Chemical Modification and Sugar Binding Properties of Two Major Lectins from Pinhão (*Araucaria brasiliensis*) Seeds

Pradip K. Datta,[†] M. Oriana R. Figueroa, and Franco M. Lajolo*

Faculdade de Ciências Farmacêuticas—USP, Departamento Alimentos e Nutrição Experimental, Caixa Postal 66355, CEP 05389-970, São Paulo, SP, Brazil

Araucaria brasiliensis lectins, lectin I and lectin II, were subjected to various chemical modifications to detect the amino acid residues present in their carbohydrate binding sites. Modification of tyrosine and arginine residues did not affect binding activities of lectins. However, modification of tryptophan and histidine led to a complete loss. Modification of amino and carboxyl groups of both lectins also abolished completely their hemagglutinating capacity. The carbohydrate moiety of lectins was not involved in maintaining their hemagglutinating activities. Binding of monosaccharides was studied by UV difference spectroscopy which showed that both lectins have two binding sites with different affinities for sugars. Each lectin molecule may bind from 6 to 14 sugar residues. The association constants and free energy of binding for different sugars are presented.

INTRODUCTION

Recently we purified and partially characterized two lectins from the seeds of *Araucaria brasiliensis* (Gymnospermae) (Datta et al., 1991). They are glycoproteins in nature containing 6.3 (lectin I) and 2.9% (lectin II) of neutral sugars. Both lectins have the same molecular weight: 200 000 and subunits of 20 000 and 34 000, suggesting they are, respectively, decameric and hexameric in nature. They interact strongly with *p*-nitrophenyl α-D-mannoside and *p*-nitrophenyl β-D-glucoside (lectin I) and with *p*-nitrophenyl α-D-mannoside (lectin II) and to a lesser extent with D-mannose and D-glucose. These lectins differ from all other plant lectins with respect to their very high specific activity and subunit composition (Datta et al., 1991). We have also demonstrated that these two lectins increase their specific activities in the presence of high molecular weight substances such as arabinogalactan and poly(vinylpyrrolidone) and also in the presence of metal ions such as 40 mM MgCl₂ (Datta et al., 1991).

To elucidate the role of functional groups of the purified lectins in erythroagglutination, in the present study we determined the effect of chemical modifications of amino acid residues on their biological properties. The binding constants of these two lectins were investigated by ultraviolet difference spectroscopy.

MATERIALS AND METHODS

Pinhão seeds (*A. brasiliensis*) were obtained from a local supermarket in São Paulo, Brazil. Arabinogalactan, D-mannose, *p*-nitrophenyl α-D-glucose, *p*-nitrophenyl β-D-glucose, *p*-nitrophenyl α-D-mannoside, succinic anhydride, citraconic anhydride, sodium borohydride, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, diethyl pyrocarbonate, histidine, *N*-bromosuccinimide, D-galactose, and 2,4,6-trinitrobenzenesulfonic acid were all from Sigma Chemical Co. (St. Louis, MO). Sodium periodate was from May and Baker Ltd. (Dagenham, England). Glycine methyl ester hydrochloride and *N*-acetylimidazole were from Aldrich Chemical Co. (Milwaukee, WI). Cyclohexane-1,2-dione was from Pierce Chemicals Co. (Rockford, IL). Formaldehyde and acetic anhydride were from E. Merck (Darmstadt, Germany). 1-Octanol was from J. T. Baker Co. (São Paulo, Brazil). All other reagents used were of analytical grade of highest purity.

* Author to whom correspondence should be addressed.

† Present address: Jadavpur University, Calcutta 700032, India.

Purification of *A. brasiliensis* Lectins. Lectin I and lectin II were prepared from *A. brasiliensis* seeds as previously described (Datta et al., 1991). The purity of preparations was determined by HPLC and PAGE.

Protein Determination. Protein concentrations were determined by spectrophotometric measurements at 280 nm by use of $E^{1\%}_{1cm}$ values 3.8 for lectin I and 4.7 for lectin II as determined by Datta et al. (1991).

Hemagglutinating Activity. Hemagglutination was assayed in V-microtiter plates by serially diluting 50 μL of sample into 50 μL of 8 mM phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS). Then, 50 μL of 2% rabbit erythrocyte suspension in the same buffer was added to each well, and hemagglutination was determined after 1 h of incubation at 37 °C as the reciprocal of the maximal dilution that gave visible aggregation.

Effect of arabinogalactan and MgCl₂ on hemagglutination was examined by preincubation of the modified lectin with the test substance at 37 °C for 30 min followed by incubation at 37 °C for 1 h after addition of eythrocyte suspension.

Chemical Modification of the Lectins. The amino groups of the lectins were modified by reductive methylation (Means and Feeney, 1968). To the lectin solution (0.5 mg/mL) at 0 °C in 0.2 M sodium borate buffer, pH 9.0, was added 0.5 mg of NaHB₄ followed by rapid addition of 20 μL of aqueous formaldehyde (37%); these additions were repeated twice at 15-min intervals. The reaction mixture was incubated for 1 h and dialyzed extensively with PBS.

Acetylation of the amino groups was carried out according to the method of Rice and Etzler (1975). To the lectin solution (0.5 mg/mL in 0.3 M *N*-ethylmorpholine acetate buffer, pH 8.5) was added 20 μL of acetic anhydride/mL four times at 15-min intervals, with constant stirring, at 0 °C. The pH was maintained at 8.5 with 0.5 M NaOH. The sample was dialyzed exhaustively against PBS.

Succinylation was carried out as described by Habeeb et al. (1958). The lectin (0.5 mg/mL in 0.3 M *N*-ethylmorpholine acetate buffer, pH 8.5) was stirred in an ice bath for 1 h with simultaneous addition of 12 mol of succinic anhydride/mol of free amino groups. The pH was maintained at 8.5 by 0.5 M NaOH. After 3 h, the sample was dialyzed exhaustively against PBS.

Citraconylation was conducted according to the method of Dixon and Perham (1968). To the lectin solution (0.5 mg/mL in 0.1 M Tris-HCl buffer, pH 8.0) were added three 4-μL portions of citraconic anhydride with constant stirring at 20 °C over a period of 30 min. The solution was then stirred for 1 h at 20 °C and dialyzed against 5 mM Tris-HCl buffer, pH 8.6, in the cold. The decitraconylation was carried out by dialyzing the citraconylated protein overnight at 4 °C in 50 mM sodium acetate buffer, pH 3.0.

The extent of amino group modification was measured by determining the unmodified amino groups with 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1967).

For modification of arginine residues the lectin (0.5 mg/mL) was dissolved in 0.2 M sodium borate buffer, pH 9.0, containing 0.05 M cyclohexane-1,2-dione (500 μL). The reaction vessel was flushed with N₂ and incubated at 37 °C, in the dark, for 4 h. The reaction was terminated by the addition of 1 mL of 5% (v/v) acetic acid. The mixture was then dialyzed against the same acid and then with PBS. This method was described by Patthy and Smith (1975).

Carboxyl groups were modified by following the method of Carraway and Koshland (1972). To the lectin solution (0.5 mg/mL) in PBS, containing 7.5 M urea, was added 50 mg of glycine methyl ester hydrochloride, the pH was adjusted to 4.7, and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide was added as a solid to bring its concentration 0.1 M. The solution was adjusted in the pH range 4.7–4.8 with 0.1 M NaOH and 0.1 M HCl. The reaction was stopped after 1 h of incubation at room temperature by adding an excess of 1.0 M acetate buffer, pH 4.7. After that, the sample was dialyzed against PBS.

Tyrosine residues were modified according to the procedure of Riordan et al. (1965). The lectin solutions (0.5 mg/mL) were treated with a 100-fold molar excess of N-acetylimidazole in 8 mM phosphate buffer containing 0.15 M NaCl, pH 7.5, at room temperature for 1.5 h. The excess of reagents was removed by dialysis against PBS. The number of tyrosine residues modified was determined spectrophotometrically by the decrease in absorbance at 278 nm using a molar absorption coefficient of 1160 M⁻¹ cm⁻¹ (Riordan and Vallee, 1967).

Modification of histidine residues was carried out according to the method of Melchior and Fahrney (1970). Diethylpyrocarbonate was added to the lectin solution (0.5 mg/mL in PBS) to a final concentration of 3-fold molar excess over the histidine content. The reaction was allowed to proceed with stirring at 25 °C for 2 h and was terminated by adding an equal volume of 10 mM cold histidine in PBS; the mixture was dialyzed in PBS to remove excess reagent. The extent of modifications of histidine residues was determined as described by Anderson and Ebner (1979).

Tryptophan residues were modified with N-bromosuccinimide (NBS), and the reaction was monitored spectrophotometrically both in the presence and in the absence of 8 M urea (Spande et al., 1966). Aqueous NBS (10 mM) was added in 5-μL portions to a solution of protein (0.5 mg/mL) in 50 mM acetate buffer, pH 4.0, at 25 °C. After the reaction, the samples were dialyzed extensively with PBS to remove excess reagents. The number of tryptophan groups modified was calculated according to the method of Spande and Witkop (1967) using the empirical factor 1.31.

NaIO₄ oxidation of carbohydrates was done according to the method of Weber et al. (1972). The oxidation was performed at a concentration of 0.05 M for 6 h at 4 °C in the dark. The concentration of protein was 0.5 mg/mL in PBS. The reaction was terminated by the addition of glycerol in 10-fold molar excess. The mixture was then dialyzed against PBS.

The conditions for chemical modifications of lectins were often extremely mild, and more drastic treatment was followed by adequate controls.

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra were recorded on a Beckman DU 70 spectrophotometer. Aliquots (1 mL) of lectin solutions, 436 μg for lectin I and 356 μg for lectin II, in PBS, were added separately to both sample and reference cuvettes. Small aliquots (2–20 μL) of sugar solutions under study were added to the sample cuvette, while the same amount of PBS was added to the reference cuvette. The difference spectra were recorded. The total volume of ligand solution varied from 15 to 25 μL, rendering concentration corrections for dilution unnecessary. Control experiments were conducted with D-galactose, which is not an inhibitor of A. brasiliensis lectins (Datta et al., 1991). All of the measurements were made at room temperature, 25 °C.

Association constants, K_a, were determined from Scatchard plot (Neurohr et al., 1982), according to

$$\frac{\Delta A}{\Delta A_{max}} \frac{1}{[S]_f} = K_a \left(1 - \frac{\Delta A}{\Delta A_{max}}\right) \qquad (1)$$

$$[S]_f = [S]_t - (\Delta A/\Delta A_{max})n[P] \qquad (2)$$

where $[S]_f$ is the free sugar concentration, $[S]_t$ is the total sugar concentration, $n[P]$ is the subunit concentration, ΔA is the difference of absorption spectra at 280 nm, and ΔA_{max} is the maximum difference of absorption spectra at 280 nm.

Values of ΔG, the standard free energy change in the formation of lectin–sugar complexes, were obtained from the equation $\Delta G = -RT \ln K_a$.

RESULTS AND DISCUSSION

Effect of Chemical Modifications on Lectins Activity. The extent and effects of chemical modifications on the hemagglutinating activity of lectin I and lectin II are summarized in Table I. These studies revealed that tryptophan, histidine, carboxy, and lysine residues may be involved in binding activity of Pinhão lectins.

Acetylation, succinylation, citraconylation, and reductive methylation modified 65–89% for lectin I and 72–92% for lectin II of the ϵ-amino groups of lysine residues. Only acetylated lectin I maintained part of the hemagglutinating activity, while modification of the amino groups by other methods abolished it completely in both lectins. Acetic anhydride is not a specific reagent for lysine residues only; sometimes, it can also modify tyrosine, whereas succinic anhydride and citraconic anhydride preferentially react with lysine residues (Dixon and Perham, 1968; Klotz, 1967). Decitraconylation at pH 3.0 restored the original activity of both lectins.

Similar effects on hemagglutinating activity have been reported for pea (Trowridge, 1973), peanut (Nonnenmacher and Brossmer, 1981), Vicia faba (Datta et al., 1984), and soybean lectins (Desai et al., 1988).

When carboxyl groups were esterified, both lectin I and lectin II lost their hemagglutinating capacity.

It was reported earlier that carboxyl groups of aspartate and glutamate residues are necessary for hemagglutination with V. faba lectin (Datta et al., 1984). Hassing et al. (1971) also demonstrated the involvement of carboxyl groups in sugar binding of Con A. They showed that these groups were masked when Con A was titrated in the presence of methyl α-D-mannopyranoside (a specific sugar).

The guanidino groups of arginine were specifically modified by cyclohexane-1,2-dione, and the resulting complex was stabilized by borate ions. The modification led to change in the hemagglutinating activity but did not abolish it completely. On the other hand, there was total loss of activity after modification of histidine residues in both lectin I and lectin II.

This conclusion, though, must be made cautiously, because nonspecific conformational changes may also produce inactive forms of lectins unable to bind sugars.

From our data it can be suggested that some of the charged residues we discussed above may be either adjacent to or present in the saccharide binding sites as happens for other lectins (Jordan et al., 1977).

These results are different from observations made with other lectins. For instance, we verified that histidine modification of Phaseolus vulgaris lectin did not affect hemagglutinating activity. A similar observation was made by Desai et al. (1988) for soybean lectins.

A similar inactivating effect was also observed after modification of tryptophan residues. The oxidation of A. brasiliensis lectins with NBS at acidic pH, as normally observed by other authors, modifies both tyrosine and

Sugar Binding Properties of Pinhão Seed Lectins

J. Agric. Food Chem., Vol. 41, No. 11, 1993 **1853**

Table I. Chemical Modification, Residues Modified, and Agglutinating Activity of Lectin I and Lectin II

| modifn | residue modified | modifn (%) | | agglutinating act.[a] |
		L-I	L-II	
acetylation	Lys	65	72	(−)*
succinylation	Lys	78	77	(−)
red. methylation	Lys	84	80	(−)
citraconylation	Lys	89	92	(−)
decitraconylation	Lys			(+)**
N-bromosuccinimide	Trp	45	50	(−)
+ urea 8 M	Trp	70	80	(−)
N-acetylimidazole	Tyr	80	75	(+)
cyclohexane-1,2-dione	Arg	nd[b]	nd	(±)
diethyl pyrocarbonate	His	85	90	(−)
glycine methyl ester	Glu, Asp	nd	nd	(−)
sodium periodate	sugar oxid			(+)

[a] (+), (±), and (−), no, partial, and total loss of agglutinating activity. *, acetylation of lectin I produces partial loss of activity. **, activity restored. [b] nd, not determined.

tryptophan residues (Viswanath and Lawson, 1961), but under the experimental conditions we describe the reagent modified only the tryptophan residues. The oxidation of tryptophan was investigated under both denaturing and nondenaturing conditions. NBS oxidized many more tryptophan residues in the presence of denaturing substance than in native form. In the presence of 8.0 M urea, 6 of 8 residues of tryptophan in lectin I and 28 of 37 residues in lectin II were modified. Treatment of native lectins with NBS also produced an inactive preparation, although only 4 residues for lectin I and 18 residues for lectin II were oxidized. Urea alone had no effect on agglutinating activity.

From this observation it can be assumed that some tryptophan residues are hidden in the structural configuration and are exposed only after denaturation.

Tryptophan, lysine, histidine, aspartyl, and glutamyl residues may be either present in or adjacent to the sugar binding sites like in potato lectin (Ashford et al., 1981), wheat germ agglutinin (Privat et al., 1976), and pea (Cermakova et al., 1976) or are important in maintaining a structural conformation for hemagglutinating activity.

The treatment with N-acetylimidazole modified both lectins but without producing any change in their activity.

Oxidation with sodium periodate did not lead to any change of hemagglutinating activity, indicating that the carbohydrate moiety is not involved.

In a previous paper (Datta et al., 1991) we observed that the presence of arabinogalactan or $MgCl_2$ increased agglutinating activity of both lectins. On the basis of this fact we postulated for *A. brasiliensis* lectins the existence of binding sites for metal ions as happens for Con A (Bittiger and Schnebli, 1975) or binding sites for high molecular weight substances as in *V. faba* lectin (Datta et al., 1988).

The presence of $MgCl_2$ increased slightly the agglutinating activity of acetylated, succinylated, and citraconylated lectin I and acetylated and tryptophan-modified lectin II (results not shown), but the experimental error of the hemagglutination assay did not allow a definitive conclusion. Arabinogalactan also had no significant effect on the modified lectin I or lectin II, contrary to what happens with the native forms, where they can change their conformation after binding with metal ions or high molecular weight substances and increase the activity (Datta et al., 1991).

Thermodynamics and Binding Constants. The difference spectra of *A. brasiliensis* were obtained after addition of specific sugars. All four sugars examined, mannose, p-nitrophenyl α-D-glucoside, p-nitrophenyl β-D-

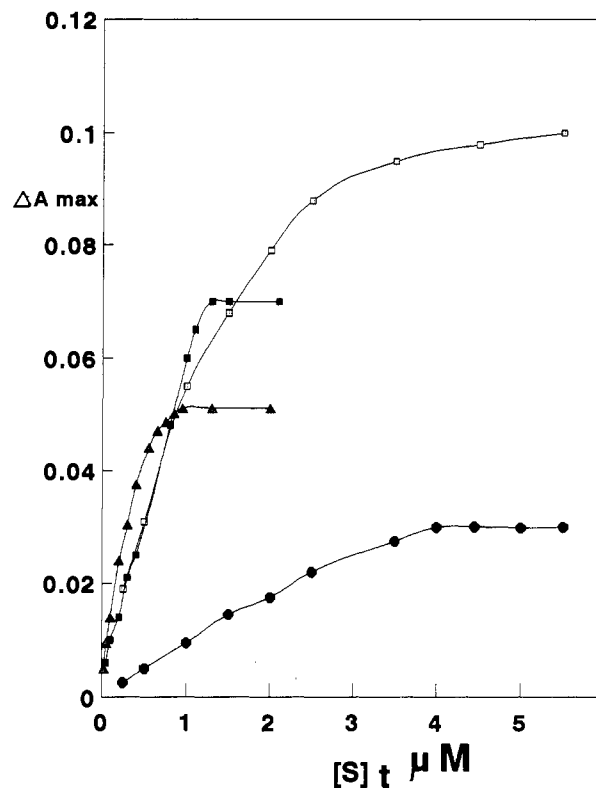**Figure 1.** Spectrophotometric titration of lectin I (436 μg/mL) with mannose (●), p-nitrophenyl α-D-glucoside (▲), p-nitrophenyl β-D-glucoside (■), and p-nitrophenyl α-D-mannoside (□) at 280 nm, 25 °C.

glucoside, and p-nitrophenyl α-D-mannoside, perturbed the UV spectra of the lectins.

In the presence of specific sugars, p-nitrophenyl β-D-glucoside for lectin I and p-nitrophenyl α-D-mannoside for lectin II, the difference spectrum showed a peak in the 278–280-nm region, suggesting that aromatic residues (tryptophanyl and tyrosyl) were perturbed in the interaction. The results of the chemical modification, however, appear to eliminate the direct involvement of tyrosyl residues in binding of carbohydrates in both lectins; on the other hand, acetylation of lectin I and lectin II with N-acetylimidazole resulted in the modification of 80 and 75% of the tyrosyl residues, respectively, without any loss of agglutinating activity, thus showing the importance of tryptophan residues.

These modifications of spectra were observed usually with specific sugars but not with nonspecific ones such as D-galactose, indicating they are due to the specific interaction between lectins and sugars.

The titration curve for binding of the four sugars to lectin I and lectin II, obtained from the difference maximum at 280 nm, for various sugar concentrations at constant lectin concentration was obtained (Figures 1 and 2). The plateau obtained at higher sugar concentration was assumed to correspond to 100% saturation of the protein binding sites with the sugar. The association constants and free energy of reaction were then calculated as mentioned under Materials and Methods, considering a molecular weight of 200 000 for both lectins and the existence of 10 subunits in lectin I and 6 in lectin II (see Table II). The binding of the β-anomer of p-nitrophenyl D-glucoside with lectin I was stronger than that for the α-anomer. For lectin II, in contrast, the α-anomer had a higher binding constant.

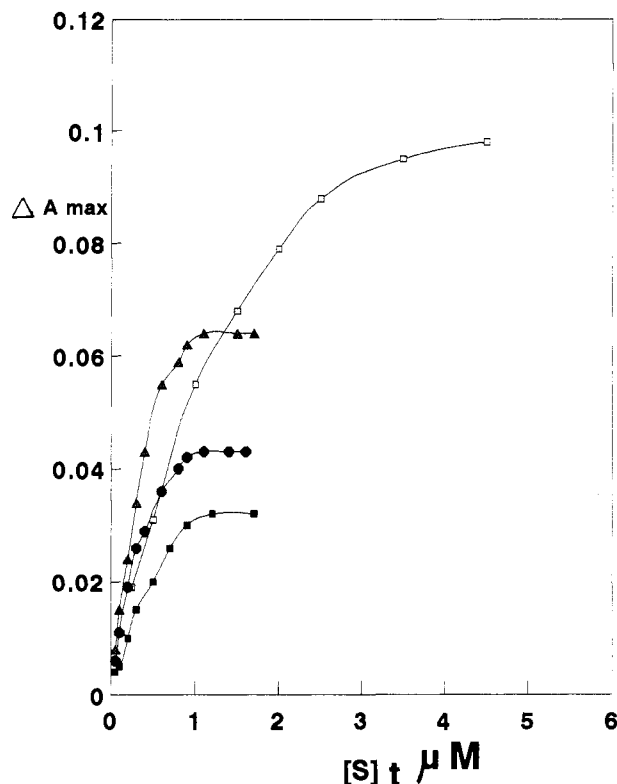The results are in accordance with data we presented previously on the carbohydrate binding capacity obtained

Figure 2. Spectrophotometric titration of lectin II (356 μg/mL) with mannose (●), p-nitrophenyl α-D-glucoside (▲), p-nitrophenyl β-D-glucoside (■), and p-nitrophenyl α-D-mannoside (□) at 280 nm, 25 °C.
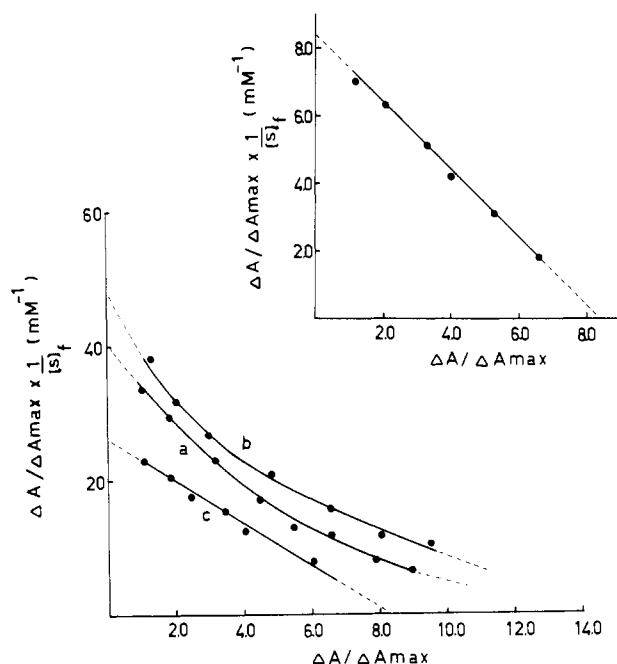

Figure 3. Scatchard plots of $\Delta A/\Delta A_{max} \times 1/[S]_f$ vs $\Delta A/\Delta A_{max}$ by the UV difference spectra method of lectin I at pH 7.2 and 25 °C. (a) p-Nitrophenyl α-D-mannoside; (b) p-nitrophenyl β-D-glucoside; (c) p-nitrophenyl α-D-glucoside. (Inset) D-mannose.

by agglutination inhibition studies (Datta et al., 1991). For lectin I p-nitrophenyl α-D-mannoside and p-nitrophenyl β-D-glucoside were the most potent inhibitors of agglutination, while for lectin II p-nitrophenyl α-D-mannoside was the most potent inhibitor.

The valence of the lectins was determined with the help of Scatchard plots, as shown in Figures 3 and 4 using $\Delta A/\Delta A_{max}$ 1/[S]$_f$ vs $\Delta A/\Delta A_{max}$.
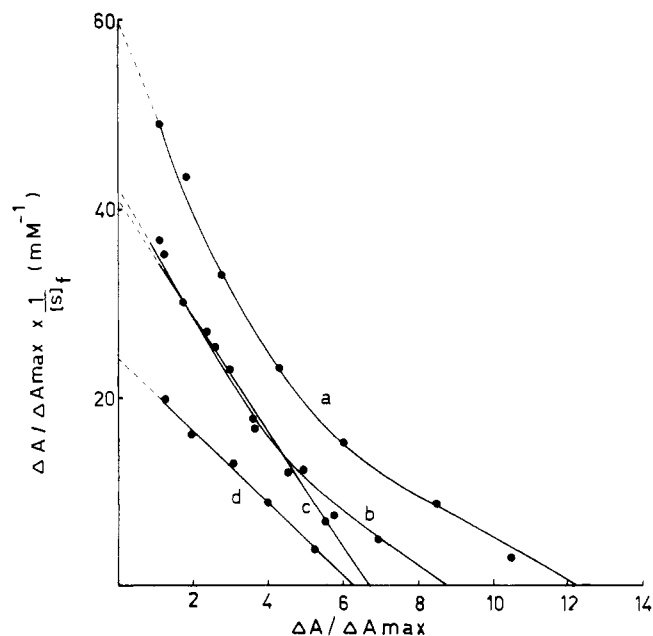

Figure 4. Scatchard plots of $\Delta A/\Delta A_{max} \times 1/[S]_f$ vs $\Delta A/\Delta A_{max}$ by the UV difference spectra method of lectin II at pH 7.2 and 25 °C. (a) p-Nitrophenyl α-D-mannoside; (b) p-nitrophenyl α-D-glucoside; (c) D-mannose; (d) p-nitrophenyl β-D-glucoside.

Table II. Association Constants and Free Energy for the Binding of *A. brasiliensis* Lectin I and Lectin II with Sugars[a]

sugar[b]	$K_a \times 10^{-6}$ (M^{-1})		free energy ($-G°$ KJ mol^{-1})			
	L-I	L-II	L-I	L-II	L-I	L-II
A	1.05	8.34	34.37	39.50	8	6
B	3.31	8.75*	37.21	39.62	8	6
		3.44**		37.31		9
C	5.25*	5.00	38.36	38.24	8	6
	2.14**		36.13		14	
D	4.75*	9.75	38.11	39.89	8	6
	2.08**	2.36	36.06	36.38	12	12

[a] Values are averages of three determinations. *, the value is for primary or high-affinity binding site. **, the value is for secondary or low-affinity binding site. [b] A, mannose; B, p-nitrophenyl α-D-glucoside; C, p-nitrophenyl β-D-glucoside; D, p-nitrophenyl α-D-mannoside.

The nonlinearity of the Scatchard plot is an indication that both lectins seem to have two binding sites, high affinity and low affinity. After binding the high-affinity sites with sugars, the lectins may open the low-affinity site by reorientation of their structures depending upon the sugar affinity.

In lectin I, only a high-affinity binding site was found when mannose and p-nitrophenyl α-D-glucoside were used in the system, although both high-affinity and low-affinity binding sites were observed when p-nitrophenyl β-D-glucoside and p-nitrophenyl α-D-mannoside were used. The highest valency of lectin I was revealed in the presence of p-nitrophenyl β-D-glucoside. It seems that mannose and p-nitrophenyl β-D-glucoside only expose the high-affinity binding sites, whereas p-nitrophenyl α-D-glucoside and p-nitrophenyl α-D-mannoside can produce both high- and low-affinity binding sites in lectin II.

The valency of lectin II was highest in the presence of p-nitrophenyl α-D-mannoside (Table II).

From these experiments it seems that the lectins have one or two binding sites per subunit, a fact that explains the high agglutinating activity of these molecules alone or in the presence of MgCl$_2$ and high molecular weight substances as we observed before (Datta et al., 1991).

Sugar Binding Properties of Pinhão Seed Lectins

J. Agric. Food Chem., Vol. 41, No. 11, 1993 **1855**

Conclusions. It seems that on the basis of chemical modifications, spectral changes, and binding constants it may be suggested that tryptophan, histidine, lysine, and carboxyl groups are involved in sugar binding for lectin I and lectin II.

The high agglutinating activities of both lectins may be explained by the existence of more than one binding site for sugars, high- and low-affinity binding sites per subunit, giving a high valence number. The total number of valencies varies from 6 for lectin II to 14 for lectin I.

ACKNOWLEDGMENT

LITERATURE CITED

Anderson, T. T.; Ebner, K. E. Reaction of the hsitidine of prolactin with ethoxyformic anhydride. *J. Biol. Chem.* **1979**, *254*, 10995–10999.

Ashford, D.; Menen, R.; Allen, A. K.; Neuberger, A. Studies on the chemical modification of potato (*Solanum tuberosum*) lectin and its effect on hemagglutinating activity. *Biochem. J.* **1981**, *199*, 399–408.

Bittiger, H., Schnebli, H. P., Eds. *Concanavalin A as tool*; Wiley: New York, 1975; pp 1–475.

Carraway, K. L.; Koshland, D. E., Jr. Carbodiimide modification of proteins. *Methods Enzymol.* **1972**, *25*, 616–623.

Cermakova, M.; Entlicher, G.; Kocourex, J. Studies on phytohemagglutinins, XXVII A study of the pea lectin binding site. *Biochim. Biophys. Acta* **1976**, *420*, 236–245.

Datta, P. K.; Basu, P. S.; Datta, T. P. Influence of chemical modification and low temperature on the biological properties of *Vicia faba* lectin. *IRCS Med. Sci.* **1984**, *12*, 687–688.

Datta, P. K.; Basu, P. S.; Datta, T. P. Enhancement of lectin erythrocyte agglutination by gums. *Biochim. Biophys. Acta* **1988**, *957*, 164–167.

Datta, P. K.; Figueroa, M. O. R.; Lajolo F. M. Purification and characterization of two major lectins from *Araucaria brasiliensis* syn *Araucaria angustifolia* seed (Pinhão). *Plant. Physiol.* **1991**, *97*, 856–862.

Desai, N. N.; Allen, A. K.; Neuberger, A. Studies on the chemical modification of soybean agglutinin. *Carbohydr. Res.* **1988**, *178*, 183–190.

Dixon, H. B. F.; Perham, R. N. Reversible blocking of amino groups with citraconic anhydride. *Biochem. J.* **1968**, *109*, 312–314.

Habeeb, A. F. S. A. Preparation of enzymatically active water insoluble derivates of trypsin. *Arch. Biochem. Biophys.* **1967**, *119*, 264–268.

Habeeb, A. F. S. A.; Cassidy, H. G.; Singer, S. J. Molecular structural effects produced in proteins by reaction with succinic anhydride. *Biochim. Biophys. Acta* **1958**, *29*, 587–593.

Hassing, G. S.; Goldstein, I. J.; Marini, M. The role of protein carboxyl groups in carbohydrate-concanavalin A interaction. *Biochim. Biophys. Acta* **1971**, *243*, 90–97.

Jordan, F.; Bassett, E.; Redwood, W. R. Proton magnetic resonance studies on wheat germ agglutinin-amino sugar interaction. Evidence for involvement of a tryptophan residue in the binding process. *Biochem. Biophys. Res. Commun.* **1977**, *75*, 1015–1021.

Klotz, I. M. Succinylation. *Methods Enzymol.* **1967**, *11*, 576–580.

Means, G. e.; Feeney, R. E. Reductive alkylation of amino groups in proteins. *Biochemistry* **1968**, *7*, 2192–2201.

Melchior, W. B., Fahrney, D. Ethoxyformylation of proteins. Reaction of ethoxyformic anhydride with α-chymotrypsin, pepsin, and pancreatic ribonuclease at pH 4. *Biochemistry* **1970**, *9*, 251–258.

Neurohr, K. J.; Bundle, D. R.; Young, N. M.; Mantsche, H. H. Binding of disaccharides by peanut agglutinin as studied by ultraviolet difference spectroscopy. *Eur. J. Biochem.* **1982**, *123*, 305–310.

Nonnenmacher, D.; Brossmer, R. Effects of chemical modification on the conformation and biological activity of peanut agglutinin. *Biochim. Biophys. Acta* **1981**, *668*, 149–159.

Patthy, L.; Smith, E. Reversible modification of arginine residues. *J. Biol. Chem.* **1975**, *250*, 557–564.

Privat, J. P.; Lotan, R.; Bouchard, P.; Sharon, N.; Mansigny, M. Chemical modification of the tryptophan residues of wheatgerm agglutinin. *Eur. J. Biochem.* **1976**, *68*, 563–572.

Rice, R. H.; Etzler, M. E. Chemical modification and hybridization of wheat germ agglutinins. *Biochemistry* **1975**, *14*, 4093–4099.

Riordan, J. F.; Vallee, B. L. O-acetyltyrosine. *Methods Enzymol.* **1967**, *11*, 570–576.

Riordan, J. F.; Wacker, W. E. C.; Vallee, B. L. N-acetylimidazole: A reagent for determination of "free" tyrosyl residues of proteins. *Biochemistry* **1965**, *4*, 1758–1765.

Spande, T. F., Witkop, B. Determination of the tryptophan content of proteins with N-bromosuccinimide. *Methods Enzymol.* **1967**, *11*, 498–506.

Spande, T. F.; Green, N. N.; Witkop, B. The reactivity toward N-bromosuccinimide of tryptophan in enzymes. *Biochemistry* **1966**, *5*, 1926–1933.

Trowridge, I. S. Mitogenic properties of pea lectin and its chemical derivates. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3650–3654.

Viswanath, T.; Lawson, W. B. The action of N-bromosuccinimide on chymotrypsin. *Arch. Biochem. Biophys.* **1962**, *93*, 128–130.

Weber, T. H.; Aro, H.; Nordman, C. T. Characterization of lymphocyte-stimulating blood cell-agglutinating glycoproteins from red kidney beans (*Phaseolus vulgaris*). *Biochim. Biophys. Acta* **1972**, *263*, 94–105.