Recent Progress on Kinins

Biochemistry and Molecular Biology of the Kallikrein-Kinin System

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Recent Progress on Kinins

IMMUNOASSAYS FOR THE DETERMINATION OF HUMAN TISSUE KALLIKREIN (TK) IN DIFFERENT BODY FLUIDS BASED ON MONOCLONAL ANTIBODIES

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SUMMARY: A monoclonal antibody produced against human tissue kallikrein was used to develop solid phase immunoassays for the determination of total immunoreactive tissue kallikrein, of the complex of tissue kallikrein with α_1-proteinase inhibitor, and of enzymatically active tissue kallikrein. The assays permit the specific determination of various forms of tissue kallikrein in body fluids and should be very useful in studies on the biological function of tissue kallikrein-kinin systems.

INTRODUCTION

The determination of tissue kallikrein (TK) in body fluids and tissues can be achieved by a number of methods, e. g. radioimmunoassays, enzyme immunoassays, determination of kinin-releasing activity, or hydrolysis of synthetic substrates. Due to the fact that TK is present in the organism in different forms, such as enzymatically inactive proenzyme, active TK or complexes with inhibitors, no single assay technique alone can accomplish the specific quantification of each of the TK species.

Here we present assays which are based on a monoclonal antibody for capturing the TK antigen and various detection systems permitting the specific determination of different forms of TK.

MATERIALS AND METHODS

Materials

Human urinary kallikrein (HUK) isolated according to (1) and rabbit polyclonal antibodies to HUK were gifts of Dr. R. Geiger (Me-

dor, Herrsching, Germany). Polyclonal antibodies to human α_1-proteinase inhibitor (α_1PI): Calbiochem, Frankfurt, Germany; alkaline phosphatase labeled anti-rabbit IgG: BioRad, Munich, Germany; substrate for kallikrein: H-D-ValLeuArg-p-nitroanilide (S-2266): KABI, Freiburg, Germany. Microplate reader MR 700: Dynatech, Denkendorf, Germany.

Buffers. A: 0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6;
B: 0.01 M monosodium phosphate, 0.15 M sodium chloride, pH 7.4;
C: 25 g/l casein in buffer B;
D: 20 g/l bovine serum albumin, 0.5 ml/l Tween 20 in buffer A;
E: 0.01 M monosodium phosphate, 0.14 M sodium chloride, 0.03 M EDTA, 3 mM o-phenanthroline, pH 7.4;
F: 0.01 M monosodium phosphate, 0.14 sodium chloride, 0.03 M EDTA, 3 mM o-phenanthroline, 0.5 g sodium azide/l, 0.2 g thimerosal/l, 0.4 ml Tween 20/l, pH 7.4;
G: 1.0 M diethanolamine/HCl, 100 mg/l $MgCl_2$*6 H_2O, pH 9.6.

Standards. The protein concentration of a stock solution of highly purified human urinary kallikrein was determined by amino acid analysis. Standard solutions of TK were prepared by diluting the stock solution appropriately. A stock solution of TK-α_1PI was prepared by incubating an aliquot of the TK stock solution with an excess of α_1PI for 24 h at room temperature.

Murine monoclonal antibodies against human tissue kallikrein were developed using human urinary kallikrein (1) as antigen by employing conventional hybridoma techniques. Antibodies of six different clones were produced, isolated and characterized, one of them was used for the immunoassays.

Coating of solid phase with monoclonal antibody. The wells of microtiter plates (Immunolon F, Dynatech, Denkendorf, Germany) were coated with monoclonal antibody by incubation (16 h, 4 °C) with 0.2 ml of a solution containing 3 µg/ml monoclonal antibody in buffer A. After washing the plate six times with 30 ml buffer B the remaining protein-binding sites in each well were blocked by incubation with buffer C for 1 h. After removing the buffer the plate was ready for use.

Capturing of immunoreactive TK. 0.2 ml of TK standard solutions or unknown samples containing TK and/or TK-α_1PI, diluted in

buffer D, were pipetted into the wells, then the microtiter plate was incubated for 2 h at 37 °C and washed.

Determination of captured antigen by second antibody. For the determination of total immunoreactive TK 0.2 ml of a solution of a rabbit anti-TK antibody, 1 μg/ml in buffer D, was added, the plate was incubated for 2 h at 37 °C and washed. Then 0.2 ml of alkaline phosphatase labeled anti-rabbit IgG (diluted 1:3000 in buffer D) was pipetted into each well. After washing 0.2 ml 4-nitrophenylphosphate, 1-2 g/l in buffer G was added and after 10-30 min incubation at 37 °C the absorbance at 405 nm was read. The complex of TK with α_1-proteinase inhibitor (TK-α_1PI) captured by the monoclonal antibody was determined by the same technique but using as second antibody a rabbit antiserum to α_1PI diluted 1:3000 with buffer D.

Determination of captured enzymatically active tissue kallikrein with a synthetic substrate. After the capturing step 0.2 ml of freshly prepared substrate solution, 0.4 mM H-D-Val-Leu-Arg-p-nitroanilide in 0.01 M Tris, pH 8.2 were pipetted into each well. After 24 h incubation at 37 °C absorbance at 405 nm was read using the microplate reader.

Determination of captured enzymatically active tissue kallikrein with kininogen as substrate. Into each well with TK bound to the coating monoclonal antibody 0.2 ml of a solution of dog kininogen (Marin-Grez and Carretero, 1972), 2 mg/ml in buffer E, was pipetted. After 4 h at 37 °C 0.1 ml of the solution was removed and mixed with 1.0 ml ethanol, the mixture was centrifuged for 10 min at 10,000 x g and 1.0 ml of the supernatant was dried in a SpeedVac concentrator. The dry residue was dissolved in buffer F and the kinin was determined by radioimmunoassay (2).

RESULTS AND DISCUSSION

A system of assays based on one monoclonal antibody was developed by which various forms of tissue kallikrein present in body fluids can be determined.

The antibody is bound to a solid phase and captures the TK present in the sample. By using different detection systems different species of TK, i. e. total (= free + complexed) tissue kallikrein, the complex of the enzyme with α_1-proteinase inhibitor and enzymatically active TK can be determined. Total TK and

the TK-α_1PI complex captured by the monoclonal antibody are detected using polyclonal antibodies to TK and α_1PI, respectively as second antibody in a sandwich ELISA.

Enzymatically active TK captured by the monoclonal antibody was determined either by using the synthetic substrate H-D-Val-Leu-Arg-p-nitroanilide or by incubation with kininogen and subsequent measuring of released kinin by a radioimmunoassay.

The lower levels of detection and the inter- and intraassay coefficients of variation are given in Table 1 for all assay systems. The activity determination with S-2266 as substrate is by a factor of 25-50 less sensitive then the other assays. However, speed and/or sensitivity of this assay can be improved by employing fluorimetric substrates.

	Total TK	TK-α_1PI	Active TK	
			Kininogen	S-2266
Lower limit of detection	0.2 ng/ml	0.2 ng/ml	0.1 ng/ml	5.0 ng/ml
Intraassay variation	3.3 %	4.8 %	13.1 %	4.4 %
Interassay variation	5.2 %	11.6 %	16.4 %	6.7 %

Table 1. Lower levels of detection and intra- and interassay coefficients of variation. Values for TK-α_1PI concentrations represent concentrations of the TK bound to α_1PI.

The recovery of tissue kallikrein added to samples of biological fluids (final concentrations: 2 ng/ml; for the assay with S-2266: 10 ng/ml) was between 92 and 99 % for urine, seminal plasma and saliva. The recovery in serum of total TK and of the complex of TK with α_1PI was 79-81 %. Recovery of active TK in serum was in the range of only 40 % reflecting the inactivation of the added tissue kallikrein by complex formation with α_1PI and possibly other plasmatic inhibitors.

The assays were successfully employed to determine the concentrations of the various tissue kallikrein species in human serum, urine, seminal plasma and saliva (Table 2). In all the fluids the concentrations of all TK species varied over a wide range. The

lowest concentrations of total TK were found in serum and virtually all of the TK was complexed with α_1PI, enzymatically active kallikrein could not be detected. In the individual urine samples the concentrations of total and enzymatically active TK differed by up to 75 %. This difference could not be explained by the presence of TK-α_1PI since this was found at low concentrations only. The difference was obviously due to the presence of high portions of tissue prokallikrein in urine as it disappeared when the urine samples were treated with trypsin prior to the activity assay (data not shown).

	Total TK [ng/ml]	TK-α_1PI [ng/ml]	Active TK	
			Kininogen [ng/ml]	S-2266 [ng/ml]
Serum (n = 10)	0.2 - 2.2	0.2 - 2.0	n.d.	n.d.
Urine (n = 5)	195 - 558	4 - 32	56 - 212	71 - 210
Seminal plasma (n = 20)	13 - 201	1 - 32	3 - 204	n.d. - 188
Saliva (n = 5)	1141 - 6116	44 - 1043	798 - 5183	1129 - 5351

Table 2. Contents of total TK, TK-α_1PI and active TK in human serum, urine, seminal plasma and saliva. Values for TK-α_1PI concentrations represent concentrations of the TK bound to α_1PI; n.d. = not detectable.

CONCLUSION

The assay system described here is highly suitable for the specific determination of different forms of human tissue kallikrein in body fluids. In addition, it can be easily adapted to the determination of complexes of TK with proteins other than α_1PI, such as kallikrein binding protein (3) or protein C inhibitor (4), by employing as second antibody a specific antibody directed towards the respective complex-forming protein.

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