Effect of Chemical Modifications of *Phaseolus vulgaris* Lectins on Their Biological Properties

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Two fractions with agglutinating activity, F-I and F-II, were isolated from Jalo beans (*Phaseolus vulgaris*). The effect of chemical modification on biological activity was studied in fraction F-I. Polyacrylamide gel electrophoresis showed that fraction F-I migrated more slowly to the anode and had higher agglutinating activity and toxicity, whereas fraction F-II migrated more quickly toward the anode and had lower agglutinating activity and toxicity. Both fractions lost their agglutinating activity and maintained their toxicity after modification of amino groups. Tryptophan oxidation with *N*-bromosuccinimide eliminated the agglutinating, mitogenic, and toxic activities of the two fractions.

Keywords: *Chemical modifications; lectins; Phaseolus vulgaris; proteins*

INTRODUCTION

Lectins are proteins or glycoproteins widely distributed in nature. They present a variety of biological properties attributed to their ability to specifically bind to carbohydrates (Liener et al., 1986). These characteristics of lectins permit their use in various types of biological research (Lis and Sharon, 1986). The specificity for sugars of lectins from leguminous plants is due to the conservation of both the topography of the binding site and some amino acids present there, which are directly involved in H bridges and in van der Waals interactions with sugars (Sharon, 1993).

Leguminous plants are an abundant source of lectins that represent the major factor responsible for the toxic effects observed after the ingestion of raw beans (*Phaseolus vulgaris*) (Liener and Wada, 1956; Pusztai, 1987). The toxic action of *P. vulgaris* lectins has been characterized on the basis of the deleterious effects observed on the intestinal wall of rats, which suffers structural disorganization with enterocyte rupture and decreased activity of some enzymes (King et al., 1982; Figueroa et al., 1984; Lafont et al., 1988; Rouanet et al., 1988; Pusztai et al., 1981). These lectins bind to enterocytes, cross the intestinal wall, and reach the circulatory system, provoking a series of toxic effects at the systemic level that have been extensively studied in experimental animals (Pusztai et al., 1983, 1989; Pusztai, 1987). The toxicity of lectins is routinely associated with their binding to cells of the intestinal wall and their resistance to enzymatic hydrolysis. However, tomato (*Lycopersicon esculentum*) lectin binds to the intestinal wall of the rat *in vivo* and is not toxic (Naisbett and Woodley, 1990). On the other hand, there are reports of lectins that do not bind to the intestinal wall and, even so, resist hydrolysis in the intestine and can inhibit the growth of the animals that consume them (Pusztai et al., 1990).

Different approaches have been used to clarify the mechanism of action of lectins at the molecular level, but little progress has been made. Chemical modification of proteins using reagents that are specific for certain amino acids has been widely used to elucidate the structure of the active site of enzymes by observing the changes provoked by these modifications in enzyme activity or kinetics. In the case of lectins, this technique has been little explored for this purpose and, when used, it was only in assays limited to the observation of the red blood cell agglutinating activity or mitogenic activity, without a specific determination of what occurs in terms of the *in vivo* toxic action (Liener and Wada, 1956; Hassing and Goldstein, 1972; Rice and Etzler, 1975; Hammarstrom et al., 1982; Datta et al., 1984, 1993; Yamasaki et al., 1985).

The objective of the present study was to modify some specific lectin residues and to determine the effect of this procedure on their biological action on the basis of red blood cell agglutination and mitogenicity *in vitro* and the ability to inhibit intestinal disaccharidases *in vivo*.

MATERIALS AND METHODS

Jalo bean (*P. vulgaris*) lectins were obtained as described previously (Figueroa et al., 1984). Briefly, a 20% bean flour suspension in 0.85% NaCl was shaken for 12 h and centrifuged at 4068*g* for 20 min. The supernatant, the pH of which was adjusted to 4.2 with 0.5 N HCl, was shaken for 1 h and centrifuged as in the previous step. The pH of the supernatant was increased to 7.0 with 0.5 N NaOH, and a mixture of Celite/bentonite (1:1) at the proportion of 10% of the initial flour weight was added, followed by shaking for 12 h. After a further centrifugation, the supernatant was submitted to fractionated precipitation with ammonium sulfate using the precipitated fraction at 50−75% saturation. The lectins were then purified on DEAE-cellulose as described by Moreira and Perrone (1977), except that we used phosphate buffer, pH 7.2.

Protein concentration was determined according to the method of Lowry et al. (1951), and lectin purification was monitored by polyacrylamide gel electrophoresis according to the method of Davis (1964). Agglutinating activity was determined by plate microtitration using a 2% rabbit red blood cell suspension. The agglutinating titer was determined as the reciprocal of the last dilution at which agglutination was observed. Red cells were previously incubated with 0.1% trypsin at 37 °C for 1 h for activation.

Mitogenicity was determined using spleen cells from normal C_{57}BL/6 mice in RPMI1640 medium (Sigma Chemical Co., St. Louis, MO) containing 10% fetal calf serum (FCS) (Guarandy, Cuiaba, Brazil). Viable cells were placed on flat-bottom

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Table 1. Agglutinating and Enzyme Inhibitory Activities of Fractions F-I and F-II

group[b]	specific agglutng activity	enzyme activity (units·mg^{-1} protein × 10^2) expt 1[a] invertase	expt 1[a] maltase	expt 2[a] invertase	expt 2[a] maltase
control	(−)[c]	2.00 ± 1.07[a]	25.41 ± 9.10[a]	1.40 ± 0.57[a]	21.44 ± 4.13[a]
F-I	21440	0.86 ± 0.39[b]	14.57 ± 5.34[b]	0.69 ± 0.14[b]	8.65 ± 2.02[b]
F-II	320	2.20 ± 0.74[a]	25.32 ± 6.36[a]	0.66 ± 0.46[b]	12.74 ± 4.18[b]

[a] Experiment 1: F-I and F-II were administered at the concentration of 1 mg/mL. Experiment 2: F-I and F-II were administered at different concentrations (F-I = 1 mg/mL; F-II = 10 mg/mL). Means along a vertical column with different superscripts are significantly different, $P < 0.001$. [b] Groups of five animals. [c] Nonagglutinating activity.

microtiter plates, 4 × 10^5 cells per well. Cultures were stimulated with phytohemagglutinin-Difco (PHA-Difco, Difco Laboratories, Detroit, MI) and with fractions F-I and F-II (modified or not with NBS) at the concentrations indicated. The total volume was 200 μL, and culture time was 72 h in a humidified oven at 37 °C in a 5% CO_2 atmosphere. Cultures were pulsed with 0.5 μCi of [^{3}H]thymidine (The Radiochemical Center, Amersham, U.K.) and collected on filter paper 18 h later using a cell harvester. After drying, the paper disks were placed in scintillation fluid and counted with a Beckman LS 100C scintillator. The results are reported as mean counts per minute (± SD) of cultures in triplicate.

Amino groups were modified by acetylation with acetic anhydride in a saturated solution of sodium acetate (Fraenkel-Conrat, 1957). Tyrosine residues may also react; however, in alkaline medium they form highly unstable derivatives. SH groups, also sensitive to reaction, are scarce in Jalo bean lectins. Amino groups were also modified by succinylation with succinic anhydride (Sigma) as described by Habeeb (1967). The number of amino groups was determined using trinitrobenzenesulfonic acid (TNBS, Sigma) (Fields, 1972). Histidine residues were modified with diethyl pyrocarbonate (Sigma), and the reaction was monitored on the basis of increased absorbance at 240 nm according to the method of Mühlrad et al. (1969). It was found that in the proteins treated with diethyl pyrocarbonate at pH 6.0, arginine, lysine, tyrosine, and cystine residues remained unchanged. The data suggested that the hystidyl side chain of the lectins reacted completely and specifically with diethyl pyrocarbonate (Ovádi et al., 1967). Lectin modification with NBS was performed as described by Spande and Witkop (1967), with determination of decreased absorbance at 280 nm. Arginine was modified with cyclohexane-1,2-dione (Pierce Chemical Co., Rockford, IL) in borate buffer, pH 9.0 (Patthy and Smith, 1975). The reaction of tyrosine with *N*-acetylimidazole (Aldrich Chemical Co., Milwaukee, WI) was carried out in 0.01 M Tris buffer, pH 7.5, in the presence of 8 M urea according to the method of Riordan and Vallee (1965). Carboxyl groups were modified using ethyldiaminopropylcarbodiimide (Sigma) and taurine (Sigma) according to the method of Carraway and Koshland (1972). In acidic medium, the protein groups that predominantly react with carbodiimides are carboxyls, sulfhydryls (which are rare in Jalo bean lectins), and tyrosine (more slowly). A control with carbodiimide in the absence of the nucleophile was also carried out in parallel since most side reactions are provoked by carbodiimide.

Lectin toxicity was tested in acute assays using isogenic albino Balb/c mice. This method has been developed in our laboratory and will be published in more detail elsewhere. Briefly, adult animals weighing 16−20 g were fasted from 9:00 a.m. until 5:00 p.m. of the same day, at which time they received the first lectin dose by gavage (0.1 mL/animal, 5 mg/mL, a concentration that was changed in some experiments). At 9:00 a.m. of the following day the animals received another dose identical to the first one and were sacrificed 2 h later. The small intestine was removed and frozen for analysis of intestinal enzymes.

Intestinal disaccharidase activity (maltase and invertase) were determined according to the method of Dahlqvist (1964).

RESULTS

The protein fraction with the highest agglutinating activity against rabbit red blood cells was that precipitated with ammonium sulfate at 50−75% saturation and resolved into two active fractions, denoted F-I and F-II, through a DEAE-cellulose column. The two fractions differed in electrophoretic pattern, agglutinating activity, and *in vivo* toxicity.

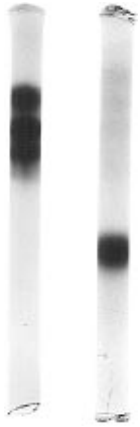

Figure 1. PAGE profile of fractions F-I and F-II isolated from Jalo bean (*P. vulgaris*). Electrophoresis was carried out on 7.5% gels in basic buffer system: lane 1 (left), F-I; lane 2 (right), F-II.

PAGE showed that F-I presented three protein bands that migrated more slowly, while F-II showed only one protein and migrated more quickly to the anode (Figure 1). The agglutinating activity of F-I was much higher than that of F-II (Table 1). F-I was also more toxic than F-II. When the two fractions were administered at low concentration (1 mg of protein/mL), only F-I inhibited the intestinal disaccharidases (Table 1). The toxicity of F-II was demonstrable only when the fraction was used at higher concentrations (Table 1).

The characteristics of the proteins present in fractions F-I and F-II made us believe that they may correspond to different isolectins present in *P. vulgaris* (Felsted et al., 1975, 1977; Pusztai and Watt, 1974). It was also observed through SDS−PAGE that F-II is a dimer with a molecular weight of 75 000 (not shown). Separation of the proteins composing fraction F-I was possible using other purification methods (Bio-Gel P-150), and all three had similar and elevated agglutinating activities. However, the protein yield was insufficient to permit chemical modifications and to test the toxicity from each protein *in vivo* .

Since F-I had higher agglutinating activity and higher toxicity, we focused our attention on it for the chemical modifications. After modification, excess reagent was removed by dialysis in water and in saline solution. The agglutinating activity and the toxicity of this fraction are presented in Table 2.

Modification of the amino groups caused variable losses in the agglutinating activity of the lectins as a function of the number of groups modified, with the possible occurrence of total loss. However, even after a

Table 2. Effect of Chemical Modifications in the Biological Activity of Lectins F-I (Agglutinating Activity and Inhibition of Intestinal Disaccharidases)

modification (modified residue)	% modification[b]	specific agglutng activity	enzyme activity[c] (units·mg^{-1} protein × 10^2)	
			invertase	maltase
control (without lectins)		(−)[d]	3.15 ± 0.65[a]	26.77 ± 6.15[d]
control (with lectins)	0	16168	1.05 ± 0.25[b]	10.87 ± 1.53[e]
acetylation (amino)	65	320	1.14 ± 0.08[b]	13.00 ± 2.26[e]
succinylation (amino)	59	(−)	1.06 ± 0.20[b]	10.89 ± 1.85[e]
taurine (Glu/Asp)	ND[e]	6301	1.17 ± 0.40[b]	12.20 ± 4.05[e]
NBS (Trp)	15	(−)	2.82 ± 0.28[a]	26.80 ± 4.10[d]
NBS/urea (Trp)	60	(−)	2.72 ± 0.42[a]	27.80 ± 5.60[d]
diethyl pyrocarbonate (His)	100	16168	0.75 ± 0.18[c]	5.20 ± 0.52[f]
N-acetylimidazole (Tyr)	75	74472	0.68 ± 0.25[c]	8.43 ± 1.85[f]
cyclohexane-1,2-dione (Arg)	ND	1040	0.43 ± 0.06[c]	6.20 ± 0.93[f]

[a] Similar or different superscripts in a vertical column indicate, respectively, similar or different activities values: $0.01 < P < 0.001$. [b] We consider molecular weight to be 130 000. [c] Groups of five animals. [d] Nonagglutinating activity. [e] ND, not determined.

total loss of agglutinating activity, the lectins still inhibited the intestinal enzymes.

Whereas modification of the carboxyl groups provoked a partial loss of agglutinating activity of the lectins, modification of histidine had no effect and modification of tyrosine increased the agglutinating titer of the lectins about 5-fold. However, none of these modifications changed the effect of the lectins on the intestinal disaccharidases.

Modification of tryptophan with NBS eliminated both the agglutinating and toxic activities of the lectins. On fraction F-I without the presence of urea, 3 mol of tryptophan/mol of lectin was modified; meanwhile, in the presence of 6−8 M urea, 12 mol of tryptophan/mol of lectin was modified. In both cases loss of biological activity of the lectins occurred. After observing the effect of tryptophan modification on fraction F-I, we submitted fraction F-II to the same treatment. In this case, in which modification could be performed only in the presence of urea, 5 mol of tryptophan/mol of lectin was oxidized. The effect on biological activity was similar to that observed in F-I, i.e., loss of agglutinating and toxic activity.

The mitogenic activity of fractions F-I and F-II (modified or not with NBS) is presented in Figure 2. These results are representatives of three assays. Tryptophan oxidation with NBS caused the loss of mitogenicity in both fractions. F-I and F-II presented different optimum doses of stimulation.

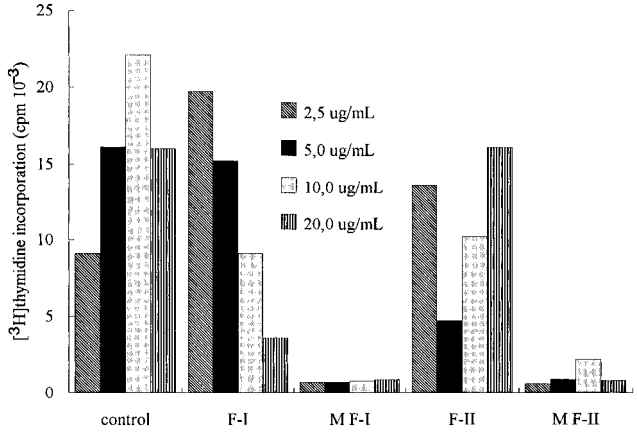

Figure 2. Variation in [^{3}H]thymidine incorporation as a function of dose: PHA-Difco (control), F-I and F-II modified with NBS (M F-I and M F-II), and not modified.

DISCUSSION

By purification of bean (*P. vulgaris*) lectins on a DEAE-cellulose column we obtained two protein fractions with agglutinating activity, which were denoted F-I and F-II (Figure 1). The lectins of *P. vulgaris* usually are isolectins of variable biological activities according to the composition of their subunits (Pusztai and Watt, 1974; Felsted et al., 1975, 1977). Electrophoresis showed that fraction F-I had three protein bands that migrated more slowly to the anode (Figure 1), with greater agglutinating activity and toxicity (Table 1). Fraction F-II corresponded to one protein band (Figure 1), which migrated more quickly to the anode upon electrophoresis and showed lower agglutinating activity and toxicity than fraction F-I (Table 1). However, when we increased the dose of fraction F-II administered by gavage to the animals, the toxic effects of the two fractions became similar (Table 1). This was possibly due to the existence of a similar mechanism of toxic action for the two fractions, with differences only in the number of binding sites encountered by each on the intestinal wall.

Some studies on *P. vulgaris* lectins labeled with ^{125}I have determined that the number of receptor sites for subunits E and L on the intestinal wall of rats varies (Boldt and Banwell, 1985). This may be due to the fact that the oligosaccharide that binds more strongly to each subunit is slightly different (Hammarstrom et al., 1982; Yamashita et al., 1983). The smaller size of fraction F-II determines a lower agglutinating activity and toxicity.

Due to its greater toxicity, fraction F-I was selected for the chemical modifications, with specific reactions for certain amino acids under the conditions previously described in the literature for other proteins. These are moderate reactions that provoke few side reactions in the protein. However, when necessary, we used controls to determine the occurrence of secondary reactions. The modified lectins were dialyzed with saline solution, their agglutinating activities were determined, and they were simultaneously administered to the animals to determine possible changes in their toxicities. Since we did not have a control for the stability of the products of chemical modification, these tests were carried out within the shortest possible time.

The experimental model developed to determine the toxicity of the lectins presents several advantages compared to the animal growth assays commonly used for this purpose. Only a small amount of purified lectin was needed because of the small size of the animals used, a fact that also facilitates handling during administration by gavage. It is also a sensitive and time-

saving procedure, because only 2 days are necessary to detect the toxic action of the lectins on the intestine, while at least 7−12 days is necessary for a routine experiment of growth with rats (Figueroa et al., 1984).

Lectin acetylation with acetic anhydride reached 22%, 65%, and 72% of the amino groups of the protein. This percentage varies as a function of the amount of acetic anhydride added and the duration of the reaction. A progressive loss in agglutinating activity occurred with increasing numbers of modified amino groups, with full disappearance of activity in the presence of 72% acetylated amino groups. A similar effect occurred with amino group succinylation, which reached 22%, 59%, and 81%. However, even with partial or total loss of agglutinating activity, the lectins continued to inhibit intestinal disaccharidases. These results were surprising since the loss of agglutinating activity is usually considered to be a sign of lectin detoxification, whereas in our study the lectins maintained their intestinal toxicity unchanged.

Modification of the amino groups of proteins causes changes in the charge equilibrium of the molecule that probably result in conformational changes possibly responsible for the behavior observed in the agglutinating activity of the lectins. A change in the number of binding sites may occur that affects only the agglutinating activity without affecting the binding to the intestinal wall or the action of the lectins on maltase and invertase. In other words, the number of binding sites needed for agglutination to occur differs from the number needed for action on the intestine.

Acetylation and succinylation of amino groups from F-II had a similar effect to that observed for F-I (data not shown).

Histidine is an important amino acid for the activity of many enzymes, as part of their active site (Feeney, 1987). Diethyl pyrocarbonate, at pH 6.0, acted exclusively on the imidazole group of histidine. The modification was monitored by the increase in absorbance at 240 nm. No change in agglutinating activity occurred. Soybean lectin modified with diethyl pyrocarbonate maintained its agglutinating activity (Desal et al., 1988). The modification of arginine caused a significant loss of agglutinating activity, whereas the modification of tyrosine provoked a significant increase. These modifications possibly caused changes in the lectin conformation, which was reflected by an increase or decrease in the ability to bind to cells. Modification of carboxyl groups provoked a partial loss of the agglutinating activity. Modification of histidine, arginine, tyrosine, glutamic acid, and aspartic acid residues did not cause any changes in lectin toxicity in the intestine. It seems that these residues are not important to the binding mechanism at the enterocytes.

Tryptophan appears to be the most important amino acid for the biological activity of lectins. Its oxidation with NBS causes the loss of both agglutinating and toxic activities. Modification of fraction F-I with NBS occurred both in the absence and in the presence of urea, with only a variation in the number of modified residues. A control carried out to observe the effect of urea showed that the latter did not change lectin activity. Due to the importance of tryptophan for the activity of fraction F-I, we submitted fraction F-II to the same modification and observed a similar behavior, except that modification could be effected only in the presence of urea. This means that the tryptophan groups of F-II are protected by the tridimensional structure of the molecule and become exposed after the disorganization of the tertiary structure of the protein.

The mitogenic activity of F-I and F-II showed that the optimum dose for stimulation differed for the two fractions, which presented a slightly lower activity than that of standard phytohemagglutinin from Difco. Modification of tryptophan caused the loss of mitogenicity in both fractions. When the loss of biological activity is total, it is easier to attribute the responsibility to a given amino acid, as in the case of tryptophan. Furthermore, hydrophobic bonds are known to be important for lectin activity, thus supporting the hypothesis of the importance of tryptophan for the biological activity of lectins.

ABBREVIATIONS USED

DEAE-cellulose, (diethylaminoethyl)cellulose; NBS, *N*-bromosuccinimide; PAGE, polyacrylamide gel electrophoresis; PHA-Difco, phytohemagglutinin- Difco; SDS, sodium dodecyl sulfate; TNBS, trinitrobenzenesulfonic acid.

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