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ASSAY OF FACTOR XII IN HUMAN PLASMA USING PREKALLIKREIN OR THE CHROMOGENIC PEPTIDE S-2222 AS SUBSTRATES - SIGNIFICANCE OF THE FUNCTIONAL STATE OF PLASMA KALLIKREIN

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ABSTRACT

Factor XII was assayed in acetone-treated and kaolin-activated human citrated plasma (total plasma dilution 1.0 + 0.3 v/v during activation with kaolin, 1.8 mg/ml incubate). Measurements were performed with the tetrapeptide Bz-Ile-Glu-Gly-Arg-pNA (S-2222) and with prekallikrein as substrates. The correlation of both methods to another S-2222 based method recently described was good, r = 0.90 and 0.85 for the two methods respectively. Under the assay conditions used, FXIIa was present as a S-2222 amidase that could be blocked by corn inhibitor, whereas plasma kallikrein was found to be present partly as an amidase blocked by a low concentration of soybean trypsin inhibitor, and partly in a functional state not inhibited and adding to the measured level of FXII. The presence of benzamidine 0.7 to 2.1 mM during acetone treatment increased the measured level of FXII assayed both as prekallikrein activator and as S-2222 amidase.

INTRODUCTION

Determination of factor XII (FXII) in human plasma by means of chromogenic peptide substrates can be carried out either by direct measurement of activated factor XII (FXIIa) (1, 2), or by assay of the kallikrein generated by FXIIa in plasma (3, 4, 5) or in purified prekallikrein (PK) (6, 7). The present report describes measurement of FXIIa in acetone-treated and kaolin-activated plasma, applying both a direct and an indirect method with the tetrapeptide Bz-Ile-Glu-Gly-Arg-pNA (S-2222) and PK as substrates respectively. The plasma samples examined were also assayed by another S-2222 based direct method recently described (1, 2). In a previous study Hoem and Briseid (8) found that the presence of benzamidine during the acetone-treatment of plasma, significantly increased the yield of kaolin-activated FXIIa

Key words: Factor XII-assay, prekallikrein activator, S-2222 amidase

assayed as prekallikrein activator (PKA). In the present work it was investigated whether such an increase could also be observed in connection with the S-2222 substrate.

MATERIALS AND METHODS

Materials: Benzamidine hydrochloride, TCI-grade, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan. Methylamine hydrochloride, purum, Fluka AG, Buchs, Switzerland. EDTA·2Na (Titriplex) and Triton X-100, E. Merck AG, Darmstadt,W.-Germany. SP-Sephadex C-50 and Protein A-Sepharose CL-4B, Pharmacia AB, Stockholm, Sweden. Anti-human IgG (gamma-chain specific)-Agarose, Sigma Chem. Co., St. Louis, MO., U.S.A. The peptide substrates H-D-Pro-Phe-Arg-pNA·2HCl (S-2302) and Bz-Ile-Glu-Gly-Arg-pNA·HCl (S-2222) were from Kabi-Vitrum, Stockholm, Sweden. Kallikrein inhibitor (a soybean trypsin inhibitor preparation) and Kallikrein activator, lyophilized in vials, Channel Diagnostics, Walmer, Kent, England. Corn inhibitor, KabiVitrum, Stockholm, Sweden. Trypsin inhibitor from soybean (SBTI), Type I-S, Sigma Chem. Co., St. Louis, MO., U.S.A. Plasma deficient in both kininogens, GK1603-926B, and plasma deficient in prekallikrein, GK1703-726, George King Biomedical Inc., Overland Park, Kansas City, Kansas, USA. Lyophilized human plasma deficient in FXII, Merz + Dade AG, Dübingen, Switzerland. Chemicals for the casting of polyacrylamide gels, SDS-PAGE standards (40 - 250 KD MW) and Immuno-Blot Assay kit - Goat Anti-Rabbit IgG (H+L) Alkaline Phosphatase Conjugate were from Bio-Rad laboratories, Richmond, CA., U.S.A. SDS, Sigma Chem. Co., St. Louis, MO., U.S.A. Nitrocellulose blotting membrane, Hoefer Scientific Instruments, San Francisco, CA., U.S.A. Antiserum v. Kaninchen gegen Gerinnungsfaktor XII K.-Nr. 750701, Behringwerke AG, Marburg, W.-Germany. Other reagents were of analytical grade.

Purified proteins: High molecular weight kininogen (HMWK) was purified according to Dittman *et al.* (10) and FXII as described by Laake and Österud (11). Prekallikrein prepared according to Laake and Vennerød (9), was further purified by adsorption onto a SP-Sephadex A-50 column (100 mM Na-acetate, pH 5.2 at 4°), elution with a linear gradient of NaCl (0 - 350 mM), and removal of immunoglobulins on an anti-IgG agarose column and a Protein A-Sepharose column operated in tandem (100 mM K-phosphate, 150 mM NaCl, pH 7.2).

Prekallikrein substrate for assay of factor FXIIa (prekallikrein activator, PKA) was partially purified from human plasma (9). Kallikrein occasionally generated during the purification procedure was removed by adsorption to a SBTI-Sepharose 4B column (220 mM phosphate buffer, pH 7.0). The activity in the PK preparation was adjusted to 10 S-2302 U/ml.

Plasma samples were obtained from 10 men (26 to 46 years old) without any known diseases. Especial care was taken to exclude persons with a history of allergy. Blood was collected by clean venepuncture into 100 mM sodium citrate dihydrate solution (one volume of anticoagulant and nine volumes of blood) and centrifuged 15 minutes twice at 3000 x g and 22°. The citrated plasma (CPL) was stored in aliquots fresh-frozen at +70°. The plasma specimens were used within 14 days and once only after thawing.

Acetone-treated plasma (CPLa). CPL was diluted 1.00 + 0.20 with EDTA·2Na in saline to yield 5 mM, rapidly mixed with 0.40 ml acetone and kept at 22° for about 17 hours. The acetone was removed by vacuum evaporation, and loss of water corrected for. Preparations were kept on ice until used.

Acetone-treated plasma with benzamidine was prepared as described for CPLa, but benzamidine was added in the saline to yield 1.0 mM in the preparation.

Photometric measurements of p-nitroaniline (p-NA) released by amidolysis of peptide substrates were performed at 405 nm. Concentrations were calculated from absorbance values using a molar extinction coefficient of 9800 for p-NA. Activities were expressed as U/ml (or mU/ml), one unit being the amount of enzyme that splits one μmole p-nitroanilide per minute. Each assay result stated represents the average value of at least 4 single measurements based on two parallels of CPLa.

Buffer solutions in the assays of FXIIa. A: 50 mM tris, 150 mM NaCl, 0.01% (v/v) Triton X-100, pH 7.9 B: 5 mM phosphate, 150 mM NaCl, 5 mM benzamidine, 0.01% (v/v) Triton X-100, pH 7.4.

Direct assay of FXIIa as S-2222 amidase. At 37° 10 μl kaolin incubate (CPLa mixed 1.0 + 0.1 with a suspension of kaolin 20 mg/ml in saline and kept agitated for 30 minutes at 25°) were diluted with 10 μl buffer A in a polypropylene tube and incubated with 30 μl kallikrein inhibitor (K.I.) solution (one vial dissolved in 10 ml distilled water and diluted 1 + 49 in 50 mM tris pH 7.9), found to be equivalent to about 5 μg/ml SBTI in the incubate. After exactly one minute 100 μl peptide substrate (2 mM) and 150 μl buffer A were added. This mixture was then maintained at 37° for 30 minutes in a temperature controlled shaker. The reaction was stopped by adding 100 μl 50% (v/v) acetic acid. The kaolin was removed by centrifugation at 14.500 x g for 10 minutes, the supernatant diluted 1 + 1 with distilled water and released p-NA measured. Blanks were prepared the same way, but the reagents were mixed in opposite order.

Control experiments without K.I. and experiments in the presence of C.I. were carried out as follows: Fifteen μl kaolin incubate were mixed with 3 μl distilled water or 3 μl corn inhibitor (C.I.) (0.5 mg/ml in distilled water) respectively, and kept at 25° for 5 minutes. Preheated (37°) substrate (100 μl) and buffer A (182 μl) were added, and the subsequent steps carried out as described above.

Indirect assay of FXIIa as PKA. Released kallikrein assayed as S-2302 amidase. Activator preparation: CPLa was mixed 1.0 + 0.1 with a suspension of kaolin 20 mg/ml in saline, kept agitated for 30 minutes at 25° and then diluted 1 + 23 with buffer A. At 25° 80 μl of PK substrate were incubated with 20 μl activator preparation. Aliquots (5 μl) were withdrawn after 20 sec. and after 3 minutes and 20 sec. and added to polypropylene tubes containing 100 μl S-2302 (2 mM) and 195 μl buffer A. The hydrolysis of S-2302 was stopped after 6 minutes by adding 100μl 50% (v/v) acetic acid. The released p-NA was measured and the rate of activation of S-2302 amidase determined. The amount of FXIIa was calculated as PKA-U/ml plasma, one PKA-unit being the amount of activator that activates one S-2302 amidase unit of PK per minute at 25°.

Acetone-treated plasma with benzamidine was used in a modification of the PKA method described. Activator preparation: CPLa with benzamidine was mixed 1 + 1 with a suspension of kaolin 20 mg/ml in saline, kept agitated for 5 minutes at 25° and diluted 1 + 11 with buffer B. At 25° 80 μl PK substrate were incubated with 20 μl activator preparation, and the further assay carried out as described above.

Amidolytic assay of FXIIa according to Gallimore *et al.* was performed as specified in the paper (1). In the method citrated plasma was incubated with acetone, treated with FXII activator and K.I., and FXIIa was assayed as S-2222 amidase.

Immunoblotting. Proteins were separated in a discontinuous polyacrylamide gel (T=10% and C=2.7%) electrophoresis system (with SDS) essentially by the method of Laemmli (12), and electroblotted onto a nitrocellulose sheet (25 mM tris, 192 mM glycine, 20% MeOH). Immunological detection of proteins was carried out using an immunoblot assay kit from Bio-Rad. The blot was washed and quenched and then overlaid with human FXII specific rabbit antiserum (immunoadsorbed with FXII deficient plasma). Bound antibodies were finally detected with alkaline phosphatase-conjugated goat antibodies against rabbit IgG.

RESULTS

Significance of the FXII concentration in the amidase and the PKA assay. Assays were carried out in mixtures of acetone-treated normal citrated plasma (CPL) and acetone-treated CPL congenitally deficient in FXII. Activities registered after activation with kaolin are demonstrated in fig. 1. Curve I shows the total S-2222 amidase activities obtained, whereas curve II shows the activities remaining after elimination of part of the kallikrein activity by incubation with K.I. Not all residual activity could be inhibited by C.I.(FXIIa, curve III). A three times higher concentration of C.I. did not increase the inhibition (data not shown). The difference between curves II and III represents kallikrein activity that was not inhibited. Results in accordance with those demonstrated in fig. 1 were obtained in experiments with FXII-deficient plasma of other origin.

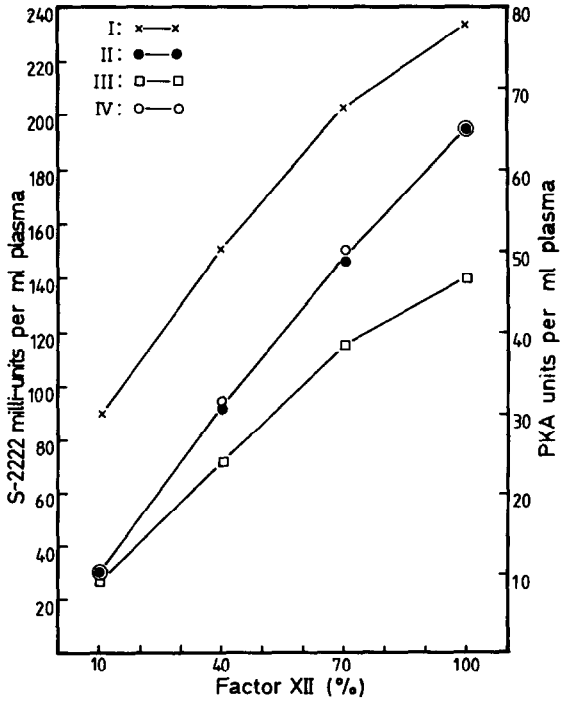


FIGURE 1

Concentration-effect curves for FXII as S-2222 amidase and as prekallikrein activator (PKA). Activities were measured after kaolin incubation in mixtures of acetone-treated normal citrated plasma (CPLa) and CPLa congenitally deficient in FXII.

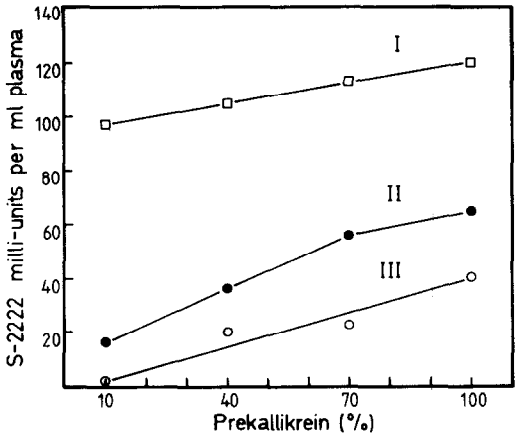
I: Total S-2222 activity
II: S-2222 activity not inhibited by K.I. (FXIIa and part of kallikrein activity)
III: S-2222 activity inhibited by C.I. (FXIIa)
IV: PKA activity

For details see METHODS

FIGURE 2

Concentration-dependence of prekallikrein on the assay of FXII as S-2222 amidase. Activities were measured after kaolin incubation in mixtures of acetone-treated normal citrated plasma (CPLa) and CPLa congenitally deficient in prekallikrein.

I: Activity inhibited by C.I. (FXIIa)
II: Activity not inhibited by C.I. (total kallikrein)
III: Kallikrein activity not inhibited by K.I.



FXIIa was also assayed as PKA in the same mixtures of normal and deficient plasma. The assay values are reflected by curve IV in fig. 1, which was found to coincide with curve II. No PKA activity was left after incubation with C.I. (data not shown). The PKA activities in fig. 1 were scaled by considering the FXII 100% value to be identical with the corresponding value for amidase activity in curve II (residual activity after K.I.), 65 PKA units being equivalent to 195 S-2222 milli-units.

In immunoblot studies CPL and CPLa with or without benzamidine all yielded one major band with an apparent MW about 80 KD, a band which was also present as the main band in purified FXII, and which was absent in FXII deficient plasma (not shown).

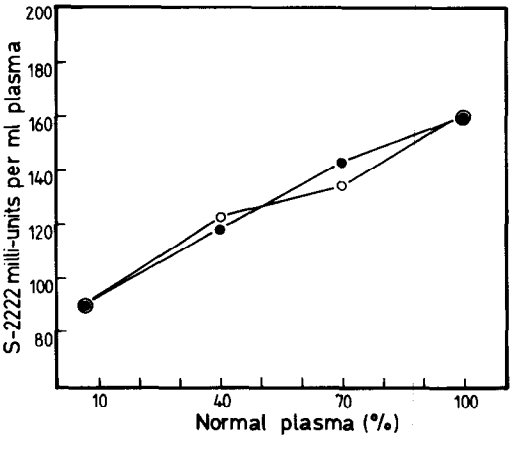
Significance of the PK concentration in the amidase assay. The results of assays carried out in mixtures of acetone-treated CPL and acetone-treated CPL congenitally deficient in PK are shown in fig. 2. Curve I shows the amounts of FXIIa present when measured as the S-2222 amidase activities blocked by C.I. Curve II demonstrates the total of amidase activity left after incubation with C.I., whereas curve III shows the part of this activity that could not be blocked by K.I. The curves indicate that both these fractions of S-2222 amidase activity originate from PK.

FIGURE 3

Concentration-dependence of high molecular weight kininogen on the assay of FXII as S-2222 amidase. Activities not inhibited by K.I. were measured after kaolin incubation in mixtures of acetone-treated normal citrated plasma (CPLa) and CPLa congenitally deficient in HMWK or PK.

● —— ● Normal plasma and HMWK-deficient plasma

O —— O Normal plasma and PK-deficient plasma



Influence of the HMWK concentration in the amidase assay. In assays carried out in mixtures of acetone-treated CPL and acetone-treated CPL congenitally deficient in HMWK, it was found that the registered level of FXII was reduced to an extent closely corresponding to that obtained by reducing PK alone (fig. 3). In addition to the lack of HMWK the plasma used contains only about 30% PK as compared to normal, which means that the mixture of 40% normal plasma and 60% HMWK deficient plasma contained 40% HMWK and 58% PK. The addition of either purified HMWK or purified PK to yield normal levels in such a mixture did not increase the assayed level of FXII. The addition, however, of both substances increased the level of FXIIa inhibited by C.I. to normal (data not shown).

Influence of benzamidine in the amidase and the PKA assay. Assays of FXIIa were carried out in the presence of different concentrations of benzamidine. Fig. 4 shows the results of experiments in which benzamidine was added to acetone-treated CPL prior to the treatment with acetone. Curve I shows that the S-2222 amidase activities were slightly increased by the lower concentrations of benzamidine (0.7 and 2.1 mM in the plasma samples corresponding to 30 and 90 μM in the final S-2222 incubates), whereas the highest concentration (6.0 mM in the plasma sample corresponding to 270 μM in the S-2222 incubate) caused a clear inhibition of the amidase activity present. Factor XIIa was not inhibited by benzamidine. The 40 to 50 % inhibition of the remaining amidase activity reflected a benzamidine effect in accordance with its inhibition of plasma kallikrein (data not shown). As shown in figure 4, curve II, the presence of benzamidine during the acetone treatment caused significant increases in PKA activity over the whole concentration range tested, amounting to 25% at the highest benzamidine concentration. No increase in S-2222 amidase activity or in PKA activity was registered when benzamidine was added after the acetone treatment of plasma (data not shown).

Correlation of chromogenic substrate assays and PK substrate assays. Plasma samples from 10 normal males were assayed for levels of FXII by two different methods based on S-2222 as substrate and two modifications of the PKA method. The results are shown i table 1. Citrated plasma was used throughout, but in method II benzamidine was was added prior to the acetone treatment, corresponding to 1.0 mM in the preparation. The variation in the level of FXII as estimated by the S.D. of the means were roughly the same for the four methods,

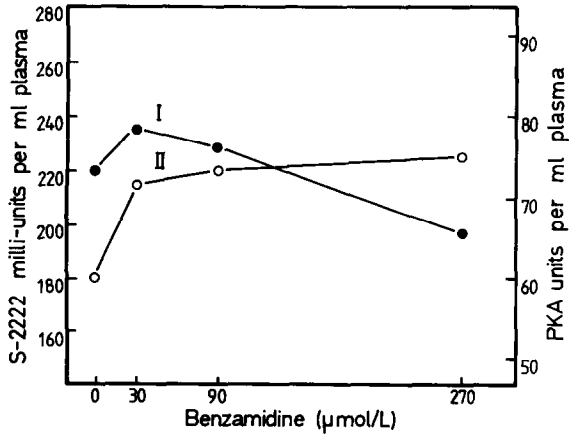


FIGURE 4

Concentration-dependence of benzamidine on the assay of FXIIa as S-2222 amidase and as PKA. Activities after kaolin incubation were measured in normal CPLa to which had been added benzamidine ***prior to*** acetone treatment.

I: S-2222 activity
II: PKA activity

For details se METHODS and legend to fig. 1.

TABLE 1

Correlation of FXII Measurements in Plasma Samples from 10 Normal Males in S-2222 Substrate Assays and in PK Substrate Assays

Method	Substrate	PKA U/ml plasma S-2222 mU/ml plasma ± S.D.	Coefficient of correlation
I	PK	63.8 ± 16.3	I-IV r=0.85
II	PK	71.6 ± 17.8	II-IV r=0.85
III	S-2222	172.2 ± 45.2	III-IV r=0.90
IV	S-2222	148.7 ± 50.9	- -

All methods based on acetone-treated citrated plasma, but in method II benzamidine was added to 1.0 mM before acetone treatment.
Activation: Methods I and III: Kaolin 1.8 mg/ml for 30 minutes at 25°. Method II: Kaolin 10 mg/ml for 5 minutes. Method IV (Gallimore *et al.* (1)): Kallikrein activator for 10 min. at 37°.

ranging from 25 to 34%. The average level of FXII was measured to be slightly higher (13%) in the modification of the PKA method based on CPL with benzamidine added (method II in table 1) than in the method based on CPL only (method I in table 1). The average level of FXII was also found to be somewhat higher (16%) in the S-2222 based method described in the present work as compared to the method of Gallimore *et al.* (1), methods III and IV in table 1 respectively. The level of correlation between method IV (considered as reference) and each of the other three methods was satisfactory and equal ($r \approx 0.85 - 0.90$).

DISCUSSION

This report describes a functional assay for FXII in human plasma based on kaolin activation of acetone-treated plasma, and measurement of the S-2222 amidase activity obtained. Under the chosen conditions the acetone-treatment will remove known inhibitors for FXIIa and kallikrein (7, 13). Only one fraction of the kallikrein activity present was eliminated by use of the low concentration of SBTI present in K.I., found to be equivalent to about 5 μg/ml in the incubate. The remaining amidase activity was found to be composed of FXII, about 2/3, as measured by the inhibition with C.I., whereas 1/3 was resistant to C.I. (kallikrein activity resistant to K.I.). Assays carried out in mixtures of normal plasma and plasma deficient in FXII, showed that the last mentioned activity was not present in the deficient plasma, and it was included in the suggested S-2222 amidase assay. The finding that assays in the plasma mixtures of FXIIa as PKA yielded results in close correspondence with those obtained by the amidase method, underlines the significance of the part of the kallikrein activity resistant to K.I. for the estimation of the plasma level of FXII. An important feature of C.I. is its inability to inhibit plasma kallikrein (14), a fact strongly supported in a recent paper (15). In the present work, assays carried out in mixtures of normal plasma and plasma deficient in PK provided evidence that not only did the S-2222 amidase activity blocked by K.I. originate from PK, but also the

activity neither blocked by C.I. nor K.I. Considered together, our results obtained in mixtures of normal plasma and the two kinds of deficient plasma, indicate a functional correlation between FXIIa and part of the plasma kallikrein present in our preparations. The possibility of such a correlation is supported by results described in a recent publication on the effect on contact activation of a monoclonal anti-human plasma prekallikrein antibody (16). The results provided evidence of a previously unrecognized site on prekallikrein (heavy chain) required for its interaction with factor XIIa, a site sterically related to the active site (light chain).

Experiments in which the level of HMWK was reduced in mixtures of normal plasma and plasma congenitally deficient in HMWK cannot alone evaluate the significance of HMWK for the assayed level of FXII. HMWK-deficient plasmas have been found to contain only about 20 - 30% of normal of PK (17, 18). This also applies to the HMWK-deficient plasma that was employed in this work (8), so that a simultaneous reduction in both plasma factors will take place. The fact, however, that the reduction in the assayed level of FXII was not more extensive in mixtures of normal plasma and HMWK-deficient plasma than it was in corresponding mixtures with PK-deficient plasma, emphasizes the importance of PK. Addition of either purified HMWK or PK to normalize their levels did not increase the assayed level of FXII. When both substances were added together, the level of FXIIa was brought to normal.

Previous results that the presence of benzamidine during the acetone treatment increased the level of FXIIa measured as PKA (8), were confirmed in the present work. We also found that the level of FXIIa assayed as S-2222 amidase was increased at low concentrations of benzamidine, whereas the amidase activity was inhibited at a higher concentration due to inhibition of the kallikrein present.

When plasma samples from 10 healthy males were compared in our S-2222 based method and a S-2222 method recently described by Gallimore *et al.* (1), a good correlation between the two assay procedures was obtained, as shown in table 1. It should be pointed out that the last mentioned method had been found to correlate well with a clotting assay for FXII. In the present report the level of FXII was also assayed by two modifications of a PKA method described previously (8). Both PKA methods showed satisfactory correlation with the reference method (1), but the measured level of FXII as PKA was somewhat higher in the modification based on the presence of benzamidine during the acetone treatment.

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