Biochemical Pharmacology, Vol. 30, No. 12, pp. 1659–1664, 1981.
Printed in Great Britain.

0006-2952/81/121659-06 $02.00/0

ENZYME IMMUNOASSAY OF BRADYKININ USING β-D-GALACTOSIDASE AS A LABELING ENZYME*

AKINORI UENO,† SACHIKO OH-ISHI, TSUNEHIRO KITAGAWA‡ and MAKOTO KATORI
Department of Pharmacology, Kitasato University School of Medicine, Sagamihara, Kanagawa 228, Japan, and ‡ Department of Microbiological Chemistry, Faculty of Pharmaceutical Sciences, Nagasaki University, Nagasaki 852, Japan

(*Received* 9 *June* 1980; *accepted* 21 *November* 1980)

Abstract—An enzyme immunoassay of bradykinin, using β-D-galactosidase from *Escherichia coli* as a labeling enzyme, is described. Bradykinin, conjugated to the enzyme with a hetero bifunctional type of coupling agent, *N*-(*m*-maleimidobenzoyloxy)succinimide, was prepared as a labeled antigen. Antisera against bradykinin were obtained from male rabbits immunized with bradykinin linked to albumins (ovalbumin or bovine serum albumin) with toluene 2,4-diisocyanate or 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. These antisera were tested for their abilities to bind the labeled antigen and for their sensitivities. The antigen–antibody reaction was performed in an ice bath for 18 hr; this was incubated for another 4 hr, after addition of anti-rabbit IgG antiserum from goat (double antibody method) to separate the bound antigen from free antigen; the enzyme activity in the precipitate was measured with a fluorogenic substrate. Some of the antisera showed good sensitivity when assayed by this method, the sensitivity having been comparable to that of radioimmunoassays of bradykinin. With this method, 30 pg/tube (0.2 ml) of bradykinin could be measured, and the standard curve was obtained in the range of 30 pg to 10 ng of bradykinin. The kininogen level in human plasma was determined by conversion of kininogen to bradykinin by trypsin after heating plasma at 60°. Kininogen levels obtained from six human subjects were in good agreement with those obtained by bioassay.

Bioassays have been used as sensitive, specific, and sophisticated tools to determine minute amounts of biologically active substances in various biological samples. Recently, many active substances have also been measured by radio-receptor assays or radioimmunoassays, which may have advantages in sensitivity and simplicity over bioassays.

A radioimmunoassay for bradykinin (BK)§ has been developed [1–8], with a sensitivity within the range suitable for measurement of free kinin in blood [6, 8]. Although the radioimmunoassay is excellent, some difficulties when using radioisotopes were inevitable.

Recently, enzymes, instead of radioisotopes, have been used for the labeling of antigens [9]. Enzyme immunoassays, using β-D-galactosidase as a labeling enzyme and *N*-(*m*-maleimidobenzoyloxy)succinimide as a coupling agent, have been developed by Kitagawa and his colleagues and applied to insulin [10], viomysin [11] and angiotensin [12]. The working ranges of these assays were reported to be 20–200, 1.5–200, and 100–4000 pg respectively.

This paper describes an enzyme immunoassay of bradykinin using β-D-galactosidase and *N*-(*m*-maleimidobenzoyloxy)succinimide.

Some of the preliminary work for this paper was presented at the International Symposium on Kinins (Kinin II, 1978, Tokyo) [13].

MATERIALS AND METHODS

Conjugation of bradykinin to β-D-galactosidase

A solution of bradykinin triacetate (1.5 mg, 1.5 μmoles, Peptide Institute Inc., Minoh, Osaka) in 75 mM sodium phosphate buffer (pH 7.0, 1 ml) was incubated at 30° for 30 min with 0.38 ml of a tetrahydrofuran solution of *N*-(*m*-maleimidobenzoyloxy)succinimide (MBS) (1.88 mg, 6.0 μmoles) that had been prepared by a method reported previously [10, 11]. The MBS-acylated bradykinin that formed was precipitated from the solution by the addition of 2.5 ml ethanol and then *ca.* 10 ml ether. The precipitate was washed three times, each time with 3 ml of ether, and then was dissolved in 1.1 ml of 0.1 M sodium phosphate buffer (pH 6.5) containing 23% ethanol. The maleimide content of the solution (68 nmoles/ml) was determined by back titration of the thiol content after addition of an excess of mercaptoethanol. MBS-acylated BK (3.6 nmoles of maleimide residue content) was conjugated to β-D-galactosidase from *Escherichia coli* (EC 3.2.1.23, Boehringer Mannheim GmbH, West Germany, 0.93 nmole) in 75 mM sodium phosphate buffer at room temperature for 2 hr. The reaction mixture was then directly chromatographed on a Sepharose 6B column (2 × 35 cm) using 0.02 M sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 0.1% (w/v) NaN_3,

* Partially supported by a Grant-in-Aid for Developmental Scientific Research and a Grant-in-Aid for Special Project Research from the Ministry of Education, Science and Culture (310507 and 387061).

† Address all correspondence to: Akinori Ueno, Department of Pharmacology, Kitasato University, School of Medicine, 1-15-1 Kitasato, Sagamihara, Kanagawa 228, Japan.

§ Abbreviations: BK, bradykinin; OA, ovalbumin; BSA, bovine serum albumin; TC, toluene 2,4-diisocyanate; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MBS, *N*-(*m*-maleimidobenzoyloxy)succinimide; and NRS, non-immunized rabbit serum.

0.1% (w/v) bovine serum albumin (BSA, Fraction V, Sigma Chemical Co., St. Louis, MO) and 2 mM $MgCl_2$ (buffer A) as an eluent. The fractions containing the peak of the enzyme activity were used for this assay. The solution of β-D-galactosidase–BK conjugate was stable for over a year at 4° in buffer A. The enzyme activity of β-D-galactosidase was not reduced by conjugation with BK. A K_m value of the enzyme conjugate of BK was 0.31 mM which was not different from that of the original enzyme. One unit of β-D-galactosidase activity was defined as the amount that hydrolyzed 1 μmole of 4-methylumbelliferyl-β-D-galactoside (Nakarai Chemicals Ltd., Kyoto) per minute under the conditions described below. The amount of the conjugate used for this assay was 5 μunits/assay tube.

Antisera

Preparation of immunogens. Immunogens to raise antisera against BK were synthesized by two different methods: (1) the method of Talamo *et al.* [14] and (2) the method of Goodfriend *et al.* [15]. According to method 1, BK triacetate (32.7 mg) was linked to ovalbumin (OA, 20 mg, Grade VI, Sigma) or BSA (20 mg, Fraction V, Sigma), using 75 μl of toluene 2,4-diisocyanate (Nakarai) as a coupling agent, as described in Refs. 1, 4, 7 and 14. These products are abbreviated as BK-TC-OA and BK-TC-BSA. After each reaction mixture had been dialyzed against 2 liters of 0.1 N ammonium bicarbonate solution to remove free BK, it was lyophilized. The yield of BK-TC-OA was 20 mg, and that of BK-TC-BSA was 21 mg. With method 2, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Nakarai) was used as a coupling agent. EDC (150 mg) was dropped into the mixture of BK triacetate (14 mg) and BSA (14 mg). The yield of this conjugate (abbreviated as BK-BSA) was 17 mg. These immunogens were stored at −75° until used.

Immunization. BK-TC-OA or BK-TC-BSA (2 mg) was dissolved in 0.5 ml of saline, emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, MI), and then injected subcutaneously into the foot pads of male rabbits. BK-TC-OA was injected into two New Zealand albino rabbits (AU-1 and AU-2: antisera obtained from these rabbits are referred to as AU-1 and AU-2 and so forth), and BK-TC-BSA was injected into a New Zealand albino rabbit (AU-3) and a Japanese white rabbit (AU-4). After 3–5 months, the emulsion of the incomplete Freund's adjuvant (Difco) containing 1 mg of the immunogen was injected into multiple subcutaneous sites on the back of each animal. The second and third booster injections (100 μg of each immunogen per animal) were given at 2–4 week intervals. The other immunogen coupled with EDC was injected into four Japanese white rabbits (AU-6 to AU-9). The initial injection was given with 1 mg of the immunogen per rabbit, and then 0.5 mg of the immunogen was injected in the same way as a booster every 3 months. One or two weeks after the last booster, rabbit blood was collected from a central artery of an ear and allowed to clot completely in a plastic tube (Falcon No. 2005, Falcon, Oxnard, CA). Serum was separated and stored at −75° until use.

Procedure of enzyme immunoassay

The buffer used for the antigen–antibody reaction consisted of 0.02 M sodium phosphate (pH 7.0) containing 0.1 M NaCl, 0.1% (w/v) BSA, 0.1% (w/v) NaN_3, 2.6 mM EDTA–$2Na^+$, and 3 mM 1,10-phenanthroline (Wako Pure Chemicals Industries, Ltd., Osaka) (buffer B). Buffer B was used for all dilutions of antiserum, for the labeled antigen, and for samples or BK standard solution.

Fifty μl of the solution of the enzyme-labeled antigen, 100 μl of an unknown sample solution or BK standard solution, and 50 μl of the diluted solution of the anti-BK antiserum were mixed in a polypropylene tube (Falcon No. 2006). The mixture (0.2 ml) was first incubated in an ice bath for 18 hr. Then, 10 μl of a 10-fold dilution of non-immunized rabbit serum (NBS) and 10 μl of a 4-fold dilution of anti-rabbit IgG antiserum from goat (Medical Biological Lab. Co., Nagoya) were added. At the end of the second incubation for 4 hr in an ice bath, 1 ml of buffer A was added and thoroughly mixed; then the mixture was centrifuged at 2000 *g* for 30 min at 4° and the supernatant fraction was removed by decanting. The activity of β-D-galactosidase in the precipitate was assayed by addition of 300 μl of buffer A containing 0.2 mM 4-methylumbelliferyl-β-D-galactoside (Nakarai Chemicals Ltd.) as a substrate. After incubation for 2 hr at 37° with shaking, 3 ml of 0.2 M sodium phosphate–NaOH buffer (pH 10.3) was added to terminate the reaction. The amount of 4-methylumbelliferone released was measured with a spectrofluorophotometer (Hitachi MPF-3, Hitachi Ltd., Tokyo). Fluorescence was read at 448 nm (emission), with excitation at 365 nm. Each assay was run in triplicate, and one set of tubes was assayed in the absence of the anti-BK antiserum as a blank, to estimate the non-specific binding of the enzyme to the precipitate and the wall of the tube.

Extraction of BK released from human kininogens in plasma

BK, released from human plasma by trypsin (Worthington Biochemical Corp., Freehold, NJ), was extracted by the method of Uchida and Katori [16]. Blood was collected in a plastic tube with 1/10 volume of 3.8% (w/v) sodium citrate. Plasma was obtained by centrifugation (1000 *g* for 15 min at 25°) and then immediately treated for 1 hr at 60°. A mixture containing 0.2 ml of heated plasma, 1.7 ml of distilled water, 0.5 ml of 0.2 M Tris–HCl buffer (pH 7.8), 0.1 ml of trypsin solution (2 mg/ml), and 0.2 ml of 1,10-phenanthroline solution (2 mg/ml) was incubated for 30 min at 37° in a siliconized glass tube to release BK. Then, 5 ml of hot ethanol was added to the tube. After heating at 70° for 15 min, the tube was centrifuged for 15 min at 2000 *g*. The supernatant fluid that collected was evaporated to dryness at 35° under reduced pressure in a 50 ml siliconized round-bottomed flask. The residue was dissolved with 2 ml of distilled water, acidified with 0.1 ml of 0.01 N HCl, and twice washed with 10 ml of diethylether to remove lipid [6]. The aqueous phase was evaporated to dryness under the reduced pressure. The dried samples were stored at −20° until use. The samples were redissolved with 40 ml

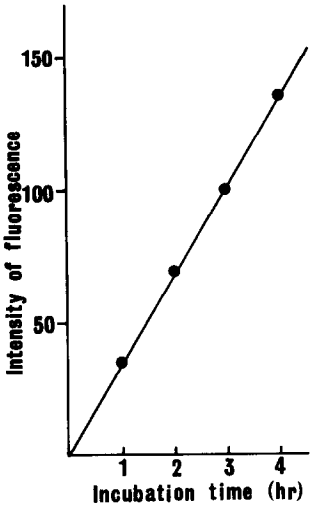

Fig. 1. Relative amounts of 4-methylumbelliferone released by BK–β-D-galactosidase after 1, 2, 3 and 4 hr of incubation. Five μunits of BK–β-D-galactosidase was incubated at 37° with 0.2 mM 4-methylumbelliferyl-β-D-galactoside in buffer A. The amounts of 4-methylumbelliferone released by the enzyme were measured by fluorescence at 448 nm (excitation 365 nm), and expressed as the intensity of the fluorescence in arbitrary units on the ordinate.

of buffer B when used for enzyme immunoassay. Soybean trypsin inhibitor was purchased from Worthington.

RESULTS

Optimal conditions for enzyme immunoassay

The amount of BK–β-D-galactosidase and substrate concentration were first varied to find the proper conditions for this enzyme immunoassay. When 5 μunits of the enzyme-labeled antigen were assayed, the fluorescence intensity observed was twenty-five times that of the reagent blank. Figure 1 indicates the amount of 4-methylumbelliferone

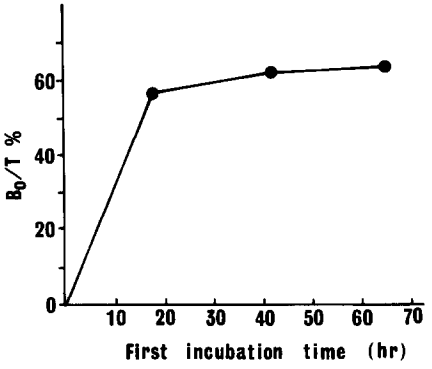

Fig. 3. Per cent binding of labeled antigen after various hours of incubation of the first antibody reaction at 0°. The ordinate shows the percentage of enzyme activity bound to antibody (B_0) with respect to the total enzyme activity added (T). The abscissa indicates the incubation time after the addition of antibody (AU-4. 1:20,000 in final dilution). The second incubation time was fixed at 4 hr with 10 μl of a 4-fold dilution of the second antiserum.

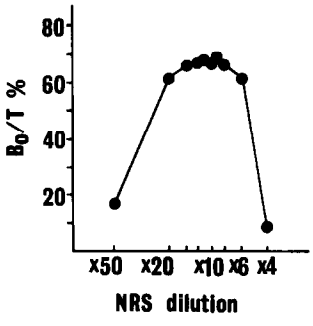

Fig. 4. Ratios of carrier to second antibody. The ordinate is the same as that of Fig. 2. Binding of the labeled antigen was examined by using 10 μl of a 4-fold dilution of anti-rabbit IgG antiserum from goat (second antibody). The abscissa indicates dilution of non-immunized rabbit serum (NRS) added as a carrier. The incubation time was 4 hr at 0°. The first incubation was 18 hr at 0° with AU-4 at final dilution of 1:20,000.

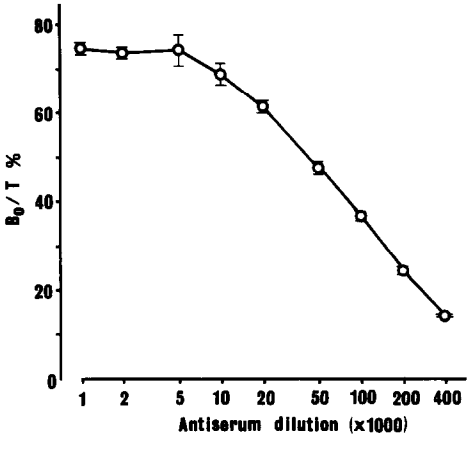

Fig. 2. Binding of enzyme-labeled antigen to antiserum AU-4. The abscissa indicates dilution of antiserum AU-4. The ordinate shows the percentage of enzyme activity bound to antibody (B_0) [100% was the total enzyme activity added (T)], corrected for non-specific binding (assay blank).

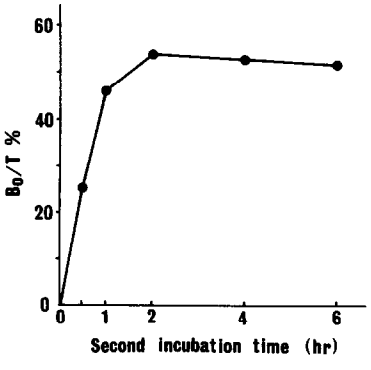

Fig. 5. Effect of incubation time after addition of second antibody. The dilution of the second antiserum was fixed at 4-fold (10 μl/tube), and NRS used was 10-fold dilution. The ordinate and the first incubation conditions are the same as in Fig. 2.

Table 1. Binding and sensitivity of labeled antigen to various anti-bradykinin antisera obtained from immunized rabbits*

Antiserum	Immunogen	Final serum dilution	Bound % (B_0)	BK ID_{50} (ng/tube)
AU-1	BK-TC-OA	1:20,000	31	4.0
AU-2	BK-TC-OA	1:20,000	40	2.2
AU-3	BK-TC-BSA	1:2000	51	1.3
AU-4	BK-TC-BSA	1:20,000	50	0.74
AU-6	BK-BSA	1:2000	15	233
AU-7	BK-BSA	1:2000	50	18.0
AU-9	BK-BSA	1:2000	23	22.0

* BK-TC-OA or BK-TC-BSA denotes immunogen, ovalbumin (OA), or bovine serum albumin (BSA) coupled to bradykinin (BK) through toluene 2,4-diisocyanate (TC). BK-BSA denotes BSA conjugated to BK with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. B_0 is the percentage of labeled antigen that was antibody-bound. The individual values of B_0 and 50 per cent inhibition doses were obtained from the experiment of the final antiserum dilution described at each antiserum.

released by 5 μunits of BK–β-D-galactosidase from 0.2 mM 4-methylumbelliferyl-β-D-galactoside as a substrate, when incubated at 37°. The intensity of fluorescence of the product increased linearly with incubation time up to 4 hr. This substrate concentration, therefore, was considered to be sufficient. A 2-hr incubation time at this substrate concentration was also chosen for the following experiments.

Figure 2 shows the binding of the enzyme-labeled antigen to the antibody using serial dilutions of the antiserum (AU-4) with the fixed amount of the enzyme-labeled antigen (5 μunits/tube). Approximately 75 per cent of the enzyme-labeled antigens was bound to an excess of AU-4.

The enzyme activity of antibody-bound labeled antigen in the absence of unlabeled bradykinin (B_0) was twelve times higher than that of the assay blank, when antiserum AU-4 was used at a dilution of 1:20,000. This amount of the enzyme gave sufficient sensitivity for the assay.

As shown in Fig. 3, the effect of incubation time on the percentage of enzyme-labeled antigen bound to antibody was examined after addition of the first antibody to the incubation mixture which was kept in an ice bath. The values at 18, 42 and 64 hr almost reached the plateau. Therefore, 18 hr of incubation was employed throughout this assay.

A double-antibody method was employed to separate the antibody-bound antigen from free antigen. The optimum ratio of anti-rabbit IgG antiserum from goat (the second antibody) to the non-immunized rabbit serum (NRS) as carrier protein was investigated (Fig. 4). When 10 μl of a 4-fold dilution of the second antibody was used, the addition of 10 μl of an 8- to 20-fold dilution of NRS resulted in the highest percentage bound in the 4 hr of the second incubation. A 10-fold dilution of NRS, therefore, was used throughout this assay. Using the mixture that produced this optimum ratio of the second antibody to NRS, the second incubation time was examined between 0.5 and 6 hr. As indicated in Fig. 5, the enzyme activity in the precipitate reached a maximum at 2 hr and maintained a plateau for 6 hr. Thus, 4 hr was considered sufficient for the second incubation time.

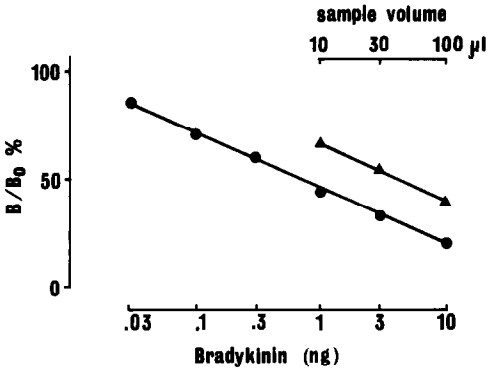

Fig. 6. Standard inhibition curves of synthetic bradykinin and of a sample solution. The ordinate indicates percentage of enzyme activity of the enzyme–antibody complex with competitive bradykinin added (B) over that without bradykinin (B_0). Amounts of bradykinin per tube are shown on the abscissa in a logarithmic scale. The sample curve (the solution processed for kininogen determination described in Materials and Methods) (▲) is plotted in arbitrary units on the top abscissa.

Standard curve and sensitivities of several antisera obtained from immunized rabbits

The binding of the enzyme-labeled antigen to several antisera and the sensitivity of these antisera are shown in Table 1. AU-1, AU-2 and AU-4 had the highest titres and the greatest sensitivity. AU-3 was sensitive to inhibition by BK even though this serum had a rather low titre of 1:2000 dilution. AU-6 to AU-9 antisera had low sensitivities and did not yield results as good as those obtained with BK-TC conjugates, under the conditions used for evaluation.

Figure 6 depicts a standard curve that was obtained by adding 10 pg to 10 ng of synthetic BK to the serum at a dilution of 1:20,000. As seen in the figure, 30 pg/tube (0.2 ml) of unlabeled BK inhibited significantly the binding of labeled antigen (B_0). The dose–response curve showed a linear relationship in the range of 30 pg to 10 ng of BK, when the logarithmic dose was plotted on the abscissa and percent inhibition (B/B_0%) on the ordinate.

The coefficient of variation (C.V.) was calculated for different doses of BK from fifteen measurements. As shown in Table 2, the C.V. was from 12.9 to 19.1 per cent between 0.1 and 10 ng of BK.

Table 2. Coefficient of variation for bradykinin standard curve*

BK dose (ng)	C.V. (%)
0.1	19.1
0.3	16.9
1.0	12.9
3.0	14.9
10.0	14.8

* The values of coefficient of variation (C.V.) were calculated from an experiment with fifteen assay tubes at each dose.

Determination of kininogen level in human plasma

When the kininogen sample, treated by the method mentioned above except for the ether-washing, was applied to enzyme immunoassay, the slope of the inhibition curve was not parallel to that of standard bradykinin. Therefore, the lipid removal by ether-washing was carried out for all kininogen samples. As shown in Fig. 6, a typical displacement curve of the kininogen sample ran parallel to the standard curve.

To test the validity of the extraction method, the recovery rates of bradykinin were obtained in the following way. A sufficient amount of soybean trypsin inhibitor (1 mg) was added to plasma to prevent spontaneous release of bradykinin by plasma kallikrein. After plasma was treated by hot ethanol, bradykinin (0.1 to 10 ng) was added to the samples, and these samples were treated with the usual extraction procedure as described above. Recovery was from 85.3 to 111.5 per cent as shown in Table 3.

Table 3. Recovery of bradykinin added to the extracted sample*

BK added (ng)	Recovery (%)
0.1	85.3
0.3	100.3
1.0	85.4
3.0	111.5
10.0	104.0

* Bradykinin (BK) was added to the sample that contained soybean trypsin inhibitor and extracted by hot ethanol in the same way as the kininogen sample. Per cent recovery was calculated from the mean value of the measurements, with fifteen assay tubes at each dose.

Table 4. Kininogen level as bradykinin equivalent in human plasma*

Sample No.	Kininogen (μg BK/ml plasma)	
	Enzyme immunoassay	Bioassay
1	3.75 ± 0.31	3.85 ± 0.35
2	3.50 ± 0.92	3.19 ± 0.32
3	4.19 ± 0.72	4.29 ± 0.58
4	3.47 ± 0.49	4.02 ± 0.92
5	3.01 ± 0.68	3.11 ± 0.24
6	3.51 ± 0.44	4.31 ± 0.66
Mean	3.57 ± 0.38	3.79 ± 0.53

* Each value of kininogen level expresses the mean ± S.D. of three different experiments. The bottom row shows the mean ± S.D. of six individuals.

The amounts of total kininogen assayed as BK equivalent in six healthy individuals by means of both the enzyme immunoassay and the bioassay are shown in Table 4. Each value is the mean of three experiments that were performed on different days. The standard deviation of each sample expresses the variation between assays. The bottom line shows the mean of six individual values and its deviation. Total kininogen level in human plasma was 3.57 μg BK equiv./ml by enzyme immunoassay and 3.79 by bioassay.

DISCUSSION

There has been a spate of papers in recent years on radioimmunoassay of several compounds in biological fluids, including the development of a radioimmunoassay of kinin [1–8]. Although radioimmunoassay has the advantages of ease, sensitivity, and multiple measurements compared to bioassay, there are some disadvantages that come chiefly from the use of radioisotopes. Radioisotopes not only are a direct hazard to human beings but also pollute the globe with radioisotope waste. Further, radioimmunoassay requires special, expensive equipment, which is not readily available in all clinical laboratories. Isotope labeling of bradykinin ^{3}H or ^{14}C incorporation is not common, and the incorporation of ^{125}I by bradykinin can be achieved only when a tyrosine-containing analog is available. It is necessary to prepare iodine-labeled antigen every 2 months, but the enzyme conjugate is very stable. The β-D-galactosidase-labeled antigen was found to lose little activity over at least 20 months when it was stored at 4° in buffer A. Thus, one can use the enzyme conjugate for a long time, once it is prepared.

Enzyme immunoassay systems, using β-D-galactosidase-labeled antigen conjugated with MBS, have been developed to quantitate insulin [10], viomysin [11], and angiotensin [12]. The MBS used in this assay is a hetero bifunctional type of crosslinking reagent and is used in a two-step reaction to link the peptide with β-D-galactosidase [10]. In the first step, the active ester of MBS acylated the amino group of the N-terminus of bradykinin. In the second step, the maleimido group of MBS carrying bradykinin reacted with the sulfhydryl groups of the enzyme. It can be assumed that no polymerization of bradykinin or of the enzyme occurred, because bradykinin, which reacts in the first step, contains no sulfhydryl group. Thus, bradykinin molecules, which are conjugated with the enzyme by MBS, are assumed to keep the carboxyl terminus intact. The activity of the enzyme was not changed by conjugation with bradykinin, as described in Materials and Methods. Use of this labeled antigen may be important to the measurement of biological active bradykinin, since bradykinin is rapidly inactivated in biological systems by hydrolysis of one or two amino acids located at the carboxyl terminus [17].

The system for assay of enzyme activitity in this method is similar to the method described by Kato *et al.* [18], except for the enzyme reaction stopping solution, 0.2 M sodium phosphate–NaOH buffer.

As for the enzyme amount of 5 μunits in each tube, this was the least enzyme activity that could be measured practically and still maintain high sensitivity of the immunoreaction. The concentration of the substrate used, 0.2 mM, was sufficient to measure 5 μunits of the enzyme for 2 hr of incubation. The fluorescence intensity generated with this amount of enzyme was about 150 times higher than that of the reagent blank [19].

Talamo and Goodfriend [19] reviewed the conditions of radioimmunoassay of bradykinin and recommended an incubation mixture of 0.1 M sodium phosphate buffer containing 3 mM 1,10-phenanthroline and 0.01% casein. The assay conditions described here were similar to the conditions they described, except for an incubation temperature of 0°, instead of 4°, because use of an ice bath is convenient to keep the temperature constant for long periods. We used 0.1% BSA instead of 0.01% casein to prevent adsorption of bradykinin to the tube. They also recommended that the antiserum be heated at 60° for 30 min, to reduce the activity of contaminating proteolytic enzymes. For the present work, antiserum AU-4, treated at 60° for 30 min, was compared with untreated antiserum, and neither showed a significant difference in the standard curve.

When the kininogen samples, treated as described above, were examined, the inhibition curve with various sample volumes from 10 to 100 μl did not show a slope identical to that from the standard bradykinin. The slope of the former was steeper at the large volumes; this might have been due to lipid contamination in the sample, as mentioned by Odya *et al.* [8]. When samples were treated with ether-washing, as described by Mashford and Roberts [6], the displacement curve was parallel to the standard curve of bradykinin. Using this treatment, the values obtained for kininogen samples showed good agreement with those by bioassay, as shown in Table 4.

The values in the present experiments also were not significantly different from those reported by Sipila and Louhija [20]. In preliminary experiments, bradykinin analogs, lacking arginine at the C-terminus, hardly interfered with the binding of the enzyme-labeled antigen, but the antisera did not differentiate kallidin from bradykinin in binding. These results will be published separately. In the kininogen assay of this experiment, physiological degradation products of bradykinin that contaminated the biological fluids, if any, did not seem to interfere with the assay of bradykinin by enzyme immunoassay. It may have been that the extraction procedure of bradykinin released from kininogen with 80% ethanol, which was done before the enzyme immunoassay of the present experiments, excluded most of the protein from the biological samples (kinin-free kininogens, plasma enzyme, etc.), including, if any, β-D-galactosidase.

Among the antisera obtained from rabbits that were immunized with BK-TC-OA, BK-TC-BSA, or BK-BSA, three antisera—AU-2, AU-3 and AU-4—had sufficient sensitivity for bradykinin measurement in kininogen samples, as shown in Table 1. Using the most sensitive antiserum, AU-4, the 50 per cent inhibition dose in this enzyme immunoassay was not as low as that of the radioimmunoassay reported: 740 pg/tube (3.7 ng/ml) for the present data compared with 125 pg/ml for Odya *et al.* [8] and 375 pg/ml for Mashford and Roberts [6]. The minimum detectable dose in the present data was 150 pg/ml (= 30 pg/tube) compared with 10 pg/ml (3–5 pg/tube) for Odya *et al.* [8] and 62.5 pg/ml (25 pg/tube) for Mashford and Roberts [6].

These results clearly indicate that the enzyme immunoassay method, using β-D-galactosidase as a labeling enzyme, is practically comparable to radioimmunoassay and has the additional benefits of a stable labeled antigen, easily available equipment, and no hazard from the labeled compounds.

Acknowledgements—The authors are grateful to Drs. Sadaaki Iwanaga and Hisao Kato, Department of Biology, Faculty of Science, Kyushu University, for their valuable advice.

REFERENCES

1. J. Spragg, K. F. Austen and E. Haber, *J. Immun.* **96**, 865 (1966).
2. H. Linderknecht, B. J. Haverback and F. Aladjem, *Nature, Lond.* **213**, 1130 (1967).
3. T. L. Goodfriend and D. L. Ball, *J. Lab. clin. Med.* **73**, 501 (1969).
4. R. C. Talamo, E. Haber and K. F. Austen, *J. Lab. clin. Med.* **74**, 816 (1969).
5. M. E. Webster, J. V. Pierce and M. U. Sampaio, in *Advances in Experimental Medicine and Biology*, Vol. 8 (Eds. F. Sicteri, M. Rocha e Silva and N. Back), p. 57. Plenum Press, New York (1970).
6. M. L. Mashford and M. L. Roberts, *Biochem. Pharmac.* **21**, 2727 (1972).
7. J. Spragg, R. C. Talamo, B. U. Wintroub, E. Haber and K. F. Austen, in *Methods in Immunology and Immunochemistry*, Vol. 5 (Eds. C. A. Williams and M. W. Chase), p. 149. Academic Press, New York (1976).
8. C. E. Odya, T. L. Goodfriend, J. M. Stewart and C. Pana, *J. Immun. Meth.* **19**, 243 (1978).
9. A. H. W. M. Sohuurs and B. K. Van Weeman, *Clinica chim. Acta* **81**, 1 (1977).
10. T. Kitagawa and T. Aikawa, *J. Biochem., Tokyo* **79**, 233 (1976).
11. T. Kitagawa, T. Fujitake, H. Taniyama and T. Aikawa, *J. Biochem., Tokyo* **83**, 1493 (1978).
12. T. Aikawa, S. Suzuki, M. Murayama, K. Hashiba, T. Kitagawa and E. Ishikawa, *Endocrinology* **105**, 1 (1979).
13. A. Ueno, S. Oh-ishi, T. Kitagawa and M. Katori, in *Advances in Experimental Medicine and Biology*, Vol. 120-A (Eds. S. Fujii, H. Moriya and T. Suzuki) p. 195. Plenum Press, New York (1979).
14. R. C. Talamo, E. Haber and K. F. Austen, *J. Immun.* **101**, 333 (1968).
15. T. L. Goodfriend, L. Levine and G. D. Fasman, *Science* **144**, 1344 (1964).
16. Y. Uchida and M. Katori, *Biochem. Pharmac.* **27**, 1463 (1978).
17. E. G. Erdös, in *Handbook of Experimental Pharmacology*, Vol. XXV suppl. (Ed. E. G. Erdös), p. 428. Springer, New York (1979).
18. K. Kato, Y. Hamaguchi, H. Fukui and E. Ishikawa, *J. Biochem., Tokyo* **78**, 235 (1975).
19. R. C. Talamo and T. L. Goodfriend, in *Handbook of Experimental Pharmacology*, Vol. XXV suppl. (Ed. E. G. Erdös), p. 301. Springer, New York (1979).
20. R. Sipila and R. Louhija, *Biochem. Pharmac.* **25**, 543 (1976).