Binding of Human Liver Hydrolases by Immobilized Lectins

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The binding of 22 human liver hydrolase activities by immobilized lectins of six different carbohydrate specificities, namely α -D-mannose (glucose), D-N-acetylglucosamine, D-N-acetylgalactosamine, L-fucose, α -D-galactose and β -D-galactose, were examined. Differences in binding among these enzymes and within specific enzymes were observed. For example, the neutral forms of α -mannosidase and β -xylosidase were bound by the *Ulex europaeus* lectin I (specific for L-fucose), whereas the acidic forms were not. *Bandiera simplicifolia* lectin (specific for α -galactose) bound 65% of β -glucuronidase activity; recycling experiments demonstrated complete binding of the enzyme that had been eluted with the competitor D-galactose and no binding of the fraction that was not initially bound. These results suggested the presence of two forms of this enzyme. Similar data were obtained for acidic β -galactosidase activity. These experiments may provide the basis for the expanded use of immobilized lectins for purification and characterization of hydrolases and other glycoproteins.

Increasing awareness that many proteins are post-translationally modified to glycoproteins has stimulated investigations of both the structure and function of protein-linked carbohydrates. The requirement, however, for large quantities of glycoproteins has often limited the characterization of these macromolecules. Such investigations might be greatly facilitated by the use of lectins as a screening system. This group of plant proteins has the property of combining with carbohydrates and oligosaccharides in a manner analogous to antigen-antibody reactions (Sharon & Lis, 1972).

Lectins are being used extensively to characterize cell surfaces and membrane interactions. Although insoluble concanavalin A has been used for the purification of various lysosomal enzymes (Beutler *et al.*, 1975), lectins have not been systematically used for the analysis of glycoprotein enzymes. Therefore we have investigated the binding of 22 human liver hydrolase activities involved in inborn errors of metabolism to immobilized lectins of different specificities and examined the potential usefulness of lectins as an analytical tool for the characterization of glycoprotein enzymes.

Materials and Methods

Specimens of human liver, obtained at autopsy, were utilized fresh or after storage at -20°C . Experiments were carried out with a soluble fraction prepared as follows: 1 g of liver was homogenized in 10 ml of 0.01 M-sodium phosphate buffer, pH 6.4;

for some experiments, 0.1% Cutscum (v/v) (Fisher Scientific, Fairlawn, NJ, U.S.A.) was added to this and subsequent buffers. The homogenate was sonicated three times for 30 s at 4°C with a Branson sonifier equipped with a microtip. The sonicated homogenate was centrifuged at 26600g for 30 min (type 40 rotor, Beckman Ultracentrifuge) and the supernatant used for the lectin affinity experiments.

For some experiments, lectins (concanavalin A, wheat-germ agglutinin, soya-bean agglutinin, *Tetragonolobus purpurea* lectin) were purchased from Sigma (St. Louis, MO, U.S.A.) and immobilized on Sepharose 4B (Pharmacia, Piscataway, NJ, U.S.A.) after CNBr activation (Porath *et al.*, 1967); all lectins were conjugated with an efficiency of 95% or greater. For other experiments, agarose-bound lectins (concanavalin A, wheat-germ agglutinin, soya-bean agglutinin, *Ulex europaeus* agglutinin I, *Bandiera simplicifolia* lectin, *Ricinus communis* agglutinin I) were purchased from Vector Laboratories (Novato, CA, U.S.A.). Columns (1 ml) of each immobilized lectin, containing 3.5–4.5 mg of protein/ml of agarose, were prepared in Pasteur pipettes stoppered with glass wool; the columns were equilibrated with 0.01 M-sodium phosphate buffer, pH 6.0, containing 0.002 M- CaCl_2 , 0.002 M- MgCl_2 and 0.002 M- MnCl_2 before use.

Binding of human liver hydrolase activities to each lectin at 25°C was determined as follows: 1 ml of liver supernatant was applied to each column, followed by 2 ml of the equilibrating buffer. Columns were washed with 5 ml of the same buffer supplied

mented with 0.5M-NaCl. Eluates were collected as 2ml fractions and assayed for hydrolytic activities as described below. The binding of activities by the lectins was calculated from the initial enzymic activity in the 1ml liver supernatant minus the activity recovered in the eluates.

Elution of adsorbed enzymic activities was carried out by passing 4ml of 0.01M-Tris/HCl buffer, pH7.0, containing 0.5M-NaCl and 1M-monosaccharide competitor; 2ml fractions were collected. Table 1 summarizes the lectins and the respective competitors utilized in these experiments. Although glycoside or polysaccharide derivatives of these monosaccharides were not tested, both larger volumes of competitor and incubation of the columns at 37°C for 2h before further elution were tested to improve elution yields. Inhibitor-eluted fractions were dialysed against 0.01M-Tris/HCl, pH7.0, before enzymic assays.

Table 2 presents the various hydrolase activities examined for binding by the immobilized lectins. These activities were assayed using various artificial or natural substrates as indicated in the Table. Also

indicated in Table 2 are the substrate concentrations and buffer systems utilized in each assay as well as the initial activity in 1ml of human liver supernatant applied to each lectin column. Those activities assayed with 4-methylumbelliferyl substrates (Sigma) were measured at 37°C for up to 2h as 50µl portions of enzyme source plus 200µl of the appropriate substrate. β -N-Acetyl hexosaminidases A and B were differentiated by using the heat-

Table 1. Lectins and competitors examined for binding and elution of liver hydrolases

Lectin	Competitor
Concanavalin A	Methyl α -D-mannoside
Soya-bean agglutinin	D-N-Acetylgalactosamine
Wheat-germ agglutinin	D-N-Acetylglucosamine
<i>U. europaeus</i> agglutinin I	L-Fucose
<i>T. purpurea</i> lectin	L-Fucose
<i>B. simplicifolia</i> lectin	D-Galactose
<i>R. communis</i> agglutinin I	D-Galactose

Table 2. Liver hydrolase activities examined for binding by immobilized lectins

Enzymic activity	Substrate* (concentration)	Buffer†	pH	Initial‡ activity (nmol/h)
α -Galactosidase (EC 3.2.1.22)	4-MU α -D-galactoside (2mM)	0.1 M-Citrate/phosphate	4.3	78
β -Galactosidase (EC 3.2.1.23)	4-MU β -D-galactoside (1mM)	0.1 M-Citrate/phosphate	4.3	344
α -Glucosidase (EC 3.2.1.20)	4-MU α -D-glucoside (2mM)	0.1 M-Citrate/phosphate	4.3	1182
β -Glucosidase (EC 3.2.1.21)	4-MU β -D-glucoside (1mM)	0.1 M-Citrate/phosphate	4.3	95
			6.2	238
β -Glucuronidase (EC 3.2.1.31)	4-MU β -D-glucuronide (10mM)	0.1 M-Acetate	4.3	436
α -N-Acetylglucosaminidase (EC 3.2.1.50)	p-NP 2-acetamido-2-deoxy- α -D-glucoside (10mM)	0.5 M-Acetate	4.3	3095
β -N-Acetylhexosaminidase A (EC 3.2.1.52)	4-MU 2-acetamido-2-deoxy- β -D-glucoside (1mM)	0.1 M-Citrate/phosphate	4.3	6675
β -N-Acetylhexosaminidase B (EC 3.2.1.52)	4-MU 2-acetamido-2-deoxy- β -D-glucoside (1mM)	0.1 M-Citrate/phosphate	4.3	4469
β -N-Acetylhexosaminidase C (EC 3.2.1.52)	4-MU 2-acetamido-2-deoxy- β -D-glucoside (1mM)	0.1 M-Phosphate	7.0	1421
α -Mannosidase (EC 3.2.1.24)	4-MU α -D-mannoside (5mM)	0.1 M-Citrate/phosphate	4.3	443
			6.2	578
β -Xylosidase (EC 3.2.1.37)	4-MU β -D-xyloside (2mM)	0.1 M-Citrate/phosphate	4.3	17
			6.2	65
α -Arabinosidase (EC 3.2.1.55)	4-MU α -L-arabinoside (2mM)	0.1 M-Citrate/phosphate	4.3	133
α -Fucosidase (EC 3.2.1.51)	p-NP α -L-fucoside (20mM)	0.5 M-Acetate	4.3	1296
Arylsulphatase A (EC 3.1.6.1)	p-NC sulphate (10mM)	0.5 M-Acetate	5.2	379
Arylsulphatase B (EC 3.1.6.1)	p-NC sulphate (50mM)	0.5 M-Acetate	6.0	342
Lactosylceramidase I (EC 3.2.1.46)	[³ H]Lactosylceramide (0.15mM)	0.1 M-Citrate/phosphate	4.3	22
Lactosylceramidase II (EC 3.2.1.23)	[³ H]Lactosylceramide (1mM)	0.1 M-Acetate	4.8	147
Sphingomyelinase (EC 3.1.4.12)	[¹⁴ C]Sphingomyelin (0.1mM)	0.1 M-Acetate	5.0	37

* Substrate abbreviations: 4-MU, 4-methylumbelliferyl; p-NP, p-nitrophenyl; p-NC, p-nitrocatechol.

† All buffers utilized sodium as the counter-ion.

‡ Initial activity in 1 ml of liver supernatant applied to each immobilized lectin.

inactivation assay of O'Brien *et al.* (1970). Enzymic reactions were terminated by the addition of 0.75 ml of 0.1 M-glycine/Na₂CO₃, pH 9.8; release of the reaction product, 4-methylumbelliferone, was measured with an Aminco-Bowman spectrofluorimeter at an excitation wavelength at 365 nm and emission absorbance at 450 nm. Arylsulphatases A and B were determined by the method of Baum *et al.* (1959) as modified by Shapira & Nadler (1975). α -N-Acetylhexosaminidase and α -fucosidase activities were determined in reaction mixtures of 100 μ l of enzyme source plus 100 μ l of the substrates (Sigma) indicated in Table 2; 30 min incubations were carried out at 37°C and reactions were terminated by addition of 1 ml of 1 M-NaOH. The reaction products of the *p*-nitrocatechol and *p*-nitrophenol assays were measured at 515 nm ($\epsilon = 12400 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) and 410 nm ($\epsilon = 14200 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$) respectively on a Gilford spectrophotometer. The assays of lactosylceramidases I and II utilized lactosylceramide (Miles Laboratories, Elkhart, IN, U.S.A.) labelled with ³H in the terminal galactose residue (Radin *et al.*, 1969). Lactosylceramidase I was assayed in the presence of oleic acid and taurocholate (Calbiochem, LaJolla, CA, U.S.A.) by the method of Wenger *et al.* (1974). Lactosylceramidase II was assayed in the presence of 0.05 M-NaCl and taurodeoxycholate (Calbiochem) (Tanaka & Suzuki, 1975; Wenger *et al.*, 1975). Sphingomyelinase was assayed as described by Pentchev *et al.* (1977) by using [*choline-methyl*-¹⁴C]sphingomyelin (New England Nuclear Corp., Boston, MA, U.S.A.).

When necessary, dilutions of enzyme were made with a solution of bovine serum albumin (3 mg/ml; Sigma), previously determined to be free of the enzymic activity being assayed.

Protein was measured by the method of Bradford (1976) by using the reagent kit supplied by Bio-Rad (Richmond, CA, U.S.A.); bovine γ -globulin was used as protein standard.

Results

The binding and recovery after competitor elution of 22 hydrolase activities are shown in Fig. 1. The results are presented as percentages of initial activity (see Table 2) bound by the immobilized lectins and the percentages of initial activity (yield) recovered after elution. These data represent the mean value for three independent experiments; values ranged within $\pm 10\%$ of the value in the Figure. The binding of hydrolases was calculated from the difference between the initial activity applied to each column and the activity that was detected as unadsorbed; the validity of this calculation is borne out by the 100% recovery of initial activity from some lectins, indicated as 0% bound in the Figure

(e.g., α -hexosaminidase on the lectins soya-bean agglutinin or *U. europaeus* agglutinin I).

Among the six lectins, concanavalin A and wheat-germ agglutinin demonstrated the most consistent capacity to bind the hydrolases assayed. In general, those activities bound by concanavalin A were also bound by wheat-germ agglutinin, although the magnitude differed in several cases (e.g., neutral β -galactosidase and arylsulphatase A). In contrast, *R. communis* agglutinin I only bound sphingomyelinase to an appreciable extent (58%), and *B. simplicifolia* lectin demonstrated the greatest variability among the lectins for binding these enzymes. Of the four enzymes bound by *U. europaeus* agglutinin I, two were activities assayed at near neutral pH, designated as 'n' in Fig. 1 (α -mannosidase and β -xylosidase), whereas these activities assayed at acidic pH, designated as 'a' in Fig. 1, were not significantly adsorbed by this lectin. The results of experiments with immobilized *T. purpurea* lectin, which has the same carbohydrate specificity as *U. europaeus* agglutinin I, were identical with those with the latter lectin, and only results for *U. europaeus* agglutinin I are presented in Fig. 1.

The profiles of lectin binding for each activity differed markedly among some of the hydrolases. For example, α -galactosidase was significantly bound by concanavalin A, soya-bean agglutinin and wheat-germ agglutinin, whereas acidic α -mannosidase was significantly bound by concanavalin A, wheat-germ agglutinin and *B. simplicifolia* lectin and neutral β -xylosidase only by *U. europaeus* agglutinin. Discrimination of acidic and neutral forms of these latter two hydrolase activities was also observed; the acidic form of α -mannosidase was bound by concanavalin A, wheat-germ agglutinin and *B. simplicifolia* lectin as compared with wheat-germ agglutinin (47%) and *U. europaeus* agglutinin of the neutral activity; the acidic form of β -xylosidase was bound by concanavalin A, wheat-germ agglutinin and *B. simplicifolia* lectin as compared with only binding by *U. europaeus* agglutinin of the neutral activity. Another example of a relatively unique profile among the activities is found for β -glucuronidase; this activity was bound by concanavalin A, soya-bean agglutinin, wheat-germ agglutinin and *B. simplicifolia* lectin. Finally, sphingomyelinase was the only activity to be bound by all the immobilized lectins with an efficiency greater than 50%.

The profiles for β -galactosidase, assayed both fluorimetrically and with lactosylceramide, the natural substrate assay for this activity, were almost identical, as would be expected if these activities reflected the same enzyme protein. The pattern for neutral β -galactosidase probably reflects the acidic form of this enzyme, as the proportion of acidic activity in human liver is considerably greater than the neutral and there is marked overlap of the acidic

Fig. 1. Binding and elution of hydrolase activities on immobilized lectins

Human liver supernatant (1 ml) was passed through 1 ml columns of the following immobilized lectins: (a) concanavalin A; (b) soya-bean agglutinin; (c) wheat-germ agglutinin; (d) *U. europaeus* agglutinin I; (e) *B. simplicifolia* lectin; (f) *R. communis* agglutinin I. See the Materials and Methods section for experimental details. Binding (%) of the enzymic activities is indicated by the top line of each bar graph and was calculated as: $100 \times (\text{initial activity applied} - \text{unadsorbed activity}) / (\text{initial activity applied})$. Acidic and neutral enzymic activities are designated as 'a' and 'n' respectively. Total enzymic activities (nmol/h) applied to each lectin column are given in Table 2. Elution of enzymic activity by competitor (see Table 1) represents the percentage yield relative to the initial activity applied to each column; these results are shown as the stippled portion of each bar graph. Percentage yield was calculated as: $100 \times (\text{bound activity eluted}) / (\text{initial activity applied})$.

activity when assayed at pH 6.2 (Ben-Yoseph *et al.*, 1977). For similar reasons, the apparent acidic β -glucosidase activity is probably a reflection of the neutral activity. Neither of these latter two activities showed significant binding to any of the lectins; it is likely that the acidic form of β -glucosidase was not sufficiently solubilized by the homogenization and

sonication procedures to be present in independently measurable quantities (Pentchev *et al.*, 1973).

Two types of recycling experiments were carried out. The portion of β -glucuronidase activity that was not initially bound by *B. simplicifolia* lectin was not bound by a second passage over a fresh column of this lectin. In contrast, the activity that was initially bound

and eluted by competitor was subsequently bound over 98% by *B. simplicifolia* lectin on re-application to a fresh column. Similarly, the acidic β -galactosidase activity that was not bound by soya-bean agglutinin and *U. europaeus* agglutinin I was also not bound when passed over fresh columns of these lectins. Greater than 90% efficiency of re-adsorption by concanavalin A and *B. simplicifolia* lectin of the enzyme initially bound and eluted by competitor from these columns was observed.

The elution of activities bound to the six lectins varied considerably from a high efficiency for concanavalin A by the glycoside methyl α -D-mannoside to inconsistent but generally low efficiency for the other lectins with monosaccharides; acidic α -mannosidase, however, was eluted from *B. simplicifolia* lectin by D-galactose with a 97% efficiency. The generally low recoveries probably reflect the need for polysaccharides (e.g., soya-bean agglutinin, wheat-germ agglutinin or glycosidic derivatives). Increased competitor volumes and heating to 37°C improved the elution yields for some of the activities (not reflected in Fig. 1), and thus individual elution procedures may be required for each lectin-enzyme combination if these agglutinins are to be used for purification purposes. Of the total protein applied to each column (13.7 mg), 86–93% was unadsorbed and the extent of purification was often considerable. Of those enzymes retained by concanavalin A with an efficiency greater than 65% of initial activity, the degree of purification was 35–54-fold; approx. 4% of the initial protein was recovered after elution. Some 2.0–3.2% of the initial protein was recovered from the remaining lectins. Lactosylceramidase II and acidic β -mannosidase were purified 75- and 93-fold respectively on wheat-germ agglutinin; acidic α -mannosidase activity was purified 40-fold on *B. simplicifolia* lectin. Only sphingomyelinase was sufficiently eluted from *U. europaeus* agglutinin for its 51-fold purification to be considered significant.

The results of these experiments were identical when either fresh or frozen liver specimens were used. Similar results were obtained when Cutscum was added to the buffers, although several activities were obtained in higher yields from the original homogenate when this detergent was present. As a control for all experiments, no activity was bound to Sepharose 4B.

Discussion

The results of these experiments suggest that immobilized lectins of various carbohydrate specificities may be a useful tool for characterizing enzymes that are glycoproteins. The conformation of the enzyme molecule, its glycosidic linkages, or the position of a carbohydrate within an oligosac-

charide may prevent its detection and thereby limit the interpretation of negative results. Therefore the inability of a lectin to bind to a given enzyme under the conditions used here cannot be interpreted as the absence of a given carbohydrate on that enzyme protein. But the presence of that carbohydrate and its linkage configuration can be inferred from positive results.

In general, these data suggest that there is heterogeneity in the carbohydrate composition of individual enzymes and among the hydrolases examined. Less than complete binding of a given enzyme by a lectin may result from one of several factors: incomplete glycosylation of the protein, the presence of isoenzymes, or differential effects of degradation. However, since fresh and stored liver specimens from two individuals were examined with very similar results, the effects of degradation or storage do not appear to contribute significantly to the heterogeneity. Furthermore, recycling of unbound enzymes (e.g., β -galactosidase and β -glucuronidase) over fresh lectin columns without further binding suggests that individual enzyme molecules may not be identical with respect to glycosylation, perhaps as a result of either incomplete post-translational processing or isoenzyme-like formation; these results are similar to those of Norden & O'Brien (1974), who examined the binding of β -galactosidase to wheat-germ agglutinin.

As a group, the acidic hydrolase activities were bound by concanavalin A, demonstrating the presence of mannose (or glucose); similarly many of the activities were bound by wheat-germ agglutinin, which has an affinity for repeating *N*-acetylglucosamine residues. *N*-Acetylglucosamine is usually found at the reducing end of oligosaccharides as the link to the protein through asparagine (Sharon, 1975). The binding of these hydrolases by wheat-germ agglutinin may represent a more distal localization of this sugar in these proteins.

Differences among the enzymes were also detected. The differential binding of activities by the lectins soya-bean agglutinin and *B. simplicifolia* lectin suggests a heterogeneity among the hydrolases, with the above qualification about negative binding results preventing a firm conclusion. Those enzymes that were bound by *B. simplicifolia* lectin, however, appear to contain D-galactose or galactose-like residues in an α -linkage. Although this sugar and linkage configuration is found in various glycolipids (e.g. blood-group B antigen), it is apparently rare among glycoproteins [although the issue of ABH-blood-group-specific glycoproteins is not resolved (Sharon, 1975)]. A similarly surprising finding was the overall lack of binding by *R. communis* agglutinin I, specific for β -D-galactose; β -galactose is commonly detected in serum and mucin-type glycoproteins (Sharon, 1975). Specific chemical and conformational

analyses of oligosaccharide side chains of individual hydrolases would confirm these suggestions.

The binding of several enzymes by *U. europaeus* agglutinin (and *T. purpurea* lectin), demonstrating the presence of L-fucose, usually a terminal sugar at the non-reducing end, is also of interest. Two of the three neutral activities assayed showed significant binding to this lectin, whereas only two of the acidic hydrolases were retained at or above 50% efficiency. It is possible that fucose may be a part of the biochemical mechanism that differentiates neutral (non-lysosomal?) from acidic lysosomal hydrolases. Further studies with other similarly related enzymes are required to clarify this question.

The present work may prove useful as a guide for the use of lectins in the purification of hydrolases. Immobilized concanavalin A has already become a common step in the isolation of lysosomal enzymes. These data provide a basis for more widespread use of lectins, some of which may be particularly useful in purifying individual enzymes from the majority of other lysosomal enzymes, which often co-purify because of similar properties (e.g., β -glucuronidase on soya-bean agglutinin, acidic α -mannosidase on *B. simplicifolia* lectin). Although some of the lectins used here are relatively expensive when obtained commercially, improved affinity techniques are making the isolation of lectins an inexpensive and rapid procedure (Baues & Gray, 1977). The extensive use of lectins, however, will depend on improved methods of elution discussed above. Some enzymes may also require specific detergents throughout the binding and elution procedures; the effect of several detergents on immobilized lectins has been examined (Lotan *et al.*, 1977) with general guidelines for their use evolving from this work.

The use of lectins as described in the present paper may also provide supportive evidence where controversies exist about the molecular identities of similar hydrolase activities. Marked differences in the binding of lactosylceramidases I and II by soya-bean agglutinin and *U. europaeus* agglutinin I provide additional evidence to that reported about the separate identity of these two enzymes despite their capacity to cleave the same substrate (Tanaka & Suzuki, 1975). Similarly, although there may have been slight differences in the binding of β -hexosaminidases A and B by *B. simplicifolia* lectin, the profiles of these two enzymes were nearly identical, consistent with the relatedness of these two enzymes (Geiger & Arnon, 1976). In contrast, the profile for β -hexosaminidase C was different from the A and B enzymes; this too is consistent with other reports supporting the unrelatedness of enzyme C to enzymes A and B (Braidman *et al.*, 1974).

Finally, it should be noted that only a small percentage (<10%) of the total protein in the liver supernatant was bound by any one of the lectins, whereas the majority of the hydrolases were bound by one or more lectins. These hydrolases, primarily of lysosomal origin, may be glycosylated as a means of differentiation at the biochemical level, perhaps as part of a mechanism that directs their subcellular localization. It is also possible that some inherited enzymic deficiencies may result from a defect in post-translational processing, which results in altered glycosylation of one or more enzymes. The use of a lectin screen may efficiently identify the effects of this kind of mutation.

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