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ISOLATION OF THROMBOSPONDIN RELEASED FROM THROMBIN-STIMULATED HUMAN PLATELETS BY FAST PROTEIN LIQUID CHROMATOGRAPHY ON AN ANION-EXCHANGE MONO-Q COLUMN

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SUMMARY

Thrombospondin, a glycoprotein found in human platelet alpha granules, is thought to play a major role in platelet haemostatic functions. A rapid method to isolate thrombospondin for functional and structural studies was developed. Freshly prepared supernatants from thrombin-stimulated platelets were separated on an anion-exchange Mono-Q column on a fast protein liquid chromatography system. Detection of thrombospondin in the eluted peaks was performed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis combined with silver staining and a solid-phase radioimmunoassay with monoclonal antibodies directed against thrombospondin and other platelet granule glycoproteins. Thrombospondin was isolated rapidly to a high degree of purity using the fast protein liquid chromatography Mono-Q system (20 min), compared with the time taken with other techniques.

INTRODUCTION

Thrombospondin, a 420 kdaltons glycoprotein found in human platelet alpha granules, is thought to play an important role in platelet haemostatic functions[1], and

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has also been located recently in endothelial cells, fibroblasts and smooth muscle cells[2,3]. The function of thrombospodin in these cells remains unknown[3]. A polyspecific antibody directed against human thrombospondin cross-reacted with thrombospondin in the serum of several animal species[4].

Exposure of human platelets to thrombin, a potent physiological stimulator, rapidly induces the secretion of thrombospondin and other granule proteins including fibrinogen, fibronectin, FVIII: von Willebrand factor (vWf), platelet factor 4, β-thromboglobulin, platelet-derived growth factor and albumin[5]. Thrombospondin in the presence of calcium binds to the platelet membrane and is only partially released to the surrounding medium[6]. In the absence of calcium, thrombospondin undergoes a conformational change and becomes highly susceptible to degradation by thrombin.

Thrombospondin has been purified from the material released from thrombin-stimulated platelets, in the presence and absence of EDTA, by gel filtration and/or heparin–Sepharose affinity chromatography[7,8]. These techniques, while yielding thrombospondin with a high degree of purity, are slow and may allow denaturation. For studying the physiological role of thrombospondin in platelets and in other cells, it would be preferable to isolate and use this glycoprotein rapidly after its secretion from platelets. The aim of this study was to develop a fast method (20 min) to isolate thrombospondin for functional and structural studies. The isolation of thrombospondin was performed using a fast protein liquid chromatography (FPLC) system with a Mono-Q anion-exchange column. Thrombospondin was detected in the eluted peaks by using sodium dodecyl sulphate–polyacrylamide gel electrophoresis combined with silver staining and a solid-phase radioimmunoassay system (SPRIA) with monoclonal antibodies directed against thrombospondin and other platelet granule glycoproteins.

EXPERIMENTAL

The FPLC separation system, heparin–Sepharose 4B, Mono-Q HR5/5 prepacked anion-exchange column (50 × 5 mm I.D.) and the Superloop injection system were purchased from Pharmacia (Uppsala, Sweden). Human α-thrombin, a generous gift from Dr. D. L. Aronson (Bureau of biologics, USDA, Bethesda, MD, U.S.A.), was dissolved in water at 100 U/ml and stored at −70°C. Hirudin, L-histidine and diisopropyl fluorophosphate were obtained from Sigma (St. Louis, MO, U.S.A.), triethanolamine from Merck-Schuchardt (Darmstadt, F.R.G.) and carrier-free $Na^{125}I$ from Amersham International (Amersham, U.K.). Wells for SPRIA were supplied by Flow Laboratories (Asnieres, France). Other chemicals were as previously indicated[9] or purchased from Merck-Schuchardt.

Thrombin stimulation of washed platelets

Platelet concentrates 1–2 h old were prepared from 4 units of blood (400 ml of blood per unit) by the blood transfusion centre. The procedures used for isolating, washing and stimulating platelets by thrombin were similar to those described by Lawler and co-workers[7,8] with a few modifications. Briefly, platelets were washed in 15 volumes of 102 m*M* NaCl, 3.9 m*M* KH_2PO_4, 3.9 m*M* Na_2HPO_4, 22 m*M* NaH_2PO_4 and 5.5 m*M* glucose (pH 6.5) buffer. The washed platelets were resus-

pended at a concentration of $5 \cdot 10^{10}$ platelets per 15 ml in 0.015 *M* Tris–HCl (pH 7.6), 14 m*M* NaCl, 5 m*M* glucose buffer. The washed platelets were then stirred and treated with α-thrombin (1 U/ml) at 22°C for 2 min, after which hirudin (2 U/ml) was added. The aggregates were allowed to settle at 0°C. The supernatant was carefully removed and centrifuged at 48,000 *g* for 30 min after adding 1 m*M* diisopropyl fluorophosphate. The centrifuged supernatant was carefully removed and stored at 4°C in siliconized bottles in the presence of 0.02% NaN_3.

Heparin–Sepharose CL-6B affinity chromatography

The supernatant from thrombin-stimulated platelets was applied to a heparin–Sepharose CL-6B column in the presence of $CaCl_2$ following the method of Lawler *et al.*[8]. Flow-through proteins and proteins eluted at different NaCl concentrations were separated on sodium dodecyl sulphate–polyacrylamide gels, which were then silver-stained[10,11]. In some instances these fractions were analysed by rocket immunoelectrophoresis using antiserum directed against fibrinogen, fibronectin or FVIII: vWf antigen (results not shown).

Ion-exchange chromatography by FPLC

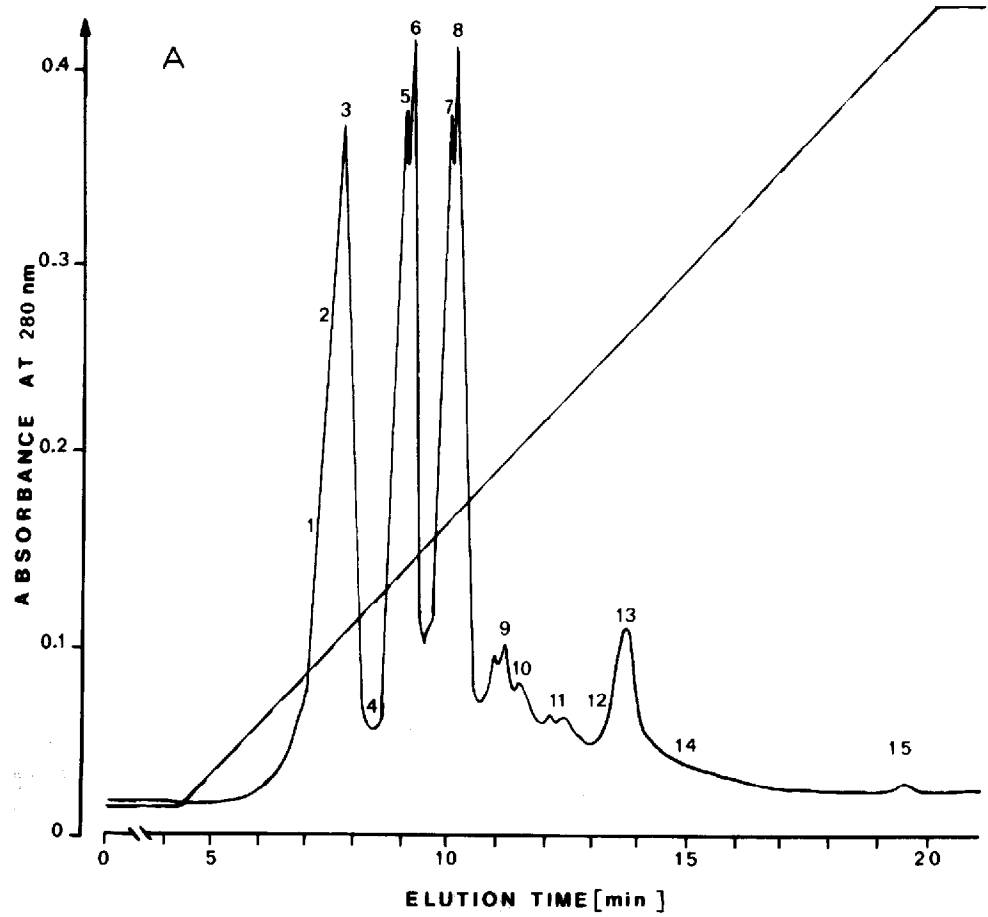
The supernatant (15 ml, containing approximately 2 mg of proteins) from thrombin-stimulated platelets was diluted (1:3) in 0.02 *M* triethanolamine buffer (pH 7.4) and applied, via a 50-ml siliconized Superloop (335 × 30 mm), to a Mono-Q column (50 × 5 mm I.D.) on an FPLC system. Flow-through material and thrombospondin, eluted from the heparin–Sepharose CL-6B column, were separately applied to the Mono-Q column. The starting buffer was 20 m*M* triethanolamine (pH 7.4) and the final buffer was 20 m*M* thiethanolamine (pH 7.4) containing 1 *M* NaCl. In initial experiments the pH of the buffer was varied to find the best separation conditions.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

Following the method of Lawler and co-workers[7,8], peak fractions were mixed with one-quarter volume of a buffer containing 25 m*M* Tris, (pH 7.4), 5% sodium dodecyl sulphate, 1.25 *M* sucrose, 100 m*M* dithiothreitol, 10 m*M* EDTA and 10 m*M* N-ethylmaleimide and heated for 1 min before separation on a 7% sodium dodecyl sulphate–polyacrylamide Laemmli gel[10]. Gels were fixed in 40% methanol–7% acetic acid and then silver-stained by the method of Merril *et al.*[11].

Solid-phase radioimmunoassay

The SPRIA procedure used was similar to that described by Howard *et al.*[12], with minor modifications. Briefly, antigens contained in each peak fractions were attached to poly(vinyl chloride) microtitre plates by incubation overnight at 4°C, followed by extensive washing with phosphate-buffered saline containing 1% horse serum and 0.02% NaN_3. The antigens bound to the microtitre plates were then identified using ^{125}I-labelled monoclonal antibodies directed against different granule glycoproteins. The monoclonal antibodies used were directed against thrombospondin[13], fibrinogen[14] and FVIII:vWf (a generous gift from Immunotech, Marseille, France). Radiolabelling of monoclonal antibodies was performed by the method of Mason and Williams[15].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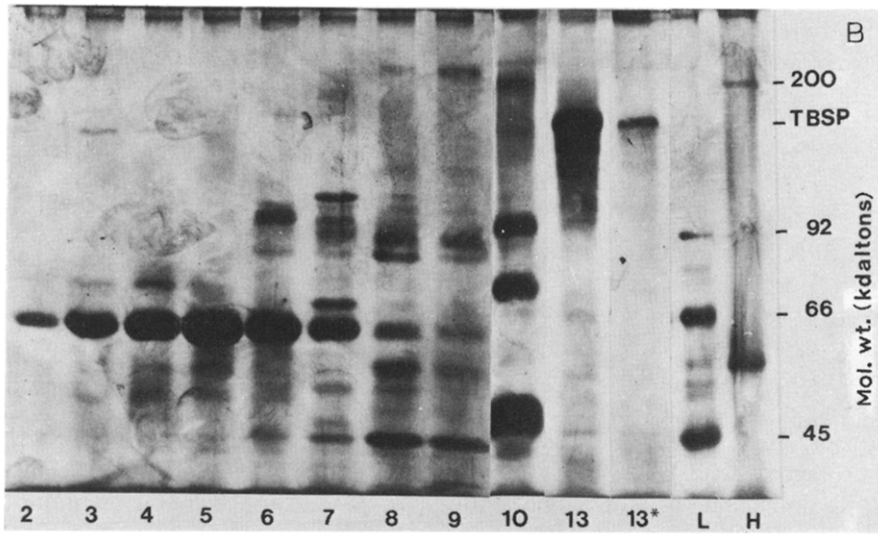
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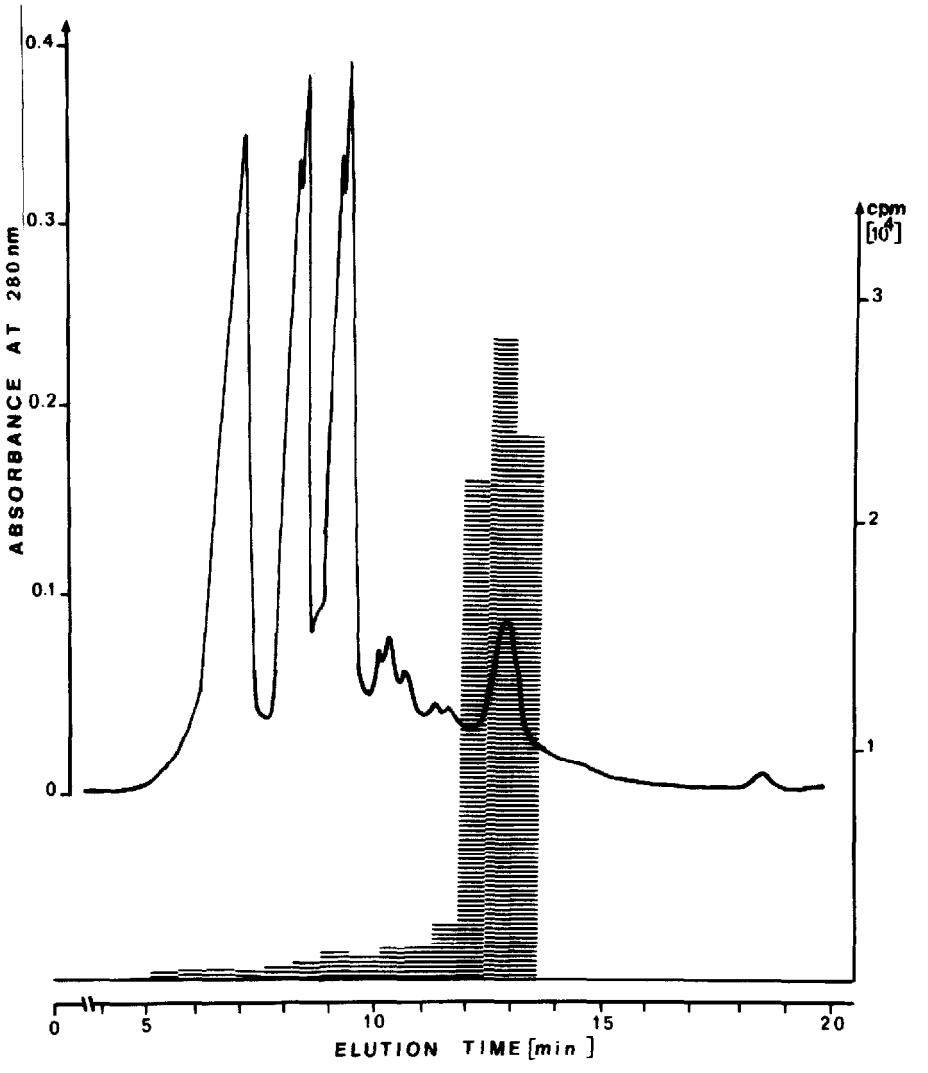

Fig. 2. SPRIA detection of the thrombospondin in each peak fraction of the Mono-Q chromatographic profile using a ^{125}I-labelled monoclonal antibody directed against thrombospondin, by SPRIA. The upper part shows the chromatographic separation and the bottom part shows the binding of the ^{125}I-labelled antibody to the peak fractions.

Fig. 1. (A) Separation of supernatant released from thrombin-stimulated platelets on a Mono-Q column connected to an FPLC system. Supernatant (2 mg of proteins per 15 ml) was injected into the column via a 50-ml Superloop. The 0–1 *M* NaCl gradient in 20 m*M* triethanolamine buffer (pH 7.4) was generated over 20 min. (B) Eluted peaks were treated with sodium dodecyl sulphate and electrophoresed under reducing conditions on a 7.5% sodium dodecyl sulphate–polyacrylamide gel. The gel was fixed and silver-stained. Numbers shown below each lane on the sodium dodecyl sulphate–polyacrylamide gel correspond to peak numbers. Lanes 13 and 13*, which contain thrombospondin, were loaded with 46 and 5 μg of eluted material, respectively. Lanes L and H contain low- and high-molecular-weight standards.

RESULTS AND DISCUSSION

The supernatant from thrombin-treated platelets, separated on a Mono-Q column (50 × 5 mm I.D.), showed several major peaks eluted by a 0–1 *M* NaCl gradient (Fig. 1). When these fractions were electrophoresed under reducing conditions on sodium dodecyl sulphate–polyacrylamide gels after silver-staining, they contained either several protein bands with one protein predominating, or a single protein band. In typical experiments albumin and fibrinogen were eluted in peaks 6, 7 and 8, peak 10 contained both fibronectin and FVIII:vWf and thrombospondin was in peak 13 (Fig. 1). Thrombospondin is composed of three polypeptide chains of similar molecular weight, cross-linked by disulphide bridges, which migrate under reducing conditions at an apparent molecular weight of 160 kdaltons[8].

SPRIA with monoclonal antibodies directed against fibrinogen, FVIII:vWf (results not shown) and thrombospondin confirmed the presence of these proteins in these peaks (Fig. 2). High background counts observed in some of the SPRIA profiles may be due to degradation products produced after brief exposure of these proteins to released endogeneous platelet proteases. The separation of these proteins on a Mono-Q ion-exchange column is in close agreement with that obtained with urine proteins on a similar column[16].

It is possible that interactions occur between two or more proteins, such as fibronectin and FVIII:vWf, to explain their elution at the same NaCl concentration. To test this hypothesis, experiments were performed in the presence of 0.2% (w/v) Berol 185, a non-ionic detergent, in an attempt to dissociate interacting proteins. However, there was no significant change in the chromatographic profiles when this detergent was added to the Mono-Q column, to the buffers used or to the material released by platelets (the detergent was incubated for 1 h at 4°C with the released material before chromatography). Similarly, no change in chromatographic profiles

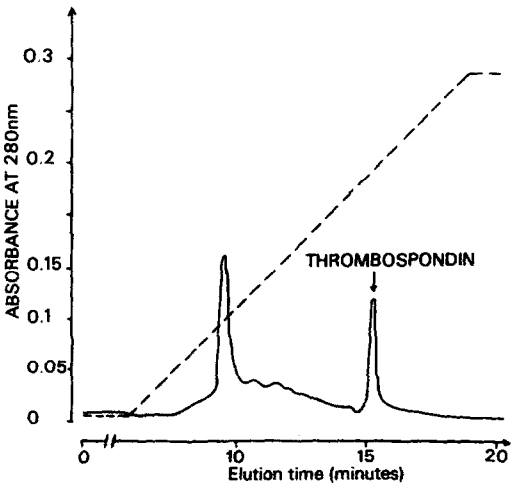

Fig. 3. Separation of thrombospondin, contaminated by other proteins, on a Mono-Q column equilibrated at pH 6.0 (A: 20 m*M* L-histidine; B: 1 *M* NaCl in A). The rechromatographed profile contained two peaks. The second peak (eluted at the same position as peak 13) contains thrombospondin.

was obtained when EDTA was added, at a final concentration of 1 m*M*, to the column, to buffers and to the released platelet material, to dissociate possible calcium-mediated interactions that are known to occur between these proteins[3,6,8].

In many experiments thrombospondin was isolated to a high degree of purity (Fig. 1, lane 13*). However, in some instances the thrombospondin was contaminated with other proteins. It was noted that when this happened the donor's platelets did not form a firm thrombus on aggregation with thrombin but a number of small aggregates. The degree of purity of a predominant protein was improved by rechromatographing the pooled peak fractions, containing this protein, on the Mono-Q column using a buffer of different pH. Previous results with Mono-Q columns have shown that under slightly different pH conditions the relative charge differences between contaminants are much greater and an improved separation can be obtained[17]. An increase in the purity of thrombospondin contaminated by various proteins was obtained by rechromatographing the collected peak fractions (peak 13), previously equilibrated in the starting buffer at pH 6.0 (Fig. 3).

Thrombospondin, isolated by heparin–Sepharose CL-6B affinity chromatography, and flow-through material (containing no thrombospondin), when chromatographed on the Mono-Q column, showed, respectively, one peak at the same position as peak 13 (previously identified as thrombospondin by sodium dodecyl sulphate–

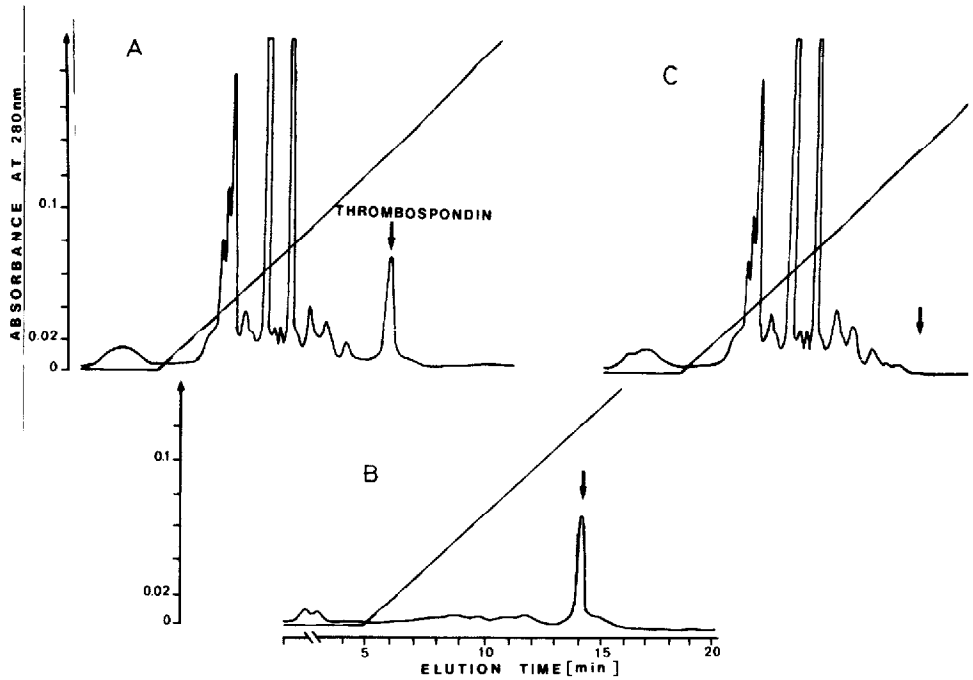

Fig. 4. Comparison of separation profiles obtained on a Mono-Q column with (A) supernatant released from thrombin-stimulated platelets, (B) isolated thrombospondin obtained by heparin–Sepharose CL-6B affinity chromatography and (C) the flow-through material (containing no thrombospondin) from the heparin–Sepharose Cl-6B column. Gradient: 0–1 *M* NaCl in 20 m*M* triethanolamine buffer (pH 7.4). These results demonstrate that all of the thrombospondin present in the supernatant from thrombin-stimulated platelets was bound by the heparin–Sepharose CL-6B column and could be eluted with NaCl. They also demonstrate that the Mono-Q column is capable of giving a better separation of thrombospondin.

polyacrylamide gel electrophoresis and using monoclonal antibodies in SPRIA), and a profile of all the separated proteins except thrombospondin (Fig. 4). These results further confirm that peak 13 in the FPLC–Mono-Q separation profile is thrombospondin and that the same molecule is purified by both methods. The FPLC–Mono-Q system can be used to separate thrombospondin and other proteins more quickly (20 min) than other techniques[7,8]. In addition, the FPLC–Mono-Q system can be used to measure rapidly the relative concentration of different proteins secreted by platelets. Such assays may be valuable in screening platelets from patients showing haemostatic problems related to platelet abnormalities.

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