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NOVEL AND SENSITIVE NONCOMPETITIVE ENZYME IMMUNOASSAY FOR PEPTIDES

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A novel and sensitive noncompetitive enzyme immunoassay for peptides is described (Fig. 1). Peptides were biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate and were trapped onto anti-peptide IgG-coated polystyrene balls. After washing the polystyrene balls to eliminate other biotinylated substances, the biotinylated peptides were eluted with HCl and were reacted with anti-peptide Fab'-peroxidase conjugate. The complex formed was trapped onto streptavidin-coated polystyrene balls. Peroxidase activity bound to the polystyrene balls was assayed by fluorimetry. The detection limit of angiotensin I as a model peptide was 13 fg (10 amol)/tube and 0.8 ng/l of plasma, which was 80 to 480-fold lower than those previously reported by competitive radioimmunoassay and competitive enzyme immunoassay. And other peptides could also be measured more sensitively by the present noncompetitive enzyme immunoassay method than by competitive immunoassays.

Peptides identified in various animal tissues have been measured by competitive radioimmunoassay using ^{125}I-labeled peptides (1-3). In some recent studies, enzymes have been substituted for ^{125}I (4,5). The detection limits of peptides by these competitive immunoassays are 0.2-4.8 fmol (1-5).

This paper describes a novel noncompetitive enzyme immunoassay for peptides, which is much more sensitive than competitive immunoassays. Angiotensin I was used as a model peptide.

MATERIALS AND METHODS

Buffer

The regularly used buffer was 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl and 1 g/l bovine serum albumin (crystallized, Miles Laboratories, Ltd., Elkhart, Indiana) (buffer A).

Antigens and Antibodies

Angiotensin I and II were obtained from Peptide Institute, Inc., Osaka, Japan.

Anti-angiotensin I serum was raised in male New Zealand White rabbits by subcutaneous injections of angiotensin I-bovine serum albumin conjugate and angiotensin I at 6- to 9-week intervals (6). Angiotensin I was conjugated to an equal amount in mg of bovine serum albumin (fraction V, Sigma, St. Louis, Missouri) using glutaraldehyde (6), and the conjugate in 2.5 ml of 0.1 mol/l

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sodium phosphate buffer, pH 7.2, was emulsified with 2.5 ml of Freund's complete adjuvant (Iatron Laboratories, Inc., Tokyo, Japan). The amounts of angiotensin I used for the first and second immunizations of two rabbits were 7 and 5 mg, respectively. For the third immunization, angiotensin I (1.5 mg) in 2 ml of saline was emulsified with 2 ml of the adjuvant. Blood was collected 13 days after the last immunization, and the antiserum was stored at -20°C. IgG was prepared from serum by fractionation with Na_2SO_4 followed by passage through a column of diethylaminoethyl cellulose (7). $F(ab')_2$ was prepared by digestion of IgG with pepsin, and Fab' was prepared by reduction of $F(ab')_2$ (7). The amount of IgG and its fragments was calculated from the absorbance at 280 nm (7).

Angiotensin I-Sepharose 4B

Angiotensin I (0.5 mg) was coupled to activated CH-Sepharose 4B (0.15 g, Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the instructions of Pharmacia.

Affinity-Purified Anti-Angiotensin I Fab'-Peroxidase Conjugate

Anti-angiotensin I Fab' was conjugated to horseradish peroxidase (Grade I, RZ=3.0, Boehringer Mannheim GmbH, Mannheim, FRG) using N-succinimidyl-6-maleimidohexanoate (Dojindo Laboratories, Kumamoto, Japan) (8). The conjugate was affinity-purified by elution from a column of angiotensin I-Sepharose 4B at pH 2.5 (9). The amount of the conjugate was calculated from peroxidase activity (7).

Treatment of Anti-Angiotensin I IgG with Charcoal

Anti-angiotensin I IgG (0.3 mg) in 180 µl of 0.1 mol/l sodium phosphate buffer, pH 7.0, was mixed with 20 µl of 1 mol/l HCl to adjust the pH to 2.5 and was incubated with 4 mg of charcoal (Norit A, Nakarai Chemicals Ltd., Kyoto, Japan) at 30°C for 5 min with continuous shaking. The mixture was centrifuged at 3,000 rpm for 3 min, and the supernatant was neutralized by addition of 30 µl of 1 mol/l sodium phosphate buffer, pH 7.0.

Biotinyl Nonspecific Rabbit IgG

Biotinyl nonspecific rabbit IgG was prepared by the reaction of maleimide-nonspecific rabbit IgG with N-biotinyl-2-mercaptoethylamine (10).

Protein-Coated Polystyrene Balls

Polystyrene balls (3.2 mm in diameter, Precision Plastic Ball Co., Chicago, Illinois) were coated with rabbit anti-angiotensin I IgG (0.1 g/l) before and after charcoal-treatment and biotinyl nonspecific rabbit IgG (0.1 g/l) by physical adsorption (11). Streptavidin-coated polystyrene balls were prepared by incubation of the biotinyl nonspecific rabbit IgG-coated polystyrene balls with streptavidin (0.1 g/l) (Bethesda Research Laboratories, Inc., Maryland) at 30°C for 4 h. The protein-coated polystyrene balls were stored in buffer A containing 1 g/l NaN_3 at 4°C.

Plasma Samples

Blood (7 ml) was collected from healthy subjects in pre-chilled tubes containing 10.5 mg of disodium ethylenediaminetetraacetate (EDTA). Plasma was separated by centrifugation at 4°C and stored at -20°C until use.

Enzyme Immunoassay A

Dilution of plasma and angiotensin I. Plasma (5 µl) was incubated with 5 µl of 0.16 mol/l sodium phosphate buffer, pH 4.6, containing 4 mmol/l 8-hydroxyquinoline, an inhibitor of angiotensin I converting enzyme (12) and 20 µmol/l pepstatin A (Peptide Institute), an inhibitor of renin (13), on ice for 20 min and was diluted 11.5-fold with chilled 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 4 mmol/l EDTA and 10 µmol/l pepstatin A. Angiotensin I (0.56 mg) dissolved in 0.5 ml of distilled water was diluted with the diluted plasma described above or with 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 0.17 mmol/l 8-hydroxyquinoline, 4 mmol/l EDTA, 1 g/l bovine serum albumin and 10 µmol/l pepstatin A. The amount of angiotensin I was calculated by taking the molecular weight as 1,297 (14).

Biotinylation. A 115 µl aliquot of the diluted angiotensin I or the diluted plasma was incubated with 5 µl of 120 mmol/l sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce Chemical Company, Rockford, Illinois) in dimethylsulfoxide at 30°C for 1 h. After incubation, the reaction mixture was incubated with 10 µl of 2 mol/l glycine-NaOH, pH 7.5, at 30°C for 1 h,

followed by addition of 20 µl of 0.1 mol/l sodium phosphate buffer, pH 7.0, containing 1 g/l bovine serum albumin, 0.1 mol/l NaCl and 7.5 g/l NaN_3. The mixture (150 µl) was subjected to enzyme immunoassay.

Enzyme Immunoassay. A polystyrene ball coated with anti-angiotensin I IgG before charcoal-treatment was incubated with biotinylated angiotensin I described above in a total volume of 150 µl at 20°C overnight. After incubation, the polystyrene ball was washed twice by addition and aspiration of 2 ml of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl, and incubated with the mixture of 100 µl of buffer A and 20 µl of 1 mol/l HCl at 30°C for 1 h. After removal of the polystyrene ball, the remaining solution was neutralized by addition of the mixture of 10 µl of 1 mol/l sodium phosphate buffer, pH 7.0, and 20 µl of 1 mol/l NaOH. The neutralized mixture was incubated with affinity-purified anti-angiotensin I Fab'-peroxidase conjugate (50 fmol) in 20 µl of buffer A at 20°C for 3 h and 4°C overnight. Subsequently, two streptavidin-coated polystyrene balls were added, and the incubation was continued at 20°C for 4 h with continuous shaking. After removal of the reaction mixture, the polystyrene balls were washed twice as described above, and peroxidase activity bound to the polystyrene balls was assayed at 30°C for 60 min using 3-(4-hydroxyphenyl) propionic acid as substrate (15). Fluorescence intensity was measured relative to 0.2 mg/l quinine in 0.05 mol/l H_2SO_4 (15).

Enzyme Immunoassay B

Dilution of plasma and angiotensin I. Plasma (50 µl) was incubated with 50 µl of 0.16 mol/l sodium phosphate buffer, pH 4.6, containing 4 mmol/l 8-hydroxyquinoline and 30 µmol/l pepstatin A on ice for 20 min. For extraction of angiotensin I, the incubated plasma (100 µl) was mixed with 50 µl of chilled buffer A containing 4 mmol/l EDTA and 3 g/l NaN_3. Angiotensin I dissolved in distilled water was diluted with buffer A containing 4 mmol/l EDTA and 3 g/l NaN_3 to a final volume of 50 µl. For extraction of angiotensin I, the diluted angiotensin I (50 µl) was mixed with the incubated plasma (100 µl) described above or 100 µl of 0.1 mol/l sodium phosphate buffer, pH 6.0, containing 1 g/l bovine serum albumin, 0.1 mol/l NaCl, 4 mmol/l EDTA, 2 mmol/l 8-hydroxyquinoline and 15 µmol/l pepstatin A.

Extraction of angiotensin I. A polystyrene ball coated with anti-angiotensin I IgG after charcoal-treatment was incubated with the diluted plasma or the diluted angiotensin I in a total volume of 150 µl at 4°C overnight. The polystyrene ball was washed twice by addition and aspiration of 2 ml of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.1 mol/l NaCl and incubated with the mixture of 75 µl of 10 mmol/l sodium phosphate buffer, pH 7.0, containing 0.5 g/l bovine serum albumin and 0.1 mol/l NaCl and 15 µl of 1 mol/l HCl at 30°C for 1 h. After removal of the polystyrene ball, the remaining solution was mixed with the mixture of 15 µl of 1 mol/l NaOH and 10 µl of 1 mol/l sodium phosphate buffer, pH 7.5, to adjust the pH to 7.5.

Biotinylation and enzyme immunoassay. Extracted angiotensin I (115 µl) was biotinylated and subjected to enzyme immunoassay as described above.

Expression of the Detection Limit of Angiotensin I

The detection limit of angiotensin I was taken as the minimal amount of angiotensin I which gave a bound peroxidase activity significantly in excess of that nonspecifically bound in the absence of angiotensin I (background). The existence of a significant difference from the background was confirmed by the *t*-test ($p < 0.001$, $n=5$).

RESULTS AND DISCUSSION

In the present noncompetitive enzyme immunoassay, peptides were biotinylated and measured using anti-peptide Fab'-peroxidase conjugate and streptavidin-coated polystyrene balls after elimination of other biotinylated substances (Fig. 1). Peptides were biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate and trapped onto anti-peptide IgG-coated polystyrene

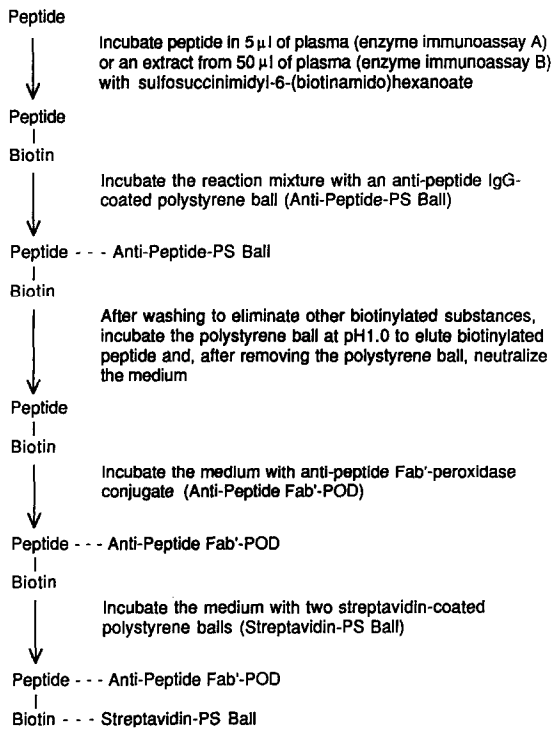

Fig. 1. Noncompetitive enzyme immunoassay for peptides.

balls. The polystyrene balls were washed to eliminate other biotinylated substances and subsequently treated at pH 1 to elute the biotinylated peptides. The biotinylated peptides eluted were reacted with anti-peptide Fab'-peroxidase conjugate and trapped onto streptavidin-coated polystyrene balls. Peroxidase activity bound to the polystyrene balls was assayed by fluorimetry. Angiotensin I was used as a model peptide.

Validation of the Present Enzyme Immunoassay for Angiotensin I

Specificity. Bound peroxidase activity in the presence of angiotensin II (13 ng/tube) was not significantly higher than that in the absence of angiotensin I (nonspecifically bound peroxidase activity) and significantly lower than that in the presence of 13 fg (10 amol, the detection limit) of angiotensin I.

Recovery. In the enzyme immunoassay A, the recoveries of angiotensin I added to plasma were 84-119 %, when angiotensin I at two different levels (260 and 780 ng/l) was added to three plasma samples (5 µl) containing 173-206 ng/l of angiotensin I. In the enzyme immunoassay B, the recoveries of angiotensin I added to plasma were 83-120 %, when angiotensin I (78 ng/l) was added to four plasma samples (50 µl) containing 97-156 ng/l.

Assay precision. The assay precision was examined at 9 different angiotensin I levels over the range of 173-1,032 ng/l in the enzyme immunoassay A and at 8 different angiotensin I levels over the range of 97-248 ng/l in the

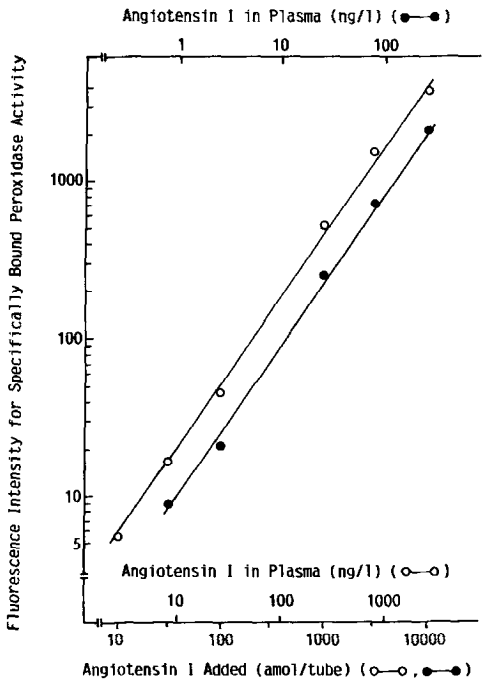

Fig. 2. Standard curves of angiotensin I by the enzyme immunoassays A (open circles) and B (closed circles). Each point is the mean of 5 determinations.

enzyme immunoassay B. The coefficients of within-assay variation in the enzyme immunoassays A and B were 5.9-9.9 % (n=5) and 4.9-13.5 % (n=5), respectively.

Detection limit and assay range. The detection limits of angiotensin I by the enzyme immunoassays A and B were 13 fg (10 amol)/tube and 39 fg (30 amol)/tube, respectively (Fig. 2). The detection limit by the enzyme immunoassay A is 80 to 480-fold lower than those previously reported by competitive radioimmunoassay (1) and competitive enzyme immunoassay (4,12). The assay range of plasma angiotensin I in the enzyme immunoassay A was 2.6-2,600 ng/l using 5 µl of plasma. In the enzyme immunoassay B, in which angiotensin I was extracted from 50 µl of plasma, the assay range of plasma angiotensin I was 0.8-260 ng/l (Fig. 2). The detection limit of plasma angiotensin I by the enzyme immunoassay B is 80 to 330-fold lower than those obtained by competitive radioimmunoassay (1) and competitive enzyme immunoassay (4,12). The present method was sufficiently sensitive to measure plasma angiotensin I in healthy subjects (154 ± 38 (SD) ng/l; range, 97-206 ng/l; n=7).

Applicability of the Present Enzyme Immunoassay

By the present enzyme immunoassay, α-human atrial natriuretic peptide (28 amino acid peptide) (16), kassinin (12 amino acid peptide) (17) and [Arg^8]-vasopressin (9 amino acid peptide) (18) have been measured 30 to 750-fold more sensitively than by competitive immunoassay using the corresponding peptides

labeled with ^{125}I or enzymes. These results will be described in detail elsewhere.

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