Isolation and Amino Acid Sequence of Dentinal Fluid Transport-Stimulating Peptide from Rat Parotid Glands

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An active peptide which stimulates dentinal fluid transport (DFT) through the dentinal tubules in the molar teeth of rats was isolated by aqueous extraction, isoelectric precipitation, ultrafiltration, gel filtration, cation exchange chromatography, a peptide mapping procedure and gel filtration from rat parotid glands. The minimum effective dose of the purified DFT-stimulating peptide was 1 μg per kg of body weight in the bioassay for DFT-stimulating activity, as indicated by the migration of a fluorescent dye through the dentinal tubules in the molar teeth of rats.

The complete amino acid sequence of DFT-stimulating peptide was determined by dansyl-Edman degradation, manual Edman degradation, carboxypeptidase digestion and tryptic peptide analysis. The final sequence was Gly–Val–Ile–Ala–Trp–Glu–Leu–Gln–His–Asn–Glu–Pro–Gly–Arg–Lys–Asp–Ser–Thr–Ala–Gly. The rat DFT-stimulating peptide thus consisted of a total of 20 amino acid residues and the calculated molecular weight was 2165.

Keywords——dentinal fluid transport (DFT); rat parotid gland; DFT-stimulating peptide; peptide isolation; molecular weight; amino acid sequence; N-termianl sequence; C-terminal sequence; carboxypeptidase Y; peptide mapping

Steinman found that the incidence of caries in rats on a cariogenic diet decreased when dentinal fluid transport (DFT) through the dentinal tubules from the pulp was stimulated, and they suggested the importance of DFT for the prevention of caries.[1] Steinman and Leonora proposed the existence of a hypothalamic-parotid gland endocrine axis as a DFT-stimulating mechanism,[2] and in 1980 Steinman et al. isolated a DFT-stimulating substance (PH-A$_\beta$) from porcine parotid glands and characterized it.[3] This active substance contained 73.3% Gly and Pro among the total amino acid residues and had a molecular weight of 8100. The N-termianl 28 amino acid sequence was also determined.

Recently the DFT-stimulating substance was isolated from bovine parotid glands; the isolated substance had a molecular weight of 30000 and consisted of a total of 263 amino acid residues per molecule.[4] Its properties were markedly different from those of the porcine substance.

In the present paper, the isolation of a DFT-stimulating substance as a homogeneous peptide from rat parotid glands and the complete amino acid sequence of the resulting active peptide are described.

Materials and Methods

Materials——Sephadex G-10, Sephadex G-50 and Sephadex LH-20 were purchased from Pharmacia Fine

Chemicals. Diaflo membranes PM30 and YC05 were purchased from Amicon Corp. Hitachi custom ion-exchanger resin 2611 was obtained from Nissei Sangyo Co., Ltd. TSK-GEL Toyopearl HW-40 (fine) and TSK-GEL LS-410 ODS were purchased from Toyo Soda Manufacturing Co., Ltd. Des-Arg^9-[Leu^8]-bradykinin, Met–Lys–bradykinin and peptide fragment 13—34 of human parathyroid hormone PTH were products of the Protein Research Foundation. Phenylisothiocyanate and trifluoroacetic acid (TFA) were obtained from Wako Pure Chemicals Ind., Ltd. Dansyl chloride was a product of Pierce Chemical Co. Carboxypeptidase Y and diphenyl carbamyl chloride (DPCC)-trypsin were obtaiend from Sigma Chemical Co. Polyamide layer sheets were obtained from Cheng Chin Trading Co., and chromatography papers from Toyo Roshi Co., Ltd. Fluorescamine (Fluram) was a product of Roche Co. Other chemicals were of analytical reagent grade from commercial sources.

Determination of Protein——Protein was determined by the method of Lowry et al.[5]

Ninhydrin Reaction——Ninhydrin reaction was carried out as described by McGrath.[6]

High-Performance Liquid Chromatography (HPLC)——Analysis by reversed-phase HPLC was performed on a Toyo Soda model HLC-803A HPLC chromatograph equipped with a stainless-steel column (4 mm × 30 cm) packed with octadecylsilane (ODS) chemically bonded on silica gel (TSK-GEL LS-410 ODS). A sample was applied to a column equilibrated with a solution of 10% acetonitrile (CH_3CN) in 0.1% TFA. The column was washed with the same solvent and eluted with a linear gradient of 10—60% CH_3CN in 0.1% TFA at a flow rate of 1.0 ml/min. The column effluent on HPLC was monitored by measuring the absorbance at 210 nm.

Estimation of Molecular Weight by Gel Filtration——Gel filtration was performed on a Sephadex G-25 column (0.9 × 156 cm) according to the method of Andrews.[7] Elution was performed with 0.2 M sodium citrate buffer (pH 3.25) containing 8% ethanol at a flow rate of 3 ml/h at 35 °C and the eluate was monitored by means of the ninhydrin reaction using a Hitachi 034 liquid chromatograph.

Amino Acid Analysis——Before amino acid analysis, 50 μg of peptide was dried under reduced pressure over P_2O_5. The dried sample was hydrolyzed with constant-boiling 6 N HCl at 110 °C in a sealed tube under a vacuum for 24 h. After evaporation of the hydrolysate, the residue was dissolved in 0.2 M sodium phosphate buffer (pH 6.5). The solution was allowed to stand for 4 h to permit air oxidation of any cysteine to cystine and was then brought to pH 2.0 by the addition of 1 N HCl as described by Moore and Stein,[8] and 0.2 M lithium citrate buffer (pH 2.2) was added to the resulting solution. For tryptophan analysis, 100 μg of sample was hydrolyzed with 2.5 N NaOH in the presence of 0.25 mg of starch at 110 °C for 20 h under a vacuum, according to the method of Hugli and Moore.[9] The hydrolysates were run on a Hitachi 034 liquid chromatograph. Norleucine was used as an internal standard in all analyses.

Carbohydrate Analysis——Hexose content was measured by the phenol–sulfuric acid method,[10] using D-glucose as a standard.

Absorption Spectrum——The ultraviolet absorption spectrum was measured in 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl.

Analysis of N-Terminal Amino Acid Sequence——A modification of the dansyl-Edman procedure of Gray and Hartley[11] was used. Approximately 100 μg of peptide was dissolved in 30 μl of 50% aqueous pyridine in a conical tube (9 × 64 mm), and 10 μl of 20% phenylisothiocyanate in pyridine was added. After flushing with nitrogen for 15 s, the tube was sealed with a glass stopper and incubated at 45 °C for 1 h. After drying in vacuo, the residue was taken up in 20 μl of ethanol and then dried again in vacuo. The residue was incubated at 45 °C for 30 min with 30 μl of TFA. After drying in vacuo, the residue was dissolved in 100 μl of water and extracted 3 times with 400 μl of n-butyl acetate. After the third extraction, the aqueous phase was freeze-dried and redissolved in 30 μl of 50% aqueous pyridine. A sample (approximately 5 μg) was removed for dansylation, and the remainder was treated with phenyl isothiocyanate exactly as before. In this way the sequence was determined for the N-terminus. For dansylation the peptide was transferred to a tube (3 × 60 mm) and dried in vacuo. Then 10 μl of 0.2 M NaHCO$_3$ and 10 μl of dansyl chloride solution (1 mg/ml in acetone) were added and the mixture was incubated at 37 C for 60 min. The solution was dried in vacuo, and 50 μl of 6 N HCl was added. The tube was sealed under a vacuum, and the dansyl peptide was hydrolyzed at 105 C for 18 h. Then the tube was cooled and opened, and its contents were evaporated to dryness over NaOH. The dansyl amino acid was determined by two-dimensional chromatography on a polyamide layer sheet (5 × 5 cm), with 1.5% formic acid in water in the first dimension and with benzene–acetic acid (9:1) in the second dimension. Trp, Asn and Gln were identified by manual Edman degradation.[12] The thiazolinone derivatives were converted to the phenylthiohydantoin derivatives by heating at 80 C for 10 min in 1 N HCl and the products were extracted with ethyl acetate. The phenylthiohydantoin derivatives were identified on polyamide layer sheets according to the procedures of Kulbe[13,14] and Summers et al.[15]

Analysis of C-Terminal Amino Acid Sequence——The C-terminal amino acid sequence was determined by carboxypeptidase digestion according to the procedure of Hayashi et al.[16] Approximately 150 μg of peptide was dissolved in 150 μl of 0.05 M sodium phosphate buffer (pH 6.5) and the solution was treated with 12 μl of 1 mg/ml carboxypeptidase Y (24 units/mg) in the same buffer. The mixtures were incubated at 25 °C for 15, 60 and 240 min, respectively. The digestion was stopped by freezing at −40 °C. Digests were diluted with 0.2 M lithium citrate buffer (pH 2.2) and analyzed with the amino acid analyzer.

Tryptic Digestion and Peptide Mapping——The peptide (2 mg) was dissolved in 200 μl of 0.2 M NH$_4$HCO$_3$ and the pH was adjusted to pH 8.2 with diluted aqueous ammonia. The solution was digested with 100 μl of trypsin

(DPCC treated, 10000 $N\alpha$-benzoyl-L-arginine ethyl ester (BAEE) units per mg protein) in 0.1 M $CaCl_2$ at 37 °C. Initially 20 μg of trypsin was added, and the same amount was again added after 3 h. After incubation for a total period of 6 h, the digestion was stopped by adjusting the solution to pH 3.0 with acetic acid and the digest was freeze-dried.

The peptide mapping procedure was performed essentially by the method of Katz et al.[17] Chromatographies were run in a descending manner with full 38 × 38 cm sheets of Toyo filter paper No. 50 for 20 h at room temperature. The paper was washed with 0.1 N HCl and redistilled 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 of the sheet at 80 °C for 30 min, high-voltage paper electrophoresis (1400 V) was carried out for 90 min in pyridine–acetic acid–water (10 : 0.4 : 90, pH 6.5) using a Toyo Kagaku HPE-406 machine. The paper was dried at 80 C for 30 min and washed with acetone and 1% triethylamine–acetone solution. The paper was then exposed to a mist of 0.003% fluorescamine and the peptides were detected under the ultraviolet lamp. The confirmed spots were extracted with 0.1 N aqueous ammonia and recovered as lyophilized samples.

Bioassay of DFT-Stimulating Peptide——According to the procedure described previously by the authors,[4] DFT-stimulating activity was assayed in terms of the ability to stimulate fluid transport, as indicated by the migration of a fluorescent dye through the dentinal tubules in the molar teeth of rats. Four-week-old Sprague-Dawley male rats were intraperitoneally anesthetized with sodium pentobarbital at a dose of 30 mg per kg. After the anesthetic had become effective, a sample dissolved in physiological saline was intravenously injected at a dose of 1.0 ml per kg. The control rats were injected with physiological saline. A fluorescent dye, acriflavine hydrochloride, dissolved in distilled water, was intraperitoneally injected at a dose of 50 mg per kg 10 min after the infusion of the sample. At 16 min after the fluorescent dye had been given, the rats were decapitated, then the upper jaws were quickly removed (in less than 1 min) and immediately frozen. The maxillary molars were sectioned with a microtome under freezing to obtain sagittal frozen sections through the middle of the occlusal surface of the molar teeth; molar sections were cut to about 100 microns thickness. The molar sections were observed with an incident illumination-type fluorescence microscope at 40 magnifications (main excitation wavelength 495 nm), and the movement of acriflavine in the molar teeth was observed. The degree of fluid movement to each molar tooth was determined by assigning a value of 0 to 6: (value 0) pulp only, (value 2) middle dentinal, (value 4) extensive dentinal and (value 6) up to enamel. If the permiation of fluorescence differed in extent among the molar sections, an intermediate value (1, 3 and 5) was used as the fluid movement value. If the fluorescent dye permeated into the dentin, the dentinal tubules showed a brilliant green fluorescence and the dentino-enamel junction appeared clearly. The fluid movement value of fluorescence was averaged among five rats. The significance of the difference between the mean values of the sample group and control group was calculated by means of the t-test. When the values of samples showed a difference at below the 5% level of significance, the sample injected was considered to be effective.

Purification of DFT-Stimulating Peptide

Step 1. Preparation of Acetone-Dried Powder——Fresh rat parotid glands (100 g) were finely ground and stirred with 1 l of acetone for 4 h. The acetone was then removed and replaced with fresh acetone. The parotid glands were then stirred with ether for 4 h. The defatted glands were recovered by filtration and dried to yield acetone-dried powder.

Step 2. Isoelectric Precipitation——Distilled water (200 ml) was added to the acetone-dried powder, and the mixture was adjusted to pH 7.0, then homogenized in a Waring blender for 1 min (10 s × 6). The homogenate was
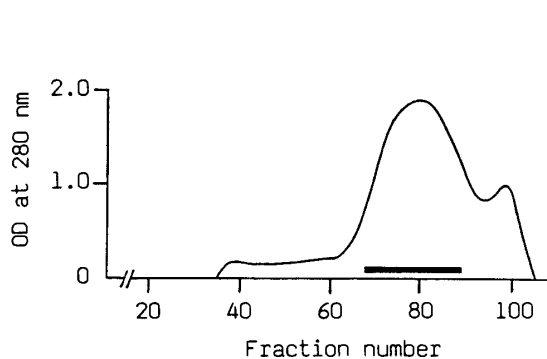
Fig. 1. Gel Filtration on a Sephadex G-50 Column

Sample: ultrafiltrate. Column size: 1.2 × 88 cm. Eluent: 0.05 M sodium phosphate buffer (pH 7.0). Flow rate: 2.5 ml/h. Fraction: 1 ml/tube. The active fractions indicated by a solid bar were pooled.
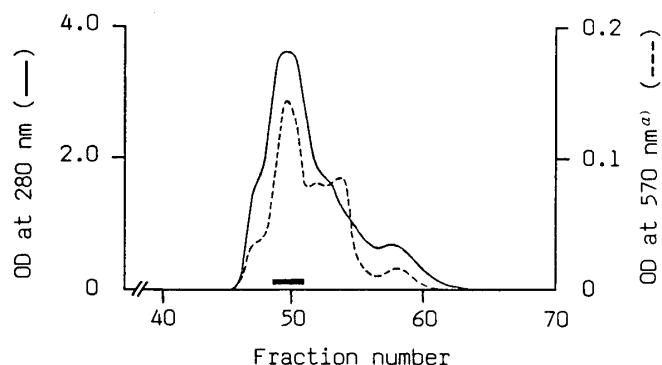
Fig. 2. Gel Filtration on a Sephadex LH-20 Column

Sample: Sephadex G-50 fraction. Column size: 3.8 × 100 cm. Eluent: ethanol–acetic acid–water (95 : 10 : 95). Flow rate: 30 ml/h. Fraction: 9 ml/tube. a) A 200 μl aliquot was taken for ninhydrin reaction. The active fractions indicated by a solid bar were pooled.

stirred at 4 °C for 4 h and then centrifuged at 10000 g for 15 min. The obtained supernatant was adjusted to pH 5.0 with 1 M H_3PO_4, stirred for 1 h and allowed to stand overnight. The resulting precipitate was removed by centrifugation and the pH 5.0 supernatant was again adjusted to pH 7.0.

Step 3. Ultrafiltration——The neutralized pH 5.0 supernatant was filtered by ultrafiltration through a Diaflow PM30 membrane (30000 MW cut-off). The filtrate was concentrated using a Diaflow YC05 membrane (500 MW cut-off), dialyzed against distilled water and lyophilized.

Step 4. Gel Filtration on a Sephadex G-50 Column——The crude ultrafiltration fraction was dissolved in 0.05 M sodium phosphate buffer (pH 7.0). The dissolved solution was passed through a column (1.2 × 88 cm) of Sephadex G-50 equilibrated with 0.05 M sodium phosphate buffer (pH 7.0) and eluted with the same buffer (Fig. 1). The absorbance of fractions at 280 nm was measured. The activity was found in the region of fractions 67—92. The pooled active fractions were desalted on a column (1.6 × 88 cm) of Sephadex G-10 and lyophilized.

Step 5. Gel Filtration on a Sephadex LH-20 Column——The active Sephadex G-50 fraction was dissolved in ethanol–acetic acid–water (95 : 10 : 95) and the insoluble material was removed by centrifugation. The solution was passed through a column (3.8 × 100 cm) of Sephadex LH-20 equilibrated with ethanol–acetic acid–water (95 : 10 : 95) and eluted with the same solvent (Fig. 2). Absorbance at 570 nm after reaction with the ninhydrin and absorbance at 280 nm were measured. Absorbance with ninhydrin reaction showed five peaks and the activity was present in the second peak of fractions 48—51. The active fractions were pooled and lyophilized.

Step 6. Column Chromatography on a Cation Exchanger Resin——The active Sephadex LH-20 fraction (100 mg) was dissolved in 0.2 M sodium citrate buffer (pH 2.2) and the insoluble material was removed by centrifugation. The supernatant was applied to a column (0.9 × 55 cm) of a Hitachi custom ion-exchanger resin 2611 equilibrated with 0.2 M sodium citrate buffer (pH 2.2) and the column was washed with the same buffer. The column was eluted with 0.2 M sodium citrate (pH 4.25), 1.2 M sodium citrate buffer (pH 5.28) and 0.1 N NaOH. The eluates were subjected to absorbance measurement at 280 nm, ninhydrin reaction and examination by the method of Lowry et al.[5] The elution profile is shown in Fig. 3. The active substance was found in the 0.1 N NaOH eluate. The active fraction, corresponding to fractions 50—54, was pooled, desalted on a column (1.6 × 88 cm) of Sephadex G-10 and lyophilized.

Step 7. Fractionation by Peptide Mapping——A peptide mapping was carried out as described by Katz et al.[17] A sample dissolved in 5% pyridine solution (200 μl for 2—3 mg of ion exchanger resin active fraction) was centrifuged and the supernatant was used. The chromatogram was run in the descending direction with full 42 × 45 cm sheets of Toyo filter paper No. 50. Phenol red was used as a marker. The solvent was composed of n-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12) and the chromatogram was run for 20 h at room temperature. At the end of chromatography the paper was dried at 80 °C for 30 min. Paper electrophoresis at high voltage was carried out using pyridine–acetic acid–water (10 : 0.4 : 90, pH 6.5) at a constant current of 150 mA for 90 min. Methyl green was used as a marker. The paper was dried at 80 °C for 30 min and washed with acetone and 1% triethylamine–acetone solution (it had previously been washed with 0.01 N HCl and redistilled water). The paper was then exposed to a mist of 0.003% fluorescamine and the peptides were detected under the ultraviolet lamp. The confirmed spots were extracted with
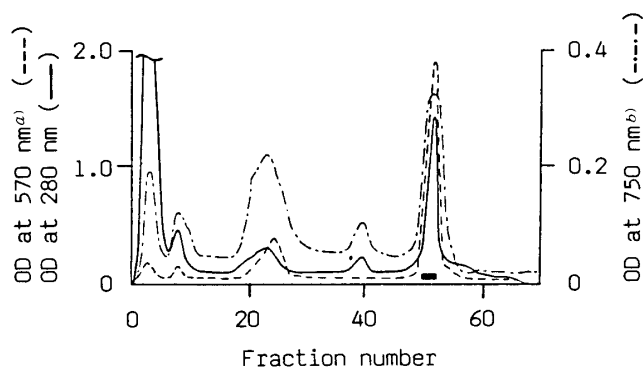

Fig. 3. Column Chromatography on a Cation Exchanger Resin

The column (0.9 × 55 cm) was developed by step-wise elution [0.2 M sodium citrate buffer (pH 4.25), 1.2 M sodium citrate buffer (pH 5.28) and 0.1 N NaOH] at 55 °C. Flow rate: 30 ml/h. Fraction: 2.5 ml/tube.

a) A 50 μl aliquot was taken for ninhydrin reaction.
b) A 50 μl aliquot was examined by the method of Lowry et al. The active fractions indicated by a solid bar were pooled.
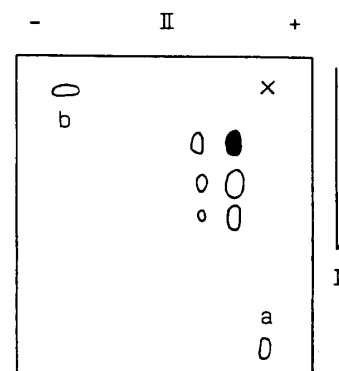

Fig. 4. Peptide Map Pattern

Mapping was accomplished by chromatography in the first dimension (I) and then by electrophoresis in the second dimension (II).

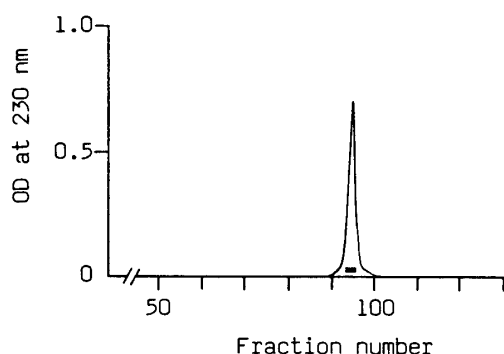×, origin; a, phenol red; b, methyl green. An active spot is shown in black.

Fig. 5. High-Performance Gel Filtration on a TSK-GEL Toyopearl HW-40 (Fine) Column

Sample: peptide mapping fraction. Column size: 1.5×88 cm. Eluent: 0.1 M $NH_4HCO_3-NH_4OH$ (pH 8.0). Flow rate: 30 ml/h. Fraction: 1 ml/tube. The active fractions indicated by a solid bar were pooled.

0.1 N aqueous ammonia and recovered as lyophilized samples. The active cation exchanger resin fraction showed six spots and the DFT-stimulating activity existed as a single spot (Fig. 4).

Step 8.   High-Performance Gel Filtration on a TSK-GEL Toyopearl HW-40 Column——The active peptide-mapping fraction was dissolved in 0.1 M $NH_4HCO_3-NH_4OH$ (pH 8.0). The solution was passed through a column (1.5×88 cm) of Toyopearl HW-40 (fine) equilibrated with 0.1 M $NH_4HCO_3-NH_4OH$ (pH 8.0) and eluted with the same buffer (Fig. 5). The optical absorption of fractions at 230 nm was measured. The activity was found in the region of fractions 93—96, which were pooled and lyophilized.

Results

Purification of DFT-Stimulating Peptide

A total of 0.43 mg of the purified DFT-stimulating peptide was obtained from 100 g of rat parotid glands. Yield and DFT-stimulating activity at each step of the purification procedure are shown in Table I. The final preparation was purified approximately 100-fold from the pH 5.0 supernatant. The minimum effective dose of the purified preparation was 1 μg per kg of body weight in the bioassay.

Homogeneity of Purified DFT-Stimulating Peptide

The polyamide layer chromatography of the dansyl (DNS)-amino acid obtained after dansylation of the purified peptide yielded only DNS-Gly as a fluorescent spot other than $DNS-NH_2$ and DNS-OH (Fig. 6). Reversed-phase HPLC of the purified peptide gave a single peak, suggesting that the substance consists of a homogeneous peptide (Fgi. 7).

Absorption Spectrum

An absorption maximum, λ_{max} was seen at 280 nm, and the extinction coefficient, $E_{1\,cm}^{1\%}$ at 280 nm was 22.6.

TABLE I.   Yield and Stimulating Activity of the Fractions at Each Purification Step

Fraction	Yield[a] (mg)	Minimum effective dose (μg/kg)	Fluid movement[b]
Ultrafiltrate	250	100	3.00 ± 0.32[c]
Sephadex G-50	128	20	3.44 ± 0.25[d]
Sephadex LH-20	32.3	10	4.60 ± 0.47[d]
Cation exchanger resin	4.90	5	5.00 ± 0.65[d]
Peptide mapping	0.49	1	4.25 ± 0.37[d]
Toyopearl HW-40	0.43	1	3.58 ± 0.29[c]
Control	—	—	1.41 ± 0.25

a) Results from 100 g of parotid glands.   b) Fluid movement was expressed as the degree of migration of a fluorescent dye through the dentinal tubules in the molar teeth of rats. Mean $\pm$ standard error.   c) $p < 0.05$, d) $p < 0.01$; versus control.
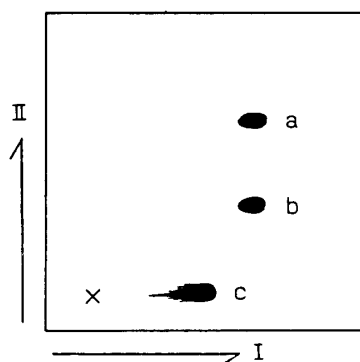
Fig. 6. N-Terminal Amino Acid Analysis of DFT-Stimulating Peptide on a Polyamide Layer Sheet

The DNS–amino acid was applied at the origin (×) and chromatographed in the direction of the arrows with the following solvents: I, 1.5% formic acid; II, benzene–acetic acid (9 : 1).

a, DNS–NH₂; b, DNS–Gly; c, DNS–OH.

Fig. 7. Reversed-Phase HPLC of Purified DFT-Stimulating Peptide
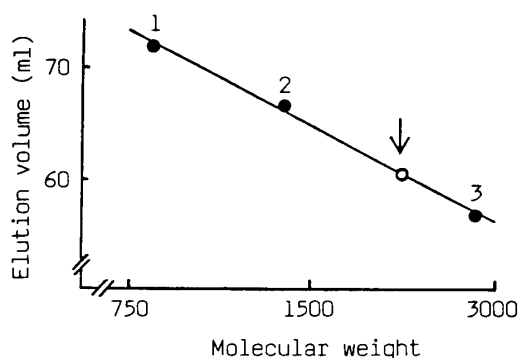
The solid bar indicates DFT-stimulating activity.

Fig. 8. Estimation of the Molecular Weight of DFT-Stimulating Peptide on a Sephadex G-25 Column

The standard peptide used for calibration were: (1) Des-Arg⁹-[Leu⁸]-bradykinin (870), (2) Met–Lys–bradykinin (1330) and (3) peptide fragment 13—34 (2808) of human parathyroid hormone PTH. The position of DFT-stimulating peptide is indicated by the arrow.
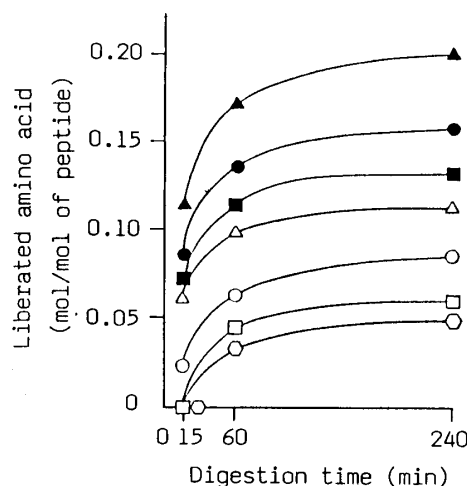
Fig. 9. Time Courses of Amino Acids Liberated from DFT-Stimulating Peptide on Digestion with Carboxypeptidase Y

▲, Gly; ●, Ala; ■, Thr; △, Ser; ○, Asp; □, Lys; ⊙, Arg.

Estimation of Molecular Weight by Gel Filtration

The molecular weight of the DFT-stimulating peptide was estimated to be 2200 by gel filtration on a Sephadex G-25 column, as shown in Fig. 8.

Amino Acid Composition

Table II shows the result of amino acid composition analysis. The numbers of residues per molecule of peptide were calculated assuming a molecular weight of 2200. The DFT-stimulating peptide consisted of a total of 20 amino acid residues per molecule. The amino acid composition was as follows: Asp_2, Thr_1, Ser_1, Glu_3, Pro_1, Gly_3, Ala_2, Val_1, Ile_1, Leu_1, Lys_1, His_1, Arg_1, Trp_1. No half-Cys, Met, Tyr or Phe was found. There was no detectable carbohydrate in this peptide.
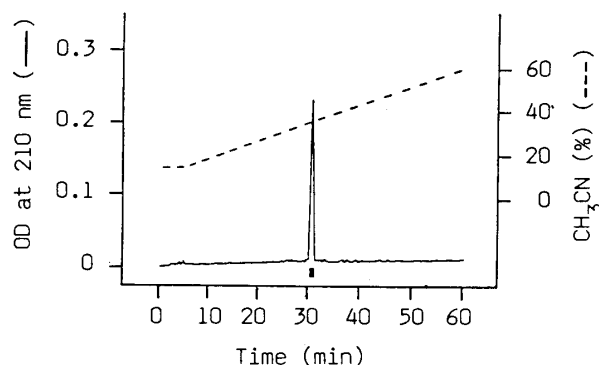
Fig. 10. Peptide Map Pattern of the Tryptic Digest of DFT-Stimulating Peptide

Mapping was accomplished by chromatography in the first dimension (I) and then by electrophoresis in the second dimension (II).

×, origin; a, phenol red; b, methyl green.

TABLE II. Amino Acid Compositions of DFT-Stimulating Peptide and Its Tryptic Peptides

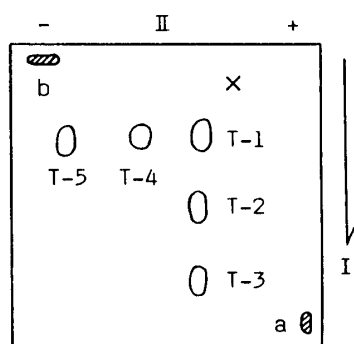| Amino acid	DFT-stimulating peptide	Tryptic peptide	
		T_1	T_3
Asp	1.92 (2)	2.05 (2)	1.13 (1)
Thr	1.03 (1)	1.09 (1)	0 (0)
Ser	1.11 (1)	1.31 (1)	0 (0)
Glu	2.82 (3)	2.87 (3)	3.17 (3)
Pro	1.15 (1)	1.02 (1)	0.92 (1)
Gly	2.85 (3)	2.94 (3)	2.16 (2)
Ala	1.78 (2)	2.09 (2)	1.14 (1)
Half-Cys	0 (0)	0 (0)	0 (0)
Val	1.07 (1)	1.15 (1)	0.98 (1)
Met	0 (0)	0 (0)	0 (0)
Ile	1.01 (1)	0.91 (1)	0.87 (1)
Leu	1.21 (1)	0.88 (1)	1.05 (1)
Tyr	0 (0)	0 (0)	0 (0)
Phe	0 (0)	0 (0)	0 (0)
Lys	0.87 (1)	0.91 (1)	1.01 (1)
His	0.91 (1)	1.05 (1)	0.86 (1)
Arg	1.04 (1)	1.12 (1)	1.04 (1)
Trp	0.91 (1)	0.88 (1)	1.13 (1)
Total	20	20	15

Numbers in parentheses represent the nearest integers.

Terminal Amino Acid Sequence

The N-terminal four residues of the purified peptide were identified as Gly-Val-Ile-Ala- by the dansyl-Edman method. The 5th N-terminal amino acid residue was identified as Trp by manual Edman degradation.

The time course of the release of amino acids from the peptide on treatment with carboxypeptidase Y is shown in Fig. 9. Gly was released first, followed by Ala, Thr, Ser and Asp. Lys and Arg were detectable after 60 min.

Based on the these results, it was concluded that the N-terminal sequence of rat DFT-stimulating peptide was Gly-Val-Ile-Ala-Trp-, and the C-terminal sequence was -Arg-Lys-Asp-Ser-Thr-Ala-Gly.

Amino Acid Sequence of Tryptic Peptides

The tryptic digest of rat DFT-stimulating peptide was separated into five spots, T-1 to T-5, by peptide mapping as shown in Fig. 10. The separated spots were extracted from the paper with 0.1 N aqueous ammonia and freeze-dried. All extracted peptides were hydrolyzed in 6 N
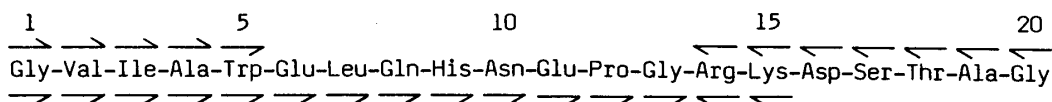
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1         5              10              15              20
—>  —>  —>  —>  —>                        <—  <—  <—  <—  <—  <—
Gly-Val-Ile-Ala-Trp-Glu-Leu-Gln-His-Asn-Glu-Pro-Gly-Arg-Lys-Asp-Ser-Thr-Ala-Gly
—>  —>  —>  —>  —>  —>  —>  —>  —>  —>  —>  —>  —>  <—  <—
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Fig. 11. Amino Acid Sequence of DFT-Stimulating Peptide

Symbols used are as follows. —➤, dansyl-Edman degradation; —➛, manual Edman degradation; ⟵—, carboxypeptidase Y digestion.

Arrows above each residue indicate results obtained by Edman degradation or carboxypeptidase Y digestion of the intact peptide, while those under the residues represent results for peptide fragments.

HCl at 105 °C for 18 h. The hydrolysates were analyzed on the amino acid analyzer.

It was concluded that T-1 was the unhydrolyzed peptide and T-3 was the N-terminal 15 amino acid residues, judging from the results of amino acid composition analyses, as shown in Table II. However, T-2, T-4 and T-5 could not be identified because of the low recoveries. The N-terminal amino acid sequence of T-3 was determined to be Gly–Val–Ile–Ala–Trp–Glu–Leu–Gln–His–Asn–Glu–Pro–Gly– by manual Edman degradation. The C-terminus of T-3 was determined to be –Agr–Lys by carboxypeptidase Y digestion. Subsequently, smaller amounts of Gln, Gly, Pro, Asp and Glu were released, but they were released at the same time, so the other sequence could not be identified.

Amino Acid Sequence of DFT-Stimulating Peptide

The complete amino acid sequence of rat DFT-stimulating peptide determined from the above results is shown in Fig. 11. The molecular weight calculated from the amino acid sequence was 2165.
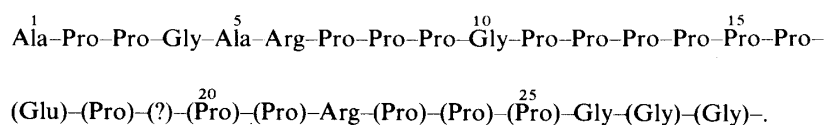
Discussion

In the bioassay of DFT-stimulating activity, Steinman et al.[3] expressed fluid transport as FMR (fluid movement ratio), that is, the ratio of the number of occlusal grooves in which the migration of fluorescence was observed to the total number of occlusal grooves present. However, the occlusal grooves sometimes exhibit autofluorescence owing to dietary components or microorganisms, causing errors in the assay. Moreover, it is necessary to use thin molar sections which are technically difficult to prepare, because the molar sections are observed with a transmitted illumination-type fluorescence microscope. In order to overcome these problems, an incident illumination-type microscope was used and the strength of DFT-stimulating activity was quantitatively measured from the whole images of the molar teeth.[4] This method allows easy observation of the migration of a fluorescent dye in whole molar sections even if the sections are thick. Therefore, this modified method was used in this study.

The minimum effective dose of the rat DFT-stimulating peptide determined by the bioassay was 1 µg per kg of rat body weight, which is 10^4 times lower than that of porcine PH-A_β reported by Steinman et al.[3] Recently, the DFT-stimulating substance was also isolated from bovine parotid glands; the minimum effective dose of this active substance was 10 µg and the specific activities of the two substances are nearly equal in molar terms.

The porcine PH-A_β contained 73.3% Gly and Pro in total and had a molecular weight of 8100. The bovine DFT-stimulating substance was found to have a molecular weight of 30000, and its amino acid composition was markedly different from that of the porcine compound.[4] The rat DFT-stimulating peptide consisted of a total of 20 amino acid residues contained only three Gly residues and one Pro residue per molecule, and thus did not show the Gly- and Pro-rich characteristic of the porcine PH-A_β. As shown in Fig. 11, the rat DFT-stimulating peptide is a novel peptide which has hydrophobic amino acids and hydrophilic amino acids in the N-terminal sequence and the middle region to the C-terminal sequence, respectively.

The N-terminal amino acid sequence of the porcine PH-A$_\beta$ was:

$$\overset{1}{\text{Ala}}\text{-Pro-Pro-Gly-}\overset{5}{\text{Ala}}\text{-Arg-Pro-Pro-Pro-}\overset{10}{\text{Gly}}\text{-Pro-Pro-Pro-Pro-}\overset{15}{\text{Pro}}\text{-Pro-}$$

$$\text{(Glu)-(Pro)-(?)-}\overset{20}{\text{(Pro)}}\text{-(Pro)-Arg-(Pro)-(Pro)-}\overset{25}{\text{(Pro)}}\text{-Gly-(Gly)-(Gly)-.}$$

Judging from the reported amino acid sequence, the porcine PH-A$_\beta$ contains approximately 17 Pro residues in the N-terminal 28 amino acid residues and approximately 54 Gly residues in the remaining C-terminal 77 amino acid residues. The possibility remains that an amino acid sequence similar to that of the rat DFT-stimulating peptide might exist in the unknown C-terminal regions of the porcine PH-A$_\beta$. However, it seems most probable that the substances purified from rat and porcine parotid glands, although they have the same DFT-stimulating activity, have different primary structures. However, it is also possible that the rat DFT-stimulating peptide is the active component itself and that the bovine DFT-stimulating substance with a molecular weight of 30000 exists as a complex of a carrier protein and a DFT-stimulating peptide with a molecular weight of 2165, or as a precursor of the low-molecular-weight form.

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