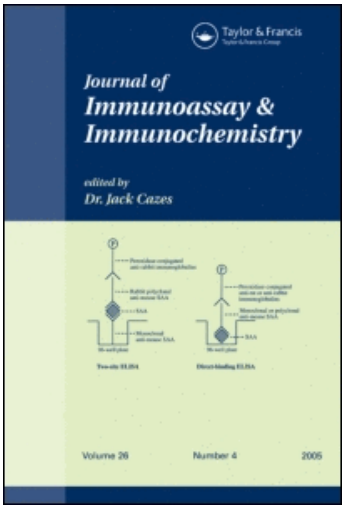

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Myrset, Astrid Hilde and Helgeland, Liv(1991) 'A Novel Enzyme Immunoassay for Quantitation of Rat Prothrombin in Microsomal Subfractions', Journal of Immunoassay and Immunochemistry, 12: 4, 597 — 609
To link to this Article: DOI: 10.1080/01971529108053282
URL: http://dx.doi.org/10.1080/01971529108053282

JOURNAL OF IMMUNOASSAY, 12(4), 597-609 (1991)

A NOVEL ENZYME IMMUNOASSAY FOR QUANTITATION OF RAT PROTHROMBIN IN MICROSOMAL SUBFRACTIONS.

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ABSTRACT

An enzyme linked immunoassay (ELISA) for quantitation of rat prothrombin, based on a biotin-streptavidin alkaline phosphatase system is described. The assay utilizes rabbit antiserum raised against purified rat prothrombin. The assay was twenty fold more sensitive than a rat prothrombin assay based on amidolytic activity following activation by Echis carinatus venom. Results obtained with the two assays show good correlation. The ELISA is a valuable tool for quantitation of minute amounts of prothrombin in subcellular fractions and large series of plasma samples.

(KEY WORDS: ELISA, prothrombin purification, antiserum, IgG-purification, amidolytic activity).

INTRODUCTION

Rat prothrombin is a plasma protein with a m_r of 75-77 000 (1) and with a concentration of approx. 0.06 mg/ml in plasma (2). Prothrombin is modified during biosynthesis by a vitamin K-dependent carboxylation at

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about ten glutamic acid residues in the N-terminal region of the protein (3). The resulting gamma carboxy glutamic acid residues, are responsible for the ability to bind calcium ions, necessary for the biological activity of prothrombin. The ability to bind divalent cations, is frequently used for isolation of prothrombin from different species (1, 4). During blood coagulation, prothrombin is cleaved to the biologically active α-thrombin, which finally cleaves fibrinogen to fibrin. Traditional methods for quantitation of prothrombin make use of this enzymatic activity, in various assays based on coagulation- or amidolytic activity (5).

Here we describe an ELISA for quantitation of rat prothrombin. The sensitivity of the assay makes it valuable for quantitation of minute amounts of prothrombin, and the assay is further a tool for correlation between immunologically and amidolytically active prothrombin precursors in subcellular fractions.

MATERIALS AND METHODS

Materials

$BaSO_4$ was obtained from Baker, D-biotinyl- ε -aminocaproic acid N-hydroxy-succinimide ester and

Streptavidin-alkaline phosphatase conjugate from BRL (Bethesda Research Lab.), p-nitrophenylphosphate from KPL (Kirkegaard & Perry Lab. Inc.) and microtiterplates MaxiSorp with certificate from Nunc. Echis carinatus venom was from Sigma, and substrate TH-1 (D-cyclohexylglycyl-L-alanyl-L-arginine-p-nitroanilide dihydroacetate) from Nycomed AS, Norway.

Animals

Male wistar rats, 240-280 g from Möllegaard, Havrup, Denmark, were used. The rats were fasted prior to experimental use.

Purification of Rat Prothrombin for Preparation of Antiserum

Oxalated rat plasma (0.015 M K-oxalate) was centrifuged for 5 min to remove insoluble material, and heparin was added to 1 I.U./ml plasma. The plasma was adsorbed on $BaSO_4$, 35 mg/ml, gently mixing for 1 hour. After centrifugation the unadsorbed material was removed, and the $BaSO_4$ with adsorbed prothrombin was washed twice with 0.15 M NaCl/0.1 M K-oxalate and once with 1.15 M

NaCl. Adsorbed material was eluted twice by 0.2 M Na-citrate, mixing for 30 min, in a total volume 1/10 of the original plasma volume. Each centrifugation was carried out at 4300 x g for 20 min. The combined eluates were centrifuged at 20 000 x g for 5 min, to remove $BaSO_4$, and dialyzed overnight against 0.05 M imidazole-HCl/0.001 M benzamidine-HCl/0.1 M NaCl (pH 6.2). Prothrombin was further purified by anion exchange chromatography on FPLC, Mono Q column (Pharmacia), with a nonlinear gradient: 0.1-0.32 M NaCl in 10 ml, 0.32-0.40 M in 10 ml and 0.40-0.45 M NaCl in 3 ml. The prothrombin peak eluted at approx. 0.36 M NaCl. Prothrombin was finally purified by preparative electrophoresis on a 5 % polyacrylamide gel during non-denaturating conditions, and eluted from the gel in 0.9 % NaCl. The entire procedure was carried out at 4 ^{o}C. From A_{280}, the recovery was estimated to be 1.35 mg purified prothrombin from 25 ml rat plasma. Antiserum was raised in rabbit by five subcutaneous injections at 14 days intervals. 100 μg prothrombin emulsified with Freunds complete adjuvant was injected in the primary injection, and 50 μg prothrombin with Freunds incomplete adjuvant in the subsequent injections. The serum was taken two weeks after the last injection, and

stored at -20 °C. The specificity of the antiserum was verified by immunoelectrophoresis. By immunoprecipitation in solution, 100 µl antiserum was sufficient to precipitate prothrombin from 50 µl rat plasma.

Purification of IgG, and Conjugation with Biotinyl Ester.

The antiserum was initially precipitated by ammonium-sulphate (50 %) on ice for 45 min. The precipitate was solubilized in 0.005 M sodium-phosphate buffer (pH 7.6), and dialyzed against the same solvent overnight, at 4 °C. IgG was further purified by an anion exchange chromatography on DEAE-Sephacel equilibrated with 0.005 M sodium phosphate buffer (pH 7.6), and eluted by a linear gradient 0.005-0.05 M sodium phosphate. The fractions containing primarily IgG as judged from SDS-polyacrylamide gel electrophoresis, were chosen for use in the ELISA. 0.2 ml biotinyl ester (1 mg/ml dimethyl-sulphoxide) was added to 1 ml IgG of approx. 1 mg/ml. After 2 hours at room temperature the sample was dialyzed overnight at 4 °C, against PBS (1.47 mM KH_2PO_4/2.68 mM KCl/6.46 mM $Na_2HPO_4 \cdot 2H_2O$/0.137 M NaCl, pH 7.4), and stored at -20 °C.

ELISA for Determination of Prothrombin

Each sample was added in a volume of 100 µl. The microtiterplate was coated with antibody at a concentration of 2.5 µg protein/ml in 0.05 M sodium carbonate buffer (pH 9.5), in a humid chamber at 4 ^{o}C overnight. The wells were blocked with PBS-Tween (0.05 %, v/v) at 37 ^{o}C for two hours, before addition of antigen sample in PBS-Tween.. Between each addition, the wells were washed five times with PBS-Tween. The antigen-antibody reactions proceeded for 1 hour at 37 ^{o}C. The third layer in the sandwich consisted of biotinylated antibody in PBS-Tween at a concentration of 1.5 µg/ml. The enzyme used was alkaline phosphatase conjugated with streptavidin. The biotin-streptavidin reaction was carried out during 15 min incubation at room temperature. The wells were finally washed, before addition of p-nitrophenylphosphate, 1mg/ml in 1.0 M diethanolamine buffer/0.5 M $MgCl_2$ (pH 9.8). The colour evolved during 30-45 min at 37 ^{o}C, and was measured at A_{410} in a Dynatech MR 700 microplate reader. Normal plasma (pooled plasma from six normal rats) in dilutions from 1/3125 to 1/50 000, corresponding to approximately 2×10^{-9} to 1.2×10^{-10} g prothrombin/well, was used as standard.

Amidolytical Determination of Prothrombin

Prothrombin was determined amidolytically essentially as described by Tollersrud and Helgeland (1986). 500 µl 8mM veronal-HCl/0.13 M NaCl pH 8.4, and 25 µl Echis carinatus venom (50 µg/ml) was added to 100 µl sample diluted in the same solvent containing 0.1 % Triton X-100 (w/v). After incubation at 37 ^{o}C for 5 min, 50 µl of 2 mM TH-1 were added, and the mixture was incubated for 15 min. The reaction was stopped by 0.4 ml 40 % acetic acid, and A_{405} was measured. Normal plasma dilutions from 1/500 to 1/2000 were used as standard.

Preparation of Rough Microsomes

Rough microsomes and microsomal subfractions were prepared as described (6). For solubilization, microsomes from 5 g of liver were resuspended in 2.5 % Triton X-100 (w/v) for 30 min, and centrifuged at 100 000 x g for 90 min to remove the ribosomes and non-solubilized membrane fragments.

RESULTS AND DISCUSSION

The aim of this study was to develop a sensitive assay to quantitate minute amounts of prothrombin in sub-

cellular fractions from rat liver. During subcellular fractionation and membrane solubilization, it is impossible to avoid considerable dilution of the fractions. As the concentration of prothrombin in solubilized rough microsomes is about 0.3 μg/ml, the need for a sensitive assay is obvious. The amidolytical assay for determination of total prothrombin (4) can measure prothrombin in this range of concentration. During subfractionation of rough microsomes, however, the concentration of prothrombin will be further reduced, and a more sensitive assay is required.

It was necessary to optimize the ELISA with respect to concentrations of coat and conjugate IgG. Recommended concentrations of coat are 2-10 μg/ml. Concentrations chosen were therefore 2.5, 5.0 and 10.0 μg/ml of coat IgG, and 1.5, 3.0, 4.5 and 6.0 μg/ml of antibody conjugated with biotin. The optimal combination was defined as that resulting in maximal signal-/noise ratio. The "noise" was defined as the absorbance in a well where the antigen was substituted with PBS-Tween (=sample blank). 1/50 000 dilution of normal rat plasma after subtraction of the sample blank, was arbitrarily chosen as the "signal". There was a clear tendency for both increased blank, and decreased signal/noice ratio, with increasing concentrations of the coat IgG. This was even more apparent with

increased concentration of the conjugate, although the numerical values might differ somewhat between different experiments. The concentrations chosen for coat and conjugate, were therefore 2.5 and 1.5 μg/ml, respectively. This coat concentration is somewhat higher than the 0.5-1.0 μg/ml chosen by Schwerer et al. (7) for quantitation of albumin. A blocking step by 3 % defatted dried milk in PBS-Tween was tried. The results obtained were very similar to those obtained with PBS-Tween, but with a slight reduction in all signals. The signal/noise ratio was not improved.

It was necessary to compare the typical rat plasma standard curve to that from solubilized rough microsomes to ensure that the intracellular prothrombin precursor behaved as normal plasma prothrombin in our assay. Fig. 1 shows that there is excellent correspondence between different standard curves, obtained from diluted rat plasma, solubilized rough microsomes, and purified prothrombin. The signal/noice ratio in the experiment was 6.

The correlation between ELISA determinations and amidolytic determinations after activation by Echis Carinatus venom is shown in Fig. 2. The regression line follows the equation

$$y = 0.995x - 0.671$$

with correlation coefficient $r = 0.910$, which represents a satisfactory correlation.

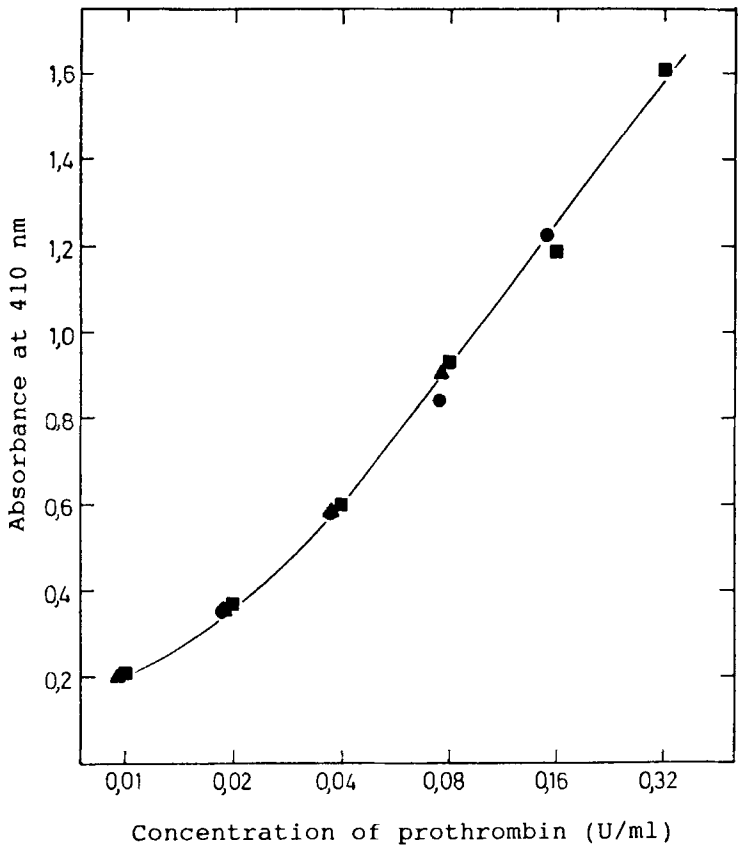

FIGURE 1. Standardcurves for determination of rat prothrombin. Dilutions of normal rat plasma (- ■ -), solubilized rough microsomes (-▲-) and purified prothrombin (-●-). The dilutions are expressed as units prothrombin/ml (U/ml). 1 U/ml is defined as the concentration of prothrombin in normal rat plasma diluted 1/1000.

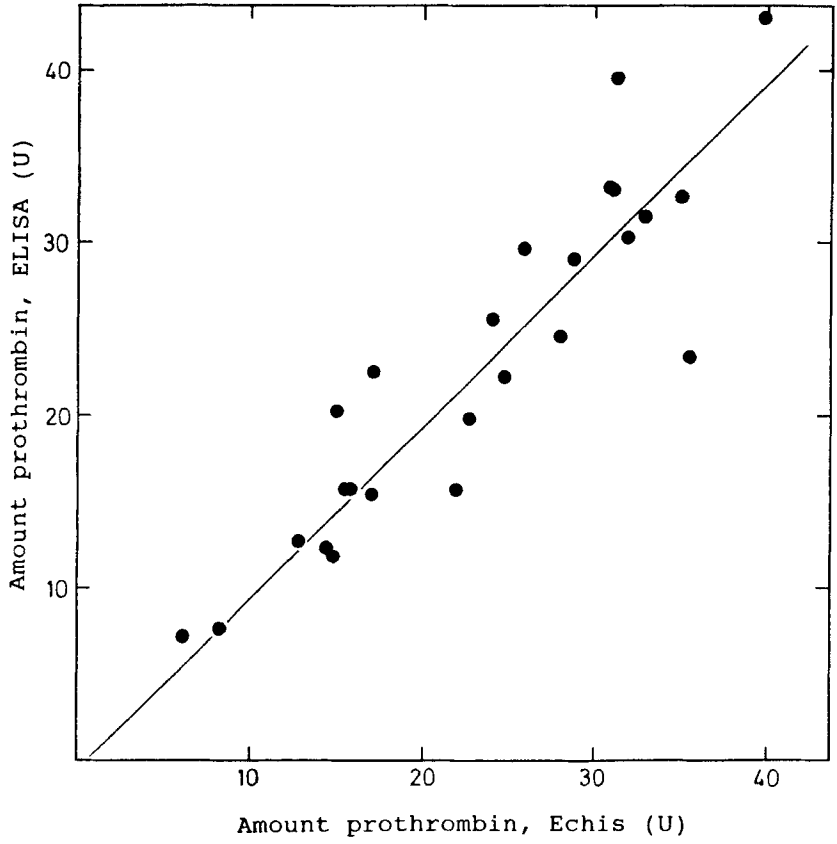

FIGURE 2. Correlation curve for determination of prothrombin in 25 samples from microsomal subfractions according to the amidolytic assay (Echis) and the ELISA. The amount of prothrombin in a subfraction is expressed as units (U). 1 U is the amount of prothrombin in 1 μl normal rat plasma.

A prerequisite for the detection by ELISA, is that the prothrombin precursor has attained sufficient antigenic determinants to be recognized by the antiserum against mature prothrombin. For the amidolytic assay, on the other hand, the prothrombin must be folded to a certain extent, to be cleaved by Echis carinatus venom, and show thrombin activity against the substrate. The correlation between the two assays (Fig. 2), indicate that these properties appear at approximately the same stage in biosynthesis.

It is important to note that the samples used in the immunosorbent assay were diluted 10-20 fold more than samples used in the amidolytic assay. The sensitivity of the ELISA assay is comparable to that of the ELISA for human prothrombin described by Ofosu et al. (8), and is sufficient for our purpose. The assay thus opens the possibility for quantitation of prothrombin in further subfractionated microsomes and other cellular organelles.

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