ANALYTICAL BIOCHEMISTRY **130**, 437–444 (1983)

Enzyme-Linked Lectin Assay (ELLA): Use of Alkaline Phosphatase-Conjugated *Griffonia simplicifolia* B_4 Isolectin for the Detection of α-D-galactopyranosyl End Groups

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Received October 25, 1982

Alkaline phosphatase has been coupled to *Griffonia simplicifolia* I B_4 isolectin using a one-step glutaraldehyde conjugation procedure. This enzyme–lectin conjugate (AP–GS I-B_4) has been used to specifically detect plastic-bound natural and synthetic glycoproteins bearing α-D-galactopyranosyl end groups. The extent of reactivity of the AP–GS I-B_4 with the glycoproteins appears to be proportional to the number of terminal galactosyl residues present. Furthermore, this assay, termed ELLA (enzyme-linked lectin assay), is specifically inhibitable by low-molecular-weight sugars containing terminal α-D-galactosyl groups. The ELLA reactions may be assayed rapidly and objectively by the use of commercially available ELISA-plate readers using standard filters.

KEY WORDS: lectin; glycoproteins; α-D-galactosyl end groups; *Griffonia simplifolia;* enzyme immunoassay; enzyme-linked lectins.

Griffonia simplicifolia was first shown to be the source of an α-D-galactopyranosyl-binding lectin by Hayes and Goldstein (1). Later it was discovered that this lectin was actually composed of five individual isolectins, each of which was a tetrameric arrangement of various combinations of the two glycoprotein subunits A and B (2). Determination of the fine specificity of the two subunits showed that the A subunit reacted primarily with α-D-GalNAc*p*[2] units but also with α-D-Gal*p*, while the B subunit showed a sharp specificity for α-D-Gal*p* units. Therefore the tetrameric isolectin B_4 (GSI-B_4) may be employed as a probe for the detection of α-D-Gal*p* end groups.

The GS I-B_4 isolectin has been conjugated to fluorescein isothiocyanate for use as a probe for α-D-Gal*p* end groups in tissue sections (3), live cells (4), and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoretograms (5). Additionally it has been possible to couple the I-B_4 isolectin to cyanogen–bromide-activated Sepharose for use in affinity purification of glycoproteins bearing α-D-Gal*p* end groups (5), e.g., laminin.

In the present study, we have conjugated alkaline phosphatase to the GS I-B_4 isolectin and have used the resulting enzyme–lectin complex as a specific probe for α-D-Gal*p* end groups on immobilized glycoproteins. We have termed this assay system ELLA (enzyme-linked lectin assay). ELLA assays are conceptually similar to the common ELISA (enzyme-linked immunosorbent assay) but are unique in that they rely upon nonimmunologic reagents.

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[2] Abbreviations used: GS I-B_4, *Griffonia simplicifolia* B_4 isolectin; AP–GS I-B_4, alkaline phosphatase-conjugated *Griffonia simplicifolia* B_4 isolectin; Me α-D-Gal*p*, methyl α-D-galactopyranoside; D-GalNAc*p*, *N*-acetyl-D-galactosamine; D-GlcA, D-gluconic acid; PBS, 0.01 M phosphate-buffered saline, pH 7.2; PBS-T, phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate.

0003-2697/83 $3.00
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METHODS AND MATERIALS

Conjugation of alkaline phosphatase to Griffonia simplicifolia I-B_4. Alkaline phosphatase from calf intestine (Type VII-S: 5000 units in 0.74 ml with 6.4 mg protein/ml) was obtained from Sigma Chemical Company, St. Louis, Missouri. *Griffonia simplicifolia* I-B_4 isolectin was prepared according to the method of Delmottee and Goldstein (6). Alkaline phosphatase, 1500 U, was conjugated to 500 μg of the GS I-B_4 lectin using modifications of the procedure for conjugation of enzymes and antibodies described by Engvall and Perlmann (1972). Briefly, 1500 U of alkaline phosphatase suspended in ammonium sulfate was centrifuged at 1000*g* for 10 min and the supernatant discarded. Five hundred micrograms GS I-B_4 in 250 μl PBS was added to the alkaline phosphatase pellet. The enzyme-lectin mixture was brought to a final volume of 500 μl with PBS containing 10 mM methyl α-D-galactopyranoside (Me α-D-Gal*p*), a haptenic sugar used to protect the lectin's binding sites. The mixture was dialyzed overnight at 4°C against PBS containing 10 mM methyl α-D-galactopyranoside. After dialysis, glutaraldehyde was added to the mixture to a final concentration of 0.3% and the mixture allowed to incubate at room temperature for 2 h at 37°C with gentle shaking every 30 min. Then the alkaline phosphatase-*G. simplicifolia* I-B_4 (AP-GS I-B_4) conjugate was dialyzed overnight at 4°C against PBS containing 5 mM Me α-D-Gal*p*. The AP-GS I-B_4 was diluted with PBS, to give a final protein concentration of 250 μg/ml and Me α-D-Gal*p* concentration of 2.5 mM.[3] One drop of 1% NaN_3 was added to this stock solution of AP-GS I-B_4 and the conjugate was stored at 4°C.

Preparation of glycoproteins and carbohydrate moieties. Laminin was prepared from EHS sarcoma tissue grown *in vivo* using the method of Timpl *et al.* (8). The purity of the laminin was assessed by SDS-gel electrophoresis using a 5% polyacrylamide gel and, consistent with published reports (5,8), the purified laminin, upon SDS-gel electrophoresis showed only 2 bands of M_r 200,000 and 400,000. Guaran, prepared in this laboratory, was available from a previous study.

Methyl α-D-galactopyranoside, methyl α-D-glucopyranoside, bovine serum albumin, and ovalbumin were purchased from Sigma Chemical Company. Feline IgG was obtained from Pel-Freeze Biologicals (Rogers, Ark). Raffinose, melibiose, and *N*-acetyl-D-galactosamine were purchased from Pfanstiehl Laboratories, Waukegan, Illinois. *N*-Acetyllactosamine was available from a previous study. Ovarian cyst substances of types A and B were kindly provided by Dr. E. A. Kabat of Columbia University.

The carbohydrate-BSA conjugates (melibionate-BSA, lactonate-BSA, and maltonate-BSA), prepared by the aldonate coupling reaction, were available from a previous study (9). The manninotrionate-BSA was prepared from manninotrionate (α-D-Gal-(1 $\rightarrow$ 6)-α-D-Gal-(1 $\rightarrow$ 6)-GlcA) and will be reported in a separate communication. The carbohydrate content of the conjugates was determined by the phenol-sulfuric acid assay (10) and was used to calculate the degree of substitution (DS = molar ratio of sugar to protein in product). The melibionate-BSA conjugates had degrees of substitution of 40, 32, 22, and 12. The Gal-(1 $\xrightarrow{\alpha}$ 3)-Gal-BSA conjugate was a gift of Dr. D. A. Baker, Chembiomed Ltd., Edmonton, Alberta, Canada. Asialoorosomucoid and asialofetuin were the gift of Dr. G. W. Jourdian, Department of Biological Chemistry, University of Michigan.

Enzyme-linked lectin assay (ELLA). Glycoproteins to be assayed for the presence of terminal α-D-galactopyranosyl groups (specified below) were diluted to a final concentration of 10 μg/ml in 0.1 M carbonate buffer, pH 9.6. The diluted glycoproteins (50 μl) were added to the wells of a plastic 96-well micro-

[3] Storage of the AP-GS I-B_4 stock in the absence of Me α-D-Gal*p* led to the formation of a precipitate, which was resolubilized by the addition of Me α-D-Gal*p*. This phenomenon, which presumably is an indication of reactivity between GS I-B_4 and calf intestine alkaline phosphatase, is currently under investigation.

titer plate (Falcon 3070 Lot 1342278, Becton–Dickinson, Oxnard, Calif.). Attachment of the glycoproteins to the plastic wells was effected by incubation at 37°C for 3 h. Following incubation, the fluid was removed from the wells and the wells washed three times with PBS containing 0.05% Tween 20 (PBS-T). Immediately after washing, 50 μl of dilutions of the AP–GS I-B_4 stock in PBS were added to the wells of the microtiter plates. Dilutions of the stock used in this study ranged from 1:100 (2.5 μg/ml) to 1:1600 (0.156 μg/ml). The appropriately diluted AP–GS I-B_4 was incubated for 30 min at 37°C in the microtiter wells. After the incubation, the wells were again washed with three changes of PBS-T. Following the last wash, 100-μl aliquots of *p*-nitrophenyl phosphate (Sigma 104, Sigma) (1 mg/ml in 0.1 M carbonate buffer, pH 9.6) were added to each well. Absorbance at 405 nm of each well was determined at various time intervals using a Titertek Multiskan ELISA plate reader (Flow Labs, McClean, Va.).

After titration of the enzyme–lectin conjugate, as described above, the ELLA reactions were also conducted using a fixed amount of AP–GS I-B_4 and 10-fold dilutions of the glycoproteins. This variation of the ELLA was performed as described above except the AP–GS I-B_4 was used at a concentration of 1.67 μg/ml in all wells, and the glycoconjugates were assayed, in duplicate in 10-fold dilutions ranging from concentrations of 1 mg/ml to 0.1 μg/ml.

Inhibition reactions in ELLA. To determine the specificity of the enzyme-linked lectin assay using the AP–GS I-B_4 conjugate, a series of carbohydrate moieties, including the haptenic sugars methyl α-D-galactopyranoside and methyl α-D-glucopyranoside, were added directly to the wells along with the AP–GS I-B_4. Other carbohydrate haptens tested for their inhibitory activity included raffinose, melibiose, *N*-acetyllactosamine, *N*-acetyl-D-galactosamine, and types A and B blood group substances.

The inhibition experiments using the ELLA technique were performed as described above except for the following modification: one concentration of AP–GS I-B_4 (1.67 μg/ml) was used, to which various sugars (potential inhibitors) were added. The inhibitors were added to the AP–GS I-B_4 at final concentrations ranging from 0.01 to 50 mM and the mixture, was immediately added to the microtiter plate. The inhibitory effect of a carbohydrate moiety was calculated as follows:

$$\frac{A_{405}(\text{lectin diluted in PBS}) - A_{405}(\text{lectin in inhibitor})}{A_{405}(\text{lectin diluted in PBS})} \times 100 = \%\ \text{inhibition}.$$

Pretreatment of laminin with α-galactosidase. To further examine the specificity of the AP–GS I-B_4 for α-D-galactopyranosyl end groups, laminin, a glycoprotein which reacted with the enzyme-conjugated lectin, was treated with coffee bean α-galactosidase (Sigma) prior to incubation with the AP–GS I-B_4. Laminin, bound to the microtiter plates as described above, was washed three times with PBS, pH 6.0. Each well was then incubated with 50 μl of different dilutions of an α-galactosidase solution (0.42, 0.21, 0.105, 0.053 U/ml) in PBS, pH 6.0 for 1 h at 37°C. Controls consisted of wells incubated with PBS, pH 6.0, with no enzyme. After incubation, the enzyme solution was removed and the wells washed three times with PBS-T. The ELLA assay was then performed as described above.

RESULTS

Binding of AP–GS I-B_4 to Immobilized Glycoproteins

The alkaline phosphatase-conjugated GS I-B_4 was used in the ELLA technique to screen both naturally occurring and synthetic glycoproteins for the presence of α-D-galactopyranosyl end groups. Figure 1 shows the results of the titration of AP–GS I-B_4 against bovine serum albumin and the natural glycoproteins laminin, ovalbumin, guaran, and feline IgG. As seen in the figure, laminin re-

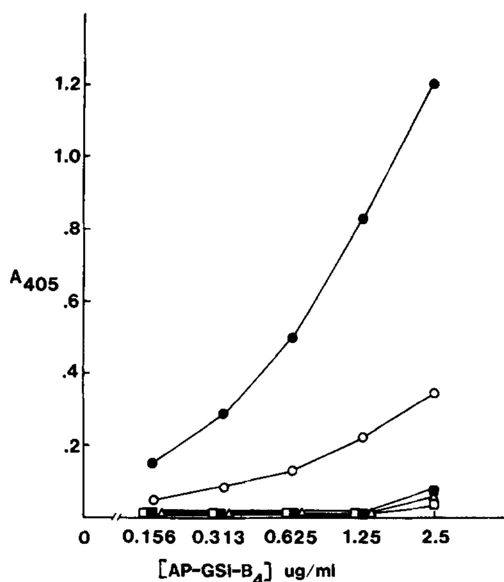

FIG. 1. Titration of alkaline phosphatase-conjugated GS I-B_4 (AP–GS I-B_4) by ELLA against naturally occurring glycoproteins. Wells are coated with 50 μl of the glycoproteins at a concentration of 10 μg/ml. The glycoproteins used were: laminin (●); guaran (○); ovalbumin (△); and feline IgG (□). Bovine serum albumin is indicated by (■).

acted significantly with the AP–GS I-B_4 and this reactivity was diminished by increasing dilution of the enzyme-conjugated lectin. Guaran, a galactomannan containing α-D-galactopyranosyl end groups, also reacted with AP–GS I-B_4 but to a much lesser extent than laminin. As with laminin, reactivity of guaran with AP–GS I-B_4 was diminished by increasing the dilution of the enzyme-conjugated lectin. Bovine serum albumin, ovalbumin, and feline IgG were all nonreactive with AP–GS I-B_4.

The results of assaying various synthetic glycoconjugates for the presence of terminal α-D-galactopyranosyl groups is shown in Fig. 2. Melibionate–BSA and manninotrionate–BSA preparations reacted with the enzyme-conjugated lectin compared to background levels obtained with native BSA controls. Lactonate–BSA, maltonate–BSA (data not shown), and asialofetuin did not react with AP–GS I-B_4 above the levels obtained with nonconjugated BSA. To ensure that all glycoconjugates had bound to the wells of the microtiter plates, glycoconjugates (with the exception of asialofetuin) presumably bound to the wells were screened by ELISA using rabbit anti-BSA. The results (data not shown) indicated that all of the carbohydrate–BSA conjugates had bound evenly to the wells.

Figures 3 and 4 illustrate the results obtained by the ELLA technique using 10-fold dilutions of natural and synthetic glycoproteins, respectively, and a constant concentration of AP–GS I-B_4 (1.67 μg/ml). Laminin and guaran reacted with the AP–GS I-B_4 when coated onto the microtiter plates only in concentrations greater than 1 μg/ml while ovalbumin, BSA, and feline IgG were virtually nonreactive at all concentrations (Fig. 3). Similarly, melibionate–BSA (DS-40) and manninotrionate–BSA clearly reacted with the

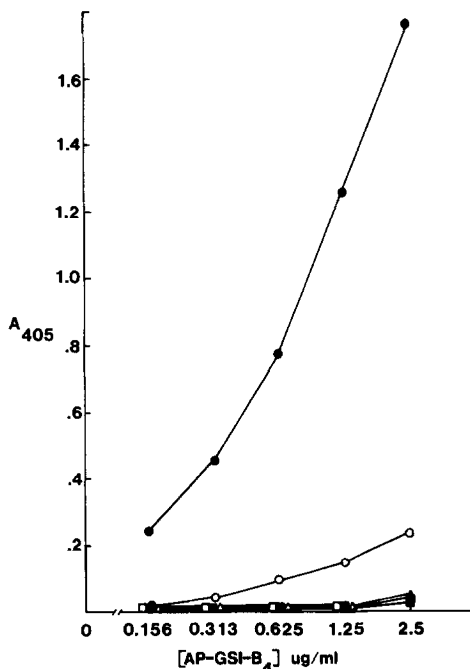

FIG. 2. Titration of alkaline phosphatase-conjugated GS I-B_4 (AP–GS I-B_4) by ELLA against synthetic glycoconjugates. Wells are coated with 50 μl of the glycoconjugates at a concentration of 10 μg/ml. The glycoconjugates used were: melibionate–BSA (DS-32) (●); manninotrionate–BSA (○); lactonate–BSA (■); native BSA (△); and asialofetuin (□).

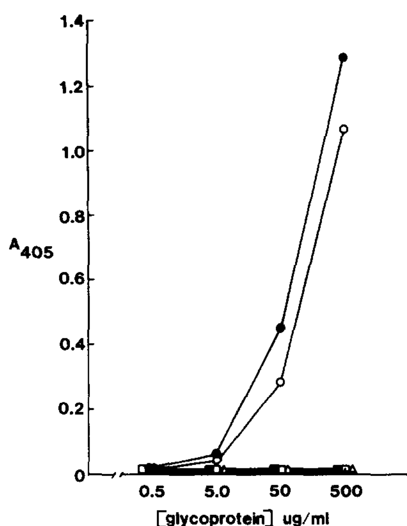

FIG. 3. Titration of naturally occurring glycoproteins by ELLA against alkaline phosphatase-conjugated GS I-B_4 (AP–GS IP-B_4). All wells are developed with 50 μl AP–GS I-B_4 at a concentration of 1.67 μg/ml. The glycoproteins used were: laminin (●); guaran (○); ovalbumin (△); and feline IgG (□). Bovine serum albumin is indicated by (■).

AP–GS I-B_4 during the ELLA reaction. Figure 6 shows the inhibitory effects of these sugars in ELLAs against the melibionate–BSA (DS 40) conjugate previously found to react in ELLA. As can be seen methyl α-D-galactopyranoside was strongly inhibitory in concentrations over 0.1 mM, whereas methyl α-D-glucopyranoside showed insignificant inhibition. Specific inhibition by Me α-Gal*p* was also observed in ELLAs directed against bound laminin and guaran. Two additional sugars containing α-D-Gal*p* end groups, raffinose and melibiose, were also tested for their inhibitory activity (Fig. 6). Both were found to significantly inhibit the ELLA reaction against melibionate–BSA when present in concentrations greater than 0.1 mM. In contrast, *N*-acetyl-D-galactosamine and *N*-acetyllactosamine were much less inhibitory, requiring concentrations approximately 100 times

enzyme–lectin conjugate when bound to the wells in concentrations above 1 μg/ml (Fig. 4). The Gal-($1 \xrightarrow{\alpha} 3$)-Gal–BSA conjugate (100 μg/ml) also reacted (data not shown). Maltonate–BSA, lactonate–BSA, asialofetuin, and native BSA all showed no reactivity at any concentration (Fig. 4). Asialoorosomucoid was also nonreactive with the AP–GS I-B_4 (data not shown).

Correlation of AP–GS I-B_4 Binding with Degree of Substitution

Melibionate–BSA conjugates with degrees of substitution of 40, 32, 22, 12, and 0 (native BSA) were tested by ELLA for AP–GS I-B_4 binding. As seen in Fig. 5, the degree of substitution of the melibionate–BSA conjugate was directly proportional to the absorbance at 405 nm obtained in the ELLA technique.

Inhibition of ELLA by Various Carbohydrate Residues

To test the specificity of the ELLA reaction, a series of carbohydrates were added to the

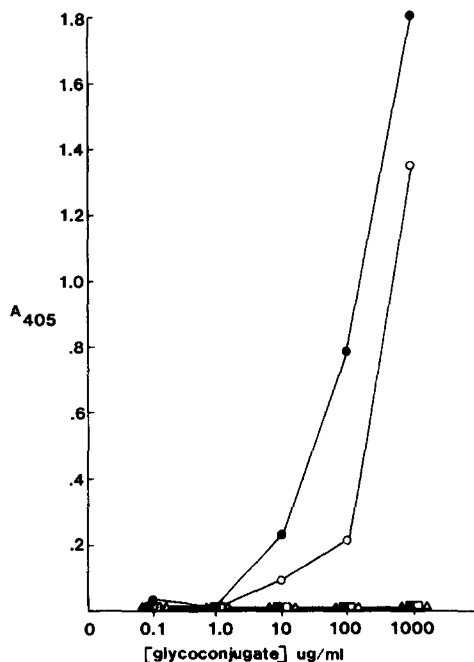

FIG. 4. Titration of synthetic glycoconjugates by ELLA against alkaline phosphatase-conjugated GS I-B_4 (AP–GS I-B_4). All wells are developed with 50 μl AP–GS I-B_4 at a concentration of 1.67 μg/ml. The glycoconjugates used were: melibionate–BSA (DS-32) (●); manninotrionate–BSA (○); lactonate–BSA (■); native BSA (△); asialofetuin (□); and maltonate–BSA (▲).

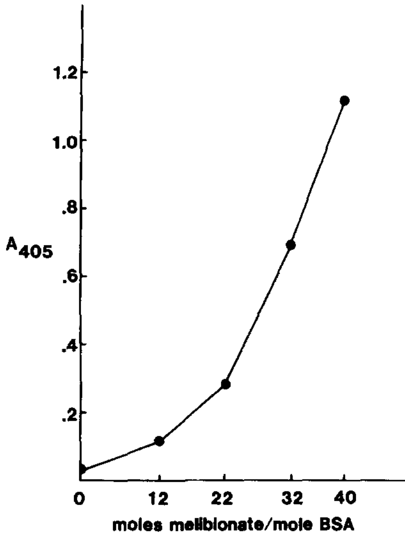

FIG. 5. Reactivity of AP–GS I-B_4 (1.67 μg/ml) against BSA substituted with various amount of melibionate.

higher to achieve the same inhibition observed with 0.1 mM methyl α-D-galactopyranoside. Lactose, which contains a β-D-galactopyranosyl end group, was not inhibitory at any concentration tested (data not shown). Type A and B blood group substances were similarly tested for inhibitory activity in concentrations between 60 and 0.06 μg/ml. The B blood group substance was strongly inhibitory at concentrations greater than 6 μg/ml, while Type A blood group substance was virtually non-inhibitory at concentrations as high as 60 μg/ml.

Effect of α-Galactosidase Pretreatment

To further determine the specificity of the ELLA reaction, immobilized laminin was treated with α-galactosidase in PBS, pH 6.0, or with PBS, pH 6.0, alone before incubation with AP–GS I-B_4. As seen in Table 1, galactosidase pretreatment markedly reduced the binding of the lectin to laminin compared to the control pretreated with the PBS, pH 6.0.

DISCUSSION

The data presented here clearly indicate the feasibility of preparing an enzyme–lectin conjugate and using the conjugate as a specific probe for certain carbohydrate moieties on immobilized glycoproteins. Conjugation of enzymes to lectins is not a new concept; peroxidase–lectin conjugates have been used for over a decade as specific probes for carbohydrate moieties using insoluble substrates in electron microscopy (11,12). These studies, however, have only described the detection of the carbohydrate moieties on cells or tissue

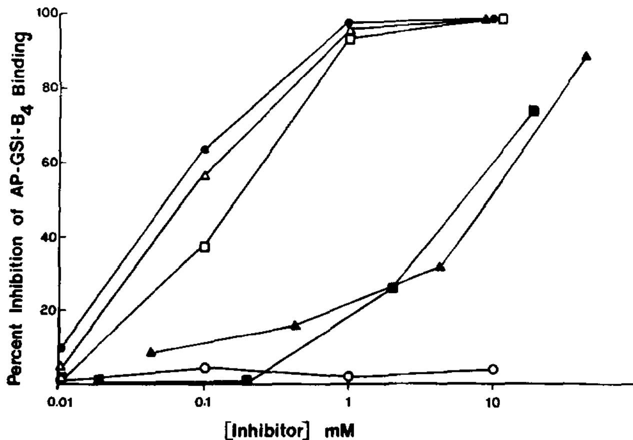

FIG. 6. Inhibition of the reactivity of AP–GS I-B_4 with melibionate-BSA (DS-40) in ELLA by low-molecular-weight sugars. The low-molecular-weight sugars used were: methyl α-D-galactopyranoside (●); methyl α-D-glucopyranoside (○); raffinose (□); melibiose (△); *N*-acetyl-D-galactosamine (▲); and *N*-acetyllactosamine (■).

sections and have required manual determination of the lectin binding. Other studies have been reported describing enzymatic analyses of lectin–carbohydrate interactions (13,14), but only the present study utilizes an enzyme directly conjugated to a lectin.

In this investigation we have used the enzyme–lectin conjugate (AP–GS I-B_4) for the specific detection of α-D-galactopyranosyl end groups on purified glycoproteins and synthetic glycoconjugates. The data using the glycoconjugates is particularly convincing since native BSA (the carrier protein) and conjugates consisting of BSA and sugars not containing α-D-galactosyl end groups were uniformly negative, in sharp contrast to the glycoconjugates of BSA containing terminal α-D-galactopyranosyl groups. The fact that rabbit anti-BSA bound equally to wells coated with the various glycoconjugates was a strong indication that the differences in binding of the AP–GS I-B_4 to the carbohydrate–BSA conjugates were due to the presence or absence of α-D-Gal*p* end groups and not merely the result of differential binding of the various glycoconjugates to the wells of the microtiter plates. Furthermore the intensity of the ELLA reaction (expressed as A_{405}) and the degree of substitution of melibionate residues onto BSA were directly proportional and indicated the potential of using the ELLA technique in a quantitative manner. Additionally, a clear distinction between carbohydrate moieties with and without α-D-galactopyranosyl end groups was possible by using these moieties to inhibit the ELLA reaction against immobilized α-D-Gal*p*-containing glycoproteins. Therefore, the ELLA technique may be used to detect α-D-Gal*p* end-groups in either immobilized glycoproteins or soluble sugar residues.

Caution must be exercised in comparing day to day A_{405} values of the ELLA technique. While replicate samples within a single experiment were generally very close, day to day variability occurred, presumably due to the slow breakdown of the enzyme–lectin conjugate. It is estimated that the activity of the conjugate decreases by 50% in 30 days. The decreasing activity of the conjugate may be compensated by increasing the time for the colorimetric development of the *p*-nitrophenyl phosphate substrate before measuring the A_{405}, thereby giving a relatively constant value for day to day comparisons of a given glycoprotein. Thus, by carefully controlling every experiment with standard control proteins, day to day comparisons of ELLAs may be possible.

TABLE 1

EFFECT OF COFFEE BEAN α-GALACTOSIDASE PRETREATMENT OF LAMININ ON REACTIVITY OF AP–GS I-B_4 IN ELLA[a]

Pretreatment	Percentage inhibition[b]
PBS, pH 6.0	0
0.42 U/ml α-galactosidase in PBS, pH 6.0	75
0.21 U/ml α-galactosidase in PBS, pH 6.0	83
0.105 U/ml α-galactosidase in PBS, pH 6.0	70
0.053 U/ml α-galactosidase in PBS, pH 6.0	64

[a] Enzyme and control pretreatments were conducted at 37°C for 1 h.

[b] Inhibition in comparison to reactivity of AP–GS I-B_4 with laminin pretreated with PBS, pH 7.2.

In summary, the ELLA technique is a simple microassay which may be used for the detection of specific carbohydrate units (in this study α-D-galactopyranosyl end units), either directly on immobilized glycoproteins or indirectly by inhibition of soluble sugars. The technique may be performed in a semiautomated and quantitative manner with the use of commercially available ELISA plate readers. It is apparent that different lectins may be conjugated in a similar fashion to alkaline phosphatase and employed for the detection and quantitation of other carbohydrate units. It should also be noted that cell surface carbohydrate units may also be quantified using a modification of this approach, an undertaking which is now in progress.

ACKNOWLEDGMENTS

This work was supported by Institutional Research Grant IN-40V to the University of Michigan from the American Cancer Society and by Grants CA29550, CA29551, CA20424, and GM29470 from the U. S. Public Health Service. We gratefully acknowledge the technical assistance of Mr. William Schade and the secretarial assistance of Ms. Kim Drake.

REFERENCES

1. Hayes, C. E., and Goldstein, I. J. (1974) *J. Biol. Chem.* **249,** 1904–1914.
2. Murphy, L. A., and Goldstein, I. J. (1977) *J. Biol. Chem.* **252,** 4739–4742.
3. Peters, B. P., and Goldstein, I. J. (1979) *Exp. Cell Res.* **120,** 321–324.
4. Varani, J., Lovett, E. J., McCoy, J. P., Shibata, S., Maddox, D., Goldstein, I. J., and Liotta, L. (1983) *Amer. J. Pathol.* **111,** 27–34.
5. Shibata, S., Peters, B. P., Roberts, D. D., Goldstein, I. J., and Liotta, L. A. (1982) *FEBS Lett.* **142,** 194–198.
6. Delmotte, F. M., and Goldstein, I. J. (1980) *Eur. J. Biochem.* **112,** 219–223.
7. Engvall, E., and Perlmann, P. (1972) *J. Immunol.* **109,** 129–135.
8. Timpl, R., Rohde, H., Robey, P. G., Rennard, S. I., Foidart, J. M., and Martin, G. R. (1979) *J. Biol. Chem.* **254,** 9933–9937.
9. Lönngren, J., Goldstein, I. J., and Niederhuber, J. E. (1976) *Arch. Biochem. Biophys.* **175,** 661–669.
10. Hodge, J. E., and Hofreiter, B. T. (1962) *Methods Carbohydr. Chem.* **1,** 380–394.
11. Gonatas, N. K., and Avrameas, S. (1973) *J. Cell Biol.* **59,** 436–443.
12. Stobo, J. D., and Rosenthal, A. S. (1972) *Exp. Cell Res.* **70,** 443–447.
13. Borrebaeck, C., and Mattiasson, B. (1980) *Anal. Biochem.* **107,** 446–450.
14. Guesdon, J. L., and Avrameas, S. (1980) *J. Immunol. Methods* **39,** 1–13, 1980.