THROMBOSIS RESEARCH 14; 551-559

0049-3848/79/0415-0551 $02.00/0

FLUOROMETRIC ASSAY FOR PLASMA PREKALLIKREIN USING PEPTIDYLMETHYL-COUMARINYL-AMIDE AS A SUBSTRATE

Sachiko Oh-ishi and Makoto Katori

Department of Pharmacology, Kitasato University
School of Medicine, Sagamihara, Kanagawa 228, Japan

(Received 18.9.1978; in revised form 5.12.1978.
Accepted by Editor S. Iwanaga)

ABSTRACT

The content of prekallikrein in human plasma was determined by activation with acetone and kaolin, and subsequent measurement of the enzyme activity using carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide as substrate. The kallikrein generated was stable for 60 min after activation and at least 10% of the original concentration of both Hageman factor and high molecular weight kininogen are required to get complete activation of prekallikrein within this time period. When this method was compared to the radiochemical method employing ^{3}H-TAME a similar sensitivity could be demonstrated. Plasmin and hog pancreatic kallikrein less readily hydrolyzed the amide substrate.

INTRODUCTION

The role of the plasma kallikrein-kinin system in pathological states has long been studied (1,2). Meanwhile, recent findings have shown that both prekallikrein and high molecular weight (HMW) kininogen are essential cofactors in the initiation of the contact phase of the intrinsic pathway of blood coagulation (3). To elucidate the involvement of prekallikrein in pathological states, it is necessary to measure its concentration accurately. Plasma prekallikrein once activated in biological fluids is inactivated by proteinase inhibitors thus making it difficult to measure the active enzyme in blood or inflammatory fluids. Currently prekallikrein is measured in plasma by activation with kaolin, celite or ellagic acid and the kallikrein so formed measured using ester or amide substrates (4).

Recently chromogenic substrates have been used to measure many enzymes including plasma kallikrein (5). Morita et al (6) synthesized a new fluorogenic substrate for plasma kallikrein, carbobenzoxy-L-phenylalanyl-L-arginine 4-methylcoumarinyl-7-amide (Z-Phe-Arg-MCA). This report describes an MCA-assay for plasma prekallikrein.

MATERIALS AND METHODS

Z-Phe-Arg-MCA, L-propyl-L-phenylalanyl-L-arginine MCA (Pro-Phe-Arg-MCA), tert-butoxycarbonyl-L-valyl-L-propyl-L-arginine MCA (Boc-Val-Pro-Arg-MCA) and 7-amino-4-methylcoumarin (AMC) were purchased from Peptide Institute Inc., Osaka. Soybean trypsin inhibitor (SBTI) and limabean trypsin inhibitor (LBTI) were products of Worthington Biochem. Co., Freehold, New Jersey. Kaolin (K-5) was purchased from Fisher Scientific Co., Pittsburgh, Pennsylvania; bovine α-thrombin from Parke Davis-Sankyo, Tokyo; N-α-p-tosyl-L-arginine ^{3}H methyl ester hydrochloride (^{3}H-TAME, 213 mCi/mmol) from The Radiochem. Center Ltd., Amersham, England; and human plasmin (15 casein units/mg) from AB KABI, Stockholm.

Normal human plasma was collected from healthy volunteers into plastic tubes containing 1/10 volume of 3.8% sodium citrate. The authors are indebted to Dr. M.E. Webster, Escola Paulista de Medicina, Brazil, for Hageman factor deficient plasma and Fletcher trait plasma, to Drs. H. Hayashi and Y. Kimura, Okayama University, for Fujiwara trait plasma and Dr. C. Kuzbach, Bayer A.G., for highly purified hog pancreatic kallikrein (KZC, 1290 kallikrein units/mg).

Human plasma prekallikrein was prepared from dialysed human plasma by filtration through DEAE-sephadex in a manner similar to that previously described (7). After freezing and thawing the prekallikrein gradually activated upon storage at 4°C for several weeks. The kallikrein so formed was further purified by affinity chromatography on Trasylol-sepharose (10 mg Trasylol/g gel) prepared from CNBr-activated sepharose 4B (Pharmacia) and Trasylol (Bayer A.G., kindly supplied). The column was washed with 0.2 M AcOH and eluted with 1×10^{-3} M HCl. The fractions containing kallikrein were neutralized and concentrated with Minicon B 15 (Amicon Far East Ltd., Tokyo) (3.2 TAME units/mg).

Z-Phe-Arg-MCA determination of prekallikrein:

(1) Activation: Citrated human plasma (50 µl) was mixed with 850 µl acetone-buffer I solution (700 µl Buffer I and 150 µl acetone) and allowed to stand for 10 min at room temperature (25°C). Then 100 µl kaolin suspension (10 mg/ml Buffer I) was added and mixed vigorously using an electric mixer for 15 sec. At certain time intervals after the addition of kaolin, 20 µl aliquots of the reaction mixture were taken. Buffer I (0.02 M tris·HCl, 0.15 M NaCl, pH 8.0). This method of activation is identical to that employed by Imanari et al (8).

(2) Amidolysis: 20 µl of the above solution was then incubated for 10 min at 37°C with 1 ml of 5×10^{-5} M Z-Phe-Arg-MCA in Buffer II (0.05 M tris·HCl, 0.1 M NaCl, 0.01 M $CaCl_2$, pH 8.0) containing either 40 µg LBTI (Tube A) or 40 µg SBTI (Tube B). The reaction was terminated by the addition of 2 ml 17% acetic acid and the fluorescence read at 460 (emission) and 380 (excitation) nm in a Hitachi MPF-3 fluorescence spectrophotometer. The difference between the values from Tube A and Tube B is calculated as prekallikrein activity. The results are reported in arbitrary units where one unit is defined as that amount of enzyme which releases 1×10^{-7} M AMC/10 min under the described conditions. Zero order kinetics were obtained up to 6 units. Km value for partially purified plasma kallikrein was 2.5×10^{-4} M. The substrate was most stable when dissolved at 1×10^{-2} M in dimethylsulfoxide, stored frozen and diluted in Buffer II just prior to assay.

MCA assay of the other substrates was performed in a similar manner to that described above. For the ^{3}H-TAME assay the enzymes were incubated with the substrate for 30 min at room temperature in Buffer I as previously described (9).

RESULTS

Specificity of the substrates: Plasma kallikrein, hog pancreatic kallikrein and plasmin were compared for their abilities to hydrolyze Z-Phe-Arg-MCA and Pro-Phe-Arg-MCA, and TAME. As shown in Fig. 1, when these three enzymes were compared at concentrations which gave similar hydrolysis of TAME, plasma kallikrein (pl-KK) more readily hydrolyzed Z-Phe-Arg-MCA than did plasmin or hog pancreatic kallikrein (KZC). Pro-Phe-Arg-MCA, a substrate for glandular kallikrein, was hydrolyzed equally well by either kallikrein although it, like Z-Phe-Arg-MCA, was a poor substrate for plasmin.

Thrombin was also capable of digesting Z-Phe-Arg-MCA but when compared with its ability to hydrolyze its specific substrate (Boc-Val-Pro-Arg-MCA), it was 0.6% as active. An aliquot of kaolin activated plasma (20 µl) formed 0.26 arbitrary units of AMC when Boc-Val-Pro-Arg-MCA was used as substrate but this corresponded to only 0.0016 units of AMC when Z-Phe-Arg-MCA was the substrate.

Optimal conditions for activation of human plasma kallikrein: The effect of dilutions of plasma and concentration of kaolin on the activation of prekallikrein were examined at various time intervals (Fig. 2). At 1 : 50 dilution of plasma the formation of kallikrein was delayed reaching a maximum only after 60 min. At 1 : 20 dilution of plasma maximum formation occurred within

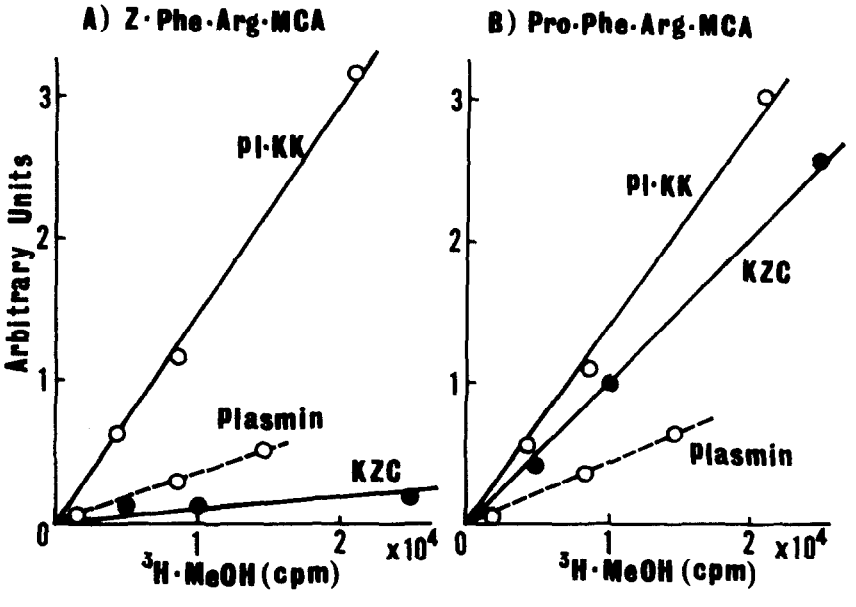

FIG. 1

Specificity of the substrates compared with TAME.

Ordinate: Amount of released AMC, shown as arbitrary unit (10^{-7} M AMC/10 min = 1 unit). Substrates used were Z-Phe-Arg-MCA (A) and Pro-Phe-Arg-MCA (B). Abscissa: Counts of ^{3}H-MeOH released from ^{3}H-TAME. pl-KK: Partially purified human plasma kallikrein. KZC: Highly purified hog pancreatic kallikrein.

30 min and remained unchanged for 2 hrs. At 1 : 10 dilution, however, maximum formation was somewhat lower and the kallikrein formed was unstable. When the concentration of kaolin was varied from 1.0 mg/ml to 0.5 mg/ml little difference could be seen in the formation of kallikrein. In the following studies a 1 : 20 dilution of plasma and kaolin at 1.0 mg/ml were used routinely.

Dose-response curve with Fletcher trait plasma or heated plasma: When Fletcher trait plasma (deficient in prekallikrein) was assayed for prekallikrein it, as expected, formed no detectable kallikrein (Fig. 3). When this deficient plasma was mixed with varying proportions of normal plasma the prekallikrein content of the normal plasma could readily be detected. The lower concentrations of normal plasma activated more slowly but all dilutions reached a maximum in 60 min. When the data at 60 min were replotted as shown in

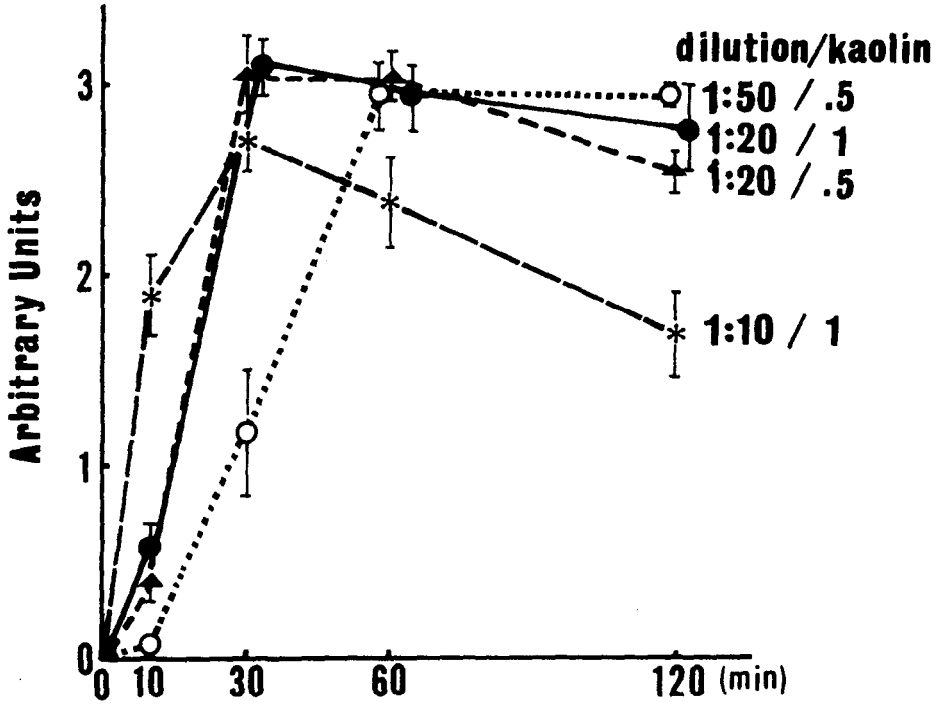

FIG. 2

Effect of the dilution of plasma and the amount of kaolin on the activation of prekallikrein.

Abscissa: Time after kaolin addition (min). At the time indicated, 10, 30, 60 and 120 min after kaolin addition, aliquots from the mixture (50 µl from 1 : 50 dilution, 20 µl from 1 : 20 and 10 µl from 1 : 10) were taken and incubated with the substrate solution as described in the text. The fluorescence of released AMC was measured and expressed as arbitrary units on the ordinate (10^{-7} M AMC/10 min = 1 unit). Each point shows the mean of 4 - 7 samples with standard error in vertical bar. Plasma concentrations are shown on the right hand side of each curve with kaolin concentration, 0.5 or 1 mg/ml.

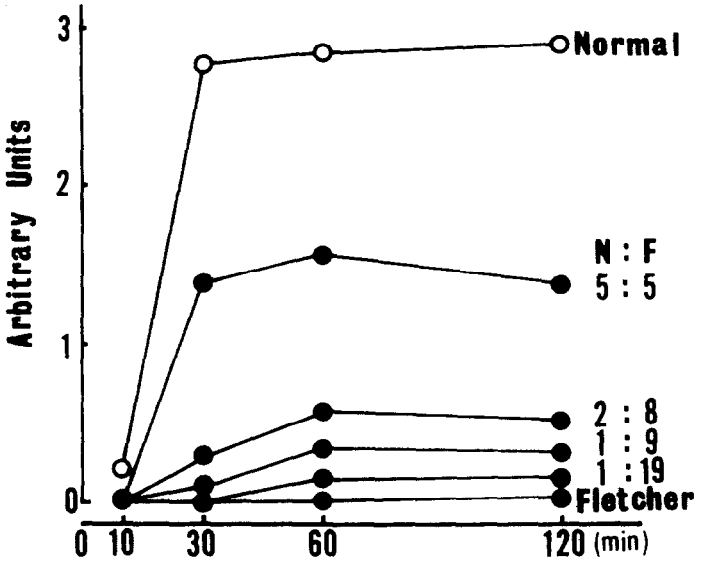

FIG. 3

Prekallikrein activity of the mixture of normal and Fletcher trait plasmas in various ratios.

Abscissa and Ordinate are the same as in Fig. 2. Ratios of normal (N) and Fletcher (F) plasmas in the mixtures are shown on the right hand side of each curve.

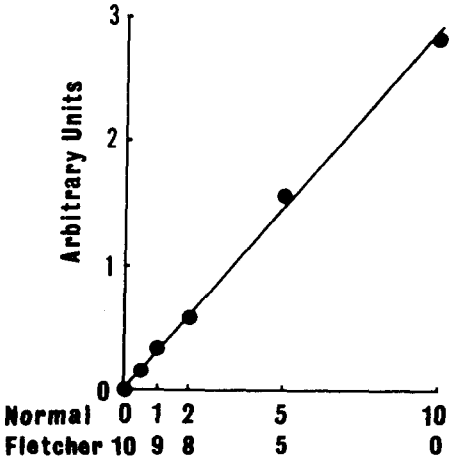

FIG. 4a

A dose-response curve of prekallikrein using Fletcher trait plasma.

Abscissa indicates the ratios of the mixtures of normal and Fletcher plasmas. The values of activities at 60 min in Fig. 3 were replotted on a dose-response curve.

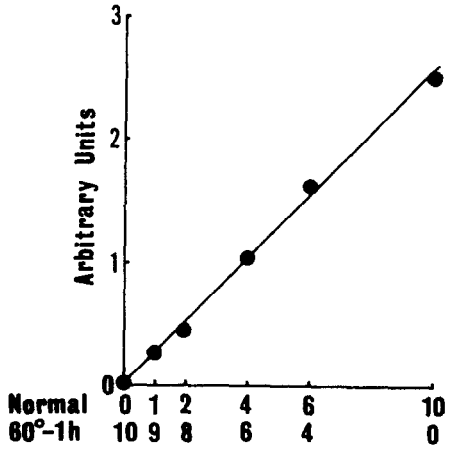

FIG. 4b

A dose-response curve of prekallikrein in using heated plasma.

Abscissa indicates the ratios of normal and heated plasmas (60°, 1 h). The activities at 60 min are plotted against the ratios of normal to heated plasma.

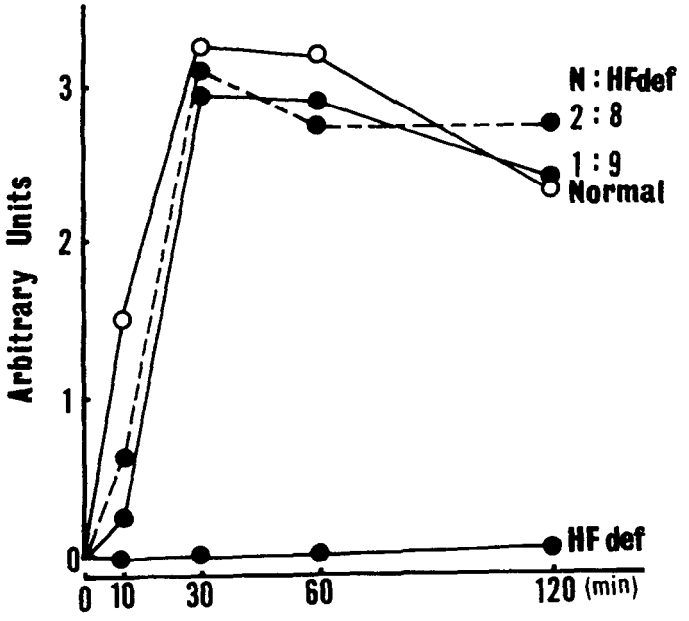

FIG. 5

Effect of Hageman factor contents on prekallikrein activation.

Abscissa and ordinate are the same as in Fig. 2. Normal plasma (N) and Hageman factor deficient plasma (HFdef) are mixed in various ratios shown on the right hand side of each curve.

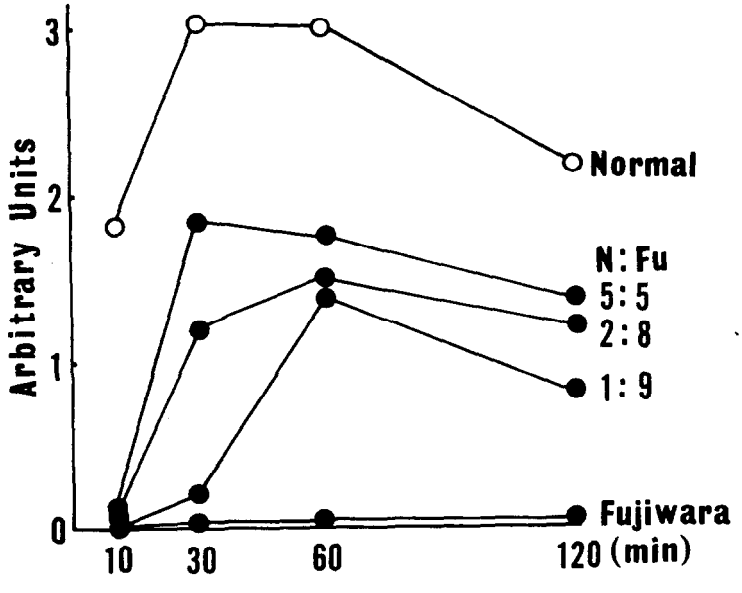

FIG. 6

Effect of HMW-kininogen contents on prekallikrein activation.

Abscissa and ordinate are the same as in Fig. 2. The ratios of normal plasma (N) and Fujiwara trait plasma (Fu) are shown on the right hand side of each curve.

Fig. 4a, a linear relationship was obtained between the content of prekallikrein in normal plasma and hydrolysis of Z-Phe-Arg-MCA. Normal human plasma heated at 60°C for 1 hr, like Fletcher trait plasma, formed no prekallikrein (Fig. 4b) and mixtures of this heated plasma with normal plasma formed kallikrein at levels which were proportional to the content of prekallikrein in the normal plasma.

Requirements of other factors for activation of prekallikrein: Hageman factor deficient plasma, like prekallikrein deficient plasma, formed no active kallikrein. However, mixtures of this plasma with normal plasma (Fig. 5) could easily activate the prekallikrein and it was apparent that only 10% of the Hageman factor in normal plasma is sufficient to activate the prekallikrein found in Hageman factor deficient plasma.

In a similar manner, plasma deficient in HMW-kininogen (Fujiwara trait) (10) was unable to activate its prekallikrein. Again replacement of 10% of normal plasma could completely activate the prekallikrein content of Fujiwara trait plasma (Fig. 6). From these data the content of prekallikrein in this deficient plasma was calculated as 17 - 38% of normal plasma.

DISCUSSIONS

A method for the assay of plasma kallikrein requires high sensitivity and high specificity as well as a simple procedure for routine studies. The fluorometric assay of the enzyme approaches in sensitivity that of the radiochemical method (8) and its specificity is certainly as great and may even be better since plasmin hydrolyzes Z-Phe-Arg-MCA at a lower rate than TAME. Also the use of SBTI and LBTI (8) may further increase the specificity of the method. However, in our experience with normal human plasma and the amidase method, the addition of LBTI gave only slightly lower values (about 5%) than without the inhibitor and all of the activity we measured during the 60 min period was inhibited with SBTI. However, when the incubation was prolonged for longer than 120 min some amidase activity appeared which was not inhibited by SBTI (i.e. plasma kallikrein-α_2-macroglobulin complex). Also in some patient's plasma a SBTI resistant enzyme has been encountered. As yet no LBTI inhibited enzyme (i.e. plasmin) has been detected and thrombin may be excluded because of its low activity on the amidase substrate.

Normal human plasma heated for 1 hr at 60°C looses its prekallikrein content and this treatment yields a plasma which, like Fletcher deficient plasma, is deficient in prekallikrein. Normal plasma added to these deficient plasma activates prekallikrein but only the prekallikrein content of normal plasma could be detected. On the other hand both Hageman factor and HMW-kininogen were required for the activation of prekallikrein, since plasma deficient in these components failed to form active kallikrein. However, when mixed with normal plasma it could be clearly shown that the addition of 10% of normal plasma was sufficient to activate the prekallikrein in these plasmas. Plasma deficient in Hageman factor contains normal levels of prekallikrein. However the plasma deficient in HMW-kininogen (Fujiwara trait) which is known to be deficient in both high and low molecular weight kininogens (10) also had a decreased level of prekallikrein --- a finding which is in agreement with those of other investigators (11,12), who also found a lower concentration of prekallikrein in patients deficient in HMW-kininogen. Further, although data was not shown here, when Fujiwara plasma and Fletcher trait plasma was mixed (1 : 1), kallikrein activity generated by this method was 15.5% of the normal value. By this value the authors could calculate the content of prekallikrein in Fujiwara trait as 31% of normal plasma.

These results suggest that this assay can be applied to the measurement of prekallikrein in deficient plasma or to those obtained under pathological

conditions provided these plasmas are examined in the presence and absence of normal plasma.

ACKNOWLEDGEMENTS

The authors are grateful to Drs. S. Iwanaga and H. Kato, Institute for Protein Research, Osaka University, for valuable discussions and supply of a part of the substrates, and to Dr. M.E. Webster, Escola Paulista de Medicina Brazil, for kind help to preparing this manuscript in English.

This work was supported partly by a Naito Research Grant for 1976 (76-108) and by Scientific Research Grants from the Ministry of Education, Sciences and Culture (310507).

REFERENCES

1. ERDOS, E.G. and WILDE, A.F. Handbook of Experimental Pharmacology vol 25, Bradykinin, Kallidin and Kallikrein. Springer-Verlag, Berlin, New York, 1970.

2. PISANO, J.J. and AUSTEN, K.F. Chemistry and Biology of the Kallikrein-kinin System in Health and Diseases. Fogarty International Center Proceedings No. 27. NIH Pub. Bethesda, Maryland, 1974-76.

3. HOUGIE, C. Hemophilia and related conditions — Congenital deficiencies of prothrombin (factor II), factor V, and factors VII to XII. In: Hematology. W.J. Williams, E. Beutler, A.J. Erslev, and R.W. Rundles (Eds.) New York, McGraw-Hill, 1977, p.1404.

4. COLMAN, R.W. and BAGDASARIAN, A. Human kallikrein and prekallikrein. In: Method in Enzymology vol. XLV part B. Proteolitic Enzymes. L. Lorand (Ed.) New York and London, Academic Press. 1976, p.303.

5. SVENDSEN, L., BLOMBACK, B., BLOMBACK, M., and OLSSON, P.I. Synthetic chromogenic substrates for determination of trypsin, thrombin and thrombin-like enzymes. Thrombosis Res. 1, 267, 1976.

6. MORITA, T., KATO, H., IWANAGA, S., TAKADA, K., KIMURA, T., and SAKAKIBARA, S. New fluorogenic substrates for a α-thrombin, factor Xa, kallikreins and urokinase. J. Biochem. 82, 1495, 1977.

7. WEBSTER, M.E., BEAVEN, V.H., NAGAI, Y., OH-ISHI, S., and PIERCE, J.V. Interaction of Hageman factor, prekallikrein activator and plasmin. Life Sci. 13, 1201, 1973.

8. IMANARI, T., KAIZU, T., YOSHIDA, H., YATES, K., PIERCE, J.V., and PISANO, J.J. Radiochemical assays for human urinary, salivary and plasma kallikreins. In: reference 2. p.205.

9. OH-ISHI, S. and WEBSTER, M.E. Vascular permeability factors (PF/Nat and PF/Dil) — Their relationship to Hageman factor and the kallikrein-kinin system. Biochem. Pharmacol. 24, 591, 1975.

10. HAYASHI, H., KOYA, H., KITAJIMA, K., KIMURA, I., KATORI, M., and OH-ISHI, S. Fujiwara trait: Kininogen deficiency in identical Japanese female twins. Proceedings of XVII Congress of the International Society of Hematology, (July 26-29, 1978, Paris) p.517.

11. SAITO, H., RATNOFF, O.D., WALDMANN, R., and ABRAHAM, J.P. Fitzgerald trait. Deficiency of a hitherto unrecognized agent, Fitzgerald factor, participating in surface mediated reactions of clotting, fibrinolysis, generation of kinins, and the property of diluted plasma enhancing vascular permeability (PF/Dil). J. Clin. Invest. 55, 1082, 1975.

12. COLMAN, R.W., BAGDASARIAN, A., TALAMO, R.C., SCOTT, C.F., SEAVEY, M., GUIMARAES, J.A., PIERCE, J.V., and KAPLAN, A.P. Williams Trait Human kininogen deficiency with diminished levels of plasminogen proactivator and prekallikrein associated with abnormalities of the Hageman factor-dependent pathways. J. Clin. Invest., 56, 1650, 1975.