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Immobilized *Marasmius oreades* agglutinin: use for binding and isolation of glycoproteins containing the xenotransplantation or human type B epitopes

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The type B–specific lectin from the mushroom *Marasmius oreades* was immobilized onto Sepharose 4B. The immobilized lectin bound murine laminin and bovine thyroglobulin, glycoproteins that contain the Galα1,3Galβ1,4GlcNAc epitope. This epitope is responsible for hyperacute rejection of xenotransplants from lower mammals to humans, Old World monkeys, or apes. The immobilized lectin also bound a fraction of serum proteins from type B human serum but little or none from type A or O(H) serum. The major protein bound from human B serum was a portion of the α_2-macroglobulin. Treatment of this fraction with N-glycosidase F resulted in decreased molecular weight of bands associated with α_2-macroglobulin and loss of their *M. oreades* lectin reactivity, whereas on treatment with coffee bean α-galactosidase, this bound fraction also lost reactivity with *M. oreades* lectin but became reactive with *Ulex europaeus* I lectin, suggesting the presence of L-fucosyl-α1,2-terminated structures. The presence of blood group epitopes on α_2-macroglobulin has been detected previously by immunological methods, but this is the first isolation and characterization of the specifically glycosylated fraction of this serum protein. The immobilized lectin also bound a number of proteins from pig, rabbit, and rat serum that were distinct in electrophoretic mobility from the human B-serum components and presumably contain the xenotransplantation epitope among their glycan structures. This report further demonstrates the utility of immobilized lectins in isolating and characterizing glycan structures of naturally occurring glycoproteins.

Key words: agglutinin/Galα1,3Galβ1,4GlcNAc/*Marasmius oreades*/mushroom lectin/serum glycoproteins

Introduction

The blood group B–like determinant, Galα1,3Galβ1, 4GlcNAc, is widely expressed in mammalian species but absent in humans and catarrhines (Old World monkeys and apes) (Galili *et al*., 1988). Naturally occurring antibodies toward this trisaccharide epitope are regularly found in human serum, constituting as much as 1% of the circulating IgG molecules (Galili, 1988). These antibodies play vital roles, including the identification and subsequent removal of senescent human red blood cells and pathogenic erythrocytes (Galili *et al*., 1988), immune protection by complement lysis of certain bacteria, and inactivation of mammalian type C retroviruses, thereby inhibiting transmission of oncoviruses from other mammals to humans (Schenkel-Brunner, 2000). More important, these antibodies cause hyperacute rejection of grafted tissue, posing a major barrier for xenotransplantation to human recipients (Galili *et al*., 1988).

We have recently elucidated the carbohydrate binding specificity of a blood group B–specific lectin from the mushroom *Marasmius oreades* (MOA) (Winter *et al*., 2002). Binding studies by the techniques of hapten inhibition of agglutination and precipitation, and hapten binding in solution by isothermal titration calorimetry established that MOA possesses an extended binding site for the Galα1, 3Galβ1,4GlcNAc structure, in contrast to other B-specific lectins, such as the *Griffonia simplicifolia* B_4 isolectin (Goldstein and Winter, 1999), which recognize only the α-galactosyl end group. Carbohydrate binding studies also revealed a high affinity of MOA for the type B branched trisaccharide (Galα1,3[Fucα1,2]Gal) and for the α1,3- but not the regioisomeric α1,2-, α1,4-, or α1,6-galactobioses.

Lectin affinity chromatography has served as a valuable technique for the separation and purification of glycans and glycoconjugates (Goldstein *et al*., 1997). This article describes the application of immobilized MOA for the fractionation of glycoconjugates from human sera and affords further insight into the carbohydrate specificity of this unique lectin.

Results and discussion

Interaction of glycans and glycoconjugates with MOA-Sepharose 4B

Various glycans and glycoconjugates were initially applied to the MOA-Sepharose column to confirm the carbohydrate binding specificity of the immobilized agglutinin. Laminin purified from the EHS sarcoma and bovine thyroglobulin, both of which contain multiple Galα1,3Galβ1, 4GlcNAc end groups (Spiro and Bhoyroo, 1984; Knibbs *et al*., 1989), bound tightly to immobilized MOA and were eluted with 20 mM diaminopropane (Figure 1). The appearance of two peaks in the elution profile of laminin reveals heterogeneity in its glycan structure. After degalactosylation with

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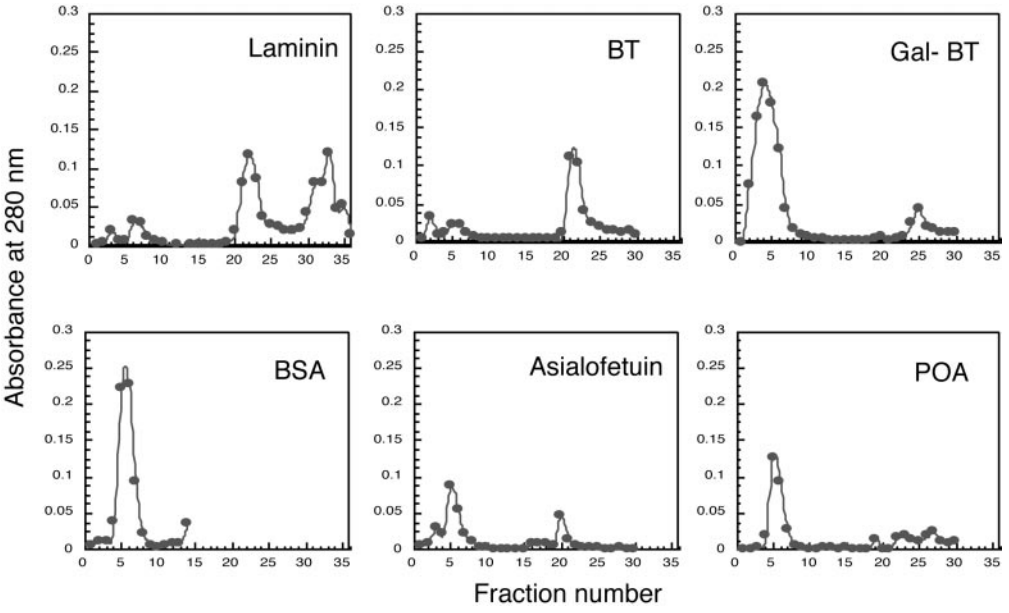

Fig. 1. Elution profile of glycans and glycoconjugates from MOA-Sepharose 4B. Elution with 1,3-diaminopropane begun at fraction 16. BT, bovine thyroglobulin; Gal-BT, α-galactosidase-treated BT; POA, pigeon ovalbumin.

coffee bean α-galactosidase, bovine thyroglobulin passed through the column, as did asialofetuin, both of which contain terminal Galβ1,3/4GlcNAc saccharide residues that are thus not recognized by the lectin. Pigeon ovalbumin, a glycoprotein that has recently been shown to contain Galα1,4Gal-terminated *N*-glycans (Suzuki *et al.*, 2001), similarly did not bind to the MOA-Sepharose column. Blood group H-2 substance, which has terminal Fucα1,2Galβ1,4GlcNAc chains, also failed to bind to MOA-Sepharose (data not shown), although the trisaccharide Fucα1,2Galβ1,4Glc exhibited low but measurable binding in solution (Winter *et al.*, 2002). Considered together, these results indicate the trisaccharide specificity of immobilized MOA, that is, its requirement of the Galα1,3Gal linkage for binding. Galactomannan, which has Galα1,6Man branches on a linear Manβ1,4Manβ backbone, is known to react strongly with GS I-A_4 and -B_4 isolectins (Murphy and Goldstein, 1977). The failure of galactomannan to bind to immobilized MOA further demonstrates this lectin's extended binding site specificity.

Interaction of various blood sera with MOA-Sepharose 4B

Serum samples obtained from human individuals belonging to blood groups A_1, A_2, B, and O as well as from animals (pig, rat, and rabbit) were obtained for study. All human individuals were of secretor type. After applying the serum sample to the column and washing with phosphate buffered saline (PBS) to remove the unbound components, subsequent elution with 20 mM diaminopropane in 0.15 M NaCl afforded the bound components, collected in 15 fractions of 1-ml volume (Figure 2). As expected, the amount of bound protein was minimal for the blood group A_1 and A_2 sera, whereas a significant amount was bound in the case of four blood group B individuals (designated B-1, -2, -3 and -4; data shown for 1 and 2 only), and a somewhat lesser amount for type O.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the bound fractions of the various sera revealed both qualitative and quantitative differences in the protein components. Most important, the major band seen in the case of the four type B serum samples corresponds to a protein with a molecular weight of ~185 kDa (Figure 3A). Protein sequencing of this component led to its identification as α_2-macroglobulin. Its identity was confirmed by the formation of a sharp precipitin band on immunodiffusion against rabbit anti-α_2-macroglobulin (data not shown), as well as strong staining on a western blot probed with the rabbit antibody and alkaline phosphatase–labeled anti-rabbit IgG (Figure 3B). It was further noted that three to four prominent bands having molecular weights between about 90 kDa and 185 kDa stained with the anti-α_2-macroglobulin, which were also apparent in an authentic sample of human α_2-macroglobulin. These same bands also stained with biotinylated MOA and with blends of monoclonal anti-B antibodies (data not shown).

To determine the nature of the linkage of the blood group B epitope to α_2-macroglobulin, the glycoprotein was treated with N-glycosidase F. A distinct shift of bands to lower molecular masses was observed on SDS–PAGE (Figure 4A). Staining of these bands on western blots with biotinylated MOA/streptavidin phosphatase was strongly attenuated, indicating removal of the type B epitope (Figure 4B). This is not surprising inasmuch as α_2-macroblobulin is known to contain eight *N*-linked chains per 170-kDa monomer with no *O*-linked chains (Sottrup-Jensen *et al.*, 1984). Treatment of a duplicate western blot with alkaline borohydride prior to MOA staining had no

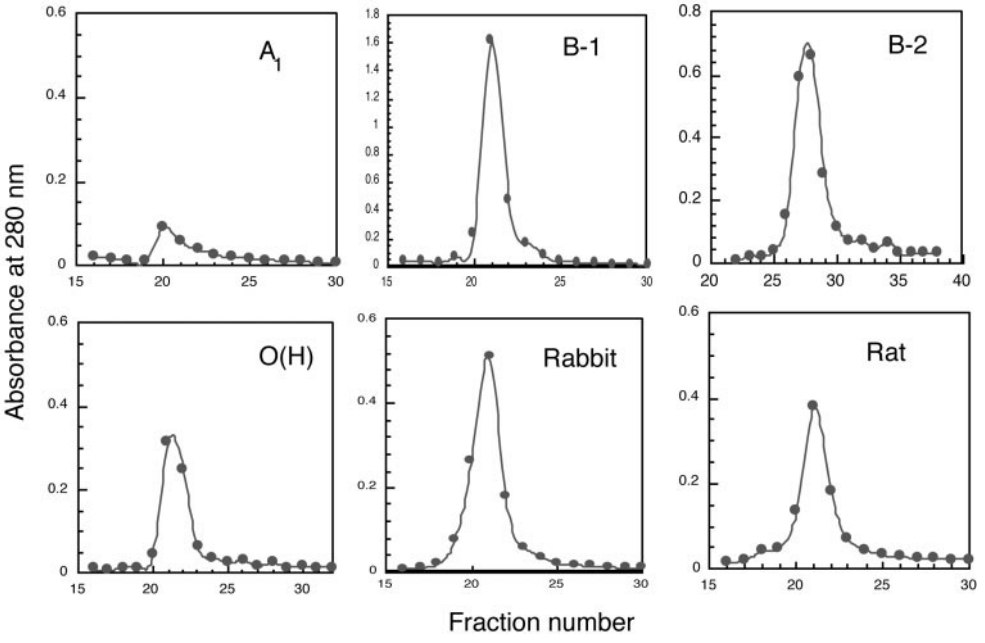

Fig. 2. Elution profile of human and animal serum components from MOA-Sepharose using 20 mM 1,3-diaminopropane, pH 11. Flow-through and wash fractions not shown. Elution profiles for serum samples B-3 and B-4 were similar to sample B-1.

noticeable effect on the undigested lanes but did eliminate a few weakly staining bands that remained in some samples after N-glycosidase F digestion (data not shown). Most likely, the weakly staining bands remaining after N-glycosidase F digestion represent small quantities of other proteins containing *O*-linked α-Gal epitopes, because α_2-macroglobulin itself contains no *O*-linked carbohydrate. Treatment of the fractions containing mostly α_2-macroglobulin with coffee bean α-galactosidase abolished its binding to the immobilized MOA column, as well as its reactivity on western blots to biotinylated MOA (Figure 5), or to monoclonal anti-B antibodies (data not shown), also indicating the presence of Galα1,3Gal end groups on these *N*-linked glycans.

To further probe the nature of the glycosyl moiety reactive with the MOA lectin, we used three additional lectins: *Euonymus* [syn. *Evonymus*] *europaeu*s (EEA), *Ulex europaeus* I (UEA I), and *Lotus tetragonolobus* (LTA) lectins. EEA has complex specificity and recognizes, among others, terminal Gal linked α1,3 to a subterminal sugar (Petryniak and Goldstein, 1986). Indeed, this lectin gave precipitin bands on agar gel diffusion against the α_2-macroglobulin from B-2 and B-3 sera. Binding of α_2-macroglobulin to immobilized MOA and its precipitation with EEA indicates the presences of Galα1,3Galβ1,3/4GlcNAc groups as a minimal structure. Digestion of the bound fraction from sample B-3 with α-galactosidase abrogated its reactivity with both lectins, as expected. The digested fraction acquired the capacity to precipitate with UEA-I, but it still failed to react with LTA.

These results indicate the presence of fucosylated blood group B structures on the fraction of α_2-macroglobulin binding to immobilized MOA. Although UEA-I reacts weakly with H-1 structures (Fucα1,2Galβ1,3GlcNAc) (Petryniak and Goldstein, 1986; Mollicone *et al*., 1996), it cannot precipitate H-1 containing glycoconjugates, indicating that the majority of H glycans remaining after α-galactosidase digestion are undoubtedly of type 2 structure (Fucα1,2Galβ1,4GlcNAc), which interact strongly with this lectin. LTA also reacts with H-2 structures, but not at all with H-1 (Pereira and Kabat, 1974), so its lack of reactivity in this case may reflect an insufficient quantity and/or improper presentation of H-2 structures. Indeed, Yan *et al.* (1997) showed that certain glycoconjugates containing H-2 ligands reacted very poorly with immobilized LTA. Interestingly, sample B-2, after α-galactosidase digestion, failed to react with either fucose-binding lectin, suggesting that there may be individual differences in the quantity and/or structure of the αGal determinants on the serum proteins, such as a preponderance of H-1 structures.

The presence of covalently linked ABO(H) blood group antigens on human plasma α_2-macroglobulin and von Willebrand factor was reported by Matsui *et al.* (1993), using antibodies to the blood group substances. We have now confirmed this finding for α_2-macroglobulin by isolation and partial characterization of this protein containing blood group B epitopes from the serum of type B individuals.

In some of the MOA-Sepharose-bound serum samples, relatively weak bands in the 50–60 kDa range were identified by *N*-terminal amino acid sequence and by antibody staining of western blots as IgG heavy chains and antithrombin III. These bands, however, were present in type A and O serum also and did not stain on blots with biotinylated MOA. Furthermore, controls of fractions of serum bound to and eluted by diaminopropane from underivatized Sepharose also contained these two proteins (data not shown), indicating that a fraction of IgG and

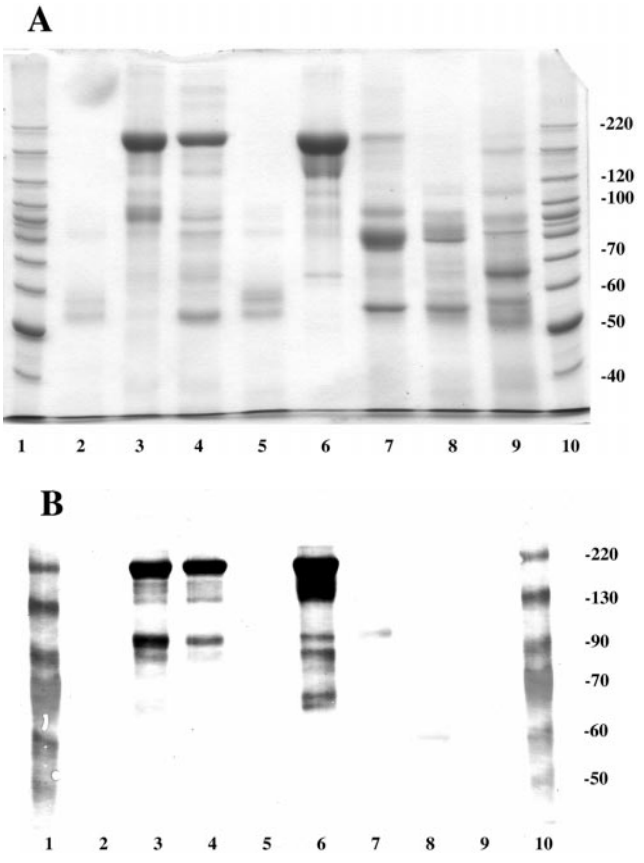

Fig. 3. SDS–PAGE (7.5% gel) and corresponding western blot of serum fractions bound to immobilized MOA. Samples in lanes are: 1 and 10, standard protein ladder; 2, A_1 serum; 3, B-3 serum; 4, B-1 serum; 5, O serum; 6, standard α2-macroglobulin; 7, pig serum; 8, rabbit serum; 9, rat serum. (**A**) Coomassie-stained gel (standard protein ladder); (**B**) western blot (prestained standard protein ladder) probed with rabbit anti-human α2-macroglobulin followed by alkaline phosphatase–labeled goat anti-rabbit IgG, and visualization with SigmaFast BCIP/NBT reagent.

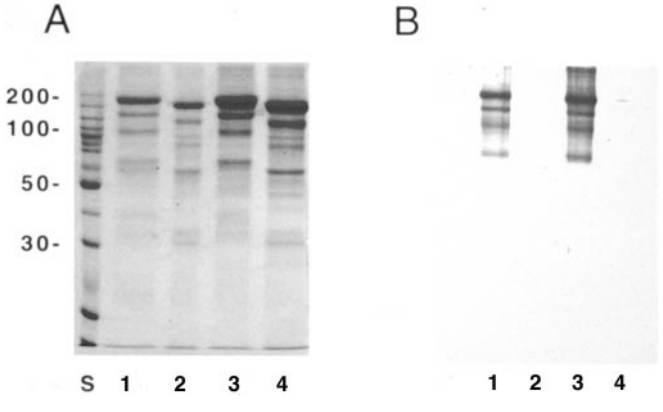

Fig. 4. Effect of N-glycosidase F digestion of type B serum fractions bound to immobilized MOA. SDS–PAGE (10% gel) and corresponding western blot of undigested (lanes 1, 3) and digested (lanes 2, 4) MOA-bound fraction from sample B-4 (lanes 1, 2) and B-2 (lanes 3, 4). (**A**) Coomassie-stained gel (lane S, Benchmark protein ladder [Invitrogen]); (**B**) western blot probed with biotinylated MOA followed by alkaline phosphatase–labeled streptavidin and development with SigmaFast BCIP/NBT reagent.

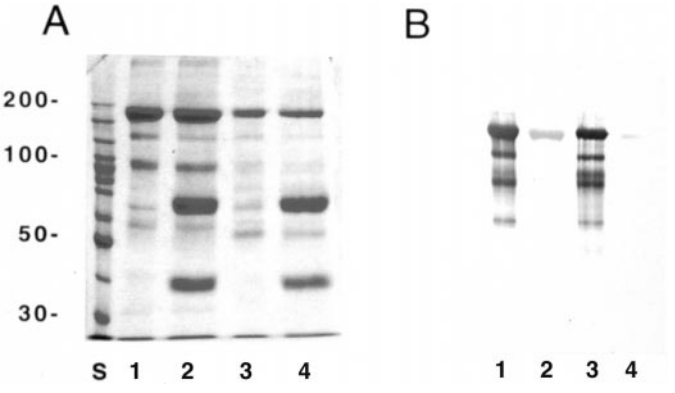

Fig. 5. Effect of α-galactosidase digestion of type B serum fractions bound to immobilized MOA. SDS–PAGE (7.5% gel) and corresponding western blot of undigested (lanes 1, 3) and digested (lanes 2, 4) MOA-bound fraction from sample B-4 (lanes 1, 2) and B-1 (lanes 3, 4). Panels and treatments as for Figure 4. Extra bands at 40 kDa and 66 kDa are the α-galactosidase and bovine serum albumin present in the commercial preparation.

antithrombin III may exhibit affinity for the Sepharose matrix itself. Thus when using Sepharose-immobilized lectins to isolate lectin-bound components from serum or other biological fluids, a pretreatment of the serum with underivatized Sepharose is indicated.

The present article clearly demonstrates the utility of immobilized MOA for the affinity purification of glycoconjugates containing the Galα1,3Galβ1,4GlcNAc trisaccharide sequence and serum components from human and animal sera. Immobilized MOA, with a unique and extended specificity for the Galα1,3Galβ1,4GlcNAc trisaccharide, should prove to be a very valuable probe for the detection, separation, and characterization of these biomedically important glycoconjugates.

Materials and methods

Materials

MOA was purified as reported earlier (Winter *et al.*, 2002). Cyanogen bromide–activated Sepharose 4B, bovine serum albumin, asialofetuin, bovine thyroglobulin, coffee bean α-galactosidase, rabbit antibody against α_2-macroglobulin, alkaline phosphate–labeled secondary antibodies, and SigmaFast BCIP/NBT alkaline phosphatase substrate were obtained from Sigma (St. Louis, MO). Alkaline phosphatase–streptavidin was obtained from Zymed Laboratories (San Francisco, CA). Galactomannan and laminin were available from previous studies. EEA was purified on Synsorb H (Chembiomed, Edmonton, Alberta). UEA was purchased from EY Laboratories (San Mateo, CA), and LTA was available from previous studies. Pigeon ovalbumin was the gift of Dr. Y. C. Lee (Johns Hopkins University). Serum samples were prepared from the blood of pig, rat, rabbit, and seven human volunteers, one each of types A_1, A_2, and O(H) and four of type B. All were Le^a-negative and Le^b-positive and therefore assumed to be secretor-positive, except for donor B-1, who was Le^b-negative but assayed directly as secretor-positive. For serum recovery, blood

drawn into a plain vacutainer tube was placed at 37°C for 20 min. The resultant clot was dislodged using an applicator stick and removed by centrifugation at $1000 \times g$ to afford clear serum.

Immobilization of MOA on Sepharose 4B

Cyanogen bromide–activated Sepharose 4B (1.5 g) was allowed to swell in 100 ml of 1 mM HCl solution for 30 min. The supernatant was removed, and the swollen beads (6 ml) were filtered on a sintered glass funnel, washed several times with 100 ml of 1 mM HCl and finally with 20 ml of 0.1 M sodium bicarbonate buffer, pH 8.3, containing 0.5 M sodium chloride. The beads were quickly transferred to a plastic bottle containing 16.2 mg MOA in the same buffer (10 ml) and 0.2 M lactose. The plastic bottle was shaken at room temperature for 6 h and at 4°C overnight. The beads were filtered on a sintered glass funnel, washed with the coupling buffer, and then shaken in 1 M ethanolamine (12 ml) at room temperature for 2.5 h to cap all of the unreacted iminocarbonate groups. The beads were filtered, washed alternately with the coupling buffer then sodium acetate buffer (0.1 M, pH 4.0, containing 0.5 M NaCl) four times. The beads contained 2.3 mg MOA per ml Sepharose 4B, based on MOA remaining in solution. A column (1×7 cm) packed with the beads was washed with 20 mM 1,3-diaminopropane to remove any noncovalently bound lectin, and finally washed with PBS containing 0.04% sodium azide.

Binding of various glycans, glycoconjugates, and serum samples to MOA-Sepharose: general procedure

The MOA-Sepharose column was loaded with glycan, glycoconjugate, or serum sample (0.6–5 ml) and washed with PBS until 280 nm absorbance was < 0.01 (15 ml). The bound components were eluted with 20 mM 1,3-diaminopropane in 0.15 M NaCl (15 ml). The fractions collected (1 ml) were neutralized immediately with 1 M phosphoric acid (~16 μl) and analyzed either by absorbance at 280 nm for protein or by phenol-sulfuric acid assay (Dubois *et al.*, 1956) for glycan. The column was finally washed thoroughly with PBS. The fractions containing the bound components were pooled and concentrated by Amicon ultrafiltration (Millipore, Bedford, MA) using a membrane (YM 10) of 10,000 molecular weight cutoff.

Digestion of proteins or serum fractions with green coffee bean α-galactosidase or N-glycosidase F

For N-glycosidase F digestion, 20 μl of a solution of the MOA-bound serum fraction (at about 1 mg protein/ml) was heated 10 min at 80°C in 1% SDS. After cooling, 4 μl of 0.5 M phosphate buffer, pH 8.7, containing 2.5% Triton X-100 was added, followed by 1 μl N-glycosidase F from *Chryseobacterium meningosepticum* (5000 U/ml, Calbiochem, San Diego, CA). After overnight incubation at room temperature, 40 μl of SDS–PAGE sample buffer was added, followed by heating in boiling water 5 min for SDS–PAGE. Control samples were treated similarly, except for omission of the enzyme. For digestion with green coffee bean α-galactosidase, the enzyme, 1 μl containing 50 IU/ml in ammonium sulfate suspension (Sigma G-8507) was added to 20 μl of MOA-bound serum fractions adjusted to pH 6.5 with 4 μl of 1.5 *N* citrate buffer and incubated at 37°C. The reaction was allowed to proceed for at least 24 h, and immediately denatured for gel electrophoresis. A solution of bovine thyroglobulin (2 mg) in pH 6.5 phosphate buffer was digested for 8 h with 18 μl (1 IU), followed by storage at 4°C.

Electrophoresis and western blotting

SDS–PAGE analysis was carried out in 7.5% gels (0.8% cross-linked) using Tris/glycine buffer, pH 8.8, by the procedure of Laemmli (1970). The samples were denatured by boiling 5 min in buffer containing 1% SDS and 1% 2-mercaptoethanol. For western blotting, gels were equilibrated in 10 mM sodium carbonate buffer, pH 10, containing 20% methanol, electroblotted overnight at 10 V in the same buffer onto polyvinylidenedifluoride membranes (Immobilon-P, BioRad, Hercules, CA) using a BioRad Mini Trans-Blot cell. Blotted membranes were blocked with 5% nonfat dry milk and 0.5% Tween 20 in PBS for antibody probes or with 1% bovine serum albumin and 0.5% Tween 20 in PBS for lectin probes.

Ouchterlony double diffusion assays

Agar gel diffusion was carried out as described by Oudin (1980) in 50×9 mm snap lock petri dishes containing a layer of 0.85% agar in PBS ~3 mm thick in which 6 mm diameter wells were cut, holding ~50 μl of lectin or glycoprotein. Precipitin bands were observed visually with side lighting against a dark field.

Acknowledgments

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Abbreviations

EEA, *Euonymus europaeus* agglutinin; LTA, *Lotus tetragonolobus* agglutinin; MOA, *Marasmius oreades* agglutinin; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UEA I, *Ulex europaeus* I agglutinin.

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