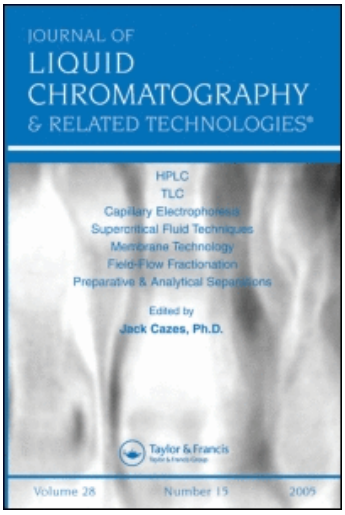

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC CHARACTERIZATION OF TWO LICHEN LECTINS WITH ARGINASE ACTIVITY DIFFERING IN THEIR GLYCOSYL MOIETY

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ABSTRACT

Two isolectins from the lichen *Xanthoria parietina*, one of them retained by the thallus and another secreted from the thallus to the medium, both showing arginase activity, have been purified to homogeneity. Ethanol-soluble products obtained after acidic hydrolysis of both proteins have been analyzed by HPLC under isocratic conditions using acetonitrile-water (80:20, v/v) as mobile phase. Detection is performed by measurement of UV absorbance at 195 nm, using a highly sensitive detector. Purification of standard sugars by filtering aqueous solutions through an activated alumina column is absolutely required. The glycosyl moiety of secreted arginase is composed by galactose and glucose whereas that of thalline enzyme contains N-acetyl-D-glucosamine and glucose.

INTRODUCTION

Plants and animals are able to synthesize lectins responsible for several cell surface interactions.[1,2] Many of these glycoproteins are enzymes that act like lectins[2,3] and even several isoforms of only one protein, differing in their glycosyl moiety, are able to bind to the same receptor in the cell surface. The identification and classification of animal proteoglycans is based on the structure of the glycosaminoglycan moiety,[2] which can be studied by analyzing the unsaturated disaccharide units that are produced by controlled, partial hydrolysis with a particular enzyme, such as chondroitinases ABC and AC[4,5] or *Streptomyces* hyaluronidase.[6] Disaccharides obtained after enzymatic digestion are usually separated by HPLC in isocratic mode on NH_2 columns, monitoring the analytes by absorbance at 232 nm.[4-6]

These separations have been improved by Numura et al.[7] by using a TSK Gel Amide 80 column which avoids the overlapping of several peaks corresponding to different disaccharides. Recently, the analysis of these oligosaccharides has been performed by capillary electrophoresis as pyridylaminated isomaltooligosaccharide[8] or 1-phenyl-3-methyl-5-pyrazolone derivatives.[9] The sodium borate buffer used in these analyses is proposed to play a key role in the separation by preferentially complexing with the diols of specific carbohydrate moieties[10] on the corresponding glycoprotein.

Lichenized fungi produce lectins which have been related to the recognition of the compatible algal partner.[11,12] Some of these lectins act as specific enzymes, such as glycosylated arginase from *Xanthoria parietina*.[13] However, lichen arginase can be produced as several different isoforms differing in both amino acid composition or glycan moiety and, in addition, they can be retained by the thallus or secreted to the medium.[14]

In this paper, we study two different isolectins of the lichen *X. parietina* by analyzing their sugar composition by HPLC after total hydrolysis of their glycosyl residues.

MATERIALS

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, no longer than two weeks.

Reagents

Sugars (D-fructose, D-fucose, D-glucose, D-galactose and D-mannose), amino sugars (D-glucosamine, N-acetyl-D-glucosamine and N-acetyl-D-galactosamine) and ribitol were provided from Sigma (St. Louis, MO, USA). Acetonitrile (HPLC grade, Carlo Erba, Milan, Italy) was used as received and doubly distilled water was filtered through Millipore GS filters (0.22 μm pore diameter) before use.

A Micropack SP NH_2-5 column (Varian, Palo Alto, CA, USA), packed with 10 μm spherical silica which has a chemically bounded phase containing aminopropyl groups and supplied in hexane for use in normal phase, was prepared for sugar analysis in reversed-phase mode.[15]

Acrylamide 2x from Serva (Heidelberg, Germany) and N,N'-methylene-*bis*-acrylamide from Sigma were used for PAGE, employing N,N,N',N'-tetramethylethylenediamine (Sigma) and ammonium persulfate (E. Merck) as polymerizing agents. Size-exclusion chromatography on column was achieved by using Sephadex G-150 from Pharmacia (Uppsala, Sweden).

Glucose oxidase, galactose oxidase, and horseradish peroxidase from Sigma were used to identify glucose and galactose. Fetuin was also obtained from Sigma. Other chemicals were obtained from E. Merck (Darmstadt, Germany).

METHODS

Purification of Glycosylated Arginases

Samples of 15 g of *X. parietina* thalli were floated on 150 mL 10 mM Tris-HCl, pH 9.1, for 1 h at 26°C in the dark. Secreted arginase (SA) was purified from the incubation media according to Planelles and Legaz[16] by precipitation with ammonium sulfate at 50% saturation, adsorption on calcium phosphate gel (SA was desorbed with 220 mM Tris-HCl) and filtration through a Sephadex G-150 column (30 x 3 cm I.D.). The algal-binding protein (ABP) was pre-purified from recently collected thalli according to Bubrick et al.[11] by two successive precipitations with ammonium sulfate (20% and 40% saturation, respectively) and later purified as above(16). ABP was desorbed from hydroxyapatite with 180 mM Tris-HCl. To test the homogeneity of both proteins, 150 μL of the corresponding solution, containing about 6.0 μg protein, were mixed with 75

μL of aqueous glycerol (v/v) and applied onto 12% polyacrylamide gels. The running buffer was 50 mM Tris-glycine, pH 8.3 and, at this pH, the current generated at 180 mV was about 25 mA at 4°C after equilibration.

Acidic Hydrolysis and Sugar Extraction

Samples of 4.0 μg of purified lectins were hydrolyzed with 0.5 mL 6 N HCl for 2 h at room temperature.[16] Mixtures were dried in air flow 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 to dryness.

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 3000*g* for 15 min.[17] The supernatants were loaded into the chromatographic column.

Samples of 40 μg fetuin (a glycoprotein of well defined monosaccharide composition) were hydrolyzed in the same way with 5.0 mL 6 N HCl in order to confirm that the sugars from lectins have been quantitatively released without destruction by HCl. Glycidic moiety of fetuin is composed of galactose, mannose, glucosamine, galactosamine and sialic acids,[18] such as N-acetylneuraminic acid to which a residue of N-acetylgalactosaminitol is linked.[19]

Purification of Standards

Samples of 5.0 mL of solutions standards in doubly distilled, filtered water, containing 40 mg of sugar, were applied onto a column of activated alumina (11 cm x 1.0 cm I.D.). The samples were eluted with doubly distilled, filtered water. Fractions of 5.0 mL volume were monitored for the sugar content according to Dubois et al.[20] The fraction containing the highest amount of the corresponding sugar was dried in air flow, redisolved in 1.0 mL 80% ethanol and analyzed by HPLC.

Purified fractions of both glucose and galactose were identified by action of specific enzymes. Aliquots of 0.5 mL of the fraction containing the highest amount of the corresponding monosaccharide were incubated for 30 min at 30°C with 1.0 mg of glucose oxidase[21] or 1.0 mg of galactose oxidase,[22] 0.5 mg peroxidase and guaiacol. Increase of absorbance was measured at 475 nm.

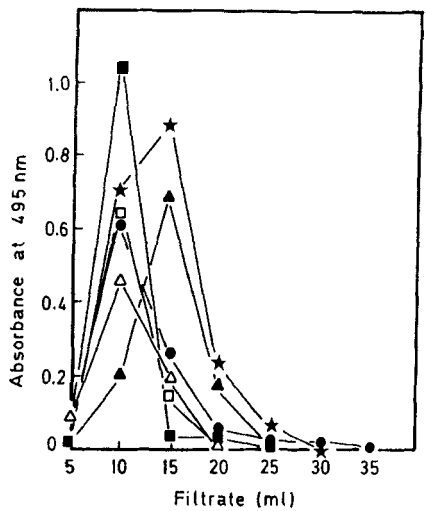

Figure 1. Elution profiles of standard monosaccharides, glucose (filled circles), fructose (empty squares), glucosamine (filled squares), mannose (empty triangles), galactose (filled triangles), and fucose (filled stars) filtered through an alumina column.

Table 1

Retention Times of Standards Filtered Through an Alumina Column

Standard	Retention Time (min)
N-Acetylgalactosamine	3.24
N-Acetylglucosamine	3.04
Glucosamine	3.65
Galactose	2.86
Glucose	4.93
Fructose	3.26
Fucose	2.70
Mannose	3.49
Glucuronic Acid	2.03

HPLC Separation of Sugars

HPLC was performed with a Varian Model 5060 liquid chromatograph equipped with a SpectraSystem UV2000 detector (SpectraPhysics, Fremont, CA, USA) and a Vista CDS 401 (Varian) computer. The chromatographic conditions were as follows: column, MicroPack NH_2 10P/N (30 cm x 3 mm I.D.) from Varian: sample loading, 10 μL; 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).

Quantitation was performed by injecting different amounts of standards after filtration through the alumina column, estimated according to Dubois et al.[20] Indirect calibration was achieved by using ribitol as internal standard.

RESULTS

ABP was purified about 110-fold with an overall yield of 27.8%. SA was purified about 67-fold with an overall yield of 31.7%. Each purified protein gave only one anodic band in PAGE. These proteins, purified at homogeneity, were subjected to complete acidic hydrolysis and ethanol-soluble fractions were extracted.

Elution of some standards from alumina column was carried out by using twice distilled, Millipore-filtered water. Results are shown in Fig. 1, and summarized in Table 1. Fructose, glucose, mannose and glucosamine eluted at 10 mL filtrate (fraction 2) whereas galactose and fucose eluted at 15 mL filtrate (fraction 3). Fractions containing the highest amount of the corresponding monosaccharide were used to be chromatographed by HPLC. Figs. 2 and 3 show two examples of the purity of commercial standards.

Standard galactose (Fig. 2A) was resolved into two main peaks with retention time values of 2.82 min and 3.25 min., respectively. However, after filtration through the alumina column, fraction 3, eluted at 15 mL filtrate, yielded only one peak with a retention time of 2.86 min (Fig. 2B). The contaminating substance could be fructose (retention time 3.26 min) or N-acetyl-D-galactosamine (retention time 3.24 min). An aliquot of this fraction 3 was incubated with 1.0 mg of galactose oxidase for 30 min at 30°C and the formation of the corresponding D-galacto-hexodialdose was confirmed. Standard glucose was resolved into three main peaks at 2.05 min, 3.10 min and 4.87 min, respectively (Fig. 3A). After filtration through the alumina column, fraction 2, filtered at 10 mL, was resolved as only one peak at 4.93 min (Fig. 3B).

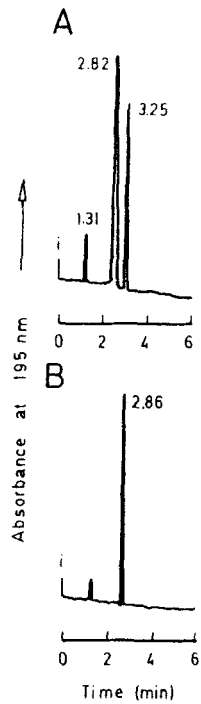

Figure 2. HPLC elution profiles of standard galactose (A) before and (B) after filtration through an activated alumina column. Number near the peak indicates retention time in min.

The contaminating substances were identified as N-acetyl-D-glucosamine (retention time 3.04 min) and glucuronic acid (retention time 2.03 min). This fraction 2 behaved as a very good substrate for glucose oxidase, since formation of gluconic acid was confirmed. Standards of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine were revealed as pure substances in HPLC.

Table 1 shows the retention time values for the different standards after their filtration through the alumina column. The peak with a retention time of about 1.3 min was always identified as acetonitrile which individualized from that contained in the mobile phase after sugar dilution. Figure 4 shows the chromatographic traces of an acidic hydrolysate of pure ABP.

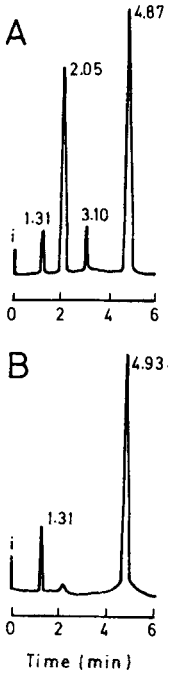

Figure 3. HPLC elution profiles of standard glucose (A) before and (B) after filtration through alumina.

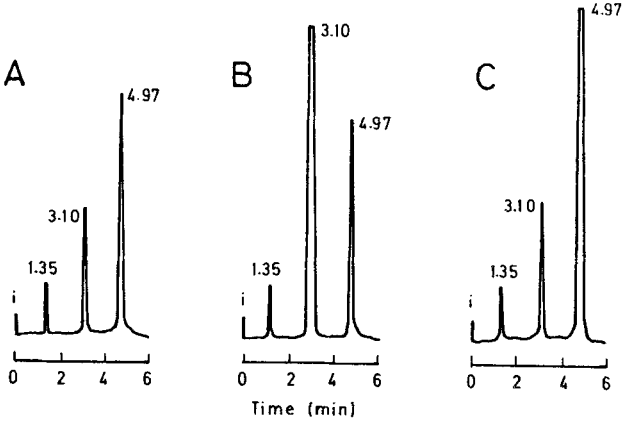

Figure 4. HPLC elution profiles of (A) ethanol-soluble fraction obtained from ABP lectin hydrolysate, (B) the same sample loaded with N-acetyl-D-glucosamine and (C) loaded with glucose.

Two main peaks were detected, with retention time values of 3.10 min and 4.97 min, respectively (Fig. 4A). After loading with the different filtered standards, the peak at 3.10 min only quantitatively increased after loading the sample with standard N-acetyl-D-glucosamine (Fig. 4B) whereas that at 4.97 min only increased after loading the sample with glucose (Fig. 4C).

Figure 5 shows the chromatographic traces of an acidic hydrolysate of secreted arginase. Two main peaks were revealed, with retention time values of 2.94 min and 4.98 min, respectively (Fig. 5A). This last peak quantitatively increased after loading the sample with standard glucose (Fig. 5B). However, two well defined peaks appeared after loading the sample with standard N-acetyl-D-glucosamine (t_R = 3.10 min), one of them at 2.92 min and another at 3.14 min (Fig. 5C). The quantitative increase of the original peak at 2.94 min was only achieved by loading the sample with standard galactose (Fig. 5D). Peaks of galactose and N-acetyl-D-glucosamine were also revealed as different after chromatographying an equimolar mixture of both standards (Fig. 5E).

Baseline correction was always applied. This was constructed from the start of the first peak to the lowest valley point at the end of the same peak and, in this way, after a baseline segment was constructed, the area of the peak was corrected. The result was stored in a time and area file and then, the next baseline segment was calculated for the included peak. Each successive baseline segment started at the end of the preceding one, to include all the peaks.[15]

The response of the detector appeared to be almost linear in the range from 0.01 μg to 2.5 μg of mass injected (Fig. 6), although sensitivity for N-acetyl-D-glucosamine was about 50 times higher than that found for glucose. Error inherent to direct calibration was estimated as standard error in six repeated injections for each one concentration of standard solutions. Equation for the calibration straight line was obtained by linear regression, r^2 of which measured the goodness of fit.

Quantitative analysis of the glycosyl rest of both lectins revealed that ABP contained 6 residues of N-acetyl-D-glucosamine and 6 residues of glucose per molecule of enzyme whereas SA contained 4 residues of galactose and 8 of glucose per enzyme molecule. The number of residues was calculated over a molecular weight of 21.6 kDa for both arginase isoforms (Molina et al., in press). Quantitative analysis of the glycosyl residue of fetuin revealed that the numbers of residues per mole of protein were 12.88 for galactose, 9.07 for mannose and 18.6 for glucosamine, calculated on the basis of a molecular weight of 48.4 kDa, whereas those found by Spiro[18] were 12.4, 8.1 and 13.2, respectively (data are not shown).

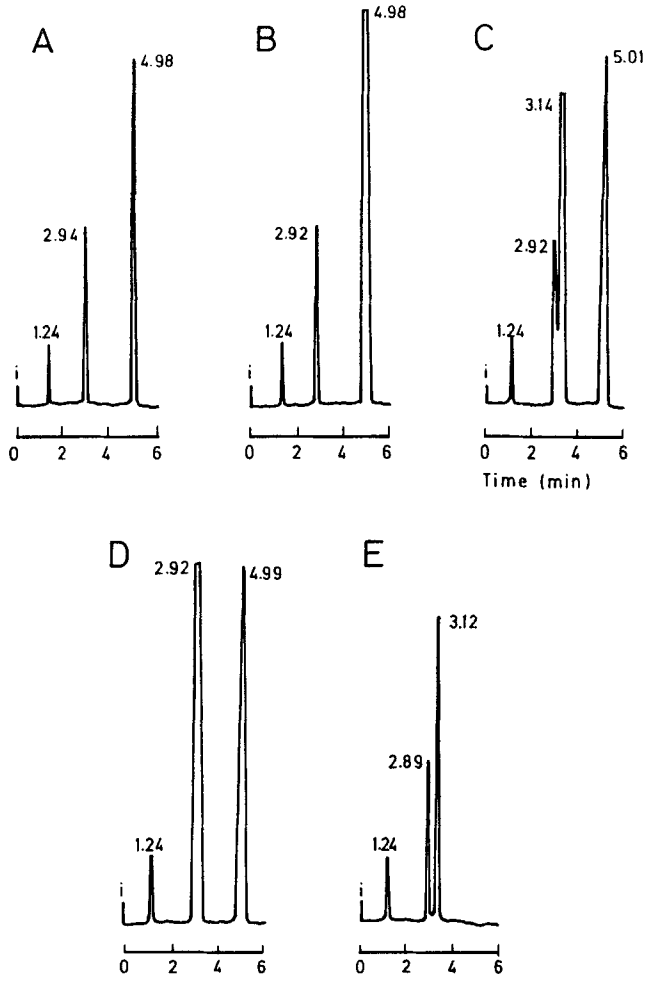

Figure 5. HPLC elution profiles of (A) ethanol-soluble fraction obtained from SA lectin hydrolysate, B) the same sample loaded with glucose, (C) with N-acetyl-D-glucosamine, (D) with galactose and (E) an equimolar mixture of standards galactose and N-acetyl-D-glucosamine.

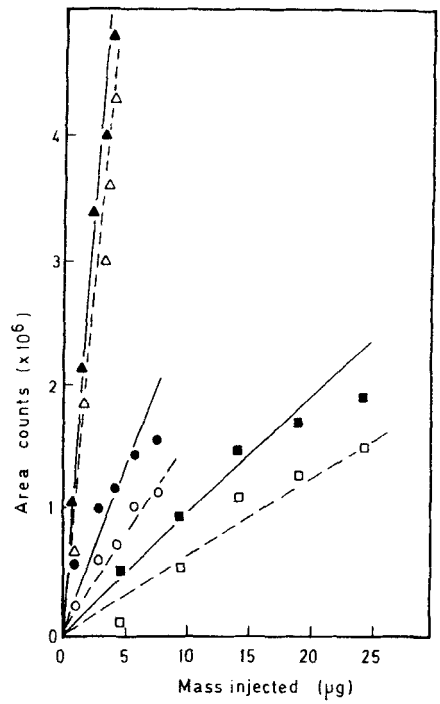

Figure 6. Calibration lines of sugar standards by HPLC. Direct calibration of galactose, $y = 246351x + 24796$; $r^2 = 0.97$ (filled circles); glucose: $y = 89367x + 32987$; $r^2 = 0.96$; (filled squares), and N-acetylglucosamine: $y = 9253970x + 343023$; $r^2 = 0.99$ (filled triangles), and indirect calibration with respect to an internal standard (2.0 mg mL^{-1} ribitol) of galactose: $y = 124297x - 11322$; $r^2 = 0.97$ (empty circles); glucose, $y = 55466x + 7654$; $r^2 = 0.99$ (empty squares), and N-acetylglucosamine, $y = 9253967x - 70878$; $r^2 = 0.99$ (empty triangles). Data are the mean of six replicates. The standard error was never larger than the symbols.

DISCUSSION

Results obtained here concern the rapid identification of lichen isolectins by analyzing the composition of their polysaccharide moiety by HPLC after acidic hydrolysis. The use of a UV detector, such as the SpectraSystem UV2000, 6 to 10 times more sensitive that other previously used (for example, VariChrom TM VUV/10),[15] reveals the appearance of contaminating substances in standard solutions in spite of their analyticial purity degree (Figs. 2A and 3A). However, other standards, such as N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, remain uncontaminated for long time periods. Thus, a re-purification step of standards is required to remove contaminating products

before loading the sugar solution onto the chromatographic column (Fig. 1). When possible, the fraction from the alumina column, containing the highest amount of sugar, has been analyzed by specific enzymatic reaction. The identity of glucose and galactose, eluted from alumina, has been confirmed by reaction with glucose oxidase[21] and galactose oxidase.[22] Anyway, the HPLC peaks of standards, after filtration through alumina, coincide with the main peak obtained from non-filtered substances.

The method for analyzing sugars used here has been revealed as highly sensitive, accurate and repetitive.[15] Both isolectins clearly differ in one of their sugar components (Figs. 4 and 5), although previous reports on *Xanthoria* lectin do not describe protein heterogeneity[11] because only thalline lectin has been isolated. In spite of this, it has recently been described the occurrence of, at least, two thalline arginases, one of which does not contain the glycosyl moiety.[13] In addition, SA from *X. parietina* is quite different from that purified from another lichen species, *Evernia prunastri*, the glycosyl residue of which is composed of glucose, fructose and mannose.[16]

The presence of galactose in *Xanthoria* SA is very interesting in order to explain the binding capabilities of this secreted lectin[13] and sufficiently differs in its chromatographic behaviour from N-acetyl-D-glucosamine, since the α coefficient between standards gives a value of 1.07. N-acetyl-D-glucosamine is not deacylated since no glucosamine appears in the chromatographic analysis. On the other hand, acidic hydrolysis of the glycoproteins with 6 N HCl seems to be a technique that does not produce chemical modification of sugars, since the recovering of sugars from fetuin, used as a control, after hydrolysis is coincident with the well known monosaccharide composition of this glycoprotein.[18,19]

Although most of papers concerning lectin structure and activity are devoted to the identification of the polysaccharide sequence of the lectin receptor,[23,24] even those related to non-glycosylated lectins,[25] sometimes the sugar composition of their glycosyl rest has been analyzed by affinity chromatography[24] or specific enzyme digestion.[27] In this way, it has been described that lectin from *Datura stramonium* seeds contains 37% carbohydrate by weight of which 93% is arabinose and 7% galactose.[28-30] Other plant oligosaccharides which do not bind to protein have been analyzed by GLC[31] or ion-exchange chromatography.[32] Although good resolutions were obtained, procedures and analyses are complicated and tedious. In addition, more than 1.0 mg pure protein is required for analysis.

The rapid and sensitive HPLC procedure described here can be developed by using 4-5 μg of pure lectin and offers highly repetitive results for time analysis no longer than 9 min, including the elution time of the internal standard.

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REFERENCES

1. A. D. Elbein, "The Structure and Biosynthesis of Lipopolysaccharides and Glycoproteins", *in* **The Phytochemistry of Cell Recognition and Cell Surface Interactions**, F. A. Loewus, C. A. Ryan, eds., Plenum Press, New York and London, 1981, pp. 1-24.

2. A. R. Poole, Biochem. J., **236**, 1-14 (1986).

3. L.M. Shannon, and C.N. Hankins, "Enzymatic Properties of Hemagglutinins" in **The Phytochemistry of Cell Recognition and Cell Surface Interactions**, F. A. Loewus, C. A. Ryan, eds., Plenum Press, New York and London, 1981, pp. 93-114.

4. J. Macek, K. Krajícková, M. Adam, J. Chromatogr., **414**, 156-160 (1987).

5. K. Murata, Y. Yokoyama, J. Chromatogr., **423**, 51-61 (1987).

6. T. Gherezghiher, M. C. Koss, R. E. Nordquist, C. P. Wilkinson, J. Chromatogr., **413**, 9-15 (1987).

7. Y. Nomura, H. Tade, K. Takahashi, K. Wada, Agric. Biol. Chem., **53**, 3313-3315 (1989).

8. S. Honda, A. Makino, S. Suzuki, K. Kakehi, Anal. Biochem., **191**, 228-234 (1990).

9. S. Honda, T. Ueno, K. Kakehi, J. Chromatogr., **608**, 289-295 (1992).

10. J. P. Landers, R. P. Oda, B. J. Madden, T. C. Spelsberg, Anal. Biochem., **205**, 115-124 (1992).

11. P. Bubrick, A. Frensdorff, M. Galun, Symbiosis, **1**, 85-95 (1985).

12. N. Kardish, L. Silberstein, G. Fleminger, M. Galun, Symbiosis, **11**, 47-62 (1991).

13. M. C. Molina, E. Muñiz, C. Vicente, Plant Physiol. Biochem., **31**, 131-142 (1993).

14. M. E. Legaz, Symbiosis, **11**, 263-277 (1991).

15. C. Vicente, J. L. Mateos, M. M. Pedrosa, M. E. Legaz, J. Chromatogr., **553**, 271-283 (1991).

16. V. Planelles, M. E. Legaz, Plant Sci., **51**, 9-16 (1987).

17. M. E. Legaz, M. de Torres, M. I. Escribano, Photosynthetica, **19**, 230-236 (1985).

18. R. G. Spiro, J. Biol. Chem., **235**, 2860-2869 (1960).

19. R. G. Spiro, V. D. Bhoyroo, J. Biol. Chem., **249**, 5704-5717 (1974).

20. M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, F. Smith, Anal. Chem., **28**, 350-356 (1956).

21. E. Raabo, T. C. Terkildsen, Scand. J. Clin. Lab. Invest., **12**, 402-411 (1960).

22. G. Avigad, D. Amaral, C. Asensio, B. L. Horecker, J. Biol. Chem., **237**, 2736-2743 (1962).

23. C. Wood, E. A. Kabat, L. A. Murphy, I. J. Goldstein, Arch. Biochem. Biophys., **198**, 1-11 (1979).

24. M. V. Krishna-Sastry, P. Banarjee, S. R. Patanjali, M. J. Swamy, G. V. Swarnalatha, A. Surolia, J. Biol. Chem., **261**, 11726-11733 (1986).

25. G. R. Gunther, J. L. Wang, I. Yahara, B. A. Cunningham, G. M. Edelman, Proc. Natl Acad. Sci. USA, **70**, 1012-1016 (1973).

26. J. F. Crowley, I. J. Goldstein, J. Arnarp, J. Lönngren, Arch. Biochem. Biophys., **231**, 524-533 (1984).

27. D. C. Kilpatrick, M. M. Yeoman, Biochem. J., **175**, 1151-1153 (1978).

28. N. N. Desai, A. K. Allen, A. Neuberger, Biochem. J., **197**, 345-353 (1981).

29. J. F. Crowley, I. J. Goldstein, FEBS Lett., **130**, 149-152 (1981).

30. D. Ashford, N. N. Desai, A. K. Alle, A. Neuberger, M. A. O'Neill, R. R. Selvendran, Biochem. J., **201**, 199-208 (1982).

31. W. R. Raymond, C. W. Nagel, Anal. Chem., **41**, 1700-1703 (1969).

32. C. W. Nagel, T. M. Wilson, J. Chromatogr., **41**, 410-416 (1969).

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