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Comparative cross-linking activities of lactose-specific plant and animal lectins and a natural lactose-binding immunoglobulin G fraction from human serum with asialofetuin

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Plant and animal lectins bind and cross-link certain multiantennary oligosaccharides, glycopeptides, and glycoproteins. This can lead to the formation of homogeneous cross-linked complexes, which may differ in their stoichiometry depending on the nature of the sugar receptor involved. As a precisely defined ligand, we have employed bovine asialofetuin (ASF), a glycoprotein that possesses three asparagine-linked triantennary complex carbohydrate chains with terminal LacNAc residues. In the present study, we have compared the carbohydrate cross-linking properties of two Lac-specific plant lectins, an animal lectin and a naturally occurring Lac-binding polyclonal immunoglobulin G subfraction from human serum with the ligand. Quantitative precipitation studies of the Lac-specific plant lectins, *Viscum album* agglutinin and *Ricinus communis* agglutinin, and the Lac-specific 16 kDa dimeric galectin from chicken liver demonstrate that these lectins form specific, stoichiometric cross-linked complexes with ASF. At low concentrations of ASF, 1:9 ASF/lectin (monomer) complexes formed with both plant lectins and the chicken lectin. With increasing concentrations of ASF, 1:3 ASF/lectin (monomer) complexes formed with the lectins irrespective of their source or size. The naturally occurring polyclonal antibodies, however, revealed a different cross-linking behavior. They show the formation of 1:3 ASF/antibody (per Fab moiety) cross-linked complexes at all concentrations of ASF. These studies demonstrate that Lac-specific plant and animal lectins as well as the Lac-binding immunoglobulin subfraction form specific stoichiometric cross-linked complexes with ASF. These results are discussed in terms of the structure–function properties of multivalent lectins and antibodies.

Key words: asialofetuin/Lac-specific lectins/immunoglobulin subfraction

Introduction

Lectins are carbohydrate-binding proteins that are widely distributed in nature, including in plants and animals (Gabius and Gabius, 1996). They can serve various functions, including receptor-mediated endocytosis of glycoproteins or in cellular recognition processes with clinical relevance (Sharon and Lis, 1989; Gabius, 1991, 1996; Drickamer and Taylor, 1993; Mody *et al.*, 1995; Gabius and Gabius, 1996). The cellular ligands for plant and animal lectins, in many cases, are the carbohydrate chains of glycoproteins and glycolipids. Lectin binding to cell surface glycoproteins and glycolipids can result in the formation of cross-linked complexes which are often associated with the biological responses of cells. For example, cross-linking of animal cell surface glycoconjugates has been implicated in the mitogenic activities of lectins (Nicolson, 1976) and in the arrest of bulk transport in ganglion cell axons (Edmonds and Koenig, 1990). Lectin–carbohydrate cross-linking interactions have also been implicated in triggering apoptosis of activated human T-cells (Perillo *et al.*, 1995). Thus, understanding the nature of these cross-linking interactions is required to gain insight into the structure–function properties of lectins and their glycoconjugate ligands.

We have observed that many asparagine-linked (N-linked[1]), serine- and threonine-linked (O-linked), and glycolipid-derived oligosaccharides are multivalent and can cross-link and precipitate with lectins (Bhattacharyya *et al.*, 1987a,b, 1988a,b, 1989; Bhattacharyya and Brewer, 1989). These interactions lead to a new dimension of specificity in carbohydrate–protein interactions, namely, the formation of homogeneous cross-linked lattices between specific multivalent carbohydrates and certain lectins, even in the presence of mixtures of the molecules. For example, quantitative precipitation studies of the Man/Glc-specific lectin concanavalin A (ConA) in the presence of binary mixtures of a series of closely related divalent, N-linked oligomannose type glycopeptides and a bisected hybrid glycopeptide suggest that each glycopeptide forms its own unique cross-linked lattice with the lectin (Bhattacharyya *et al.*, 1988b). Similar studies have shown that multivalent complex oligosaccharides possessing terminal LacNAc residues bind to multivalent Gal-specific lectins and form homogeneous cross-linked complexes (Bhattacharyya and Brewer, 1992).

Recent studies have suggested that lectins also form homogeneous cross-linked complexes with specific glycoproteins. For example, quantitative precipitation studies have provided evidence that the Man/Glc-specific lectin concanavalin A forms homogeneous cross-linked complexes with five different glycoproteins, even in the presence of mixtures of the molecules (Mandal and Brewer, 1992b). Similar studies have suggested that the Lac-specific 14 kDa lectin from calf spleen (galectin-1) forms homogeneous cross-linked lattices with asialofetuin (ASF), a bovine plasma glycoprotein possessing three N-linked complex triantennary carbohydrates with terminal LacNAc residues (Gupta and Brewer, 1994). Thus, evidence suggests that, in principle, both plant and animal lectins with different specificities are able to form homogeneous cross-linked complexes with specific glycoproteins.

In the present study, we have investigated whether lectins from different sources and naturally occurring heterogeneous IgG antibodies with the same nominal monosaccharide specificity display similar quantitative cross-linking activities with a common ligand. We have thus compared the cross-linking activities of two Lac-specific plant lectins, the mistletoe (*Viscum album L.*) agglutinin (VAA) and the agglutinin from *Ricinus communis* (RCA-I), the Lac-specific 16 kDa dimeric galectin from chicken liver (16 kDa lectin), and a human natural Lac-binding immunoglobulin G subfraction (Lac-IgG) with ASF. The results indicate differences in the formation of specific stoichiometric cross-linked complexes of the plant and animal lectins in relation to the Lac-binding antibodies with the glycoprotein.

Results and discussion

Properties of the proteins

The Lac-specific mistletoe lectin from *Viscum album* (VAA) belongs to the class of ribosome-inactivating proteins from plants that are composed of a toxic A chain and a saccharide-binding B chain (Gabius *et al.*, 1992; Barbieri *et al.*, 1993; Read and Stein, 1993). The overall structure of VAA is comprised of two noncovalently associated pairs of AB dimers which are held together by disulfide bonds. The biological effects of the lectin include eliciting immunomodulatory responses *in vitro* and *in vivo* upon binding to cell surface glycoligands with positive cooperativity (Gabius *et al.*, 1992, 1995; Gabius, 1994). Studies of the binding specificity of VAA indicate that it binds to both α- and β-galactosides with no marked specificity for the preterminal sugar and its linkage type to Gal (Lee *et al.*, 1992, 1994; Wu *et al.*, 1992).

RCA-I is a lectin isolated from *Ricinus communis* and possesses a molecular mass of 120 kDa. RCA-I is a heterotetrameric lectin like VAA-1, consisting of two A- and two B-chains with each heterodimer held together by a disulfide bond (Barbieri *et al.*, 1993; Read and Stein, 1993). Each B-chain possesses one carbohydrate-binding site specific for lactose (Podder *et al.*, 1974), and therefore the lectin is a dimeric carbohydrate binding protein.

The 16 kDa Lac-specific lectin from chicken liver is a dimeric lectin with a monomer molecular mass of 14,976 Da as determined by mass spectrometry analysis, as expected by calculations on the basis of the sequence (Sakakura *et al.*, 1990; Schneller *et al.*, 1995). It is a member of the family of Gal/Lac-specific lectins (galectins) that have been widely conserved throughout evolution (Barondes *et al.*, 1994; Kasai and Hirabayashi, 1996). The binding specificity of galectins appears to be generally directed toward LacNAc and polylactosamine chains (Barondes *et al.*, 1994; Kasai and Hirabayashi, 1996).

ASF is a monomeric bovine plasma glycoprotein possessing a molecular mass of 48 kDa. ASF displays three triantennary N-linked oligosaccharide chains with terminal LacNAc residues (74%) (Figure 1A), a small amount of isomer (9%) with a Galβ(1,3) linkage in the outer Manα(1,3) arm, a biantennary chain with terminal LacNAc residues (17%) (Green *et al.*, 1988) and three O-linked disaccharide chains (Galβ(1,3)GalNAcα-) (Figure 1B) (Nilsson *et al.*, 1979). The physiological function of ASF is not known.

Precipitation of VAA with ASF

VAA was previously shown to bind to the major N-linked triantennary complex glycopeptide of ASF shown in Figure 1A

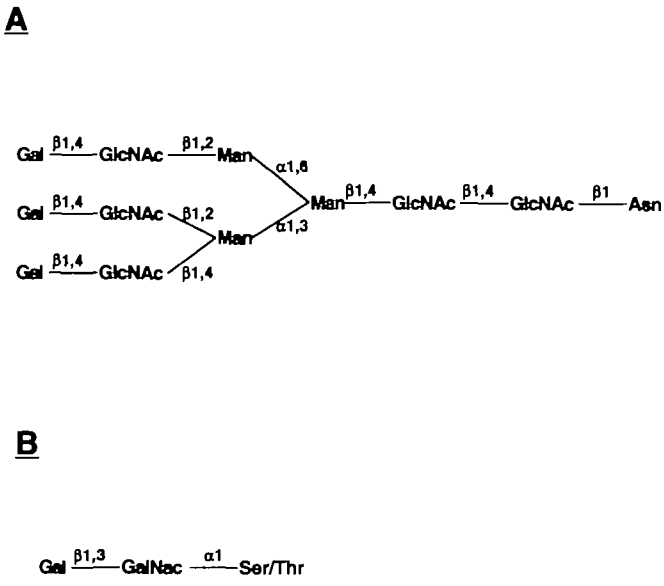

Fig. 1. Structures of the (**A**) major N-linked triantennary complex glycopeptide of ASF and (**B**) O-linked glycopeptide of ASF.

(Lee *et al.*, 1992). The lectin also binds to the triantennary chains of intact ASF as determined by quantitative precipitation experiments. The quantitative precipitation profile of VAA (175 μM) in the presence of increasing concentrations of ^{3}H-ASF is shown in Figure 2A. The A_{280} profile for total protein precipitated is the sum of the two profiles of the lectin and ASF. The precipitation profile of ^{3}H-ASF in c.p.m. is also shown. From the specific activity of ^{3}H-ASF, the concentration of ASF precipitated was calculated. Knowing the contribution of ASF to the total A_{280} profile allowed calculation of the contribution of VAA to the profile and hence its concentration in the precipitate which is also shown in Figure 2A. Thus, the mole ratios of precipitated VAA and ASF could be determined in the profile.

Figure 2B shows the ASF/VAA mole ratio (per monomer) in the precipitates with increasing concentration of ASF. Up to a concentration of 30 μM of ASF, the ASF/VAA mole ratio is 1:9. Increasing the concentration of ASF leads to a decrease in the ratio to 1:3 up to an ASF concentration of 80 μM, after which it remains constant. These results indicate that, at relatively low concentrations of ASF, VAA forms a 1:9 ASF/VAA cross-linked complex in which each of the individual carbohydrate chains of the three N-linked oligosaccharide of ASF bind to VAA. A schematic diagram of this complex is shown in Figure 3A. Under these conditions, each N-linked carbohydrate of ASF is trivalent, and ASF is functionally nonavalent for VAA binding. At higher concentrations of ASF, a 1:3 ASF/VAA cross-linked complex forms. This indicates that each N-linked oligosaccharide chain is bound by one VAA molecule, as shown in Figure 3B. Under these conditions, each N-linked triantennary carbohydrate is univalent for VAA binding, and ASF is functionally trivalent for VAA binding. VAA is divalent for ASF binding under all conditions, indicating, as expected, that each B-chain of VAA is univalent for carbohydrate binding.

Precipitation of RCA-I with ASF

RCA-I has also been shown to bind to the major triantennary complex glycopeptide of ASF (cf. Bhattacharyya *et al.*, 1988).

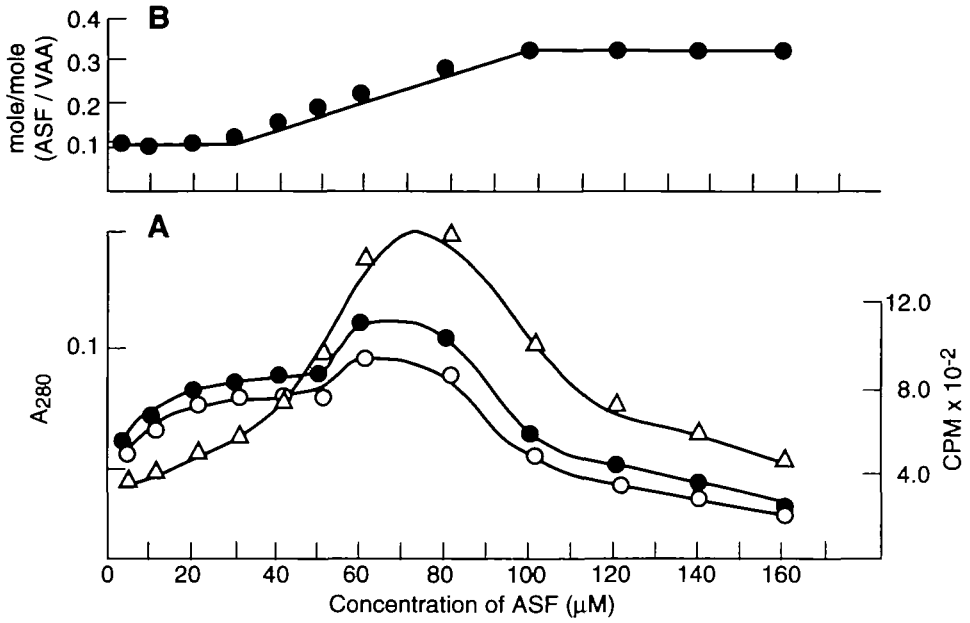

Fig. 2. Quantitative precipitation profile of VAA with ASF at 22°C. (**A**) A_{280} of total protein precipitated (•), A_{280} of VAA precipitated (○) and c.p.m of ^{3}H-ASF in the precipitates (△), (**B**) ratio of moles of ASF precipitated per mole of VAA monomer (•). The concentration of VAA was 175 μM. The specific activity of ^{3}H-ASF was 1.5 × 10^3 c.p.m./nmol. The buffer was 0.1 M Hepes at pH 7.2, containing 0.9 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$

The quantitative precipitation profile of RCA-I (86 μM) in the presence of increasing concentrations of ^{3}H-ASF is shown in Figure 4A. The A_{280} profile for total protein precipitated is the sum of the two profiles of RCA-I and ASF. The precipitation profile of ^{3}H-ASF in c.p.m. is also shown. From the specific activity of ^{3}H-ASF, the concentration of ASF precipitated was calculated, and from the total A_{280} profile of the two proteins in the precipitate the A_{280} profile of RCA-I was determined and is shown in Figure 4A. Thus, the mole ratio of precipitated RCA-I and ASF could be determined in the profile.

Figure 4B shows the ASF/RCA-I mole ratio (per monomer) in the precipitates with increasing concentration of ASF. Up to an ASF concentration of 12 μM, a 1:9 ASF/RCA-I cross-linked complex forms. With increasing ASF concentrations, a 1:3 ASF/RCA-I cross-linked complex spontaneously forms, similar to that observed with VAA. In the 1:9 complex, nine molecules of RCA-I bind to the three N-linked triantennary chains of ASF (Figure 3A). In the 1:3 complex, three RCA-I molecules bind to the three N-linked triantennary chains (Figure 3B). These results indicate that ASF possesses a valency of nine for RCA-I in the 1:9 complex, and that ASF possesses a valency of three for RCA-I in the 1:3 complex. RCA-I is divalent in both complexes, as expected from the number of B-chains in the molecule.

RCA-I has previously been shown to bind and form a cross-linked complex involving all three arms of the N-linked triantennary complex glycopeptide from ASF (Figure 1A) (Bhattacharyya *et al.*, 1988; Bhattacharyya and Brewer, 1992). The present results indicate that RCA-I can also bind and cross-link all three chains of each triantennary carbohydrate of ASF in the 1:9 cross-linked complex. Thus, the presence of the protein moiety of ASF does not hinder the ability of RCA-I to bind to all three arms of the triantennary carbohydrate.

Precipitation of the 16 kDa animal lectin with ASF

The precipitation profile of the 16 kDa ^{14}C-lectin (200 μM), isolated from chicken liver, in the presence of increasing concentrations of ^{3}H-ASF is shown in Figure 5A. The A_{280} profile shows the sum of the total protein precipitated. The c.p.m. for precipitated 16 kDa ^{14}C-lectin and for precipitated ^{3}H-ASF are also shown in Figure 5A. From their respective specific activities the mole ratio of each protein precipitated was determined.

Figure 5B shows that the ASF/16 kDa lectin (monomer) mole ratio is 1:9 up to an ASF concentration of 15 μM, and that the ratio decreases to 1:3 with further increases in the ASF concentration up to 60 μM after which it remains constant. (Similar results were obtained using lower salt concentrations in the buffer, i.e., 0.02 M sodium phosphate containing 1 mM DTT at pH 7.2.) The results indicate that the 16 kDa lectin, like VAA and RCA-I, forms two different stoichiometric cross-linked complexes with ASF depending on their relative concentrations. At lower concentrations of ASF, the 16 kDa lectin forms a 1:9 cross-linked complex (Figure 3A), and at higher concentrations of ASF, a 1:3 complex forms (Figure 3B). The cross-linking properties of the 16 kDa lectin from chicken liver with ASF are thus similar to the activities of the two plant lectins, VAA and RCA-I. The cross-linking activity of the 16 kDa lectin from chicken liver with ASF is also similar to galectin-1 from calf spleen with ASF (Mandal and Brewer, 1992a; Gupta and Brewer, 1994).

Precipitation of Lac-IgG with ASF

The quantitative precipitation profile of Lac-binding IgG (250 μM) in the presence of increasing concentrations of ^{3}H-ASF is shown in Figure 6A. The A_{280} for total protein precipitated is shown in Figure 6A, as well as the A_{280} for immunoglobulin G precipitated and c.p.m. for ^{3}H-ASF precipitated. The profiles for these parameters are different from those observed for the two plant lectins and the chicken lectin. Two peaks are observed in Figure 6A for all three parameters. These results suggest that at least oligoclonal antibodies are composed of at least two general populations with different affinities and precipitation activities to this glycoprotein. However, Figure 3B shows that the mole ratio of ASF/Lac-binding IgG (monomer)

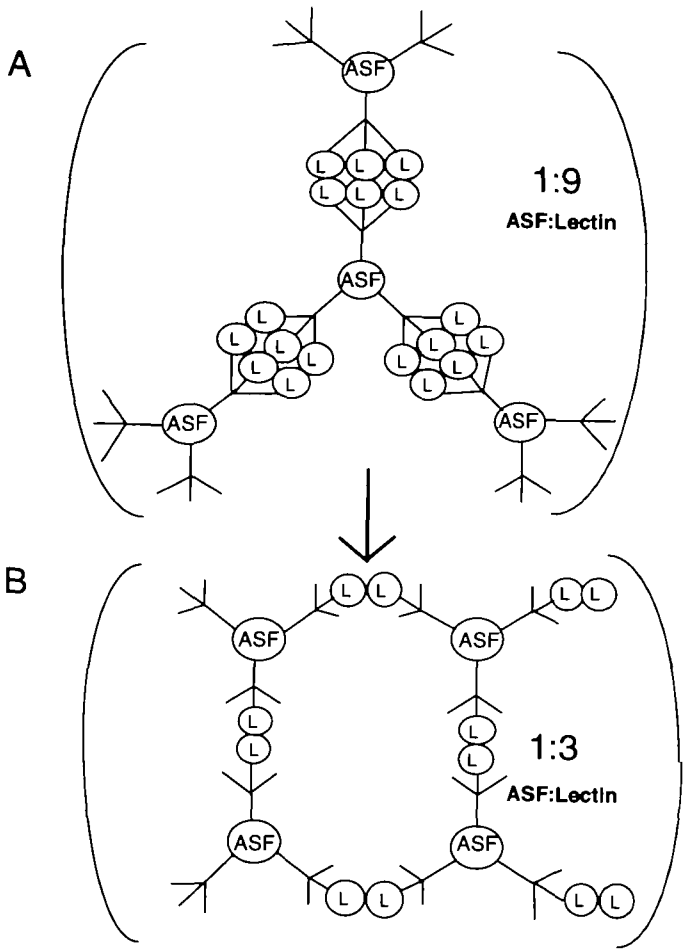

Fig. 3. Schematic representation of the (**A**) 1:9 and (**B**) 1:3 ASF/VAA cross-linked complex. The small circles marked with L represent lectin monomer. In the 1:9 complex, each arm of the three triantennary complex oligosaccharides of ASF is cross-linked by a lectin dimer to another similar oligosaccharide of a neighboring ASF molecule.

is 1:3 at all concentrations of ASF. (Lower salt concentrations also give the same results.) These results demonstrate that Lac-binding IgG forms only one stoichiometric cross-linked complex with ASF, independent of the relative concentrations of the two proteins. In the 1:3 complex, three antibody molecules bind to the three N-linked triantennary chains of ASF. The valency of each triantennary carbohydrate is one under these conditions. The valency of the Lac-IgG antibodies is two in the cross-linked complexes, as expected for IgG antibodies (Kabat, 1976).

These results differ from those for VAA, RCA-I, and the 16 kDa lectin in that the 1:9 cross-linked complexes observed for the latter three lectins with ASF are absent in the ASF/Lac-binding IgG cross-linked complexes. The absence of this cross-linked complex but presence of the 1:3 cross-linked complex has previously been observed for the soybean agglutinin (SBA) cross-linked complex with ASF (Mandal and Brewer, 1992a; Gupta and Brewer, 1994). In this case, a 1:3 ASF/SBA (monomer) complex forms at all concentrations of ASF. The explanation for the formation of only the 1:3 complex is the large size of the SBA tetramer which has a molecular mass of ~120 kDa. This can be compared to the lower molecular masses of galectin-1 from calf spleen (~30 kDa), and the 16 kDa lectin from chicken liver (~30 kDa) which both form 1:9 cross-linked complexes as well as 1:3 cross-linked complexes with ASF. Recombinant *Erythrina corallodendron*, which is a dimeric Lac-specific lectin with a molecular mass of ~60 kDa, also forms 1:9 and 1:3 cross-linked complexes with ASF (Gupta *et al.*, 1994). The fact that RCA-I and VAA have molecular masses of ~120 kDa, which are similar to that of SBA, indicates that the shape of the proteins must also be a factor in their ability to form the higher ratio complex with ASF. The absence of the 1:9 cross-linked complex for Lac-IgG is likely to be due to the large molecular mass (~180 kDa) of IgG antibodies.

Conclusions

The present study demonstrates that two plant lectins, RCA-I and VAA, the 16 kDa dimeric chicken lectin and heterogeneous human Lac-binding IgG antibodies all form specific, stoichiometric cross-linked complexes with the N-linked carbohydrate chains of the bovine plasma glycoprotein, ASF. The formation of specific stoichiometry cross-linked complexes between several related plant lectins such as *Erythrina indica*, *Erythrina cristagalli* and SBA, and galectin-1 from calf spleen, with ASF has been shown to correlate with the ability of these lectins to form homogeneous cross-linked complexes with the glycoprotein, even in the presence of mixtures of the molecules (Gupta *et al.*, 1994). The fact that natural polyclonal Lac-IgG antibodies also form a specific, stoichiometric cross-linked complex with ASF raises the question as to whether these antibodies also form homogeneous cross-linked complexes with the glycoprotein. Importantly, the cross-linking activities of IgG antibodies with antigen are known to be important for their immunological functions (cf. Kabat, 1976).

The present study thus demonstrates a conserved feature of plant and animal lectins as well as polyclonal anti-carbohydrate IgG antibodies. The formation of specific stoichiometry cross-linked complexes with a certain glycoprotein appears to be a common structure–function property of these multivalent proteins, which, in turn, may be related to their biological activities, for example, in cell surface ligand aggregation-dependent signaling.

Materials and methods

Materials

VAA and the 16 kDa lectin from chicken liver were prepared as previously described (Beyer *et al.*, 1980; Gabius, 1990; Schneller *et al.*, 1995). RCA-I was purchased from Sigma Chemical Co. (St. Louis, MO). Lac-IgG was obtained from the serum of healthy donors. Approximately 400 ml was coagulated and the supernatant, after centrifugation, passed over an unmodified Sepharose 4B column to remove any protein with affinity for the matrix. The resulting fraction was then passed through a lactose-Sepharose 4B column (1.8 × 20 cm; 50 ml bed volume), which was derived by divinyl sulphone activation (Gabius, 1990), at a flow rate of 25 ml/h. The Lac-binding immunoglobulin G fraction was eluted with 20 mM phosphate buffer (pH 7.2) containing 0.9% NaCl and 0.3 M Lac, as described previously (Dong *et al.*, 1995; Wawotzny *et al.*, 1995). This main fraction was further refined with respect to its anomeric selectivity by passage through a melibiose–Sepharose 4B column. The resulting flow-through fraction (approximately one-half of the applied amount of protein) is the immunoglobulin G (primarily G_2) subfraction used in the analysis.

Protein concentration of RCA-I was determined spectrophotometrically at 280 nm using an extinction coefficient ($A^{1\%,1cm}$) of 11.8 (Olsnes *et al.*, 1974). Concentration of VAA, Lac-IgG and 16 kDa lectin was calculated by Lowry estimation using BSA as standard. Monomer molecular masses of the lectins are 60 kDa for RCA-1, 60 kDa for VAA, 16 kDa for the chicken lectin, and 90 kDa for Lac-IgG.

ASF was prepared from fetuin obtained from Sigma Chemical Co. (St. Louis, MO) and purified by FPLC on a Superdex G-75 column, as described previously (Spiro and Bhoyroo, 1974). Its concentration was determined by modification of the phenol–sulphuric acid method (Saha and Brewer, 1994)

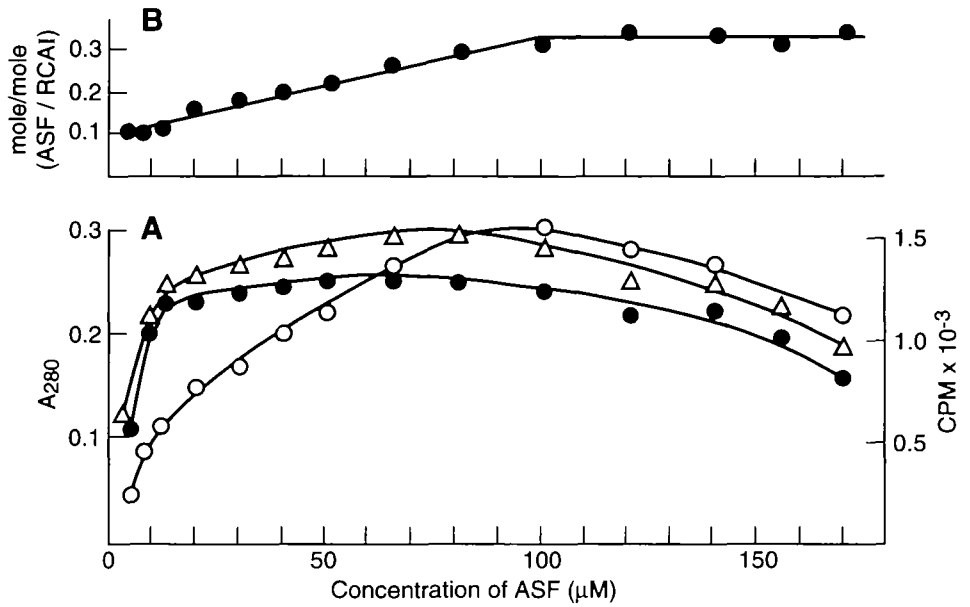

Fig. 4. Quantitative precipitation profile of RCA-I with ASF at 22°C (**A**) A_{280} of total protein precipitated (△), A_{280} of RCA-I precipitated (•) and ^{3}H-ASF in the precipitates (○); (**B**) ratio of moles of ASF precipitated per mole of RCA-I monomer (•). The concentration of RCA-I was 86 μM. The specific activity of ^{3}H-ASF was 2.7×10^3 c.p.m./nmol. The buffer was 0.1 M Hepes at pH 7.2, containing 0.9 M NaCl, 1 mM $CaCl_2$, and 1 mM $MnCl_2$.

with Man as standard using 21 mol of hexose (a mixture of 9 Man/12 Gal) per mole of protein (Spiro, 1960, Nilsson *et al.*, 1979). The structures of the oligosaccharides were confirmed by 500 MHz ^{1}H NMR.

Radiolabeling of proteins

ASF was radiolabeled in 0.1 M sodium phosphate buffer, pH 7.2 with ^{3}H-formaldehyde. The 16 kDa lectin was radiolabeled with ^{14}C by reductive methylation in 0.1 M sodium phosphate buffer, pH 7.2, containing 1 mM DTT and 0.1 M Lac, and labeled lectin was purified on an ASF-Sepharose column (Mandal and Brewer, 1992a). VAA and RCA-I could not be radiolabeled by reductive methylation without loss of their activities. The specific activities of the 16 kDa lectin and ASF are reported in the figure captions for each experiment.

Quantitative precipitation assay

Quantitative precipitation profiles were performed in 100 μl of 0.1 M Hepes buffer, pH 7.2 containing 0 9 M NaCl, 1 mM $CaCl_2$, and 1 mM of $MnCl_2$ as described previously (Mandal and Brewer, 1992a). Precipitates were inhibited from forming or dissolved by addition of 0.1 M Lac. Nonspecific sugars such as Glc or Fuc had no effect

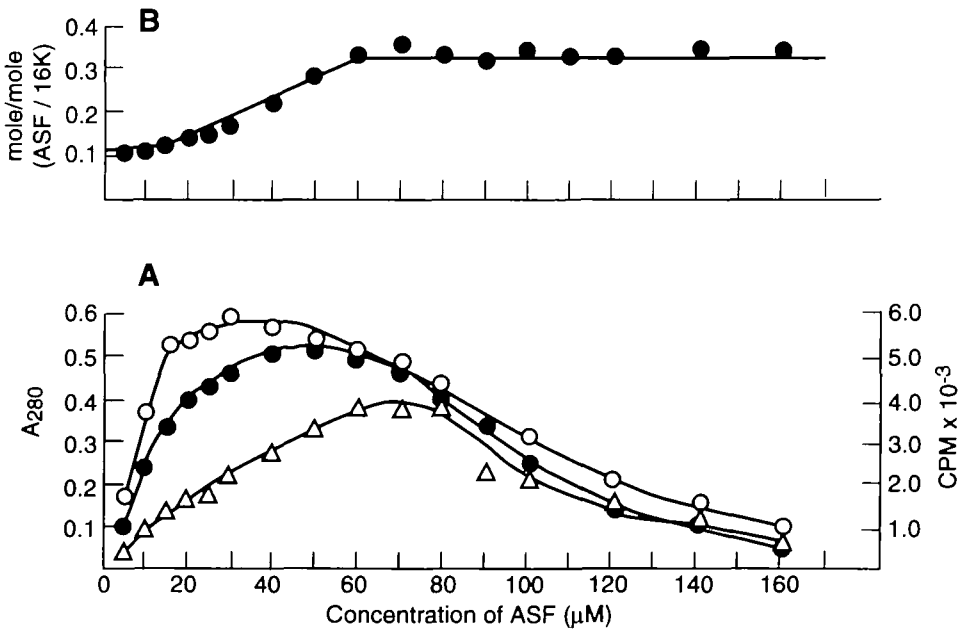

Fig. 5. Quantitative precipitation profile of the 16 kDa lectin with ASF at 22°C. (**A**) A_{280} of total protein precipitated (•), c.p.m. of ^{14}C-16 kDa lectin (○) and c.p.m. of ^{3}H-ASF (△) in the precipitates; (**B**) ratio of moles of ASF precipitated per mole of the 16 kDa lectin monomer (•) The concentration of the 16 kDa lectin was 200 μM. The specific activities of the ^{14}C-16 kDa lectin and ^{3}H-ASF were 3.0×10^3 and 6.5×10^3 c.p.m./nmol, respectively. The buffer was 0.02 M sodium phosphate containing 0.9 M NaCl and 1 mM DTT at pH 7.2.

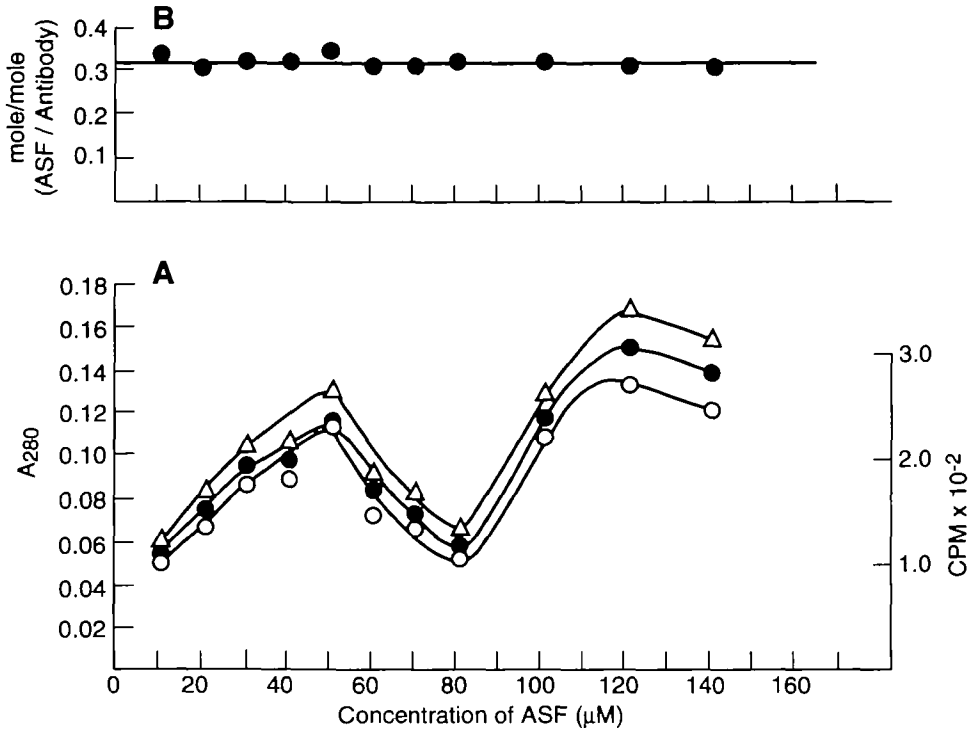

Fig. 6. Quantitative precipitation profile of the Lac-binding IgG polyclonal antibodies with ASF at 22°C: (**A**) A_{280} of total protein precipitated (△), A_{280} of total antibody precipitated (•) and c.p.m. of ^{3}H-ASF in the precipitates (○); (**B**) ratio of moles of ASF precipitated per mole of monovalent antibody (•). The concentration of Lac-binding IgG was 250 μM. The specific activity of ASF was 7.0 × 10^3 c.p.m./nmol. The buffer was 0.02 M sodium phosphate containing 0.9 M NaCl and 1 mM DTT at pH 7.2.

Acknowledgments

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Abbreviations

RCA-I, agglutinin-I from *Ricinus communis* (molecular mass 120 kDa); VAA, agglutinin isolated from mistletoe (*Viscum album L.*) plant (molecular mass 120 kDa); Lac-binding IgG, Lac-binding polyclonal immunoglobulin G subfraction; 16 kDa lectin, the dimeric lectin from chicken liver; SBA, lectin from soybean (*Glycine max*); Lac, Galβ(1,4)Glc; LacNAc, Galβ(1,4)GlcNAc; MeβGal, methyl β-galactopyranoside; N-linked, asparagine-linked; O-linked, serine- and threonine-linked. All sugars are in the D-configuration.

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