Novel and sensitive noncompetitive enzyme immunoassay for kassinin in rat plasma

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Received 21 November 1988; accepted 17 January 1989

Summary. A novel and sensitive noncompetitive enzyme immunoassay for kassinin is described. Kassinin was biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate. The biotinylated kassinin was trapped on anti-kassinin IgG-coated polystyrene balls and, after washing to eliminate other biotinylated substances, was eluted with HCl. The biotinylated kassinin eluted was reacted with anti-kassinin Fab'-peroxidase conjugate and trapped onto streptavidin-coated polystyrene balls. Peroxidase activity bound to the polystyrene balls was assayed by fluorimetry. The detection limit of kassinin was 0.13 pg (0.1 fmol)/tube or 0.065 µg/l of rat plasma, which was 750-fold or 15-fold lower than that for competitive radioimmunoassay.
Key words. Enzyme immunoassay; peptide; kassinin; biotinylation.

Kassinin, a dodecapeptide isolated from the skin of the African frog, *Kassina senegalensis*, is similar to substance P in structure[1] and biological activity[2]. Kassinin and substance P share an identical sequence of three amino acids at the C-terminus[1]. Both kassinin and substance P stimulate the secretion of growth hormone, prolactin and follicle-stimulating hormone in rats, suggesting the physiological role of kassinin as a neuropeptide[2]. For further investigation of the possible physiological roles of kassinin, this paper describes a novel and sensitive noncompetitive enzyme immunoassay for kassinin, which is 15- to 750-fold more sensitive than competitive radioimmunoassay.

Materials and methods. Buffers. The regularly used buffers were 10 mmol/l sodium phosphate buffer, pH 7.0, containing 1 g/l bovine serum albumin and 0.1 mol/l NaCl (buffer A) and 50 mmol/l sodium phosphate buffer, pH 7.0, containing 0.15 mol/l NaCl, 1 g/l gelatin and 0.2 g/l NaN_3 (buffer B). Bovine serum albumin (fraction V) was obtained from Armour Pharmaceutical Co., Kankakee, Illinois, USA. Gelatin was obtained from Nakarai Chemicals Ltd., Kyoto, Japan.

Antigens and antibodies. Kassinin and tyrosyl-kassinin were synthesized[3] and generously donated by Dr Yajima (Kyoto University, Kyoto, Japan). Substance P was obtained from Peptide Institute, Inc., Osaka, Japan.

Anti-kassinin serum was raised in male New Zealand white rabbits by subcutaneous injections of kassinin-bovine serum albumin conjugate five times at 2-week intervals. Kassinin (4 mg) was conjugated to bovine serum albumin (6 mg, Armour) using *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce Chemical Company, Rockford, Illinois, USA)[4]. The conjugate (125 µg) in 1 ml of 0.1 mol/l sodium phosphate buffer, pH 7.0, was emulsified with 1 ml of Freund's complete adjuvant (Nakarai) for the first to third immunizations and with 1 ml of Freund's incomplete adjuvant (Nakarai) for the other immunizations. The amount of the conjugate injected each time per rabbit was 125 µg. Blood was collected a week 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[5]. $F(ab')_2$ was prepared by digestion of IgG with pepsin, and Fab' was prepared by reduction of $F(ab')_2$[5]. The amount of IgG and its fragments was calculated from the absorbance at 280 nm[5].

Kassinin-Sepharose 4B. Kassinin (0.45 mg) was coupled to activated CH-Sepharose 4B (0.17 g, Pharmacia Fine Chemicals AB, Uppsala, Sweden) according to the instructions of Pharmacia.

Affinity-purified rabbit anti-kassinin Fab'-peroxidase conjugate. Rabbit anti-kassinin 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)[6]. The conjugate was affinity-purified by elution from a column of kassinin-Sepharose 4B at pH 2.5[7]. The amount of the conjugate was calculated from peroxidase activity[5].

Affinity-purified rabbit anti-kassinin IgG. Rabbit anti-kassinin IgG was affinity-purified by elution from a column of kassinin-Sepharose 4B at pH 2.5[7].

Biotinyl nonspecific rabbit IgG. Biotinyl nonspecific rabbit IgG was prepared by the reaction of maleimide-nonspecific rabbit IgG with N-biotinyl-2-mercaptoethylamine[8].

Protein-coated polystyrene balls. Polystyrene balls (3.2 mm in diameter, Precision Plastic Ball Co., Chicago, Illinois, USA) were coated with rabbit anti-kassinin IgG (0.1 g/l) before and after affinity-purification and biotinyl nonspecific rabbit IgG (0.1 g/l) by physical adsorption[9]. 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, Life Technologies, Inc., Maryland, USA) 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 and dilution of kassinin. Blood (7 ml) was collected from male Wistar rats in prechilled tubes containing 10.5 mg of disodium ethylenediaminetetraacetate (EDTA). Plasma was separated by centrifugation at 4 °C and stored at −20 °C until use. Before use, plasma was diluted 25-fold with 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 4 mmol/l EDTA.

Kassinin (0.5 mg) was dissolved in 1 ml of 0.1 mol/l sodium borate buffer, pH 8.5, and diluted with 0.1 mol/l sodium phosphate buffer, pH 7.5, containing 4 mmol/l EDTA and 1 g/l bovine serum albumin or the diluted plasma described above.

Biotinylation of kassinin. A 50-µl aliquot of the diluted kassinin in the presence of bovine serum albumin or plasma was incubated with 3 µl of 53 mmol/l sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin, Pierce) in dimethylsulfoxide at 30 °C for 1 h. After incubation, the reaction mixture was incubated with 7 µl of 1 mol/l glycine-NaOH, pH 8.0, at 30 °C for 1 h, followed by addition of 90 µl of buffer A containing 1 g/l NaN_3 and 4 mmol/l EDTA. The mixture (150 µl) was subjected to the present enzyme immunoassay. The amount of kassinin was calculated by taking the molecular weight as 1335 [1].

Present enzyme immunoassay for kassinin. Two rabbit anti-kassinin IgG-coated polystyrene balls were incubated with the biotinylated kassinin described above in a total volume of 150 µl at 20 °C overnight. After incubation, the polystyrene balls were 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 balls, 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 0.9 mol/l NaOH. The neutralized mixture was incubated with affinity-purified rabbit anti-kassinin Fab'-peroxidase conjugate (500 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 bound peroxidase activity was assayed at 30 °C for 60 min using 3-(4-hydroxyphenyl) propionic acid as substrate [10]. Fluorescence intensity was measured relative to 0.2 mg/l quinine in 0.05 mol/l H_2SO_4 using 320 nm for excitation and 405 nm for emission with a Shimadzu fluorophotometer (RF-510, Shimadzu Seisakusho, Ltd., Kyoto, Japan).

Iodination of kassinin. Tyrosyl-kassinin (5 µg) was iodinated with $Na^{125}I$ (Amersham, Buckinghamshire, UK) by the chloramine T method [11]. ^{125}I-tyrosyl-kassinin was purified by gel filtration on a column of Sephadex G-10 (Pharmacia) using buffer B. The specific activity of ^{125}I-tyrosyl-kassinin was 11 µCi/µg.

Radioimmunoassay for kassinin. Kassinin (0.5 mg) was dissolved in 1 ml of 0.1 mol/l sodium borate buffer, pH 8.5, and diluted with buffer B. Rabbit anti-kassinin serum was diluted 1000-fold with buffer B. The diluted kassinin (0.1 ml) was incubated with 0.2 ml of the diluted anti-kassinin serum, 0.1 ml of ^{125}I-tyrosyl-kassinin (10,000 cpm) in buffer B and 0.2 ml of buffer B at 4 °C for 18 h. After incubation, 0.2 ml of buffer B containing 25 g/l Norit A (Nakarai) and 2.5 g/l Dextran T-70 (Pharmacia) was added to the reaction mixture and the incubation was continued at 4 °C for 15 min, followed by centrifugation at 3000 rpm at 4 °C for 20 min. The radioactivity in the supernatant was counted by a Packard gamma counter (PRIAS-240CGD, Packard Instrument Co., Inc., Downers Grove, Illinois, USA).

Expression of the detection limit of kassinin. The detection limit of kassinin in the present enzyme immunoassay was taken as the minimal amount of kassinin which gave a bound peroxidase activity significantly in excess of that nonspecifically bound in the absence of kassinin (background). The existence of a significant difference from the background was confirmed by the t-test ($p < 0.01$, $n = 5$).

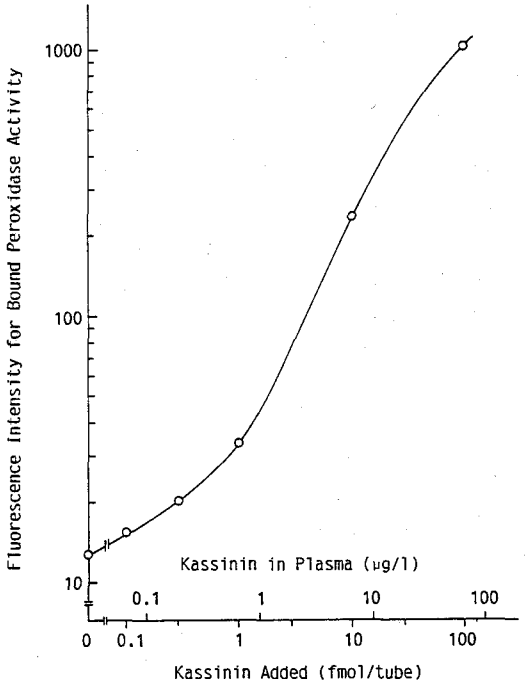
Results and discussion. In the present noncompetitive enzyme immunoassay, kassinin was biotinylated and measured using anti-kassinin Fab'-peroxidase conjugate and streptavidin-coated polystyrene balls after elimination of other biotinylated substances. Kassinin in buffer, which contained bovine serum albumin, or in plasma, was biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate. The biotinylated kassinin was trapped on anti-kassinin IgG-coated polystyrene balls. The polystyrene balls were washed to eliminate other biotinylated substances and subsequently treated at pH 1 to elute the biotinylated kassinin. The biotinylated kassinin eluted was reacted with anti-kassinin Fab'-peroxidase conjugate and trapped on streptavidin-coated polystyrene balls. Peroxidase activity bound to the polystyrene balls was assayed by fluorimetry.

Validation of the present enzyme immunoassay for kassinin. Specificity. Bound peroxidase activity in the presence of substance P (1.3 ng/tube) was not significantly higher than that in the absence of kassinin (nonspecifically bound peroxidase activity) and significantly lower than that in the presence of 0.13 pg (0.1 fmol, the detection limit) of kassinin.

Recovery. When kassinin at three different levels (0.65–19.5 µg/l) was added to a rat plasma sample (2 µl), the recoveries of added kassinin were 101–119%.

Assay precision. The assay precision was examined at 6 different kassinin levels over the range of 0.84–26.7 µg/l. The coefficient of within-assay variation was 7.9–13% ($n = 5$).

Detection limit and assay range. The detection limit for kassinin was 0.13 pg (0.1 fmol)/tube. This was 750-fold lower than the detection limit by competitive radioimmunoassay. The assay range of plasma kassinin was 0.065–65 µg/l using 2 µl of plasma (fig.). The detection limit for plasma kassinin by the present method was



Standard curve for kassinin by the present enzyme immunoassay. Each point is the mean of 5 determinations.

15-fold lower than that obtained by competitive radioimmunoassay. Therefore, the present method appeared to be sufficiently sensitive to measure the level of plasma kassinin, when rats were intravenously injected with 0.3–3.0 µg of kassinin per 100 g of body weight to stimulate the secretion of growth hormone[2]. (The detection limit for kassinin using affinity-purified rabbit anti-kassinin IgG-coated polystyrene balls was 40 fg (30 amol)/tube or 20 ng/l using 2 µl of plasma.)

Plasma kassinin in rats. The levels of plasma kassinin in two rats, measured by the present method, were found to be 0.84 and 0.95 µg/l. However, the exact nature of the substance(s) measured by the present method remains to be investigated.

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0014-4754/89/050470-03$1.50 + 0.20/0

The effects of beta-endorphin on arginine-8-vasopressin and oxytocin levels in rat brain areas

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Received 6 June 1988; accepted 19 January 1989

Summary. Measurements were made of the effects of intracerebroventricular treatment with beta-endorphin (BE; 100 ng) on the arginine-8-vasopressin (AVP) and oxytocin contents of rat hypothalamic and limbic brain areas (hippocampus, amygdala and septum). The hormone concentrations were determined by radioimmunoassay. The administration of BE resulted in a significant reduction of the AVP level in the amygdala in a naloxone-reversible manner. Naloxone (Nal) administered subcutaneously significantly increased the AVP content in the septum. The results revealed that BE and Nal had regionally specific effects on the activity of the vasopressinergic system but not on that of the oxytocinergic system in the brain.
Key words. Beta-endorphin; vasopressin; oxytocin; brain regions.