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Practical application of a chromogenic FXIIa assay

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Abstract

Autohydrolysis of blood factor XII (FXII + FXIIa → 2FXIIa) is found to be a facile reaction in neat-buffer buffer solutions of FXII but an insignificant reaction in the presence of plasma proteins. Autohydrolysis causes a chromogenic assay for FXIIa in buffer solution to strongly deviate from the traditional plasma-coagulation assay. Autohydrolysis can be accommodated by performing chromogenic detection of FXIIa as a rate assay in swamping concentrations of FXII. Rate-assay results performed in this way are shown to be in analytical agreement with the plasma-coagulation assay. Autohydrolysis can be used as a means of amplifying FXIIa produced by contacting neat-buffer solutions of FXII with biomaterials, suggesting a route to highly sensitive measurement of biomaterial hemocompatibility.

Keywords: Blood coagulation; Factor XII; Hageman factor; Autoactivation; Autohydrolysis

1. Introduction

Thrombosis remains a significant barrier to the development and implementation of advanced in-dwelling blood pumps and ventricular assist devices [1,2]. Thrombosis can be usually traced to adverse cell-and-protein interactions with biomedical devices that occur in the acute phase of blood contact [3]. Among these adverse protein-biomaterial interactions, surface-contact activation of blood factor XII is important because this is the first step in a cascade of linked zymogen-enzyme conversions (the intrinsic pathway of blood-plasma coagulation) that ultimately leads to coagulation (clotting) of blood in the presence of artificial materials. Thus, a full understanding of the surface biochemistry leading to FXII activation is critical to the prospective development of biomaterials with improved hemocompatibility.

Contact activation, also termed autoactivation in the hematology literature [4], occurs through a poorly understood contact or binding step with procoagulant surfaces [4,5] that is a matter of continued investigation in our laboratories [6–11]. Our work has substantially relied on a plasma-coagulation-time-assay [9,12] to detect and quantify surface activation. Plasma can also be used to quantify FXIIa by using a calibration curve relating observed coagulation time (CT) to exogenously added FXIIa concentrations (a.k.a. FXIIa titration). Although plasma coagulation offers a number of distinct advantages as a traditional-hematology assay for FXIIa (simple, reproducible, sensitive, biomedically relevant, etc.), it does not directly detect FXIIa but rather measures the response of the whole coagulation cascade to an exogenous FXIIa bolus that presumably includes kallikrein amplification [6]. Also, there is the unavoidable problem of lot-to-lot variability inherent in donated blood.

Commercial chromogenic assays, first introduced in the mid 1980s [13,14], are an alternative to plasma coagulation that offer direct and specific detection of various coagulation factors such as FXIIa and FXIIf [15]. In the process of applying a chromogenic assay in our studies of FXII activation *in neat buffer solutions*, we discovered that autohydrolysis (FXII + FXIIa → 2FXIIa) is an important contributor to signal that must be properly accounted for to obtain agreement with traditional plasma-coagulation

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assays in which autohydrolysis is apparently insignificant. The outcome is an improved chromogenic-assay protocol that has utility in measuring activation properties of biomaterials [15–19].

2. Methods and materials

2.1. Plasma and coagulation proteins

Human platelet poor plasma (PPP) was prepared from outdated (within 2 days of expiration) lots obtained from the M.S. Hershey Medical Center Blood Bank. This work was performed with a single lot of pooled plasma aliquoted into 15 mL polypropylene tubes (Falcon, Becton Dickinson) and frozen at −20 °C until use. We observe consistent results with plasma prepared and stored in this manner over about 1 year of experimentation. Factor XII depleted plasma (12dPPP) with a dysfunctional contact activation system was used as received from George King Biomedical, Inc., Overland Park, KS. 12dPPP was optionally reconstituted with variable FXII concentrations (R12dPPP) with the same FXII used to prepare neat-buffer solutions. Experience has shown that different lots of plasma yield quantitatively different but qualitatively similar results. Human FXII and FXIIa were used as received from Haematologic Technologies, Inc. Essex Junction, VT and Enzyme Research Laboratories, South Bend, IN, respectively. FXIIa activity was measured in Plasma Equivalent Units, PEU/mL [14]. CT of recalcified R12dPPP supplemented with 30 μg/mL FXII (physiologic concentration [20,21]) exceeds 40 min, demonstrating that FXII was not contaminated with measurable quantities of FXIIa (estimated to be $\sim 10^{-3}$ PEU/mL based on the standard deviation of multiple FXIIa measurements [22]).

2.2. Plasma coagulation-time assay

The basic protocol for the CT assay has been described in detail elsewhere [7,9–11] and applied in this work as a "FXIIa titration" calibration curves that relate CT to exogenously added FXIIa concentrations. FXIIa titrations were carried out by equilibrating 0.5 mL of thawed plasma in 15 × 75 mm polystyrene tubes (VWR) and diluting with 0.4 mL of FXIIa solution in PBS at concentrations ranging from 0 to 5 PEU/mL. Coagulation was induced by recalcification with 0.1 mL of 0.1 M $CaCl_2$ and contents were mixed on a slowly turning hematology mixer (Roto-shake Genie, Scientific Industries, Inc.). CT after recalcification was noted by a distinct change in fluid-like rheology to gel formation, allowing determination of the end point of the coagulation process to within 10 s or so [9]. CT was observed to vary sharply with FXIIa concentration (see Fig. 1) but yield linear calibration curves on logarithmic [FXIIa] axes [22], where [FXIIa] is FXIIa concentration in PEU/mL.

2.3. Chromogenic assay

Amidolytic activity of FXIIa was measured using a chromogenic substrate s-2302 (H-D-Pro-Phe-Arg-pNA··· 2HCl, MW = 611.6 used as received from Diapharma Group Inc., Columbus, OH). The chromogenic assay was used to quantify FXIIa for comparison to plasma-coagulation assays. A 4.0 mM stock solution of the chromogen was prepared in 18 MΩ water (obtained from a Millipore Simplicity Unit) and stored at 2–8 °C for no more than 6 months. A 0.4 mM working solution was prepared by diluting the stock solution 10-fold with buffer (0.05 M Tris/Hcl, 0.012 M NaCl, 0.003 M EDTA, PH 7.8). The actual FXIIa assay was performed by mixing 450 μL of working solution with 150 μL of test solution in a ultra-microcuvette (VWR) held in the chamber of an automatically recording UV-VIS spectrophotometer (DU® Series 500, Beckman Coulter, Inc., Fullerton, CA). Absorbance change at 405 nm was recorded for 5 min to determine the initial velocity of color development, as further described in the Results section. FXIIa concentrations of unknown solutions were determined by reference to a FXIIa titration curve in which known concentrations of FXIIa were mixed with working solution to determine initial velocities.

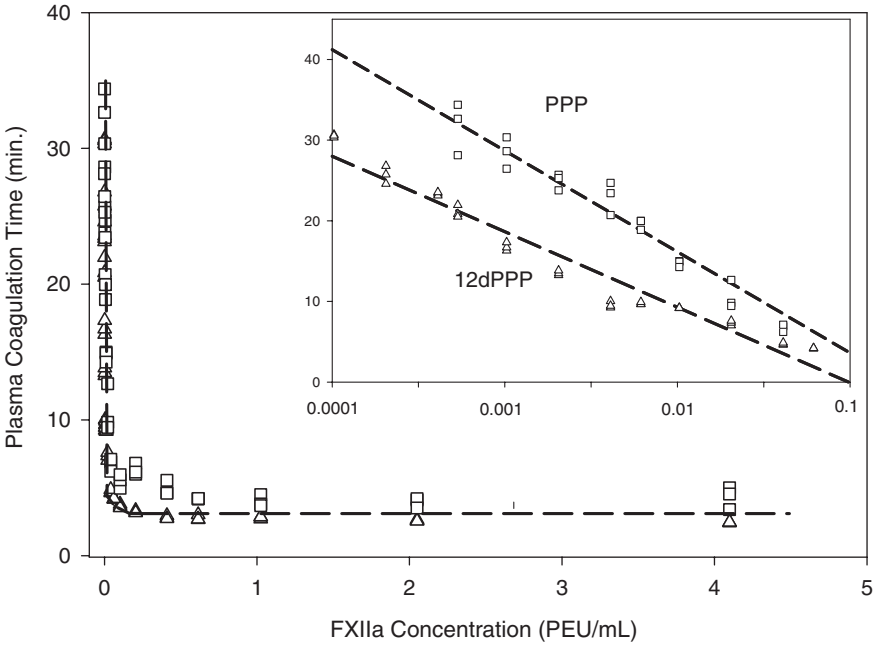

Fig. 1. FXIIa titration of normal platelet-poor plasma (PPP, squares) and FXII-depleted plasma (12dPPP, triangles). Inset plots data on log-normal axes and serves as a calibration curve relating observed plasma coagulation time to FXIIa concentrations. The curve drawn through the data of the main figure is a guide to the eye whereas lines through data of the inset are the result of linear-least-squares regression through the data interval shown; (12dPPP: $CT = (-9.36 \pm 0.35)[FXIIa] - 9.45 \pm 0.96$, $R^2 = 95.4\%$; PPP: $CT = (-12.50 \pm 0.53)[FXIIa] - 8.95 \pm 1.22$, $R^2 = 95.7\%$).