckert, *J. Biol. Chem.*, **244**, 5074 (1969).
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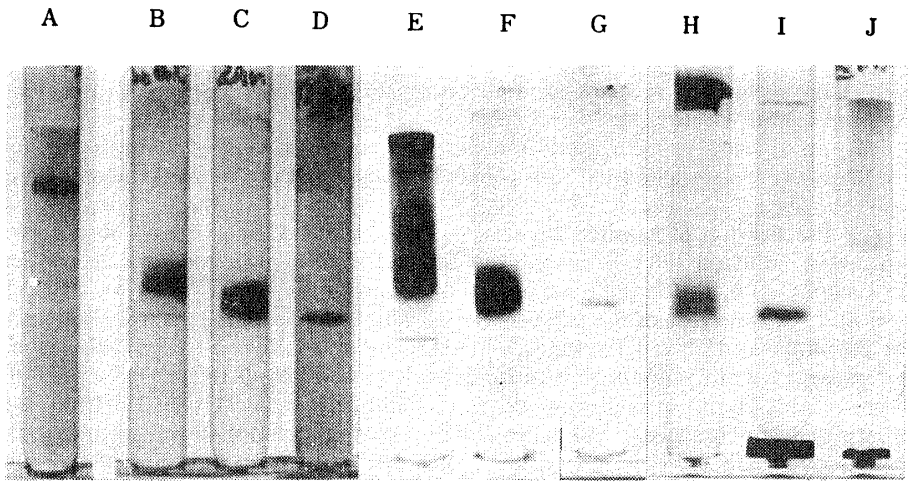

Fig. 1. Polyacrylamide Gel Electrophoretic Patterns

A-I, disc electrophoresis; A, the native hypocalcemic protein; B, the sample obtained by the digestion with chymotrypsin for 10 min; C, the sample digested for 2 hr; D, the sample digested for 7 hr; E, G-I in Fig. 3A; F, G-II in Fig. 3A; G, G-IV in Fig. 3A; H, CPD-III in Fig. 4; I, CPD-P; J, CPD-P by SDS electrophoresis.

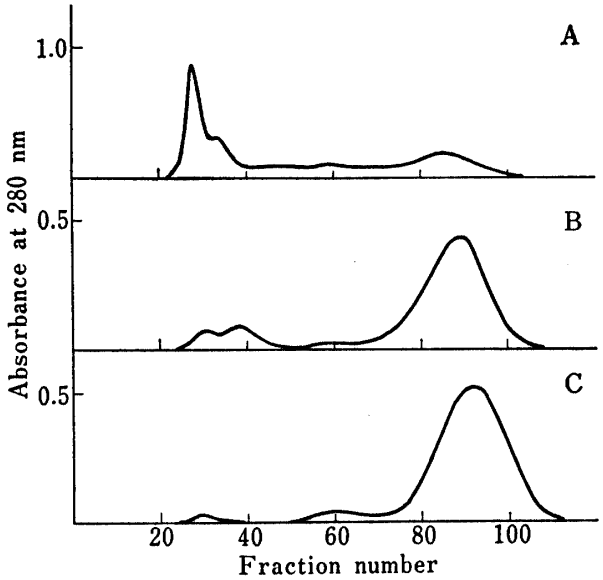

Fig. 2. Elution Patterns of Digests with Chymotrypsin in Gel Chromatography on Sephadex G-100

Digests (100 mg) were chromatographed in 0.05 M phosphate buffer (pH 7.4) on a column (4×70 cm) and fraction volume was 10 ml. A, the sample digested for 10 min; B, for 7 hr; C, for 45 hr.

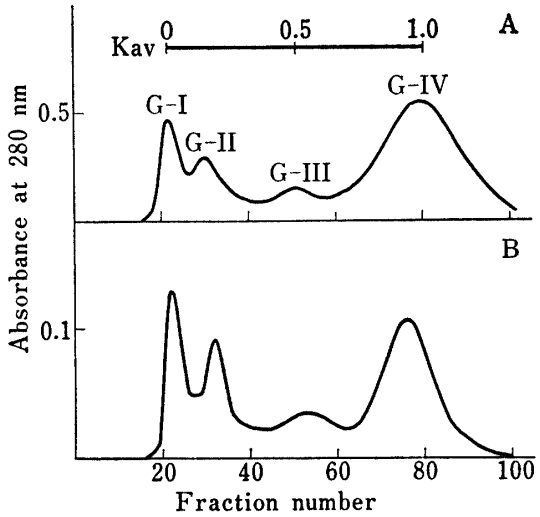

Fig. 3. Elution Patterns of Limited Chymotrytic Digests in Gel Chromatography on Sephadex G-100

Products obtained by the digestion for 2 hr were chromatographed in 0.05 M phosphate buffer (pH 7.4) on a column (4×70 cm). A, the sample (400 mg) of 90% purity; B, pure sample (100 mg).

elution pattern of the products in the digestion for 2 hr using purified hypocalcemic protein is shown in Fig. 3A. The digest contained the substances eluted at Kav 0.2 (tubes 28—35) on the column and this fraction was of a limited chymotryptic digest. The disc gel patterns of the fractions in Fig. 3A are shown in Fig. 1E—G. The pattern of a limited digest of the partially purified protein (90% purity), which was obtained by gel chromatography on Sepharose 6B,[3a] is also shown in Fig. 3B and the disc gel patterns were identical with that of Fig. 3A. Therefore, we used the protein of 90% purity for a large scale preparation of an active limited digest. G–I contained an undigested protein and a limited digest of large

molecular weight as shown in Fig. 1E. G–II eluted at 0.2 of Kav value contained a limited digest which had the relative mobility of 0.8 on a disc gel, but this fraction gave a relatively broad band as shown in Fig. 1F. The yield of G–II was 8—10% of total protein applied on the column. G–IV was of the fragments moved at the top on a disc gel (Fig. 1G). Chymotrypsin was eluted at 0.5 of Kav value, which was ascertained from the digestion of BSA with the fraction. The mobility of digested BSA was changed from that of native BSA. Hypocalcemic activity of G–I, G–II, G–IV, and chymotrypsin are shown in Table I. Chymotrypsin did not show the hypocalcemic activity in a dose of 0.8 mg/kg and G–IV was inactive in a dose of 0.8 mg/kg but G–II was active in a dose of 0.3 mg/kg. Fig. 4 shows the elution pattern of G–II on Sepharose 6B in 6M Gdn-HCl containing 0.1 M Tris-HCl (pH 8.0). Main peak (CPD–III) eluted at 0.49 of Kd value (tubes 51—56 in Fig. 4) was collected, dialyzed, and lyophilized (yield: 23%). Although the stained gel pattern of G–II (Fig. 1F) shows

Table I. Hypocalcemic Activities and Yields of the Fractions obtained from Gel Chromatography

Fractions	Dose (mg/kg)	Mean ±S.E. ($n=6$) Percent decrease of serum calcium	Yield (%)
Native hypocalcemic protein	0.03	6.6±0.9[a]	
G–I	0.3	6.5±0.6[b]	15[c]
G–II	0.3	11.7±2.1[b]	8[c]
G–IV	0.8	0.8±0.6[d]	70[c]
Chymotrypsin	0.8	2.4±1.7[d]	
CPD–P (Lot 1)	0.06	6.5±1.1[b]	2.3[e]
(Lot 1)	0.03	3.7±1.8[d]	
(Lot 2)	0.06	7.7±0.7[b]	3.2[e]
Control		1.6±0.5	

a) significantly different from saline control, $p<0.05$.
b) significantly different from saline control, $p<0.01$.
c) The percent yields were estimated on the basis of total proteins applied on gel chromatography.
d) not active >0.05.
e) The yields were estimated on the basis of total proteins applied on Sepharose 6B in 6M Gdn-HCl.

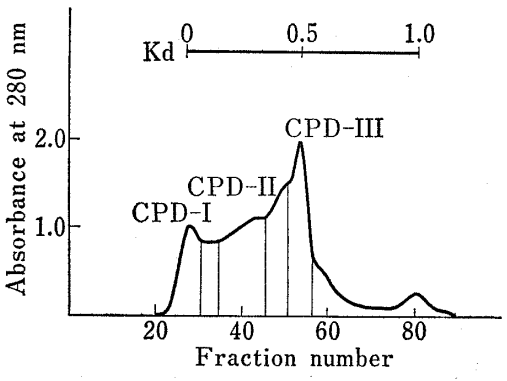

Fig. 4. Elution Pattern of G–II in Gel Chromatography on Sepharose 6B

G-II (30 mg) was chromatographed in 6M Gdn-HCl-0.1M Tris-HCl pH 8.0 on a column (1.1×61 cm) and fraction volume was 0.7 ml.

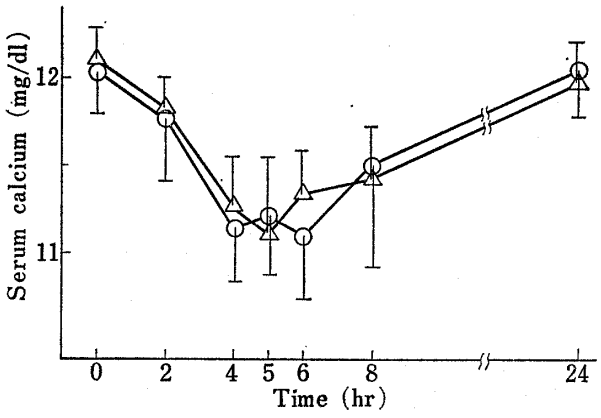

Fig. 5. Time Courses of the Hypocalcemic Proteins on Serum Calcium Level in Rabbits

Rabbits were subjected to 24 hr fasting and injected intravenously with CPD-P (0.06 mg/kg body weight, triangles) or native hypocalcemic protein (0.03 mg/kg body weight, open circles). After administration, the treated rabbits were bleed at the times indicated in the figures and then the serum calcium concentration was determined. The values are means ±S.E. determined in six rabbits.

overlapping of major three protein species, the pattern of the stained disc gel (Fig. 1H) indicates that CPD–III is composed of one major protein band moving at relative mobility of 0.8 and other minor two bands. CPD–III was thoroughly purified by preparative polyacrylamide gel electrophoresis and main protein fraction in CPD–III was sliced out from the original gel, using the stained gel specimen as a reference.[3a] After extraction of protein from the gel, gel particles in the extract were removed by gel chromatography on Sepharose 6B in 0.05 M phosphate buffer and the yield of the pure substance isolated by preparative gel electrophoresis and second gel chromatography was 2.3% on the basis of total protein applied on Sepharose 6B in 6 M Gdn-HCl. The purified substance was designated as CPD–P. The patterns obtained by analytical disc electrophoresis and SDS-gel electrophoresis are shown in Fig. 1I and J, and these results show that CPD–P is uniformly homogeneous. After preservation of CPD–P for long time in a vacuum desiccator, the sample was still easily soluble, although the solubility of native hypocalcemic protein decreased during long preservation in a desiccator. CPD–P was also more soluble in buffers than native protein.

Characterization of CPD-P

The hypocalcemic activity and the yield of CPD–P are shown in Table I and CPD–P is active in a dose of 0.06 mg/kg (Ca lowering rate 6.5±1.1%) but inactive in a dose of 0.03 mg/kg. This activity was found in another lot of CPD–P (the rate 7.7±0.7%). The time courses of CPD–P and native hypocalcemic protein are shown in Fig. 5 and the both proteins caused the maximum hypocalcemia at 4—6 hr after the administration. These results suggest that the hypocalcemic protein of parotid gland is not a precursor of calcitonin, because the maximum lowering time of calcitonin was at 1 hr after administration in rabbits.[14] Molecular weight of CPD–P obtained by SDS gel electrophoresis and by gel chromatography on Sepharose 6B in the Gdn-HCl solution is shown in Fig. 6 and the values obtained were 11000

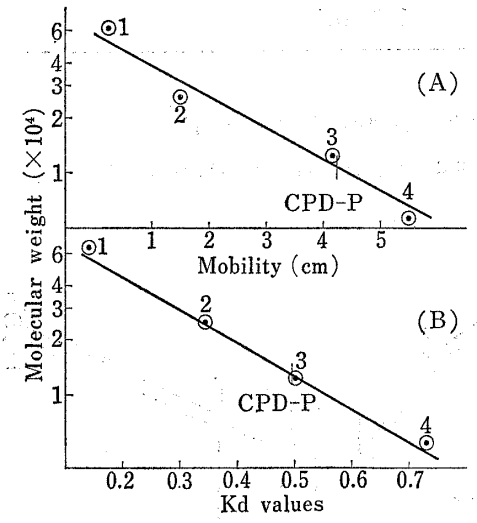

Fig. 6. Molecular Weight Determination of CPD–P

Figure A shows the results obtained by SDS gel (20%) electrophoresis and B by gel chromatography on Sepharose 6B in 6M Gdn-HCl-0.1M Tris-HCl (pH 8.0). Calibration curve shows in terms of semilogarithmic plots of molecular weight *versus* mobility for figure A and Kd values for figure B. CPD-P is represented by longitudal bar. The protein standards are represented by circles: 1, bovine serum albumin; 2, chymotrypsinogen A; 3, cytochrome c; 4, insulin.

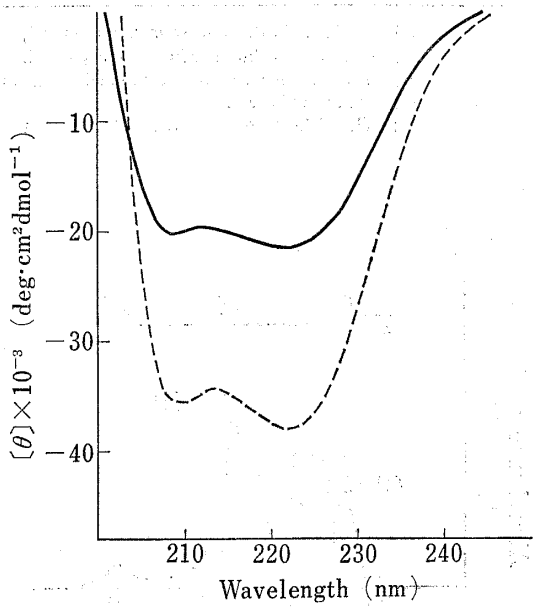

Fig. 7. CD Spectra of the Hypocalcemic Proteins

Spectra of the proteins dissolved in 0.2M NaCl and 0.01M phosphate buffer (pH 7.3) were measured at 20°. Broken line, CPD-P; straight line, native hypocalcemic protein.

14) E. Ogata, H. Suzuki, E. Shimazawa, K. Nakanowatari, and H. Asano, *Endocrinol. Japan*, **18**, 235 (1971).

and 12500, respectively. Molecular weight of native protein was 46500±1500,[3b] and hence molecular weight of CPD–P was approximately one-fourth of native protein. The results of amino acid analyses of CPD–P are shown in Table II. CPD–P did not contain methionine, histidine, cystine, and tryptophan, but CPD–P contained large amount of aspartic acid, glutamic acid, isoleucine, and alanine. Amino acid composition of CPD–P was different from that of calcitonin,[15] especially calcitonin did not contain lysine and isoleucine which were contained in CPD–P. The CD spectra of CPD–P and native protein are shown in Fig. 7 and the pattern indicated that the secondary structure of CPD–P was almost composed of α-helix (about 90%). This rigid α-helix core in native protein must not be digested by limited digestion with chymotrypsin. The amino acids, such as glutamic acid and alanine which

Table II. Amino Acid Composition of the Hypocalcemic Proteins

Amino acid	Mol/mol of protein		
	CPD–P	Native hypocalcemic protein[a]	Calcitonin[b]
Aspartic acid	16.9	48.8	4
Threonine	5.0	19.2	2
Serine	3.5	20.8	4
Glutamic acid	25.6	66.2	1
Proline	1.1	3.4	2
Glycine	4.0	24.2	3
Alanine	6.7	37.9	1
Half cystine	0	2.8	2
Valine	4.7	19.6	1
Methionine	0	7.3	1
Isoleucine	7.5	22.0	0
Leucine	3.7	48.1	3
Tyrosine	2.8	8.4	1
Phenylalanine	2.9	8.1	3
Tryptophan	0	5.1	1
Lysine	8.0	25.6	0
Histidine	0	3.9	1
Arginine	6.3	25.1	2

a) The results from Mizutani, *et al.*[3a]
b) Cited from reference 15.

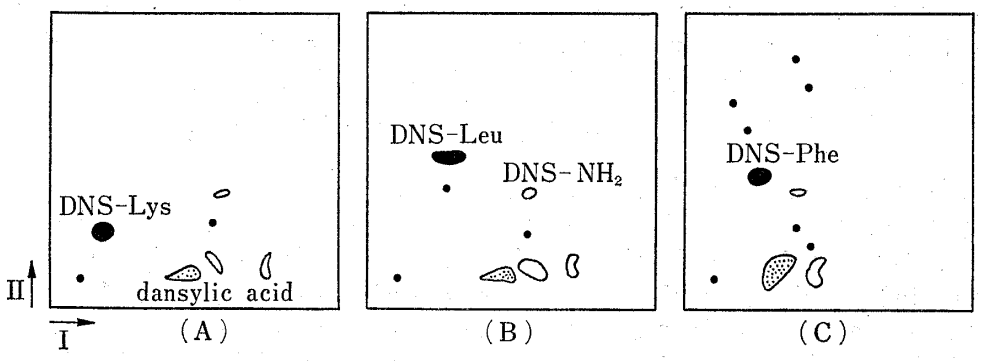

Fig. 8. Chromatograms of Dansyl-amino Acid obtained by the Dansyl-Edman Method and Hydrazinolysis-dansylation Procedures on Polyamide Plates

A, dansyl-amino acid obtained from the amino-terminus of modified CDP-P; B, dansyl-amino acid of the second amino acid from the terminus of CDP-P; C, dansyl-amino acid obtained from the carboxyl-terminus of CDP-P by hydrazinolysis-dansylation procedures.

15) O.K. Behrens and E.L. Grinnan, *Ann. Rev. Biochem.*, **38**, 83 (1969).

were contained in CPD–P in large amount as described above, might contribute to forming α-helix. The amino-terminal sequence was determined as Lys-Leu- as shown in Fig. 8A and B and this sequence was identical with that of native protein. Carboxyl-terminal amino acid was determined as phenylalanine as shown in Fig. 8c.

Discussion

Some properties of CPD–P and native parotid hypocalcemic protein are compared in Table III. The amino-terminal sequences of both proteins were identical Lys-Leu- and those results suggest that CPD–P might be situated at the amino-terminal of native protein and the active core of parotid hypocalcemic protein was at the amino-terminal of the protein. Parathyroid hormone also have an active core on the amino-terminal.[16] Since the amino-terminal amino acid of G–II was also ascertained as Lys, we supposed that CPD–I and II were larger limited digests containing the amino acid sequence of CPD–P. G–II also contained the major fraction of molecular weight of 12000 and the minor fractions of molecular weight of 25000.

Table III. Comparison between CPD–P and Native Hypocalcemic Protein

	CPD–P	Native hypocalcemic protein[a]
Molecular weight	12000±1000	46500±1500
Helix content	90%	54%
Amino-terminal	Lys-Leu-	Lys-Leu-
Carboxyl-terminal	-Phe	-Leu
Kav values	Sephadex G-100 (Kav=0.2)	Sepharose 6B (Kav=0.3)
Relative mobility to BPB on disc gel	0.8	0.3
Maximum Ca lowering time	4—6 hr	4—6 hr
Minimum effective dose	0.06 mg/kg	0.01—0.03 mg/kg
Isoelectric point	pH 5.5	pH 5.3

a) cited from references 3*a*, 3*b*, and 3*c*.

Molecular weight of CPD–P was one-fourth of native hypocalcemic protein which was active in a dose of 0.03 mg/kg; therefore the specific activity of CPD–P was approximately one tenth of native protein. This decrease of the specific activity might be due to the collapse of the conformation of the active state by gel chromatography in the Gdn-HCl solution or the removal of the site necessary to reinforce the hypocalcemic activity by digestion with chymotrypsin. However, the purified fraction of limited digest, CPD–P, clearly has hypocalcemic activity and CPD–P supposedly is an active core contained in native hypocalcemic protein. In previous report, the sample dialyzed after incubation in 6 M Gdn-HCl showed no activity by administration of the same dose of the sample as the minimum effective dose of native protein.[3d] However, if the ten-fold of minimum effective dose of native protein was used like the case of CPD–P, the sample treated with Gdn-HCl might be effective.

We reported in this paper about an active core of molecular weight of 12000, probably derived from the amino-terminus of the bovine parotid hypocalcemic protein. Meanwhile, Aonuma, *et al.* recently isolated two active fragments produced from similar bovine parotid hypocalcemic protein by digestion with trypsin.[17] One active fragment of molecular weight

16) J.T. Potts, G.H. Tregear, H.T. Keutmann, H.D. Niall, R. Sauer, L.T. Deftos, B.F. Dawson, M.L. Hogan, and G.D. Aurbach, *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 63 (1971).

17) S. Aonuma, Y. Kohama, Y. Kamiyama, S. Nakajin, and Y. Yamada, *Nippon Naibumpi Gakkai Zasshi*, **53**, 821 (1977).

of 9100 had an antigenic activity and an increasing activity in the rabbit-circulating leucocyte number but had not hypocalcemic activity. Another active fragment of a pentapeptide had only the hypocalcemic activity. Since amino-terminal amino acids of those fragments were leucine and aspartic acid, the amino terminuses of those fragments were different from that of CPD–P which we described in this paper as to be lysine of amino-terminus. We suppose that the active pentapeptide might be contained in CPD–P molecule if both the native hypocalcemic substances were identical.