Homogeneous Enzyme-Linked Binding Assay for Studying the Interaction of Lectins with Carbohydrates and Glycoproteins

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A simple and rapid homogeneous enzyme-linked binding assay method for studying lectin-carbohydrate interactions is described. The method is based on the homogeneous inhibition of appropriate enzyme-saccharide conjugates by specific carbohydrate-binding lectins. In the presence of carbohydrate structures recognized by the lectins, enzyme activity is regained in an amount of proportional to the concentration of carbohydrate. The new method can be used to rapidly assess the relative carbohydrate specificity of the various lectins and for the selective analytical detection of simple saccharides and complex glycoproteins. Indeed, when Jacalin lectin is used in conjunction with a malate dehydrogenase-galactose conjugate, selective measurement of human IgA (immunoglobulin A) at microgram per milliliter levels in <10 min is possible. The potential for using this analytical methodology for determining changes in the carbohydrate structure of intact recombinant glycoproteins is also discussed.

The use of enzyme labels is now commonplace in the development of competitive and noncompetitive binding immunoassays for the detection of a wide variety of biomolecules at trace levels (*1-5*). Enzyme-linked competitive binding assays may be classified as either heterogeneous or homogeneous. While less sensitive, homogeneous methods do not require time-consuming washing steps to separate bound and free labels and are therefore more rapid and amenable to automation. In the EMIT (enzyme-multiplied immunoassay technique) approach pioneered by Ullman et al. (*6*) for the detection of lower molecular weight ligands (i.e., drugs and hormones), analytical signals result from either the activation or inhibition of the catalytic activity of an appropriate enzyme-ligand conjugate in solution by antiligand antibodies. Such modulation of enzyme activity is reversed in an amount proportional to the concentration of analyte ligand present in the sample. In previous work (*7-12*), we have demonstrated the theoretical and practical advantages of using endogenous binding proteins rather than antibodies in the development of EMIT type assays for several biomolecules. We now extend this concept further by describing how natural carbohydrate-specific binding lectins can be used in conjunction with enzyme-saccharide conjugates to devise a very simple and rapid homogeneous assay method that can be used to both evaluate the specificity of lectin carbohydrate interactions and selectively detect certain carbohydrates and glycoproteins in solution.

Although the EMIT type homogeneous enzyme immunoassays and, more recently, the fluorescence polarization homogeneous immunoassays (*13*) have gained widespread use in clinical chemistry laboratories for measurement of low molecular weight drugs, hormones, etc., in physiological samples, there has been relatively little progress on the development of analogous homogeneous methods for the selective detection of high molecular weight proteins. At the same time, with the increased use of recombinant DNA methods to prepare commercial therapeutic protein prducts, there is a growing need to determine the carbohydrate structure of intact glycoprotein products produced by these methods (e.g., tissue plasminogen activator (TPA), etc.). Indeed, although the amino acid sequence of the protein can be fixed, cell cultures producing these biotechnology products can often incorporate erroneous carbohydrate structures, thereby decreasing the biological activity of the protein product.

Lectins, carbohydrate-selective binding proteins of nonimmune origin, are potentially useful analytical tools to study, characterize, and quantitate simple carbohydrates as well as complex glycoconjugates. Relative carbohydrate specificity of lectins has been determined traditionally via a precipitin assay method in which a protein-monosaccharide conjugate is precipitated by the multivalent lectin (*14, 15*). The amount of precipitin formed after long-term incubation (i.e., 24-48 h), as measured by a total protein colorimetric assay, decreases as the concentration of free carbohydrate added to the assay mixture increases. The concentration of different carbohydrate species required to yield a 50% decrease in the total precipitin formed (ED_{50} value) is often used to assess the relative specificity of the lectin. As analytical reagents, lectins have also been used in blood-typing agglutination tests (*16*), purification of glycoproteins by affinity chromatography (*17*), and quantitation of carbohydrates by the above competitive precipitation method (*18, 19*). Schultz et al. demonstrated that an immobilized lectin (Conconavalin A), in conjunction with fluorescein-labeled dextran, can be used in a competitive binding assay type optical sensor for the detection of blood glucose (*20*). Ineke et al. studied the competitive binding of enzyme-labeled lectin to a glycoprotein (ovalbumin) bound to a solid phase (heterogeneous method) in order to detect the presence and relative affinities of various lectin receptors (*21*).

In this paper, we describe a novel homogeneous enzyme-linked binding assay (ELBA) to rapidly detect the presence of specific carbohydrate structures, either as mono- and oligosaccharides in solution or within intact glycoproteins. The proposed method utilizes malate dehydrogenase (MDH)- and/or glucose 6-phosphate dehyrogenase (G6PDH)-saccharide conjugates (with mannose and galactose) that are inhibited in solution by lectins that recognize these saccharide groups appended to the enzymes. In the presence of selected analyte saccharides or glycoproteins, the activity of the enzyme conjugates is increased in an amount proportional to the concentration of carbohydrate present. By comparison of the concentrations of glycospecies required to reduce the homogeneous inhibition of labeling enzyme activity, the relative specificity of the lectin for carbohydrate species with differing structures can be determined in less than 15 min. In addition, using the interaction of Jacalin lectin from jackfruit with human IgA (immunoglobulin A) as a model system, it is shown that such homogeneous lectin-based ELBAs can also be used to rapidly detect given glycoproteins (IgA) with high selectivity over other proteins.

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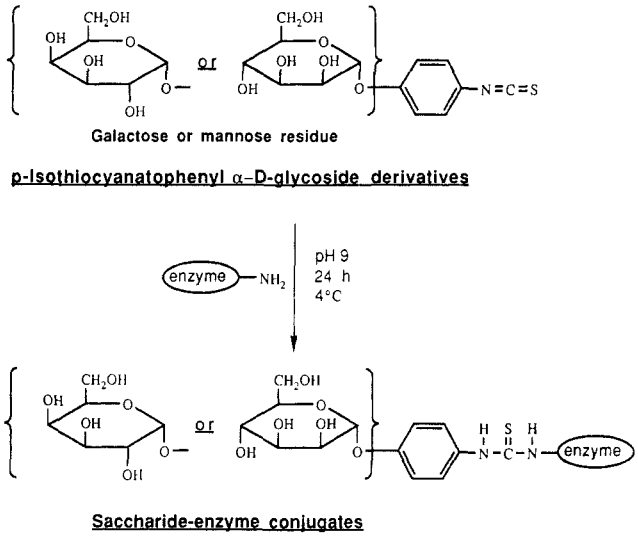

Figure 1. Reaction sequence used for preparing enzyme–saccharide conjugates.

EXPERIMENTAL SECTION

Apparatus. Enzymatic activities were measured with a Gilford-Stasaar-III spectrophotometer equipped with a vacuum-operated sampling system and temperature-controlled cuvette (maintained at 30 °C throughout the experiments). This spectrophotometer was connected to a Syva CP-5000 EMIT Clinical Processor for automatically setting the reading intervals and recording the absorbance values. A Perkin-Elmer Lamda Array 3840 UV/vis spectrophotometer operated by a model 7300 professional computer was used to obtain the spectra of enzyme–ligand conjugates to determine the degree of saccharide conjugation. A microdialyzer System 500 from Pierce was used for dialyzing enzyme–ligand conjugates.

Reagents. Jacalin was obtained from Pierce. *p*-(Isothiocyanato)phenyl α-D-mannopyranoside, *p*-(isothiocyanato)phenyl α-D-galactopyranoside, malate dehydrogenase (MDH) from pigeon breast muscle, glucose 6-phosphate dehydrogenase (G6PDH) from *Leuconostoc* mensenterides, as well as the lectins Concanavalin A (Con A), *Bandeiraea smplicifolia* (BS-1), *Bauhinia purpurea* (BP), *Lens culinaris* (Lentil), and *Pisum sativum* (PS), were obtained from Sigma Chemical Co. (St. Louis, MO). The substrates glucose 6-phosphate (G6P), oxaloacetic acid, NAD^+, and NADH were also Sigma products. The working assay buffer for G6PDH and G6PDH–saccharide conjugates was a 0.05 mol/L tris(hydroxy)aminomethane–hydrochloric acid buffer (Tris–HCl), pH 7.8, containing 0.01% (w/v) NaN_3 and 0.3% (w/v) gelatin (assay buffer). Activity measurements and dilutions of MDH and MDH–saccharide conjugates were performed in a 0.10 mol/L sodium phosphate buffer, pH 7.5, containing 0.01% (w/v) NaN_3 and 0.30% (w/v) gelatin (assay buffer). Deionized water was used to prepare all buffers.

Preparation of Enzyme–Saccharide Conjugates. Enzyme–saccharide conjugates were synthesized by using *p*-(isothiocyanato)phenyl saccharide derivatives with MDH and G6PDH (see Figure 1). *p*-(Isothiocyanato)phenyl α-D-mannopyranoside or galactopyranoside was added in small portions to enzyme solutions which were buffered at pH 9.0 (HCO_3^-/CO_3^{2-}). The coupling reaction was run for 24 h at 4 °C with stirring. The active site of G6PDH was protected during the reaction by adding an excess of G6P and NADH. After extensive dialysis against the working assay buffer for the respective enzymes, the resulting conjugates were characterized by their residual activities, degrees of saccharide conjugation and percent inhibition induced by excess amount of lectins. The degree of conjugation was determined by the phenol–sulfuric acid method (*22*).

Determination of Enzymatic Activity and Maximum Percent Inhibition. The activity of G6PDH–mannose conjugates was determined by measuring the rate of increase of NADH at 340 nm after addition of 100 μL of NAD^+ (0.06 mol/L in 0.05 mol/L Tris–HCl–gel assay buffer, pH 7.8), 100 μL of glucose 6-phosphate in Tris–HCl–gel assay buffer, and 100 μL of the appropriate diluted conjugate solution to a gelatin-coated disposable plastic sample tube containing 700 μL of Tris–HCl–gel buffer. On the other hand, the rate of decrease of NADH concentration was used to determine the activity of MDH–ligand conjugates. This assay involved the addition of 100 μL of NADH (6.4×10^{-4} mol/L in 0.10 mol/L phosphate–gel buffer, pH 7.5), 100 μL of oxaloacetic acid (1.9×10^{-3} mol/L in phosphate–gel buffer), and 100 μL of diluted MDH–ligand conjugate to a gelatin-coated disposable sample tube containing 700 μL of phosphate–gel assay buffer. All solutions were kept at 0 °C until the addition of the reagents. For each assay, after reagents were mixed and subsequently agitated on a vibrator for 2 s, the reaction mixture was aspirated into the thermostated flow cell of the spectrophotometer and the absorbance at 340 nm was measured over 1-min period after an initial 20-s delay (ΔA, min^{-1}).

In order to determine the maximum percent inhibition value for each conjugate, 100 μL of the assay buffer was replaced by 100 μL of lectin solution prepared in assay buffer. In addition, the conjugates were incubated first with the lectin for 10–15 min before addition of the substrate solutions.

Association Kinetic Study. The rate of binding between the enzyme–saccharide conjugates and corresponding lectins was measured as follows: 100-μL portions of appropriately diluted MDH–mannose (Mman16) conjugate in assay buffer plus 100 μL of Con A solution (266 μg/mL), or 100-μL portions of diluted MDH–galactose (Mgal5) conjugate with 100 μL of Jacalin solution (100 μg/mL), were mixed and incubated for varying time periods. After each incubation period, enzymatic activity of the conjugates was determined as described above.

Effect of Specific Lectins and Concentration on Inhibition of Enzyme–Saccharide Conjugates. Aliquots (100 μL) of solutions containing various concentrations of different lectins were incubated with 100 μL of appropriately diluted Mman16 or Mgal5 conjugate for 15 and 10 min, respectively. After incubation, the enzymatic activity was measured as described above. Binder dilution curves were prepared by plotting percent inhibition vs amount of lectin added.

Dose Response Curves for Carbohydrate Species. Standard solutions of different saccharides and glycoproteins were prepared in the appropriate assay buffer. In the assay protocol, 100 μL of standard solution and 100 μL of conjugate solution were added to a gelatin-coated disposable tube containing 100 μL of Jacalin solution (50 μg/mL) or 100 μL of Con A (133 μg/mL), and the mixtures were incubated for 10 or 15 min on a shaker. The resulting enzymatic activity was measured as described above. Dose response curves were prepared by plotting percent inhibition vs the logarithm of the concentration of the carbohydrate species in the 100 μL of standard solutions added to the assay mixture.

RESULTS AND DISCUSSION

In the design of a homogeneous ELBA for studying lectin–carbohydrate interactions, it is essential to synthesize enzyme–carbohydrate conjugates that can be inhibited substantially upon binding of given lectins to the appended saccharide structures. To this end, in preliminary studies, a number of enzyme–mannose and –galactose conjugates were prepared in accordance with the reaction scheme shown in Figure 1. Glucose 6-phosphate dehydrogenase (G6PDH) and malate dehydrogenase (MDH) were chosen as potential labeling enzymes, since both have been used previously in the design of analytically useful homogeneous ELBAs and EMIT methods (*7–12*), and neither enzyme contains endogenous carbohydrate structure. However, MDH is available commercially with a specific activity (5600 units/mg) higher than that of G6PDH (860 units/mg) and, thus, all of the galactose conjugates were prepared exclusively with this enzyme. The resulting conjugates were characterized by their degrees of conjugation, percent residual enzyme activities, and percent inhibition induced by excess of the lectins (Con A for enzyme–mannose conjugates and Jacalin for enzyme–galactose conjugates). These results are summarized in Tables I and II for conjugates prepared by using different initial molar ratios of the *p*-(isothiocyanato)phenyl α-D-glycoside derivatives to the enzyme. The active sites of G6PDH were protected

Table I. Characteristics of Enzyme–Mannose Conjugates

conjugate[a]	initial ratio (ligand/enzyme)	degree of conjugation	% residual activity	% inhibition[b]
Gman6	100	9.7	87.6	13.5
Gman	250	13.7	71.3	29.1
Gman8	500	24.6	59.4	34.8
Gman9	1000	36.8	37.3	42.0
Mman15	100	8.8	72.7	35.1
Mman16	250	13.2	69.0	56.1
Mman17	500	22.1	64.0	54.2
Mman18	1000	28.7	26.5	67.0

[a] Gman represents the G6PDH–mannose conjugate, and Mman represents the MDH–mannose conjugate. [b] For all conjugates, a fixed amount of Con A (26.6 μg/tube) was incubated with a fixed concentration of enzyme conjugate (1.51×10^{-6} M).

Table II. Characteristics of MDH–Galactose Conjugates

conjugate	initial ratio (ligand/enzyme)	degree of conjugation	% residual activity	% inhibition[a]
Mgal1	0	0	100	2.0
Mgal2	50	4.3	96.7	8.6
Mgal3	100	10.9	83.9	24.0
Mgal4	250	16.7	55.0	42.6
Mgal5	500	26.6	35.5	60.7

[a] For all conjugates, 10 μg/tube of Jacalin was incubated with a fixed amount of enzyme conjugate (1.51×10^{-6} M).

with excess G6P substrate and NADH product during the coupling reaction to enhance the residual enzymatic activity of the resulting G6PDH–mannose conjugates. However, the conjugation reactions with MDH were carried out without active-site protection, since conjugation of MDH in the presence of excess substrates/products can yield conjugates that do not display the required binder-induced homogeneous inhibition (*23*).

As Tables I and II show, increasing the initial molar ratio of ligand/enzyme results in a higher degree of saccharide conjugation and greater percent inhibition by the lectins. However, these desirable characteristics come at the expense of a decrease in the percent residual catalytic activity and thus enzyme detectability. This has been observed previously when other ligand structures have been covalently linked to MDH and G6PDH (*9*, *10*). It was found that mannose conjugates prepared with MDH could be inhibited to a greater degree (up to 67%) by Con A than similarly substituted G6PDH conjugates (34–42%). This may be due to an enhanced steric effect when Con A binds to MDH–mannose conjugates and not to a decrease in Con A affinity for mannose appended to G6PDH relative to mannose attached to MDH (see below). On the basis of residual activities and maximum percent inhibition, conjugates Mman16 and Mgal5 were selected as enzyme-labeled conjugates for use in all the subsequent homogeneous binding studies.

The kinetics of the association between the lectins and the enzyme–saccharide conjugates were first studied in order to optimize the incubation time required for competitive binding assays. As shown in Figure 2, relatively fast association kinetics were observed for both lectin/enzyme–saccharide systems employed as models in these studies. Indeed, >80% of the maximum final value of inhibition was obtained after a 2-min incubation period for the MDH–mannose/Con A system, while only 30 s of incubation were required to achieve a comparable extent of inhibition for the MDH–galactose/Jacalin system. These fast binding kinetics between the

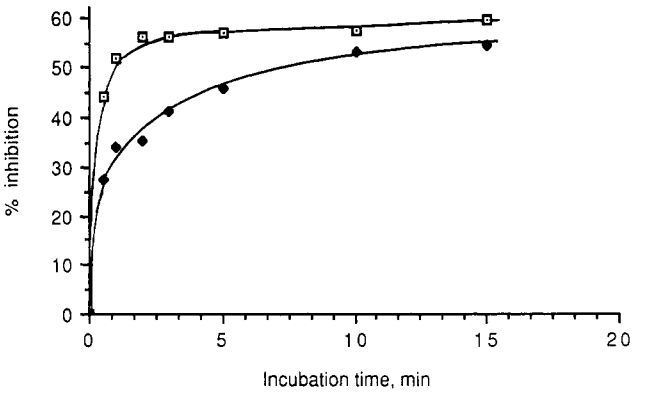

Figure 2. Kinetics of association between MDH–saccharide conjugates and lectins (1.51×10^{-6} M of enzyme concentration): (●) Mman16 conjugate with Con A (13.5 μg/tube); (⊡) Mgal5 with Jacalin (5 μg/tube).

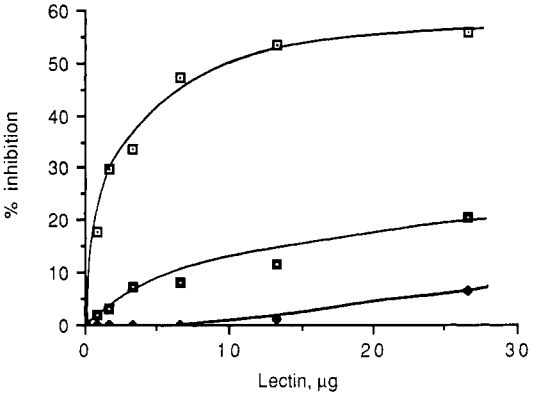

Figure 3. Binder dilution curves for Mman16 conjugate (1.51×10^{-6} mol/L) with different lectins: (⊡) Con A; (◘) PS; (◆) Jacalin.

Table III. Characteristics of Lectins Examined[a]

Lectin	source of lectin	mol formula	mol wt	binding specificity
Con A	Jack bean	α_4	106 000	αMan > αGlc > GlcNAc
Pisum sativum	pea	$\alpha_2\beta_2$	50 000	αMan > αGlc = GlcNAc
Bauhinia purpurea	*B. purpurea* seed	α_4	195 000	GalNAc > Gal
Bandeiraea simplicifolia	*B. simplicifolia* seed	α_4	114 000	Gal
Jacalin	jackfruit seed	α_4	40 000	αGal

[a] From refs 16, 24, and 25.

macromolecular reagents make homogeneous ELBAs involving lectins and enzyme–saccharide conjugates potentially much faster than enzyme-linked heterogeneous and/or precipitin-based assay methods (*14*, *15*). In subsequent experiments, incubation periods of 10 and 15 min were used in studies involving the MDH–galactose/Jacalin and MDH–mannose/Con A systems, respectively.

The effect of varying amounts of different lectins on percent inhibition observed with each of the enzyme–saccharide conjugates was also examined. Table III lists some lectins employed in these studies, their reported specificity, and other characteristics. As shown in Figure 3, relatively little of Con A is required to inhibit a given amount of the Mman16 conjugate (1/1000 dilution of original enzyme conjugate preparation; 1.5×10^{-6} M), and the plot of percent inhibition vs amount of lectin actually plateaus at Con A levels >15 μg/tube. However, when the same amount of Mman16 conjugate is incubated with varying amounts of *P. sativum*, another mannose-specific lectin, significantly less total inhibition is

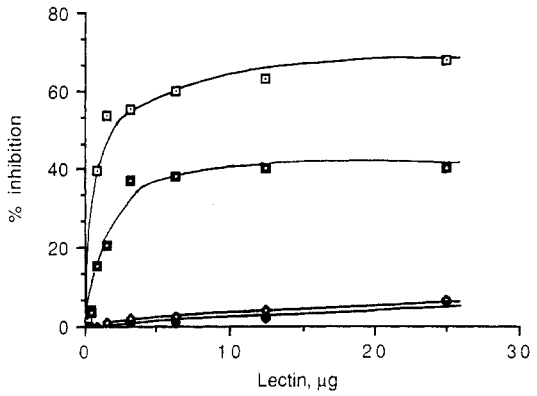

Figure 4. Binder dilution curves for Mgal5 conjugate (1.51×10^{-6} mol/L) with different lectins: (⊡) Jacalin; (■) BS-1; (◇) BP; (◆) ConA.

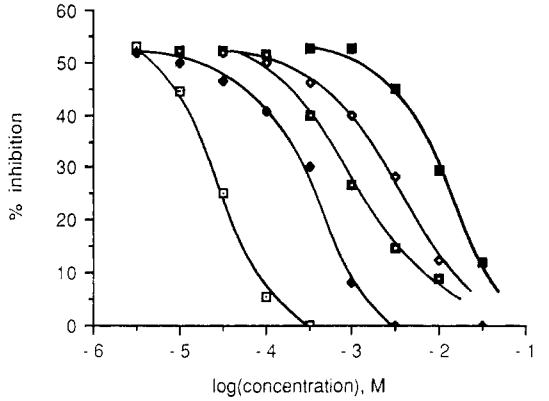

Figure 5. Dose response curves obtained for saccharides with Mman16 conjugate (1.51×10^{-6} mol/L) using Con A (13.6 μg/tube): (⊡) *p*-aminophenyl α-D-mannopyranoside; (●) methyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside; (■) D(+)-melezitose; (◇) D-mannose; (■) D-glucose.

observed, and the steepness of the binder dilution cuve is much shallower. Binder dilution curves for the Mgal5 conjugate with three different galactose and/or *N*-acetylgalactosamine-specific lectins were also prepared (see Figure 4), and significant differences are observed.

The shape of the binder dilution curves for different lectins with the same enzyme–saccharide conjugate depends on several factors. To achieve a substantial degree of inhibition at relatively low levels of lectin, the lectin must have a reasonably high association constant for the saccharide structure appended to the enzyme. At the same time, the specific three-dimensional structure and size of the lectin will also affect the maximum degree of inhibition (in presence of excess lectin). Since the homogeneous inhibition of MDH- and G6PDH–ligand conjugates in the presence of antiligand antibodies has been attributed to steric exclusion of substrates and/or conformational changes in the labeling enzyme's structure induced by the binding reaction, the magnitude of such effects will likely differ for different lectins, even if the actual association constant between the lectins and conjugate are similar. Thus, the shape and steepness of the binder dilution curves shown in Figures 3 and 4 provide some information about affinity constants for lectin conjugate binding, while the percent maximum inhibition in the presence of excess lectin is solely dependent on the size and structure of the lectin. Indeed, as noted above, the percent inhibition of Mman16 plateaus at 15 μg/tube of Con A (Figure 3), while that with *P. sativum* lectin is significantly less and still increasing consistently over this concentration range, reaching a maximum inhibition of 49% at 200 μg/tube (not shown). This behavior is apparently due to the fact that the affinity constant for *P. sativum* with mannose is lower than that of Con A by 1 order magnitude (*16*). Similar results are obtained when Jacalin, a galactose-specific binding lectin, is utilized in conjunction with the MDH–mannose conjugate (see Figure 3). These results clearly demonstrate one way in which the proposed homogeneous assay method can be utilized to gain information about relative binding affinities of different lectins toward the appended saccharide structures. Indeed, with further efforts, it may be possible to develop a mathematical model that will allow the initial slope of the binder dilution curves to be used to gain more quantitative association constant data (i.e., utilize percent inhibition to quantitate bound and free concentrations of labels for each binder concentration).

Results in Figure 4 illustrate similar types of behavior for different lectins interacting with the MDH–galactose conjugate. Galactose-selective Jacalin is the most potent inhibitor (on the basis of maximum percent inhibition) and binds the appended galactose with the highest affinity (on the basis of the slope of the binder dilution curve). However, *B. simplicifolia* lectin appears to have a binding affinity toward galactose similar to Jacalin (taking into account the difference in molecular weight; see Table III), but the magnitude of inhibition observed is considerably less. This suggests that the large size of this lectin may prevent a number of galactose residues on the enzyme from being bound simultaneously by several lectin molecules (i.e., larger size sterically prevents, via crowding, the number of lectins bound per enzyme conjugate molecule). The rather limited inhibition found with *B. purpurea* (approximately 10% even at 100 μg/tube; not shown), a lectin with known specificity for *N*-acetylgalactosamine and galactose (*16*), suggests that the even larger size of this lectin, coupled with its somewhat weaker affinity for galactose, renders it an ineffective inhibitor of the MDH–galactose conjugate. Note that when mannose-specific Con A is employed as the binder with the MDH–galactose conjugate, very limited inhibition is also observed over the range of lectin concentrations examined. This further confirms the usefulness of the method for assessing relative saccharide specificity and binding affinity of given lectins.

The relative affinities of the various lectins for a wider range of carbohydrate structures (other than the simple monosaccharides appended to the enzymes) can be readily determined by utilizing the reagents in a competitive binding assay arrangement. For this purpose, in accordance with the recommendations of Kabakoff and Greenwood for the antibody-based EMIT type of competitive assay (*26*), the amount of binder utilized to achieve optimum dose-responses should correspond to that which yields approximately 85% of the maximum inhibition with a given amount of enzyme–ligand conjugate. Therefore, on the basis of the binder dilution curves shown in Figures 3 and 4, 5 μg/tube of Jacalin and 13.6 μg/tube of Con A were used in the subsequent competitive assay studies involving the MDH–galactose and MDH–mannose conjugates, respectively.

Competitive dose response curves for a variety of different saccharides and glycoproteins were constructed by plotting percent inhibition vs the concentration of species added to the assay mixtures (see Experimental Section). In the case of the Con A/MDH–mannose system, mono-, di-, and trisaccharide structures and related derivatives were tested (see Figure 5). Among the species examined, *p*-aminophenyl α-D-mannopyranoside is the best competitive analyte and thus appears to bind with Con A most tightly. It is known that branched glycoconjugates exhibit an enhanced affinity for lectins in comparison to corresponding simple monosaccharide structures (*27*, *28*). Brewer (*29*) suggests that the most plausible reason for this is a statistical effect due to the increased number of nonreducing terminal saccharide residues present within such glycostructures. As shown in Figure 5,

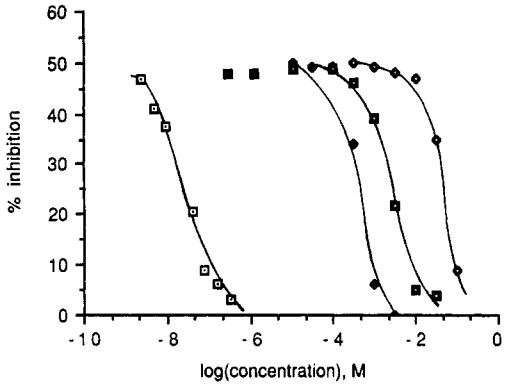

Figure 6. Dose response curves obtained for saccharides and glycoproteins with Mgal5 conjugate (1.51×10^{-6} mol/L) using Jacalin at 5 μg/tube: (□) IgA, (■) IgG; (◆) *p*-aminophenyl α-D-galactopyranoside; (◘) methyl α-D-galactopyranoside; (◇) D-galactose.

methyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside and D(+)-melezitose, a trisaccharide composed of 2 mol of glucose and 1 mol of fructose, also compete for Con A binding sites better than methyl α-D-mannopyranoside, presumably for similar reasons. A variety of other species such as D-galactose, methyl α-D-galactopyranoside, and heparin competed very poorly for Con A binding sites, with little reversal in homogeneous inhibition even at concentrations approaching 100 mmol/L (not shown). For the most part, these data are in good agreement with the known binding specificity of Con A as determined by the conventional precipitin assay method (*16*). Human IgA, a glycoprotein, was found to bind only very weakly to Con A, with a 0.6 μmol/L dose still resulting in 45% inhibition (compared to 61% without any competitor) of the MDH–mannose conjugate (not shown in Figure 5).

Figure 6 illustrates the results obtained when the Jacalin/MDH–galactose system was employed in a competitive binding assay mode. In contrast to the Con A system, human IgA binds Jacalin rather tightly, as evidenced by the very sensitive dose response toward this glycoprotein. Neither human IgG nor goat IgA (not shown) exhibited similar behavior. Human IgA is known to contain several galactose residues in the hinge region of the molecule (*30*), and on the basis of previously reported studies, as well as the results shown in Figure 6, these residues appear to be readily accessible for binding by galactose-specific Jacalin. The fact that human IgG or goat IgA does not compete effectively for Jacalin binding sites indicates that these glycoproteins do not contain galactose residues on their structure or, if present, these residues are not accessible to the binding sites of lectin. In comparing the dose response for D-galactose and methyl α-D-galactopyranoside, which only differ in the configuration at the anomeric position, the α-anomer appears to have stronger binding potency toward Jacalin than the anomeric mixture. D-Mannose and D-glucose are isomers of D-galactose; however no significant reversal in enzyme inhibition was observed with these species, even at 100 mmol/L levels.

In general, for either the Jacalin/MDH–galactose or Con A/MDH–mannose systems, the concentration of carbohydrate required to achieve a 50% reduction in the maximum enzyme inhibition values can be related to the relative affinity of the given lectin toward that particular structure. For example, Table IV summarizes some of these so called "ED_{50}" values obtained with the Jacalin/MDH–galactose reagent system and compares these results to those found previously with Jacalin within a latex particle agglutination assay method (*31*) (based on inhibition of Jacalin-induced agglutination of particles coated with human IgA). As Table IV indicates, the molar concentrations for 50% inhibition of maximum values by the homogeneous ELBA method were consistently lower than those obtained with the latex agglutination assay, but the

Table IV. Comparison of Homogeneous ELBA and Latex Agglutination Method

saccharides or IgA	concn for 50% inhibition of max value (mM in the final reacn mixture)	
	homogeneous ELBA	latex agglutination[a]
p-aminophenyl α-D-galactopyranoside	3.2×10^{-2}	0.20
methyl α-D-galactopyranoside	0.30	1.0
D-galactose	4.5	38
IgA	3.0×10^{-6}	3.0×10^{-5}

[a] From ref 31.

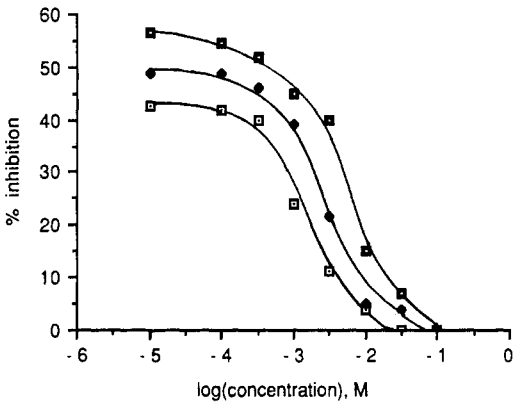

Figure 7. Effect of various Jacalin levels on the dose response curve for methyl α-D-galactopyranoside using Mgal5 conjugate (1.51×10^{-6} mol/L); (◘) 10.0 μg; (◆) 5.0 μg; (□) 2.5 μg.

relative order of Jacalin affinity toward these species was essentially the same in both cases. Naturally, the exact ED_{50} values obtained by the new ELBA method, or any other competitive approach, will be highly dependent on the concentration of reagents utilized in the assay mixture. For example, Figure 7 illustrates how the dose response (and thus, the ED_{50} value) toward methyl α-D-galactopyranoside varies by changing the amount of Jacalin utilized with a fixed amount of MDH–galactose conjugate. As expected, dose response curves are shifted to a lower concentration range when less binder is employed. However, this shift occurs at the expense of decreasing the maximum percent inhibition, which is the signal-generating parameter used to assess the binding reaction.

The reproducibility of the competitive lectin-based assay arrangement is comparable to that observed in previous homogeneous ELBA and EMIT methods involving either natural binding proteins or antibodies. Generally, percent inhibition values are reproducible to ±1.4% (sd for $n = 3$).

In summary, we have described the performance of a novel homogeneous enzyme-linked binding assay that is useful for studying the interactions of various lectins with a variety of carbohydrates and glycoprotein structures. By determination of the degree of inhibition of suitable enzyme–saccharide conjugates in the presence of varying concentrations of different lectins, the relative binding affinities of the lectins for the given saccharide structure can be determined. Moreover, when the assay is utilized in a competitive binding mode, the relative selectivity and affinities of lectins for a broader range of saccharide and glycoprotein structure can be readily assessed. By utilizing the varying dose responses obtained for a given glycoprotein within several lectin/enzyme–saccharide conjugate assay systems, it should be possible to employ the assay principle to devise a "fingerprint" method capable of probing the specific carbohydrate structure of intact glyco-

proteins. That is, changes in the carbohydrate content/structure may result in a more or less sensitive dose response with each of the homogeneous lectin-based assays (i.e., change in the ED_{50} value for given glycoprotein). Such an approach is currently under study with the development of additional assays (e.g., sialic acid) and the specific application of this methodology for detecting changes in carbohydrate structure within recombinant glycoprotein therapeutic products.

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Influence of Particle–Wall and Particle–Particle Interactions on Retention Behavior in Sedimentation Field-Flow Fractionation

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In sedimentation field-flow fractionation (SdFFF) experiments carried out with carrier solutions of low surfactant concentrations or distilled water, the retention ratio of the sample particles increased from the values predicted by the theory of Giddings and his coworkers, in which the steric effect has been taken into consideration. To explain this phenomenon, this paper discusses theoretically the perturbation behavior in the retention by the particle–wall and the particle–particle interactions due to electrostatic repulsive and van der Waals' attractive forces. The influence of these interactions was not significant at low sample concentrations below 0.1% and at high ionic strengths of the carrier solution above 10^{-3} M. However, under low ionic strength conditions of the carrier solution, the concentration profiles in the channel spread widely, and the estimated retention time decreases from the Giddings' theory. The contribution of the particle–particle interaction increases at high sample concentration. The retention time calculated by the theory described in this paper agreed fairly well with the experimental data at various ionic strengths of the eluent.

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INTRODUCTION

Field-flow fractionation (FFF) is classified as a one-phase chromatography technique in which an externally adjusted force field is applied to the suspended particles under motion in a channel (*1-3*). Sedimentation FFF (SdFFF) is one FFF technique that uses a centrifugal force field as the external field. It is suitable for the characterization and the fractionation of colloidal particles in the submicron range. The theoretical basis of SdFFF was originally presented by Giddings and his co-workers (*4-6*). This "standard" theory by Giddings et al. has advantages because it requires only the densities of particles and the eluent and experimental operation conditions, such as the flow rate, the strength of external field, temperature, and the channel dimensions. The average particle sizes converted by this theory from the retention times measured by using an aqueous solution with 0.1% Aerosol OT (AOT) surfactant as the eluent were generally found to agree well with the values obtained from the suppliers, quasi-elastic light-scattering spectroscopy (QELS), and/or electron microscopy, although SdFFF tended to give sizes somewhat smaller than other techniques (*7-11*).

Recently, Hoshino et al. reported that the retention behavior depended on the kinds and the concentration of surfactant added in the eluent (*12*). This fact was explained by the authors to be due to the different states of the interface

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