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SEED GLYCOPROTEINS OF *LUPINUS ANGUSTIFOLIUS*

CHRISTOPHER A. EATON-MORDAS* and KEITH G. MOORE†

*School of Plant Biology, University College of N. Wales, Bangor; †School of Biological Sciences, Univeristy of Bath, Claverton Down, Bath BA2 7AY, U.K.

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Abstract—The seed globulins of *Lupinus angustifolius* are glycoproteins containing 1.4–1.9% (α-conglutin), 2.8–6.4% (β-conglutin) and 1.2–3.8% (γ-conglutin) carbohydrate. The highest values were obtained after acid hydrolysis and determination by phenol–H_2SO_4 (α, γ-conglutins) or by methanolysis and sugar determination by GLC (β-conglutin). TCA denaturation of β- and γ-conglutins was necessary to remove adsorbed galactomannans before determination of glycoprotein carbohydrates. All 3 conglutins contained mannose, galactose and glucosamine, though the ratio of mannose to galactose, and to a lesser extent neutral sugars to hexosamine varied. Small amounts of fucose were found associated only with γ-conglutin.

INTRODUCTION

Reports have indicated that in a number of legume species [1, 2] and in some non legumes [3–5] the seed storage proteins contain small amounts of bound carbohydrate, suggesting their glycoprotein nature. In general, the oligosaccharide moieties are small although there is some evidence that the amount may vary between the different reserve proteins of the seed [2] and also during seed development and germination [6]. In a survey of the seed proteins of a range of species for the presence of glycosylated storage proteins (Eaton-Mordas and Moore, unpublished) it was observed that the seed proteins of *Lupinus angustifolius* contained appreciable bound carbohydrate and the present work was therefore carried out to characterise the bound carbohydrates of these proteins.

The low levels of carbohydrate in seed glyco-proteins pose difficulties in both qualitative and quantitative analysis, and in the absence of comparative data on different methods of analysis it is often difficult to assess the reliability of values reported. This report establishes the glycoprotein nature of the 3 major lupin seed globulins and indicates some differences in values of individual sugars, due to the method of quantification employed.

RESULTS AND DISCUSSION

The 3 lupin seed globulin fractions subjected to cellulose acetate electrophoresis produced single bands and may therefore be considered as distinct proteins, although β-conglutin in particular is known to be complex and heterogeneous [7]. Separation of the conglutins on acrylamide gels gave essentially similar results, although the resolution is rather poor by this method [7], and each conglutin gave a positive reaction for carbohydrate when the gels were stained with periodic acid–Schiff reagent.

Quantitative determination of the carbohydrate content of the conglutins (Table 1) confirmed that all 3 fractions contained appreciable amounts of carbohydrate.

Table 1. Total carbohydrate content of native and TCA-denatured conglutins

Conglutin	Carbohydrate content (% of anhydrous protein wt)	
	Phenol–H_2SO_4	Anthrone
α-native	1.3	1.0
α-denatured	1.4	1.5
β-native	6.5	5.4
β-denatured	3.1	2.8
γ-native	7.7	5.4
γ-denatured	2.2	1.2

Denaturation of the proteins resulted in considerable loss of carbohydrate from the β- and γ-conglutins but not from α-conglutin. The possibility that β- and γ-conglutin may contain some carbohydrate covalently linked by bonds labile to cold 5% TCA cannot be ruled out, but it would seem more likely that this material is adsorbed onto the protein either before or during extraction. The latter conclusion is supported by the more intense reaction of lupin seed proteins to periodic acid–Schiff's reagent on acrylamide gels after extraction from whole seed than after extraction from protein-body preparations, and emphasises that particular care is needed to avoid interference by adsorbed carbohydrate when quantitative and qualitative studies of the low levels of sugars in seed storage glycoproteins are being investigated.

Analysis of the carbohydrate released by denaturation showed that it was a galactomannan of very high MW, which was largely excluded by Sephadex G200 and the MW must therefore exceed *ca* 500000. The specific binding of this carbohydrate to two of the conglutins may indicate a lectin-like activity by these globulins, or alternatively the galactomannan itself might be a highly glycosylated lectin of the type found in the cell walls of

many legumes [8]. The possibility of globulin/lectin interactions during isolation of seed proteins has been discussed recently [2].

The presence of carbohydrates in the denatured conglutin preparations showed that some sugars were more tightly linked and suggested that the conglutins might be glycoproteins. This was confirmed by the isolation of the major subunits of the conglutins by preparative PAGE in the presence of SDS and 2-mercaptoethanol, followed by quantitative analysis of the sugars by GLC, which showed that all the major conglutin subunits are glycosyalted, though to different degrees. This work will be reported in a subsequent paper.

In order to avoid contamination by adsorbed carbohydrate material, the further analysis of the conglutin bound sugars was carried out on TCA denatured proteins.

The results of carbohydrate estimations on the unhydrolysed proteins (Table 1, Table 2a, b) indicated that the phenol–H_2SO_4 method gives consistently higher values than anthrone for β- and γ-conglutins, perhaps due to the relatively greater mannose content of these proteins (Table 3). Values of total sugars measured after acid hydrolysis were higher than on the unhydrolysed protein, probably due to interference by the large excess of unhydrolysed protein with the chromogenic reaction in the latter. Methanolysis and GLC determination of individual sugars gave the highest value for β-conglutin (Table 2) and resulted in higher individual values for glucosamine and for mannose/galactose ratio (Table 3) compared with acid hydrolysis and GLC. This is consistent with reports that methanolysis results in less sugar degradation than acid hydrolysis [9] and emphasises the importance of the methods employed to estimate the sugars present in lightly glycosylated proteins.

Analysis of the composition of the conglutin carbohydrates showed that glucosamine, galactose and mannose could be positively identified in all the proteins. Fucose was present only in γ-conglutin, and glalactosamine could not be detected in any. The relative proportions of the constituent sugars differed between the proteins, with β-conglutin showing the highest mannose level and mannose/galactose ratio, and α-conglutin the least. The ratios of neutral sugars to glucosamine varied only slightly (β 5.4; α 5.0; γ 4.3) and were quite close to the ratio of *ca* 4 reported for vicilin in *Phaseolus vulgaris* [10] and *Glycine max* [11], in which similar levels of carbohydrate have been found. Much higher ratios (*ca* 10–20) have been found in seed glycoproteins in which carbohydrate contents are smaller such as *Vicia faba* vicilin [12], *Phaseolus aureus* vicilin [13] and *Pisum sativum* legumin [6].

Table 2. Total carbohydrate content of TCA denatured conglutins by various procedures

	Carbohydrate content (% of anhydrous protein wt)				
	a	d	c	d	e
α-Conglutin	1.45	1.36	1.63	1.86	1.57
β-Conglutin	2.78	3.06	3 71	4.56	6.40
γ-Conglutin	1.22	2.23	3.23	3.77	2.62

Carbohydrate analysis by (a) anthrone on hydrolysed protein; (b) phenol–H_2SO_4 on unhydrolysed protein; (c) phenol–H_2SO_4 after acid hydrolysis; (d) value from (c) with total hexosamines added; (e) total neutral sugars and hexosamines from GLC of methyl glycosides after methanolysis. All values except hexosamines in mannose equivalents.

Table 3. Monosaccharide composition of TCA denatured conglutins

	Sugar (% of anhydrous protein wt)					
	Man	Gal	GlcN	Fuc	Man/Gal Ratio	
α-Conglutin	0.71(a)	0.60(a)	0.26(a) 0.23(b)	0.00(c)	1.2(a)	1.0(d)
β-Conglutin	4.20(a)	1.20(a)	1.00(a) 0.85(b)	0.00(c)	3.5(a)	2.4(d)
γ-Conglutin	1.40(a)	0.69(a)	0.53(a) 0.54(b)	0.10–0.20(c)	2.0(a)	1.7(d)

Monosaccharide values derived from (a) GLC of TMSi ethers of Me glycosides after protein methanolysis [9]; (b) Elson–Morgan estimation of hexosamine in acid-hydrolysed protein [26]; (c) fucose estimated by Dische–Shettles method on unhydrolysed protein [21]; (d) Man/Gal ratio by acid hydrolysis and GLC of neutral sugars [24]

Despite their common subcellular location in the protein bodies of the lupin seed [7] the conglutins clearly differ quite considerably in their total glycosylation and in the relative proportions of the constituent sugars present. In common with a number of other legume species [2], the largest amounts of carbohydrate are present in the vicilin-like globulin β-conglutin, whilst smaller amounts are present in the legumin-like α-conglutin. γ-Conglutin, which is very different from the other conglutins in its physical properties, and high content of cysteine [7] is apparently also distinct in its high content of bound galactomannan, and in containing detectable fucose.

EXPERIMENTAL

Dry seeds of *Lupinus angustifolius* (cv New Zealand Bitter Blue) were milled (20 mesh) and defatted with hexane.

Protein fractionation. Preliminary purification of α,β- and γ-conglutin was performed as in ref. [7]. Further purification procedures were modified from ref. [7] as follows. twice precipitated α-conglutin was dissolved in 0.15 M NaPi buffer, pH 7 (to 0.25% concn w/v). $(NH_4)_2SO_4$ added to 50% satn, stirred 2 hr room temp. and centrifuged 20000 *g* for 30 min. The supernatant was made 66% satd with $(NH_4)_2SO_4$ and the ppt. collected. Twice pptd β-conglutin was dissolved to 0.25% concn in buffer, $(NH_4)_2SO_4$ added to 70% satn, centrifuged, the supernatant made to 90% satn, and the ppt. collected. γ-Conglutin was not purified beyond the preliminary state. All prepns were dissolved in buffer, dialysed extensively against H_2O at 4° and lyophilised.

Electrophoresis. The conglutin prepns were examined by electrophoresis on cellulose acetate strips impregnated with 0.15 M NaPi pH 7 [7]. Disc electrophoresis was carried out on 7.5% acrylamide gels pH 8.5 [14] and proteins located with coomassie blue [15] or amido black [14]. Presence of carbohydrate was detected by periodic acid-Schiff reagent [16]. SDS gel electrophoresis of 2-mercaptoethanol reduced conglutins was carried out by the method of [15] and, after alkylation, [17].

Estimation of total sugar in the presence of protein Total protein-bound carbohydrates were estimated on the lyophilised proteins and also after cold TCA denaturation (final TCA concn 5%. Pellets washed with 50% EtOH–H_2O, $CHCl_3$ and Me_2CO, and solubilised in 0.1 N NaOH prior to sugar determination). Carbohydrates were measured by anthrone [18]

and by phenol–H_2SO_4 [19] with D-mannose as standard. Protein was measured by the method of ref. [20] relative to standard curves prepared for each prepn and also to casein. Specific determination of fucose was carried out on the unhydrolysed proteins by the cysteine–H_2SO_4 method [21] with L-fucose as standard.

Hydrolysis, identification and estimation of neutral sugars. TCA denatured proteins (1–2 mg/ml) were hydrolysed in N H_2SO_4 in sealed tubes for 8 hr at 100° [22], passed through Dowex 50 × W (200–400 mesh, H^+) and Dowex 1 × 8 (200–400 mesh, formate$^-$) coupled columns, and neutral sugars determined by the method of ref. [19] on water eluates after drying *in vacuo*. Sugars were identified after TLC on NaPi–Si gel using iso-PrOH–Me_2CO–lactic acid 0.1 M (4:4:2) solvent and aniline diphenylamine and *p*-anisidine sprays [23]. Molar ratios of neutral sugars were determined by GLC of TMSi derivatives prepared according to [24] and separated on 150 cm × 4 mm columns of 3% SE30, N_2 carrier (40 ml/min) with temp. programmed from 160–220° at 15°/min using dual FID. Myo-inositol was used as int. stand.

Hydrolysis, identification and estimation of hexosamines. TCA denatured proteins (1–2 mg/ml) were hydrolysed in 4N HCl in sealed tubes for 8 hr at 100° [22]. Hydrolysates concd *in vacuo* at 50° were taken up in H_2O and separated from interfering chromogens by passage through a column of Dowex 50XW (200–400 mesh, H^+) as in ref. [25]. HCl eluates were dried *in vacuo* at 50°, taken up in H_2O and total hexosamines measured by the method of ref. [26], using the neutralisation procedure of ref. [27] and glucosamine–HCl as standard. Hexosamines were chromatographed by PC on Whatman No. 1 paper, developed in *n*-BuOH–Py–H_2O (9:5:8) for 3 × 3 hr with drying between each stage [28]. Papers were sprayed with *p*-anisidine–EtOH (1% in EtOH–H_2O, 7:3) heated at 105° 10 min, then dipped in 10 ml of 0.1 M Na periodate in 100 ml Me_2CO. Ion exchange chromatography of hexosamines were performed on Dowex 50 XW (200–400 mesh, H^+) by the method of [29] with 1 ml fractions estimated by *p*-dimethylaminobenzaldehyde reagent [26]. Glucosamine–HCl and galactosamine–HCl were chromatographed separately and in mixture as standards. Fractions corresponding to resolved hexosamine peaks were subjected to PC on Whatman No. 1 using Py–EtOAc–HOAc–H_2O (5:5:3:1) solvent and Py–EtOAc–H_2O (11:40:6) vapour phase, using glucosamine–HCl and galactosamine–HCl as standards [30].

Methanolysis and estimation of sugars by GLC. Simultaneous separation and estimation of protein bound neutral sugars and hexosamines were carried out essentially as in ref. [9]. Dry glycoprotein (1–6 mg) together with arabitol and mannitol (0.1 mol each) and 0.5 ml of anhydrous MeOH containing 0.5 M HCl were placed in a glass ampoule, gassed (N_2), sealed and heated to 85° for 24 hr. Acid was neutralised by addition of $(Ag)_2CO_3$ and hexosamines acetylated with 0.05 ml Ac_2O (6 hr room temp.). The contents were triturated, centrifuged, extracted with 3 × 0.5 ml MeOH and the supernatants dried *in vacuo* at 35°. The residue was silylated (TMCS–HMDS–Py 1:1:5) for 30 min at room temp., and the TMSi ethers separated on 150 cm × 4 mm columns packed with 3% SE30, N_2 carrier (30 ml/min) with temp. programming from 130–180°, at 0.5°/min, using dual FID. Absolute quantities were calculated from peak area ratios with reference to previously determined molar response factors for each monosaccharide relative to each of the two int. stand.

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REFERENCES

1. Millerd, A. (1975) *Ann. Rev. Plant Physiol.* **26**, 53.
2. Derbyshire, E., Wright, D. J. and Boulter, D. (1976) *Phytochemistry* **15**, 3.
3. Goding, L. A., Bhatty, R. S. and Finlayson, A. J. (1970) *Can. J. Biochem.* **48**, 1096.
4. Sawai, H. and Morita, Y. (1970) *Agr. Biol. Chem. (Tokyo)* **34**, 53.
5. Sawai, H. and Morita, Y. (1970) *Agr. Biol. Chem. (Tokyo)* **34**, 61.
6. Basha, S. M. M. and Beevers, L. (1976) *Plant Physiol.* **57**, 93.
7. Blagrove, R. J. and Gillespie, J. M. (1975) *Australian. J. Plant Physiol.* **2**, 13.
8. Jermyn, M. A. and Yeow, Y. M. (1975) *Australian J. Plant Physiol.* **2**, 501.
9. Clamp, J. R. (1974) *Biochem. Soc. Symp.* **40**, 3.
10. Pusztai, A. and Watt, W. A. (1970) *Biochim. Biophys. Acta* **207**, 413.
11. Koshiyama, I. (1968) *Cereal Chem.* **45**, 394.
12. Bailey, C. J. and Boulter, D. (1972) *Phytochemistry* **11**, 59.
13. Ericson, M. C. and Chrispeels, M. J. (1973) *Plant Physiol.* **52**, 98.
14. Davis, B. J. (1964) *Ann. NY Acad. Sci.* **121**, 404.
15. Weber, K. and Osborne, M. (1969) *J. Biol. Chem.* **244**, 4406.
16. Zacharius, R. M., Zell, T. E., Morrison, T. H. and Woodcock, J. J. (1969) *Anal. Biochem.* **30**, 148.
17. Stephens, R. E. (1975) *Anal. Biochem.* **65**, 369.
18. Roe, J. H. (1955) *J. Biol. Chem.* **212**, 335.
19. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
20. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
21. Dische, Z. and Shettles, L. (1948) *J. Biol. Chem.* **175**, 595.
22. Spiro, R. G. and Spiro, M. J. (1965) *J. Biol. Chem.* **240**, 997.
23. Hansen, S. A. (1975) *J. Chromatogr.* **107**, 224.
24. Phillips, D. V. and Smith, A. E. (1973) *Anal. Biochem.* **54**, 95.
25. Boas, N. (1953) *J. Biol. Chem.* **204**, 553.
26. Elson, L. A. and Morgan, W. T. J. (1933) *Biochem. J.* **27**, 1824.
27. Rondle, C. J. M. and Morgan, W. T. J. (1955) *Biochem. J.* **61**, 586.
28. Veiga, L. A. and Chandelier, E. L. (1967) *Anal. Biochem.* **20**, 419.
29. Gardell, S. (1953) *Acta Chem. Scand.* **7**, 207.
30. Fischer, F. G. and Nebel, H. J. (1955) *Z. Physiol. Chem.* **302**, 10.