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Note

Human platelet glycocalicin purification by phenyl boronate affinity chromatography coupled to anion-exchange high-performance liquid chromatography

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The membrane platelet glycoprotein Ib (Gp Ib) plays an important role in the physiological processes of platelet aggregation and adhesion [1,2]. The Gp Ib molecule consists of two subunits (α and β) with relative molecular masses of ca. 140 000 and 20 000, respectively [3]. Glycocalicin (Gc), which belongs to the α-subunit, contains the binding sites for α-thrombin and Von Willebrand factor [4,5]. Gc has a very high sugar content (60% by weight) with a predominance of sialic acid and galactose [6]. Generally, Gc is purified most efficiently by using wheat germ–Sepharose affinity chromatography.

In this study we demonstrate that affinity chromatography on phenylboronate–Agarose resin (PBA), followed by anion-exchange high-performance liquid chromatography (HPLC) allows the preparation of Gc of high purity. Since phenylboronate interacts with 1,2-*cis*-diols [7] it has, in comparison with wheat germ agglutinin, an affinity for a broader range of carbohydrates such as galactose, mannose and sialic acid [8]. PBA affinity chromatography is especially useful when the sialic acid content of Gc is reduced in certain physiological [9] or pathological processes [10,11].

EXPERIMENTAL

Chemicals

All chemicals were analytical grade and were purchased from Merck (Darmstadt, F.R.G.), Carlo Erba (Milan, Italy), Pharmacia (Uppsala, Sweden) and

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Sigma (St. Louis, MO, U.S.A.). Matrex gel PBA-60 and Sepharose 2B resins were obtained from Amicon (Denver, CO, U.S.A.) and Pharmacia, respectively. Neuraminidase (*Clostridium perfrigens*, fraction V), obtained from Sigma, contained less than 0.1 mU/ml protease activity.

Preparation of washed platelets

Blood samples from healthy donors were collected using plastic syringes containing 3.8% sodium citrate. The ratio of blood to citrate was 9:1 (v/v). Blood was then immediately transferred to polystyrene tubes. Platelet-rich plasma (PRP) was obtained by centrifugation at 120 *g* for 20 min. Washed platelets were obtained by a double gel-permeation of PRP (10 ml) on 25 cm × 1.5 cm I.D. Sepharose 2B columns equilibrated with a solution containing 10 mmol/l Tris–HCl, 0.15 mol/l sodium chloride, 0.6 mmol/l Na_2EDTA and 5.5 mmol/l glucose (pH 7.60).

Glycocalicin purification

A 6–8 ml volume of platelet suspension (containing $3 \cdot 10^8$–$5 \cdot 10^8$ platelets per ml) obtained by gel permeation was pelleted by centrifugation at 2000 *g* for 15 min. The pellet was resuspended in 3 ml of 3 mol/l of potassium chloride at 37°C for 15 min. After centrifugation at 8000 *g* for 10 min, the supernatant (potassium chloride extract) was dialysed for 18 h against a solution containing 10 mmol/l HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid), 0.2 mmol/l Na_2EDTA, 0.1% sodium azide and 50 mmol/l potassium chloride (pH 8.20). Elution conditions for PBA affinity chromatography were previously tested using samples containing purified catalase preparation (Pharmacia) and α_1 acid glycoprotein (α_1 Gp), which is a glycoprotein with high sialic content.

The dialysed and lyophilised potassium chloride extract was then loaded onto a 15 cm × 1 cm I.D. column of PBA equilibrated with a starting buffer containing 10 mmol/l HEPES and 50 mmol/l potassium chloride, pH 8.20 (buffer A). Protein elution was monitored by recording the absorbance at 280 nm with an LKB 2238 UVICORD SII. The column was washed with 30 ml of buffer A, which allowed the complete elution of the unadsorbed material, as checked by recording the absorbance at 280 nm. The adsorbed material was then eluted by stepwise elution with buffer B (0.1 mol/l sorbitol in buffer A). An absorbance peak was recorded after ca. 10 ml elution with buffer B. The fractions corresponding to this peak were pooled, dialysed overnight against 0.02% sodium azide at 4°C and lyophilised. The protein content of these preparations was determined by the method of Lowry et al. [12].

Anion-exchange high-performance liquid chromatography

The HPLC apparatus used was a Perkin-Elmer two-pump Series 10 liquid chromatograph equipped with an LC 85 spectrophotometric detector and an LKB 210 two-channel recorder. The chromatographic column was an AX 300 semi-preparative cartridge (Brownlee Labs., Santa Clara, CA, U.S.A., 220 mm × 4.6 mm I.D., 10 μm particle diameter, connected to a 30 mm × 4.6 mm I.D. guard cartridge containing the same packed material. Chromatography was performed at 30°C. The elution buffers were (A) 25 mmol/l sodium phosphate (pH 7.55)

and (B) 0.5 mol/l potassium bromide in A. The gradient was linear from 0 to 25% B in 25 min and the flow-rate was 1 ml/min. The eluate was monitored at 254 nm. The injected sample volumes ranged from 6 to 20 μl on the basis of the protein concentration.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on 7% acrylamide rod gels, using Laemmli's buffer system under reducing and non-reducing conditions [13]. The gels were stained with Coomassie blue and periodic acid Schiff's reagent, as reported by Nurden et al. [14].

Neuraminidase treatment of platelets isolated by gel permeation

A 10-ml volume of gel-filtered platelet suspension was centrifuged at 2000 *g* for 15 min and then resuspended in the same volume of a solution containing 0.1 mol/l sodium acetate, 0.30 mol/l sodium chloride and 9 mmol/l calcium chloride (pH 5.60) (neuraminidase buffer). After addition of neuraminidase (200 mU per 10^{12} platelets), the platelet suspension was incubated at 37 °C for ca. 15 min. Platelets were then pelleted and washed once with neuraminidase buffer. The effect of this treatment on the platelet sialic acid content was checked by measuring the platelet sialic acid before and after neuraminidase treatment by Warren's method [15]. A platelet sialic acid loss of ca. 50% was consistently found.

RESULTS AND DISCUSSION

A typical elution pattern obtained by PBA affinity chromatography of a potassium chloride extract of platelets separated by gel permeation is shown in Fig. 1. Soluble proteins extracted with potassium chloride were separated into two fractions. The retarded fraction was found to contain Gc as the predominant glyco-

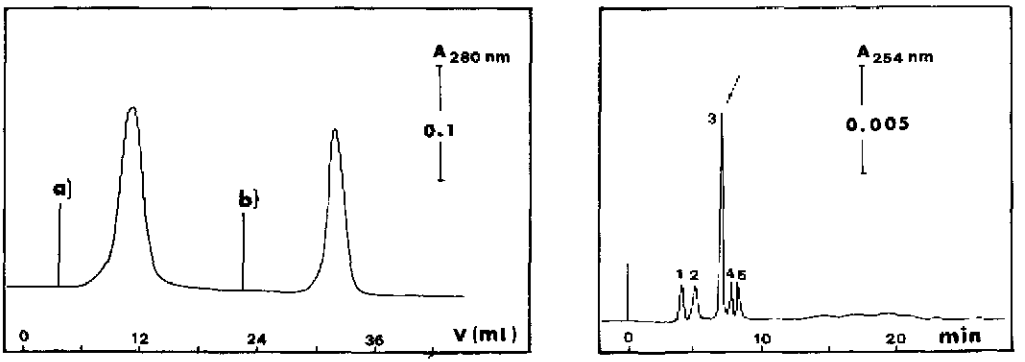

Fig. 1. PBA affinity chromatography of a 3 mol/l potassium chloride extract of washed platelets. Flow-rate, 0.9 ml/min; (a) 10 mmol/l HEPES, 50 mmol/l potassium chloride pH 8.20; (b) 0.1 mol/l sorbitol in buffer a.

Fig. 2. Anion-exchange HPLC of retarded fraction of PBA affinity chromatography (Fig. 1). The arrow indicates the fraction collected and analyzed by disc-SDS-PAGE. Chromatographic conditions are described in the text. Peaks 1 and 2 and 4 and 5 are unidentified protein contaminants.

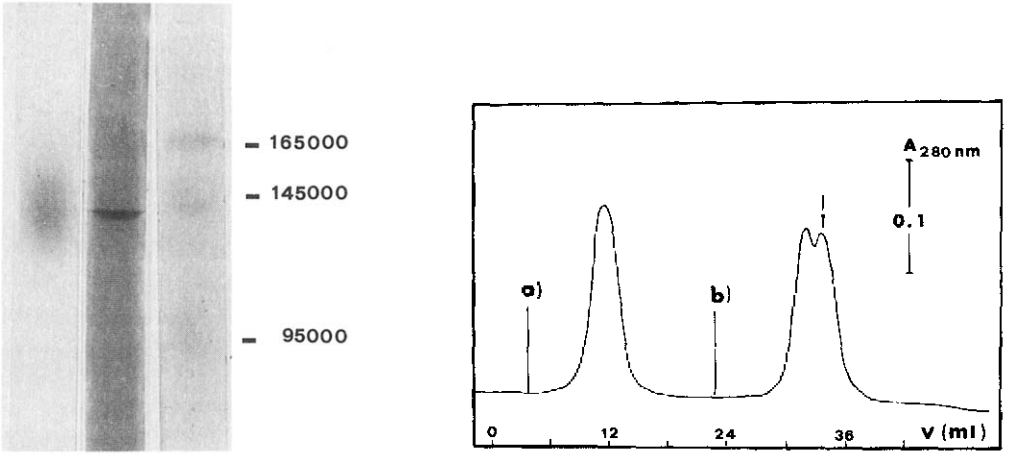

Fig. 3. SDS-PAGE of: (left) purified Gc (PAS staining); (centre) purified Gc (Coomassie blue staining); (right) platelet glycoproteins Ib, IIb and IIIa (the corresponding M_r values are shown on the right). SDS-PAGE was performed under non-reducing conditions.

Fig. 4. PBA affinity chromatography of a 3 mol/l potassium chloride extract of washed and neuraminidase-treated platelets. The arrow indicates the retarded fraction containing the desialylated Gc. Flow-rate, 0.9 ml/min; for a and b, see legend to Fig. 1.

protein. This is demonstrated by the anion-exchange HPLC pattern of this fraction shown in Fig. 2. By coupling PBA affinity chromatography to anion-exchange HPLC a very pure preparation of Gc can be obtained. The fractions corresponding to peak 3 of the anion-exchange HPLC profile (Fig. 2) were collected, dialysed and lyophilised, and tested by SDS-PAGE. Both Coomassie blue and periodic acid Schiff's staining of the gels demonstrated a single band of ca. 135 000 (Fig. 3) This value for the relative molecular mass is in agreement with that previously reported for Gc [3]. The yield of purified Gc was ca. 50 μg per 100 ml of whole blood. The purification of Gc is commonly performed by wheat-germ agglutinin–Sepharose affinity chromatography. The Gc binding site for this lectin is represented by sialic acid residues. Therefore this method could not allow the preparation of Gc variants lacking such carbohydrate.

As previously demonstrated by gas chromatographic–mass spectrometric analysis [6,16], the major O-linked oligosaccharide of Gc is a hexasaccharide alditol, NeuAc-α2–3-Gal-β1–4-Gal-1–4-GlcNAc-β1–6-(NeuAc-α2–3-Gal-β1–3)-N-acetylgalactosaminitol. This Gc hexasaccharide can also interact with borate anion when terminal sialic acid is lacking. Galactose has, in fact, two coplanar hydroxyl groups, which bind to PBA. As demonstrated in Fig. 4, desialylation of Gc does not prevent the binding of this glycoprotein to PBA. A defect of sialylation of Gp Ib has been demonstrated in myeloproliferative disorders and malignancies [11,17]. In other pathological conditions, such as uraemia, a severe reduction of Gp-conjugated carbohydrates has been hypothesized [11]. In addition, a physiological loss of sialic acid from membrane glycoproteins during platelet aging has been recently demonstrated [10].

In conclusion, the use of PBA coupled to anion-exchange HPLC can allow the purification of both normal Gc and of Gc variants deficient in sialic acid content.

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