Purification and Isotype Identification of Glucose Binding Protein in Normal Human Platelets

Trombositlerde Glukoz Bağlayıcı Protein Saflaştırılması ve İzotipinin Belirlenmesi

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Abstract

In this study glucose binding protein was isolated and purified from the normal human platelets and its was determined to identify of isoform.

The specific activities of the glucose binding protein in the shock fluid and after Sephadex G-200 and DEAE-Sepharose column chromatographies were found to be 26.4, 37.9, and 84.45 nmolglucose/mg protein, respectively.The molecular weight of glucose binding protein was found to be 29,000 Da by SDS-PAGE. This protein does not correspond to any of the isoforms of the GLUT family described in the literature. After the purification steps, it was determined that platelet glucose binding protein was purified 3.2 times when compared the initial phase.

Key words: Glucose binding protein, Platelet, Glucose transporter

Introduction

The most important energy source in platelets is glucose and the energy needed for platelet functions, aggregation and secretion, comes from glycolysis. The first step in the metabolism of blood glucose is the transport across the platelet membrane to the platelet cytoplasma (Leoncini and Maresca, 1986; Mürer, 1969; Yardımcı and Ulutin 1986). This step is carried out by a group of membrane carrier proteins called glucose transporters. Two main classes of glucose transporters have been identified in humans: Na^+/glucose cotransporters and facilitative glucose transporters (Devaskar and Muecler,1988; Pinches, *et al.*, 1993;Kayano, *et al.*, 1990). Na^+/glucose cotransporters actively concentrate glucose inside the intestinal and renal cells using the electrochemical potential of Na^+ as their energy source. A second class of transporters, the facilitative glucose transporters, are not concentrative and transfer glucose down to its own concentration gradient (Elsas and Longo, 1992; Mueclerr, 1994).

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Sodium-glucose cotransporters: They are expressed by specialized epithelial cells, i.e. the mature enterocytes lining the intestinal microvillus type, and the brush border of the proximal tubule of the kidney. D-glucose is absorbed from the intestinal tract by the Na^+/dependent transporter (SGLTs) located in the brush border. Following Na^+ and glucose uptake, Na^+ inside the cell is actively extruded by the Na^+/K^+ ATPase in the basolateral membrane, maintaining almost a constant Na^+ gradient across the brush border membrane of the intestinal cells (Elsas and Longo, 1992; You *et al.,* 1995).

Facilitative glucose transporters: A second major type of glucose transporter is described in nearly all mammalian cells. Recently six types of the facilitative glucose transporter have been identified. These proteins are Glut 1-5 and Glut 7. Glut 6 is the pseudogene. These classes of transporters are encoded by different genes and exhibit distinct tissue distributions (Devaskar and Muecler,1998; Elsas and Longo, 1992; Muecler, 1994). Researches concerning glucose transport in platelets have been carried out since 1970 (Solomon and Gaut, 1970). Different transport mechanisms in platelets were performed in our laboratory and their responses to chemicals in normal or pathologic conditions were observed. In these studies the glucose transport system of platelets obtained from atherosclerotic patient was shown proved demonstrated to be defective (Yardımcı and Ulutin, 1986). In this study our aim was to determine the similarities and differences between glucose transporters in other tissues and platelet glucose transporter.

Materials and Methods

Isolation of platelets: Venous blood was with drawn from normal donors into tubes and mixed with 1/9 EDTA (0,077 M). After 15 min at 4°C these were centrifuged at 1500 rpm at room temparature for 8 min to isolate the platelet rich plasma (PRP). PRP was recentrifuged for 20 min at 6000 rpm. Platelet pellet was washed and then Cold Osmotic Shock (COS) procedure was applied.

Isolation of glucose specific binding protein by cold osmotic shock (COS) procedure (Yardımcı and Ulutin, 1986): Washed platelet pellet was suspended ratio in %30 glucose solution (1:40), stirred at room temperature for 20 min and then centrifuged at 10,000 rpm for 15 min. The supernatant was decanted and replaced by distilled water with 1:80 ratio and homogenous suspension was obtained. This was stirred magnetically at 4^0C for 30 min, then centrifuged a speed of 10 000 rpm for 15 min. COS liquid was grouped as the supernatant and pellet was grouped as the shocked platelets. The supernatant was precipitated with 90% cross sectioned $(NH_4)_2SO_4$ and centrifuged with at 10,000 rpm for 15 min.The precipitate was dissolved in 5 ml Tris-NaCl buffer, then dialysed in the same buffer for 48 hours. The glucose binding specific activity in the shock fluid, Sephadex G-200, DEAE-Sepharose column chromatographies and HPLC procedures was determined. The purification degree was controlled by SDS-PAGE and the molecular weight was determined using a standart protein curve.

Purification of glucose spesific binding protein by gel filtration, ion exchange chromatography and HPLC (Baldwin, 1989): The material to be purified applied to Sephadex G-200 column (0.9x23 cm), the eluate was collected as fractions and liyophilized. In addition to this, 250 μl taken from fractions 7,8,9 and 2.5 μl ^{14}C- glucose (10^{-3} mol/L) was added and incubated at room temperature for 10 minutes followed by filtration through nitrocellulose membrane. Glucose binding activities of the fractions were determined. by reading the fluid cintillation gauge.

Fractions which showed glucose binding activity were subjected to DEAE-Sepharose ion-exchange chromatography. Elution was performed with 0-0.25 mol/L sodium chloride solution in order to

obtain salt gradient and 1 ml fractions were collected. The activities of these fractions were studied by ^{14}C- glucose and the protein amounts was determined according to Lowry (Lowry, 1951). The fraction showing high activity was diyalized through Tris-HCl (pH: 7.4) for 24 hours. Glucose binding protein purified by the Cold Osmotic Shock procedure, gel filtration and ion exchange chromatographies were applied to HPLC where BIOSEP SEC-S 3000 (300x75 mm) column was used.

SDS-Polyacrilamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970): After each purification step, protein fractions were applied to SDS-PAGE and the band corresponding to the glucose binding protein and standart protein were studied. From the Rf values of protein bands in the sample, corresponding molecular weights were estimated by means of the standart curve.

Results and Discussion

The glucose binding specific activities in the shock fluid, Sephadex G-200 and DEAE- Sepharose column chromatographies were found to be 26.4 nmol glucose/ mg protein, 37.9 nmol glucose/ mg protein, 84.45 nmol glucose/ mg protein respectively (Table 1). The molecular weight was calculated to be 29 000 Da by SDS-PAGE (Table 2, Fig.4).

Table 1. Purification of glucose binding protein.

Purification steps	Protein (mg.ml^{-1})	Spesific Activity (nmolgluko./mg^{-1} protein)	Purification coefficient
Cold osmotic shock fluid	1.75	26.4	1.00
Sephadex G-200	1.47	37.9	1.44
DEAE-Sepharose	0.475	84.45	3.2

Table.2. SDS-PAGE value of standart protein and glucose binding protein.

Standart proteins	Molecular weight	ln MW	Length	Rf
Bovine Albumin	66.000	11.11	0.8	0.13
Egg Albumin	45.000	10.70	2	0.33
Karbonic Anhidrase	29.000	10.28	3	0.50
Tripsin Inhibitor	20.100	9.91	3.5	0.58
α-Lactalbumin	14.200	9.56	5	0.83
Glucose binding protein*	29.000	10.20	3	0.50

*One band of DEAE-Sepharose column chromatography

Because of the low ratio of binding protein to total cell protein, and diffuculties in defining the binding protein, COS procedure was used in the isolation of glucose binding protein. It was composed of COS platelets' material which were loosely bound to membrane and a small amount of

protein. It has been shown that platelets of COS impair active transport system and that glucose binding protein passes to COS fluid (Yardımcı and Ulutin, 1986).

Research concerning the glucose transport in the platelets have been carried out since 1970. In 1970 Solomon and Gaut showed that 2- deoxyglucose, which could not be metabolised beyond phosphorilation, used active transport system and in 1975 Schneider reported that glucose was transported by facilitated diffusion. Challenging results were found as the sensitivity of the diffusion's to insulin was studied (Yardımcı and Ulutin, 1986; Solomon and Gaut, 1970; Schneider,1975; Karpatkin and Charmatz, 1970).

Glucose transport in isolated platelets possessing high concentration of glucose was carried out through facilitated diffusion. In low concentrations it was observed by Yardımcı that Na^+/K^+ pump which could be inhibited by inhibitors like DNP, KCN, arsenic and ouabain has an active transport linked to it (Yardımcı and Ulutin, 1986).

Similar to previous studies platelet- glucose binding protein was purified and its kinetics were studied in our laboratory. By pooling the proteins of 10 substances, the synthesized binding protein was acquired as Km:$7.5x10^{-5}$ mol/L, Vmax:12.5 nmol glucose/mg protein according to Lineweaver Burk and Scatchard graphs (Göker, et al., 1991). In platelets which have gone through COS and as a result lost its glucose binding protein, the lack of glucose active transport and the harmony between glucose binding protein and glucose kinetics proved the importance of binding protein in transport processes.

Glucose transporters are found distributed all over erythrocytes, brain, fibroblast, liver, kidneys, small intestine, fat, skeletal muscles and the heart (Devaskar and Muecler, 1988; Kayano et al., 1990; Elsas and Longo, 1992). Glucose transporters which are distributed all over the tissues are grouped as facilitated glucose transporters (GLUTs) and Na^+/glucose cotransporters (SGLTs). In human tissues the Km values of this protein were calculated as Glut1≅5-30 mM, Glut2≅60 mM, Glut3≅10 mM, Glut4≅2-5 mM. The Km and Vmax values of this 4 isoforms for glucose transport actively possess different values (Elsas and Longo, 1992; Nagamatsu, et al., 1993). In the present work glucose binding protein was isolated from the platelets by COS procedure and purified 3.2 times compared to the initial phase by Sephadex G-200 column chromatography and DEAE-Sepharose ion exchange chromatographies (Figs 1,2), The glucose binding proteins obtained from each chromatographic step, showed similar Rt values by HPLC analysis. Each fraction acquired from DEAE-Sepharose column chromatography was observed to have the same Rt value with a single peak on the HPLC chromatogram. (Fig.3). Spectrophotometric values were determined, for each of these fractions. When the glucose binding protein activity was measured, it was clear that glucose binding protein activity was distributed fairly even among the fractions. The proteins obtained from each chromatogram, were applied to SDS-PAGE and 4 bands in COS fluid, 2 bands in Sephadex G-200 and 1 band in DEAE-Sepharose were observed. As a result the molecular weight was calculated to be 29.000 Da by SDS-PAGE. (Table 2 and Fig.4).

Our further studies which will help to clarify the subject will involve the use of more sophisticated instrumental techniques and determination of the amino acid sequence of the synthesized binding protein.
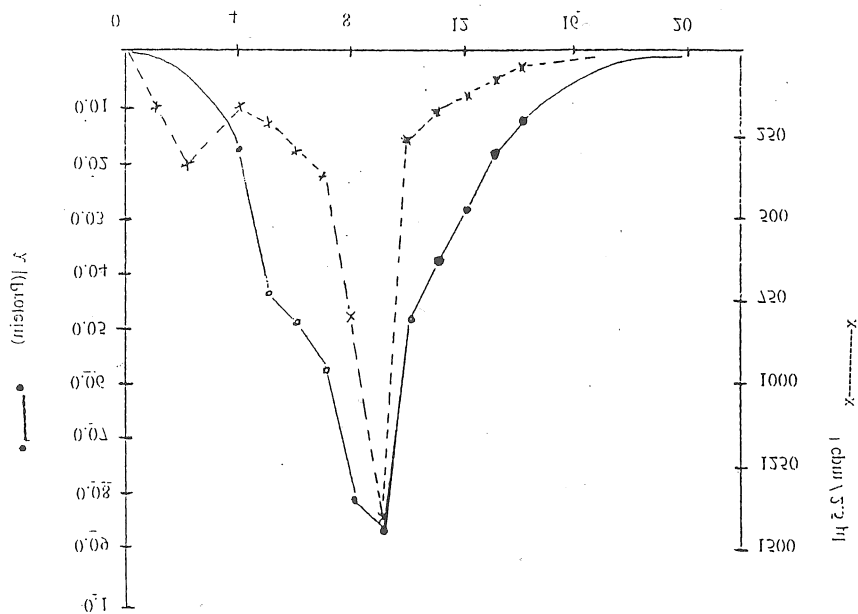
Elution volume (ml)

Fig. 1. Sephadex G-200 column chromatography of ammonium sulfaphate ppt. of cold osmotic shock-treated platelets (Column dimension: 0.9x23cm, flow rate: 21.6 ml/h, equilibrium and elution buffer: 10mmol/L Tris-HCl, pH: 7.4).
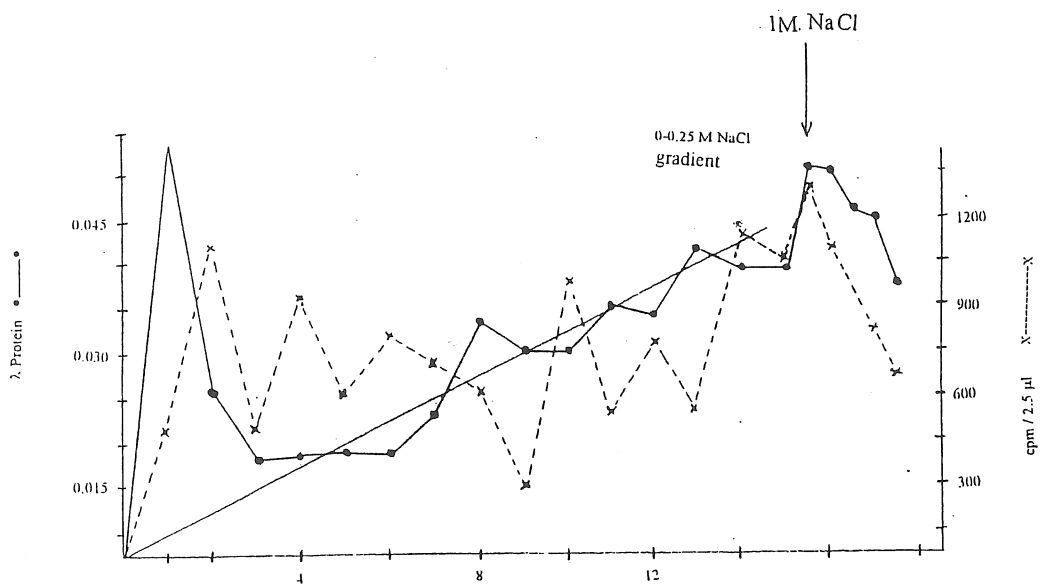
Elution volume (ml)

Fig.2. DEAE-Sepharose column chromatography of the glucose binding protein pool (fract. 7,8,9) which are synthesised from Sephadex G-200 column chromatography (column dimension: 0.6x7cm, flow rate: 54ml/h, elution buffer: 0-0.25mol/L NaCl, linear gradient between 10mmol/L Tris-HCl).
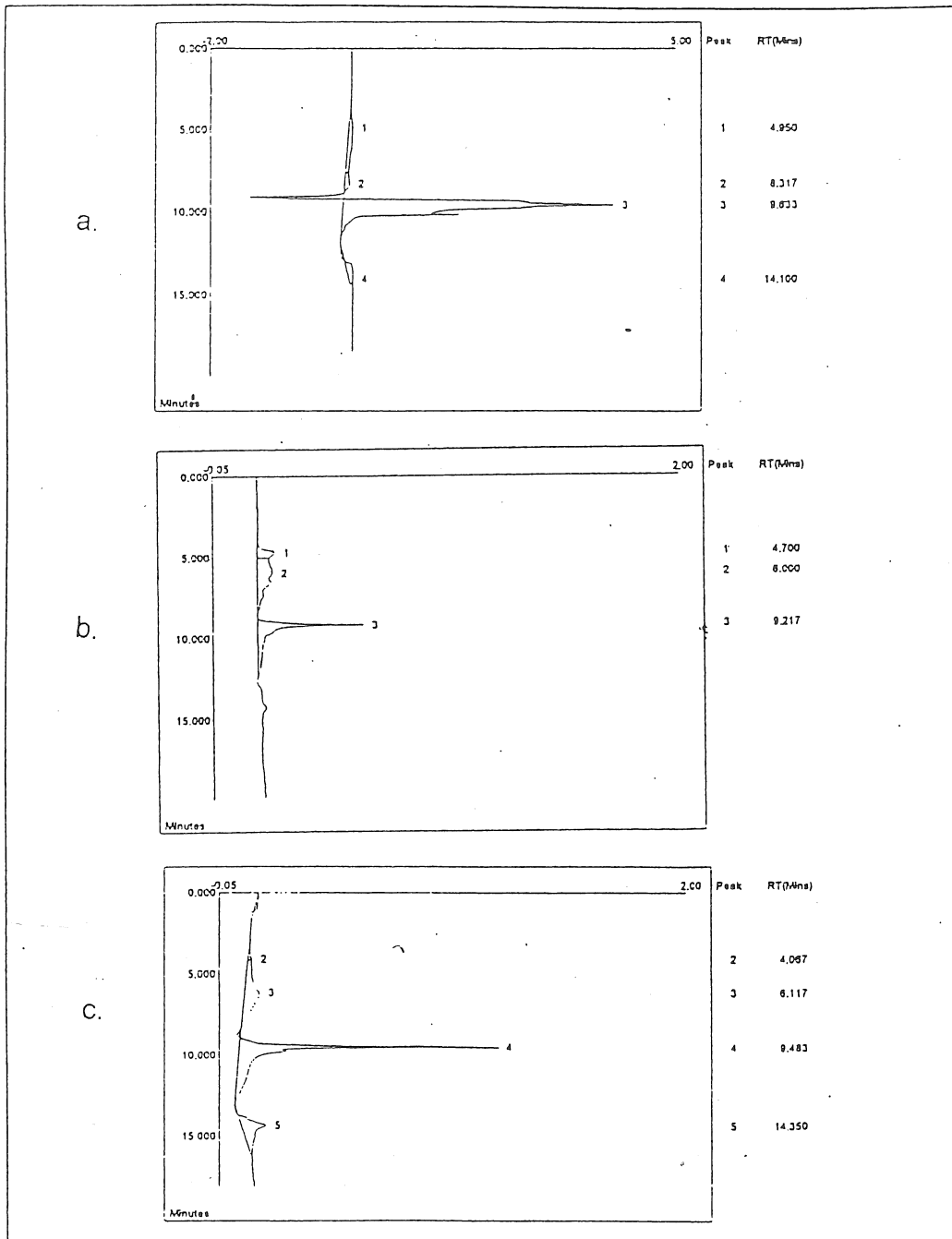
Peak	RT (Mins)
1	4.950
2	8.317
3	9.633
4	14.100

Peak	RT (Mins)
1	4.700
2	8.000
3	9.217

Peak	RT (Mins)
2	4.067
3	6.117
4	9.483
5	14.350

Fig.3. HPLC Diagrams.
a. COS Fluid b. Sephadex G-200 c. DEAE-Sepharose.
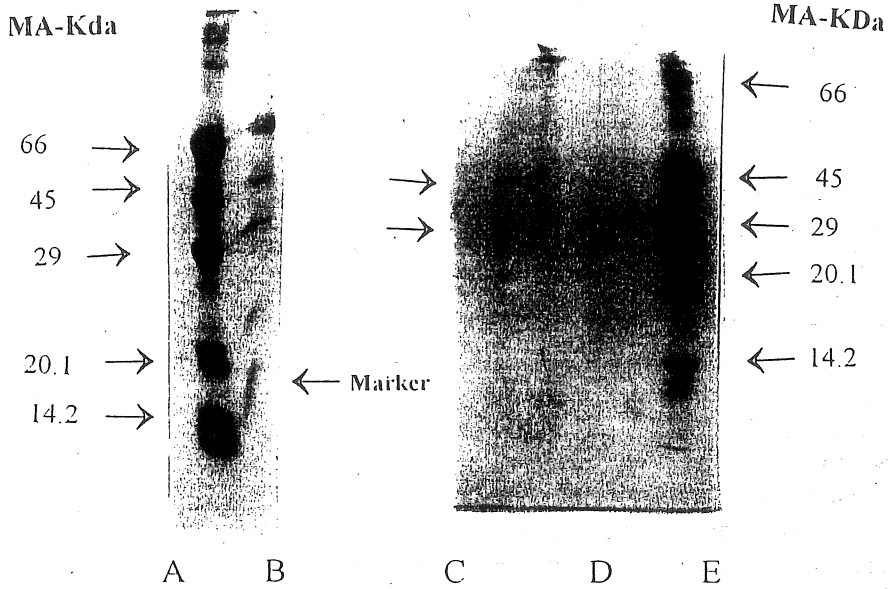
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a. COS Fluid b. Sephadex G-200 c. DEAE-Sepharose.

Fig.4. SDS-PAGE (%10) of
A. SDS-PAGE Molecular weight standarts
B. Protein fractions synthesised from COS fluid
C.Protein pool which are glucose binding protein activated, and it have been synthesis from Sephadex G-200
D. Protein band synthesised from DEAE-Sepharose ion   exchange column chromatography
E. SDS-PAGE Molecular weight standarts.

Özet

Bu çalışmada sağlıklı insan trombositlerinden glukoz bağlayıcı protein saflaştırılarak izotipi belirlendi. Glukoz bağlayıcı proteinin spesifik aktivitesi sırası ile ozmotik şok sıvısı, Sefadeks G-200 ve DEAE-Sefaroz kolon kromatografisinde 26,4 nmol glukoz/ mg protein, 37,9 nmol glukoz/mg protein, 84,45 nmol glukoz/ mg proteindir.SDS-PAGE ile yapılan çalışmalar sonucunda elde edilen glukoz bağlayıcı proteinin molekül ağırlığı 29,00 Da olarak saptandı. Buna göre insan glukoz taşıyıcılarının molekül ağırlığı ile karşılaştırıldığında trombositteki bağlayıcı proteinin diğer dokulara dağılmış halde bulunan taşıyıcı izoformuna uymadığı belirlendi. Trombosit glukoz bağlayıcı proteinin saflaştırma basamakları kullanıldıktan sonra başlangıca göre 3.2 kez saflaştırıldığı saptandı.

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