Ann. Hum. Genet. (1982), **46**, 293–298
Printed in Great Britain

The use of fluorogenic peptide substrates for the detection of coagulation factors II and X after electrophoresis

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SUMMARY

The use of synthetic oligopeptides with a fluorescent detector group for the localization of thrombin and activated factor X activity after electrophoresis is described. This new technique provides high specificity and resolution. This approach to the detection of blood coagulation factors II and X can be extended to other factors, where their specific peptide cleavage sequence is known.

INTRODUCTION

The coagulation of blood is a result of an interdependent sequential series of proteolytic reactions. After an initiating event, each active blood coagulation enzyme activates the subsequent zymogen in the series, resulting eventually in the generation of thrombin and the proteolytic conversion of fibrinogen to fibrin which polymerizes to form the clot. While many of the coagulation factors were discovered through the study of genetically inherited deficiencies, and the last twenty years has seen the biochemical elucidation of the cascade mechanism, the proteins of the coagulation cascade have not been the subject of extensive biochemical genetic investigations. The notable absence of published results in this area appears to be due to the lack of suitable techniques for the study of these proteins by the biochemical geneticists' principal tool, electrophoresis.

Most proteins of the coagulation system have been studied to a limited extent by crossed immunoelectrophoresis, but that method only allows a limited comparison of different samples and, in many cases, exhibits a low degree of resolution. Immunofixation techniques originally described by Alper & Johnson (1969) have been used by Board (1979, 1980) and Board, Coggan & Pidcock (1982) to demonstrate the existence of genetic polymorphisms at the structural loci coding for both factor XIII and prothrombin (factor II). Unfortunately, these immunological tests do not give any indication of the functional activity of any structural variants discovered. Only two methods for the functional detection of coagulation factors after electrophoresis have been described; Bird & Rizza (1975) described a technique which allowed the identification of a zone of factor VIII coagulant activity after electrophoresis by the generation of fibrin in an overlay of factor VIII deficient plasma. Although this technique identified a zone of functional factor VIII, the electrophoretic resolution obtained was not high. Board (1979) described a technique for the functional detection of factor XIII A subunits after electrophoresis. This method allowed high resolution and has been of considerable value for the characterization of inherited factor XIII deficiencies (Board, Coggan & Hamer, 1980; Castle, Board & Anderson, 1981) and in population genetic studies (Board, 1979; Board & Coggan, 1982). Unfortunately that technique can only be applied to the study of factor XIII A subunits.

The recent development of synthetic oligopeptide substrates with chromogenic or fluorescent detector groups for the measurement of proteolytic enzyme activity (Huseby & Smith, 1980) has provided a new strategy for the biochemical genetic study of the coagulation system. We describe here the application of synthetic oligopeptide substrates with a fluorescent detector group for the functional localization of factor II and factor X after electrophoresis. This approach can potentially be utilized for the detection of other coagulation factors.

MATERIALS AND METHODS

Reagents

Two synthetic peptide substrates were used in these studies. Both peptides, Boc-Ile-Glu-Gly-Arg-MCA and Boc-Val-Pro-Arg-MCA were obtained from Peninsular Laboratories, San Carlos, California and contained 7-amino-4 methylcoumarin (MCA) as a fluorescent detector group. A stock solution of each peptide was prepared by dissolving 5 mg of Boc-Ile-Glu-Gly-Arg-MCA or 1 mg of Boc-Val-Pro-Arg-MCA in 0·1 ml dimethylsulphoxide which was then diluted to a final volume of 2 ml with 150 mM-NaCl and stored at −20 °C in small aliquots. Several products were used for the activation or inhibition of factors II and X. Russel's viper venom in cephalin (RVV), factor X activating enzyme (XAE) and hirudin were obtained from Sigma, Saint Louis, Missouri and Australian Taipan venom (TV) (*Oxyuranus scutelatus*) was obtained as desiccated crystals from the Gosford Reptile Park, Gosford, Australia.

Electrophoresis

Electrophoresis was carried out in agarose gels prepared as previously described by Board *et al.* (1982). Plasma samples of approximately 5 μl were placed in sample slots with a micro syringe. Two different buffer systems were used. Buffer A was used for the separation of factor II and the electrode tank buffer contained 186 mM Tris, 530 mM glycine, 31 mM sodium 5,5-diethyl-barbiturate, 5·6 mM barbituric acid and 2 mM calcium lactate. The final pH was 8·8. The gel buffer was a 1-in-4 dilution of the electrode buffer. Electrophoresis was carried out at 26 V/cm until a haemoglobin marker had migrated 5 cm down the gel. Buffer B was used for the electrophoresis of factor X. The electrode buffer contained 100 mM Tris, 100 mM maleic anhydride and 10 mM-$CaCl_2$. The pH was adjusted to 7·4 with NaOH. The gel buffer was a 1-in-10 dilution of the electrode buffer and electrophoresis was carried out at 26 V/cm for one hour. Under these conditions factor X was found to migrate towards the anode at approximately the same rate as a bromophenol blue stained serum albumin marker.

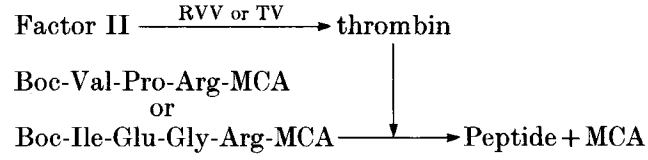
Detection of factors II and X

A reaction mixture was prepared by combining 1 ml of 100 mM Tris/HCl pH 8·5, 0·1 ml 50 mM-$CaCl_2$ and 25 μl of the appropriate peptide substrate stock solution, with various activating agents. Normally RVV was added at a final rate of 2·5 mg/ml, XAE at 25 μg/ml and TV at a rate of 10 μg/ml. The reagent solution was spread evenly over the surface of the gel with a glass rod and the gel incubated in a humid environment at 37 °C for 30–60 minutes. Fluorescent zones of activity were identified by viewing the gel under long-wave UV light. Longer incubation periods may be required if weakly reacting samples are used.

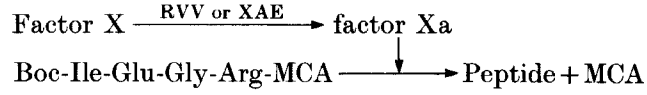
RESULTS

The differential detection of factors II and X can be achieved by the selective use of either different peptide substrates, different activating agents or different anticoagulants. The principle of the detection method described here rests on the recognition of specific amino-acid sequences by the active forms of factor II (thrombin) and factor X (factor Xa) and the subsequent release of free 7-amino-4-methylcoumarin (MCA) which fluoresces strongly in the free form. The reactions required for the detection of factor II or X are given below.

(*A*)



(*B*)



Thus, the detection of factor II or X after electrophoresis requires the application of a suitable specific peptide with MCA linked to a carboxy terminal arginine residue and an agent capable of activating factor II or factor X.

Several agents for the activation of factor II or factor X have been evaluated. Crude RVV contains separate enzymes which are capable of activating either factor and, therefore, can be used for the detection of both. Purified XAE may be used for the specific activation of factor X. In comparison, the venom of the Australian Taipan snake contains a factor II activating agent but has no effect on factor X.

Choice of peptide substrate is another variable to be considered. Boc-Ile-Glu-Gly-Arg-MCA was designed specifically as a substrate for factor X since it duplicates the known sequence in prothrombin which is cleaved by active factor Xa. Although this peptide does act as an excellent substrate for factor Xa, our results indicate it is also a substrate for thrombin. In comparison, Boc-Val-Pro-Arg-MCA reacts readily with thrombin, but is not a suitable substrate for factor Xa.

Further selectivity can also be achieved by the choice of anticoagulant used in the preparation of plasma. Collection of blood on to citrate or EDTA prevents coagulation by removing free calcium, but does not interfere with the functional detection of factors II and X. In comparison, heparin inhibits factor X but has little effect on factor II.

The detection of factor II, after electrophoresis in buffer A and using Boc-Ile-Glu-Gly-Arg-MCA as a substrate, is illustrated in Fig. 1. This Figure shows the position of thrombin activity generated from various factor II variants described previously by Board *et al.* (1982). The plasma samples used in this experiment were collected on heparin and factor II was activated to thrombin by the use of RVV. Figure 1 therefore demonstrates the finding that, although RVV activates factor X and the substrate Boc-Ile-Glu-Gly-Arg-MCA is an excellent substrate for factor Xa, the use of heparin as an anticoagulant makes this detection system specific for factor II. Although not shown here, factor X is not well resolved by electrophoresis in buffer A

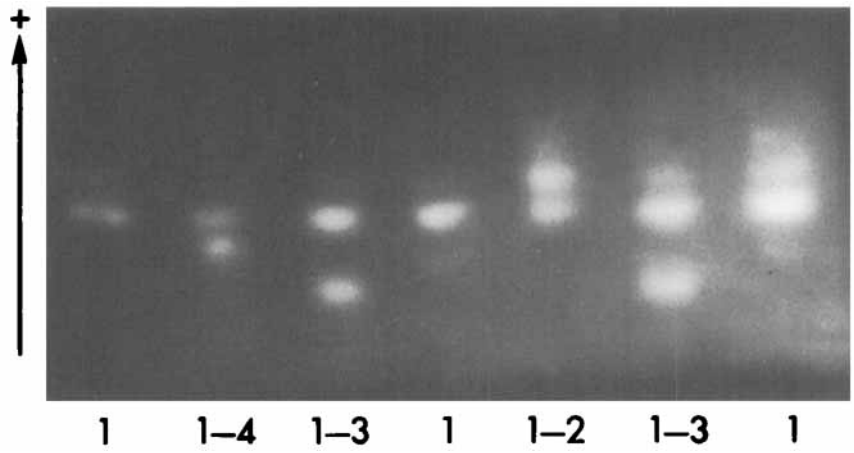


Fig. 1. Electrophoretic phenotypes of factor II in buffer A detected by the reaction of thrombin with Boc-Ile-Glu-Gly-Arg-MCA, as described in the text.

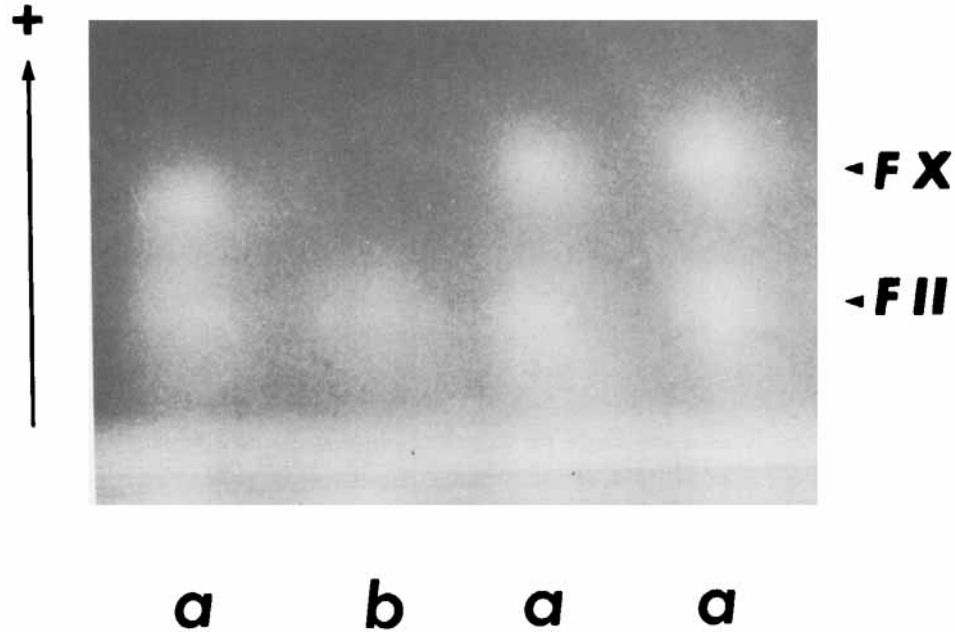


Fig. 2. Electrophoresis of factor II (F II) and factor X (F X) in buffer B. Samples in tracks marked a are normal. The track marked b is deficient in factor X.

and, if citrated samples had been used, a smeared zone of factor Xa activity would be observed slightly anodal to the normal type 1 factor II band.

The detection of both factor X and factor II, after electrophoresis in buffer B and using Boc-Ile-Glu-Gly-Arg-MCA as a substrate, is illustrated in Fig. 2. This Figure shows the relative position of both thrombin and factor Xa activity in plasma samples collected on to citrate. The absence of the anodal zone of activity in a sample from an individual with congenital factor X deficiency clearly indicates that the most anodal of the two zones is that of factor Xa. Factor X can be specifically observed without the development of thrombin activity if highly specific

XAE is used as an activating agent, or if hirudin is added to specifically inhibit thrombin activity.

In general, for the localization of factor II we have obtained the best results by using a combination of Buffer A, heparinized samples, either RVV or TV as activating agents, and Boc-Ile-Glu-Gly-Arg-MCA as a substrate. Although Boc-Val-Pro-Arg-MCA is a far more sensitive and specific substrate for thrombin, in our experience it has proved to be less stable. Acceptable electrophoretic resolution of factor X has been difficult to obtain. The best results were gained using buffer B, citrated plasma samples, either RVV or XAE as activating agents and Boc-Ile-Glu-Gly-Arg-MCA as a substrate.

DISCUSSION

Many of the activated blood coagulation factors are proteolytic enzymes with a high degree of sequence specificity. Knowledge of the specific amino acid sequences cleaved by the different factors has led several groups to synthesize and test small peptides as substrates for particular coagulation factors. The development and application of these techniques to the measurement of blood coagulation factors has been reviewed by Huseby & Smith (1980). Chromogenic or fluorogenic peptide substrates have been largely used for the measurement of enzymatic activity and their potential use as histochemical reagents has yet to be fully exploited.

Previously utilized immunological methods such as crossed immunoelectrophoresis and immunofixation can demonstrate the presence of a particular protein, but give no indication of the functional integrity of the antigen so identified. The methods described here have the considerable advantage of specifically identifying factors II and X after electrophoresis but also indicate the relative activity of any structural variants that may be discovered. The results presented here indicate that the variant forms of factor II previously described by Board *et al.* (1982) have normal activity. Although this fact was previously predicted from the quantitative evaluation of prothrombin times of plasma from heterozygotes with the variant forms, further investigation with plasma from homozygotes, or highly purified material would normally have been required to confirm that finding.

Some caution in the interpretation of results with abnormal variants may, however, be required. Girolami *et al.* (1980) have shown that some patients with congenital dysprothrombinaemias gave deceptively high reaction rates with the chromogenic peptide substrate 2238 (Kabi Diagnostics, Sweden) when compared with conventional clotting assays. Alternatively, it is conceivable that other mutations may give rise to variants which have normal clotting activity, but low reactivity with synthetic substrates.

The results obtained in these studies clearly indicate that both factor Xa and thrombin react with Boc-Ile-Glu-Gly-Arg-MCA. This substrate was originally designed as a substrate for factor Xa since the sites on prothrombin cleaved by factor Xa are preceded by the sequence Ile-Glu-Gly-Arg. Morita *et al.* (1977) demonstrated that thrombin hydrolysed Boc-Ile-Glu-Gly-Arg-MCA at only 8·3% of the rate achieved by factor Xa on an equivalent weight basis. However, since there is considerably more factor II than factor X in normal plasma, it seems likely that the contribution of factor II to the total hydrolysis of this substrate in plasma may be greater than has previously been considered. The overlap in the specificity of the substrate does not severely detract from its use for the detection of factor Xa activity after electrophoresis since either factor II or factor X can be selectively activated or inhibited.

The results presented here clearly show that synthetic peptides can be used to identify coagulation factors II and X after electrophoresis and can be applied to extensive population and biochemical genetic studies of these factors. Since synthetic peptides with specificities for other components of the coagulation system, such as Kallikrein, factor XIa and plasmin, have been described and are commercially available, it is likely that this method can also be applied to the electrophoretic and genetic evaluation of these proteins.

The author wishes to acknowledge the technical assistance of Ms M. Coggan and the constructive comments of Dr N. M. Blake.

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