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An improved lectin-based method for the detection of mucin-type *O*-glycans in biological samples

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Mucins and mucin-type glycoproteins, collectively referred to as mucin-type *O*-glycans, are implicated in many important biological functions and pathological conditions, including malignancy. Presently, there is no reliable method to measure the total mucin-type *O*-glycans of a sample, which may contain one or more of these macromolecules of unknown structures. We report the development of an improved microassay that is based on the binding of lectins to the unique and constant GalNAc-Ser/Thr structural feature of mucin-type *O*-glycans. Since the sugar–amino acid linkage in the mucin-type *O*-glycans is invariably cryptic, we first chemically removed the heterogeneous peripheral and core saccharides of model glycoconjugates before examining for their interactions using an enzyme-linked lectin assay (ELLA). Desialylation of the model glycoconjugates led to maximal binding of the lectins but additional treatments such as Smith degradation did not result in increased binding. Of the lectins tested for their ability to probe the desialylated *O*-glycans, jacalin showed the highest sensitivity followed by champedak galactose binding (CGB) lectin and *Vicia villosa* agglutinin. Further improvement in the sensitivity of ELLA was achieved by using microtiter plates that were pre-coated with the CGB lectin, which increased the specificity of the assay to mucin-type *O*-glycans. Finally, the applicability of the developed sandwich ELLA to crude samples was demonstrated by estimating trace quantities of the mucin-type *O*-glycans in the human serum.

Introduction

Mucins are large glycoproteins containing many clusters of glycosylated serines and threonines in tandem repeat regions.[1–3] Mucins and mucin-type glycoproteins, referred to as mucin-type *O*-glycans†, are implicated in many important biological functions and pathological conditions including malignancy.[1,4,5] In cancer, the over-expression and aberrant glycosylation of cell surface glycoconjugates are among the phenotypic changes that are widely recognized.[6] The tumor cells of cancer patients actively secrete and/or shed, during cell turnover, a repertoire of *O*-glycans into the circulation.[7,8] Hence, specific detection and quantitation of circulating tumor-associated *O*-glycans will be extremely beneficial for the early diagnosis and treatment of cancer. In fact, immunological methods are available for detection of cancer-associated *O*-glycans such as MUC1, CA125 and CA19-9.[9–11] However, these assays based on monoclonal antibody recognition of one specific epitope are of very narrow specificities and therefore not suitable for detection of the total *O*-glycan content of samples such as serum, saliva, urine or tissue extracts.

A problem in developing a single assay for *O*-glycans is the heterogeneity of their saccharides.[12] However, in contrast to the high variability of the peripheral saccharides, which are based on eight different core structures, the sugar–amino acid linkage in *O*-glycans is a unique and constant feature. Previously, one of us (VPB) had developed an assay for the estimation of *O*-glycans that was based on the specific chemistry of the GalNAc-Ser/Thr linkage.[13] Since the sensitivity of this colorimetric assay is very limited, in this study we explored the use of lectins to estimate trace amounts of *O*-glycans.

The aim of the present study was to develop a sensitive assay for the determination of the *O*-glycan content of a sample. This

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† The nomenclature in this field is very confusing and keeps changing as new members of the glycoconjugates family are discovered. The term, *O*-glycan or *O*-linked glycoprotein, has been traditionally used to refer to glycoconjugates in which the saccharides are attached to the protein *via* an *O*-glycosidic linkage. The major class of *O*-glycans consists of those having saccharides linked *via* the reducing end of GalNAc to the hydroxyl group of serine and threonine. However, there are also glycoconjugates having saccharides, including GlcNAc-, mannose-, fucose-linked *O*-glycosidically to proteins. Therefore, the term "mucin-type *O*-glycan" has to be used to distinguish glycoconjugates having GalNAc-Ser/Thr linkage from other *O*-glycans. In this article the term *O*-glycans is used synonymously with mucin-type *O*-glycans.

task was complicated by the fact that the saccharides in this class of glycoconjugates are highly heterogeneous.[14,15] Firstly, the saccharides in *O*-glycans are formed by chain elongation, branching and chain terminations of eight different core structures resulting in a bewildering array of structures.[12] Secondly, the total *O*-glycan content of a biological sample is likely to be constituted by a mixture of molecules of unknown saccharide structures. However, all *O*-glycans have a unique structural feature that distinguishes them from other classes of glycoconjugates (*N*-glycans, glycolipids and proteoglycans/glycosaminoglycans), *i.e.*, the presence of a GalNAc residue at the reducing termini of the saccharides, which is to be linked to specific serine or threonine residues in the core protein. In fact, the sensitivity of these GalNAc-Ser/Thr linkages to mild alkaline conditions has been very valuable in the characterization[16,17] and for colorimetric estimation of *O*-glycans.[13]

Our strategy was firstly to maximize the exposure of the cryptic sugar–amino acid linkage by removal of the heterogeneous peripheral and core saccharides. Secondly, we determined the best lectin to quantitate the exposed linkage region of *O*-glycans by Enzyme-Linked Lectin Assay (ELLA). Towards this objective, model glycoconjugates (PGM, BSM, PSM, and fetuin) were chemically treated to remove peripheral saccharides. The untreated and treated glycoconjugates were then tested for their interaction with jacalin and VVA as they are known to best bind to Galβ1,3GalNAcα-Ser/Thr (T-antigen) and GalNAcαSer/Thr (Tn-antigen), respectively.[18,19] CGB lectin, previously isolated in this laboratory and found to interact with the T-antigen,[20] was also tested with the model glycoconjugates. The modifications and lectin combination that gave the highest sensitivity were then used to develop an assay. Finally, the assay was evaluated for its ability to quantitate trace quantities of *O*-glycans in serum samples.

Experimental

Materials

Porcine gastric mucin (PGM) was partially purified from commercial preparations (Sigma-Aldrich, St. Louis, MO, USA) as described.[21] Bovine submaxillary mucin (BSM) and porcine submaxillary mucin (PSM) were prepared from fresh glands as previously described.[22] Alpha1-acid glycoprotein (AGP) and ovomucoid (trypsin inhibitor) were obtained from Sigma-Aldrich, St. Louis, MO, USA. Serum samples were obtained from healthy donors with the consent of the individuals. Jacalin, VVA, biotinylated jacalin and biotinylated VVA were purchased from E-Y Labs (San Mateo, CA, USA). CGB lectin was extracted and purified from the seeds of champedak in the laboratory as previously described.[20,23] Streptavidin-alkaline phosphatase and *p*-nitrophenyl phosphate were obtained from Sigma-Aldrich. ELISA immunoassay plates with medium binding capacity were purchased from Jet-Biofill (Guangzhou, China). EZ-Link sulfo-NHS-biotin and Pierce bicinchoninic acid (BCA) protein assay kit were purchased from Thermofisher Scientific (Rockford, IL, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Modification of *O*-glycans

Chemical desialylation was performed by treatment with mild acid hydrolysis (0.1 N sulfuric acid, 80 °C, 60 minutes) and the asialo product was recovered by neutralization with sodium hydroxide followed by dialysis against distilled water and lyophilization. The lyophilized asialo sample was reconstituted in ultra pure water. The protein concentration was determined by the BCA microassay.

Native as well as desialylated mucins were subjected to Smith degradation,[24,25] which involved mild periodate oxidation of the sample with 0.05 M sodium periodate in 0.05 M sodium acetate, pH 4.5 buffer at 4 °C for 18 hours. The aldehyde groups generated on the susceptible saccharides were reduced by treatment with 0.15 M sodium borohydride in 0.1 M sodium borate buffer. After incubation for three hours at room temperature, the reaction was terminated by neutralization with acetic acid. The samples were finally subjected to mild acid hydrolysis (0.025 M sulfuric acid at 80 °C for 60 minutes) and neutralized with 1.0 N sodium hydroxide. After each treatment the sample was recovered by dialysis *versus* distilled water at 4 °C and lyophilized. The Smith-degraded samples were reconstituted and the protein concentration determined by the BCA microassay.

Analysis of monosaccharides of native and modified glycoproteins

For neutral sugars and hexosamines, the samples were hydrolyzed with 2 N trifluoroacetic acid at 100 °C for six hours. The hydrolysates were dried in a vacuum centrifuge (Speed-Vac) (Labconco Corporation, Kansas City, Missouri), reconstituted in HPLC grade water and analyzed on a Carbopac PA10 high pH anion-exchange column (HPAEC) using a Dionex BioLC HPLC by isocratic elution with 16 mM sodium hydroxide and pulsed amperometric detection.[26] Sialic acids were determined after hydrolysis with 0.1 N sulfuric acid at 80 °C for one hour. The hydrolysates were neutralized with barium carbonate, centrifuged and the supernatant was dried, reconstituted as described above and analyzed by HPAEC as described by Manzi *et al.*[27] The response factors for the monosaccharides were determined using standard sugars.

Biotinylation of lectins

Biotinylated CGB lectin was prepared by adding sufficient volume of 10 mg ml^{-1} EZ-Link sulfo-NHS-biotin reagent to a solution of CGB lectin (5 mg ml^{-1} in PBS). The solution was mixed gently at room temperature for 60 minutes. The reaction mixture was dialyzed for two days with two changes of distilled water. The amount of biotinylated CGB lectin recovered in the retentate was determined by using BCA protein microassay.

Microtiter plate lectin binding assay

ELLA was performed either on samples directly coated on 96 well microtiter plates or on samples bound to lectin pre-coated plates. For coating of plates, 50 μl of the sample or lectin diluted in coating buffer (0.05 M sodium carbonate, pH 9.6) was added

to each well and incubated overnight at 4 °C. The wells were washed thrice with TBS-T (0.01 M Tris–HCl, pH 8.0, 0.05% Tween-20 and 0.05% sodium azide) and blocked by incubating with the wash buffer for 30 minutes. In the case of the lectin pre-coated plate, 50 μl test sample in phosphate buffered saline (PBS) was added to each well, incubated for 90 minutes at 20 °C and the unbound material was removed by washing as described above.

In both cases, the quantity of *O*-glycans in the test sample bound to the plate was determined as follows: 50 μl of diluted biotinylated lectin in TBS-T was added to each well and incubated for 90 minutes. The solution was removed and the wells washed three times. Fifty μl of avidin alkaline phosphatase (1 : 10 000) in TBS-T was then added to the wells and incubated for 90 minutes. The plate was washed thrice before adding 50 μl of 1 mg ml^{-1} *p*-nitrophenyl phosphate in substrate buffer (0.05 M sodium carbonate, pH 9.6, 1 mM $MgCl_2$). After appropriate incubation time, typically 20–30 min at 20 °C, the absorbance was read at 415 nm on a microplate reader (Bio-Rad, USA). Checkerboard assays were performed with serial dilutions of each ligand and lectin to determine optimal concentrations.[28,29] All ELLAs were done in triplicate and the mean values plotted. Typically, the variation (standard deviation) between the replicates was between 5 and 10% of the mean values.

Results

Effects of chemical treatment on the glycoproteins

The effects of acid hydrolysis and Smith degradation on the oligosaccharide structures were tested using PGM, BSM and PSM as well as fetuin. The concentrations of the monosaccharides remaining in the treated samples were determined as described in the Experimental section. The results of the analysis, expressed as molar ratios relative to GalNAc, of PGM before and after treatment are summarized in Table 1. Mild acid hydrolysis, as expected, removed all of the sialic acids from PGM and all the test glycoconjugates. This treatment also removed varying amounts of fucose for example, about 38% from PGM (Table 1) and 40% from PSM (not illustrated). Mild acid hydrolysis did not significantly affect the GlcNAc, galactose and GalNAc contents of PGM (Table 1). Subjecting the desialylated samples to Smith degradation resulted in removal of varying amounts of the now exposed penultimate saccharides. Thus, Smith-degradation of asialoPGM resulted in the loss of 100% of fucose, 58% of galactose and 9% of GlcNAc (Table 1). When PGM was directly subjected to Smith degradation, about 8% of GlcNAc, 49% of galactose, 100% of fucose and 33% of sialic acid were lost. This indicates that in PGM, while all of the terminal fucose is susceptible to periodate cleavage, about two-thirds of sialic acid is protected as a result of *O*-acetylation.[30]

Table 1 Carbohydrate composition of pig gastric mucin (PGM) after different chemical treatments to modify the saccharide chains

	Molar ratio of monosaccharides (with GalNAc as 1.00)				
	GalNAc	GlcNAc	Gal	Fuc	Sialic acid
PGM	1.00	2.03	1.25	0.65	0.15
asialoPGM	1.00	1.87	1.23	0.40	0.00
Smith-degraded PGM[a]	1.00	1.86	0.64	0.00	0.10
Smith-degraded asialoPGM[a]	1.00	1.71	0.52	0.00	0.00

[a] Sample subjected to Smith degradation as described in the "Experimental" section.

Lectin binding of native and chemically treated mucins

ELLA was performed to evaluate the ability of PGM, BSM, PSM and fetuin to interact with biotinylated jacalin, VVA and CGB lectin before and after the saccharide modifications as described above. In all cases, 50 μl of a solution of the test glycoconjugates at 1 μg protein per ml of coating buffer was used to coat the wells. Fig. 1 illustrates results of the experiments, which involved 12 different combinations of lectins and chemical treatments. Of the three lectins tested, jacalin demonstrated the highest detection sensitivity for all the native glycans tested. Binding of all three lectins was enhanced after desialylation by mild acid hydrolysis. For example, the substantially increased binding of VVA to asialoBSM compared to BSM and CGB to asialoPSM compared to PSM can be noted in Fig. 1b3 and 1c2, respectively. The influence of Smith-degradation on the ability of the desialylated glycans to interact with the three lectins was highly variable. While Smith degradation substantially reduced the binding of VVA to asialoBSM (Fig. 1b3) and asialoPSM (Fig. 1c3), it had no notable effect on the binding of jacalin to asialoPGM (Fig. 1a1). In contrast, the binding of CGB to asialoPGM was significantly increased after Smith degradation (Fig. 1a2). We also examined the interaction of the lectins to glycans that were directly subjected to Smith-degradation to determine whether prior desialylation was essential. Again the results were variable; for example, binding of jacalin to PGM and VVA to BSM was improved after Smith degradation (Fig. 1a1 and b3) but binding of jacalin to BSM (Fig. b1) and to fetuin (Fig. 1d1) was drastically reduced. An important observation was that virtually in all cases, the binding of the lectins to the Smith-degraded glycans was less than that to the corresponding asialo glycans. Based on these and other experiments further studies were carried out primarily using asialoPGM and asialoBSM as ligands and biotinylated jacalin as the probe lectin.

Direct *versus* sandwich ELLA

Because of the high level of glycosylation, *O*-glycans, in general, are known to bind to plastic microtiter plates rather poorly. Thus, we performed a series of experiments to compare the binding of biotinylated jacalin to *O*-glycans that were directly coated onto microtiter plates *versus* those that interacted with lectins that were pre-coated onto the plates (*i.e.* direct ELLA *versus* sandwich ELLA). The optimal concentration of lectin for coating the plates was first determined. In a typical experiment, the wells of the microtiter plates were coated by incubating with 50 μl of solutions containing 0.25, 0.50, 1.00 and 2.00 μg of jacalin in the coating buffer. After washing to remove unbound lectin, the wells were incubated for 90 minutes with 50 μl of a solution of

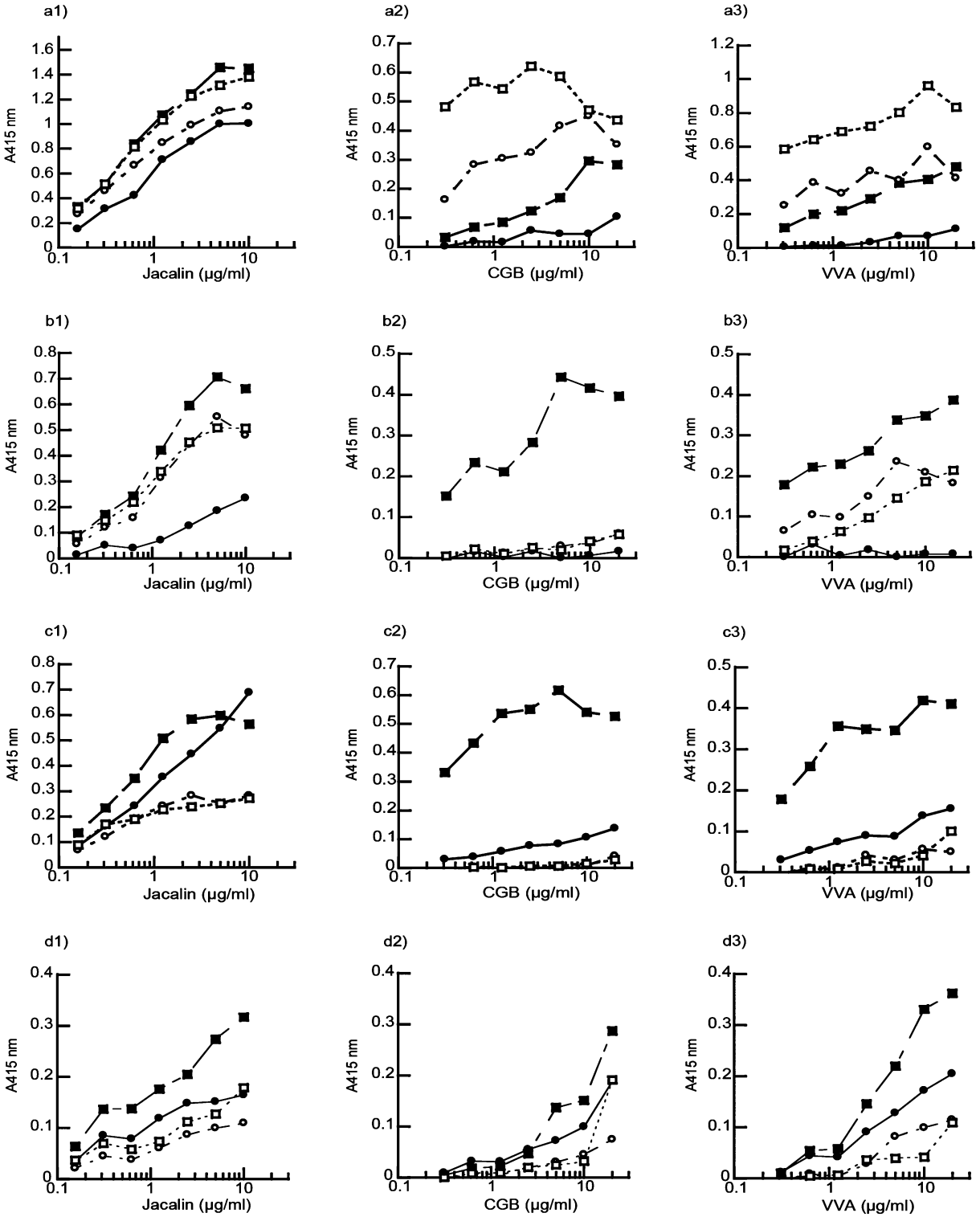

Fig. 1 Binding of serially diluted biotinylated lectins to various glycoconjugates. Panels A, B, C and D refer to PGM, BSM, PSM and fetuin, before (●) and after chemical treatment to modify the saccharide chains. The glycoconjugates were subjected to desialylation by mild acid hydrolysis (■), Smith degradation (○) and both desialylation and Smith degradation (□).

asialoPGM (1 μg protein per ml PBS). They were washed, incubated with a serial dilution of biotinylated jacalin at a starting concentration of 5 μg ml^{-1} and further processed as described above. The results revealed that the absorbance increased by 47.0, 92.1 and 110.5% when the coating concentration of jacalin was increased from 0.25 to 0.50, 1.00 and 2.00 μg per well, respectively. Based on this, 1 μg lectin per well was chosen as the coating concentration in all subsequent experiments.

We next performed a series of experiments to compare the binding of biotinylated jacalin to asialoBSM and asialoPGM that were directly coated onto microtiter plate wells *versus* those that interacted with 1.0 μg of jacalin, CGB lectin or VVA that was pre-coated to the wells. Our results showed that pre-coating of wells with a lectin substantially increased the sensitivity of the ELLA for the ligands tested (Fig. 2). Thus, the binding of biotinylated jacalin to asialoBSM and asialoPGM was substantially increased when they were bound to the lectin pre-coated wells compared to the uncoated wells (Fig. 2a and b). CGB lectin coated wells gave the highest reading followed by jacalin in both cases of asialoBSM and asialoPGM. When VVA was used as the coating lectin, increased sensitivity was obtained with asialoBSM but not with asialoPGM.

Effect of *N*-glycans on sandwich ELLA for *O*-glycans

The interference of *N*-glycans in our sandwich ELLA was tested using asialoAGP and ovomucoid, a lactosaminyl and an oligomannosyl *N*-glycan respectively. These *N*-glycans did not show significant interaction in the CGB lectin–jacalin (Fig. 3a) and jacalin–jacalin sandwich ELLA (Fig. 3b) even at a concentration of 1000 ng protein per ml. We also evaluated the presence of 500 ng protein per ml of asialoAGP during CGB–jacalin sandwich ELLA of 5 ng protein per ml of asialoBSM. The results presented in Fig. 3c clearly show that the presence of a 100-fold excess of an *N*-glycan had hardly any effect in the CGB lectin–jacalin sandwich ELLA of an *O*-glycan.

Detection of *O*-glycoproteins in serum

To evaluate the applicability of the developed sandwich ELLA for the estimation of *O*-glycans in crude samples, we carried out a few preliminary experiments using human serum. Since desialylation increased binding of the lectins to all the test glycoconjugates, the serum samples were also first subjected to mild acid hydrolysis. The desialylated serum samples were then subjected to direct ELLA and sandwich ELLA using biotinylated jacalin as the probe lectin and CGB lectin, jacalin and VVA as the coating lectins. Typical results of the binding of biotinylated jacalin to the desialylated *O*-glycans in serum are illustrated in Fig. 2c. It can be seen that the absorbance readings are highest when wells were precoated with CGB lectin. Relatively high absorbance readings were also obtained with wells coated with jacalin but the readings were very low when wells were uncoated or coated with VVA.

Next, three human serum samples (N1, N2 and N3) were desialylated and subjected to sandwich ELLA using biotinylated jacalin as the probe lectin, and jacalin and CGB lectin as the coating lectins. In each assay, asialoBSM in the range of 0.08 to 5.0 μg protein per ml was included as the standard. Typical results obtained for one serum sample (N2) and the asialoBSM standard by jacalin–jacalin sandwich ELLA and CGB lectin–jacalin sandwich ELLA are illustrated in Fig. 4. Using the results of the jacalin–jacalin sandwich ELLA (Fig. 4a and b) the *O*-glycan contents of N1, N2 and N3 were calculated to be 36.0, 36.1 and 36.8 pg asialoBSM protein per μg serum protein, respectively. The corresponding values calculated from the

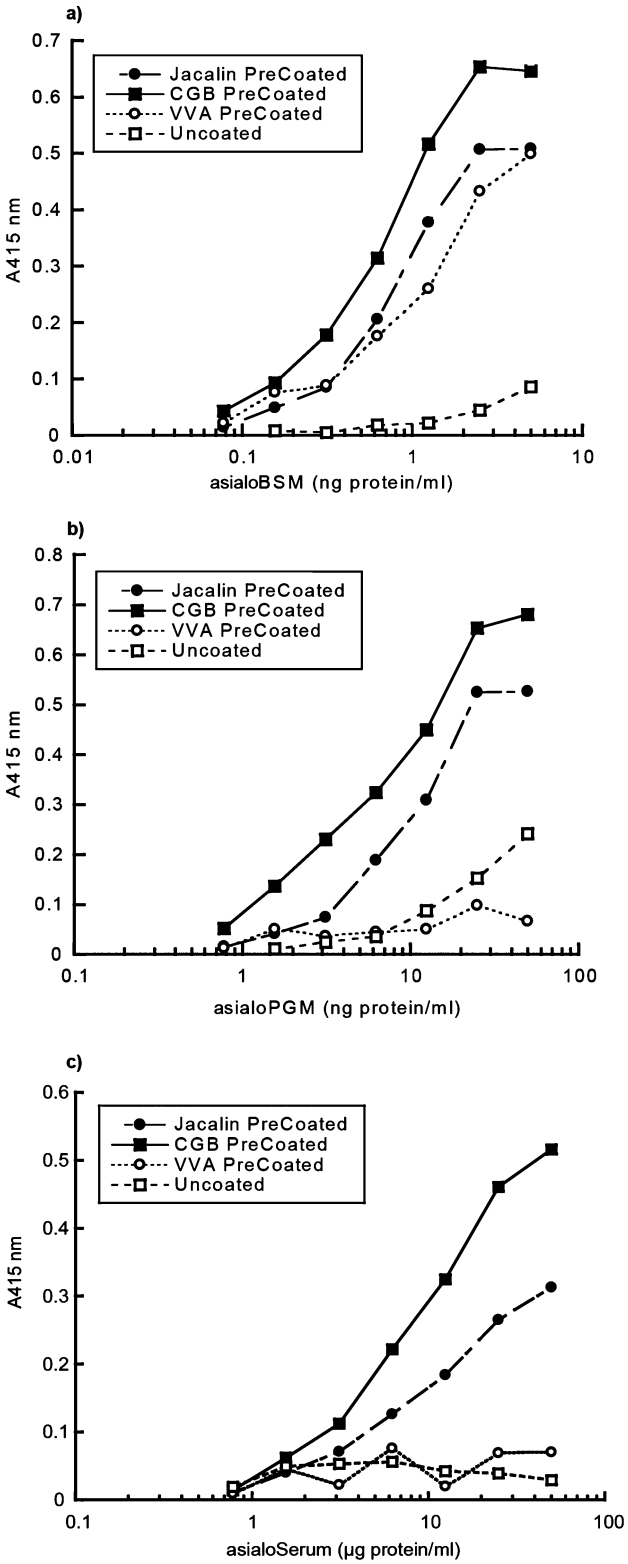

Fig. 2 Development of ELLA using microtitre plates precoated with different lectins. BSM (panel a), PGM (panel b) and serum (panel c) were subjected to mild acid treatment to desialylate the glycoconjugates. Binding of serially diluted biotinylated jacalin to desialylated BSM, PGM and serum was tested using microtitre plates that were either not precoated (□) or precoated with jacalin (●), CGB lectin (■) and VVA (○).

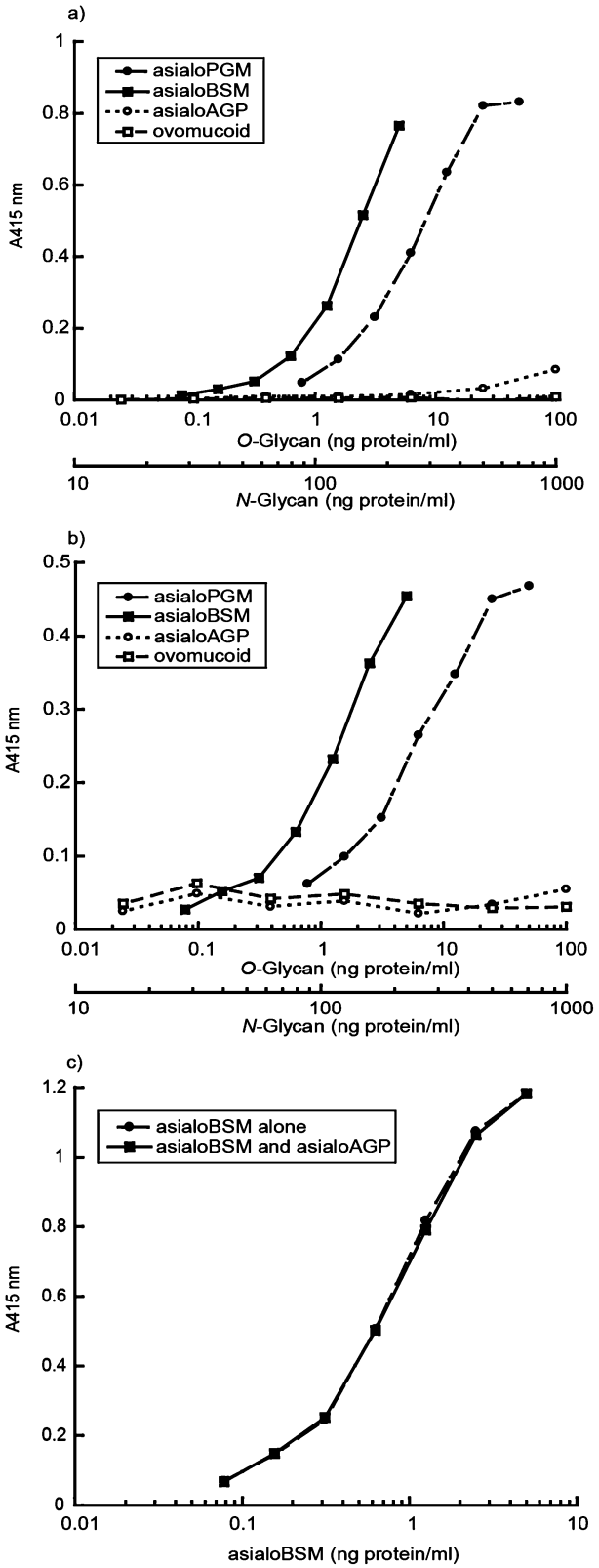

Fig. 3 Influence of *N*-Glycans on the sandwich ELLA for *O*-glycans. AsialoAGP and ovomucoid show minimum binding in CGB lectin–jacalin (panel a) and jacalin–jacalin (panel b) sandwich ELLA for asialoPGM and asialoBSM. Panel c illustrates the results of CGB lectin–jacalin sandwich ELLA on serially diluted solution of asialoBSM (5 ng ml^{-1}) and a mixture of asialoBSM (5 ng protein per ml) and asialoAGP (500 ng protein per ml).

results of the CGB lectin–jacalin sandwich ELLA were 27.7, 30.3 and 31.1 pg asialoBSM protein per μg serum protein, respectively.

Discussion

In the present study, we have targeted the binding of lectins to the unique GalNAc-Ser/Thr linkage feature for the development of an assay to quantify *O*-glycans in crude biological samples. Since the GalNAc-Ser/Thr linkages are invariably cryptic in *O*-glycans, it was necessary to first remove at least some of the peripheral saccharides. One approach to do this is by treatment with enzymes. However, this will require a supply of several highly purified glycosidases. Another approach is using chemical treatment such as mild acid hydrolysis and periodate oxidation.[24] The rationale for periodate oxidation was that it would cleave peripheral saccharides but not the linkage GalNAc. The GalNAc-Ser/Thr will be preserved in the majority of the *O*-glycan saccharides since they are based on core structures 1 to 5 in which position 3 of GalNAc is substituted making it resistant to periodate oxidation.[24,25,29] However, our experiments with model glycoconjugates revealed that desialylation by mild acid hydrolysis alone was sufficient for maximal binding and periodate oxidation did not further increase binding of the lectins selected for this study. These experiments also showed that of the three lectins tested, jacalin provided the highest sensitivity of binding to the desialylated *O*-glycans. This is probably due to the reported broad specificity of jacalin for *O*-glycans.[14,31,32] Therefore, biotinylated jacalin was selected as the most suitable detecting reagent in all subsequent experiments.

As expected, our experiments showed that the sandwich binding approach in which a lectin was used to capture the *O*-glycans is superior to direct coating of the sample onto the plastic microtiter plates. This is particularly relevant for crude samples having high levels of non-glycosylated proteins, such as serum, which are more likely to bind to plastic than the heavily glycosylated *O*-glycan molecules that are of much lower concentrations. When different lectins were tested as the coating capture reagent, the best sensitivity in the developed sandwich ELLA was obtained using CGB lectin. This is in agreement with our earlier data suggesting that the CGB lectin and jacalin, despite having close structural similarities,[33,34] demonstrated slightly distinct binding specificities.[20]

When the developed CGB lectin–jacalin and jacalin–jacalin sandwich ELLAs were used to estimate the *O*-glycan content of three human serum samples, the values obtained using the two assays were slightly different. Clearly, the developed assays are not likely to yield absolute values for the *O*-glycan content of test samples such as body fluids containing one or more *O*-glycans of unknown structures. Aside from the type of *O*-glycan used as the standard, the values obtained are also dependent on the lectins used in the sandwich ELLA. In the present study, the higher values obtained using the jacalin–jacalin sandwich ELLA compared to CGB lectin–jacalin sandwich ELLA may be due to the non-specific interaction of jacalin since the lectin has previously been reported to show weak interaction with

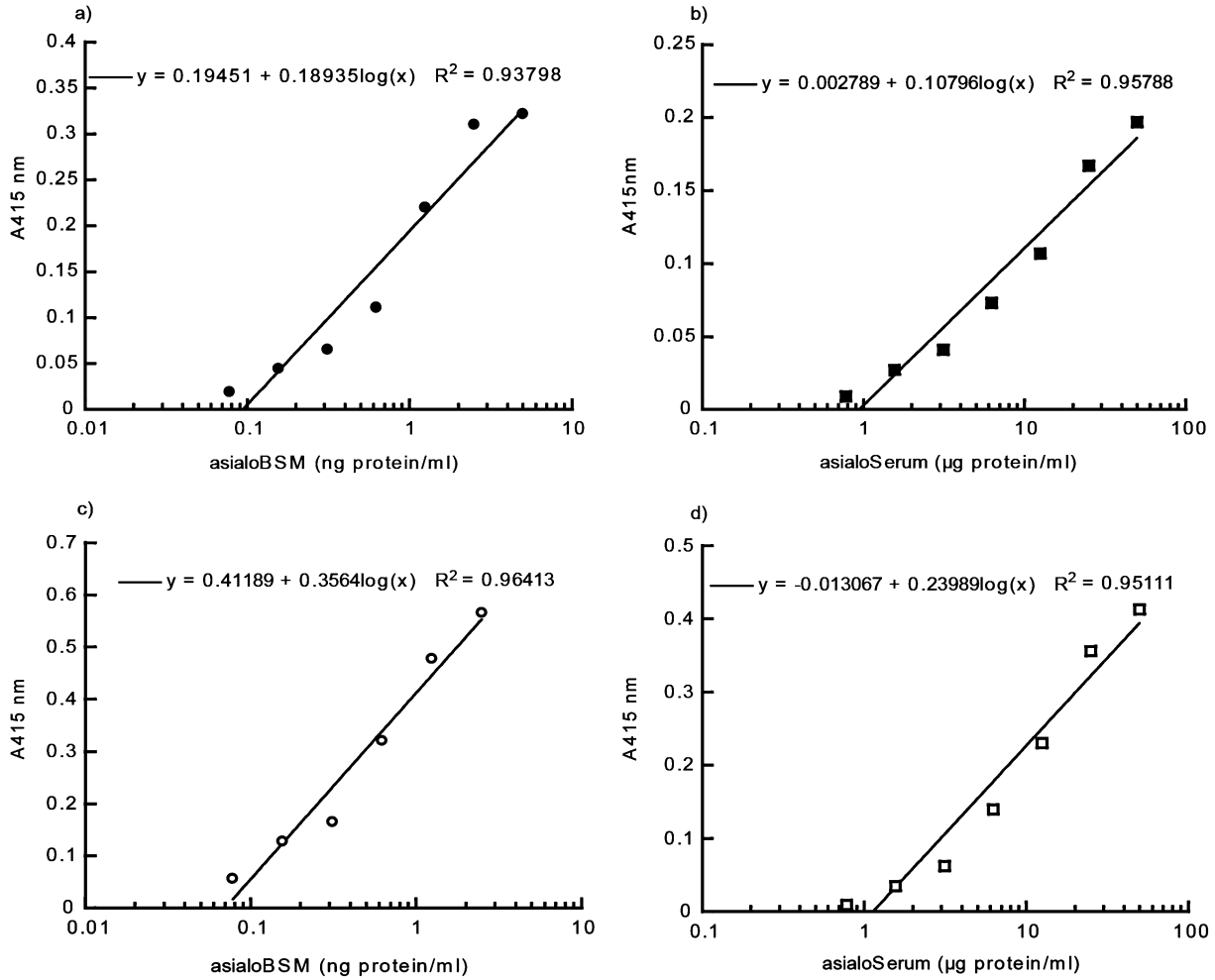

Fig. 4 Estimation of *O*-glycans in human serum samples subjected to mild acid treatment. Sandwich ELLA was performed using asialoBSM as the standard *O*-glycan (panels a and c). The *O*-glycans of asialo-serum samples were estimated using jacalin (panels a and b) or CGB lectin (panels c and d) as the coating lectin and biotinylated jacalin as the probe lectin.

non-*O*-glycosylated glycoproteins.[31,35] But more importantly, the results illustrated in Fig. 2 clearly demonstrate that the sandwich assay using CGB as the coating lectin gave the highest sensitivity not only with model *O*-glycans (BSM and PGM) but also with serum, a crude biological sample. Further, in a sandwich assay it is preferable to use two different lectins rather than the same lectin jacalin, for both coating and probing purposes. Hence, we conclude that for the analysis of crude samples such as serum the sandwich assay using CGB lectin as the capture lectin and jacalin as the probe lectin is more suited than the one using jacalin as both the capture and probe lectin.

Conclusions

In the present study, a method to detect asialo mucin type *O*-glycans was developed using champedak galactose binding lectin and biotinylated jacalin. This method was more sensitive when tested on serum samples and may be easily applied in the analysis of a large number of biological fluid samples. The CGB lectin–jacalin sandwich ELLA that is reported here will be most valuable in studies comparing the levels of *O*-glycans in biological fluid samples of healthy individuals with patients with a variety of diseases.

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