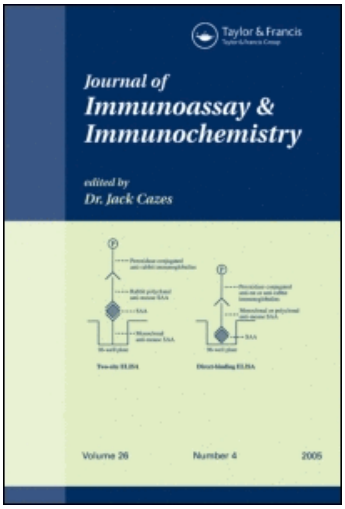

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Development of a Peptide-Based Sandwich Elisa for Human Tissue Prokallikrein with No Cross-Reactivity from Mature Kallikrein

Stefan Mauer[a]; Armin Maidhof[b]; Michael Kemme[a]
[a] Institute for Biochemistry, Darmstadt University of Technology, Germany [b] Institute of Physiological Chemistry, University of Mainz, Germany

To cite this Article Mauer, Stefan , Maidhof, Armin and Kemme, Michael(2000) 'Development of a Peptide-Based Sandwich Elisa for Human Tissue Prokallikrein with No Cross-Reactivity from Mature Kallikrein', Journal of Immunoassay and Immunochemistry, 21: 4, 411 — 426
To link to this Article: DOI: 10.1080/01971520009349546
URL: http://dx.doi.org/10.1080/01971520009349546

JOURNAL OF IMMUNOASSAY, 21(4), 411–426 (2000)

DEVELOPMENT OF A PEPTIDE-BASED SANDWICH ELISA FOR HUMAN TISSUE PROKALLIKREIN WITH NO CROSS-REACTIVITY FROM MATURE KALLIKREIN

Stefan Mauer, Armin Maidhof* and Michael Kemme
Institute for Biochemistry, Darmstadt University of Technology, Germany;
*Institute of Physiological Chemistry, University of Mainz, Germany
e-mail: mkemme@pop.tu-darmstadt.de

ABSTRACT

Human tissue prokallikrein is the enzymatically inactive zymogen of a serine proteinase involved in the liberation of vasoactive kinin peptides, and it is supposed that an impaired prokallikrein-to-kallikrein conversion is closely related to certain hypertensive and inflammatory disorders. Progress in understanding the biological role of the proenzyme has been limited by the absence of an accurate assay for the kallikrein precursor. We describe a sandwich enzyme-linked immunosorbent assay to measure human tissue prokallikrein using monospecific anti-peptide antibodies raised against propeptide derivatives. This method could detect a minimum concentration of 60 pg/ml prokallikrein and displayed no cross-reactivity or interference with mature tissue kallikrein. The intra- and inter-assay precision varied from 8-15%, respectively, indicating a reasonable reproducibility of the method. The level of prokallikrein was defined in different human urine samples, and the corresponding dilution curves showed good linearity. The mean recovery of added zymogen was 104%. Prokallikrein immunoassay is the first reported tool for the direct and sensitive quantification of the precursor of tissue kallikrein and should facilitate the precise determination of prokallikrein levels in a variety of biological specimen.
(KEY WORDS: Anti-peptide antibody; Sandwich ELISA; Tissue prokallikrein; Urine; Zymogen)

Address for reprint requests: M. Kemme, Institute for Biochemistry, Darmstadt University of Technology, Petersenstr. 22, 64287 Darmstadt, Germany; e-mail: mkemme@pop.tu-darmstadt.de

INTRODUCTION

Tissue prokallikrein (TproK) represents the inactive precursor or zymogen of a serine proteinase that plays a key role in a reaction cascade liberating vasoactive and proinflammatory kinin peptides (reviewed in (1)). Human TproK is excreted in urine (2) and saliva (3) and is present on the surface of circulating neutrophils (4). The primary structure of TproK has been deduced from DNA (5) and protein sequence analyses (6), respectively, to yield a typical proenzyme architecture comprising the active form of tissue kallikrein (TK) with an additional heptapeptide sequence at the amino terminus (Figure 1). The zymogen can be converted rapidly to mature TK by limited proteolysis using model activators with tryptic-like specificity, although the physiological mechanism of the activation step remains unknown (1).

It has been shown clinically that the ratio of TK-to-TproK was decreased in various hypertensive disorders including essential hypertension (7) and pre-eclampsia (8). The reduced amount of urinary TK in such patients was interpreted as the direct consequence of a primary defect in zymogen conversion (9). On the other hand, a markedly increased TK activity in different body fluids of subjects with interstitial cystitis (10) and Sjögren's syndrome (11) has been determined, suggesting that an enhanced activation of TproK may be involved in the pathogenesis of these local inflammatory disorders. Thus, the TproK level in biological fluids, cells and tissue homogenates could be an important indicator of physiological or pathophysiological conditions.

Usually, TproK is measured after in vitro conversion of the zymogen as the difference in amidolytic activity of total kallikrein and active TK using chromogenic peptide substrates (10). The low specificity of this method and the presence of TK inhibitors, however, may interfere with the reliable data of TproK levels. In addition, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA)

procedures have been designed based on antibodies that recognized either mature or total (pro- plus mature) TK, but none of the assay techniques alone can achieve the specific quantification of the TK precursor (12). Therefore, it will be crucial to establish an accurate immunological method for the estimation of human TproK in clinical samples.

Most commonly, parts of a protein which are not dominant epitopes for antibodies induced by the whole protein can often be recognized by anti-peptide antibodies (13). This approach utilizes short synthetic peptides corresponding to predetermined amino acid sequences of the intact molecule as immunogen, thereby obtaining site-specific immunological probes. Anti-peptide antibodies may be used for distinguishing between closely related proteins that differ by only a single or a few amino acids. In such cases, immunization with the complete protein will elicit cross-reactive antisera, while the selection of appropriate peptides will allow the production of antibodies that specifically bind the native protein from which the peptide was derived. Recent results have shown that this powerful strategy has been applied successfully to analyse different enzyme isoforms (14) and to develop ELISA systems for the quantification of proproteins (15-16).

The purpose of this study was to design a quantitative and reliable immunoassay for human TproK without cross-reactivity from mature TK. Using monospecific antibodies raised against synthetic peptides copying the amino terminus of the TK precursor, we have developed a sensitive sandwich ELISA for the evaluation of TproK levels in complex biological fluids. This is the first accurate assay for direct measurements of human TproK alone.

MATERIALS AND METHODS

Antigens and Antibodies

Descriptions of the peptides used in this study are summarized in Figure 1. CAP-11 was synthesized on a solid-phase peptide synthesizer using standard N-(9-

fluorenyl)-methoxycarbonyl chemistry; APP-11 was supplied by Eurogentec (Seraing, Belgium). After analysis by Edman degradation, both peptides were covalently coupled to maleimide-activated keyhole limpet hemocyanin (KLH; Pierce, Rockford, IL, USA) according to the instructions of the manufacturer. Rabbit immunization with the CAP-11 conjugate followed a previously described protocol (17); anti-APP-11 serum has been raised in rabbits as a custom service (Eurogentec).

Recombinant human TproK, expressed in baculovirus-infected insect cells (18), and mature TK, derived from it by in vitro activation, were tested as standards in the ELISA. A polyclonal antiserum (anti-TproK) directed against the purified recombinant zymogen has been raised in rabbits (Prof. Krantz, Department of Biochemistry, Greifswald) and characterized by immunoblotting to confirm specific binding of TproK (4).

Antibody Purification and Biotinylation

Polyclonal antibodies were purified from hyperimmune sera by ammonium sulfate precipitation (1 ml antiserum/ml 3.2 M $(NH_4)_2SO_4$, pH 7) for 30 min at 4°C and subjected to affinity chromatography. The cognate peptide antigens were covalently coupled to agarose (6 mg/column) using the SulfoLink kit (Pierce), and 10 ml antibody solution per ml of gel was applied for 2 h at 4°C. After extensive washing of the affinity matrix with phosphate-buffered saline (PBS; 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.4), bound antibodies were eluted with 0.1 M glycine, pH 2.5, and immediately neutralized with 1 M Tris-HCl, pH 9. Anti-TproK IgG were isolated on a protein A-agarose column (Boehringer Mannheim, Mannheim, Germany) using the low salt method (19).

Biotinylation of anti-TproK IgG with biotin hydrazide followed the EZ-Link technology (Pierce) according to the manufacturer's recommendations.

Enzyme Immunoassays

If not stated otherwise, the following buffers were used for all ELISA procedures: 12 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6 (coating buffer); phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20 (PBST); PBST including 2% (w/v) bovine serum albumin (BSA-PBST); 1 mg/ml 2,2′-azino-di-(3-ethylbenzthiazolin-6-sulfonate) (ABTS; Boehringer Mannheim) in 0.1 M citric acid, 0.1 M Na_2HPO_4, pH 4.5 containing 0.012% (v/v) H_2O_2 (substrate solution). Five washings with 250 µl PBST/well were performed between each incubation step, and residual solution was gently tapped on absorbent tissue paper.

Indirect ELISA

Synthetic peptides and recombinant proteins were adsorbed overnight at 4°C to microtiter plates (Greiner PS, Nürtingen, Germany) at a concentration of 50 nM (200 µl/well) in coating buffer, respectively. After a warm-up period at 37°C, wells were incubated for 3 h with 200 µl samples of affinity-purified anti-peptide antibodies, serially diluted form 160 µg/ml to 78 pg/ml in BSA-PBST. Detection of bound antibodies occurred for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma, Deisenhofen, Germany), diluted 1/1000 in BSA-PBST, followed by an incubation step with substrate solution for 30 min. The absorbance in each well was read at 405 nm using a 340 ATCC ELISA reader (SLT Labinstruments, Crailsheim, Germany). The potency of the antibodies was expressed as titer values reflecting working dilutions which yielded 1 absorbance unit in the indirect ELISA.

Sandwich ELISA

Greiner PS 96 well-plates were coated overnight at 4°C with 5 µg/ml affinity-purified anti-APP-11 antibodies (200 µl/well). Each plate was then saturated for 1 h at 37°C with BSA-PBST. Properly diluted, the samples or TproK standards (100

µl/well) were incubated for 2 h at 37°C, before addition of biotin-labeled anti-TproK IgG at a concentration of 800 ng/ml in BSA-PBST (100 µl/well). Following a further incubation step at 37°C, 200 µl streptavidin-HRP conjugate (2 µg/ml; Dianova, Hamburg, Germany) was pipetted into each well and after 30 min, HRP activity was established as desribed above.

Samples

Spot urine samples were collected from 6 healthy volunteers (age 25-40 years) and immediately chilled on ice. The urine was centrifuged at 3000 g for 10 min and the supernatants were stored at -30°C until analyzed.

RESULTS

Antibody Specificity

Two synthetic peptides covering the entire amino acid sequence of the activation peptide and the first three residues of mature TK, covalently coupled to the carrier protein KLH by an additional cysteine residue either at the amino-terminal (CAP-11) or the carboxy-terminal end (APP-11), were selected for immunization of rabbits (Figure 1). Indirect ELISA studies indicated that both affinity-purified polyclonal anti-peptide antibodies reacted at comparable titers with the uncoupled immunizing peptides (Figure 2). In addition, both antibody preparations recognized the zymogen molecule TproK with titer values of 0.04 µg/ml and 40 µg/ml for anti-APP-11 and anti-CAP-11, respectively. Analysis of cross-reactivity with the mature enzyme TK demonstrated the absence of binding for anti-APP-11 over the whole concentration range (Figure 2B) whereas anti-CAP-11 cross-reacted with TK at low dilutions (Figure 2A). These findings were previously confirmed by Western blot data (4,20) showing the unique selectivity of anti-APP-11 antibodies for TproK. Thus, anti-APP-11 was used as capture antibody specific for the zymogen form of human TK.

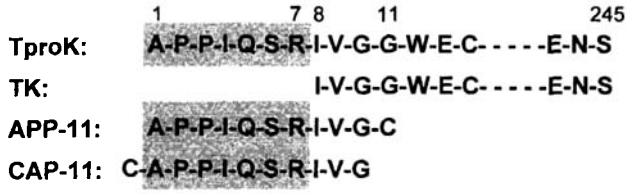

FIGURE 1: Amino acid sequences of human TproK, mature TK, and the synthetic peptides used in this study. Numbering is according to the zymogen sequence. Peptides are identified by their three amino-terminal residues using the one-letter code, following by the total number of residues constituting the peptide. The common prosequence motif is depicted by grey boxes.

Assay Conditions, Sensitivity and Specificity

A sandwich ELISA was developed using immunoselected anti-APP-11 antibodies as the coating/capture antibody, biotinylated anti-TproK IgG as the detection antibody, and recombinant human TproK as the standard. The resulting assay procedure allowed for the quantification of TproK in a working range between 80 pg/ml and 20 ng/ml established from the linear section of a typical standard curve (Figure 3). The detection limit of the TproK ELISA in buffer was approximately 60 pg/ml, as assessed by the value corresponding to three standard deviations above the mean of the zero response measured in three independent assays.

To determine whether TproK activation has an effect on the present ELISA, the assay was performed with recombinant TK in a concentration range between 40 pg/ml and 80 ng/ml. However, no cross-reactivity with the mature enzyme was detected even at concentrations up to 80 ng/ml (Figure 3).

Precision

The precision of the sandwich assay was assessed using diluted recombinant TproK at concentrations of 0.4, 3 and 15 ng/ml, respectively. The intra-assay

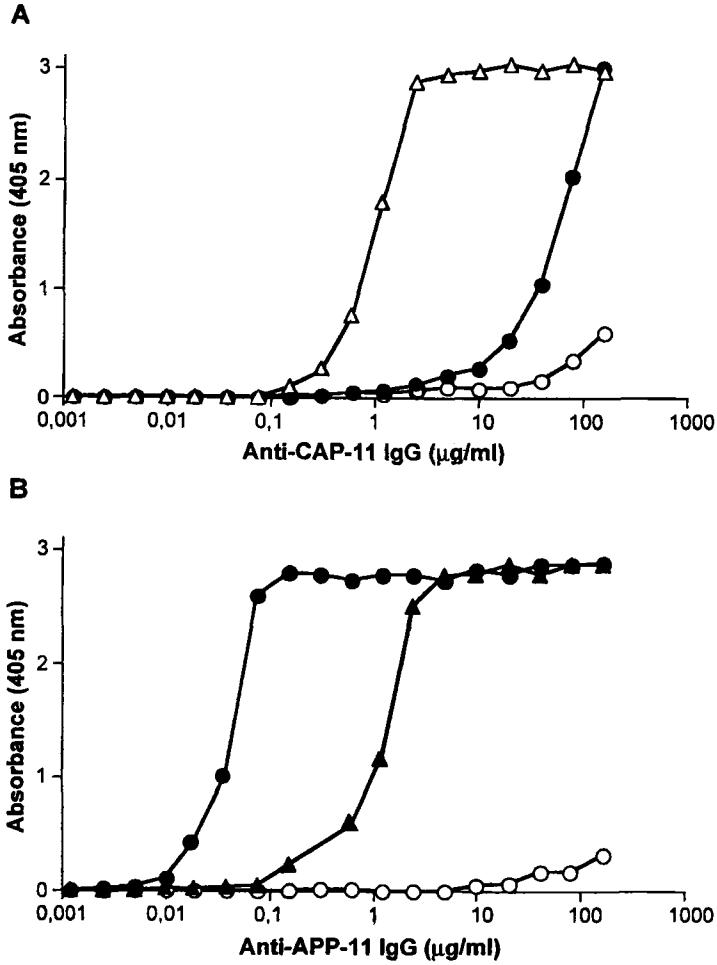

FIGURE 2: Binding analysis of anti-CAP-11 (A) and anti-APP-11 (B) antibodies by an indirect ELISA. Plates were coated with 10 pmol CAP-11 (Δ), APP-11 (▲), TK (○) and TproK (●), respectively, and bound anti-peptide antibodies were detected using HRP-labeled secondary antibodies.

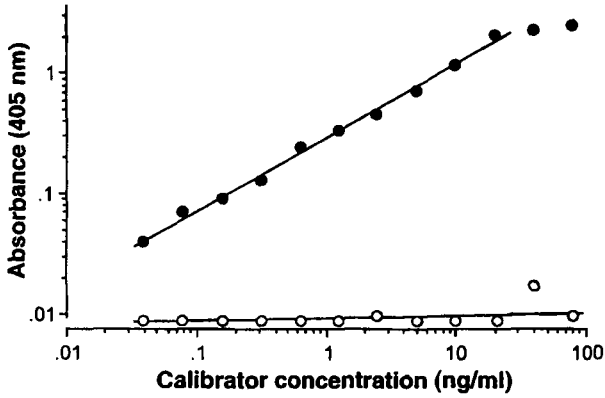

FIGURE 3: Representative calibration curve for the TproK sandwich ELISA. Known amounts of recombinant TproK (●) in buffer served as primary standards in serial dilutions from 80 ng/ml to 40 pg/ml. The absorbance was plotted logarithmically against the calibrator concentration to yield a linear fit with r^2 of 0.995. The assay does not detect mature TK (○).

coefficient of variation (CV), calculated from four replicate measurements, ranged from 8.0-15.4%. Inter-assay variability was derived by evaluating the low, medium and high controls in quadruplicate in six independent assays to yield a CV range from 9.3-13.5% (Table 1).

Analytical Recovery and Linearity of Dilution

To evaluate the recovery of TproK in urine, we added recombinant TproK to final concentrations of 1.2, 6 and 12 ng/ml, respectively, into samples from three normal donors. The amount of endogenous zymogen had to be subtracted before calculating the recovery levels by comparison of the expected versus the observed concentrations (Table 2). Recoveries of exogenously supplemented TproK from urine were complete in all samples and ranged from 83-133%. The mean recovery was found to be 104%. Additionally, the calculated values indicate the absence of urine matrix effects.

TABLE 1

Precision of the TproK Sandwich ELISA

	Within-run (n = 4)			Between-run (n = 6)		
	Mean (ng/ml)	SD (ng/ml)	CV (%)	Mean (ng/ml)	SD (ng/ml)	CV (%)
Low controls	0.39	0.06	15.4	0.40	0.05	12.5
Medium controls	3.38	0.27	8.0	3.01	0.28	9.3
High controls	14.92	1.95	13.1	17.22	2.32	13.5

(SD, standard deviation)

TABLE 2

Recovery of TproK in Human Urine

Sample matrix	TproK (ng/ml)		Recovery (%)
	Expected*	Observed	
Female urine	1.2	1.0	83
	6.0	5.2	87
	12.0	11.9	99
Male urine	1.2	1.3	108
	6.0	6.4	107
	12.0	14.2	118
Male urine	1.2	1.6	133
	6.0	6.2	103
	12.0	11.4	95

*Concentration of exogenously added recombinant TproK

A parallelism study served to identify any changes in the assay performance due to different analyte configurations in the standard and test sample. Human urine was serially diluted to the levels encompassing the working range of the TproK ELISA and the linearity was evaluated by comparing the measured values with the calibration curve (Figure 4). The relation between TproK concentration

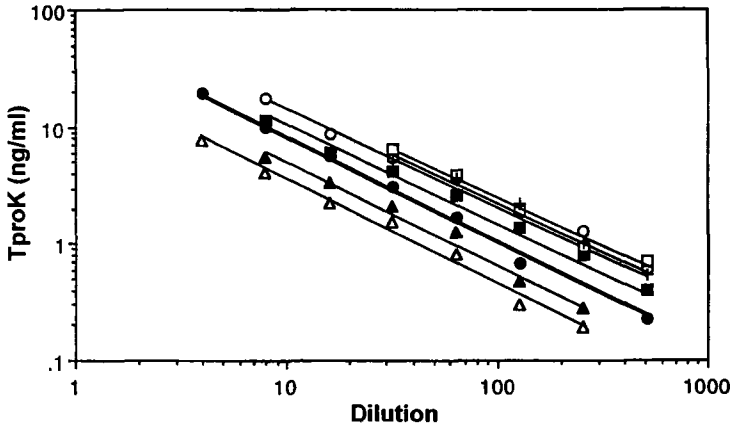

FIGURE 4: Dilution curves for TproK in human urine. Serial dilutions of four male urine samples (1, ○; 2, ■; 3, ❑; 4, +) and two female urine samples (1, ▲; 2, Δ) were compared to TproK standards (●).

TABLE 3

Concentrations of TproK in Human Urine

Sample	No.	TproK (ng/ml)
Female urine	1	32
	2	43
Male urine	1	144
	2	180
	3	246
	4	136

and dilution of all samples tested did not significantly deviate from linearity over the whole concentration range.

Detection of TproK in Human Urine

Concentrations of human TproK in urine samples obtained from normal men and women of ages between 25 and 40 were determined by the sandwich ELISA.

All samples had detectable TproK and the immunoreactive zymogen could be quantified after dilution to the linearity level (Table 3). In female urine, the TproK content is significantly lower with a mean of 38 ng/ml (range, 32-43 ng/ml) when compared to male urine with a median concentration of 177 ng/ml (range, 136-246 ng/ml). These data correspond only to a limited group of individuals and will require validation by larger clinical studies.

DISCUSSION

Abnormalities in the TK-kinin system have been proposed to contribute to the pathogenesis not only in hypertensive but also in inflammatory disorders. Using enzymatic methods and immunological assays, several groups have shown the presence of reduced urinary levels of active and/or total TK in hypertension (9,21), while increased amounts of active TK are widely believed to reflect the symptoms of inflammation (10-11). It has been noted that TK is secreted from cells as inactive precursor TproK (4,22). Thus, beside active enzyme and enzyme-inhibitor complexes, variable amounts of the zymogen could be expected to be present in extracellular fluids as an additional form to characterize certain disease states. However, a direct method for the quantification of TproK has not been reported so far.

Therefore, the major objective of this study was to develop an accurate and sensitive immunoassay for human TproK to distinguish between the mature enzyme and the zymogen form of TK in biological samples. The availability of the deduced amino acid sequence for the activation peptide of TproK has provided a means to produce anti-peptide antibodies by immunization with synthetic peptides corresponding to a unique sequence segment within the zymogen. Monospecific antibodies to rat TproK have been developed by immunization with an

undecapeptide covering the entire prosequence of seven amino acid residues and a linker sequence from the signal peptide, however, the resulting antiserum has only been used in binding studies and not for quantitative measurements (23). In order to elongate the short heptameric propeptide of human TproK, we have added three amino acids found in the mature enzyme and an additional cysteine residue at the amino terminus (CAP-11) and the carboxy terminus (APP-11) for coupling of a carrier protein, respectively (see Figure 1). Specificity of the generated anti-CAP-11 and anti-APP-11 antibodies was evaluated by their different response to TproK and TK in an indirect ELISA system. The pattern of binding suggested that immunization with the carboxy-terminal coupled peptide induced antibodies reactive specifically with the zymogen form, whereas the amino-terminal coupled peptide induced antibodies that react with an epitope common of mature TK and TproK. This finding supports an earlier report on the importance of the orientation of the immunizing peptide for raising anti-peptide antibodies (24). Compared with anti-CAP-11 antibodies, the anti-APP-11 antibodies displayed a unique selectivity for the zymogen, thus, monospecific anti APP-11 was found to be a suitable capture antibody in an ELISA system.

The sandwich ELISA we described in this study seems to be the first direct assay specific for intact human TproK. Interferences by mature TK or unrelated proteins from complex biological samples did not occur. The present method could detect TproK concentrations of 60 pg/ml which is comparable to the detection limit of 40 pg/ml for mature TK in a previously published radioimmunoassay (25). The current ELISA was further characterized by analyzing urine samples from normal individuals. All samples had detectable TproK, but the zymogen concentrations varied over a wide range, in good agreement with earlier observations (26). In the present study, zymogen levels in female urine were about fivefold lower than those measured for male urine. Whether this sex-related difference is because of

hormonal regulation of urinary TproK excretion or to different renal synthesis rates of the zymogen requires further investigation.

In conclusion, the ELISA set-up in this paper was shown to be a sensitive, precise and specific tool that permits for the first time the direct quantification of human TproK. Despite a high degree of sequence identity between the zymogen and mature TK, the assay displayed no cross-reactivity from the mature form, emphasizing the value of anti-peptide antibodies for the study of pro-proteins. The application of this sandwich ELISA for in vitro experiments and analysis of biological specimen may contribute to our understanding in the regulation of TproK, its physiological role and involvement in human disease.

ACKNOWLEDGEMENTS

The outstanding support and valuable comments of Prof. Werner Müller-Esterl (Mainz, Germany) in the development of peptide antibodies are gratefully acknowledged. Our appreciation is extended to Prof. Sven Krantz (Greifswald, Germany) for the generation of anti-TproK antiserum. We thank Prof. Hans G. Gassen (Darmstadt, Germany) for providing excellent research facilities. This work was funded by a grant (Ke 639/1-1) from the Deutsche Forschungsgemeinschaft, Bonn, Germany.

REFERENCES

1. Bhoola, K.D., Figueroa, C.D. and Worthy, K. Bioregulation of kinins: kallikreins, kininogens, and kininases. Pharmacol. Rev. 1992; 44: 1-80.

2. Spragg, J. Latent kallikrein from human urine. Methods Enzymol. 1988; 163: 96-102.

3. Jenzano, J.W., Su, H.-W., Featherstone, G.L. and Lundblad R.L. Molecular diversity of tissue kallikrein in human saliva. Agents Actions 1992; 38: 137-144.

4. Kemme, M., Podlich, D., Raidoo, D.M., Snyman, C., Naidoo, S. and Bhoola, K.D. Identification of immunoreactive tissue prokallikrein on the surface membrane of human neutrophils. Biol. Chem. 1999; 380: 1321-1328.

5. Angermann, A., Bergmann, C. and Appelhans, H. Cloning and expression of human salivary-gland kallikrein in *Escherichia coli*. Biochem. J. 1989; 262: 787-793.

6. Takahashi, S., Irie, A. and Miyake, Y. Primary structure of human urinary prokallikrein. J. Biochem. 1988; 104: 22-29.

7. Lieberthal, W., Arbeit, L., Oza, N.B., Bernard, D.B. and Levinsky, N.G. Reduced ratio of active-to-total urinary kallikrein in essential hypertension. Hypertension 1983; 5: 603-609.

8. Kyle, P.M., Campbell, S., Buckley, D. et al. A comparison of the inactive urinary kallikrein:creatinine ratio and the angiotension sensitivity test for the prediction of pre-eclampsia. Br. J. Obstet. Gynaecol. 1996; 103: 981-987.

9. Bellini, C., Ferri, C., Carlomagno, A. et al. Impaired inactive to active kallikrein conversion in human salt-sensitive hypertension. J. Am. Soc. Nephrol. 1996; 7: 2565-2577.

10. Zuraw, B.L., Sugimoto, S., Parsons, C.L., Hugli, T., Lotz, M. and Koziol, J. Activation of urinary kallikrein in patients with interstitial cystitis. J. Urol. 1994; 152: 874-878.

11. Hernandez, C.C.C., Donadi, E.A. and Reis, M.L. Kininogen-kallikrein-kinin system in plasma and saliva of patients with Sjögren's syndrome. J. Rheumatol. 1998; 25: 2381-2384.

12. Irie, A., Takahashi, S., Katayama, Y., Shibata, Y. and Miyake, Y. Reactivities of human urinary prokallikrein and kallikrein toward rabbit antibodies with special reference to enzyme immunoassay. Clin. Chim. Acta 1988; 173, 289-298.

13. Lerner, R.A. Antibodies of predetermined specificity in biology and medicine. Adv. Immunol. 1984; 36: 1-44.

14. Brown, C.D., Barnes, K. and Turner, A.J. Anti-peptide antibodies specific to rat endothelin-converting enzyme-1 isoforms reveal isoform localization and expression. FEBS Lett. 1998; 424: 183-187.

15. Barkia, A., Martin, C., Puchois, P. et al. Enzyme-linked immunosorbent assay for human proapolipoprotein A-1 using specific antibodies against synthetic peptide. J. Lipid Res. 1988; 29: 77-84.

16. Costopoulou, D., Leondiadis, L., Czarnecki, J. et al. Direct ELISA method for the specific determination of prothymosin alpha in human specimen. J. Immunoassay 1998; 19: 295-316.

17. Müller-Esterl, W. Immunological probes for the bradykinin B_2 receptor - a toolbox. In: Farmer, S.G., ed. The kinin system. San Diego: Academic Press, 1997: 99-110.

18. Angermann, A., Rahn, H.-P., Hektor, T., Fertig, G. and Kemme, M. Purification and characterization of human salivary-gland prokallikrein from recombinant baculovirus-infected insect cells. Eur. J. Biochem. 1992; 206: 225-233.

19. Harlow, E. and Lane, D. Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory, 1988.

20. Naidoo, Y., Snyman, C., Raidoo, D.M., Bhoola, K.D., Kemme, M. and Müller-Esterl, W. Cellular visualization of tissue prokallikrein in human neutrophils and myelocytes. Br. J. Haematol. 1999; 105: 599-612.

21. Maier, M., Jerabek, I., Reissert, G., Höltzl, E. and Binder, B.R. Correlation of two different assays for urinary kallikrein in normotensive and hypertensive subjects. Clin. Chim. Acta 1988; 178: 127-140.

22. Abe, M., Nakamura, F., Tan, F. et al. Expression of rat kallikrein and epithelial polarity in transfected Madin-Darby canine kidney cells. Hypertension 1995; 26: 891-898.

23. Takaoka, M., Nakamura, M., Nishii, M., Kashiwagi, Y. and Morimoto, S. Immunological identification of rat urinary inactive kallikrein as a proform of tissue kallikrein. Biochemistry Int. 1988; 16: 719-725.

24. Dyrberg, T. and Oldstone, M.B.A. Peptides as antigens: importance of orientation. J. Exp. Med. 1986; 164: 1344-1349.

25. Girolami, J.P., Pecher, C., Bascands, J.L. et al. Direct radioimmunoassay of active and inactive human glandular kallikrein. J. Immunoassay 1989; 10: 221-236.

26. Witzgall, K., Godec, G., Shimamoto, K. and Fink, E. Immunoassays for the determination of human tissue kallikrein in different body fluids based on monoclonal antibodies. Agents Actions 1992; 38: 153-158.