Glycoconjugate Journal (1997) **14:** 493–496

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A lectin method for investigating the glycosylation of nanogram amounts of purified glycoprotein

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To unravel the complexities of the glycosylation of a protein is a substantial task, which requires considerable effort and resources. However, in many situations this is unnecessary, because only a limited amount of information is required. A new lectin-binding assay is described which is rapid, cheap and versatile. A purified glycoprotein is absorbed on to the plastic surface of a microtitre plate. After removing unbound protein by washing, uncoated sites on the plate are blocked and digoxigenin or biotin-labelled lectin is added. The degree of lectin binding is measured using either an anti-DIG antibody or streptavidin conjugated enzyme, which is subsequently used to develop a colour reaction. Using this method it is possible to screen multiple specimens with high sensitivity and excellent precision. In addition, very small amounts of lectin are used, background absorbances are low, and the procedure does not require a high degree of technical skill. Because very small amounts of glycoprotein are needed, a glycoprotein can often be rapidly purified by batch affinity chromatography. The method has been successfully applied to several purified proteins using the lectins, Con A, LCA, LTA, MAA, and SNA, and the information obtained agrees with that produced by more sophisticated approaches, *eg* Dionex Carbohydrate Analyser. Using a panel of lectins, a carbohydrate structural profile is quickly built-up, and subtle differences in glycosylation identified. This method should be particularly useful for screening glycosylation in multiple clinical specimens; in specimens where very small amounts of material are available, such as membrane molecules; and in the screening of recombinant proteins produced commercially.

***Keywords*: alpha-1-proteinase inhibitor, ELISA, haptoglobin, lectin, transferrin**

Introduction

There is increasing interest in determining the glycosylation of a molecule because of the importance of glycosylation in affecting reactivity [1, 2]. This is particularly true in the production of therapeutic glycoproteins by recombinant methods, where glycosylation can be determined by the type of host cell used, or the production process employed [3]. Glycosylation is also important in disease situations where changes in carbohydrate structure could be involved in pathological processes [4].

Unfortunately, the glycosylation of proteins is very complex; there are variations in the site of glycosylation, the type of glycopeptide bond, the composition of the carbohydrate chains, and the particular carbohydrate sequences and linkages in the chains [2, 4]. In addition, within any population of molecules there is considerable heterogeneity in the carbohydrate structures that are synthesized at any one time [1]. This is typified in many molecules by changes in glycan branching, fucosylation and/or sialylation [4].

Detailed structural characterization of the glycan chains of a glycoprotein is not necessary in many situations, because only a limited amount of information is needed on a single or group of structural features. Lectins can be very useful for this purpose. They have previously been used to study glycosylation by incorporating them into existing technologies such as affinity chromatography, blotting, electrophoresis and lectin immunoassay [4].

In the assay described a purified glycoprotein is absorbed on the plastic surface of a well in a microtitre plate. A lectin labelled with dioxigenin (DIG) or biotin is added and allowed to interact with the carbohydrate on the absorbed glycoprotein. The amount of bound lectin is measured by adding anti-DIG antibody or streptavidin conjugated to an enzyme. A colour reaction is developed by addition of an appropriate substrates for the bound enzyme. A preliminary account of this procedure has already been described [5].

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Experimental

All purified proteins were obtained from Sigma UK except for α1-proteinase inhibitor (API) which was purchased from SCIPAC, UK. The DIG-labelled lectins (Con A, MAA, SNA) and anti-DIG antibody were from Boehringer-Mannheim UK. Biotin-labelled lectins (LCA and LTA) and streptavidin-alkaline phosphatase were purchased from Sigma UK. A 100 µl aliquot of protein in 25 mmol l^{-1} Tris/100 mmol l^{-1} NaCl, pH = 7.5 (TBS), was added to a microtitreplate (Immunolon 4, Dynatech) and incubated at 37 °C for 2 h. The amount of glycoprotein used to coat each well depended on the particular combination of lectin and glycoprotein investigated, and varied between 0.78–280 ng. Unbound proteins were removed by washing three times with TBS containing 0.1% Tween 20 (TTBS) and the plate was incubated with TTBS for 1 h at 37 °C and overnight at 4 °C. After washing twice with 1 mmol l^{-1} Tris pH = 7.5 containing 0.1% Tween and 1 mmol l^{-1} $CaCl_2$, $MgCl_2$ and $MnCl_2$, 200 µl of either DIG-labelled or biotin-labelled lectins was added and stood at 37 °C for 1 h. The plate was washed four times with TTBS and 150 µl of either anti-DIG antibody conjugated with horseradish peroxidase (HRP) (50 mU ml^{-1}) or streptavidin conjugated with alkaline phosphatase (ALP) (1.3 mU μl^{-1}) was added and incubated at room temperature and 37 °C respectively. The colour reaction was developed using O-phenylenediamine (BDH) as a substrate for HRP and para-nitrophenylphosphate (BDH) as a substrate for ALP, as previously described [6]. For each lectin, the assay was also carried out in the absence of protein (background).

Haptoglobin (HP) and API were isolated form sera using an affinity chromatography technique, which was reported previously [7]. Purity of isolated proteins was checked by SDS-PAGE. Protein concentration was determined using rocket immunoelectrophoresis. Following hydrolysis of purified proteins by trifluoroacetic acid, monosaccharide analysis was carried out using a carbohydrate analysis system (Dionex) [8].

Results and discussion

Table 1 shows the principal specificity of the lectins used in this study. It has been previously shown that all these lectins have much weaker affinities for other carbohydrate groups, either in different linkages or to different carbohydrates [9]; however, the specificity given is the one that predominates.

Figure 1 compares the optical density (OD) obtained for different lectins with API, HP, or transferrin (TF). For any combination of lectin and glycoprotein, there was a range of glycoprotein coating concentrations over which the amount of lectin-binding was proportional to the amount of protein used. In all cases this was at the ng level, but it varied for each lectin. Presumably, this was due to differences in the

Table 1. Carbohydrate specificity of lectins used in this study.

Lectin	*Abbreviation*	*Major specificity*
Concanavalin A	Con A	Mannose
Lens culinaris agglutinin	LCA	*a*1-6Fucose[a]
Lotus tetragonolobus agglutinin	LTA	*a*1-3Fucose[b]
Maackie amurensis agglutinin	MAA	*a*2-3Neu5Ac
Sambucus nigra agglutinin	SNA	*a*2-6Neu5Ac

[a] Requires the presence of mannose to bind.
[b] Can also bind to the other fucose sites.
Taken from reference [9].

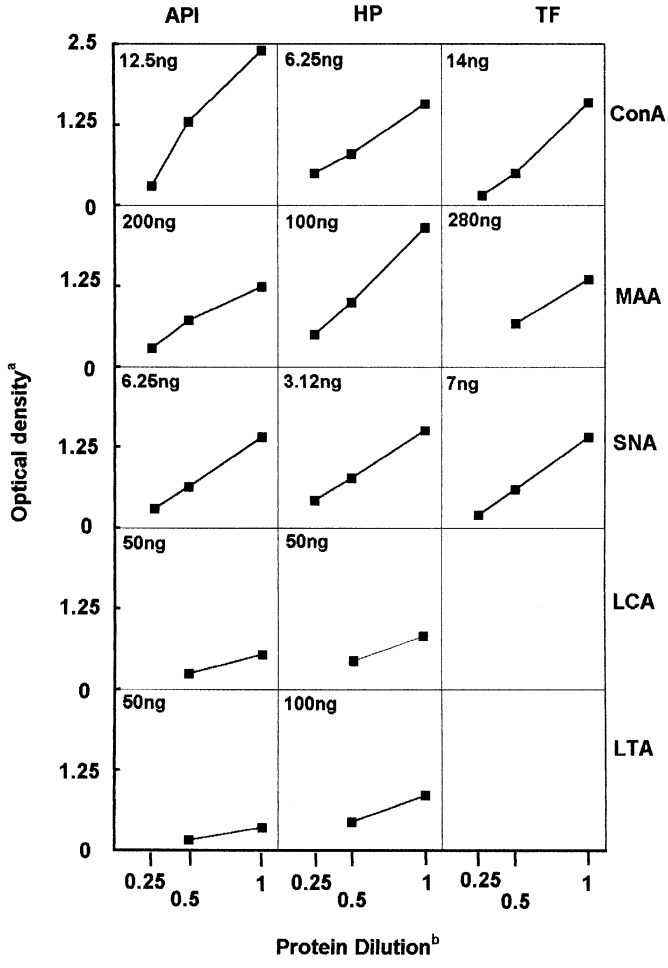

Figure 1. [a] OD was read at 492 nm for Con A, SNA, and MAA; and 405 nm for LCA and LTA. All values are the means of triplicate measurements from three experiments, except for TF which are from two experiments. [b] The maximum concentration (= 1) of glycoprotein used is given on each graph. In Figure 1 and Tables 2–4, Con A, MAA, SNA, LCA, and LTA were used at concentrations of 1 µg ml^{-1}, 1.5 µg ml^{-1}, 1 µg ml^{-1}, 0.5 µg ml^{-1}, 1 µg ml^{-1} respectively, and the background OD was subtracted from all data. Data for Con A, MAA and SNA are taken from reference [5].

affinity of the various lectins and variations in the occurrence of a particular carbohydrate grouping in the glycoprotein being studied.

When plates were coated with bovine serum albumin and probed with the different lectins, the OD values were only slightly higher than background values. The specificity of the lectins used was investigated either by probing glycoproteins in the presence of competitive sugars, *eg* Con A and α-D-methyl mannoside, or by using glycoproteins in the assay that were known not to contain the particular carbohydrate structure that was recognized by the lectin being used, *eg* carboxypeptidase Y for MAA and SNA and transferrin for LTA.

Table 2 summarizes the inter- and intra-assay precision of the method for API and HP with different lectins. The inter-assay precision of the method was obtained by measuring the same sample on a number of different occasions, and the intra-assay precision was obtained by measuring the same specimen several times on the same occasion. As can be seen from Table 2 the precision of this method is excellent.

Table 2. Precision of lectin–binding assay.

Protein	*Lectin*		*Precision (%)*	
ng per well		*ng per well*	*Intra-*	*Inter-*
Hp				
6.25	Con A	200	5.4	7.2
6.25	SNA	100	3.3	5.0
50.0	MAA	150	4.5	9.2
50.0	LCA	100	4.8	5.7
100.0	LTA	200	2.5	6.2
API				
15.0	Con A	200	1.5	2.2
15.0	SNA	200	3.0	4.5
50.0	MAA	100	5.0	6.4
50.0	LCA	100	3.7	5.6
50.0	LTA	200	7.7	8.3

All precision values (SD/mean × 100) were calculated from the mean ± SD of four to six measurements.

Table 3 shows that there is a decrease in GlcNAc content of API and an increase in GlcNAc content of HP in ovarian cancer patients compared to healthy individuals. These findings are consistent with the observed increase in Con A reactivity of API and the decrease in Con A reactivity of HP. The former result suggests a decrease in branching, and the latter vice versa.

Table 4 shows the *N*-acetyl neuraminic acid (Neu5Ac) content for API and HP in the healthy and cancer groups. For API, Neu5Ac content in the two groups is very similar, and for HP it is slightly higher in the cancer group. However, with the lectin binding assay there is an increase in the binding of SNA to API in the cancer group and a decrease in the binding to MAA. This suggests that the binding of the Neu5Ac in API is changing from α2-3 to α2-6 in cancer. With HP, the lectin-binding results suggest no changes in the α2-6 linkage but an increase in the amount of NeuAc linked α2-3 in the cancer. Thus, the lectin binding assay is able to complement monosaccharide analysis, by giving more information on a particular structural feature. LCA and LTA are also useful for distinguishing fucosylation at different sites (data not shown).

The results show that this method is very useful for monitoring the changes in a particular carbohydrate structure in different samples of the same purified glycoprotein in the same assay, *eg* a glycoprotein from healthy and disease individuals; a recombinant glycoprotein obtained on different occasions; or a glycoprotein obtained in control and test situations. It is not an absolute method because the lectin reactivity will depend upon the accessibility of the interacting ligand, which in turn will be due to many other factors.There is also the possibility of weak interaction with different carbohydrate ligands of the same type and this could sometimes lead to misinterpretation of the results. It cannot, therefore, be used to compare the content of a particular carbohydrate grouping between two different glycoproteins. Nevertheless we found a remarkable correspondence between the results we obtained for GlcNAc

Table 3. Branching of API and Hp isolated from 'healthy' and 'cancer' sera: Lectin-binding assay versus monosaccharide analysis.

	API		*HP*	
Measurement	*Healthy*	*Cancer*	*Healthy*	*Cancer*
GlcNAc content (mol mol^{-1})	13.7 ± 2.3(8)	10.9 ± 0.6(12)	23.3 ± 2.4(14)	28.5 ± 2.2(14)
	$p < 0.0001$		$p < 0.0001$	
[a]Con A binding (A_{492nm})	1.3 ± 0.3(8)	1.9 ± 0.14(12)	1.4 ± 0.35(8)	0.9 ± 0.15(11)
	$p < 0.0001$		$p < 0.0005$	

In tables 4 and 5 cancer specimens are from women with stage III/IV ovarian carcinoma; all values are mean ± SD with the number of observations in parentheses. In the lectin analyses, wells were coated with 15 ng and 5 ng for API and HP respectively. Some of the data from Tables 4 and 5 are taken from reference [7].

Table 4. Sialylation of API from 'healthy' and 'cancer' sera: Lectin-binding assay versus monosaccharide analysis.

	API		*HP*	
Measurement	*Healthy*	*Cancer*	*Healthy*	*Cancer*
Neu5Ac content (mol mol^{-1} protein)	$6.7 \pm 0.65(8)$	$6.84 \pm 0.60(12)$	$12.95 \pm 1.7(14)$	$13.9 \pm 2.20(14)$
	$p > 0.05$		$p > 0.05$	
SNA binding (A_{492nm})	$1.30 \pm 0.20(8)$	$1.95 \pm 0.29(12)$	$1.70 \pm 0.22(8)$	$1.69 \pm (12)$
	$p < 0.0001$		$p > 0.05$	
MAA binding (A_{492nm})	$0.47 \pm 0.08(8)$	$0.23 \pm 0.07(12)$	$0.18 \pm 0.07(8)$	$0.45 \pm 0.10(12)$
	$p < 0.0001$		$p < 0.0001$	

In the lectin analyses, wells were coated for SNA probing with 15 ng API and 5 ng HP and for MAA probing with 50 ng API and 12.5 ng HP. Neu5Ac content was measured using the Dionex carbohydrate analyser.

analysis and Con A binding, and the results we obtained for the fucose analysis and LCA and LTA binding (data not shown). In addition, we found that glycoproteins that are known to contain only bi-antennary groups bound to Con A well and glycoproteins that are known to contain mainly tri- and tetra-antennary groups bound Con A less well. We think that in many situations the method gives an approximate indication of the relative proportions of a particular carbohydrate structure.

In conclusion, a rapid lectin-binding method has been developed to investigate particular features of carbohydrate structure on purified glycoproteins. This method is very sensitive, has excellent reproducibility and can handle large numbers of specimens. It is useful for confirming changes in the carbohydrate structure changes indicated by other analytical techniques (*eg* Dionex), and for producing more detailed structural information.

Acknowledgement

We gratefully acknowledge the Islamic Republic of Iran for financial support.

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