Phytochemistry, 1979, Vol. 18, pp. 1775–1779. © Pergamon Press Ltd. Printed in England. 0031-9422/79/1101-1775 $02.00/0

SUBUNIT GLYCOSYLATION OF *LUPINUS ANGUSTIFOLIUS* SEED GLOBULINS

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(*Revised received* 20 *April* 1979)

Key Word Index—*Lupinus angustifolius*; Leguminosae; seed; seed glycoproteins; polypeptides; carbohydrates.

Abstract—Reduced polypeptide subunits of α-, β- and γ-conglutins from *Lupinus angustifolius* seeds were resolved by preparative SDS gel electrophoresis of the fluorescent labelled proteins, into four, six and two major components, respectively. All subunits were glycosylated, to varying degrees, containing mannose, galactose and glucosamine. The major glycopeptides released by pronase digestion of each conglutin had similar galactose/mannose ratios; the MW of the glycopeptide released from α- and β-conglutin was *ca* 5000. Although on average, each molecule of α-conglutin contains one main oligosaccharide chain, and β-conglutin two, the presence of carbohydrate in all polypeptide subunits suggests that some subunits may arise by proteolytic cleavage of a larger polypeptide after glycosylation. The presence of minor glycopeptide components indicates that modification of carbohydrate chains during seed development may also occur.

INTRODUCTION

The seed storage globulins of many cultivated grain legume species [1, 2] and some non-legumes [3, 4] are glycoproteins containing small amounts of glucosamine and mannose, together with other sugars in some species [3, 5]. The major seed globulins of *Lupinus angustifolius* contain galactose in addition to mannose and glucosamine, the three conglutins varying both in total carbohydrate content and the relative proportions of the component sugars [5].

The seed storage proteins are complex at the subunit level [2] and there is some evidence that the glycosylation of individual subunits may vary considerably. For example, only two of the four subunits of *Phaseolus aureus* vicilin resolved by PAGE were PAS positive [6], and in *Brassica* the GI fraction of the 12S globulin, resolved by Sephadex chromatography in urea, contained most of the carbohydrate [3]. Preferential labelling of particular subunits of *Pisum sativum* legumin and vicilin by glucosamine-[^{14}C] fed during seed development is also suggestive of preferential glycosylation [7].

The present work was carried out to determine the pattern of glycosylation of the major subunits of α-, β- and γ-conglutin. The isolation of individual protein subunits in sufficient quantity for characterization of their glycosylation patterns poses problems, especially when several subunits of similar size are present. Some workers have separated subunits by molecular filtration or ion-exchange chromatography in urea, e.g. carboxymethylated, and non-derivatized *Vicia* legumin, soya bean glycinin, cold-insoluble *Pisum sativum* vicilin, 12S globulins of *Brassica* spp. [3, 8–11]. Greater resolution is afforded by electrophoretic or iso-electric focusing methods, which have been utilized in separating acidic and basic subunits of *Vicia faba* globulin, *Pisum sativum* vicilin and soya bean glycinin [11–13].

In the present work, separation of the polypeptide subunits of *Lupinus* globulin was effected by preparative SDS gel electrophoresis of the fluorescent labelled proteins followed by quantitative methanolysis and GLC of the glycoprotein sugars.

RESULTS AND DISCUSSION

Preparations of α-, β- and γ-conglutins produced by isoelectric precipitation [5] from extracts of *L. angustifolius* seed were homogeneous by cellulose acetate electrophoresis. Analytical SDS–PAGE of the conglutins after reduction with mercaptoethanol revealed the presence of four major polypeptide components in α-conglutin, together with a minor component and some trace bands. An additional main band (α4) was found in α-conglutin preparations which had not been fractionated by DEAE, but was absent after fractionation. Densitometry of the Coomassie blue stained gels indicated that the lightest subunit (α6) was present in amounts 2–3 times greater than any other subunit.

Reduced conglutin β was resolved by SDS–PAGE into (usually) six major components, although in some preparations β5 and β6 were not clearly separated, and in all gels these two components ran close together. Even allowing for differences in colour yield, these two components (β5, β6) were probably present in amounts 2–3 times greater than any other.

The simplest pattern of reduced polypeptides was obtained with γ-conglutin, which gave only two main bands, with a slightly greater amount of the heavier polypeptide.

The pattern of subunit proteins found was essentially similar to that described by Blagrove and Gillespie [14] for a different cultivar of *L. angustifolius*.

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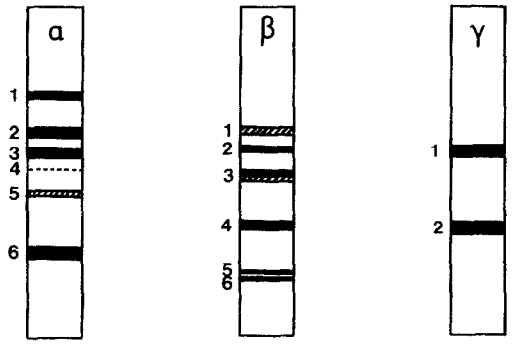

Fig. 1. SDS polyacrylamide disc gel electrophoresis of reduced conglutins.

Preparative separation of conglutin polypeptides

Attempts at preparative scale separation of the polypeptides by chromatography on Sephadex gel or hydroxylapatite were not wholly successful. In both β- and γ-conglutins, when the reduced polypeptides were separated by Sephadex gel filtration in the presence of SDS or urea, a large proportion of the protein emerged in the void volume and as a high MW complex. Less than 30% of the β-conglutin was resolved as the six major polypeptides previously identified by analytical PAGE, and the two polypeptides of γ-conglutin could not be resolved at all, due to the presence of large amounts of carbohydrate material moving in a similar position to the protein on the column. Resolution of the α-conglutin subunits showed no high MW complex, but most of the five polypeptide components were not sufficiently well separated for preparative purposes.

The anomalous chromatographic behaviour of β- and γ-conglutins may have been due to the presence of bound galactomannans which are known to occur in preparations of these proteins [5] and which, after separation from the protein by TCA precipitation, can be shown to occur as a high MW, heterogeneous complex by gel permeation chromatography. The absence of high MW complex formation in α-conglutin is correlated with the absence of galactomannan in preparations of this protein [5].

Preparative electrophoresis was therefore employed to separate the major subunit components of the conglutins, using a fluorochrome tagging technique to enable the polypeptide bands to be located on the gels, for excision, elution and analysis. This technique gave reproducible separations of the lupin conglutin polypeptides in amounts from 1 μg to 1 mg.

The pattern of bands located by fluorescence on the preparative gels, after electrophoresis of the dansylated conglutins (Fig. 1), closely matched the bands located on analytical gels by Coomassie blue staining suggesting that dansylation itself had no effect on the electrophoretic mobility of the polypeptides.

No appreciable contamination by other polypeptides could be detected when the fluorescent bands were excised, eluted, re-run individually on analytical gels and stained with Coomassie blue. Clean separation of β5 and β6 was not always possible, due to their close proximity, although the leading edge of β6 and the trailing edge of β5 were usually taken to minimize cross contamination.

Differences in glycosylation of reduced polypeptides

Analysis of the polypeptide sugars indicated that carbohydrate was associated with each of the component subunits (Table 1). Interference during GLC analysis of the sugars prevented quantitative determination of α-conglutin glucosamine, and therefore of total polypeptide sugar in this component, and it was necessary to allow for a substantial degree of error (> ± 15%) in calculating total carbohydrate values on an anhydrous protein weight basis in β-conglutin, due to the small quantities of protein involved. Nevertheless, it would appear that the carbohydrate content of the β-conglutin subunits was quite varied, with no apparent relationship with polypeptide MW, whereas both γ-conglutin subunits had similar sugar contents.

Specific sugar ratios varied considerably between the

Table 1. Properties of the *Lupinus augustifolius* conglutin subunits

Protein	Subunit	MW $\times 10^{-3}$	PAS reaction	Fluorescence	Man/Gal	Man/GlcN	Gal/GlcN	Total sugar as % anhydrous protein*
α-Conglutin	1	57–70	+	Orange	4.1	†	†	†
	2	50–61	−	Orange	1.2	†	†	†
	3	44–54	+	Orange	3.9	†	†	†
	4	42–51	+	Orange	3.9	†	†	†
	5	29–35	−	Orange	1.8	†	†	†
	6	21–26	+	Yellow	2.6	†	†	†
β-Conglutin	1	51–62	+	Yellow	0.2	0.4	1.8	3–5
	2	44–53	+	Yellow	3.2	0.8	0.2	3–5
	3	36–44	+	Yellow	0.4	0.5	1.1	6–8
	4	25–31	+	Yellow	5.6	2.4	0.4	6–8
	5	18–22	+	Yellow	0.9	0.2	0.3	2–3
	6	17–21	+	Yellow	5.1	3.4	0.7	7–10
γ-Conglutin	1	27–33	+	Orange	1.4	1.7	1.6	2.9
	2	15–18	−	Yellow	2.9	0.2	0.1	2.7

Subunit MW and reaction to PAS were determined on SDS gels after mercaptoethanol reduction. Fluorescence of dansylated reduced polypeptides recorded after preparative SDS gel electrophoresis, before elution of bands for sugar analysis.

* Range of total sugar values in each β-conglutin polypeptide is due to allowance for error in measuring the anhydrous protein weight of the small amounts of polypeptide material available from this conglutin.

† Interference with GlcN determinations prevented full sugar analysis of α-conglutin polypeptides.

Table 2. Mannose/Galactose ratio of the glycopeptides from *Lupinus angustifolius* conglutins

Protein	Glycopeptide peak	Man/gal ratio before ion-exchange	Man/gal ratio after ion-exchange
α-Conglutin	I	4.2	4.0
	II	ND	ND
	III	5.4	4.8
	IV	5.8	ND
β-Conglutin	I	3.0	3.2
	II	ND	ND
	III	6.6	6.6
	IV	9.1	ND
γ-Conglutin	I	⩾100	⩾100
	II	4.2	3.3
	III	2.8	trace

Notation of glycopeptide peaks refers to the separations shown in Fig. 2. ND = Not detected.

individual subunits of all three conglutins, suggesting that some oligosaccharide heterogeneity may be present.

This is the first attempt at quantifying the sugars of individual polypeptide constituents of a complex seed storage globulin and no other data are available for comparison. The results show that all of the subunits bear carbohydrate and thus may differ from mung bean vicilin [6] where it has been suggested, on the basis of Schiffs staining, that only some of the protein bands contain carbohydrate.

Oligosaccharide composition of conglutins

Digestion of TCA denatured conglutins with pronase [15] resulted in peptide bond hydrolysis and the resulting digest contained the oligosaccharide chains of the glycoprotein attached to small but variable amounts of peptide material. In the present study, β-conglutin glycopeptides after separation from amino acid and peptide material (Fig. 2a) were then resolved by Sephadex G50 chromatography (Fig. 2b). The α- and γ-conglutin glycopeptides were separated from digests without prior purification (Fig. 2c, 2d).

The conglutin digests were complex, containing several minor peaks of phenol–H_2SO_4 positive material together with a major glycopeptide. Calibration of the Sephadex column with carbohydrates ranging in MW from 500 to 10000 showed that the major glycopeptides of α- and β-conglutins (α_1 and β_1) were of similar MW (4000, and 5000, respectively). The major glycopeptide of γ-conglutin (γ_{II}) and some minor components gave elution volumes appropriate to a monosaccharide, probably due to some retardation on the column. Reliable MW determinations of these components therefore could not be achieved. The pale yellow colour of these glycopeptide fractions suggests that phenolic material may have been responsible for their anomalous chromatographic behaviour. Both α- and γ-conglutin were pale yellow in concentrated solution even after extensive purification (see [16] also). The protein bodies in which the conglutins are located in the seed contained substantial amounts of flavonoid material (Ryan and Moore, unpublished) which showed a similar shift in λ_{max} with pH, as did the purified α- and γ-conglutins. Some of this protein-bound phenolic material appeared as a pale blue fluorescent spot after TLC separation of the glycoprotein sugars in acid hydrolysates of the conglutins, but the identity of the component and its linkage to α- and γ-conglutins was not established.

The major glycopeptide peaks of α, β and γ (αI, βI and γII) had similar mannose/galactose ratios of 3–4 (Table 2). Peak I of γ conglutin, a minor component, had a very high mannose/galactose ratio (greater than 100),

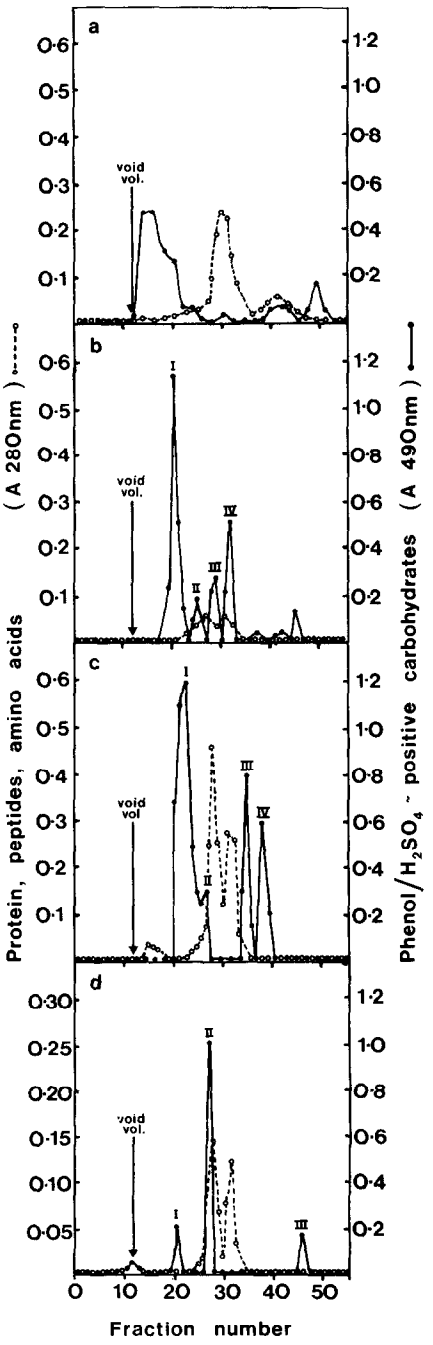

Fig. 2. Resolution of conglutin glycopeptides after pronase digestion. (a) β-Conglutin digest separated on Sephadex G25; (b) resolution of glycopeptide fractions (12–28) from (a) on Sephadex G50; (c) resolution of α-conglutin digest on Sephadex G50; (d) resolution of γ-conglutin digest on Sephadex G50.

whilst the remaining peaks of α, β and γ generally had mannose/galactose ratios varying between 2 and 10. It was not possible to determine sugars in αII and βII due to the very small amounts of material and also to interference. Glucosamine could be identified in GLC traces of αI, βI and γII glycopeptides, but identification of glucosamine in the minor components, and accurate quantitative glucosamine determination in any was prevented by interference, giving GLC base line instability. The possible presence of phenolic material in some glycopeptide fractions may have been responsible.

CONCLUSIONS

All the major reduced subunits of each conglutin bear carbohydrate, though with varying molar sugar ratios, and after digestion the conglutins all released one major glycopeptide, with mannose/galactose in the ratio of 3–4, together with several minor glycopeptide fractions with high mannose/galactose ratios.

By consideration of the MWs of α- and β-conglutins [16] together with their total per cent carbohydrate content [5], the amount of sugar in each 'molecule' of α-conglutin corresponded to a MW of *ca* 5000, and for β-conglutin 11000. The individual molecular size of γ-conglutin is unknown [14] but if the MW is taken to be *ca* 50000, which represents the size of the single unreduced subunits of which this conglutin is composed, the carbohydrate component per 50000 MW subunit would be *ca* MW 1300.

Glycopeptide analysis indicates that both α- and β-conglutin carry an oligosaccharide component of MW *ca* 5000 of similar mannose/galactose ratio, which is close to the ratio of these sugars in the intact protein [5] and in many but not all of the polypeptide subunits. This interpretation would suggest that a single oligosaccharide chain may be present per molecule of α-conglutin, and two per molecule of β-conglutin, providing that all molecules of conglutin are glycosylated.

The anomalous retention of γ-conglutin glycopeptides on Sephadex prevented MW determination, but the main glycopeptide (γII) was similar in mannose/galactose ratio to the main glycopeptides of the other conglutins. If this oligosaccharide is similar in size also, the carbohydrate content of γ-conglutin would allow one oligosaccharide chain to four 50000 subunits of the conglutin.

Measurements of subunit glycosylation show that no single polypeptide can be identified as carrying the conglutin oligosaccharide and indeed none of the subunits of β-conglutin bear sufficient carbohydrate to allow one oligosaccharide chain to each subunit molecule. Presumably only a proportion of the subunit molecules are glycosylated, but it is not possible to determine if some molecules of α- or β-conglutin are glycosylated by the attachment of an oligosaccharide chain of MW 5000 on every subunit, and some not at all, or if a uniform glycosylation of one chain per molecule, with random attachment to individual subunits takes place, or some intermediate situation.

The presence of minor glycopeptide fractions in the pronase hydrolysed conglutin preparations suggests that some smaller oligosaccharide chains are also present, often with mannose/galactose ratios greater than one. This could be due to partial hydrolysis of completed oligosaccharide chains during seed development, by the activity of glycosidases in the protein bodies [17]. Alternatively the heterogeneity may be due to the formation of incomplete or distinct chain types. Interpretation is complicated by the possibility that some of the polypeptide components of α- and β-conglutin may arise during seed development by proteolytic cleavage of larger molecules after glycosylation has taken place. The presence of small polypeptide subunits together with the main subunit in preparations of mung bean vicilin has been interpreted in this way [6], although a considerably greater degree of proteolytic modification would be required to account for the preponderance of small subunits in α- and β-conglutin.

The presence of a larger and more complex carbohydrate moiety on the lupin storage proteins than has been reported in mung bean [6] raises the interesting possibility that the intracellular pathway of storage protein transport and secretion may differ in the two species. It is generally held that glucosamine/mannose core polysaccharides are added in one piece to polypeptides in the rough endoplasmic reticulum, whilst further lengthening, for example by stepwise addition of galactose, occurs in the smooth endoplasmic reticulum and Golgi apparatus [18]. Further investigation of the deposition of lupin storage proteins may therefore help to clarify the, at present, uncertain [19] role of the Golgi apparatus in protein body formation in the developing seed.

EXPERIMENTAL

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

Protein preparation. The 3 major globulins of lupin seeds, α-, β- and γ-conglutin, were extracted from the seed meal and purified as described [5]. Further purification of the conglutins was attempted but only utilized in the case of α-conglutin. In this case; DEAE-cellulose chromatography in 25 mM Na-Pi buffer, pH 7 with a linear increase in salt concn from 25 mM to 0.5 M NaCl, gave a major and minor peak (*ca* 85 and 15%, respectively). PAGE in 7.5% gels [20] showed the peaks to be electrophoretically distinct. With 10% acrylamide–0.1% SDS gels [21], the major peak was shown to contain protein with a greater proportion of less mobile subunits than the minor peak. The major peak was initially utilized in further study, but in later expts, unchromatographed α-conglutin was used. The presence of bound polysaccharide material, and also phenolic compounds, prevented further chromatography of the other two conglutins, which were homogeneous by cellulose acetate electrophoresis [5]. Attention is also drawn to the anomalous chromatographic behaviour of γ-conglutin [14].

Reduction and alkylation of proteins. A modification of the method of ref. [22] was followed after prior removal of adsorbed polysaccharide material [5] with TCA treatment. Protein precipitated by TCA treatment was solubilized and reduced by a method adapted from ref. [23]. Pellets were stirred for 1–5 min at 100° in a soln of 8 M urea–1% v/v 2-mercaptoethanol–1.2% Tris (pH unadjusted). The final protein concn was *ca* 7 mg/ml. Protein solns were cooled and dialysed 18 hr against 8 M urea–0.12 M 2-mercaptoethanol–0.1% EDTA–0.35 M Tris–HCl, pH 8.8, at room temp. Protein solns were then alkylated as described [24] except a 10% excess of alkylating reagent was used. Alkylating reagent consisted of 1.1 M Na iodoacetate–8 M urea added in the proportion of 1 vol. to 9 vols. of protein soln. A 10% excess was then added and solns held in the dark at room temp. for 30 min. After alkylation, the reaction was

quenched by the addition of a calculated excess of 2-mercaptoethanol. The soln was then dialysed for 18 hr, with several changes, against 10 mM Na-Pi buffer, pH 7, at 4°. Solns were then made 0.005% in phenylmethylsulphonylfluoride [25] to prevent possible proteolysis, and SDS then added to 2%. An aliquot of 5–10% of total vol. was then dansylated according to ref. [26] by the addition of 20 μl of a 10% dansyl chloride–Me_2CO soln per ml of protein soln, with vigorous shaking followed by boiling for 3 min. The aliquot was then recombined with the remainder of sample and the whole was heated for 2 min at 90°. The soln was then dialysed against 100 vols. of 2.5 mM Tris–glycine buffer, pH 8.3–0.1% SDS, at room temp., for 18 hr, and finally made 10% in glycerol and frozen at −20° until required.

Preparative polyacrylamide gel electrophoresis. PAGE of reduced, alkylated subunits was carried out by the method of ref. [22] using 7.5% acrylamide gels made in 0.1% SDS. Gels were cast to 1 cm dia and 8 cm long, and 6 such gels were run together. Sample vols. were 0.25 ml of protein soln per gel and electrophoresis was carried out in 25 mM Tris–glycine buffer with 7% SDS, pH 8.3, at 5 mA per gel and 50–100 V. Migration of protein bands was monitored with a 'black' UV lamp and continued until the fluorescent front reached the bottom of the gels. Gels were then removed, zones of major fluorescence cut out with a sharp degreased razor and corresponding zones from each of the gels were stacked into electroelution tubes, as described in ref. [22]. Electroelution concn of the subunits was achieved with 2.5 mM Tris–glycine, pH 8.3, at 1–2 mA per gel tube and 350 V for *ca* 2–3 hr. Fluorescent protein was removed from bottom of dialysis tubing with a syringe and a small aliquot used for analytical gel electrophoresis, the remainder being lyophilized.

Removal of SDS from protein–SDS complexes. It was essential to remove SDS quantitatively to avoid interference during GLC of the methanolysis products. The method of ref. [27] enabled complete removal of SDS from protein before methanolysis. Lyophilized material was dissolved in 6 M urea–0.1 M NH_4HCO_3 to give a 0.1–1.0% SDS concn, passed through a microcolumn (0.5 ml) of Dowex 1 × 2 (200–400 mesh) equilibrated with 6 M urea–0.05 M NH_4HCO_3 and column washed 6 M urea–0.05 M NH_4HCO_3. The eluted protein soln was dialysed thoroughly against H_2O at 4° and lyophilized.

Methanolysis and GLC of subunit sugars. Lyophilized protein was dried over P_2O_5, weighed and to the dried residue was added 0.01–0.1 mol each of arabitol and mannitol (int. standards). After re-drying, 0.5 ml MeOH–1 M HCl was added, gassed with N_2, sealed and incubated at 80°, 24 hr. The remaining procedure was as described in ref. [5].

Analytical SDS-PAGE and Schiffs staining. Disc electrophoresis of isolated polypeptides, and also of whole reduced proteins, was carried out on 7.5% acrylamide gels of similar formulation as that employed in preparative electrophoresis. Bromophenol blue tracker dye was added and electrophoresis performed at 3.5 mA/gel 250 V. Gels were stained with Coomassie blue. Standards of BSA, lysozyme, ovalbumin and trypsin were also electrophoresed and MWs of polypeptide fractions calculated by reference to these. Periodic acid–Schiffs staining of polypeptides was carried out by the method of ref. [28] after electrophoresis of whole reduced proteins.

Glycopeptide analysis. Prepns of α-, β- and γ-conglutins (20–30 mg) in 0.15 M Na-Pi buffer were precipitated at 0° with 5% TCA, centrifuged and the pellet washed with TCA and EtOH (twice). The pellet suspended in 0.05 M Na-Pi buffer (pH 7.4) was digested with pronase (1.5–2 mg) at 37° for 24 hr with vigorous shaking, then for a further 80 hr with addition of a further 1.5 mg pronase. Toluene was included to inhibit microorganisms. Digestion was terminated by heating (100°, 3 min). Digests were applied to long columns (112 × 1 cm) of Sephadex G25 (0.1–0.3 mm) or G50 (20–80 μm) equilibrated with 0.2 M HOAc. Fractions were analysed at 280 nm for proteins, peptides and amino acids, and by phenol–H_2SO_4 treatment for carbohydrates. Fractions comprising the phenol–H_2SO_4 positive peaks were pooled, freed of peptides and amino acids by microbatch treatment with Dowex 50W-X8 resin (H^+, 20–50 mesh), then lyophilized at 50°. Determination of the carbohydrates in the fractions was by GLC separation of the TMSi ethers after methanolysis, as described earlier.

Acknowledgements—This work was supported by an SRC research studentship to C. E. M.

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