Journal of Chromatography B, 1008 (2016) 11–14

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Purification of glycocalicin from human plasma

Basma HadjKacem[a,*], Héla Mkaouar[a], Ikram Ben Amor[b], Jalel Gargouri[b], Ali Gargouri[a]

[a] Laboratoire de valorisation de la biomasse et production des protéines chez les eucaryotes, Centre de Biotechnologie de Sfax, Université de Sfax, Tunisia
[b] Centre Régional de Transfusion Sanguine de Sfax- 99/UR/08-33, université de Sfax, Tunisia

ARTICLE INFO

Article history:
Received 5 February 2015
Received in revised form
23 September 2015
Accepted 29 September 2015
Available online 14 November 2015

Keywords:
Glycocalicin purification
HPLC gel filtration
Affinity chromatography
NHS column
Blood purification

ABSTRACT

Glycocalicin (GC) is a large extracellular proteolytic fragment of glycoprotein Ib, a membrane platelet component playing an essential role in the physiological processes of platelet adhesion and aggregation. GC contains the binding sites for thrombin and von Willebrand factor. GC circulates normally in vivo in significant concentrations and the plasma level of this protein reflects a complex function of factors including platelet count or platelet turnover. It can therefore serve as a good indicator for many diseases like hypoplastic thrombocytopenia and idiopathic thrombocytopenic purpura. For this reason, several purification assays have been previously described. In this work, we describe a novel analytical method for GC purification from human platelets based on preparative HPLC gel filtration followed by immuno-affinity chromatography on NHS activated column conjugated with specific antibody. Pure GC was obtained from tiny amount of starting material. Our protocol of GC purification is simple, fast and provides a pure end product.

1. Introduction

Glycocalicin (GC) is the external portion of GPIbα glycoprotein expressed at platelet surface and plays an essential role for platelet adhesion to vascular injury [1]. GC can be rapidly cleaved by enzymes such as calpain, plasmin, trypsin, elastase, etc. [2]. When it is cleaved by trypsin, GC yields to a macro-glycopeptide (123 kDa) and a tail (45 kDa) which contains the thrombin-binding site [3]. GPIbα and GC react in the same way against specific antibodies [4].

Thrombocytopenia is a hematologic abnormality resulting from hypoproduction, hyperdestruction or sequestration of platelets. It is known as difficult to distinguish between hypoplastic and hyperdestructive forms of thrombocytopenia. The diagnosis was often based on heavy methods such as bone marrow examination or platelet life-span measurements, both presenting several drawbacks, that's why the use of simple, rapid and non-invasive tests was very important [5].

Glycocalicin plasma level is one of the simplest laboratory methods reported to be helpful for the thrombocytopenia diagnosis. GC index is the level of plasma GC normalized for the individual platelet count. It was significantly high in patients with idiopathic thrombocytopenic purpura but normal in patients with aplastic anemia or chemotherapy-induced thrombocytopenia [6].

GC can be easily measured by enzyme-linked immunosorbent assay (ELISA), serving as a good indicator for many diseases. Beer et al., have developed and standardized a sandwich ELISA which uses two monoclonal antibodies, both of which bind to the amino-terminal 45 kD fragment of glycocalicin [2]. This assay was then used to analyze the glycocalicin levels of healthy subjects (2.04 ± 0.46 pg/mL) and patients with selected diseases such as aplastic anemia and thrombocytosis. Beer et al., suggested that GC is a useful platelet marker which directly reflects platelet damage and possibly platelet dysfunction [2]. Gurney et al., showed that increased levels of GC characterized idiopathic thrombocytopenia purpura, the thrombocytopenia of HIV infection, and immune thrombocytopenia. Normal plasma levels have been reported in patients with bone marrow hypoplasia and low levels were found in umbilical vein blood [7]. Massive cleavage will ultimately result in an acquired Bernard–Soulier like bleeding disorder, and circulating glycocalicin may act as a potential inhibitor of platelet adhesion [2].

Given the interest of GC in medical analyses, several groups have focused on the purification of this protein. The first assay was performed in 1976 by Okumara et al. They purified GC by gel filtration followed by hydroxyapatite chromatography [8]. In 1982, Moroi et al., have described another method of GC purification, based on affinity chromatography on wheat germ agglutinin (WGA)

* Corresponding author at: Laboratoire de valorisation et production de la biomasse chez lez eucaryotes, Centre de Biotechnologie de Sfax, BP"K"3038 Sfax, Tunisie. Fax: 216 74 874 449.
E-mail address: hadjkacem_basma@yahoo.fr (B. HadjKacem).

http://dx.doi.org/10.1016/j.jchromb.2015.09.044
1570-0232/

sepharose and then on thrombin sepharose using selective elution by heparin [3].

The present work describes a novel, rapid and reproducible method for GC purification from, relatively, a tiny amount of starting materials.

2. Materials and methods

2.1. Sample preparation

Platelets pocket was gifted by the regional centre of blood transfusion. The pocket was aged two days and intended for transfusion. Total platelets count per pocket was 0.5×10^{11} in a total volume equal to 200 mL. Only 15 mL of platelets samples were used to purify GC (7.5% of platelet unit). Sample was crushed using a Potter tube, placed on ice in presence of protease inhibitors (PMSF, benzamidin and pepstatin).

2.2. Preparative HPLC chromatography

Crushed platelets were centrifuged at 2000 rpm. The obtained supernatant was filtered through Minisart Syringe filter 0.45 μm (Sartorius) before injection onto the gel filtration column PL aquagel-OH40 10 μm (Polymer Laboratories). Its dimension was 300×25 mm and its fractionation range was between 10 and 200 kDa. The concentration of sample was 200 mg/mL determined by Biorad assay and 1 mL was injected manually. The flow rate was fixed to 5 mL/min using 50 mM Phosphate buffer (K_2HPO_4/KH_2PO_4), pH 6.8. Column effluents were monitored by absorption at 280 nm. This step was repeated 15 times to inject the entire sample.

2.3. Slot-blot

100 μl of samples were vacuum-fixed onto PVDF membrane using the slot blot apparatus. The membrane was blocked by 5% dried skimmed milk in PBS with 0.1% Tween. After washing by PBS Tween 0.1%, the membrane was incubated during two hours with the primary antibody CD42b (N-19) (Santa Cruz) diluted in 1% skimmed milk-PBS Tween 0.1%. Peroxidase-conjugated anti-goat antibody was then added and incubated for one hour. Visualization was performed using the enhanced chemiluminescence kit (ECL plus Amersham).

2.4. Concentration of protein samples

Proteins were concentrated by two steps. First, all fraction of interest were pooled in a dialysis tubing cellulose membrane (Sigma–Aldrich) and wrapped by Poly-Ethylene-Glycol (PEG) 6000 to reduce the volume by 10 fold. The residual volume was ultimately concentrated by centricon centrifugal filter containing Ultracel YM-50 membrane (Millipore Amicon bioseparation). The centricon 50 allows a sample concentration and protein removal of size below 50 kDa.

2.5. Immuno-affinity chromatography

The GC monoclonal antibody SZ2 (Immunotech) was conjugated to HiTrap NHS-activated HP 1 mL column (GE Healthcare Life Sciences). 0.5 mg/mL of antibody was fixed in the column using a standard coupling buffer (0.2 M NaHCO3, 0.5 M NaCl, pH 8.3) and following the instructions provided by the manufacturer. The coupling efficiency was determined to be 70%. The column was operated with HPLC system. The flow rates were 1 mL/min.

To purify the GC protein, the column was washed with 3 mL binding buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.4), 3 mL elution buffer (0.2 M glycine, pH3) and then equilibrated using 10 column volumes of binding buffer. The concentrated sample was then injected onto the immunoaffinity column using HPLC system. The column was washed with 10 volumes of binding buffer. Finally, proteins were eluted using a gradient from 0 to 0.2 M glycine pH 3. Proteins were concentrated with speed vac.

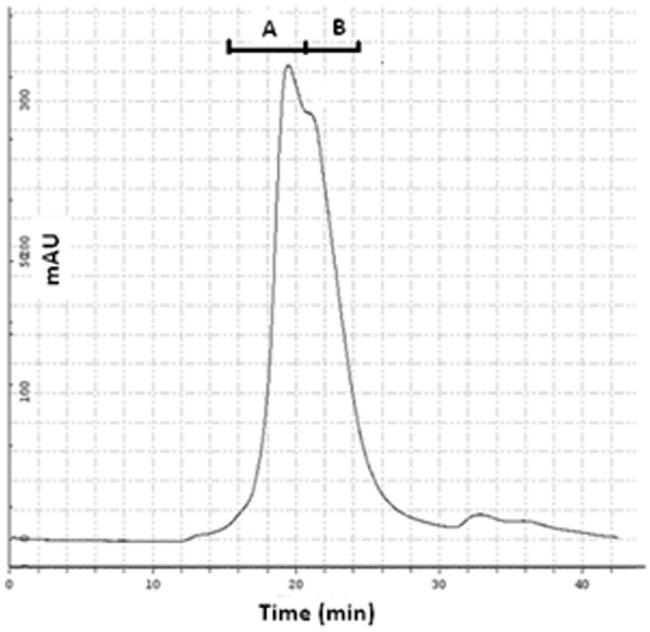

Fig. 1. Chromatogram of the preparative gel filtration on PL aquagel OH40 column. A:peak A. B:peak B. The *x*-axis represents time and the *y*-axis represents the optical density at 280 nm.

2.6. Western blot

Proteins were analyzed by electrophoresis on a 8% SDS-PAGE gel, which was either silver stained or transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk in PBS Tween 0.1%, at 4 °C overnight. A primary goat polyclonal antibody (N-19 Santa Cruz) raised against GC was added to the membrane and incubated at room temperature for 2 h. The membrane was then washed several times with PBS 0.1% Tween. A secondary anti-goat antibody conjugated with HRP was diluted at 1:10000 in PBS Tween 0.1% and added to the membrane. After 1 h, the positive signals were detected using the enhanced chemiluminescence kit (ECL plus Amersham).

3. Results

3.1. Purification of GC from fresh platelet pocket

15 mL of crushed platelets were filtered and injected onto preparative HPLC chromatography PL aquagel-OH40 column. We used 1 mL loop and we injected each time 200 mg of proteins. The chromatogram showed two peaks, named A and B (Fig. 1). To check the presence of GC, a slot blot immunostaining was performed using an antibody recognizing specifically this protein. GC was present in only one peak (peak A). To better ensure the observed results, we analyzed peak A and B by western-blot using a specific monoclonal antibody (N-19). We showed that peak A contained a band corresponding to the expected size of GC. However peak B contained only proteolytic products of small size.

Based on this result, the total volume of peak A was pooled on a dialysis tube which was thereafter covered by PEG 6000 to concentrate proteins. The remaining volume was then centrifuged