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Enzyme Immunoassay of a Substance P-like Immunoreactive Substance in Human Plasma and Saliva

Masaharu TAKEYAMA,*[,a] Kazuo MORI,[a] Fusako TAKAYAMA,[a] Kazuhiro KONDO,[a] Kouki KITAGAWA,[b] and Nobutaka FUJII[c]

Department of Hospital Pharmacy, Medical College of Oita,[a] Hasama-cho, Oita-gun Oita 879-56, Japan, Faculty of Pharmaceutical Sciences, University of Tokushima,[b] Sho-machi, Tokushima 770, Japan, and Faculty of Pharmaceutical Sciences, Kyoto University,[c] Sakyo-ku, Kyoto 606, Japan.
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A sensitive and specific double-antibody enzyme immunoassay (EIA) for a substance P (SP)-like immunoreactive substance (SP-IS) was developed. For competitive reactions, the SP-antibody was incubated with SP standard (or sample) and β-D-galactosidase labeled Tyr[8]-SP (delayed addition). Free and antibody-bound enzyme hapten were separated by using an anti-rabbit immunoglobulin G coated immunoplate. Activity of the enzyme on the plate was fluorometrically determined. The present immunoassay allows detection of 0.4 to 10 fmol/well of SP. Using the present EIA, SP-ISs in human saliva and plasma were determined. The level of SP-IS in human saliva was about 7 pmol/l, which was almost three times higher than that in human plasma.

Keywords substance P-like immunoreactive substance; human plasma; human saliva; highly sensitive enzyme immunoassay; Tyr[8]-substance P-labeled-β-D-galactosidase; fluorogenic substrate

Substance P (SP), undecapeptide, was first detected by von Euler and Gaddum[1] in extracts from the equine intestine and brain in 1931 as a principle that exhibited a hypotensive effect. The isolation of this principle from bovine hypothalami[2] and its structure was determined by Chang *et al.*[3] in 1971. It is widely distributed in the central and peripheral nerve endings,[4] and is implicated as a sensory neurotransmitter[5] and an excitatory transmitter to intestinal muscles.[6]

At present, the structures of equine,[7] cow,[8] rat,[9] human,[10] and guinea-pig[11] SP have been demonstrated to be identical to the structure of bovine SP.[2] In 1983, similar tachykinin peptides, neurokinin A[8,12] (NKA = neuromedin L) and neurokinin B[13] (NKB = neuromedin K), were found in mammalian tissues.

Radioimmunoassay (RIA) of SP has been developed by several groups[14] using N^{α}-^{125}I-Tyr-SP or ^{125}I-Tyr[8]-SP. However, in terms of safety, sensitivity and ease of handling, RIA methods are still less than satisfactory.

In 1982, Stjernschantz *et al.*[15] reported an enzyme immunoassay (EIA) for SP using a polystyrene plate coated with SP-poly-D-glutamic acid conjugate as a solid-phase antigen. Now we wish to report a highly sensitive and specific EIA for SP using β-D-galactosidase-labeled Tyr[8]-SP as a marker antigen, a second antibody coated immunoplate and 4-methylumbelliferyl β-D-galactopyranoside as a fluorogenic substrate.

Materials and Methods

Materials Synthetic SP, its sulfoxide [SP(O)] and its fragment (position 6—11),[16] Tyr[8]-SP,[17] NKA,[18] NKB,[18] porcine secretin,[19] human glucose-dependent insulinotropic peptide (GIP),[20] peptide histidine isoleucine (PHI),[21] neuromedin-C,[22] vasoactive intestinal polypeptide (VIP),[23] brain natriuretic peptide (BNP)[24] and neuropeptide Y (NPY)[25] were used. Somatostatin (SS) and gastrin were purchased from Peptide Institute Inc. (Osaka, Japan) and bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and 4-methylumbelliferyl β-D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). β-D-Galactosidase (β-Gal from *Escherichia coli*) and goat anti-rabbit immunoglobulin G (IgG) (TAGO 4120) were purchased from Boehringer Mannheim Corp. (Mannheim, Germany) and TAGO Inc. (Burlingame, CA, U.S.A.), respectively.

Antiserum to SP (RA-08-095) was purchased from Cambridge Research Biochemicals Ltd. (Cambridge, England) and the lyophilized SP-antiserum was reconstituted to 200 ml with an assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.5% BSA, 1 mM $MgCl_2$ and 250 KIU/ml aprotinin). All other chemicals were of analytical reagent grades.

Preparation of Plasma and Saliva Extracts Human plasma and saliva samples were obtained from 10 healthy volunteers. Blood was collected in a chilled tube containing 500 KIU/ml aprotinin and 1.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After centrifugation (3000 rpm, 4 °C, 20 min), plasma was stored at −40 °C until use. Human plasma (2 ml) and saliva (1 ml) samples were diluted fivefold by 4% acetic acid (AcOH), pH 4.0, and loaded on reversed-phase C_{18} cartridges (Sep-Pak C_{18}, Waters Co., Inc., Milford, MA, U.S.A.). After washing with 4% AcOH (10 ml), the SPs were eluted with 70% acetonitrile (MeCN) in 0.5% AcOH, pH 4.0 (2 ml). Eluates were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μl with the assay buffer and subjected to EIA. Recovery of this extraction procedure was 92 ± 10% (n = 6).

Preparation of Enzyme-Labeled Antigen Tyr[8]-SP was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa *et al.*[26] Tyr[8]-SP (0.50 mg) in a 0.05 M phosphate buffer, pH 7.0 (1 ml), was stirred with EMC-succinimide (0.4 mg) in tetrahydrofuran (50 μl) at 20 °C for 60 min. Then, obtained EMC-Tyr[8]-SP was purified with a Sephadex G-25 column (1.5 × 49 cm) using a 0.05 M phosphate buffer, pH 7.0, as an eluate. Purified EMC-Tyr[8]-SP fractions were combined with β-Gal (0.98 mg) by stirring at 20 °C for 30 min. The β-Gal conjugate was then applied to a Sephacryl S-300 column (1.5 × 59 cm) and eluted with a 0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$. Tyr[8]-SP-labeled β-Gal fractions

TABLE I. Structures of Substance P and Related Peptides

	1 5 10
Substance P	H–Arg–Pro–Lys–Pro–Gln–Gln–Phe–Phe–Gly–Leu–Met–NH_2
Tyr[8]-SP	H–Arg–Pro–Lys–Pro–Gln–Gln–Phe–Tyr–Gly–Leu–Met–NH_2
Neurokinin A	H–His–Lys–Thr–Asp–Ser–Phe–Val–Gly–Leu–Met–NH_2
Neurokinin B	H–Asp–Met–His–Asp–Phe–Phe–Val–Gly–Leu–Met–NH_2
Neuromedin-C	H–Gly–Asn–His–Trp–Ala–Val–Gly–His–Leu–Met–NH_2
Somatostatin	H–Ala–Gly–Cys–Lys–Asn–Phe–Phe–Trp HO–Cys–Ser–Thr–Phe–Thr–Lys

were collected and stored at 4 °C after the addition of 0.2% BSA and 0.1% NaN_3.

Assay Procedure for SP For assay, the above-mentioned assay buffer and the washing buffer, 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20, were used. Second-antibody-coated immunoplates were prepared as previously reported[27] using MicroWell Maxisorp F8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG. A test tube containing 100 μl of anti-SP-serum, RA-08-095 and each sample (or standard, 100 μl) was incubated at 4 °C for 24 h and then the diluted enzyme-labeled antigen (50 μl) was added. The test tube was further incubated at 4 °C for 24 h. The antibody–antigen solution (100 μl) from each test tube was added to the second-antibody-coated immunoplate. The plate was incubated at 4 °C overnight, then washed 4 times with the washing buffer, then 0.1 mM MUG (200 μl) in a substrate buffer (0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$) was added to each well. The plate was again incubated at 37 °C for 3 h. The resulting fluorescence intensity (λ_{ex} 450 nm, λ_{em} 360 nm) of each well was measured with a MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

High Performance Liquid Chromatography (HPLC) of Plasma and Saliva Extracts HPLC was performed using a reversed phase C_{18} column (Wakosil 5C18, 4.6 × 150 mm, Wako Pure Chemical Industries Ltd., Osaka, Japan). The HPLC consisted of a model 610 dual pump system (Waters Co. Inc., Milford, MA, U.S.A.). The samples [plasma (5 ml), saliva (3 ml)] purified by the Sep-Pak C_{18} cartridge mentioned above were reconstituted to 200 μl with 15% MeCN in 0.1% trifluoroacetic acid (TFA), and applied to the column. SP-ISs were eluted with a linear gradient of MeCN (from 15% to 50% in 35 min) in 0.1% TFA. The flow rate was 1 ml/min and the fraction size was 1 ml. The fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μl with the assay buffer and submitted to EIA.

Results and Discussion

Standard Curve The typical calibration curve for this EIA of SP is shown in Fig. 1. A linear displacement of enzyme-labeled Tyr^8-SP by synthetic SP was obtained, when plotted as a semilogarithmic function from 6 to 300 pmol/l (0.24 to 12 fmol/well) of SP. The minimum amount of SP detectable by the present EIA system was 10 pmol/l (0.4 fmol/well). An intraassay variation of 5% and interassay variation of 8% were obtained at 80 pmol/l. Serial dilution of plasma and saliva extracts showed a parallel displacement of enzyme-labeled Tyr^8-SP to synthetic SP in this EIA.

Specificity of the Antiserum (RA-08-095) Examined by EIA Immunospecificity of the antiserum (RA-08-095) was examined by EIA using Tyr^8-SP-labeled-β-Gal. The displacement curves of various SP-related polypeptides are shown in Fig. 2. Tyr^8-SP exhibited approximately 40% cross-reactivity as compared to synthetic SP, and the displacement curve was slightly different from that of SP. SP(O) inhibited the binding of Tyr^8-SP-labeled-β-Gal with the SP-antibody (approximately 30%). A carboxy-terminal fragment, SP(6—11) also inhibited the binding of Tyr^8-SP-labeled-β-Gal with the SP-antibody, but showed reduced cross reactivity (approximately 15%). SS also inhibited the binding of Tyr^8-SP-labeled β-Gal with the SP-antibody, but showed significantly reduced cross reactivity (0.01%). On the other hand, NKA, NKB and neuromedin-C hardly inhibited the binding. Thus, it was shown that the SP-antiserum, RA-08-095, can recognize precisely the central region of SP (Gln–Phe–Phe– and Gln–Phe–Tyr) and can distinguish SP from NKA, NKB and neuromedin-C, a structurally related peptide. Secretin, PHI, GIP, gastrin, VIP, BNP and NPY hardly inhibited the binding of Tyr^8-SP-labeled β-Gal with the SP-antibody.

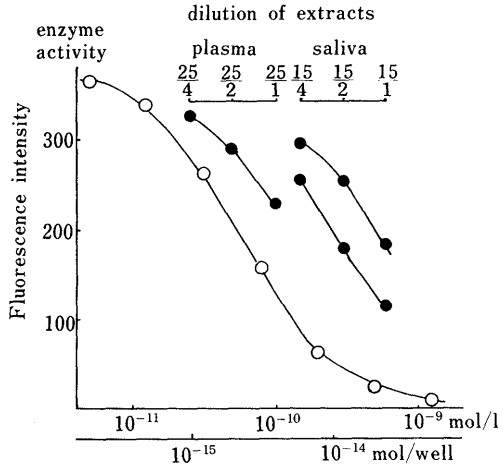

Fig. 1. Standard Curve of Synthetic SP and Dilution Curves of Plasma and Saliva Extracts Obtained with Antiserum RA-08-095 by EIA

Synthetic SP (○), plasma and saliva extracts (●).

Measurements of SP-Immunoreactive Substances (SP-IS) in Plasma and Saliva by EIA The proposed EIA was applied to the determination of SP in human plasma and saliva samples from 10 volunteers (5 men and 5 women, 24 to 43 years old). Concentrations of SP-IS in human plasma and saliva were 2.2 ± 1.1 pmol/l and 7.1 ± 4.0 pmol/l, respectively (Table II).

HPLC of Plasma and Saliva Extracts Human plasma and saliva extracts were subjected to reversed phase HPLC in order to study the molecular variants of SP-IS present

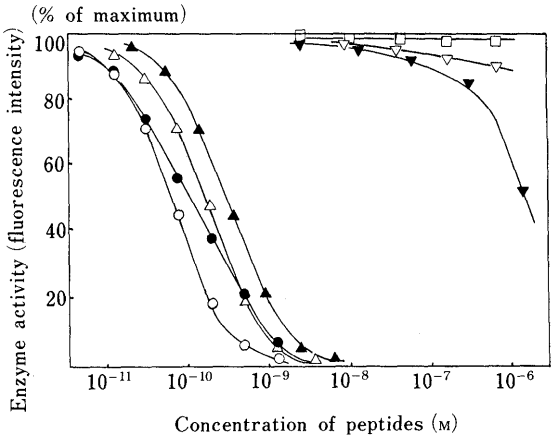

Fig. 2. Inhibition Curves of Various Peptides in EIA by Competition between Tyr^8-SP-Labeled β-D-Galactosidase and Various Peptides toward Antiserum RA-08-095

SP (○), Tyr^8-SP (●), SP (O) (△), SP (6—11) (▲), NKA (▽), somatostatin (▼), other peptides (NKB, neuromedin-C, VIP, secretin, gastrin, PHI, GIP) (□).

TABLE II. Levels of SP-IS in Human Saliva and Plasma (pmol/l)

Subject	Sex	Age	Plasma	Saliva
1	M	34	3.8	4.7
2	M	43	2.0	3.9
3	F	24	0.8	5.8
4	F	25	3.4	17.5
5	M	37	3.2	5.2
6	F	25	2.1	6.5
7	F	27	1.0	6.5
8	M	34	1.3	10.0
9	F	33	3.4	6.9
10	M	30	1.3	3.9
Mean ± S.D.			2.2 ± 1.1	7.1 ± 4.0

M, male; F, female.

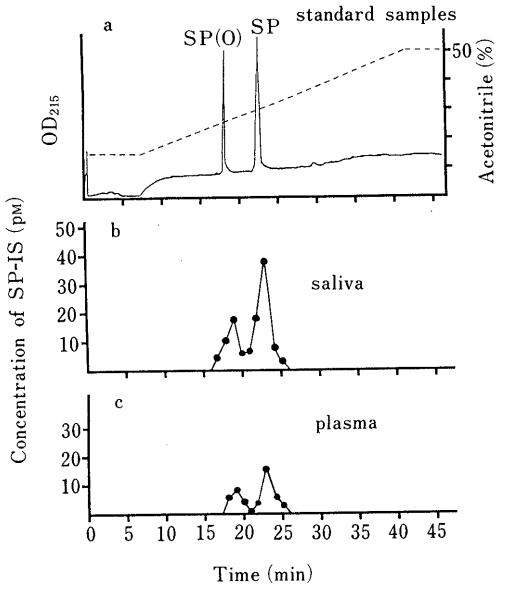

Fig. 3. Elution Profiles of Plasma and Saliva Extracts by HPLC on a C_{18} Column

SP-ISs extracted from plasma (5 ml) and saliva (3 ml) samples by Sep-Pak C_{18} were dissolved in 15% MeCN in 0.1% TFA and injected onto HPLC. The MeCN gradient is indicated by the dotted line. Fractions were lyophilized and their content of SP-IS was measured. Synthetic SP and SP(O) were run in a separate chromatography under the same conditions and their elution pattern was shown in Fig. 3a: column, Wakosil 5C18 (4.6 × 150 mm); flow rate, 1 ml/min.

in human plasma and saliva. Extracts from human plasma and saliva by a Sep-Pak C_{18} cartridge were applied to reversed phase HPLC on a C_{18} column, and the elution profiles revealed the presence of two immunoreactive peaks (Fig. 3). One was eluted at the position corresponding to that of standard synthetic SP and the other eluted earlier at a position corresponding to that of standard SP(O).

Using β-Gal-labeled Tyr^8-SP as a marker antigen, an anti-rabbit IgG coated immunoplate and MUG as a fluorogenic enzyme substrate, we developed a highly sensitive and specific EIA for the quantitation of SP. The calibration curve was linear in the range of 6 pmol/l to 200 pmol/l and maximum sensitivity was 10 pmol/l (0.4 fmol/well). By an extraction procedure with Sep-Pak C_{18} from human plasma (more than 1 ml) and human saliva (more than 0.5 ml), the present method made it possible to determine SP-levels in human plasma and saliva. The cross-reactivity of the oxidative derivative, SP(O), was found to be 30%, and that of carboxy-terminal fragment of SP, SP(6—11), 15%. Other SP-related peptides, NKA, NKB and neuromedin-C, were distinguished by this EIA.

Stjernschantz *et al.*[15] reported an EIA of SP using a polystyrene plate coated with SP-poly-D-glutamic acid conjugate as a solid phase antigen. The inhibition curve described in their report was a significantly flattened slope standard curve. The EIA reported here, using β-Gal-labeled Tyr^8-SP, anti-rabbit IgG coated immunoplate and MUG as a fluorogenic substrate, was highly sensitive and specific for the quantitation of SP, and a sharp standard inhibition curve was obtained.

The level of SP-IS in plasma from 10 healthy volunteers reported here was 2.2 ± 1.0 pmol/l, which was a lower level than that detected by RIA.[14] The level of SP-IS in saliva was 7.1 ± 4.0 pmol/l, which was more than three times higher than that in plasma. SP was first isolated as a sialogenic peptide by Chang *et al.*[2] and the SP-neuron was thought to be present in sialaden. Thus, topically released SP was diluted by saliva. As the volume of saliva was smaller than that of plasma, the level of SP in saliva was higher than that in plasma.

The molecular heterogenicity in human saliva and plasma was also examined by HPLC (Fig. 3). The main SP-IS in plasma and saliva was eluted at the same elution volume of synthetic SP with a minor peak at the same elution volume of SP(O). No other SP-ISs in plasma and saliva were detected in HPLC elution fractions by the present EIA.

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