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Optimisation of the enzyme-linked lectin assay for enhanced glycoprotein and glycoconjugate analysis.

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Abstract

Lectin's are proteins capable of recognising and binding to specific oligosaccharide structures found on glycoproteins and other biomoloecules. As such they have found utility for glycoanalytical applications. One common difficulty encountered in the application of these proteins, particularly in multi-well plate assay formats known as Enzyme Linked Lectin Assays (ELLA's), is in finding appropriate blocking solutions to prevent non-specific binding with plate surfaces. Many commonly used blocking agents contain carbohydrates and generate significant background signals in ELLA's, limiting the utility of the assay.

In this study we examined the suitability of a range of blocking reagents, including protein based, synthetic and commercially available carbohydrate free blocking reagents, for ELLA applications. Each blocking reagent was assessed against a panel of 19 commercially available biotinylated lectins exhibiting diverse structures and carbohydrate specificities. We identified the synthetic polymer Polyvinyl Alcohol (PVA) as the best global blocking agent for performing ELLA's. We ultimately present an ELLA methodology facilitating broad spectrum lectin analysis of glycoconjugates and extending the utility of the ELLA.

Key Words: Enzyme-linked lectin assay, Glycoprotein Analysis, Lectin, Glycoprotein, Blocking reagents, BSA, PVA.

Introduction

Many proteins are subject to post translation modifications (PTM's) and these modifications have significant effects on the biological properties and functions of proteins. Of all of the potential PTM's that occur in cells glycosylation, which results in the addition of oligosaccharide moieties to proteins, is one of the most abundant and biologically significant (1-3). Oligosaccharide structures are also found attached to other cellular macromolecules such as lipids, and include molecules such as lipopolysaccharides (LPS and LOS) of bacterial cell membranes, GPI proteins and glycosphingolipids found in eukaryotic cell membranes (3, 4). Glycans, therefore, constitute an important interface between a cell and its environment and play important roles in molecular recognition, usually through interactions with specific carbohydrate-binding proteins called lectins (5-7). As molecular recognition molecules, glycans mediate a vast array of biological processes including cell development and differentiation, cell adhesion and migration, innate and adaptive immune responses, cell proliferation and apoptosis, interactions between cells and soluble ligands (cell signaling), and between cells and various infectious agents such as prions, viruses and microorganisms (1, 8-10). Our ability to understand such complex biological process therefore necessitates techniques enabling the analysis of glycoproteins and other glycoconjugates.

Glycans exhibit greater structural diversity than other cellular macromolecules and often form highly complex branched structures. Their capacity to generate such diversity stems from the fact that oligosaccharide structures can (1) vary in terms of the sequence in which monosaccharide subunits are linked (2) the positions of linkages resulting in branching and the extent of branching (3) the anomeric configuration of linkages (α or β configurations) and (4) the presence or absence of additional modifications such as sulphonation (11). This capacity for structural and conformational diversity facilitates their role in expanding the diversity of biological systems, and their roles in molecular recognition, but the analysis of such complex and diverse molecules presents a significant technical challenge. Unfortunately, the development of glycoanalytical techniques lagged significantly behind the technological advances experienced in the fields of genomics and proteomics. However, as our appreciation for the biological significance of glycan's has grown the development of glycoanalytical techniques has accelerated and methods now exist that allow the detailed characterization of glycoconjugates. These include HPLC, mass spectrometry (MS), Nuclear Magnetic Resonance (NMR) and combinatorial methodologies (12-18). However, such techniques usually require expensive specialized equipment, as well as highly skilled personnel, and as such these methods are not always widely accessible to all. Analysis requires chemical or enzymatic removal of glycan's followed by their derivatization with fluorescent markers to enable their subsequent detection. These glycan removal and derivatization steps often require optimization, increase analysis times and analysis can require large sample quantities (4, 19, 20). There is therefore an urgent requirement for the development of more simplistic and accessible methods for glycoprotein and glycoconjugate analysis.

Lectins are carbohydrate binding proteins (CBP's) capable of recognizing and binding to specific glycan structures. An increasing number of lectins, particularly from plant and animal sources, have been characterised with respect to their glycan binding specificity and a large number of such lectins are now commercially available (see

Table 1). The ability of lectins to discriminate between different glycan structures makes them useful tools for performing glycoanalysis. Using panels of lectins, of known glycan binding specificities, lectin binding patterns (fingerprints) can be obtained that are indicative of the glycosylation status of a glycoprotein or glycoconjugate (21-23). One of the key advantages of using lectins is that analysis can be performed without the need for initial glycan removal and derivatization thereby greatly simplifying analysis.

Lectins can be implemented for glycoanalysis in a number of different formats including lectin microarray techniques (21-25). However, the most simplistic format in which lectins can be employed for glycoanaylsis is the Enzyme-Linked Lectin Assay (ELLA). This assay has the same basic format as a standard ELISA (see Figure 1) and allows the analysis of glycoproteins, and lectin-glycan interactions, in a standard microtitre plate format. The most common format of the ELLA requires the immobilization of glycoconjugates on the surface of an ELISA plate, subsequent blocking of the plate surface and then probing with biotinylated lectins (26). The major problem that is encountered when performing the ELLA stems from the lack of suitable blocking reagents. Many of the blocking reagents traditionally used in ELISA are unsuitable for use in ELLA as they generate high background signals, contributing to false-positive signals, due to the presence of carbohydrate in the preparations (26-29). As a result of these difficulties many of the ELLA protocols reported in the literature to date have only utilized small and specific subsets of lectins (30-32) and their utility is thereby restricted. In this paper we have examined the suitability of a number of blocking agents for broad spectrum lectin analysis in an ELLA format. Ultimately we describe an optimized ELLA methodology that allows the assay to be performed with a broad spectrum of lectins, of diverse glycan binding specificities, thereby extending and enhancing its utility and providing a broadly accessible and simple assay for glycoanalysis.

Abbreviations

BSA, bovine serum albumin; ELLA, enzyme-linked lectin assay; HRP, Horse Radish Peroxidase; PBS, phosphate buffered saline; PEG; polyethelene glycol; PVA, polyvinyl alcohol; PVP, Polyvinylpyrrolidone; TBS, tris buffered saline; TBST, tris buffered saline supplemented with 0.05% Tween-20; TMB, 3,3′,5,5′-Tetramethylbenzidine; Lectins are listed in Table 1.

Materials and Methods

Materials

Biotinylated lectins, agarose immobilized lectins and Carbo-Free Blocking (Product code SP-4050) were purchased from Vector Laboratories (Peterborough, UK). Bovine serum albumin (BSA) was obtained from Sigma Aldrich. Three grades were used in this study: product A9647 is described as having 96% purity (BSA96), product A7906 is described as being 98% purity (BSA98) and product A7030, also described as being 98% purity, is suitable for use in ELISA (ELISA Grade BSA). The BSA routinely used was BSA98 unless otherwise stated. Synblock was purchased from AbD Serotec (Oxford, UK). The glycoproteins Bovine Fetuin, Invertase (from *Saccharomyces cerevisiae*) and porcine Thyroglobulin used in this study were obtained from Sigma Aldrich. All other chemicals, including L-fucose, methyl α-D-mannopyranoside, HRP-conjugated anti-biotin, polyvinyl alcohol (molecular weight, 50,000), Polyvinylpyrrolidone (molecular weight, 360,000), PEG 8,000 and PEG 20,000 and TMB were also obtained from Sigma–Aldrich–Fluka (St. Louis, MO, USA). Polystyrene microtiter plates (Nunc MaxiSorp) were purchased from Thermo Fisher Scientific, Roskilde, Denmark.

Methods

Enzyme-Linked Lectin Assays (ELLA's).

Although certain assay parameters were varied throughout this series of experiments, the basic protocol followed is described here. Glycoprotein immobilization was performed by adding 50 μL of a glycoprotein solution to the wells of an ELISA plate and incubating overnight at 4°C. Glycoprotein solutions used were prepared by dissolving a glycoprotein, typically at a concentration of 10mg mL^{-1}, in 10 mM sodium phosphate buffer pH7.2 containing 150 mM NaCl (PBS). For negative control wells 50 μL of PBS was added. These wells would be used to monitor for cross reactivity of lectins, or detection anti-bodies, with plate surfaces that would generate background signals. All subsequent steps were performed at 25°C. The glycoprotein solution was removed by inversion, 150 μL of a blocking solution was added to all wells and the plate was left to block for 2 hours. The wells were washed four times with 20 mM Tris pH 7.6, 150 mM NaCl, 0.05% Tween-20 supplemented with 1 mM $CaCl_2$, 1 mM $MnCl_2$, 1 mM $MgCl_2$ (TBST). Lectin solutions were prepared in TBST, 50 μL was added to each well and the plates were incubated for 1 hour. When inhibition assays were performed, lectins were pre-incubated with the sugar under investigation for 15 minutes prior to application to plates. Unbound lectin was removed by washing each well four times with TBST before the addition of 50 μl of 1:10 000 diluted HRP labeled anti-biotin antibody for 1 hour. Plates were subsequently washed four times with TBST followed by one final wash with PBS. TMB solution was prepared according to the supplier's instructions. 100 μL TMB was added to each well and the reaction was stopped with 50 μl 10% H_2SO_4 after 10 min. The absorbance was measured at 450 nm using a BioTek ELx808 plate reader.

Preparation of Blocking Solutions For ELLA's

BSA blocking solutions were prepared as 3% solutions in TBS. Deglycosylated BSA (deBSA) was prepared by sodium periodate oxidation (33). This was prepared by dissolving 5% BSA and 50mM sodium periodate in 50mM sodium acetate, pH 4.0, and incubating with rotation for 2 hours in the dark at 4^{o}C. The deBSA solution was dialysed into TBS overnight with three changes of buffer prior to use in ELLA's. PVA and PVP were prepared by dissolving in heated PBS. PEG solutions were prepared by dissolving in water. All blocking solutions, with the exception of deBSA, were prepared directly before use.

Lectin Affinity Purifications – Pull Down Assays.

Lectin affinity purifications were performed using ConA-agarose and AAL-agarose. These lectin affinity resins were equilibrated prior to use by washing with TBST. Purifications were performed in the form of pull down assays whereby 100µl of an equilibrated lectin agarose resin was mixed with 1 mL of a BSA98 solution (5 $mg.mL^{-1}$ in TBST) and the suspension left to mix by inversion overnight. The suspension was then centrifuged at a low speed of 5000g for 2 minutes to sediment the lectin agarose resin and any bound glycoproteins. The supernatant, containing unbound proteins, was then recovered and retained for later analysis. Prior to the elution of bound proteins, the lectin resin was washed four times with TBST. Bound glycoproteins were then selectively eluted by the addition of 100µl of 200 mM α–methyl-mannose or 100 mM L-fucose to ConA-agarose and AAL-agarose respectively. Suspensions were left incubating for 1 hour, to permit efficient elution of bound glycoproteins, and then centrifuged as before. Supernatants, containing sugar eluted glycoproteins, were recovered and retained for later analysis. Residual bound proteins, that had not been successfully eluted from the lectin resins, were recovered by applying a final denaturing elution step. This was achieved through the addition of 50µl of SDS-PAGE loading buffer to the resin and boiling for 5 mins. Again samples were ultimately centrifuged to sediment residual insoluble material and the supernatant, containing solublized denatured proteins, was recovered for later analysis. Samples taken at each step in the pull down assays were run on 10% SDS-PAGE gels (34).

Results

Evaluation of BSA as a Blocking Reagent for use in ELLA Protocols.

BSA is the most commonly used blocking agents used in ELISA methods and its use in ELLA's has also been reported (24, 35). We evaluated the suitability of BSA for use as a blocking agent in broad spectrum lectin screens (6, 36). To do this 150 µl of a 3% BSA solution was added to pre-rinsed wells for 1 hr to block the wells. The wells were then probed with biotinylated lectins to monitor for interactions with the BSA coated surfaces. Of the 19 lectins tested, 9 of them were observed to exhibit strong interactions with the BSA blocked surfaces (see Figure 2). In particular, sialic acid binding lectins WGA, MalII and SNA showed very strong responses as did the mannose specific lectins ConA and LCA. The binding of such sugar specific subsets of lectins, and such a range of structurally diverse lectins, was suggestive that the high background signals observed were due to specific lectin-glycan interactions with the BSA blocked plate surfaces and not simply due to non-specific protein-protein interactions. The signals observed for many of these lectins, which included some of the most commonly used lectins and key glycan specificities, were of such a magnitude that it would preclude their use in ELLA assays using BSA as a blocking agent. We therefore sought to verify the basis for the high background signals observed with the intention of finding an ultimate solution to overcome this issue.

Lectin Interactions with BSA Blocked Plate Surfaces are due to Specific Lectin-Glycan Interactions.

Sugar inhibition assays were performed to establish whether the lectin interactions observed with BSA blocked wells were due to protein-protein interactions or more specific lectin-glycan interactions. Lectins displaying strong interactions with the BSA blocked wells were chosen for this experiment. In each case, when the lectins were pre-incubated with their preferred sugar ligand, binding to BSA blocked plate surfaces was completely suppressed. Conversely, when lectins were pre-incubated with a non-binding sugar, significant binding of the lectins to BSA blocked plate surfaces was still observed (see Figure 3). These results clearly demonstrated that many of the observed lectin interactions with the BSA blocked plate surfaces were due to specific lectin-glycan interactions and not due to non-specific protein-protein interactions.

Lectin Interactions with BSA Blocked Plate Surfaces are due to the Presence of Contaminating Glycoproteins in BSA Preparations.

It is known that BSA is not itself a glycosylated protein (37). It was therefore proposed that the lectin interactions with BSA blocked surfaces may be due to the presence of contaminating glycoproteins in the BSA preparation. To verify this we performed lectin affinity pull down assays on BSA preparations. These were performed with two of the lectins that had displayed the strongest interactions with BSA blocked wells, ConA-Agarose and AAL-Agarose. Using these lectin affinity resins we were able to isolate and concentrate contaminants in the BSA98 preparation. When lectin bound proteins were eluted from the ConA agarose resin and run on SDS-PAGE, multiple bands were clearly evident (see Figure 4). While fewer bands were visible in the eluted fraction of proteins from the AAL-agarose, most of those that were present were also

visible in the ConA eluted pool. This clearly demonstrated the presence of specific contaminating glycoproteins in BSA98 preparations.

Having verified that the observed interactions of lectins with BSA blocked wells were due to contaminants in the BSA we decided to examine several different purity grades of commercially available BSA. We wanted to determine how the different levels of purity would impact on the observed interactions of the lectins and if a specific commercially available BSA preparation could be identified for specific application in ELLA's. Three different commercially available BSA preparations were compared: 96% BSA (BSA96), 98% BSA (BSA98) and ELISA grade BSA. Many of the lectins showed very strong signals on all of the purity grades of BSA although the signals on the ELISA grade BSA were generally slightly lower than those obtained for plates blocked with the BSA96 and BSA98 (see Figure 5). While the signals on the ELISA grade BSA were generally lower, this grade of BSA would still be unsuitable for ELLA analysis.

Oxidative Deglycosylation of BSA Preparations – Cost Effective Generation of a Suitable Blocking Agent.

Having verified that the observed interactions of lectins with BSA blocked wells was due to specific interactions with contaminating glycoproteins in BSA preparations we sought to try and overcome this by performing a bulk deglycosylation of BSA preparations. This was achieved by treatment of BSA preparations with sodium periodate to oxidize any sugars present. Interactions of both untreated BSA and deglycosylated BSA (deBSA) with biotinylated lectins were compared by ELLA. As can be seen from Figure 6, the periodate treatment did appear to eliminate the reactivity of most lectins with BSA blocked wells, confirming that most of the lectins observed to interact with the BSA blocked wells had done so in a sugar specific fashion. However, four of the lectins, AIA (Jacalin), DSL, WGA, and to a lesser extent GSLI, did still generate significant signals against wells blocked with the deBSA.

Evaluation of ELLA Protocols Omitting Plate Surface Blocking Steps

Duk *et al* reported an ELLA protocol whereby the wells of ELISA plates were coated with glycoprotein (or a glycoconjugate) solutions and biotinylated lectins were added directly to the wells without a prior blocking step (26). They reported that once Tween-20 was included in the lectin solution, absorbance readings in lectin control wells, with no immobilized glycoprotein, was low. However this was only demonstrated for a relatively small set of lectins, all of which had Gal/GalNAc specificity. We therefore sought to evaluate this method for use with a broader range of lectins. ELISA plates were rinsed with PBS, to mimic the 'no glycoprotein' negative control wells of an ELLA, and then washed with TBST. A range of biotinylated lectin solutions was prepared at concentrations of 1-5 $\mu g.mL^{-1}$ in TBST, and added directly to the plate. Although many of the lectins tested did not generate significant signals, some, did exhibit clearly detectable signals indicating a capacity to interact non-specifically with unblocked plate surfaces under the assay conditions used (see Figure 5). Again, some of the most commonly used lectins, such as AAL, ConA, AIA and DSL, gave

extremely high signals of such a magnitude that they would not be suitable for use in ELLA's performed using this methodology.

Investigation of Synthetic Blocking Reagents

Having determined that ELLA strategies either omitting plate surface blocking steps or using BSA as a blocking agent were unsuitable for broad spectrum lectin screens, we investigated a number of synthetic polymers as blocking agents. The reagents investigated were 0.5% PVA, 0.5% PVP, 1% PEG 8,000 and 1% PEG 20,000 solutions. In an initial experiment these were used to coat the wells of ELISA plates prior to the addition of biotinylated lectins which were prepared in TBST as usual. Only those lectins that had been observed to exhibit high backgrounds in other blocking strategies were examined initially. As can be seen from the results obtained (see Figure 7A) PVA performed the best with all of the lectins tested giving very low background signals of 0.1 or lower.

To further evaluate the suitability of PVA as a blocking agent it was used in an ELLA analysis of a number of standard commercially available glycoproteins. The glycoproteins Fetuin, Invertase and Thyroglobulin were immobilized in the wells of ELISA plates by overnight incubation at 4^{o}C. Wells were washed as usual and then blocked with 0.5% PVA. Each glycoprotein was then probed with a broad spectrum of lectins (see Figure 7B). Each lectin was added at a concentration of 5 $\mu g.mL^{-1}$ and all of the lectins showed negligible signals in negative control wells in which there was no immobilized glycoproteins. Each of the lectins tested was however observed to interact with the glycoproteins in an expected fashion based on the glycan structures known to be present on these glycoproteins. For example, the sialic acid binding lectins showed high signals on Fetuin (38) and Thyroglobulin (39), but did not interact with Invertase (40). Conversely, terminal mannose binding lectins interacted strongly with Invertase, but not with Fetuin. ConA and LCA, which are known to bind to *N*-linked glycans, showed binding to all of the glycoproteins. These results indicated that while the PVA prevented non-specific interactions with the plate surface it did not interfere with the ability of the lectins to interact with the immobilized glycoproteins. PVA can therefore be used as a blocking agent enabling ELLA analysis of glycoproteins to be performed using a very broad spectrum of lectins thereby achieving more in-depth glycoanalysis of target glycoproteins.

Comparison of PVA versus Commercial Carbohydrate Free Blocking Agents.

The final blocking solutions tested were two commercially available ELISA blocking reagents Synblock (AbD Serotec) and Vector Laboratories' Carbo-Free blocking solution. PVA solution was included in the experiment for comparison. Synblock proved to be an excellent blocking reagent and gave identical results to the PVA blocked wells. Surprisingly, several of the lectins tested interacted with the Carbo-Free blocker, including AIA, MAL II, SNA, DSL, WGA and SBA (Figure 9). Since this blocking solution does not contain carbohydrate the interactions must be due to protein-protein interactions.

Signal Quenching by Inclusion of BSA in Lectin Probing Solutions

In addition to being used as a blocking agent BSA is traditionally included in antibody probing solutions in ELISA's to reduce non-specific binding. Some ELLA methods similarly use BSA in lectin probing solutions (29, 33). However, having demonstrated that many lectins can interact with glycosylated contaminants in BSA solutions, there is the possibility that inclusion of BSA in lectin probing solutions could quench interactions with the desired immobilized glycoprotein targets resulting in false negatives. This inhibitory effect was explored by immobilizing 50 ng of the glycoprotein Fetuin in the wells of ELISA plates and the wells were subsequently blocked with 0.5% PVA. Wells were then probed with lectin solutions with or without BSA included to final concentrations of 1% or 3%. Lectins were additionally incubated with inhibiting sugars to demonstrate that observed lectin-glycoprotein interactions were glycan mediated. When the plates were developed it was evident that signal strengths were affected by the presence of BSA in the probing solutions (Figure 9). AIA and WGA signals were abolished by both 1% and 3% BSA, while the SNA signal degreased with an increase in BSA concentration. The ConA signal strength was not significantly impacted by the presence of the BSA. This experiment clearly demonstrated that the inclusion of untreated BSA in lectin probing solutions can reduce the magnitude of the signal strength, thereby reducing the sensitivity of the assay and potentially resulting in false negative results.

Discussion

One of the key steps in any ELISA is the blocking step. After immobilization of a target molecule to a surface, unoccupied sites on the plate surface must be blocked off prior to addition of subsequent probing protein solutions. If this is not done non-specific interactions between the probing biomolecules with the plate surface lead to false positive signals or masking of true signals. This blocking step is equally important in performing ELLA analysis, but unlike standard ELISA assays appropriate blocking procedures are still ill defined in the literature. Many of the blocking agents described to date are only suitable for limited subsets of lectins. As a result most methods reported to date are limited in their utility and the breath of analysis that can be performed. We therefore sought to investigate and compare a range of blocking agents with the aim of establishing a generic protocol applicable to performing analysis with a broad spectrum of lectins.

Many ELISA protocols commonly use BSA solutions as blocking reagents, typically at concentrations of 0.5% to 5% (w/v). This use of BSA has also been reported in ELLA protocols in the literature (29, 33). In this study we demonstrated that when BSA is used to block plates in ELLA's, false positives can become a major problem (see Figure 2). In assay control wells, which were coated with BSA blocking solution only, high signals were consistently observed for certain lectins and interestingly these lectins could be grouped according to sugar specificity. ConA and LCA, which are reported to bind to the mannose core of *N*-linked glycans, were both observed to exhibit high binding to the BSA preparations, while NPL and GNL, which are reported to bind to terminal mannose, did not appear to interact at all. The sialic acid binding lectins in the test-set, SNA, MAL II and WGA, were also observed to interact strongly with BSA blocked wells. These lectins encompass key specificities that researchers would want to screen for in ELLA's but the observed interactions with BSA blocked wells would preclude the use of these lectins in ELLA's where BSA was used as a blocking agent.

We demonstrated that specific lectin interactions with BSA blocked wells could be inhibited if the lectin was pre-incubated with its preferred sugar ligand before being added to the wells (see Figure 3). If mixed with an alternative sugar no inhibition was observed. This sugar specific inhibition of lectin interactions, in addition to the observation that defined specificity groups of lectins had displayed reactivity to BSA blocked wells, indicated that the signals were due to specific lectin-glycan interactions and not due to non-specific protein-protein interactions. Since BSA itself is known not to be glycosylated (37) we believed that the interactions were not with BSA itself but with contaminating glycoproteins present in BSA preparations. The presence of contaminating glycoproteins in BSA preparations was confirmed by performing lectin pull down assays on BSA preparations. Using ConA and AAL agarose resins, contaminating glycoproteins were effectively purified from BSA preparations (see Figure 4). When sugar eluted fractions were examined by SDS-PAGE, the ConA eluted fraction in particular showed multiple strong bands. The major band visible is likely to correspond to the heavy chain of purified γ-globulins (IgG) which is known to be a major contaminating species in BSA preparations. However, several other strong bands were also observed indicating the presence of additional significant glycosylated contaminants. The AAL eluted fraction contained fewer purified proteins and resulted

in weaker bands on SDS-PAGE. However, some of the bands observed were of the same molecular mass as those observed in the ConA eluted fraction.

For BSA to be an effective blocking reagent, applicable to a broad range of lectins in ELLA's, a highly purified preparation would be required. Therefore, we decided to assess a number of commercially available preparations of BSA to try and identify one that might be suitable for use direct use in ELLA's. Three different BSA preparations, all available from Sigma-Aldrich, were examined (see Figure 5). BSA is primarily produced by heat treatment and alcohol precipitation from serum, resulting in purity levels of 96-98% (41). BSA96 is purified by heat treatment and organic solvent precipitation only, and the major contaminants listed are globulins. The γ-globulin, IgG, is one of the most common proteins in serum after albumin and would represent one potential glycosylated contaminant. BSA98 is further processed to remove lipid and low molecular weight substances and also contains contaminating globulins. When these two BSA preparations were subjected to ELLA analysis, they both exhibited very similar lectin responses, and can be assumed to be comparable in terms of glycan content. The ELISA grade BSA is processed to be essentially free of fatty acids and γ-globulins but it still generated significant signals against the same lectins as the other BSA samples tested (see Figure 5). This confirmed that the interaction of lectins with BSA preparations was only partly due to the presence of contaminating globulins and that a large proportion of the observed signals were due to the presence of additional contaminating glycosylated proteins. This was therefore consistent with the results obtained in the lectin pull down assays with ConA which showed the presence of multiple contaminating glycoprotein species. While the observed signals against the ELISA grade BSA were slightly lower than those observed against BSA96 and BSA98, they were still of such a magnitude that the ELISA grade BSA would not be suitable for use in broad spectrum ELLA analysis.

Sodium periodate treatment can be used to oxidize the sugars present on glycoproteins thereby preventing interactions with lectins. We evaluated the utility of this method for the preparation of deglycosylated BSA (deBSA). The treatment was found to be quite effective; dramatically decreasing background signals for most of the lectins tested and confirming that most of the lectins that had showed interactions with the BSA had done so in a sugar specific manner (see Figure 6). Of the 19 lectins tested, only 3 (AIA, WGA and DSL) showed residual binding to deBSA (42). Periodate treatment is relatively simple to perform and can be scaled for bulk preparation of BSA blocking solutions. It could also be performed on the lower grades of BSA to further reduce costs if necessary. Some protein precipitation was observed during incubation of BSA with the sodium periodate but as BSA solutions were diluted prior to use, this did not affect the reproducibility of the assay

We also evaluated methods that reported the omission of blocking steps (26). These methods simply include Tween 20 in lectin probing solutions. While we found this method to be more suitable for a broader spectrum of lectins than those using BSA blocking steps, some of the most commonly used lectins, such as ConA, AAL, DSL and AIA, still exhibited very high background signals against the unblocked wells. These signals were of such a high magnitude that they would preclude the use of these lectins in this format.

Having determined that none of the above approaches would entirely enable broad spectrum lectin screens we decided to evaluate a number of synthetic blocking agents. Synthetic blocking reagents have been successfully used in ELISA and other immunoabsorbent assays where BSA was found to be inappropriate (27, 43). In this study we examined PVA, PVP and PEG solutions as blocking agents. These were initially tested against a subset of lectins comprised of those that had displayed high background signals in the other assay protocols. All of the synthetic blockers appeared to effectively block plate surfaces for the majority of the lectins tested. Only WGA and DSL displayed significant background signals against PVP and PEG blocking agents, but none of the lectins tested showed interactions with PVA. PVA was therefore selected and used to block plates in an ELLA analysis of three commercially available glycoproteins, Fetuin, Invertase and Thyroglobulin. The assay was conducted using 19 lectins and all of the lectins showed low signals in negative control wells containing no glycoprotein. Conversely all of the lectins showed responses to the glycoproteins that were consistent with the expected glycan structures known to be present on these glycoproteins. For example, the sialic acid binding lectins showed high signals on Fetuin and Thyroglobulin, but did not interact with Invertase. Conversely, mannose binding lectins interacted strongly with Invertase which is known to contain high mannose glycans, but not with Fetuin or Thyroglobulin. ConA and LCA, which have been reported to bind to the mannose core structure of *N*-linked glycans, showed strong binding to each of the glycoproteins.

In addition to being used to block unoccupied sites on plate surfaces, BSA is commonly included in the probing solutions of ELISA's and ELLA's to reduce non-specific binding. However, glycosylated contaminants present in BSA preparations could potentially act as lectin inhibitors, out-competing and quenching interactions with surface immobilized target molecules and reducing the sensitivity of the assay. To evaluate the potential impact of utilizing BSA in this manner in ELLA's we performed using the glycoprotein Fetuin was immobilized as the target molecule. Wells were subsequently blocked with PVA and probed with lectin solutions with 0%, 1% or 3% BSA included. All of the lectin-Fetuin interactions were prevented by pre-incubating the lectins with their inhibiting sugars, demonstrating that the interactions were protein-glycan in nature. The results of the experiments with the BSA solutions varied. ConA, which interacted strongly with BSA coated wells, did not vary its signal when 0%, 1% or 3% BSA was included in the probing solution. This is probably a reflection of the strength of the interaction of this lectin with the immobilized Fetuin. In contrast, Although AIA and WGA both gave decent signals when there was no BSA in the lectin solution, both the 1% and 3% solutions abolished the lectins interaction with the Fetuin, suggesting that the lectins interacted more strongly with the contaminants in the BSA (or the BSA itself) than they did with the Fetuin. The SNA signal was also affected by inclusion of the BSA in the solution. In this case, the signal decreased with increasing BSA concentration. These results clearly demonstrated that the inclusion of BSA preparations in lectin probing solutions could severely quench the potential signal strength thereby reducing the sensitivity of the assay and potentially resulting in false negative results.

Commercially available carbohydrate free blocking reagents, such as Synblock (AbD Serotec) and Carbo-Free blocking solution (Vector Laboratories), are finding wider use for glycoanalytical applications. Under our experimental conditions the Carbo-Free blocking solution was observed to exhibit interactions with a small subset of the 19

lectins tested. We found the Synblock reagent exhibited low overall background signals against all of the lectins tested. PVA was found to perform equally as well but is significantly cheaper than the commercially available alternatives.

Ultimately this study therefore presents a global methodology for performing broad spectrum lectin based analysis of glycoproteins and glycoconjugates via an ELLA methodology implementing PVA as an effective blocking agent and making it possible to analyse glycoconjugates in a more thorough manner than had been previously possible.

Tables

Table 1. Abbreviations and sugar specificities of the lectins used in this study (6, 28, 36).

Lectin	**Abbreviation**	**Sugar specificity**
Aleuria aurantia	AAL	Fucose
Concanavalin A (*Canavalia ensiformis*)	ConA	Mannose/Glucosec
Dolichos biflorus	DBA	GalNAc
Datura stramonium	DSL	GlcNAc/LacNAc
Erythrina cristagalli	ECL	Galactose/GalNAc
Galanthus nivalis	GNL	Mannose
Griffonia simplifolia I Isolectin B_4	GSL I	Galactose
Griffonia simplifolia Lectin II	GSL II	GlcNAc
Jacalin (*Artocarpus integrifolia*)	AIA	Galactose/GalNAc
Lentil Lectin (*Lens culinaris*)	LCA	Mannose/Glucosec
Maackia amurensis Lectin I	MAL I	LacNAc
Maackia amurensis Lectin II	MAL II	Sialyllactose
Narcissus pseudonarcissus	NPL	Mannose
Peanut Agglutinin (*Arachis hypogaea*)	PNA	Galactose
Ricinus communis	RCA_{120}	Galactose/GalNAc
Soybean Agglutinin (*Glycine max*)	SBA	GalNAc/Galactose
Sambucus nigra	SNA	Neu5Ac
Ulex europaeus Lectin I	UEA I	Fucose
Wheat Germ Agglutinin (*Triticum vulgare*)	WGA	Neu5Ac; GlcNAc

Figures

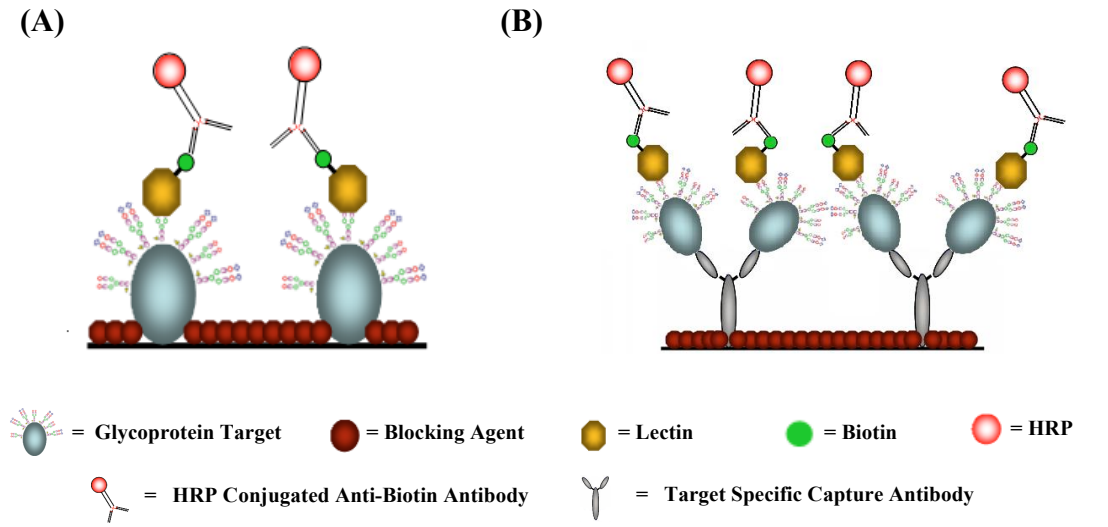


Figure 1: Potential Enzyme Linked Enzyme Assay (ELLA) formats. **(A) Direct immobilization of glycoprotein targets:** Target glycoproteins are immobilized by non-specific absorption on the surface of an ELISA plate. Plates are blocked and the target glycoproteins are then probed with biotinylated lectins. Bound lectins are ultimately detected with labeled Streptavidin or an anti-biotin antibody. **(B) Affinity capture and immobilization of glycoprotein targets:** If an affinity molecule, such as a specific antibody, is available for a desired target glycoprotein it can be pre-immobilized onto ELISA plate surfaces and the surfaces subsequently blocked. The desired target glycoprotein can then be captured and effectively immobilized on the antibody functionalised surfaces for subsequent probing with lectins. This format allows the selective capture a target molecule from complex mixtures but the capture antibody must be deglycosylated prior to use as an assay component .

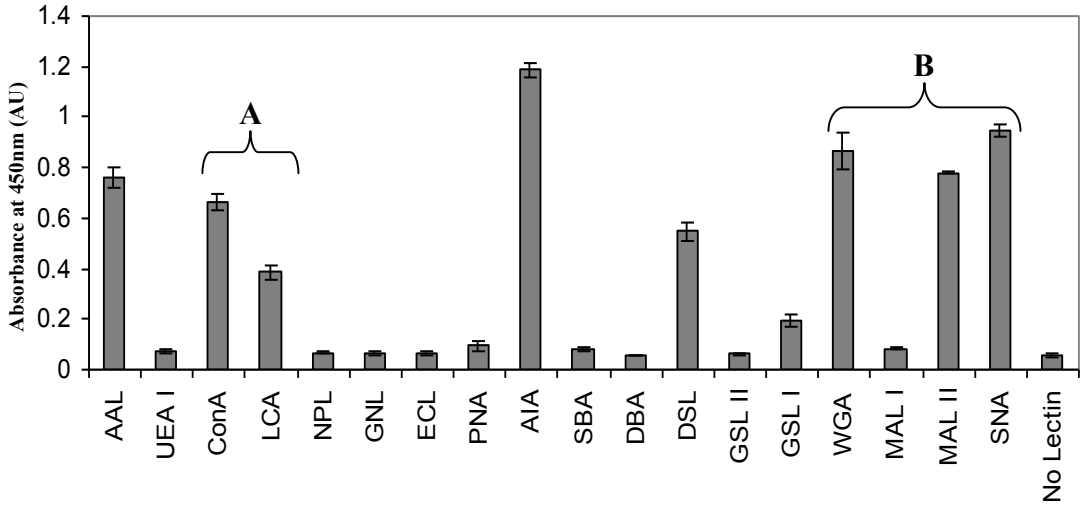
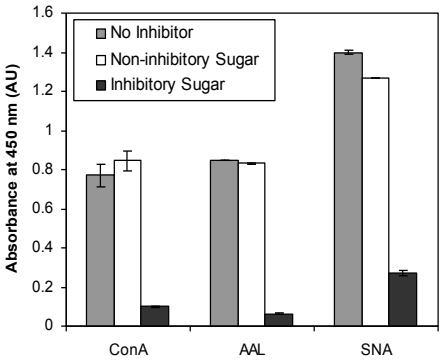


Figure 2: Lectin interactions with BSA blocked surfaces. Nunc MaxiSorp plates were blocked with 3% BSA solution to mimic negative controls used in ELLA's. Of the 19 lectins tested, nine of them showed strong signals against the BSA blocked wells. It was observed that many of the reacting lectins could be grouped according to specific carbohydrate binding specificities **(A)** ConA and LCA which bind to the mannose core structure of N-linked glycans **(B)** WGA, MAL II and SNA are sialic acid binding lectins. These interactions therefore collectively indicate the presence of specific glycan's and that the observed interactions are due to specific lectin-glycan interactions rather than non-specific protein-protein interactions. The magnitude of the observed interactions would make BSA an unsuitable blocking agent in ELLA's using these lectins.

Figure 3: Sugar inhibition of lectins interactions with BSA blocked surfaces. Incubation of ConA and AAL with 50 mM α-methyl mannose and 50mM fucose, respectively, prior to addition to BSA blocked wells resulted in a dramatic decrease in signal strength. Similarly, interaction of SNA with BSA blocked wells was inhibited by prior incubation with glycoprotein Fetuin, known to be heavily sialylated. In contrast, incubation of the lectins with 50 mM galactose resulted in no change in signal strength.



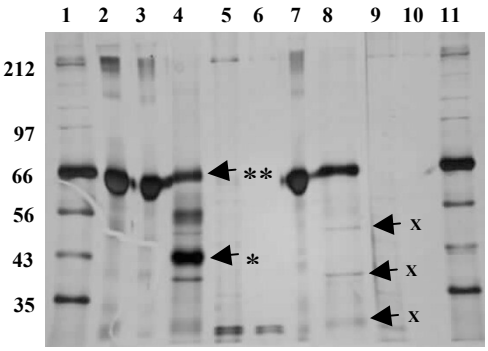


Figure 4: Enrichment of low abundance glycosylated species in BSA preparation using ConA-Agarose and AAL-Agarose: Lanes 1 & 11: Molecular weight markers; Lane 2: BSA Preparation; Lane 3: ConA unbound material; Lane 4: ConA α-methylmannose-eluted species; Lane 5: Residual material released from ConA-Agarose by boiling; Lane 6: Boiled ConA-Agarose control; Lane 7: AAL unbound material; Lane 8: AAL fucose-eluted species; Lane 9: Residual material released from AAL-Agarose by boiling; Lane 10: Boiled AAL-Agarose control.

Several proteins were eluted from the ConA-Agarose with α-methylmannose. One of the strongest bands eluting from the ConA agarose is likely to be the heavy chain of IgG (*). Some residual BSA was also observed to elute from the resin (**), potentially as a result of co-purification with specifically bound glycoproteins. Several of the bands that eluted were also observed to elute from the AAL-agarose (X).

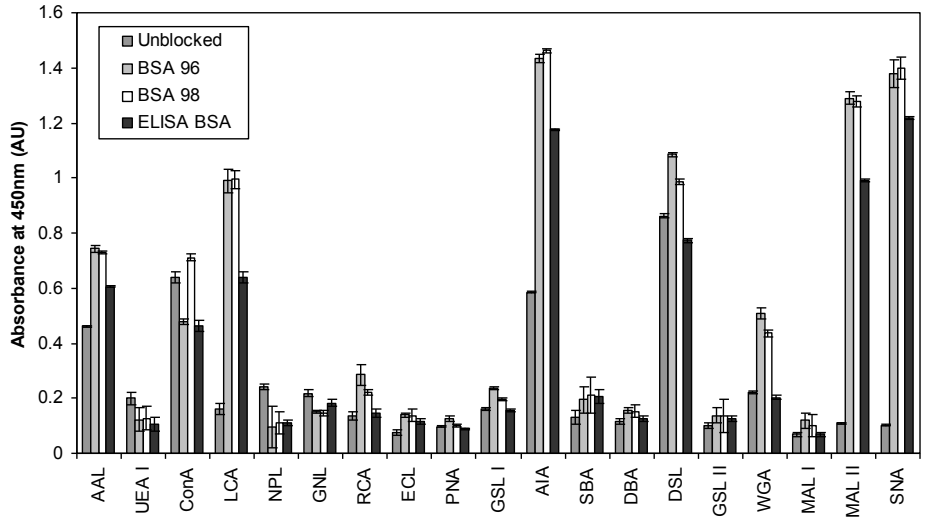


Figure 5. Evaluation of BSA purity grades for application in ELLA's. The wells of ELISA plates were blocked with three different commercially available purity grades of BSA and probed with an extensive panel of lectins. Negative control wells were not blocked prior to probing with lectins. Several lectins displayed strong interactions with unblocked wells (AAL, ConA, AIA and DSL). Additional lectins also showed lower but significant responses, around 0.2 AU, against unblocked wells (UEAI, NPL, GNL and WGA). Many lectins displayed very strong signals against all grades of BSA tested, including ELISA grade BSA. This demonstrated that using BSA, or unblocked wells, was not an effective strategy for reducing background for all lectins in the test set.

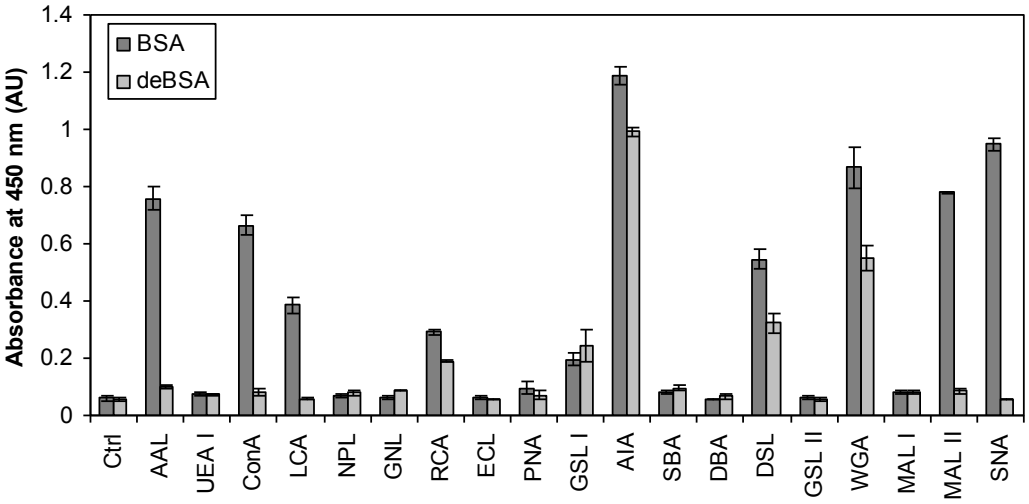


Figure 6. Comparison of the responses of lectins to ELISA plate surfaces blocked with deglycosylated BSA (deBSA). Oxidation of the contaminating glycans in the BSA preparations by sodium periodate treatment prior to use in blocking plate surfaces eliminated the interaction of the majority of the lectins with plate surfaces. Only three of the lectins tested (AIA, WGA and DSL) continued to interact with deBSA blocked surfaces, and this may be because of protein-protein interactions occurring (42)

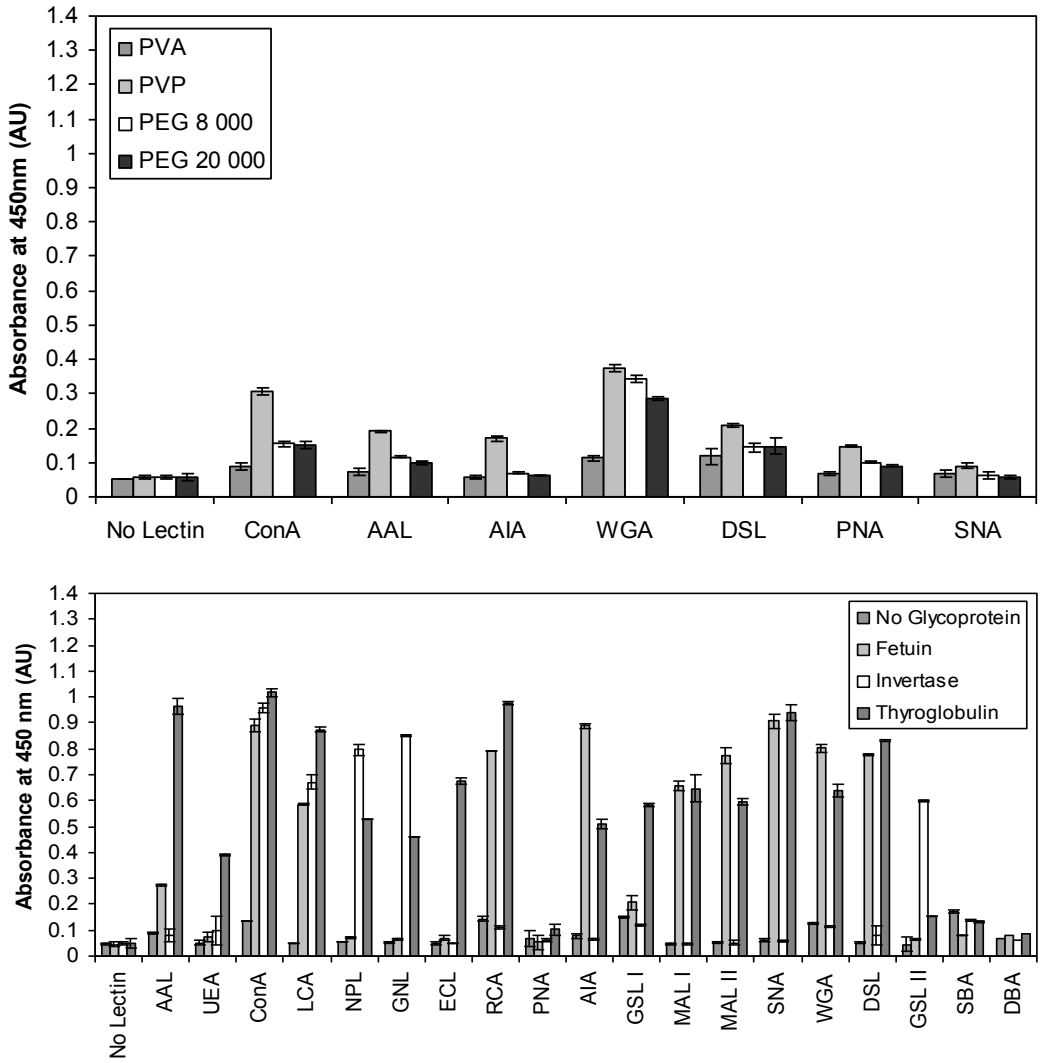


Figure 7: **Evaluation of synthetic blocking reagents for use in ELLA's**. **(A)** Four synthetic polymers were used to block the wells of ELISA plates which were then probed with a broad spectrum of lectins. All of the synthetic agents tested effectively blocked surfaces for the majority of the lectins. However, PVP was found to be unsuitable for use with the lectins WGA, ConA, AAL, AIA and DSL. The lectin WGA was also found to interact with PEG blocked wells. PVA acted as the best blocking agent and did not interact significantly with any of the lectins tested. (**B)** ELLA analysis was performed on the glycoproteins Fetuin, Invertase and Thyroglobulin using PVA as a blocking agent. No significant background signals were observed for any of the lectins used. Each of the lectins interacted with the glycoproteins in a manner that was consistent with the glycan structures expected to be present on the surface of the glycoproteins.

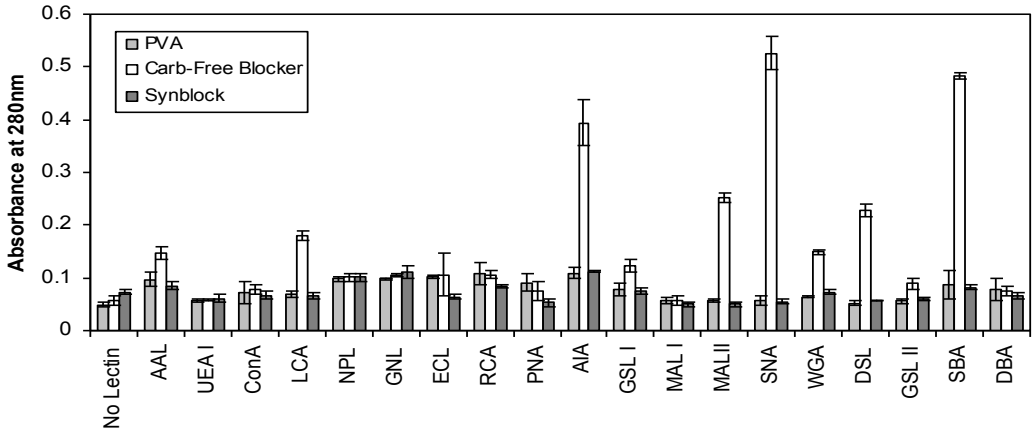


Figure 8. **Evaluation of commercially available blocking reagents.** The commercial blocking reagents Synblock produced by AbD Serotec and Carbo-Free blocker produced by Vector Laboratories were evaluated in ELLA's. Like PVA, Synblock was found to be a suitable reagent for blocking plates in ELLA's since none of the lectins in the test set interacted with Synblock coated wells. However, although acted as an effective blocking reagent for the majority of the lectins tested, several of the lectins in the set (AIA, MAL II, SNA, DSL and SBA) interacted with the Carbohydrate free blocking reagent, making this reagent unsuitable for use in assays utilizing these particular lectins.

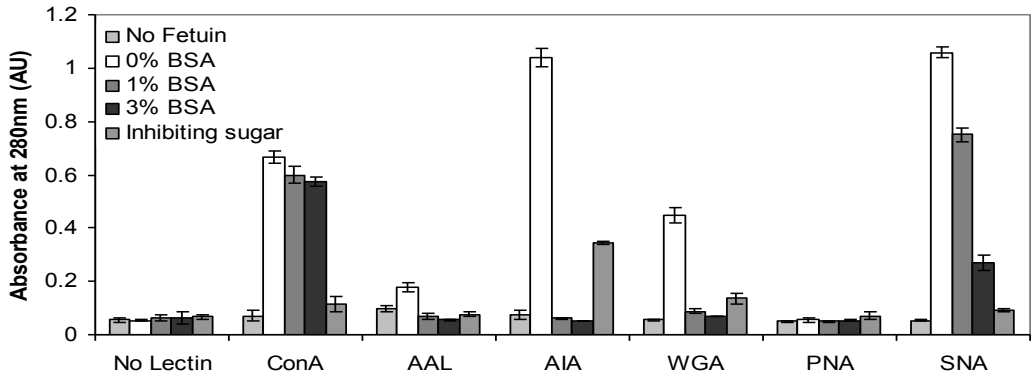


Figure 9. **Effect of including BSA in lectin probing solutions.** BSA is commonly used in multiwell plate assays to prevent non-specific binding, but the contaminants in the BSA preparations may have undesirable effects in ELLA's. To examine this, Fetuin was immobilized on a plate surface and blocked with PVA. Lectin solutions were prepared in TBST, 1% BSA in TBST or 3% BSA in TBST and added to the wells of the plate. Wells without Fetuin were used as controls. Fetuin was additionally probed with lectin solutions containing inhibiting sugars specific to each lectin used to ensure that any signals observed on were due to interactions with the glycan component of the Fetuin. When the plate was developed it was observed that the presence of BSA diminished the signal strength of each lectin tested, and in the cases of AIA and WGA abolished the signal completely. It is therefore better to prepare solutions without BSA in ELLA's to maximize the sensitivity of the assay.

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