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Characterization of the carbohydrate structures of apolipoprotein H through concanavalin A affinity chromatography

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Abstract

Apolipoprotein H, also known as β2-Glycoprotein I, is a single chain highly glycosylated polypeptide of 326 amino acids. The carbohydrate content of apolipoprotein H is approximately 19% of the molecular weight. Some studies have described the main oligosaccharides forming the glycosylated chains but the carbohydrate inner structures of apolipoprotein H has not been investigated yet. This gap should be filled being glycosylation a very important process which is able to regulate the structure and the biological functions of proteins. Lectins are proteins which specifically bind carbohydrate structures. Affinity chromatography of glycoproteins on immobilized lectins, such as Concanavalin A (Con A), has been proved to be a useful method for oligosaccharide fractionation. *N*-Linked oligosaccharide structures were shown to interact with Con A according to their branching properties. In the present study, we analyzed the patterns of Con A elution of apolipoprotein H isolated from human plasma. Using Con A affinity chromatography we show that apolipoprotein H has a high degree of heterogeneity in its glycosylated structure. It allowed one to isolate two groups of apolipoprotein H molecules bearing biantennary and truncated hybrids and high mannose and hybrid oligosaccharides. Since Con A affinity chromatography allows fractionation of molecules differing in the extent of carbohydrate branching irrespective of the sialyl residues, we can conclude that mannose residues are masked with other sugars such as galactose-β(1-4)*N*-acetylglucosamine, galactose-β(1-3)*N*-acetyl-galactosamine and sialic acid

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linked α(2-6) to galactose or to *N*-acetylgalactosamine, or capped with sulfated residues. Thus, according to our results apolipoprotein H presents truncated hybryd or hybrid-type carbohydrate chains which bear few unmasked mannose residues as terminal sugar. Moreover, isoelectrofocusing of apolipoprotein H forms fractionated on Con A demostrates that weakly bound material presents a predominance of more acidic isoforms than that firmly bound to the lectin, indicating that weakly bound fractions contain molecules which are more negatively charged and that Con A is able to separate glycosylated forms which are not discriminated by isoelectrofocusing. © 1997 Elsevier Science B.V.

Keywords: Apolipoprotein H; β2-Glycoprotein I; Concanavalin A (Con A) affinity chromatography; Glycosylation

1. Introduction

Apolipoprotein H (apo H), also known as β2-Glycoprotein I, is a human plasma protein which belongs to the very large family of glycoproteins. Apo H is a single chain polypeptide of 326 amino acids, with an apparent molecular weight of 50 KDa and is highly glycosylated (Lozier et al., 1984). The carbohydrate content of apo H is approximately 19% of the molecular weight (Day et al., 1989). The reported five glucosamine-attached oligosaccharide side chains are composed of galactose, mannose, *N*-acetylglucosamine, fucose, and *N*-acetylneuraminic acid (Kamboh et al., 1988). Compared to other human plasma proteins apo H has a high content of cysteine (6.2%) and proline (8.3%) (Lozier et al., 1984). Apo H existed as 40% β-sheet, 30% β-turn and 30% random coil (Walsh et al., 1990), but after deglycosylation the primary structure of the polypeptide chain is maintened and the secondary structure alters with greater number of β-turns, accompanied by reduction in random coil structures (David et al., 1994).

Apo H is composed of five repeating domains of about 60 amino acids each with a conserved pattern of cysteine residues (Steinkasserer et al., 1991). Carbohydrates are mainly linked to asparagine residues in the carbohydrate acceptor sequence Asn-X-Ser/Thr (Lennarz, 1980). The oligosaccharides are attached to asparagine residues at positions 143, 164, 169, 174, and 234 (Lozier et al., 1984). At asparagine-174 and asparagine-234 the acceptor sequence is Asn-Trp-Ser/Thr. This sequence is very rare among glycoproteins (Lozier et al., 1984).

Apo H exhibits a high degree of polymorphism (Sepehrnia et al., 1989). Its polymorphic variations can be classified into two types: a postsynthetic polymorphism consisting of multiple isoforms due to differences in the number of terminal sialic acid moieties and a genetical polymorphism revealing structurally different isoforms with different isoelectric points, representing a polypeptide chain with substituted amino acids (Kamboh et al., 1988).

Although its physical and chemical characteristics were well studied its physiological functions remain unknown. Nakaya (Nakaya et al., 1980) proposed that it acts as an activator of lipoprotein lipase. Schousboe (Schousboe, 1985) has presented

evidence that Apo H binds to platelet membranes, interacts with negatively charged macromolecular structures including heparin, DNA, phospholipids, modulates the activity of adenylate cyclase and may be involved in blood coagulation since it inhibits the prothrombinase activity on human platelets (Nimpf et al., 1986). Apo H is supposed to be involved in haemostasis since in vitro it displays anticoagulant properties (McNeil et al., 1990).

Some studies (Kamboh et al., 1988; Steinkasserer et al., 1991) have described the main oligosaccharides forming the glycosylated chains but the carbohydrate inner structures of apo H has not been investigated yet. This gap should be filled, glycosylation being a very important process which is able to regulate the structure and the biological functions of proteins. In particular asparagine glycosylation is very important for the appropriate folding and assembly of intact proteins (Imperiali and Rickert, 1995).

Lectins are proteins which specifically bind carbohydrate structures. Affinity chromatography of glycoproteins on immobilized lectins, such as Concanavalin A (Con A), has been proved to be a useful method for oligosaccharide fractionation (Baenziger and Fiete, 1979; Cummings and Kornfeld, 1982). *N*-Linked oligosaccharide structures were shown to interact with Con A according to their branching properties as follows: triantennary, tetraantennary, and bisecting oligosaccharides do not bind to Con A, biantennary and truncated hybrids weakly bind to Con A, and high mannose and hybrid oligosaccharides firmly bind to Con A.

In the present study, we analyzed the patterns of Con A elution of apo H isolated from human plasma in order to elucidate the microheterogeneity of glycosylations characterizing apo H structure.

2. Materials and methods

2.1. Materials

Tris, urea, Tween 20, acrylamide and *N,N'*-methylene-bis-acrylamide, analytical grade, were purchased from Bio-Rad (Richmond, CA). Gel Bond and ampholytes (pH = 4–6.5 and pH = 6.5–9) were obtained from LKB (Bromma); nitrocellulose sheets from Bio-Rad. Rabbit polyclonal antibody to apo H was kindly supplied by Behring (Scoppito). Goat anti-rabbit IgG (alkaline phosphatase conjugated) and the alkaline phosphatase conjugate substrate kit were purchased from Bio-Rad. Molecular weight standards (low M_w) were from Bio-Rad. Con A-Sepharose was purchased from Sigma (Milan).

2.2. Isolation and purification of apo H

Apo H was isolated from human plasma and purificated through a combination of affinity chromtography and continuous elution electrophoresis as previously described (Gambino et al., 1996).

2.3. Concanavalin A (Con A) lectin affinity chromatography

Lectin column (2.5 ml Con A-Sepharose, Sigma) was equilibrated with a buffer containing 10 mM Tris-HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and 0.01% Thimerosal pH 8.0 (Con A buffer) at a flow rate of 0.3 ml/min. Approximately 55 μg of apo H diluted in 1 ml was applied to the column at a flow rate of 0.1 ml/min to allow sample interaction with the lectin. After this incubation, Con A buffer was added to the column at a flow rate of 0.2 ml/min for 60 min in order to wash away unbound sample. Weakly bound apo H was eluted from the Con A column with 10 mM α-D-methylglucopyranoside (Sigma) at a flow rate of 0.3 ml/min for 60 min. Firmly bound apo H was subsequently eluted with 300 mM α-D-methylmannopyranoside (Sigma). Eluates containing unbound, weakly bound and firmly bound were collected into 0.6 ml fractions. If unbound fractions were pooled and loaded again on the same column, no bound material was recovered. Similarly, if bound fractions were pooled, dialyzed, and reloaded on Con A affinity column, neither unbound nor weakly bound material was found, and bound materials were eluted in the same proportion as the initial one. Weakly bound material was always eluted with 10 mM α-D-methylglucopyranoside. The same profile was obtained when other samples of apo H were run through the column.

2.4. Protein dot-blotting of Con A fractions

Rapid screening of Con A fractions was conducted in the Bio-Dot microfiltration apparatus (Bio-Rad). A sheet of nitrocellulose is clamped between the gasket and the 96-well sample template. Fifty microliters of sample is allowed to filter through the membrane. After the antigen is immobilized, the nitrocellulose is incubated in 4% albumin blocking solution. Nitrocellulose is subsequently incubated with rabbit IgG anti-apo H (Behring) at a 1/2000 dilution at room temperature for 1.5 h, and then with goat IgG anti-rabbit IgG labelled with alkaline phosphatase at a 1/6000 dilution (Sigma). Dots are visualized with the procedure contained in the Immuno-Blot Assay kit (Bio-Rad).

2.5. ELISA method of Con A fractions

Apo H in both plasma and column fractions was measured by an ELISA method. We analysed the fractions which resulted positive in the dot-blotting assay, since the dot-blotting assay is not suitable for a quantification especially when the level of the sample is very low. As a matter of fact, we found very low traces of apo H just in one of the three fractions which resulted positive after the dot-blotting assay suggesting that a specific absorption of antibody could occur on the nitrocellulose. This overestimation due to the enzymatic reaction could mimic large amounts of sample.

2.6. SDS-PAGE and Western blot analysis of Con A fractions

Positive fractions from dot-blotting analysis were subjected to 12% SDS-PAGE electrophoresis in a Bio-Rad Mini Protean II apparatus under non-reducing conditions. Electrophoresis buffer was 25 mM Tris, 192 mM Glycine, 0.1% SDS pH 8.3. Subsequently, proteins were transferred onto nitrocellulose by electroblotting in 25 mM Tris, 192 mM Glycine, and 20% methanol pH 8.3 at 295 mA for 100 min. The nitrocellulose membrane was then tested with the specific antibody, and the bands were visualized with a second, alkaline phosphatase labelled anti-rabbit IgG antibody in the presence of the specific substrate.

2.7. Isoelectrofocusing and immunoblotting of Con A fractions

Analytical isoelectrofocusing of unbound, weakly bound, and firmly bound fractions was performed on 5% polyacrylamide gels (Cassader et al., 1994) containing 2% ampholytes (pH 4–6.5 and 6.5–9, 3.75:1 v/v) and 3 M urea. IEF was carried out in a Bio-Phoresis TM Horizontal Electrophoresis Cell (Bio-Rad), using a Model 3000/300 power unit. The focusing was carried out at 10 W constant power for 3 h, and then the proteins were transferred overnight at room temperature onto 0.45μm pore size nitrocellulose membrane by simple diffusion. After transfer, the nitrocellulose was processed using the same procedure as for Western blot.

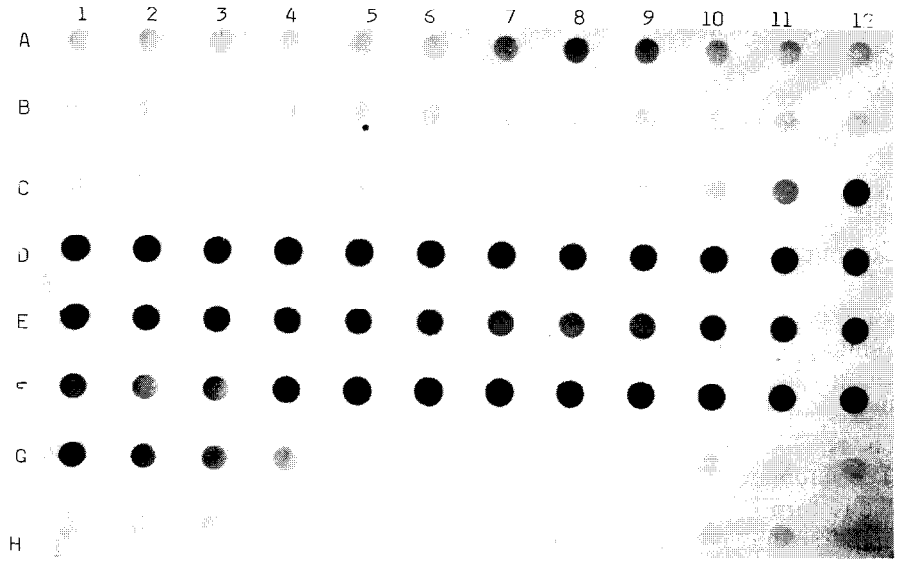

Fig. 1. Dot-blotting analysis of apo H eluted from Con A column: A1-C5 dots represent unbound fractions; C7-E12 dots are weakly bound fractions and F1-H6 dots are firmly bound fractions.

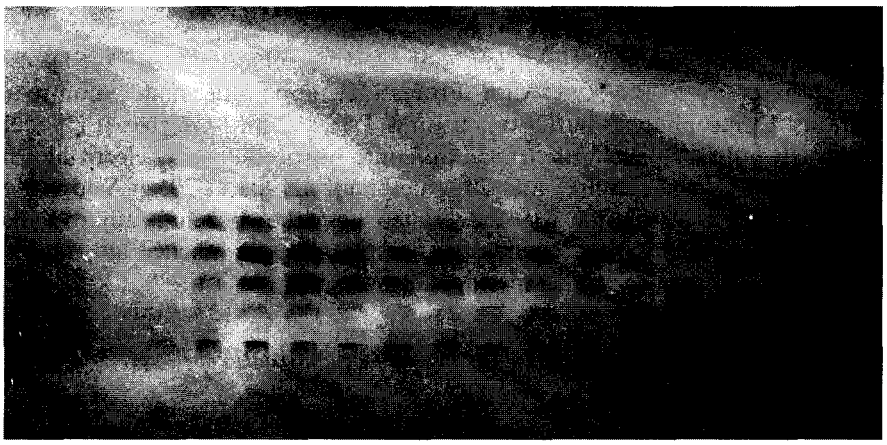

Fig. 2. Analysis of apo H isoforms separated through IEF followed by Western blot: Con A fractions were subjected to denaturated IEF in 5% acrylamide gels with pH 4–8 ampholytes, tranferred to nitrocellulose and probed with specific antibody. Going from left to right: unbound fractions (lanes 1–2). Weakly bound fractions contained molecules of apo H having a predominance of more acidic isoforms (lanes 3–18). Anode is at the top; cathode is at the bottom.

3. Results

Dot-blotting analysis mirrors the chromatographic pattern of apo H eluted from Con A column (Fig. 1). Apo H interacted with the Con A lectin. Detectable amounts of protein were not found in the first chromatographic fractions suggesting that apo H is not eluted with Con A buffer. After adding a buffer with low sugar concentration (10 mM glucoside) a large amount of apo H was recovered. These molecules of apo H weakly bound to the lectin. When a higher sugar concentration (300 mM mannoside) was added, most of the sample applied was eluted. These molecules of apo H firmly bound to the column having high affinity for the lectin.

Column fractions, which resulted positive in the dot-blotting assay, were tested for their content of apo H. For this purpose we used an ELISA method. Unbound fraction contains very low traces of apo H representing less than 0.1% of the total amount applied onto the column. Weakly bound apo H represents 18% of the total amount, whereas firmly bound apo H is 82% of the total amount. Final recovery ranges from 84 to 101%.

The results of isoelectrofocusing of Con A fractions are illustrated in Figs. 2 and 3. The unbound fraction, run in Fig. 2, lane 1 contains very low traces of some acidic isoforms, while no isoforms are detected in lane 2. On the contrary, weakly bound fractions contained molecules of apo H having a predominance of more acidic isoforms (Fig. 2, lanes 3–18), while firmly bound fractions contained molecules of apo H having a predominance of more cathodic isoforms (Fig. 3, lanes 8–13). Isoforms H3 and H4 were mostly represented in the molecules of apo H

binding weakly to the lectin. Isoforms H1, H2, and H3 are mostly present in the molecule of apo H binding firmly to the lectin (Kamboh et al., 1988).

The results of Western blot of Con A fractions are shown in Figs. 4 and 5. Weakly bound (Fig. 4, lanes 3–10) and firmly bound (Fig. 5, lanes 2–10) fractions migrated with an apparent M_w of approximately 50 kDa demonstrating that no proteolytic cleavage had occurred during chromatography. Unbound fractions didn't contain apo H (Fig. 4, lane 2). In Fig. 5 bands are stronger than those shown in Fig. 4, indicating that most of apo H firmly bound to the column.

4. Discussion

We have studied the molecular structure of the carbohydrate moiety of apo H purified from human plasma. Lectin affinity chromatography was used to analyse apo H according to the characteristics of its carbohydrate chain inner to sialic acid residues. Con A has the advantage of sialyl residues not being recognized. It allowed us to isolate two groups of apo H molecules bearing biantennary and truncated hybrids and high mannose and hybrid oligosaccharides. Under our working conditions we could avoid column overloading since just 55 μg of apo H was applied to 2.5 ml of gel.

Most of apo H bound both weakly and firmly to the column. Some material was found only in one of the unbound fractions but its concentration is extremely low when assayed in an ELISA method. Since apo H in the unbound fraction represents less than 0.1% of total apo H applied to the column this amount can be considered

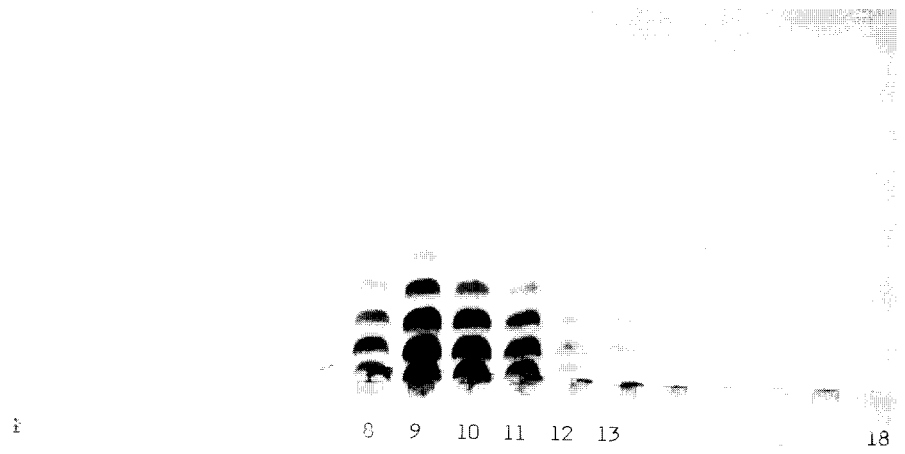

Fig. 3. Analysis of apo H isoforms separated through IEF followed by Western blot: Con A fractions were subjected to denaturated IEF in 5% acrylamide gels with pH 4–8 ampholytes, tranferred to nitrocellulose and probed with specific antibody. Going from left to right: firmly bound fractions contained molecules of apo H having a predominance of more cathodic isoforms (lanes 8–13). Anode is at the top; cathode is at the bottom.

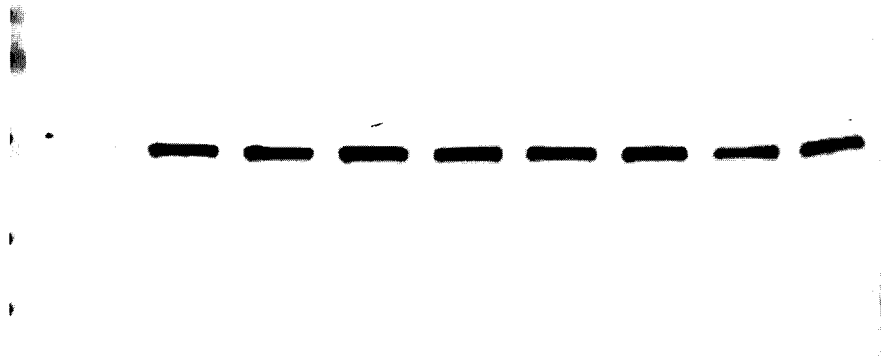

Fig. 4. Western blot of Con A fractions. Going from left to right, lane 1: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa. Lane 2: Con A unbound fraction. Lane 3–10: fractions binding weakly to Con A.

insignificant. After isoelectrofocusing electrophoresis just few acidic bands are barely detectable. Isoelectrofocusing of other apo H forms fractionated on Con A demonstrates that weakly bound material presents a predominance of more acidic isoforms than that firmly bound to the lectin, indicating that weakly bound fractions contain molecules which are more negatively charged. Biantennary and truncated hybrid rather than high mannose-rich or hybrid carbohydrate chains may bear more sialic acid residues modifying glycoprotein overall charge (Baenziger and Fiete, 1979; Cummings and Kornfeld, 1982). Sialic acid is a negatively charged terminal sugar which covers penultimate galactose residues. Firmly bound molecules have a predominance of cathodic isoforms which are less negatively charged. This group of molecules could be mainly organized in hybrid carbohydrate chains bearing fewer sialic acid residues rather than in high mannose oligosaccharide structures. On a molecular bases this heterogeneity indicates that each isoform may contain different glycosylation patterns (Green and Baenziger, 1988a; Green and Baenziger, 1988b; Papandreou et al., 1993a; Papandreou et al., 1993b; Sardanons et al., 1987). Heterogeneity of apo H molecular size and isoelectric point is due to the oligosaccharide units and to the degree of sialylation. However, the physiological significance of the heterogeneity of apo H has not been adequately investigated.

When the Con A fractions were subsequently subjected to SDS-PAGE, they resolved into a band whose molecular weight was some 50 kDa. Apparent molecular weights didn't significantly differ from each other suggesting that lectin chro-

matography was able to isolate two classes of apo H molecules. The difference between weakly and firmly bound molecules lies in the different carbohydrate organization.

In conclusion, using Con A affinity chromatography we show that apo H has a high degree of heterogeneity in its glycosylated structure. This manuscript proves evidence that apo H is differently glycosylated. Apo H molecules bear either hybrid structures or biantennary and truncated hybrid structures. High mannose and hybrid structures bear more mannose residues than biantennary and truncated hybrid structure do. Nevertheless, in our previous observation we could barely detect mannose residues. Since Con A affinity chromatography allows fractionation of molecules differing in the extent of carbohydrate branching irrespective of the sialyl residues, we can conclude that mannose residues are masked with other sugars such as galactose-β(1-4)*N*-acetylglucosamine, galactose-β(1-3)*N*-acetylgalactosamine and sialic acid linked α(2-6) to galactose or to *N*-acetylgalactosamine (our observations), or probably capped with sulfated residues (Papandreou et al., 1993b). Thus, according to our results apo H presents truncated hybryd or hybrid-type carbohydrate chains which bear few unmasked mannose residues as terminal sugar.

The expected significance of the study is that information on glycosylation is important to understand key processes involved in the regulation of the biological functions of proteins. In particular asparagine glycosylation is very important for the appropriate folding and assembly of intact proteins. As a matter of fact the potential of *N*-linked oligosaccharides for structural variation is not confined to

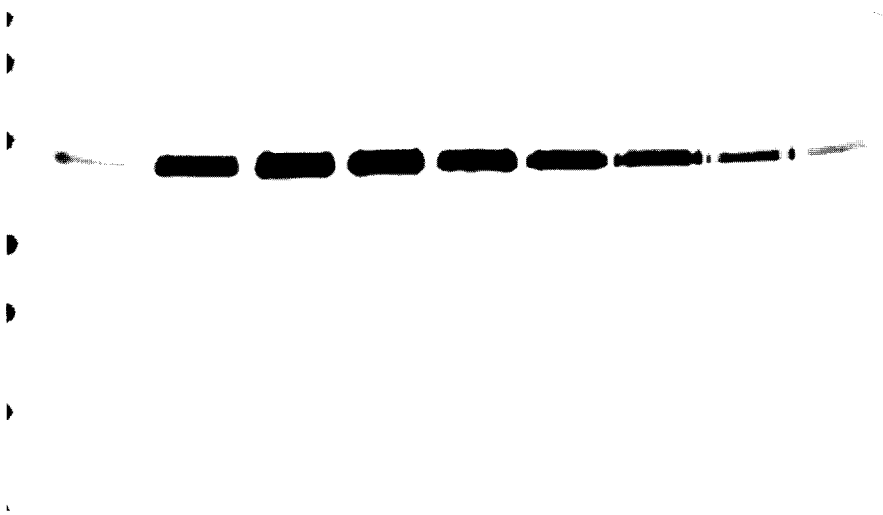

Fig. 5. Western blot of Con A fractions. Going from left to right, lane 1: prestained molecular mass markers marked with black points are (from top to bottom): 106.0, 80.0, 49.5, 27.5, 18.5 kDa. Lane 2–10: fractions binding firmly to Con A.

their chain-terminating sugars. Carbohydrate branching also affects the biological activity of glycoproteins by inducing variations in their tertiary structure (Papandreou et al., 1993a). Moreover, the high amount of sialic acid is known to regulate the blood circulation of glycoproteins by protecting them from hepatic galactose receptor (Ashwell and Harford, 1982; Morell et al., 1971). Thus, further experiments get necessary to understand whether apo H is involved into a similar physiological mechanism.

Acknowledgements

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