[Chem. Pharm. Bull. 28(2) 417—423 (1980)]

Amino Acid Sequence of an Active Fragment, Fr. AA-1, of Salivary Gland Hormone

Shigeru Aonuma, Yasuhiro Kohama, Yutaka Komiyama, Shigeko Fujimoto, and Masato Nomura

Faculty of Pharmaceutical Sciences, Osaka University[1]

(Received May 14, 1979)

The complete amino acid sequence of a biologically and immunologically active fragment, Fr. AA-1, of salivary gland hormone (parotin) has been established through the uses of combined dansyl-Edman procedure, manual Edman degradation and standard enzymatic and chemical techniques. The binding site of the carbchydrate moiety was determined from the amino acid composition of the carbohydrate moiety obtained from digests of Fr. AA-1 with pronase. Fr. AA-1 was shown to be a glycopeptide consisting of 58 amino acid residues. Its sequence is: H_2N–Leu–Tyr–Ile–Leu–Tyr–Phe–Phe–Gln–Ser–Asp–Asn–Glu–Asp–Lys–Glu–Lys–Val–Val–Arg–Gln–Glu–Glu–Gly–Glu–Glu–Arg–Ile–Thr–Ala–Leu–Leu–Met–Asn(carbohydrate moiety)–Gly–Ser–Ala–Leu–Lys–Gln–Glu–Glu–Trp–Trp–Glu–Gly–Lys–Glu–Asp–Thr–Asp–Asp–Thr–Ala–Ile–Val–Leu–Leu–Lys–COOH.

Keywords——salivary gland hormone; active fragment; amino acid sequence; cyanogen bromide cleavage; thermolytic cleavage; hypocalcemic fragment; leucocyte-increasing fragment

The salivary gland hormone, parotin, isolated from the salivary gland, shows differentiation and growth stimulating activities on various mesenchymal tissues. Since the early work in 1944,[2] however, its structure has remained obscure, largely because its molecular weight is higher than 100000. During our studies on the chemistry of parotin, we presented evidence that parotin contains a subunit which has a molecular weight of 45000 and possesses the biological and immunological activities of parotin.[3] Previously, we isolated two active fractions (Fr. A and Fr. H) from the subunit by tryptic digestion, and obtained Fr. AA-1 and Fr. H-1, respectively, as pure active fragments of the subunit.[4] Fr.H-1 was shown to be a pentapeptide, Asp-Try-Glu-Trp-Lys, which retained the ability of parotin to decrease the calcium level in rabbit serum (Ca-activity), and Fr. AA-1 was a glycopeptide which possessed Ca-activity, the ability to increase the number of circulating leucocytes in the rabbit (L-activity), specific localization in bone and incisors as determined by mouse whole-body autoradiography, and immunological specificity in common with that of parotin and its subunit.[4] Consequently, Fr. AA-1 is a functional principle with the typical activities of parotin. It is a glycopeptide having the NH_2-terminal amino acid sequence Leu-Tyr-Ile-Leu-Tyr-Phe-Phe-Glx-, the COOH-terminal amino acid sequence -Ile-Val-Leu-Leu-Lys, and a molecular weight of less than 10000. It contains one methioninyl residue and no detectable prolinyl, cystinyl or histidinyl residues.

1) Location: *133-1, Yamadakami, Suita, Osaka.*
2) A. Ogata, Y. Ito, Y. Nozaki, S. Okabe, T. Ogata, and Z. Ishii, *Igaku to Seibutsugaku*, **5**, 253 (1944).
3) S. Aonuma, Y. Kohama, S. Nakajin, S. Yashiki, and H. Egawa, *Nippon Naibunpi Gakkai Zasshi*, **50**, 1468 (1974); S. Aonuma, Y. Kohama, S. Nakajin, S. Yashiki, H. Egawa, M. Wada, and Y. Komiyama, *ibid.*, **52**, 93 (1976).
4) S. Aonuma, Y. Kohama, Y. Komiyama, S. Nakajin, and Y. Yamada, *Nippon Naibunpi Gakkai Zasshi*, **53**, 821 (1977).

The present paper reports the complete amino acid sequence of Fr.AA-1, determined by sequential degradations together with cyanogen bromide and thermolytic cleavages of the fraction.

Experimental

Preparation of Fr.AA-1——Fr.AA-1 was purified from tryptic digests of the subunit by successive chromatographies on Sephadex G-25, Sephadex G-50 and QAE-Sephadex A-25 columns, as described by the authors.[4]

Cyanogen Bromide Cleavage——The procedure was essentially that described by Gross *et al.*[5] Fr.AA-1 (150 mg) was dissolved in 15 ml of 70% formic acid. A 30-fold molar excess of cyanogen bromide (Nakarai Kagaku) over methionine was added. The reaction was allowed to proceed for 24 hr in the dark at room temperature in a tightly stoppered flask. The reaction was terminated by the addition of 10 volumes of ice-cold water, and the solution was freeze-dried.

Thermolysin Digestion——According to the procedure of Kistler *et al.*,[6] the peptide (2 μ mol) was dissolved in 2 ml of 0.1 M N-ethylmorpholine-acetate buffer (pH 8.0) containing 0.01 M $CaCl_2$ and thermolysin (91 U/mg protein, Sigma) in a molar ratio of 1/1000. The reaction mixture was incubated at 34° for 2 hr, and the solution was freeze-dried.

Digestion with Pronase——The basic procedure was as described by Fukuda *et al.*[7] Fr.AA-1 (27.3 mg) was dissolved in 2 ml of 0.1 M tris-HCl buffer (pH 7.8) containing 0.01 M $CaCl_2$ and digested with pronase (Grade E, Kaken Kagaku) at 37°. Initially 0.27 mg of pronase was added, and a further portion of 0.14 mg was added after 24 hr. After incubation for a total period of 36 hr, the digests were freeze-dried.

Amino Acid Analysis——Peptide (50—100 n mol) was hydrolyzed in 1 ml of 6 N HCl at 110° *in vacuo* for 24 hr. Before amino acid analysis, homoserine lactone was converted to homoserine by drying the hydrolysate *in vacuo*, treating the residue with 1 ml of pyridine/acetic acid/water (10: 0.4: 90, pH 6.5) at 105° for 1 hr and again drying it.[8] The hydrolysate was then redissolved in 1 ml of 0.2 M sodium citrate buffer (pH 2.2) and immediately applied to an amino acid analyzer (Hitachi KLA-III; Hitachi custom ion-exchange resin 2611). Norleucine was used as an internal standard in all analyses.

Peptide Mapping Procedure——A modification of the procedure described by Katz *et al.*[9] was used. Chromatographies were run in a descending manner with full 38×48 cm sheets of Toyo filter paper No. 50 for 16 hr at room temperature. The paper was washed with 0.1 N HCl and water before use. The origin was approximately 4 cm below the rod over which the paper was hung. The solvent was *n*-butanol/pyridine/acetic acid/water (15: 10: 3: 12). After drying the sheet at 80° for 30 min, high-voltage paper electrophoresis (1400 V) was carried out for 1 hr in pyridine/acetic acid/water (10: 0.4: 90, pH 6.5) using a Toyo Kagaku HPE-406 machine. Peptides were located using cadmium-ninhydrin and Ehrlich stains. Glycopeptides were independently stained with silver nitrate reagent. Peptides were eluted from the paper with 10% acetic acid and freeze-dried. All purified peptides isolated were examined for homogeneity by the peptide mapping procedure.

Amino Acid Sequence Analyses——All reagents were Wako sequence-grade reagents. A modification of the combined dansyl-Edman procedure of Gray *et al.*[10] was used. Approximately 20 n mol of peptide was dissolved in 30 μl of 50% aqueous pyridine in a conical tube, and 10 μl of 20% phenylisothiocyanate in pyridine was added. After flushing with nitrogen for 15 sec, the tube was sealed with a glass stopper and incubated at 45° for 1 hr. After drying *in vacuo*, the residue was added to 20 μl of ethylalcohol and then dried again *in vacuo*. The residue was incubated at 45° for 20 min with 30 μl of trifluoroacetic acid. After drying *in vacuo*, the residue was dissolved in 100 μl of water and extracted 3 times with 400 μl of *n*-butylacetate. After the third extraction, the aqueous phase was freeze-dried and redissolved in 30 μl of 50% aqueous pyridine. A sample (approximately 1 n mol) was removed for dansylation, and remainder was treated with phenylisothiocyanate exactly as before. In this way the sequence was determined from the N-terminus. For dansylation the peptide was transferred to a tube (3.0×60 mm) and dried *in vacuo*. Twenty μl of 0.2 M $NaHCO_3$ and 10 μl of dansyl chloride solution (1 mg/ml in acetone) were mixed and incubated at 45° for 30 min. The solution was dried *in vacuo*, and 10 μl of 6 N HCl was added. The tube was sealed under a vacuum, and the dansyl peptide was hydrolyzed at 105° for 18 hr. The dansyl amino acid were determined by two-dimensional chromatography on polyamide layer sheets (5×5 cm, Cheng Chin Trading

5) E. Gross and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).
6) W.S. Kistler, C. Noyes, R. Hse, and R.L. Heinrikson, *J. Biol. Chem.*, **250**, 1847 (1975).
7) M. Fukuda and F. Egami, *Biochem. J.*, **123**, 407 (1971).
8) R.P. Ambler, *Biochem. J.*, **96**, 36P (1965).
9) A.M. Katz, W.J. Dreyer, and C.B. Anfinsen, *J. Biol. Chem.*, **234**, 1856 (1959).
10) W.R. Gray and B.S. Hartley, *Biochem. J.*, **89**, 379 (1963).

Co., Ltd.).[11] Tryptophan, asparagine and glutamine were identified by mannual Edman degradation.[12] The thiazolinone derivatives were converted to the phenylthiohydantoin derivatives by heating at 80° for 10 min in 1 N HCl, and the products were extracted with ethylacetate. The phenylthiohydantoin derivatives were identified by thin-layer chromatography on silica gel (20×20 cm, DC-Fertigplatten Kiesel Gel F 254, Merck).[13]

Carboxypeptidase digestion was performed according to the procedure of Maghuin-Rogister *et al.*,[14] as follows. Peptide (0.2 μ mol) was dissolved in 100 μl of 0.1 M tris-HCl buffer (pH 8.0). A crystalline suspension (21 mg/ml) of carboxypeptidase A (47 U/mg, DFP-treated, Sigma) and a solution (6 mg/ml) of carboxypeptidase B (101 U/mg, DFP-treated, Worthington) were diluted 10-fold in 10% LiCl, and an aliquot (approximately 10 μl or 50 μl, respectively) was added to the substrate solution. Digestions were conducted at 37° and stopped by freezing at −25°. Digests were diluted with 0.2 M sodium citrate buffer (pH 2.2) and analyzed directly with the amino acid analyzer.

Bioassay——The Ca-activity and the L-activity (typical biological activities of parotin) were determined as described by the authors.[15] During the 8 hr following injection, blood samples were drawn (at 1, 4 and 7 hr for Ca-activity and at 1, 2, 4, 6 and 8 hr for L-activity). A maximum percentage decrease of calcium level in the serum of more than 5% and a maximum increase in the number of leucocytes in the blood of more than 20%, compared with the values before injection, were considered to indicate significant activity.

Results and Discussion

Isolation of Cyanogen Bromide Cleaved Fragments of Fr. AA-1 and Analysis of Their Terminal Sequences

Cyanogen bromide fragments of Fr. AA-1 were fractionated on Sephadex G-50 to provide AA-1-1 and AA-1-2 as shown in Fig. 1, and these fractions were freeze-dried. The results of sequence analyses of both termini are shown in Table I. The NH_2-terminal sequence of AA-1-1 was Asx-Gly-Ser-Ala-Leu- and the COOH-terminal sequence was -Thr-Ala-Ile-Val-Leu-Leu-Lys, which coincided with that of Fr.AA-1.[4] The NH_2-terminal sequence of AA-1-2 was Leu-Tyr-Ile-Leu-Tyr-Phe-Phe-Glx-, which coincided with that of Fr.AA-1,[4] and the COOH-terminal sequence was -Glu-Glu-Arg-Ile-Thr-Ala-Leu-Leu-Hse including the homoserinyl residue derived from cyanogen bromide cleavage. Therefore, AA-1-1 was the COOH-terminal region (residues 33—58) and AA-1-2 was the NH_2-terminal region (1—32) of the original material, as shown in Table I. As regards the biological activities of the cyanogen bromide cleaved fragments, AA-1-1 (20 μg/kg, *i.v.*) and AA-1-2 (20 μg/kg, *i.v.*) produced 1.4% and 3.6% decreases,

Table I. Sequence Analyses of AA-1-1 and AA-1-2

Peptide	No. of residues	Amino acid sequence and composition
AA-1-1	26	33 37 Asx–Gly–Ser–Ala–Leu– [Asx(3), Thr(1), Glx(5), Gly(1), Lys(2), Trp(2)] 52 58 –Thr–Ala–Ile–Val–Leu–Leu–Lys
AA-1-2	32	1 8 Leu–Tyr–Ile–Leu–Tyr–Phe–Phe–Glx– [Asx(3), Ser(1), Glx(5), Val(2), Gly(1), Lys(2), Arg(1)] 24 32 –Glu–Glu–Arg–Ile–Thr–Ala–Leu–Leu–Hse

11) K.R. Woods and K.T. Wang, *Biochem. Biophys. Acta*, **133**, 369 (1967); S. Kimura, *Bunseki Kagaku*, **23**, 563 (1974).
12) P. Edman, *Acta Chem. Scand.*, **10**, 761 (1956).
13) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953); P. Edman and J. Sjöquist, *ibid.*, **10**, 1507 (1956); J.O. Jeppsson and J. Sjöquist, *Anal. Biochem.*, **18**, 264 (1967).
14) G. Maghuin-Rogister, J. Closset, and G. Hennen, *FEBS Lett.*, **13**, 301 (1971).
15) S. Aonuma, T. Mimura, F. Hayashi, and H. Egawa, *Yakugaku Zasshi*, **82**, 429 (1962).

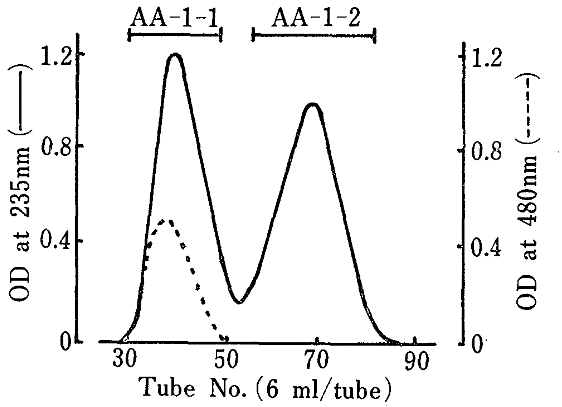

Fig. 1. Gel Filtration of Cyanogen Bromide Cleaved Fr. AA-1 on a Sephadex G-50 Column (2.6×103 cm) equilibrated with 0.05 M NH_4HCO_3

The phenol–H_2SO_4 procedure was used to determine the OD at 480 nm.

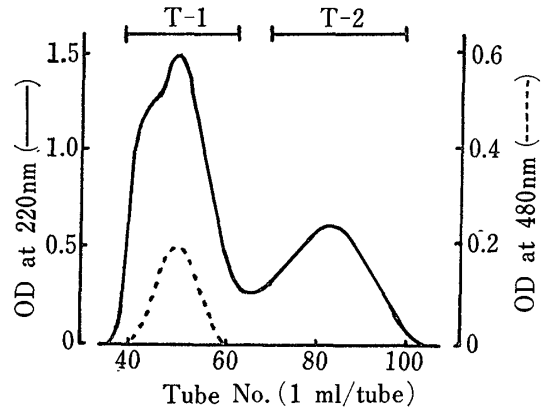

Fig. 2. Gel Filtration of Thermolytic Fragments of AA-1-1 on a Sephadex G-15 Column (1.6×44 cm) equilibrated with 0.05 M NH_4HCO_3

The phenol–H_2SO_4 procedure was used to determine the OD at 480 nm.

respectively, in serum calcium level and 17.1% and 13.8% increases, respectively, in leucocyte number. Thus, these fragments were considered to have no significant biological activities.

Purification and Amino Acid Sequence Analyses of Thermolytic Fragments of AA-1-1 and AA-1-2

Thermolytic digests of AA-1-1 were separated to three fractions, T-1, T-2, and T-3, with Sephadex G-15 as shown in Fig. 2. T-1 was further fractionated to T-1-1 and T-1-2 with Sephadex G-25 as shown in Fig. 3. T-1-1 was homogeneous. T-1-2 was separated to three homogeneous peptides, T-1-2-a, T-1-2-b and T-1-2-c, by high-voltage paper electrophoresis. T-1-1, T-1-2-a, and T-1-2-c were Ehrlich-positive, indicating the presence of tryptophan or carbohydrate. T-2 was separated into six purified components, T-2-a, T-2-b, T-2-c, T-2-d, T-2-e, and T-2-f, by paper chromatography and high-voltage paper electrophoresis. The results of sequence analyses of these fragments are given in Table II. T-1-2-c (33—35) could easily be identified as the NH_2-terminal fragment from the sequence of AA-1-1 shown in Table I. The

Table II. Amino Acid Sequence of AA-1-1

AA-1-1	33 35 40 44 Asx–Gly–Ser–Ala–Leu (Lys, Glx, Glx, Glx, Trp, Trp, Glx,
T-1-2-c	Asx–Gly–Ser
T-2-a	Ala
T-1-1	Leu–Lys–Gln–Glu–Glu–Trp–Trp–Glu–
T-1-2-a	Leu–Lys–Gln–Glu–Glu–Trp–Trp–Glu
AA-1-1	45 50 55 58 Gly, Lys, Glx, Asx, Thr, Asx, Asx) Thr–Ala–Ile–Val–Leu–Leu–Lys
T-1-1	Gly–Lys (Glx, Asx, Thr, Asx, Asx, Thr)
T-1-2-b	Gly–Lys–Glu–Asp–Thr–Asp–Asp–Thr
T-2-b	Ala–Ile–Val
T-2-a	Ala
T-2-d	Ile (Leu)
T-2-c	Val
T-2-e	Leu–Leu–Lys
T-2-d	(Ile) Leu
T-2-f	Leu–Lys

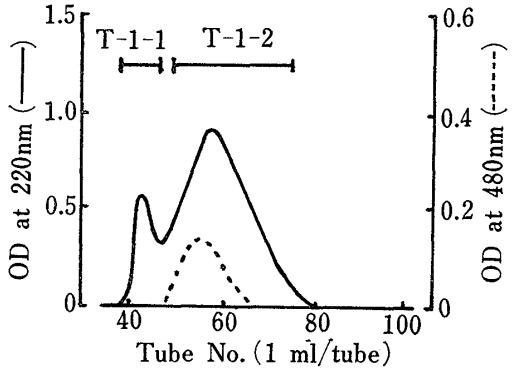

Fig. 3. Gel Filtration of T-1 on a Sephadex G-25 Column (1.6×44 cm) equilibrated with 0.05 M NH_4HCO_3

The phenol-H_2SO_4 procedure was used to determine the OD at 480 nm.

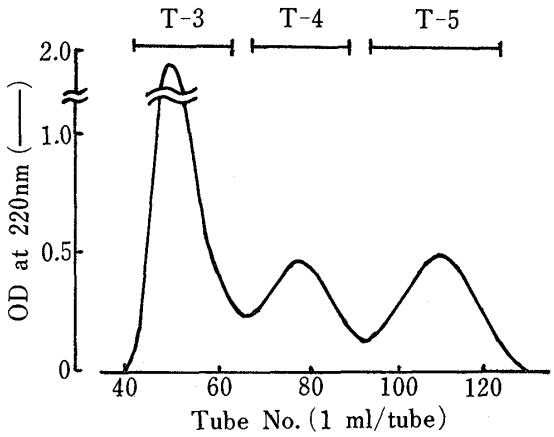

Fig. 4. Gel Filtration of Thermolytic Fragments of AA-1-2 on a Sephadex G-15 Column (1.6×44 cm) equilibrated with 0.05 M NH_4HCO_3

NH_2-terminal amino acid was identified as Asx by combined dansyl-Edman procedure, but could not be identified as asparagine or aspartic acid by manual Edman degradation. T-1-1 (37—52) was a hexadecapeptide residue containing two tryptophan residues. T-1-2-a (37—44) and T-1-2-b (45—52) were overlapping peptides of T-1-1. T-2-b (53—55) was a tripeptide which coincided with a part of the COOH-terminal sequence of AA-1-1, and T-2-e (56—58) was the COOH-terminal tripeptide of AA-1-1. Complete cleavage of the COOH-terminal region with thermolysin gave T-2-a (53), T-2-d (54 and 56), T-2-c (55) and T-2-f (57—58). The carbohydrate moiety of Fr. AA-1 was recovered in fractions AA-1-1, T-1, T-1-2 and T-1-2-c, successively, each of which was positive to silver nitrate reagent.

TABLE III. Amino Acid Sequence of AA-1-2

AA-1-2	1				5					10						16
	Leu–	Tyr–	Ile–	Leu–	Tyr–	Phe–	Phe–	Glx	(Ser,	Asx,	Asx,	Glx,	Asx,	Lys,	Glx,	Lys)
T-5-d	Leu–	Tyr														
T-5-c			Ile			(Phe)										
T-3-b				Leu–	Tyr–	Phe–	Phe–	Gln–	Ser–	Asp	(Asx,	Glx,	Asx,	Lys,	Glx,	Lys)
T-5-d				Leu–	Tyr											
T-5-c			(Ile)			Phe										
T-3-a							Phe-	Gln–	Ser–	Asp–	Asn–	Glu–	Asp–	Lys–	Glu–	Lys

AA-1-2	17			20					25					30		32
	Val,	Val,	Arg,	Glx,	Glx,	Glx,	Gly)	Glu–	Glu–	Arg–	Ile–	Thr–	Ala–	Leu–	Leu–	Hse
T-5-b	Val															
T-3-c		Val–	Arg–	Gln–	Glu–	Glu–	Gly–	Glu–	Glu–	Arg						
T-4-b		Val–	Arg–	Gln–	Glu–	Glu										
T-4-a							Gly–	Glu–	Glu–	Arg						
T-5-e										Ile–	Thr					
T-5-a											Ala					
T-5-c												Leu				
T-5-f													Leu–	Hse		

AA-1-2 was also digested with thermolysin and fractionated on Sephadex G-15 to give T-3, T-4, and T-5 as shown in Fig. 4. T-3 was separated to T-3-a, T-3-b, and T-3-c by high-voltage paper electrophoresis. T-4 gave T-4-a and T-4-b on paper chromatography. T-5 was separated to T-5-a, T-5-b, T-5-c, T-5-d, T-5-e, and T-5-f by paper chromatography and

high-voltage paper electrophoresis. The results of sequence analyses are summarized in Table III. T-5-d (1—2) was the NH_2-terminal dipeptide residue of AA-1-2. T-3-b (4—16) was a tridecapeptide residue and its NH_2-terminal sequence (4—10) was determined. T-5-d (4—5) and T-3-a (7—16) were obtained from the complete digests of T-3-b. T-3-c (18—26) was a nonapeptide including the COOH-terminal side (24—26) and was further cleaved to T-4-b (18—22) and T-4-a (23—26). The carboxyl terminal region of AA-1-2 was digested to provide T-5-e (27—28) and T-5-f (31—32). Based on the above results and the amino acid composition of thermolytic digests of AA-1-2, the isoleucine residue of T-5-c (3), phenylalanine of T-5-c (6), valine of T-5-b (17), alanine of T-5-a (29) and leucine of T-5-c (30) were fitted into appropriate positions. The presence of free amino acids in thermolytic digests of AA-1-1 and AA-1-2 is in accord with reports that thermolysin cleaves both of phenylalanine (86 and 101) in nerve growth factor[16] and leucine (35) in cytochrome c,[17] although the general susceptibility to hydrolysis by thermolysin requires the absence of free amino and carboxyl groups in the immediate vicinity of the peptide bond to be hydrolyzed, as described by Matsubara.[18]

Location of the Carbohydrate Moiety

As mentioned above, only one Asx residue (33) out of 7 Asx residues in Fr. AA-1 could not be detected either as phenylthiohydantoin asparagine or as phenylthiohydantoin aspartic acid. The same result was reported by Fletcher,[19] who found that the asparaginyl residue carrying the carbohydrate moiety in a peptide chain was cleaved to the phenylthiohydantoin derivative of the carbohydrate-asparagine complex by manual Edman degradation. It seems likely that Asx (33) of Fr. AA-1 is the binding site of the carbohydrate moiety. To determine the binding site of the carbohydrate moiety in T-1-2-c (Asx-Gly-Ser), the carbohydrate fraction was isolated on a Sephadex G-25 column (1.6×47 cm) equilibrated with 50 mM pyridine-acetate buffer (pH 5.3) from the pronase digest of Fr. AA-1. To remove peptide fractions, this fraction was further chromatographed on a DEAE-cellulose column (0.6×40 cm) using linear gradient elution from 7.5 mM tris-HCl buffer (pH 7.0) to 1.0 M NaCl in the buffer. The car-

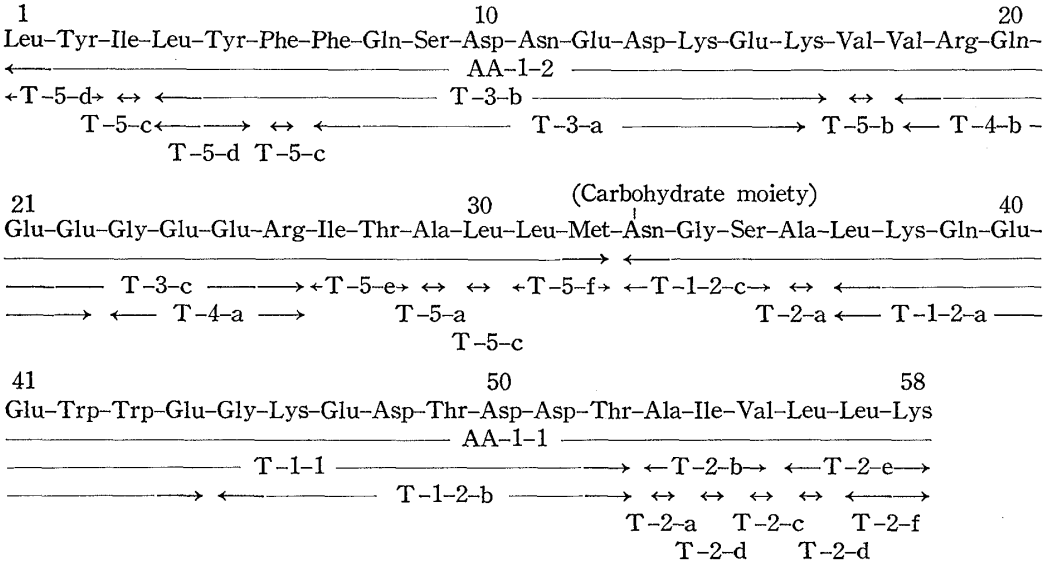

Fig. 5. Amino Acid Sequence of Fr. AA-1

The various peptides and amino acids obtained as fragments are indicated by double-headed arrows.

16) R.H. Angeletti, M.A. Hermodson, and R.A. Bradshow, *Biochemistry*, **12**, 100 (1973).
17) H. Matsubara, A. Singer, R. Sasaki, and T.H. Jukes, *Biochem. Biophys. Res. Commun.*, **21**, 242 (1965).
18) H. Matsubara, *Biochem. Biophys. Res. Commun.*, **24**, 427 (1966).
19) A.P. Fletcher, R.D. Marshall, and A. Neuberger, *Biochem. J.*, **88**, 37P (1963).

bohydrate moiety was eluted at 0.7 M NaCl, and its amino acid composition was analyzed. This moiety contained only aspartic acid. Consequently, it was concluded that the binding site of the carbohydrate moiety was the asparaginyl residue (33). The binding of the carbohydrate moiety to the asparaginyl residue in T-1-2-c is also supported by the hypothesis of Hunt *et al.*[20] that the asparaginyl residue in the tripeptide sequence Asn-X-Ser/Thr generally carries a carbohydrate moiety.

Complete Amino Acid Sequence of Fr. AA-1

Based on the results shown in Tables II, III, and the above results, it was concluded that Fr.AA-1 is a glycopeptide of 58 amino acids with leucine at the NH_2-terminal position and lysine at the COOH-terminal position (Fig. 5). The sequence of Fr. AA-1 was compared with those of several other proteins involved in calcium and mesenchyme metabolisms, or present in the salivary gland, but no similarities were found as regards sequence or size with calcitonin,[21] parathyroid hormone,[22] growth hormone,[23] statherin from saliva,[24] epidermal growth factor[25] and nerve growth factor.[16]

Acknowledgement The authors wish to thank Teikoku Zoki Pharmaceutical Co., Ltd., for supplying crude parotin.

20) L.T. Hunt and M.O. Dayhoff, *Biochem. Biophys. Res. Commun.*, **39**, 757 (1970).
21) H.B. Brewer, Jr. and R. Roman, *Proc. Nat. Acad. Sci. U.S.A.*, **63**, 940 (1969).
22) H.D. Niall, H. Keutman, R. Sauer, M. Horman, B. Dawson, G. Aurbach, and J. Potts, Jr., *Z. Phys. Chem.*, **351**, 1586 (1970).
23) C.H. Li, J.S. Dixon, and W.K. Liu, *Arch. Biochem. Biophys.*, **133**, 70 (1969).
24) D.H. Shresinger and D.I. Hay, *J. Biol. Chem.*, **252**, 1689 (1977).
25) C.R. Savage, Jr., T. Inagami, and S. Cohen, *J. Biol. Chem.*, **247**, 7612 (1972).