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Purification and Characterization of Two Isolectins with Arginase Activity from the Lichen *Xanthoria parietina*

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Two glycoproteins were purified and biochemically characterized from the lichen *X. parietina*. Both behaved as enzymes with arginase activity and haemaglutinins. Secreted arginase (SA) contained galactose and glucose in the saccharide moiety and an isoelectric point of 4.54. The algal binding-protein (ABP) had N-acetyl-glucosamine and glucose as glycosidic residues and an isoelectric point of 3.53. Both proteins had the same molecular mass (58.6 kDa) and the same qualitative amino acidic composition. The results allowed us to consider these glycoproteins as isolectins, which have significant physiological roles in the relationship between photobiont and mycobiont of symbiotic association.

Keywords: Algal binding-protein, Glycoprotein, Isolectins, Secreted arginase.

Introduction

Lectins constitute a heterogeneous group of glycoproteins of non-immune origin with non-catalytic binding sites which are capable of recognizing and reversibly binding to specific saccharide moieties (Goldstein *et al.*, 1980). Since the nineteenth century, these proteins have been described mainly in dicot (Kamemura *et al.*, 1993; Mandal *et al.*, 1994), but also in monocot (Kilpatrick and Yeoman, 1978; Crowley *et al.*, 1984), bacteria (Heerze *et al.*, 1992; Heerze and Armstrong, 1993), animals (Thiel, 1992), yeasts (Viard *et al.*, 1993), mushrooms (Yoshida *et al.*, 1994) and several symbiotic associations such as *Rhizobium*-legume, mycorrhizae (Díaz *et al.*, 1989) and lichens (Petit *et al.*, 1983, Bubrick *et al.*, 1985). The first report using lichen material showed that these glycoproteins were involved in recognition mechanisms (Lockhart *et al.*, 1978; Bubrick *et al.*, 1985). Lectins have also been considered as recognition like-proteins in other symbiosis (Díaz *et al.*, 1989, Giollant *et al.*, 1993). However, these proteins have also been related to parasitic mechanisms (Saikawa, 1982; Elad *et al.*, 1983) in attack-defense processes on the basis of their antiviral (Kumar *et al.*, 1993) or antifungal (Viard *et al.*, 1993) activity, which is a defense against animal attack (Hoedemaeker *et al.*, 1993), etc.

Biochemical studies revealed that these lectins are very conserved glycoproteins with a high degree of homology (Chrispeels and Raikhel, 1991). Chrispeels and Raikhel (1991) suggested that these proteins, encoded by tandem genes, presumably arose through duplication and divergence of an ancestral gene. They also take part of a protein family involved in plant defense mechanisms.

Although the physiological role of lectins is still subject of controversy, they have often been involved as enzymes in some metabolic pathways. Shannon and Hankins (1981) described certain galactosidases and mannosidases as phytohaemagglutinins. Molina *et al.* (1993) reported for the first time an algal-binding protein from lichens with arginase activity. Moreover, several lectins from plants can act as elicitors for the production of a phytoalexin, as found in *Pisum sativum* (Toyoda *et al.*, 1995). Some are able to bind to other enzymes in order to modify their activity. DNA polymerase a, b and g activities in human lymphocytes are stimulated by *Phaseolus vulgaris lectin* (Umekawa *et al.*, 1992). In other cases, they have been assayed as *in vitro* inhibitors of protein synthesis (Kumar *et al.*, 1993).

On many occasions lectin purification procedures revealed a variability between several isoforms or isolectins with differences in their specific agglutination activity (Petrescu *et al.*, 1993), tertiary structure (Yagi *et al.*, 1993) or post-transductional enzymatic digestion (Hoedemaeker *et al.*, 1994). Molina and Vicente (1995) purified two fungal isolectins with arginase activity from the lichen *Xanthoria parietina* and it showed a different affinity by the cell wall receptor in the phycobiont. One of them, an algal-binding protein (ABP), is almost identical to the protein located at the surface of the fungal cell wall, as described by Bubrick *et al* (1985). The

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second isolectin, however, behaves as a secreted arginase. These isolectins are phycotoxins since they induce a decrease in clorophyll concentration (Molina and Vicente, 1993), ultrastructural alterations (Molina and Vicente, 1996), an increase of the amount of endogenous polyamines (Molina and Vicente, 1995) and glucanase activity in algal cells (Molina *et al.*, 1998a). Further, Molina *et al.* (1998b) described a defense mechanism of algal cells that acted against the supply of fungal lectins, such as synthesis and the secretion of amorphous polysaccharides.

In this paper, the results of the purification and biochemical characterization of these isolectins are reported. These verify the physiological importance of lichen symbiosis.

Material and Methods

Plant material *Xanthoria parietina* (L.) Th. Fr., growing on *Robinia pseudoacacia* L. was collected in Montejo de la Sierra (Madrid). Thalli were air-dried and stored at 4°C in the dark for no longer than two weeks.

Purification of glycosylated arginases (SA) and algal binding protein (ABP) Samples of 15 g of *X. parietina* thalli were floated on 150 ml 40 mM arginine in 10 mM Tris-HCl, pH 9.1, for 1 h at 26°C in the dark (Rodriguez and Vicente, 1991). Secreted arginase (SA) was purified from the incubation media according to Planelles and Legaz (1987) by precipitation with ammonium sulfate at 50% saturation, adsorption on calcium phosphate gel, prepared in the laboratory (SA was desorbed with 220 mM Tris-HCl), and filtration through a Sephadex G-150 column (30 cm x 3 cm I.D.) stabilized with 220 mM Tris-HCl buffer, pH 9.1. The algal-binding protein (ABP) was pre-purified from recently collected thalli according to Bubrick *et al.* (1985) by two successive precipitations with ammonium sulfate (40% and 60% saturation, respectively) and later purified as above (Planelles and Legaz, 1987). ABP was desorbed from hydroxyapatite with 180 mM Tris-HCl. To test the homogeneity of both proteins 150 ml of the corresponding solution, containing about 6.0 mg protein, were mixed with 75 ml of aqueous glycerol (v/v) and applied onto 12% polyacrylamide gels (SDS-PAGE). The running buffer was 50 mM Tris-Glycine, pH 8.3, and at this pH the current generated at 180 V was about 25 mA at 4°C after equilibration. Staining was performed by using $AgNO_3$. Carbonic anhydrase (29 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa) were used as standards.

Arginase activity was measured by coupling crystalline urease to purified proteins in reaction mixtures containing arginine as a substrate (Legaz and Vicente, 1982). Ammonia production was measured by the method of Conway (Conway, 1962). Protein was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Amino acid analysis Both glycoproteins (80 µg) were used to discover the aminoacidic composition. In parallel, 80 µg and 250 µg of egg lysozyme were prepared as controls. Proteins were hydrolyzed at 108°C for 2 h with 6.0 N HCl containing 0.1% (w/v) phenol in flame seated ampoulles. Hydrolysates were analyzed on a Beckman System 6300 (System Gold) amino acid analyzer (Gavilanes *et al.*, 1982).

Cys moieties were estimated by oxidation with performic acid according to Hirs (Hirs, 1967). Tryp moieties were determined by spectrophotometric measurement at 294.4 and 280 nm (Beaven and Holiday, 1952).

Measurements of fluorescence spectra Absorbance spectra were recorded by using a dual-beam Varian DMS 90 spectrophotometer. Fluorescence spectra of isolectins were determined by using a SFM-25 Kontron spectrofluorimeter equipped with quartz cuvettes of 1 cm path-length. The wavelength of the exciting light was always 275 nm.

Capillary zone electrophoresis High Performance Capillar Electrophoresis (HPCE) was performed using the Spectraphoresis 500 system from Spectra-Physics. Microbore fused silica tubing coated with polyimide of 70 mm I.D. and 360 mm O.D. were used with a total length of 70 cm and the separation length of 63 cm. The capillary was enclosed on a cassette for easy handling. On line-detection was performed with a variable-wavelength UV-Vis absorbance detector of 6 nm bandwidth (Spectra-Physics). Detection of proteins was monitored at 280 nm and electropherograms were recorded using a SP 4290 integrator (Spectra-Physics).

A recently purchased capillary was first conditioned with 0.5 N NaOH for 5 min at 60°C, 0.05 N NaOH for 5 min at 60°C and bidistilled-deionized, filtered water for 5 min at 60°C. Then, the equilibration of the capillary was performed by washing it with 25 mM sodium carbonate buffer, pH 10, for 10 min at 25°C. After this, the capillary was washed again with the same buffer for 10 min at 25°C under 20 kV of applied voltage. This buffer system was chosen in order to produce a pH value that was higher than the isoelectric point (pI) of the protein to be separated. This renders the protein negatively charged, resulting in repulsion from the charged fused-silica capillary walls and thereby minimizing adsorption. Regeneration of the capillary surface between runs was performed by rinsing it with the following sequence: 0.05 N NaOH for 3 min, bidistilled-deionized water for 5 min and a corresponding conditioning buffer for 3 min. The buffer used as a electrolyte was a 25 mM sodium carbonate buffer, pH 10 (Legaz and Pedrosa, 1993).

Protein solutions, prepared in the electrophoresis buffer and diluted three times, were injected into the capillary by siphoning for a fixed time of 8 s. Benzol at 4% (v/v) was diluted in the same buffer and was used as neutral marker. A voltage of 20 kV was applied using positive-to-negative polarity. During electrophoresis, the temperature control was employed as indicated. The isoelectric points were calculated according to Pedrosa and Legaz (1995).

Acidic hydrolysis of lectins and sugar extraction Samples of 41.0 mg of purified lectins were hydrolyzed with 0.5 ml 6 N HCl for 2 h at room temperature (Legaz *et al.*, 1990). Mixtures were air dried and the residues were dispersed in 1.0 ml of cold 80% (v/v) ethanol and stored at -13°C for 14 h. The precipitates were then discarded and the supernatants heated at 60°C for 20 min. To these supernatants, 1.0 ml of 80% cold ethanol was added and then heated again until they were dry. This procedure was

repeated three times under the same conditions as above. The last residues were reconstituted with 1.0 ml of cold 80% ethanol and centrifuged at 3,000 × g for 15 min (Legaz *et al.*, 1985). Supernatants were analyzed for sugar content according to Dubois *et al.* (1956) and qualitatively analyzed by HPLC (High Performance Liquid Chromatography).

HPLC separation of sugars HPLC was performed on a Varian Model 5060 liquid chromatograph equipped with a SpectraSystem UV2000 detector (SpectraPhysics) and a Vista CDS 401 (Varian) computer, according to Molina and Vicente (1996). The chromatographic conditions were as follows: column, MicroPack NH_2 10P/N (30 cm × 3 mm I.D.) from Varian: sample loading, 10 ml; mobile phase, acetonitrile-water (80:20, v/v) isocratically; flow rate, 1.0 ml min^{-1}; temperature, 20°C; detector UV (195 nm), 0.005 a.u.f.s.; attenuation 64; internal standard, 2.0 mg ml^{-1} ribitol (retention time = 6.42 min). N-Acetyl-D-galactosamine, N-acetyl-D-glucosamine, fructose, fucose, galactose, glucose and mannose (2 mg ml^{-1}) from Sigma Chemical Co. were used as external standards.

Phytohaemagglutinin assay Two glass slides containing 30 ml of recently extracted human B- blood were placed under a light microscope and treated with 30 µl of saline solution (Lockhart *et al.*, 1978), or either a 30 µl ABP or SA solution containing 0.37 mg protein ml^{-1}. Haemagglutination was observed for 5 min at 25°C.

Results

Thallus samples were floated on a 40 mM arginine in 10 mM Tris-HCl for 8 h in dark or in white light (200 mmol m^{-2} s^{-1} at the level of plants) at 26°C. The activity of the secreted arginase was measured in an incubation media. A maximum of secretion in light was found at the 2 h incubation (17.02 units) and decreased later (Fig. 1). However, a maximum of arginase activity was recovered from the medium after 1 h of thalli incubation in the dark (26.57 units). These media were

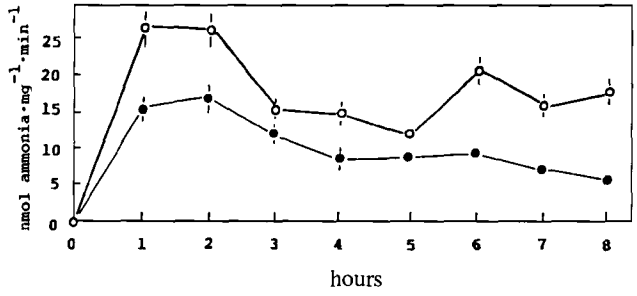

Fig. 1. Time-course of arginase activity secreted from thallus. *X. parietina* thallus incubated on 40 mM L-arginina in 10 mM Tris-HCl buffer (pH 9.15) in dark (○) or in white light (●). Values are the mean of the three replicates. Vertical bars give standard error where larger than the symbols.

then used to purify secreted arginase. ABP was purified from recently collected lichen thalli of about 73-fold with an overall yield of 5% (Table 1). Secreted arginase was purified from the incubation media 66-fold with an overall yield of about 4% (Table 2). To determine the homogeneity of these proteins, SDS-PAGE electrophoresis was performed. The samples were resolved in only one band, respectively, with a molecular mass of about 58.6 kDa (Fig. 2). There was no visible haemagglutination in the blood samples mixed with the saline solution (Fig. 3A and B). However, haemagglutination was found during the first minutes in those samples mixed with partially purified ABP. Erythrocytes move towards the first agglutinated nodules. These increase in complexity and their size seemed a function of time, as can be observed in Fig. 3C, corresponding to 5 min after ABP addition. Results for the SA haemagglutination assay are shown in Fig. 3D.

Data obtained from the analysis of both isolectins by capillary electrophoresis revealed that they are acidic proteins. The isoelectric point of ABP was determined as 3.53, whereas that of SA was 4.54 (Fig. 4). Both isolectins showed some similarities in their fluorescence spectra when the wavelength

Table 1. Purification of intrathalline arginase, ABP

Step	Vol. (ml)	Protein (mg ml^{-1})	Total protein (mg)	Specific activity (units)	Total activity (units)	Yield (%)	Purification (-fold)
Cell-free extract	135	0.42	56.70	1.60	90.72	100	---
Supernatant from 40% saturation $(NH_4)_2SO_4$	144	0.08	11.52	2.92	33.64	38.28	1.82
Pellet from 60% saturation $(NH_4)_2SO_4$	60	0.06	3.60	4.73	17.03	18.77	2.96
140 mM eluate from hydroxyapatite	15	0.03	0.45	37.65	16.94	18.67	23.53
Eluate from Sephadex G-150	5	0.008	0.04	117.43	4.70	5.18	73.39

Table 2. Purification of secreted arginase, SA

Step	Vol. (ml)	Protein (mg ml^{-1})	Total protein (mg)	Specific activity (units)	Total activity (units)	Yield (%)	Purification (-fold)
Cell-free extract	135	0.29	39.15	2.60	101.79	100	--
Supernatant from 50% saturation $(NH_4)_2SO_4$	150	0.09	13.50	7.13	96.25	94.5	2.74
180 mM eluate from hydroxyapatite	100	0.003	0.30	54.20	16.26	15.97	20.84
Eluate from Sephadex G-150	5	0.005	0.025	171.60	4.29	4.21	66.00

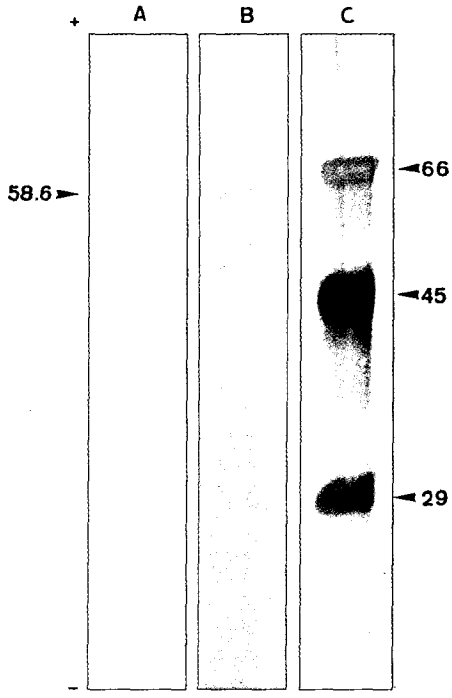

Fig. 2. PAGE of purified isolectins. ABP (lane A) and SA (lane B) indicated molecular weight with arrow. Molecular standards, carbonic anhydrase (29 kDa), egg albumin (45 kDa), and bovine serum albumin (66 kDa) are indicated with arrows (line C).

of the excitation light was 257 nm. ABP had a net maximum of fluorescence emission at 417.5 nm, a secondary maximum at 347.5 nm and a shoulder at 292.5 nm (Fig. 5A). SA also had two maxima of fluorescence emission. The main one was at 417.5 nm, whereas the secondary one was displaced at 357.5 nm. The fluorescence spectrum of SA also showed two shoulders at 445 nm and 300 nm (Fig. 5B).

The amino acid composition of both isolectins was analyzed after the acidic hydrolysis of purified SA and ABP. Both isolectins contained high amounts of Asx and Glx, although the frequency of the occurrence of Gly and Ser was in the same order or higher. Quantitative differences in the amino acid composition of both isolectins mainly affected Gly and Phe, the abundance of which was higher for ABP than for SA (Table 3). The frequency of occurrence of Met, Phe, Tyr and Ileu was very small, similar to that found for arginase isoforms of *Evernia prunastri* that contained, in contrast, more Pro than was found for ABP and SA (Legaz and Pedrosa, unpublished). The quantitative composition of the glycosyl rest was analyzed by HPLC after the acidic hydrolysis of both proteins. The rest of ABP contained equimolar amounts of both N-acetyl-D-glucosamine and glucose, whereas SA contained galactose and glucose (Table 4).

Discussion

An algal-binding protein (ABP) that developed arginase activity and a secreted arginase (SA) has been purified by size exclusion chromatography and ion exchange chromatography. Incubation of *X. parietina* thalli for 1 h in 28 mM arginine at 26°C in the dark seemed to be the best condition in which the highest yield of enzyme was recovered (Fig. 1). These results differ from those used to produce secretion of arginase from *Evernia prunastri* thallus, which requires 8 h of incubation on arginine (Planelles and Legaz, 1987). On the other hand, it is important to report that these glycoproteins tend to aggregate after elution from Sephadex, especially ABP. This particular behavior of the oligomer formation during the process of purification has been described by Sharon and Hankins (1981) for lectins and by Ikamoto *et al.* (1990) for arginases.

Both ABP and SA agglutinate human erythrocytes from the B- group (Fig. 3). Thus, they are considered as isolectins. They also have the same molecular weight. The isoelectric point of both isolectins from *X. parietina* has been determined by capillary electrophoresis, about 3.53 for ABP and 4.54 for SA. Arginase isoforms from the lichen *E. prunastri* also behave as acidic proteins (Pedrosa and Legaz, 1995) as well as some other plant lectins (Yagi *et al.*, 1994). The different isolectric points of isolectins have been explained as a consequence of the variation of only one amino acid (Mandel *et al.*, 1994). However, the analysis of the amino acid composition reveals that this is almost identical for both glycoproteins. This shows a high homology in the amino acid percentage (Table 3) and a relatively high content in acidic and polar amino acids. This is in agreement with the results obtained for many other plants (Mo *et al.*, 1993; Yagi *et al.*, 1993; Yagi *et al.*, 1994; Yoshida *et al.*, 1994) and even for animal lectins. However, the amount of both acidic and polar amino acids does not remain constant (Yamashita *et al.*, 1992; Kamemura *et al.*, 1993). The difference observed in the isoelectric point for both glycoproteins (Fig. 4) could be explained as a consequence of different saccharide moiety (Table 4). A fluorescence maxima at 300 nm, 320 nm and 350 nm for both ABP and SA reveal the occurrence of Phe, Tryp and Tyr, respectively (Fig. 4). The displacement of the emission maximum from 347.5 nm for ABP to 357.5 nm in SA indicates an externalization of Tyr from the tertiary structure, whereas a shoulder at 445 nm could be interpreted as the neighborhood of several fluorophores (mainly Tryp) in the same domain of the tertiary structure (Campbell and Dwek, 1984).

The concept of isolectin is fundamentally the consequence of the protein heterogeneity during the last steps of lectin purification. Although a definitive definition of isolectins has not been established, some authors consider two proteins as isolectins after proving their haemagglutinin activity (Yoshida *et al.*, 1994) and after verifying a high degree of homology in their amino acid composition, probably as a consequence of a differentiation from an ancestral gene (Chrispeels and Raikhel, 1991). Frequently isolectins specifically bind the same receptor on the basis of identical glycosidic moiety. Yamaguchi *et al.* (1993) reported that two galactose-specific isolectins from

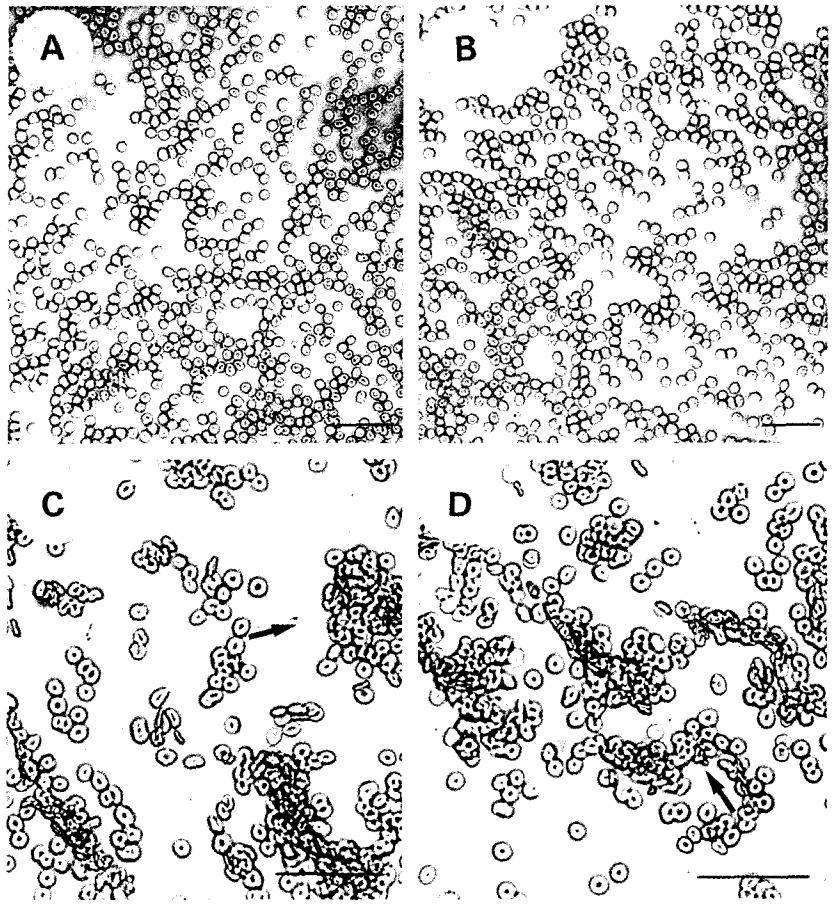

Fig. 3. Haemagglutinin activity of ABP and SA from *X. parietina* on B- human erythrocytes. (A) and (B) controls in saline solution. (C) Haemagglutination 5 min after adding ABP (C) or SA (D) solution. Arrows indicate agglutination nodules. Bar = 10 mm.

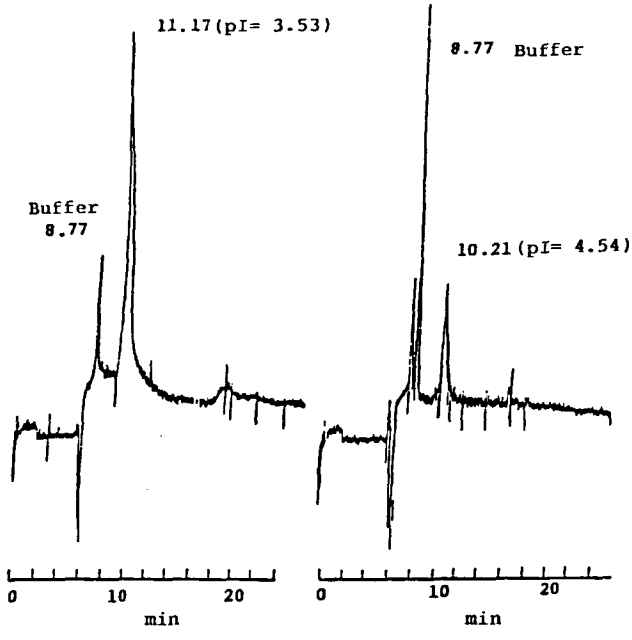

Fig. 4. HPCE profiles of ABP (A) and SA (B) with detection at 200 nm. Isoelectric points were calculated according to Pedrosa and Legaz (1995).

Erythrina variegata do not have structural differences in their sugar chains. However, Yagi *et al.* (1994) described isolectins with different specificity for the same receptor. The acidic digestion of both ABP and SA, as well as the HPLC analysis of the digestion products, reveals that SA contains a glycosidic rest composed by D-galactose and D-glucose. ABP, however, contains N-acetyl-D-glucosamine and D-glucose

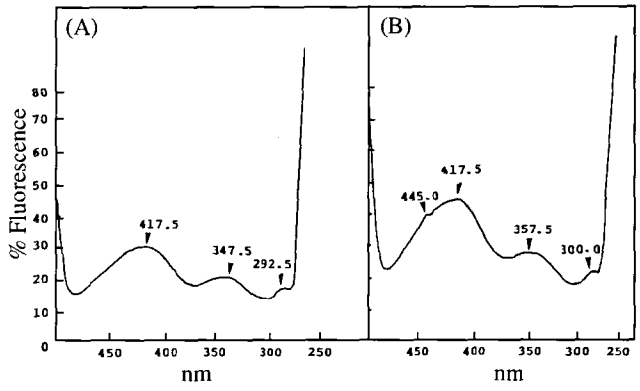

Fig. 5. Fluorescence spectra of isolectins. ABP (A) and SA (B) between 270 and 500 nm. Excitation wave length 275 nm.

Table 3. Amino acid composition of purified ABP and SA

Amino acid	ABP		SA	
	nmol	%	nmol	%
Cys*	1.20	1.25	0.87	1.13
Asx	8.78	9.15	7.63	9.88
Thr	3.26	3.40	2.75	3.56
Ser	11.38	11.86	11.26	14.58
Glx	14.92	15.55	13.93	18.04
Pro	2.30	2.40	1.86	2.41
Gly	30.05	31.32	17.29	22.39
Ala	6.08	6.34	5.51	7.13
Val	2.68	2.79	2.10	2.72
Met	0.15	0.16	0.13	0.17
Ile	1.32	1.38	1.12	1.45
Leu	1.95	2.03	1.62	2.10
Tyr	1.23	1.28	0.83	1.07
Phe	0.98	1.02	0.26	0.34
His	4.34	4.52	4.85	6.28
Lys	2.08	2.17	2.22	2.87
Arg	3.23	3.37	3.00	3.99
Tryp**	0.51		0.38	

*Estimated as cysteic acid.
**Spectrophotometrically measured, according to Beaven-Holiday method (1952).

Table 4. Quantitative composition of the glycosyl rest of both ABP and SA, determined by HPLC of the corresponding acidic hydrolysates

Sugar	Residues by molecule of	
	ABP	SA
Glucose	16	11
Galactose	--	27
N-acetyl-D-glucosamine	16	--

(Table 4). The occurrence of D-galactose in SA is very interesting in explaining the binding abilities of this secreted lectin (Molina and Vicente, 1995). The binding of SA to the cell wall ligand seems to be stronger than that observed for ABP, since SA easily displaces ABP from the phycobiont cell wall. ABP, however, removes SA from this structure in a smaller extent (Molina and Vicente, 1995). In addition, SA largely enters algal cells when they have not induced its lectin ligand. The change of N-acetyl-D-glucosamine by galactose in the SA molecule can be used to explain the highest affinity of this lectin for the cell wall ligand, since this ligand, revealed as a cell wall urease, contains a large amount of galactose (Pérez-Urria and Vicente, 1989). The consistence or inconsistency of the specific binding seems to be mediated by the occurrence of galactose in the lectin, which binds to a galactose-containing a cell wall ligand. Nagai and Yamaguchi (1993) and Nagai *et al.* (1993) suggest that the glycosyl moiety of lectins is very important in relation to the intramolecular folding, and describe how non-glycosylated proteins lose their agglutinant activity. In addition, SA from *E. prunastri* greatly differs from this purified form of *X. parietina* since its glycosyl moiety is composed of glucose, mannose, and fructose (Planelles and Legaz, 1987).

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