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Bioluminescence Enhanced Enzyme Immunoassay

New Ultrasensitive Detection Systems for Enzyme Immunoassays, II.[1])

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Summary: Ultrasensitive bioluminescence immunoassays for the determination of peptides and proteins (illustrated with human urinary kallikrein, bradykinin and the determination of human urinary kallikrein antibody titers) have been developed. The usable ranges of the standard curves are from 5 pg to 5000 pg per liter. The relative intra-assay coefficients of variation of the tests were between 2 and 6%, and the inter-assay coefficients of variation between 4 and 12%.

Introduction

Despite widespread application of radioimmunoassays, non-isotopic immunoassays have been developed during the past decade (1, 2). In particular, enzyme immunoassays (3–8) have proved to be a useful alternative to radioimmunoassays. Although both radio- and enzyme immunoassays have similar sensitivities for antigen determination, enzyme immunoassay has several advantages (9) and allows measurement of the signal in generally available spectrophotometers.

More recently, highly sensitive bioluminescent and luminescent detection systems have become available for the determination of ATP and many different analytes (10–13). Bioluminescence is a natural phenomenon found in many lower forms of life (14). Naturally ocurring bioluminescent systems differ with regard to the structure and function of enzymes and cofactors, as well as in the mechanism of the light emitting reactions (11–21).

In this communication we describe and demonstrate highly sensitive enzyme immunoassays in which bioluminescence is used as the detection system. The principle of these bioluminescence enhanced immunoassays is as follows: an antibody enzyme or antigen enzyme conjugate becomes bound in the enzyme immunoassay, then enzymatically releases *D*-luciferin from luciferin derivatives. The *D*-luciferin is quantified in a luminometric assay.

Materials and Methods

Sephadex G-15, G-150 and N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP®) were products of Pharmacia Fine Chemicals, Uppsala, Sweden.

D-luciferin (*Photinus pyralis*), carboxylic esterase, carboxypeptidases A and B, arylsulphatase, alkaline phosphatase, Tween 20, and bovine serum albumin were purchased from Sigma Chemicals, Deisenhofen, FRG.

Glutardialdehyde was purchased from E. Merck, Darmstadt, FRG.

Luciferase (*Photinus pyralis*; specific activity 8 mU/mg) was a product of Boehringer, Mannheim, FRG.

Bradykinin was a product of Novabiochem AG, Läufelfingen, Switzerland.

Human urinary kallikrein was isolated as described by *Geiger et al.* (22).

Antibodies against human urinary kallikrein and bradykinin were raised in rabbits and in goats as already described (23). IgG fractions were isolated according to *Steinbuch & Audran* (25).

[1]) I.: this j. *25*, 23–30 (1987).

Bradykinin antibodies, purified by immunoaffinity, were prepared as described in l.c. (26).

Goat anti-rabbit immunoglobulins, purified by immunoaffinity, were purchased from Miles/Yeda Ltd., Rehovot, Israel.

Microtiter plates were purchased from Dynatech, Denkendorf, FRG.

D-luciferin methyl ester, *D*-luciferyl-*L*-phenylalanine, *D*-luciferyl-*L*-N$^{\alpha}$-arginine, *D*-luciferin-O-sulphate and *D*-luciferin-O-phosphate were synthesized as described (9).

Enzyme[2]) conjugates

Bradykinin, human urinary kallikrein and immunoglobulin G were conjugated with carboxylic esterase, carboxypeptidases A and B, arylsulphatase and alkaline phosphatase. Coupling to carboxylic esterase was performed with glutardialdehyde and/or N-succinimidyl 3-(2-pyridyldithio) proprionate (27), and to carboxypeptidases A and B, arylsulphatase and alkaline phosphatase with SPDP® as described by *Carlsson* et al. (28). All conjugates synthesized were purified by gel filtration on Sephadex G-15 (bradykinin) or G-150 (human urinary kallikrein and IgG) equilibrated with 0.1 mol/l phosphate buffer, pH 7.5, containing 0.25 mol/l NaCl, at 4 °C. The concentrations of enzyme conjugates in the resulting reagent solutions were determined by measuring enzyme activities by conventional methods as described (9). Aliquots of the enzyme conjugates were stored at −30 °C until use.

Luminometric assay

Luciferase activity was measured in a luminometer (Biolumat, Fa. Berthold Typ 9500 T, Wildbad, FRG) as follows (10): 0.4 ml incubation buffer (30 mmol/l HEPES, 6.6 mmol/l $MgCl_2$, 0.66 mmol/l EDTA, 0.1 mmol/l dithiothreitol, 5 mmol/l ATP, pH 7.75 containing 1 µg luciferase) was preincubated in a cuvette at 25 °C for 5 min in the luminometer. Thereafter 0.1 ml luciferin solution or incubation solutions of the immunoassay were added and the integrated light impulses were monitored for 10 s.

Kinetics of luciferin liberation from luciferin derivatives

Microtiter plates were coated with rabbit IgG (10 mg/l buffer A) overnight at 4 °C. After washing with buffer B (5 times), 0.2 ml of goat anti-rabbit IgG enzyme conjugates were added (for concentrations of enzyme conjugate in test, see table 1) and incubated at 37 °C for 5 h. Then the plates were washed with buffer B (5 times), 0.2 ml substrate solution was added and luciferin release was measured after the time indicated.

Detection systems for bioluminescence enzyme immunoassay

Enzyme activities of the enzyme conjugates adsorbed onto microtiter plates in the assay were determined as summarized in table 2. The assays were performed as follows: 0.2 ml *D*-luciferin derivative solution was added to the microtiter plate wells and incubated for 60 min at 37 °C (alkaline phosphatase-based detection systems were incubated only for 30 min at 37 °C). Thereafter, 0.1 ml of the incubation mixture was transfered to the luminometer and light impulses were monitored for 10 s.

The luciferin derivative solutions used contained the following concentrations:
2 µmol/l *D*-luciferin methyl ester in 0.05 mol/l Tris/HCl, pH 7.5;
10 µmol/l *D*-luciferyl-*L*-phenylalanine in 0.05 mol/l Tris/HCl, 3 g/l LiCl, pH 7.5;
10 µmol/l *D*-luciferyl-*L*-N$^{\alpha}$-arginine in 0.05 Tris/HCl, 0.2 mol/l NaCl, pH 7.8;
10 µmol/l *D*-luciferin-O-sulphate in 10 mmol/l sodium acetate, pH 5.0 and
10 µmol/l *D*-luciferin-O-phosphate in 10 mmol/l diethanolamine, 0.5 mmol/l $MgCl_2$, pH 9.8.

Tab. 1. Conjugates of the bioluminescence enhanced enzyme immunoassay.

Conjugate	Conjugate activity*) in the assay (kU/l)
Bradykinin	
carboxylic esterase	5.8
carboxypeptidase A	29
carboxypeptidase B	35
arylsulphatase	1.7
alkaline phosphatase	0.12
Human urinary kallikrein	
carboxylic esterase	7.2
carboxypeptidase A	21
carboxypeptidase B	29
Anti-human urinary kallikrein	
carboxylic esterase	0.37
carboxypeptidase A	25
carboxypeptidase B	31
alkaline phosphatase	0.22
Immunoglobulin G	
carboxylic esterase	0.62
carboxypeptidase A	6.5
carboxypeptidase B	3.5
arylsulphatase	1.5
alkaline phosphatase	0.11

*) enzymatic activity was measured as described in Material and Methods using chromogenic substrates.

Tab. 2. Scheme of the detection systems of the bioluminescence enhanced enzyme immunoassay.

	Volume (ml)
Addition of *D*-luciferin derivative solution to the microtiter plate wells	0.20
	60 min at 37 °C*)
Transfer to the luminometer	0.10 light impulses were read for 10 s

*) in the alkaline phosphatase-based detection system, the system was incubated for only 30 min.

[2]) *Enzymes*
Alkaline phosphatase (EC 3.1.3.1),
arylsulphatase (EC 3.1.6.1),
carboxylic esterase (EC 3.1.1.1),
carboxypeptidase A (EC 3.4.17.1)
carboxypeptidase B (EC 3.4.17.2) and
human urinary kallikrein (EC 3.4.21.35).

Enzyme immunoassay conditions (fig. 1)

Buffer A: 15 mmol/l Na_2CO_3, 0.35 mol/l $NaHCO_3$, 0.2 g/l NaN_3, pH 9.6

Buffer B: 10 mmol/l KH_2PO_4, 15 mmol/l NaCl, 0.05 g/l, Tween 20, pH 7.4

Buffer C: 1.5 mmol/l $KH_2PO_4 \times H_2O$, 0.14 mol/l NaCl, 0.5 g/l Tween 20, 2 mg/ml bovine serum albumin, pH 7.4

Competitive human urinary kallikrein enzyme immunoassay

Microtiter plates were coated with rabbit anti-human urinary kallikrein IgG (10 mg/l) in buffer A; 0.2 ml) at 4 °C overnight. Thereafter the plates were washed (5 times) with buffer B. Then 0.15 ml of human urinary kallikrein standard or test sample and 0.05 ml of a human urinary kallikrein enzyme conjugate solution (see above) were added, and the mixture was incubated for 24 h at 4 °C. The plates were washed with buffer B, then 0.2 ml of the corresponding *D*-luciferin derivative solution was added and detected as described in "detection systems".

Human urinary kallikrein immuno enzymometric assay

Microtiter plates were coated with human urinary kallikrein (10 mg/l buffer A; 0.2 ml per well) overnight at 4 °C. In parallel to, but separately from the coating procedure, 0.5 ml anti-human urinary kallikrein IgG solution (0.1 µg/l specific antibodies in buffer C) and 0.5 ml human urinary kallikrein solution (0.01 – 10 ng/l buffer C) were incubated in capped polypropylene vials overnight at 4 °C. The microtiter plates were washed with buffer B (5 times). Thereafter, 0.2 ml of each incubation mixture was transferred from the polypropylene vials to the corresponding microtiter plate wells and incubated at 37 °C for 5 h. After washing the plates (5 times), 0.2 ml of a goat anti-rabbit IgG enzyme conjugate solution in buffer C was added to each well and the plates were incubated again for 24 h at 4 °C. The plates were washed with buffer B (5 times), then the corresponding *D*-luciferin derivative solution (0.2 ml) was added to the wells. Bound IgG conjugates were detected as described in Materials and Methods.

Human urinary kallikrein sandwich antigen assay

Microtiter plates were coated with goat anti-human urinary kallikrein-IgG (10 mg/l buffer A; 0.2 ml per well) at 4 °C overnight. The plates were then washed (5 times) in buffer B. Human urinary kallikrein standard samples and test samples were diluted with buffer C. Of these samples 0.2 ml was added to the wells and the plates were incubated at 4 °C for 24 h. After incubation, the plates were washed (5 times) with buffer B, 0.2 ml of rabbit anti-human urinary kallikrein-IgG enzyme conjugate solution was added to each well, the plates were washed (5 times), then 0.2 ml of the corresponding *D*-luciferin derivative substrate solution (see detection systems) was added to the wells, and the human urinary kallikrein-bound IgG conjugates were detected as described in Materials and Methods.

Competitive bradykinin enzyme immunoassay

Microtiter plates were coated overnight at 4 °C with immunoselected anti-bradykinin IgG solution (10 mg/l in buffer A; 0.2 ml per well). The plates were then washed with buffer B (5 times). Thereafter 0.15 ml bradykinin standard solution in buffer C and 0.05 ml bradykinin enzyme conjugate were added to the wells and incubated at 4 °C for 24 h. Then the plates were washed with buffer B (5 times), the corresponding *D*-luciferin derivative solution was added (0.2 ml) to the wells, and the bound bradykinin conjugates were measured as described in Materials and Methods.

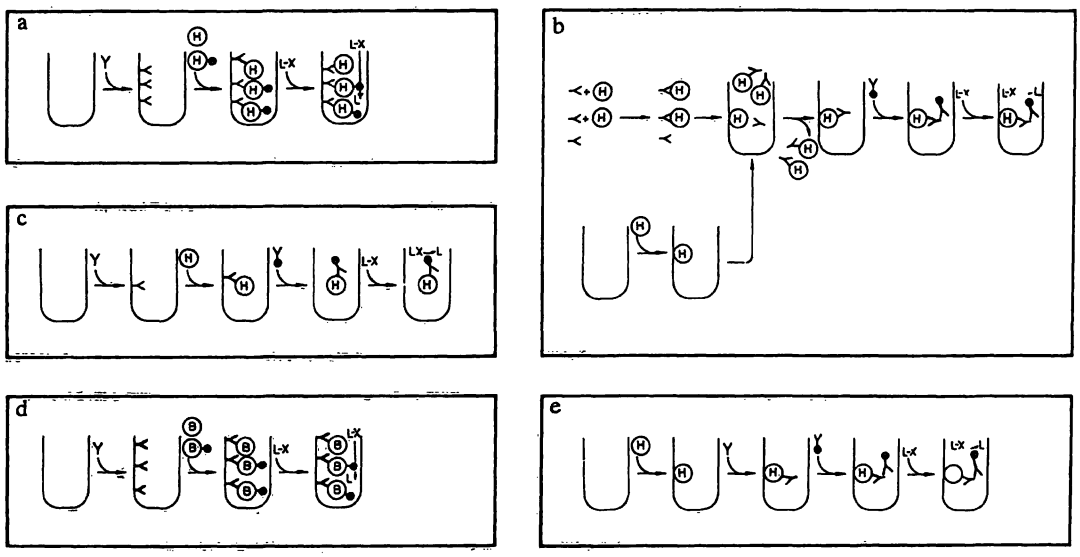

Fig. 1. Scheme of the bioluminescence enzyme immunoassays
a) competitive human urinary kallikrein assay;
b) human urinary kallikrein immuno enzymometric assay;
c) human urinary kallikrein sandwich antigen assay;
d) competitive bradykinin assay;
e) human urinary kallikrein sandwich antibody assay.
Ⓑ = bradykinin, Ⓗ = human urinary kallikrein, Y = immunoglobulin G, L-X = luciferin derivative, L = luciferin, Y-● = immunoglobulin G enzyme conjugate, Ⓑ-● = bradykinin enzyme conjugate, Ⓗ-● = human urinary kallikrein enzyme conjugate.
For details see Methods.

Enzyme immunoassay for titer determination

Microtiter plates were coated with antigen (10 mg/l human urinary kallikrein in buffer A; 0.2 ml) at 4 °C overnight. The plates were then washed with buffer B (5 times). Thereafter 0.2 ml of different antiserum dilutions (10 – 0.001 µl anti-human urinary kallikrein serum in 0.2 ml buffer C) were added and incubated for 3 h at 37 °C. The plates were washed (5 times with buffer B), 0.2 ml of goat anti-rabbit IgG enzyme conjugate were added and incubated for 2 h at 37 °C, and the plates again washed with buffer B (5 times). The corresponding *D*-luciferin derivative solution (0.2 ml) was added to the wells and the bound conjugates were measured as described in Materials and Methods.

Results and Discussion

Bradykinin, human urinary kallikrein and immunoglobulin G were conjugated to carboxylic esterase, carboxypeptidases A and B, arylsulphatase and alkaline phosphatase, according to established methods as described in Materials and Methods (27, 28). The enzymatic activities of the conjugates after synthesis were sufficiently high for their intended use (tab. 1).

Using these enzyme conjugates, a number of different variants of immunoassays with different detection systems were developed for the assay of bradykinin, human urinary kallikrein and for human urinary kallikrein antibody-titer determinations. The principles of the assays are shown schematically in figure 1. The bradykinin assay was performed as a competitive test (fig. 1 d), whereas for human urinary kallikrein a competitive (fig. 1 a), an immuno enzymometric assay (fig. 1 b), and a sandwich antigen assay (fig. 1 c) were established. Anti human urinary kallikrein titers were measured by sandwich antibody assay (fig. 1 e). The assays were performed as described in Materials and Methods.

Assay systems were optimized with respect to enzyme immunoassay conditions (binding of antigen/antibody to microtiter plates, antibody-antigen binding, etc.) as previously described (29), and to optimal detection system conditions (concentration of substrates, temperatures, etc.). Luciferin liberation at 25 °C and 37 °C (fig. 2), as well as the kinetics of the releasing reaction, was also investigated (fig. 2). At 37 °C luciferin was liberated at a higher rate. Optimal substrate concentrations for the detection systems were determined by adding increasing amounts of *D*-luciferin derivatives to the respective assay (fig. 3). As can be seen from the data, optimal substrate concentrations are in the range of about 10 µmol/l.

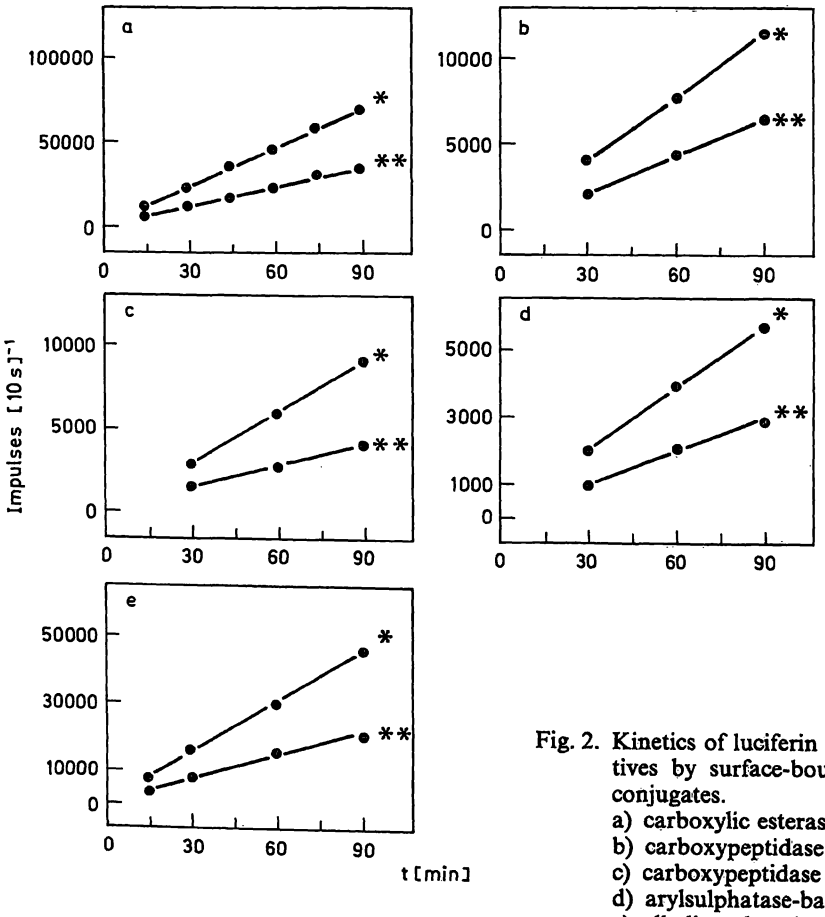

Fig. 2. Kinetics of luciferin liberation from *D*-luciferin derivatives by surface-bound goat anti-rabbit IgG enzyme conjugates.
a) carboxylic esterase-based detection system I;
b) carboxypeptidase A-based detection system II;
c) carboxypeptidase B-based detection system III;
d) arylsulphatase-based detection system IV;
e) alkaline phosphatase-based detection system.
Incubation temperature: * = 37 °C; ** = 25 °C.

In figures 4 to 8 binding curves of different types of bioluminscence enhanced enzyme immunoassays for human urinary kallikrein, bradykinin and antibody titers (anti-human urinary kallikrein serum) are shown, using different detection systems. The standard curves were constructed by plotting the fraction of light impulses of the blank and the samples against the dose of antigens either in a logit-log mode (figs. 5 and 8) or in a linear-log mode (figs. 4, 6 and 7). The sensitivity and the slope of the curves vary, depending on the detection system and the type of enzyme immunoassay. The sensitivities of the bioluminescence enhanced enzyme immunoassays were defined according to the method described by *Kaiser* (30). The lowest concentration of human urinary kallikrein that produces a response greater than that caused in the absence of human urinary kallikrein was 1 fg per well in the immuno enzymometric assay (fig. 7), corresponding to 5 pg/l. For bradykinin the lowest detectable concentration was 1 fg per well, corresponding to 5 pg/l (fig. 4). The mass of antibodies necessary for 50% binding in the assay was 200 fg per well, corresponding to 1 ng/l specific anti-human urinary kallikrein immunoglobulin G.

The determination of precision (intra- and inter-assay coefficient of variation), specificity and recovery of the assays led to the same results as previously published for enzyme immunoassays with peroxidase as label (26, 29). The relative intra-assay coefficient of variation (N between 15 and 25 for each assay; measured on different days) ranged from 2 to 6% for all assays and detection systems. The intra-assay coefficient of variation was calculated for all standard curve concentrations (e. g. fig. 4; range 10^{-15} to 10^{-12} g/tube for ●). The inter-assay coefficient of variation of identical samples (N between 8 and 22) and detection systems containing all standard curve concentrations (e. g. fig. 6; concentrations used for ○: 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8} g/tube) was 4–12%.

The sensitive bioluminescence enhanced enzyme immunoassay developed for peptides (bradykinin), proteins (human urinary kallikrein) and antibody titer determinations should be especially suitable for studies in which highly sensitive methods are needed, e. g. for detection of monoclonal antibody synthesis in cell cultures, demonstration of in vitro translation

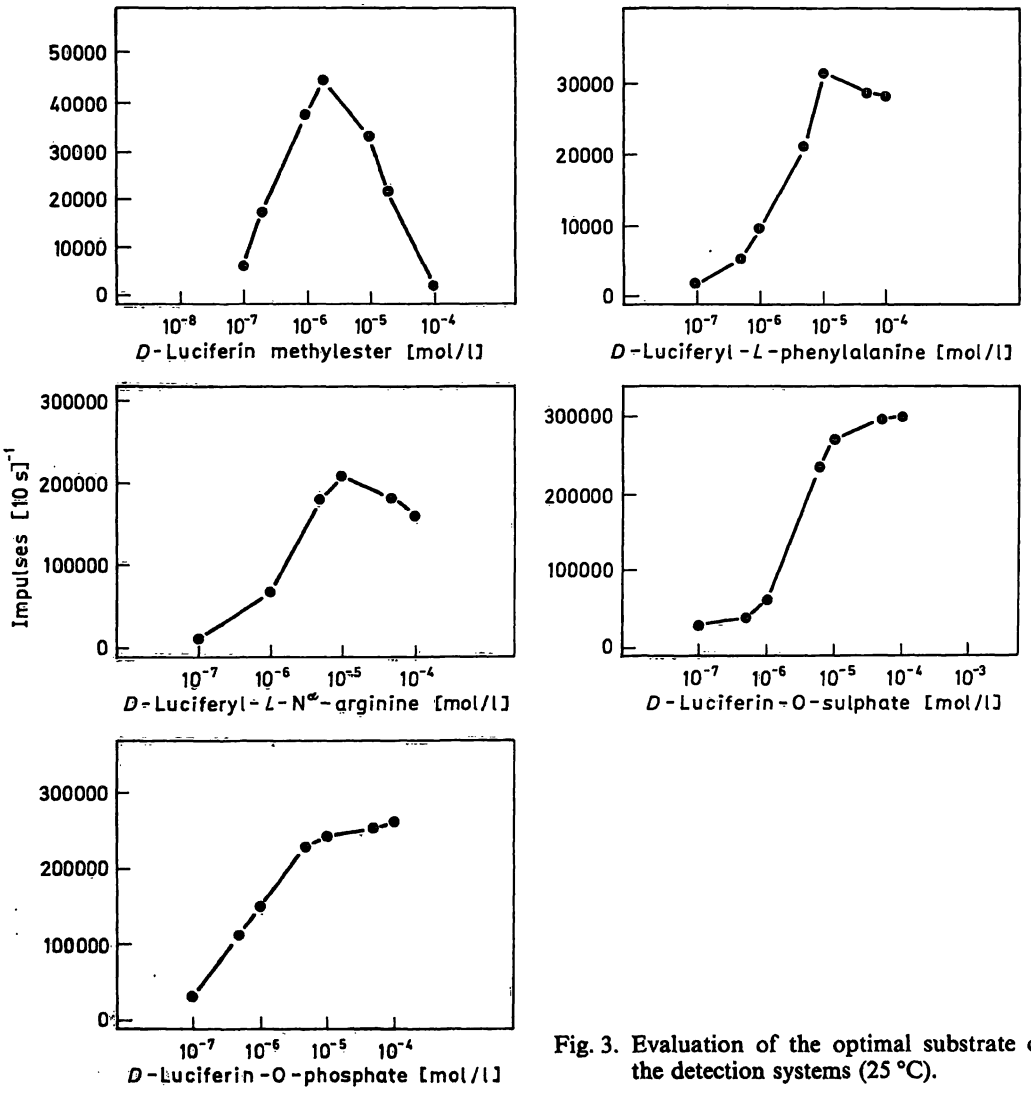

Fig. 3. Evaluation of the optimal substrate concentration in the detection systems (25 °C).

products, etc. The high sensitivity of the bioluminescence enhanced enzyme immunoassay (1 pg/l; in general radio and enzyme immunoassays reach a sensitivity of 10 to 100 ng/l; (24)) was demonstrated by employing various bioluminescence detection systems for different types of enzyme immunoassay. Depending on the enzyme conjugates and substrates used, turnover rates of enzymes ranged from a k_{cat} value of 11 s^{-1} (carboxylic esterase (9)) up to 1010 s^{-1} (alkaline phosphatase (9)). In figure 4, dose response curves obtained for detection system I to V in the competitive bradykinin bioluminescence enzyme immunoassay are shown. As can be seen, alkaline phosphatase conjugates gave a response curve with the lowest detection limit. Titer determinations (fig. 5) and the different types of human urinary kallikrein immunoassays (figs. 7, 8) showed similar good results. Alkaline phosphatase conjugates gave the best responses with the lowest limits of detection when compared with the other detection systems.

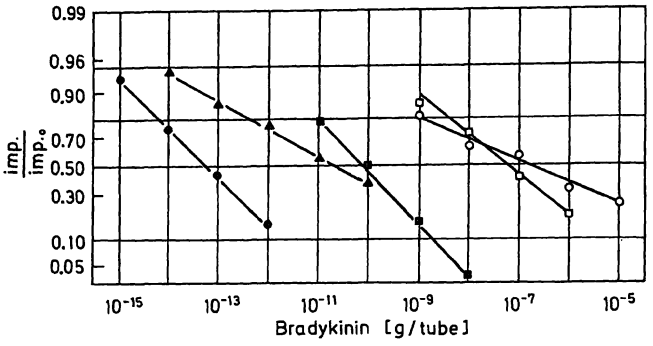

Fig. 4. Dose response curves of bradykinin in the competitive bradykinin bioluminescence enzyme immunoassay.
▲, carboxylic esterase-based detection system I;
○, carboxypeptidase A-based detection system II;
■, carboxypeptidase B-based detection system III;
□, Arylsulphatase-based detection system IV;
●, alkaline phosphatase-based detection system V.
For details of the assay procedures see Methods.

The bioluminescence enhanced enzyme immunoassays described are useful for determination of antigens in a very low concentration range (down to 10^{-15} mol/l or $0.2 \cdot 10^{-19}$ mol/test). Conjugates with alkaline phosphatase are especially useful for work requiring highest sensitivity. The assay procedure is

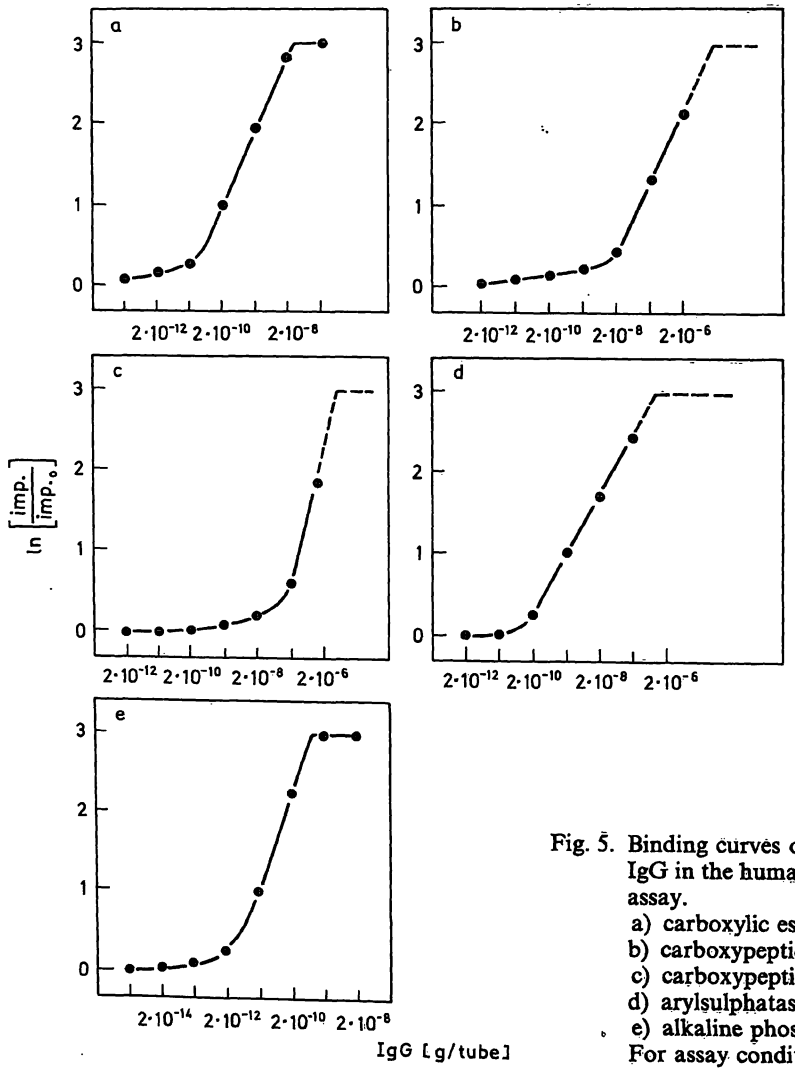

Fig. 5. Binding curves of rabbit anti-human urinary kallikrein IgG in the human urinary kallikrein sandwich antibody assay.
a) carboxylic esterase-based detection system I;
b) carboxypeptidase A-based detection system II;
c) carboxypeptidase B-based detection system III;
d) arylsulphatase-based detection system IV;
e) alkaline phosphatase-based detection system V.
For assay conditions see Methods.

simple to perform, and the substrates are stable at −20 °C in the dark for more than one year. Substrate solutions can also be stored at −30 °C for some weeks.

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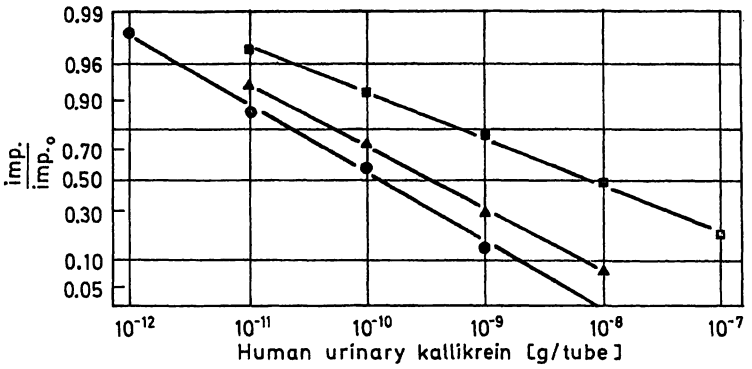


Fig. 6. Binding curves of human urinary kallikrein in the competitive human urinary kallikrein assay.
□, carboxylic esterase-based detection system I;
▲, carboxypeptidase A-based detection system II;
●, carboxypeptidase B-based detection system III.
For assay conditions see Methods.

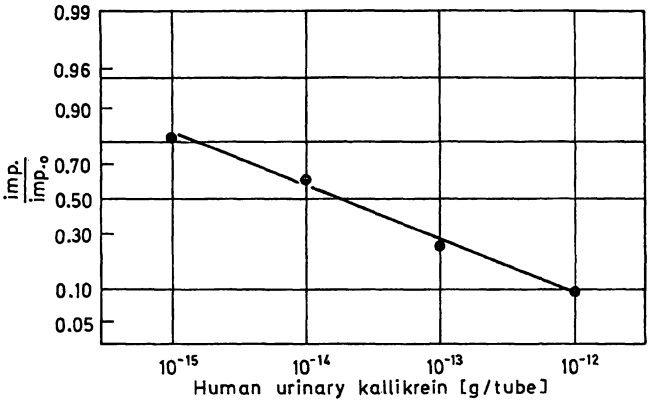
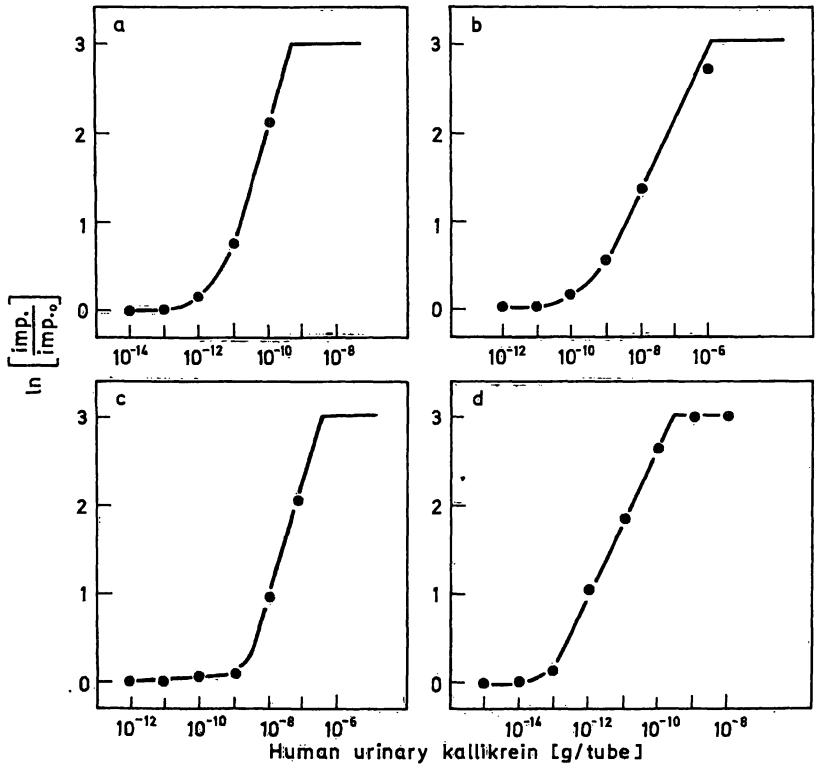


Fig. 7. Binding curve of human urinary kallikrein in the immuno enzymetric human urinary kallikrein assay.
●, alkaline phosphatase-based detection system V.
For details see Methods.



a) carboxylic esterase-based detection system I;
b) carboxypeptidase A-based detection system II;
c) carboxypeptidase B-based detection system III;
d) alkaline phosphatase-based detection system V.
For assay conditions see Methods.

Fig. 8. Binding curves of human urinary kallikrein in the human urinary kallikrein sandwich antigen assay.

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