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ARTICLES

# Quantitative Determination of Dietary Lectin Activities by Enzyme-Linked Immunosorbent Assay Using Specific Glycoproteins Immobilized on Microtiter Plates

SIMONE VINCENZI, GIANNI ZOCCATELLI, FABIO PERBELLINI, CORRADO RIZZI,* ROBERTO CHIGNOLA, ANDREA CURIONI,† AND ANGELO D. B. PERUFFO

Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie 15, 37134, Verona, Italy, and Dipartimento di Biotecnologie Agrarie, Università di Padova, Padova, Italy

An immunoenzymatic method for the quantitative determination of dietary lectin activities employing immobilized glycoproteins was studied. Lectins from wheat germ (WGA), peanut (PNA), and jack bean (ConA) were added to microtiter plates coated with ovalbumin or asialofetuin and quantified by enzyme-linked immunosorbent assay (ELISA) with lectin-specific antibodies. ELISA responses for lectin activity were dose-dependent in the concentration range 30−1000 ng/mL for WGA and 80−1000 ng/mL for both PNA and ConA. Inhibition assays carried out with different saccharides confirmed that the binding of lectins to immobilized glycoproteins was specific. The proposed method is specific and sensitive, allowing the quantitative determination of lectin activities on raw samples by simple dilution of the extracts. Examples of application to wheat germ and roasted peanut extracts are reported.

**KEYWORDS: ELISA; glycoproteins; lectins; wheat germ; peanut**

## INTRODUCTION

Lectins are proteins or glycoproteins of nonimmune origin that bind specifically to carbohydrates, this ability often resulting in the agglutination of eukaryotic cells (*1*).

For some dietary lectins, an antinutritional effect has been established, and some of them are considered as remarkably toxic (*2*). Most lectins react with the glycoconjugates present on the surface epithelium of the digestive tract, causing several clinical or subclinical effects in humans and animals, particularly when the amount of ingested lectins is large (*3*).

The edible parts of many crop plants and some foodstuff extracts have been analyzed for determining and quantifying the presence of active lectins using agglutination assays with human or animal erythrocytes, treated or not treated with proteases or other enzymes such as sialidase (*4*). These tests are based on the visual evaluation of the modifications of the cell sedimentation behavior as caused by the presence of lectins that induce reticulation of the cells. Although this detection method can be useful in some cases, a positive agglutination reaction is only indicative of the possible presence of lectin activities, because also other substances of a non-protein nature can have a similar agglutinating effect (*5*, *6*). On the other hand, a negative agglutination result does not necessarily mean that a lectin activity is absent. For example, the agglutinating activity of a lectin present in crude extracts can be completely masked, especially when the lectin level is low, by the presence of an excess of free saccharides or glycoconjugates or by the occurrence of compounds causing the lysis of the red blood cells before the agglutination becomes visible. Furthermore, agglutination assays only detect lectins having multiple binding sites (hololectins), whereas other lectins, although being able to interact with specific sugars, do not cause agglutination. Finally, the joint presence of two or more lectins cannot be deduced by a positive result of an agglutination test (*7*). For all these reasons it would be preferable to directly quantify the sugar binding ability of specific lectins, which is the basic mechanism of the biological activity of these proteins.

Quantification of the activity of lectins by immunological evaluation of their binding to specific sugars immobilized with different methods on solid phases have been used in lectin activity studies (*8*, *9*). On the other hand lectins have been utilized as tools for the study of glycoproteins after their immobilization in enzyme-linked immunosorbent assay (ELISA) systems (*10*, *11*).

Here we describe an immunoenzymatic method for the quantification of three dietary lectins which is based on their

* To whom correspondence should be addressed at the Dipartimento Scientifico e Tecnologico, Università di Verona, Strada Le Grazie, 15, 37134, Verona, Italy. Fax: 39 045 8027929. E-mail: rizzi@sci.univr.it.
† Università di Padova.

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binding to commercially available glycoproteins immobilized on microtiter plates.

## MATERIALS AND METHODS

**Materials.** Peanut agglutinin (PNA) was from Vector (Burlingame, CA). Glycoproteins (fetuin and asialofetuin from fetal bovine serum, mucin from bovine submaxillary salivary glands, conalbumin and ovalbumin from hen egg), bovine serum albumin (BSA), wheat germ agglutinin (WGA), Concanavalin A (ConA), rabbit antibodies to PNA, WGA, and ConA, alkaline phosphatase (AP)-labeled monoclonal anti-rabbit IgG (clone RG-16) and Sigma Fast *p*-nitrophenyl phosphate tablets were purchased from Sigma (Milan, Italy). Microtiter plates were from Sarstedt (Nümbrecht, Germany).

**Lectin Activity Detection by ELISA.** Flat-bottomed 96-well microtiter plates were coated with 50 $\mu$L of the different glycoproteins (fetuin, asialofetuin, mucin, conalbumin, and ovalbumin) in a 10−30 $\mu$g/mL range in 50 mM Na-carbonate buffer (pH 9.6) or with the same amount of BSA as a negative control and incubated for 4 h at 37 °C. Plates were then washed three times in phosphate buffer saline (PBS) and saturated with 200 $\mu$L of 1% (w/v) BSA in PBS (PBS−BSA) overnight at 4 °C (or 4 h at 37 °C).

Microtiter plates were then incubated at 37 °C for 1 h with 50 $\mu$L of lectin samples serially diluted in PBS−BSA. Wells were washed three times in PBS, incubated with PBS−BSA at room temperature for 30 min, and further washed three times in PBS. Then, 50 $\mu$L of the antibody (diluted 1:2000 in PBS−BSA) specific for the lectin to be detected was added to each well. Plates were incubated at 37 °C for 1 h, washed three times in PBS, incubated with PBS−BSA for 30 min, and further washed three times with PBS. To each well, 50 $\mu$L of AP-labeled monoclonal anti-rabbit IgG antibody diluted 1:5000 in PBS−BSA was added. After incubation at 37 °C for 1 h, plates were washed three times with PBS, incubated for 30 min with PBS−BSA and washed three times again with PBS. Seventy-five microliters of alkaline phosphatase substrate (Sigma Fast *p*-nitrophenyl phosphate) was finally added to each well. After 30 min at room temperature plates were read on a Microplate Reader (BioRad model 550) at a wavelength of 415 nm.

Inhibition of lectin binding to immobilized glycoproteins was performed by adding specific saccharides [*N*-acetylglucosamine (GlcNAc) or mannose (Man) for WGA, Man or galactose (Gal) for ConA, and Gal or Man for PNA (*3*)] at increasing concentrations to the lectin samples diluted in PBS−BSA at 0.5 $\mu$g/mL. The ELISA procedure was then completed as described above.

**Determination of Lectin Activity in Roasted Peanuts and Wheat Germ.** One g of roasted peanuts or wheat germ was homogenized in 10 mL of PBS, and the obtained suspension was stirred overnight at 4 °C. The homogenate was centrifuged at 9000*g* at 4 °C for 15 min, and the supernatant was clarified by filtration on Whatman no. 4 paper. The resulting solution was diluted in PBS−BSA in ratios varying from 1:100 to 1:1000. Diluted peanut or wheat germ extracts were tested by ELISA on plates coated with asialofetuin and ovalbumin, respectively. Bound lectins were detected with the corresponding antibodies, following the procedures described above. Lectin concentrations, as determined by ELISA, were compared to those measured in standard agglutination assays [on rabbit erythrocytes for WGA or sialidase-treated erythrocytes for PNA (*3*)] carried out with the same extracts.

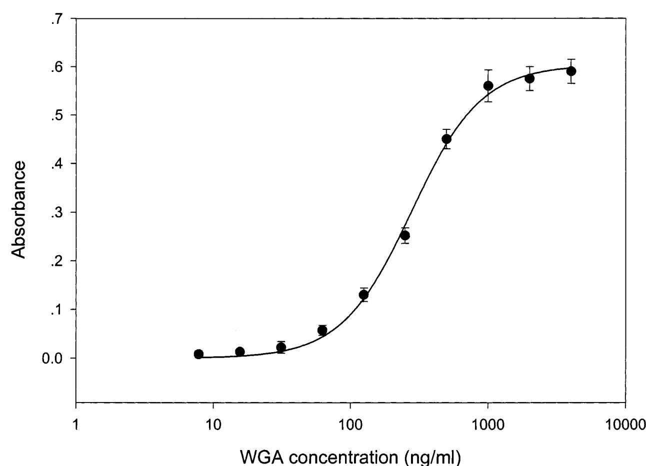


**Figure 1.** Binding of WGA to ELISA plates coated with ovalbumin.

## RESULTS

To develop an ELISA procedure for the quantitative determination of the activity of lectins by using immobilized glycoproteins, the following parameters were first studied: (a) glycoprotein type, (b) glycoprotein concentration, and (c) pH of the coating buffer.

Different glycoproteins were selected on the basis of the sugar specificity of PNA, WGA, and ConA (*3*). These were fetuin, asialofetuin, mucin, conalbumin, and ovalbumin. WGA (which binds GlcNAc residues) activity was best detected by coating the plates with ovalbumin, which is in fact rich in GlcNAc (*12*). The other glycoproteins gave poorer responses in terms of precision, detectability, and sensitivity (not shown). PNA and ConA, binding Gal and Man, respectively (*3*), were instead best detected with desialized fetuin, although ovalbumin could be used with overlapping results for the quantitative determination of ConA (not shown). Neither PNA nor ConA could be detected when fetuin [which contains a high proportion of Gal and Man (*13*)] and mucin [which is rich in GalNac, but contains a lower quantity of Gal and Man compared to fetuin (*13*)] were used.

The effect of the coating buffer was also examined by dissolving ovalbumin and asialofetuin in different buffers (50 mM K-acetate pH 4.8, 50 mM Tris-$H_3PO_4$ pH 5.5, PBS, 10 mM Tris-HCl pH 8.5, and 50 mM Na-carbonate pH 9.6). For both glycoproteins, the best results were obtained by using 50 mM Na-carbonate (pH 9.6). The optimal concentrations for coating were found to be 20 $\mu$g/mL for ovalbumin and 25 $\mu$g/mL for asialofetuin.

Under these conditions, although with different absorbance *maxima*, ELISA responses were dose-dependent for all lectins (range 30−1000 ng/mL for WGA; range 80−1000 ng/mL for PNA and ConA). Linearity of the dose/response curves was obtained in the concentration range 125−500 ng/mL for WGA and PNA (Figures 1 and 2, respectively) and 250−1000 ng/mL for ConA (Figure 3). Higher concentrations of the antilectin antibodies slightly shortened the time needed to complete the final enzymatic reaction but did not decrease the lower limit of lectin detection (not shown). Background signals were detected when BSA was coated instead of glycoproteins (not shown).

The binding specificity of the different lectins to the coated glycoproteins was established by inhibition with specific saccharides. The presence of increasing concentrations of GlcNAc, Gal, and Man during incubation clearly resulted in a progressive decreasing of the ELISA signals obtained with 500 ng/mL of WGA (Figure 4), PNA (Figure 5), and ConA (Figure 6), respectively. For the considered lectins, the maximum of

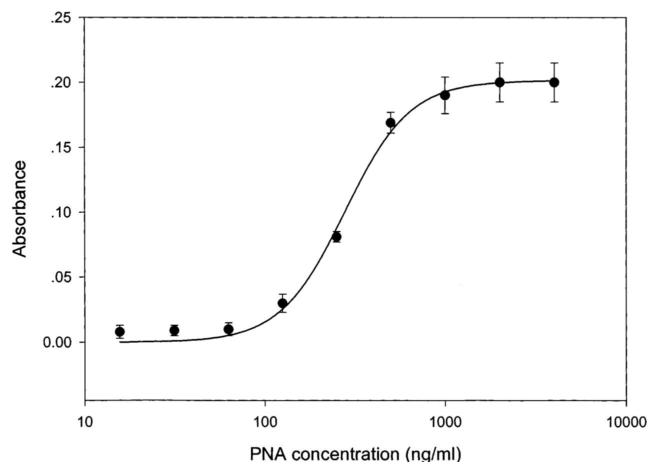


**Figure 2.** Binding of PNA to ELISA plates coated with asialofetuin.

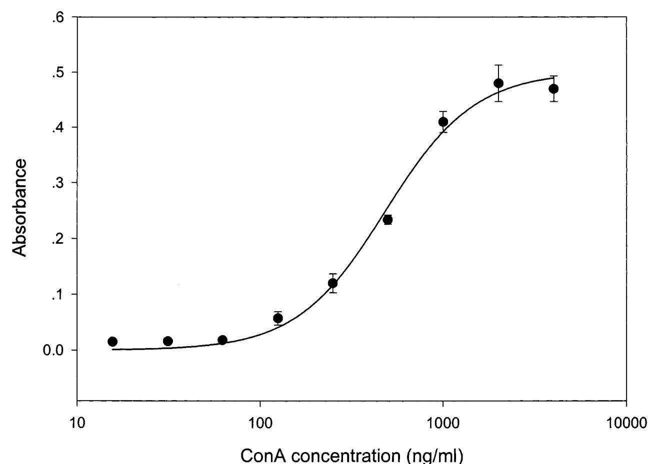


**Figure 3.** Binding of ConA to ELISA plates coated with asialofetuin.

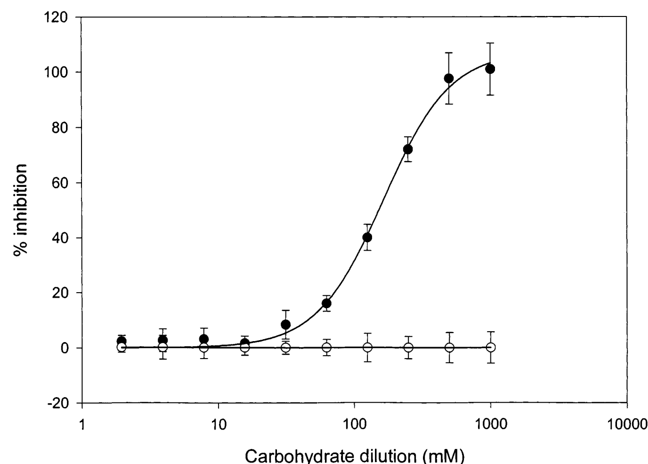


**Figure 4.** Inhibition of WGA binding to ovalbumin-coated ELISA plates by GlcNac (●) or Man (○).

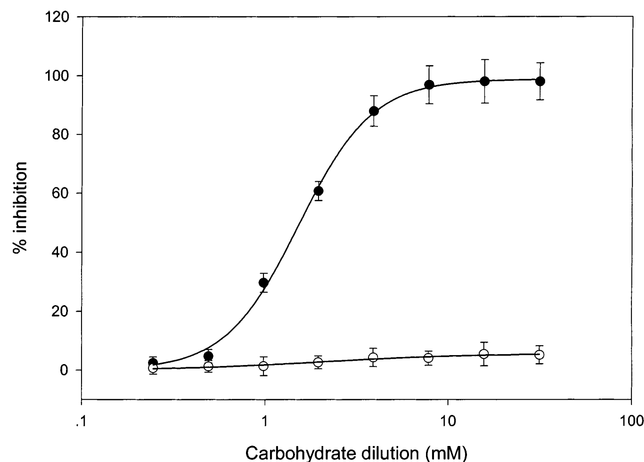


**Figure 5.** Inhibition of PNA binding to asialofetuin-coated ELISA plates by Gal (●) or Man (○).

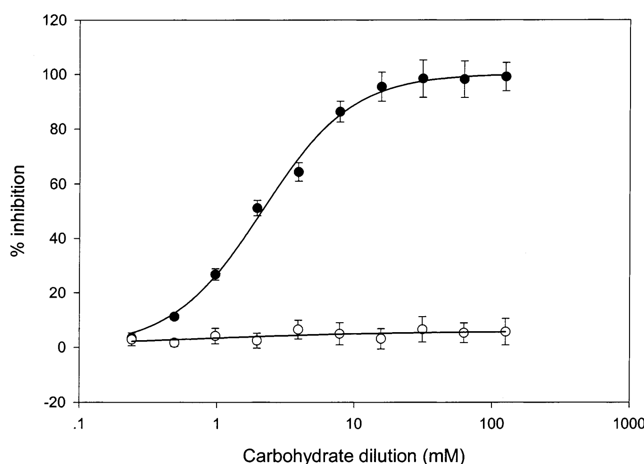


**Figure 6.** Inhibition of ConA binding to asialofetuin-coated ELISA plates by Man (●) or Gal (○).

inhibition was obtained at varying sugar concentrations, reflecting different affinity values. On the contrary, no inhibition was obtained with sugars not specific for each of the lectins tested (i.e., Man for WGA and PNA and Gal for ConA) (Figures 4−6). These results demonstrate that the lectins bound specifically coated glycoproteins.

The described method was used for the quantification of the lectins present in raw extracts from wheat germ and roasted peanuts. Lectin activities in the soluble fractions of these samples were determined by comparing data with standard curves obtained with purified WGA and PNA in the same ELISA plates; 0.30 ± 0.035 (SD) mg/g of active WGA and 0.11 ± 0.012 (SD) mg/g of active PNA were detected in examined wheat germ and roasted peanut samples, respectively. These values are in agreement with those found in agglutination assays carried out with the same extracts (0.2 and 0.12 mg/g for WGA and PNA, respectively).

## DISCUSSION

Assays for lectin studies include a variety of qualitative and quantitative methods, such as cell agglutination tests, binding to cells and tissues, lectinoblotting, affinity chromatography, affinity electrophoresis and many others (*10, 11, 14, 15*). ELISA systems based on solid phases coated with specific saccharides have been introduced in lectin activity studies (*9, 10, 16, 17*). However, the direct immobilization of oligosaccharides is usually cumbersome, ineffective, and does not always proceed in a dose-dependent manner (*17*). Lectin activity detection remains commonly performed by cell agglutination tests. Although studies on lectin-induced cell agglutination have a long history, not all the parameters controlling this complex reaction are fully understood. For example, although it is known that binding of lectins to the cell surface is a necessary prerequisite for agglutination, the extent of lectin binding does not always fully correlate with the actual proportion of cells agglutinated (*3*). In most instances, the extent of agglutination is dependent

on the specificity, number of binding sites, and size of the lectin as well as on cell properties and metabolic state (*18*). Furthermore, other factors, such as, for example the presence of agglutinating substances of nonlectin nature, can determine misinterpretation of the agglutination results (*5, 6*).

The method described in the present study is an alternative assay for the quantitative determination of lectin activities, being based on the binding of these proteins to specific glycoproteins immobilized on ELISA plates. A similar approach was previously used only for glycoprotein detection and characterization (*14, 19*) or in glycosidase activity studies (*20*). In the present study, the lowest detectable lectin concentration was 230- and 750-fold higher for WGA and ConA, respectively, as compared to cell agglutination methods (as specified in the datasheets for the commercial lectins used or as compared to our own data). PNA was instead detected at only slightly lower concentrations. However, the ELISA method allows one to avoid the desialylation of erythrocytes required for the detection of PNA by cell agglutination test (*4, 21*).

Although carrying the sugars suitable for specific lectin binding (*13*), not all the glycoproteins used in ELISA assays turned out to be useful for lectin activity determination. Besides the sugar composition, other factors, such as the properties of the aglycon, the overall structure of the glycan chain (*22, 23*), and also the interactions with the solid phase of the glycoprotein (*24*) can affect its binding to a given lectin. Therefore, the suitability of a glycoprotein to be used in lectin activity detection by ELISA cannot be assessed a priori on the basis of the mere sugar composition of its glycan chains.

In determining WGA activity the best results were obtained using ovalbumin. In this case it is easy to relate the specificity of the lectin for GlcNAc with the assay results because the glycosilation pattern of commercial ovalbumin is characterized by a high content of GlcNAc, present in the glycan chain as the terminal sugar (*12*).

When PNA [whose preferred specificity is for terminal Gal (3)] was tested, only asialofetuin from fetal bovine serum effected satisfactory ELISA detection. Since detection of PNA was not possible using fetuin, desialylation of this glycoprotein was necessary to eliminate the masking effect of sialic acid on terminal Gal (*19*). Indeed, cell agglutination by PNA can be obtained only after treating the cells with sialidase (*4, 21*).

Asialofetuin was effective even for ConA activity determination, although ovalbumin could also be used. In this case, the Man6(Man3)Man core present in the fetuin glycan chain (*25*) is likely to play a key role for binding (*23*). However, binding of ConA was not detectable when fetuin was used as the coating glycoprotein, indicating a negative effect of the sialosyl residues. The presence of terminal sialogalactosyl residues has been previously shown to reduce the affinity of ConA for oligosaccharides containing the trimannosidic core (*23*).

Inhibition of the glycoprotein binding activity was obtained for each lectin after incubation with specific monosacchararides although the affinity of a lectin for its specific free sugar might be much lower than that for a glyconjugate containing the same sugar (*23*). The results of the inhibition experiments confirmed the specificity of the ELISA responses and suggested that the proposed method could be potentially useful also in studying the affinity of each lectin for different sugars.

The ELISA method allows one to analyze the lectin content in very diluted samples. As a consequence, the possible interfering effects of compounds that might be present in crude extracts are reduced without the need for lectin purification. Experiments of lectin determination were therefore performed utilizing crude extracts from both wheat germ and roasted peanuts. The WGA activity found by ELISA in the wheat germ extracts was in good agreement with previously reported data (*21*) as well as with data obtained in standard agglutination tests. A discrepancy in PNA activity of roasted peanuts was found between the values obtained by us in ELISA assays and those obtained by Ryder et al. (*26*) in agglutination assay. This is not surprising because of the great variability in PNA content of different cultivars, or it could be a consequence of technological processing. For this reason, PNA in the same extracts was also determined by agglutination assays with good agreement.

Possible limitations of the present method rely on the limited availability of specific antibodies to different lectins. However, it is conceivable that the increasing interest for these proteins will make available antibodies to almost all edible plant lectins soon.

Overall, the described method can be exploited to quantitate in a simple and relatively inexpensive way the activity of lectins in biological extracts, thus opening the way to the development of ELISA kits to be used for routinary analyses of different food products or raw materials.

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