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Affinity Chromatography of Arabinogalactan-Proteins*

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ABSTRACT

Arabino-(1→3),(1→6)-β-D-galactan-proteins (AGPs) and related compounds from Lolium multiflorum *(ryegrass) endosperm cell suspension culture, wheat endosperm, larchwood and* Gladiolus *stigma extract were shown to bind selectively at neutral pH to a column of Sepharose to which the anti-galactan myeloma protein J539 had been covalently linked. Elution was achieved with buffer at pH 3 or with a pulse of p-nitrophenyl-β-D-galactopyranoside at neutral pH. These observations formed the basis for an affinity chromatographic purification of AGPs from natural sources. Some heterogeneity in a ryegrass AGP preparation was indicated by its incomplete elution by D-galactose.*

INTRODUCTION

Arabino-(1→3),(1→6)-β-D-galactans (AGs) and arabino-(1→3),(1→6)-β-D-galactan-proteins (AGPs) have been isolated from a great variety of plant sources, including *Acacia* type gum exudates, larchwood, endo-

* These results were presented in part at the Australian Biochemical Society Meeting, Brisbane, May 1977 (Andrew & Stone, 1977).

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sperm of wheat and ryegrass, stigma surfaces and style exudates, etc. (Clarke *et al.*, 1979; Fincher *et al.*, 1983). These molecules have a branched β-galactopyranose framework of (1 → 3), (1 → 6)- and (1 → 3), (1 → 6)-linked residues. The branches from the framework often bear terminal L-arabinofuranosyl residues and may also carry additional, less abundant monosaccharides, such as L-Rhap, D-Xylp, D-Manp, D-Glcp, D-GlcpA and D-GlcpA, often as terminal substituents.

In some gum exudates, e.g. gum ghatti, a non-galactan core bears arabinogalactan side chains of the type found in AGs and AGPs (Aspinall, 1969).

Certain of these AGs and AGPs have been shown to interact specifically with β-D-glycopyranosyl Yariv antigens (1,3,5-tris-(4-β-D-glycopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene) (Yariv *et al.*, 1967; Jermyn & Yeow, 1975). The coloured azo-dye–AGP complex is insoluble in water and has been used histochemically to locate AGPs in plant cells and tissues (Clarke *et al.*, 1975; Anderson *et al.*, 1977; Clarke *et al.*, 1978). AGPs can be isolated from aqueous plant extracts by precipitation with the β-D-glycopyranosyl Yariv antigen, followed by dissociation from the complex (Clarke *et al.*, 1975, 1978). However, the methods of recovery may affect the integrity of the AGP and last traces of Yariv antigen, which are difficult to remove, complicate monosaccharide analysis by introducing extraneous glucose. Other methods for isolating AGPs are relatively non-specific and rely on their solubility in saturated ammonium sulphate solution and subsequent chromatography (Fincher & Stone, 1974; Anderson *et al.*, 1977). An alternative simple and specific method of isolation was needed to facilitate a continuing study of the structure and biosynthesis of cereal endosperm and other AGPs. Affinity chromatographic methods have been successfully employed for the purification of a number of polysaccharides (see, for example, Ross *et al.*, 1976) and a suitable support for the affinity chromatography of AGPs was sought. Glaudemans (1975) and co-workers have investigated a number of (1 → 6)-β-D-galactan-specific IgA mouse myeloma proteins and one of these, protein J539, has been shown to bind ryegrass AGP (Baldo *et al.*, 1978) and an AG-peptide from wheat (Baldo *et al.*, 1978; M. Potter, personal communication), as well as larch arabinogalactan (Potter *et al.*, 1972) and other galactose-containing polysaccharides (Glaudemans, 1975). In this paper we report the affinity chromatography of some AGPs on a column of immobilised protein J539 (Sepharose J539).

EXPERIMENTAL

Materials

Ryegrass AGP was prepared from a late log phase culture of *Lolium multiflorum* endosperm cells, grown on a modified White's medium with sucrose as carbon source (Smith & Stone, 1973). Cells were harvested by filtration and crude AGP prepared from the filtrate by ammonium sulphate fractionation and dialysis (Anderson *et al.*, 1977). Further purification of the ryegrass AGP (I. E. P. Taylor, unpublished data) was achieved by gel filtration on Sephadex G100 (Pharmacia Fine Chemicals, Uppsala, Sweden) and chromatography on DEAE-Sephadex A50 (Pharmacia). The purified AGP contained approximately 3% protein and 90% carbohydrate. Its monosaccharide composition, as shown in Table 4, indicated an arabinose/galactose molar ratio of 1·00.

[^{3}H]-AGP was prepared from crude ryegrass AGP by J. Sabatino. The AGP was incubated with galactose oxidase (Galactostat, Worthington Biochemicals, USA) followed by sodium [^{3}H]-borohydride (Radiochemical Centre, Amersham, UK) to label carbon atom 6 of any free terminal galactosyl residues (Morell *et al.*, 1966; Jack & Sturgeon, 1976). After removal of protein by ammonium sulphate fractionation, the AGP was precipitated with ethanol and dried by solvent exchange (Green, 1963). Prior to use, it was diluted with non-radioactive AGP to a specific activity of about 0·5 μCi/mg.

Wheat AG-peptide (Fincher & Stone, 1974) and wheat arabinoxylan (Andrewartha *et al.*, 1979) were prepared in this laboratory by M. McNamara and K. Andrewartha, respectively. Lupin arabino-(1→4)-β-galactan, a purified neutral fraction from lupin cotyledons, was a gift from N. K. Matheson, Department of Agricultural Chemistry, University of Sydney, New South Wales, Australia.

A cell-free extract of ryegrass endosperm was prepared by F. Keller from cells harvested at late log phase by passage of a buffered suspension of the cells through a French pressure cell, followed by centrifugation. A *Gladiolus* stigma extract was kindly supplied by A. E. Clarke, School of Botany, University of Melbourne, Parkville, Victoria, Australia.

β-D-Glucopyranosyl Yariv antigen was kindly donated by M. A. Jermyn, Division of Protein Chemistry, CSIRO, Parkville, Victoria, Australia, and J539 ascites serum provided by M. Potter, National Institutes of Health, Bethesda, Maryland, USA.

General methods

Carbohydrate was determined by the phenol–sulphuric acid method of Dubois *et al.* (1956) as modified by Immers (1964).

Protein was determined by the method of Lowry *et al.* (1951).

Radioactivity was determined in a Packard Tricarb liquid scintillation spectrometer, using TRIPOP scintillation fluid (Anderson & McClure, 1973).

Monosaccharide analysis was performed by gas chromatography. Samples were dialysed, if necessary, and hydrolysed with 0·5 M nitric acid, containing 0·5% urea for 4 h at 100°C (Jermyn & Isherwood, 1956). After neutralisation with mixed-bed resin (BioRad AG501 X8, H^+/HCO_3^- form), reduction with sodium borohydride, acetylation (Albersheim *et al.*, 1967) and addition of myoinositol hexacetate as internal standard, the alditol acetates were separated on a 1·8 m × 2 mm internal diameter stainless steel column packed with 3% SP 2340 on 100/120 Supelcoport (Supelco Inc., Supelco Park, Bellafonte, Pennsylvania 16823, USA), in a Hewlett Packard gas chromatograph, operating isothermally at 215°C.

Preparation of affinity adsorbents

Myeloma protein J539 was purified from defatted ascites serum by affinity chromatography on acid-washed Sepharose 2B (Pharmacia), as described by Eichmann *et al.* (1976).

Purified protein J539 was coupled to Sepharose 4B (Pharmacia) using the cyanogen bromide procedure of March *et al.* (1974). The product, referred to as Sepharose J539, was equilibrated with phosphate-buffered saline (PBS), pH 7·2, ionic strength 0·24, prepared from equal volumes of 0·15 M NaCl and 0·15 M KH_2PO_4–Na_2HPO_4 buffer, pH 7·2.

The bound protein J539 was assayed by the ultraviolet absorption of a suspension of the gel in polyethylene glycol solution (Mosbach, 1974). Assuming $E^{1\%}_{1\,cm}$ (280 nm) = 13·0 for IgA (Sober, 1970), two preparations gave values of 1·8 and 2·7 mg protein/ml packed gel.

Sepharose IgG, prepared by linking non-specific human IgG to Sepharose 4B by the cyanogen bromide method, with a yield of 7 mg IgG per ml packed gel, was a gift from A. E. Clarke.

Affinity chromatography

Sepharose J539 (1–2 ml) was packed into a 6 mm diameter glass column (prepared from a Pasteur pipette) and equilibrated with PBS, pH 7·2. After applying the sample, the column was routinely washed with PBS, pH 7·2, to remove unadsorbed material and then eluted, either (i) with a gradient starting with PBS and ending with 0·1 M citric acid in 0·2 M NaCl, pH 2·0, ionic strength 0·21, or (ii) with a buffer of pH 3·0, ionic strength 0·24, prepared by mixing PBS and citric acid–NaCl in the appropriate proportions (approximately 9:10 by volume). All operations were performed at 2–4°C. Elution of AGP was monitored by a gel diffusion test with β-D-glucopyranosyl Yariv antigen (Jermyn & Yeow, 1975) as well as by determination of carbohydrates and/or radioactivity, and in some cases protein and monosaccharide composition.

RESULTS AND DISCUSSION

When a solution of purified *Lolium* AGP was applied to a column containing 1·6 ml (packed bed volume) Sepharose J539, up to 1 mg of the AGP remained bound after elution with PBS, pH 7·2, and was eluted by a pH 3 buffer of the same ionic strength (0·24) (Table 1). This corresponded to a capacity of 1 mg AGP/3 mg immobilised protein J539. As seen from the data in Table 1, however, this capacity was achieved only with considerable overloading. A maximum of about 80% of the applied AGP was adsorbed at loadings of less than 1 mg AGP/3 mg protein J539. On gradient elution (Fig. 1) the adsorbed AGP was eluted as a single, symmetrical peak.

Table 1 also indicates the binding of other arabino-(1→3),(1→6)-β-D-galactans and gums. Lupin arabino-D-galactan was not adsorbed, nor were wheat arabinoxylan, soluble starch or bovine serum albumin. A similar specificity of the protein J539 for arabino-(1→3), (1→6)-β-D-galactans was observed in gel diffusion experiments (data not shown). It was also noted in these experiments that removal of the arabinofuranose residues from AGP and gum arabic by mild oxalic acid treatment (as described by Fincher *et al.*, 1974, courtesy of J. Sabatino) enhanced the precipitation reaction, presumably due to exposure of the reactive (1→6)-linked oligo-β-D-galactopyranosyl chains. Degrada-

TABLE 1
Binding of Polysaccharides to Sepharose J539,[a] pH 7·2

Sample	*Applied mg*	*Bound*[b] *mg*	*Bound %*
Lolium AGP	0·47	0·40	84
	0·94	0·74	78
	1·88	1·00	53
Wheat AG-peptide	0·49	0·24	49
Larch arabinogalactan	0·35	0·19	54
	1·00	0·25	25
Gum Ghatti	0·25	0·14	54
Gum Arabic	1·08	0·07	7

[a] Column with 1·6 ml Sepharose J539, containing 3·0 mg protein J539.
[b] Carbohydrate which remained bound on column at pH 7·2 but was eluted at pH 3. See text for details.

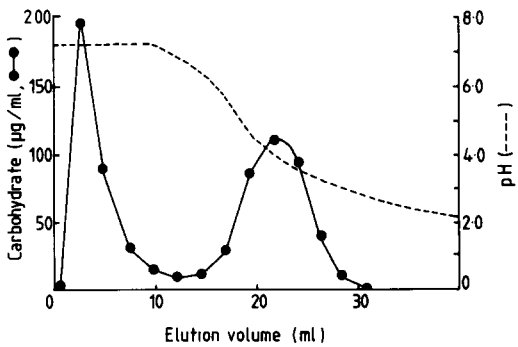

Fig. 1. Elution of AGP with pH gradient. Same column as in Table 1; 1·88 mg purified AGP applied; eluted with PBS, pH 7·2 and then with a pH gradient (see under 'Materials' and 'General Methods'). Fractions of approximately 2 ml collected and analysed for carbohydrate. Note the large peak of unbound material due to overloading of column. See text for details.

tion of the ryegrass AGP with sodium hydroxide to remove the protein (as described by Fincher *et al.*, 1974, courtesy of I. E. P. Taylor) had no detectable effect on the capacity to react with protein J539. These observations confirm the finding (Baldo *et al.*, 1978) that the presence

of terminal arabinosyl units of the AGP inhibits reaction with the protein J539 whose specificity is directed toward (1→6)-linked β-D-galactopyranosyl residues (Glaudemans, 1975).

The maximum percentage of applied ryegrass AGP which was adsorbed to the Sepharose J539 column was about 80%. This was not increased either by reducing the column loading (below 0·5 mg) or by decreasing the flow rate during application and elution, nor was it affected by prolonged washing of the loaded column with PBS, pH 7·2 (data not shown). This suggested that the greater part of the AGP sample had a high affinity for the protein J539 and that the non-quantitative binding could, at least in part, be explained by a micro-heterogeneity of the sample with respect to its affinity for the bound protein J539.

To test this hypothesis, a sample of radioactive AGP was applied to the column, and the bound and unbound fractions collected, concentrated by Diaflo (Amicon) ultrafiltration, readjusted to pH 7·2 and separately re-applied to the column. Table 2 shows that unbound and bound fractions did indeed differ in their affinity for the bound protein J539. The source of these affinity differences may arise from natural differences in arabinosylation of the AGP or may perhaps be due to alterations occurring during the labelling.

Almost quantitative elution of adsorbed AGP or arabinogalactan from the Sepharose J539 was effected with pH 3 buffer, ionic strength 0·24 (Table 3). At higher pH values, partial elution could be achieved

TABLE 2

Rechromatography on Sepharose J539 of Those Fractions of *Lolium* [^{3}H]-AGP Which Remained Unbound (Fraction U) or Bound (Fraction B) to the Column at pH 7·2 During a Previous Run

Sample applied	*% Unbound*	*% Bound*
[^{3}H]-AGP[a]	32(U)	68(B)
Fraction U	88	12
Fraction B	22	78

[a] 0·5 mg [^{3}H]-AGP applied. The column was the same as in Table 1.

TABLE 3
Elution of *Lolium* AGP from Sepharose J539[a]

Eluant	*% Bound AGP eluted*
pH gradient (pH 7·2 to 3·0)[b]	99
pH 3 buffer[b]	99
p-Nitrophenyl-β-galactoside[c]	90
Galactose (pulse)[d]	49
Galactose (gradient)[e]	54

[a] The same column was used as in Table 1; approximately 1 mg AGP applied in PBS, pH 7.2; column then washed with approximately 16 ml PBS, pH 7·2, then eluted as indicated.
[b] As described under 'Materials' and 'General Methods'.
[c] 0·5 or 1·0 ml 0·1 M *p*-nitrophenyl-β-D-galactopyranoside in PBS, pH 7·2, followed by PBS, pH 7·2.
[d] 6 ml 0.1 M D-galactose in PBS, pH 7·2, followed by PBS, pH 7·2.
[e] Linear gradient, starting with PBS, pH 7·2, ending with 1 M D-galactose in PBS, pH 7·2; see Fig. 2.

by increasing the ionic strength: e.g. at pH 4·0 (0·1 M acetate buffer) 90% was eluted at ionic strength 1·06 (1·0 M NaCl present), but only 46% if the ionic strength was decreased to 0·16 (0·1 M NaCl).

During normal use, the column was washed after each run with 0·1 M citric acid – 0·3 M NaCl, pH 2·0, and then with PBS, pH 7·2. One column so treated was used over 20 times with little loss in capacity. When a 1·6 ml column was eluted with 3 M NaCNS after the routine washing procedure, a further 5–15 μg AGP was removed which had not been eluted with acid. This tightly-bound AGP amounted to not more than 1% of the total AGP applied. The AGP binding capacity of the column was reduced by about 50% after the NaCNS treatment; hence this eluant was not routinely used during the experiments reported.

It is likely that some non-specific mechanism was, in part, responsible for the low-level acid-resistant binding of AGP to the Sepharose J539. A control column of Sepharose IgG (not galactan-specific) was shown to bind about 3–5 μg AGP/10 mg IgG. This non-specifically bound material was not eluted by the usual acid treatment,

but was eluted with 3 M NaCNS. A. E. Clarke (personal communication) has also demonstrated a low-level non-specific binding of *Gladiolus* AGP to several proteins.

The elution of AGP by *p*-nitrophenyl-β-D-galactopyranoside shown in Table 3 suggests that most of the bound AGP was specifically adsorbed to the immobilised protein J539. 90% of the bound AGP was eluted by a pulse of 50 or 100 μmol *p*-nitrophenyl-β-D-galactopyranoside in PBS, pH 7·2. Such elution was expected, since Sher & Tarikas (1971) showed that protein J539 was precipitated with protein-bound *p*-azophenyl-β-D-galactopyranoside and that *p*-nitrophenyl β-D-galactopyranoside specifically inhibited this precipitation.

On the other hand, D-galactose (in PBS, pH 7·2) was able to elute only 54% of the bound AGP, even when applied as a gradient up to 1 M concentration. Thus there was a fraction of bound AGP on the column which was resistant to elution by galactose. To test whether this reflected a further microheterogeneity of the AGP preparation similar to that observed for the [^{3}H]-AGP in Table 2, a further galactose elution experiment was run. In this experiment, ryegrass AGP (1 mg) was applied to a column (1 ml bed volume, containing 2·7 mg protein J539) and eluted with (i) PBS, pH 7·2, (ii) D-galactose (0·1 M in PBS, pH 7·2) and finally (iii) 0·1 M citric acid–0·3 M NaCl (pH 2·0). Three fractions of carbohydrate were obtained, A (unbound), B (eluted with galactose), C (eluted with acid), and each was treated for Yariv antigen binding and for monosaccharide composition. Only the bound fractions (B and C) interacted with β-D-glucopyranosyl Yariv antigen in a gel diffusion test. The three fractions also differed in their arabinose/galactose ratio, these being 1·7, 1·2 and 0·9 for fractions A, B and C, respectively. Thus it appeared that the AGP sample was quite heterogeneous, and that the most tightly bound material was that with the lowest arabinose/galactose ratio. The lower arabinose content might facilitate binding between the galactose residues of the AGP and the immobilised protein J539. Support for this suggestion comes from the observation, noted above, that mild hydrolysis of AGP or gum arabic enhances its capacity to precipitate the protein J539 in a gel diffusion test.

The specificity of Sepharose J539 suggested that it would be useful for the affinity chromatographic purification of AGP. Crude undialysed culture filtrate (2 ml) from an 11-day culture of *Lolium multiflorum* (containing 16·5 mg carbohydrate and 2·2 mg protein) was adjusted

to pH 7·2 with Na_2HPO_4, diluted to 4 ml, and applied to a 1·6 ml column of Sepharose J539 (containing 3·0 mg protein J539). Elution with PBS, pH 7·2, and then with pH 3 buffer, yielded two carbohydrate-containing fractions. About 97·5% of both the carbohydrate and the protein were found in the first fraction, eluted at pH 7·2. Only the second fraction, eluted at pH 3·0, gave precipitation lines with β-D-glucopyranosyl Yariv antigen in the gel diffusion test. Monosaccharide analyses (Table 4) suggested that the fraction eluted at pH 3·0 was virtually pure AGP. Similar purification of AGP was obtained when crude cell-free extracts of suspension-cultured *Lolium multiflorum* cells and of *Gladiolus* stigma were used.

The single step purification to apparent homogeneity observed here for *Lolium* AGP is considerably simpler and more rapid than previously published methods, and has been used in our laboratory as a routine method for small-scale purifications, in the isolation of galactan products in biosynthesis experiments (Mascara & Fincher, 1982) and the preparation of an AGP from the styles of *Nicotiana alata* (A. C.

TABLE 4

Monosaccharide Analysis of AGP and of Fractions from Affinity Chromatography of Crude Undialysed Culture Filtrate of *Lolium multiflorum* Endosperm[a]

Sugar residue	*Purified AGP*[b]	*Unbound fraction*	*Bound fraction*
Galactose	47	<1	48
Arabinose	47	7	48
Glucose	1	77[c]	3[d]
Mannose	4	9	0
Xylose	0	6	<1
Others	1	<1	<1

[a] See text for details. Values expressed as % total sugars.

[b] Purified AGP prepared as described under 'Materials' and 'General Methods'.

[c] Glucose probably arises from the sucrose supplied in the culture medium. Any fructose remaining at the time of harvesting would give peaks recorded as glucose and mannose.

[d] Glucose in the bound fraction probably represents carry-over from the unbound fraction due to insufficient washing with PBS, pH 7·2.

Gell & A. E. Clarke, personal communication). The usefulness of the method may even be extended by the use of an appropriate elution schedule leading to a sub-fractionation of the otherwise apparently homogeneous AGP. Another application of Sepharose J539 is in the purification of ryegrass protoplasts (Keller & Stone, 1978). This method is based on the tight binding between a galactose ligand on the protoplast surface and the myeloma protein J539 (Schibeci *et al.*, 1982).

Other affinity matrices are available for AGs and AGPs. Gleeson *et al.* (1979) have described a Sepharose affinity column based on the Ca^{2+}-dependent galactose-specific protein, tridacnin with a capacity for *Gladiolus* AGP comparable to that of the ryegrass AGP on Sepharose J539. The affinities of galactose-specific carbohydrate-binding proteins for different AGs and AGPs varies and depends on the detailed structure of the galactosyl determinants. For example, Sepharose J539 has a lower affinity for larchwood than ryegrass AGP. The availability of different galactosyl-specific affinity columns allows an appropriate choice to be made for different galactose-containing polymers.

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