

The $\beta 1 \rightarrow 2$ -D-xylose and $\alpha 1 \rightarrow 3$ -L-fucose substituted N-linked oligosaccharides from *Erythrina cristagalli* lectin

Isolation, characterisation and comparison with other legume lectins

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The carbohydrate moieties of *Erythrina cristagalli* lectin were released as oligosaccharides by hydrazinolysis, followed by *N*-acetylation and reduction with NaBH₄. Fractionation of the tritium-labelled oligosaccharide mixture by Bio-Gel P-4 column chromatography and high-voltage borate electrophoresis revealed that it is composed of five neutral oligosaccharides. Structural studies by sequential exoglycosidase digestion in combination with methylation analysis and two-dimensional ¹H-NMR showed that the major component was the fucose-containing heptasaccharide Man α 3(Man α 6)(Xyl β 2)Man β 4GlcNAc β 4(Fuc α 3)GlcNAc. This is the first report of such a structure in plant lectins. Small amounts of the corresponding afucosyl hexasaccharide were also identified, as well as three other minor components.

The structure of the heptasaccharide shows the twin characteristics of a newly established family of N-linked glycans, found to date only in plants. The characteristics are substitution of the common pentasaccharide core [Man α 3(Man α 6)Man β 4GlcNAc β 4GlcNAc] by (1) a D-xylose residue linked $\beta 1 \rightarrow 2$ to the β -mannosyl residue and (2) an L-fucose residue linked $\alpha 1 \rightarrow 3$ to the reducing terminal *N*-acetylglucosamine residue.

The oligosaccharide heterogeneity pattern for *Erythrina cristagalli* lectin was also found for the lectins from four other *Erythrina* species and the lectins of two other legumes, *Sophora japonica* and *Lonchocarpus capassa*.

All species of the genus *Erythrina* tested contain *N*-acetylglucosamine-specific lectins that are remarkably similar in their properties [1, 2]. These molecules are all glycoproteins (3–10% neutral carbohydrate by weight) with *M_r* approximately 60 000 and are composed of two identical or nearly identical subunits. Their amino acid composition is very similar and a high degree of homology exists in the N-terminal sequences. The carbohydrate composition of *Erythrina* lectins shows minor quantitative variations [1–3] but all contain mannose, *N*-acetylglucosamine, fucose and xylose.

The occurrence of fucose and/or xylose has been reported in a number of other legume lectins: *Dolichos biflorus* [4]; *Phaseolus limensis* (Lima bean) [5, 6]; *Griffonia simplicifolia* lectin I [7], lectin II [8] and lectin IV [9]; *Sophora japonica* [10]; *Caragana arborescens* [11]; *Crotalaria juncea* [12]; *Bauhinia purpurea* [13]; *Wisteria floribunda* [14]; *Vicia cracca* [15]; *Phaseolus vulgaris*, cv. Greensleeves [16] and Tora bean [17]; *Onobrychis viciifolia* (sainfoin) [18]; *Lonchocarpus capassa* [19]. However, with the exception of the lectins from Lima bean, Tora bean and sainfoin, for which partial structures have been proposed [6, 17, 18], no information is available on the structure of the oligosaccharide units of these lectins. Structural data has been reported for the xylose- and fucose-containing oligosaccharides from pineapple stem bromelain

[20] and more recently for sycamore cell culture laccase [21] and *Caesalpinia pulcherrima* protease inhibitor [22].

The simultaneous presence of xylose and fucose therefore appears to be a common feature of many plant glycoproteins. This combination has only been described in glycoproteins of animal origin for *Helix pomatia* α -haemocyanin [23].

In this paper we describe the isolation of the asparagine-linked oligosaccharides of *E. cristagalli* lectin, their separation by high-resolution gel permeation chromatography and high-voltage electrophoresis and their characterisation by a combination of methylation analysis, nuclear magnetic resonance and sequential exoglycosidase digestion. We also compare the oligosaccharides of *E. cristagalli* lectin with those of the lectins from four other species of *Erythrina* (*E. corallodendron*, *E. caffra*, *E. latissima* and *E. lysistemon*) and the unrelated legumes *Lonchocarpus capassa* and *Sophora japonica*.

EXPERIMENTAL PROCEDURES

Chemicals

Bio-Gel P-4 (– 400 mesh), Dowex AG-50 X12, AG-3 X4A and Chelex-100 were obtained from Bio-Rad Laboratories Ltd (Watford, Hertfordshire, UK). QAE-Sephadex A-25 was obtained from Pharmacia (Great Britain) Ltd (Milton Keynes, Buckinghamshire, UK).

Proteins and enzymes

Erythrina and *Lonchocarpus capassa* lectins were isolated from the seeds of the respective plant by affinity chromatog-

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Abbreviations. GlcNAc, *N*-acetylglucosamine; COSY, ¹H-¹H correlated spectroscopy.

Note. All sugars are of the D configuration, except fucose which is L.

raphy [3, 19]. *Sophora japonica* lectin, pronase and α -fucosidase from bovine epididymis were purchased from Sigma Chemical Co. (Poole, Dorset, UK). Sialidase (neuraminidase) from *Arthrobacter ureafaciens* (Calbiochem) was purchased from Cambridge Bioscience (Cambridge, UK). β -Mannosidase from *Achatina fulica* was a gift of Seikagaku Kogyo Co. (Tokyo, Japan). The following enzymes were purified by modifications of the published procedures; *Aspergillus saitoi* $\alpha(1 \rightarrow 2)$ -mannosidase [24], α -mannosidase and β -N-acetylhexosaminidase from *Canavalia ensiformis* (jack bean) [25] and α -fucosidase and β -xylosidase from *Charonia lampas* [26, 27]. β -Xylosidase from almonds was purified from almond β -glucosidase (Sigma) by gel filtration and cation-exchange chromatography (Ashford, D., Dwek, R. A. and Rademacher, T. W., unpublished data).

Radioactivity

NaB^3H_4 (10 Ci/mmol) was purchased from New England Nuclear (Stevenage, Hertfordshire, UK). Tritium-labelled oligosaccharides in solution were measured by scintillation counting in an LKB 1210 Ultrabeta counter (LKB Instruments Ltd, Croydon, Surrey, UK) and detected on paper chromatograms and electrophoretograms by either a Packard 7220 radiochromatogram scanner (Packard Instruments Ltd, Caversham, Berkshire, UK) or a Berthold LB 2832 linear analyser fitted with a 30-cm detector head (Lab-Impex Ltd, Twickenham, Middlesex, UK).

Exoglycosidase digestion

Digestion of tritium-labelled oligosaccharides (approximately 2×10^5 cpm) with exoglycosidases with defined specificities were carried out under the following conditions: $\alpha(1 \rightarrow 2)$ -mannosidase (*A. saitoi*), 10 μl of 5 $\mu\text{g ml}^{-1}$ in 0.1 M sodium acetate, pH 5.0; α -mannosidase (jack bean), 100–1000 mU in 10 μl 0.1 M sodium acetate, pH 5.0; α -fucosidase (bovine epididymis), 20 mU in 10 μl 0.2 M citrate/phosphate buffer, pH 6.0; α -fucosidase (*Charonia lampas*), 100 mU in 10 μl 0.1 M sodium acetate, pH 4.0 containing 0.1 M sodium chloride; β -xylosidase (almond), 30 mU in 10 μl 0.1 M sodium acetate, pH 4.0; β -xylosidase (*C. lampas*), 400 mU in 10 μl 0.1 M sodium acetate, pH 4.0 containing 0.1 M sodium chloride; β -mannosidase, 20 mU in 10 μl 0.1 M sodium acetate, pH 5.0 containing 0.2 M sodium chloride; β -N-acetylhexosaminidase, 100 mU in 10 μl 0.1 M sodium citrate, pH 5.0; sialidase, 100 mU in 10 μl 0.1 M sodium acetate, pH 5.0. In all the above experiments, one unit of glycosidase was defined as the amount of enzyme that, in control experiments, released 1 μmol 4-nitrophenol from the respective 4-nitrophenyl glycoside/min at 37°C. All incubations were carried out for 18 h at 37°C under toluene, and were terminated by heating at 100°C for 2 min.

Isolation of oligosaccharides

Lectins (1–5 mg) were dialysed exhaustively against distilled water (4°C) and cryogenically dried over activated charcoal at -196°C (< 0.1 Pa). Asparagine-linked oligosaccharides were released by treatment with fresh double-vacuum-distilled anhydrous hydrazine (25°C, 1.3 kPa), in sealed glass tubes under argon, maintaining strictly anhydrous conditions. The temperature of the reaction was raised from 30°C to 85°C at 10°C h^{-1} and then maintained at 85°C for a further 10–12 h. Hydrazine was

removed by evaporation under reduced pressure at 25°C followed by repeated co-evaporation (five times) with anhydrous toluene. Re-N-acetylation was carried out with a fivefold molar excess of acetic anhydride in saturated NaHCO_3 for 10 min at 0°C and then 50 min at room temperature. Sodium ions were removed by passage through Dowex AG-50 X12 (H^+ form). The eluate was taken to dryness (27°C), redissolved in a minimum of water and applied to Whatman 3MM chromatography paper. After development for 48 h at 27°C with butan-1-ol/ethanol/water (4:1:1, v/v/v) the oligosaccharides were recovered from the first 5 cm from the origin of the paper by elution with water. The samples were then evaporated to dryness, redissolved in 1 ml water and stored at -20°C .

Reduction of oligosaccharides

Preliminary reduction experiments (Amatayakul, S., Dwek, R. A. and Rademacher, T. W., unpublished data) have shown that pH of the medium is important for efficient incorporation of radioactivity from NaB^3H_4 into oligosaccharides and the degree of epimerisation at carbon-2 and 'peeling' of the reducing terminal residue. These effects are dependent on the substitution pattern of the reducing terminal residues.

Since the substitution patterns of the reducing terminal residues of the oligosaccharides of *E. cristagalli* lectin were unknown, the optimum pH for reduction of the oligosaccharides released by hydrazinolysis was determined as follows. Equal aliquots of oligosaccharide mixture (equivalent to 0.5 mg of lectin) containing 2 μCi [^3H]lactitol were incubated at 30°C with a fivefold excess of NaB^3H_4 (6 mM) in 0.05 M NaOH adjusted to the required pH with boric acid. After 4 h an equal volume of 1 M NaB^2H_4 in buffered 0.05 M NaOH was added and the reaction continued for a further 2 h. The reduction was terminated by acidification with 1 M acetic acid and the mixture was passed through Dowex AG-50 X12 (H^+ form) and concentrated to dryness. The residue was freed from boric acid by repeated evaporation (five times) with methanol, dissolved in water and subjected to paper chromatography as described above. Radioactivity was detected by radiochromatogram scanning. After 60 h, when [^3H]lactitol was well resolved from the reduced oligosaccharides, each radioactive region was eluted, concentrated and the reduction efficiency was calculated from the ratio of the radioactivity of the reduced oligosaccharide to that of the [^3H]lactitol internal standard.

The incorporation of radioactivity when plotted against pH was a bell-shaped curve that peaked between pH 10.5 and 11.5. Borate electrophoresis of an acid hydrolysate (see below) showed no detectable epimerisation of N-acetylglucosaminitol to N-acetylmannosaminitol under the peak conditions. Bio-Gel P-4 chromatography also showed that these conditions were maximal for recovery of intact oligosaccharides (i.e. no loss of fucose or 'peeling' of the reducing terminal residue). Reduction of oligosaccharides in subsequent experiments was therefore carried out between pH 10.9 and 11.2. Labelling for radio-analytical procedures was carried out with 6 mM NaB^3H_4 (10 Ci/mmol) and 1 mM oligosaccharide as described above. Reduction prior to methylation and NMR was carried out with 0.1 M NaB^2H_4 for 4 h at 30°C. Tracer amounts of [^3H]oligosaccharitol were added to the material reduced with NaB^2H_4 to simplify detection during the subsequent separation procedures.

Separation of neutral oligosaccharides

Reduced oligosaccharides were applied to Whatman 3MM paper and electrophoresed for 45 min at 80 V cm^{-1} in pyridine/acetic acid/water (3:1:387, v/v/v) pH 5.4. Radioactivity was detected by the linear analyser. Neutral oligosaccharides remaining at the point of application were eluted with water, passed through a column containing 0.1 ml bed volume of each of Chelex 100, Dowex AG-50 X12 (H^+ form) Dowex AG-3 X4A (OH^- form) and QAE-Sephadex A-25. The eluate was filtered through a $0.5\text{-}\mu\text{m}$ teflon filter (Millex SR, Millipore, Harrow, Middlesex, UK), evaporated to dryness and taken up in 100–200 μl water containing 0.5 mg of a mixture of isomalto-oligosaccharides which were produced by partial acid hydrolysis of dextran (this addition was omitted for samples that were intended for monosaccharide composition, NMR or methylation analysis). This solution was applied to a high-resolution gel filtration system comprising two columns of Bio-Gel P-4 (-400 mesh, $1.5 \text{ cm} \times 100 \text{ cm}$) in series. The columns were maintained at 55°C and were eluted with water. The effluent was monitored by a Berthold HPLC radioactivity flow monitor (model LB503, Lab-Impex) and an Erma refractive index monitor (model ERC7510, HPLC Technology Ltd, Macclesfield, Cheshire, UK) prior to collection. Analog signals from these instruments were digitized using a Nelson Analytical ADC interface and the digital values were collected and analysed by computer (model 9836C, Hewlett-Packard, Winnersh, Berkshire, UK).

Pooled oligosaccharides from Bio-Gel P-4 were lyophilised, redissolved in a minimum of water and then electrophoresed on Whatman 1 paper for 5 h at 8.5 kV m^{-1} in 15 mM sodium tetraborate, pH 9.5 using a custom-built 1.2-m flat-bed high-voltage electrophoresis unit (Locarte, London, UK). Radioactive regions detected by the linear analyser were eluted and evaporated to dryness. The samples were then dissolved, passed through Dowex AG-50 X12 (H^+ form) and evaporated repeatedly (five times) with 1% (v/v) acetic acid in methanol and once with water, to remove buffer salts. The samples were then evaporated to dryness, redissolved in 1 ml H_2O and stored at -20°C .

Carbohydrate composition

Composition of oligosaccharides was determined by GLC either after hydrolysis in 1 M HCl for 3 h at 100°C and conversion to per-*O*-acetylated alditols by reduction in 10 mg/ml NaBH_4 for 3 h at 25°C and acetylation in acetic anhydride for 4 h at 100°C , or as per-*O*-trimethylsilyl derivatives following methanolysis [28] or by the radio-electrophoretic method [29]. The identity of the reducing terminal monosaccharide was determined using the radio-electrophoretic method omitting the reduction step and after double hydrolysis in 1 M HCl (see above) of the tritium-labelled oligosaccharide.

Methylation analysis

Oligosaccharides reduced with NaB^2H_4 were permethylated by a modification of the method of Hakomori [30] involving two preliminary treatments prior to the main methylation.

Oligosaccharitol (50–100 nmol) was dissolved in 20 μl Me_2SO and 10 μl (10–20 μmol) sodium methylsulphonyl carbanion solution was added. The mixture was sonicated for

5 min, cooled to 0°C before addition of 5 μl (80 μmol) iodomethane and sonication for a further 5 min. These additions were repeated. For the main methylation 40 μl sodium methylsulphonyl carbanion solution was added and the mixture sonicated for 10 min. After cooling to 0°C , iodomethane (15 μl) was added and the mixture sonicated for 10 min.

The permethylated oligosaccharitol was recovered in the organic phase after partitioning following the addition of 900 μl chloroform/methanol (2:1, v/v) and 600 μl water and thorough mixing. Further isolation procedures, hydrolysis, reduction and acetylation were as described by Liang et al. [31].

Analysis of the partially methylated alditol acetates was performed on a Hewlett Packard 5996C GLC-MS system fitted with on column injection and flame ionisation detection. Separation was by capillary GLC on both bonded OV17 ($0.32 \text{ mm} \times 50 \text{ m}$, Alltech Associates, Carnforth, Lancashire, UK) and SP1000 ($0.25 \text{ mm} \times 50 \text{ m}$, Supelco, R. B. Radley and Co. Ltd, Sawbridgeworth, Hertfordshire, UK) stationary phases. The temperature programs were $90-200^\circ\text{C}$ at $20^\circ\text{C min}^{-1}$ and $90-240^\circ\text{C}$ at $10^\circ\text{C min}^{-1}$, for OV17 and SP1000 respectively. Identification was based on retention time and mass spectrum by comparison with synthetic reference compounds or published data. Quantification was carried out by calculation from the flame ionisation detector response using the effective carbon response value calculated for that compound according to Ackman, as given by Sweet et al. [32], or the molar response measured for the authentic compound.

Preparation of glycopeptides from *Erythrina corallodendron* lectin

Denatured lectin (40 mg/ml) was subjected to exhaustive digestion with pronase (5% w/w of lectin) in 1 M Tris/HCl, pH 8.5 containing 60 mM CaCl_2 at 52°C for 48 h. Another aliquot of pronase (1–2% w/w of lectin) was added and incubation continued for a further 24 h. The mixture was then centrifuged and separated on a column ($1.8 \times 140 \text{ cm}$) of Bio-Gel P-4, equilibrated and eluted with 0.1% (v/v) acetic acid. The fractions were monitored for carbohydrate content by the phenol/sulphuric acid method [33].

The major glycopeptide (65% of recovered carbohydrate) was further purified by reverse-phase HPLC on Waters apparatus (Waters, Milford, MA) employing a Lichrosorb RP-18 (Merck, Darmstadt, FRG) column. Elution was carried out with a linear gradient of 5–55% acetonitrile in 25 mM ammonium acetate, pH 6.4. Two major peaks in approximate ratio 2:1 (based on absorbance at 220 nm) were obtained, only the first of which was examined.

^1H -NMR spectroscopy

Samples were prepared for NMR studies as described previously [34]. Two-dimensional COSY experiments were performed at a sample concentration of 100 μM and a probe temperature of 303 K on a custom-built 500-MHz spectrometer. A total of 512 t_1 increments were recorded with a sweep width of $\pm 1300 \text{ Hz}$ and 1024 real data points in t_2 . Phase-sensitive data processing was achieved using the method of Müller and Ernst [35] and States et al. [36]. Both positive and negative contour levels were plotted simultaneously. The numbering of core residues in oligosaccharide A1 are as given for the biantennary complex oligosaccharide of transferrin [34].

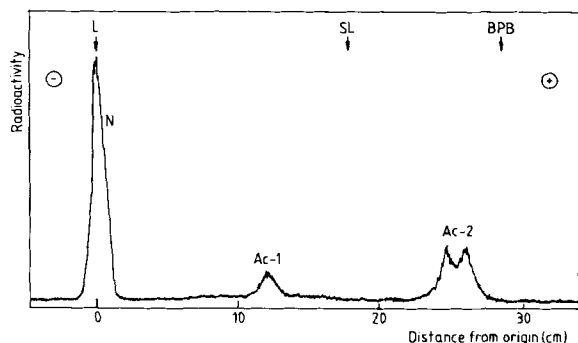


Fig. 1. High-voltage paper electrophoresis of *E. cristagalli* lectin oligosaccharides. Tritium-labelled *E. cristagalli* lectin oligosaccharides were subjected to high-voltage paper electrophoresis (80 V cm^{-1}) in pyridine/acetic acid/water (3:1:387, v/v/v) pH 5.4. The arrows indicate the positions of [^3H]lactitol (L), 6'(3')-sialyl-[^3H]lactitol (SL) and bromophenol blue (BPB) markers

RESULTS

Oligosaccharides isolated from *Erythrina cristagalli* lectin

High-voltage electrophoresis of the oligosaccharides obtained by treatment of *E. cristagalli* lectin with hydrazine, followed by acetylation and reduction with NaB^3H_4 , demonstrated three components (Fig. 1). The major component comigrated with lactose and was therefore neutral. The other components were negatively charged and migrated relative to 6'(3')-sialyllactose (R_{SL}) with values of 0.67 for Ac-1 and 1.37–1.46 for Ac-2.

The neutral fraction was subjected to gel-permeation chromatography on Bio-Gel P-4. This procedure, in which oligosaccharides are separated by differences in hydrodynamic volumes, afforded three components (Fig. 2). Peak A was eluted at 8.8 glucose (Glc) units compared to internal isomalto-oligosaccharide standards. Oligosaccharides in peak B were eluted at approximately 8 Glc units and those in peak C at 5.8. For further fractionation, oligosaccharides were pooled as marked in Fig. 2. High-voltage borate electrophoresis at pH 9.5 of pool A yielded a single component, A1 (Fig. 3a). After elution of this compound from paper and rechromatography on Bio-Gel P-4 a single homogeneous peak at 8.8 Glc units was obtained (data not shown). Pool B from Bio-Gel P-4 yielded two components on borate electrophoresis, B1 and B2 (plus some A1 overlapping from the A peak; see Fig. 3b). Upon rechromatography on Bio-Gel P-4, these components were eluted as symmetrical peaks of 8.1 and 8.0 Glc units, respectively. Pool C also contained two components, C1 and C2 (Fig. 3c) both of which when rechromatographed on Bio-Gel P-4 were eluted at 5.8 Glc units. The yields of the different neutral oligosaccharides are given in Table 1. This table also presents the monosaccharide composition on the intact lectin and the isolated reduced oligosaccharides.

Paper electrophoresis in borate buffer of the *N*-acetylation products of an acid hydrolysate of oligosaccharide A1 revealed the presence of a single radioactive component which comigrated with *N*-acetylglucosaminitol. Similar analysis of oligosaccharides B1 and C1 gave the same result, whereas in the case of C2 a component that comigrated with *N*-acetylmannosaminitol (R_{GlcNAcol} 0.92) was detected instead. The radioactive compound released from B2 did not comigrate with either *N*-acetylglucosaminitol or *N*-acetylmannosaminitol and could not be unambiguously identified. The

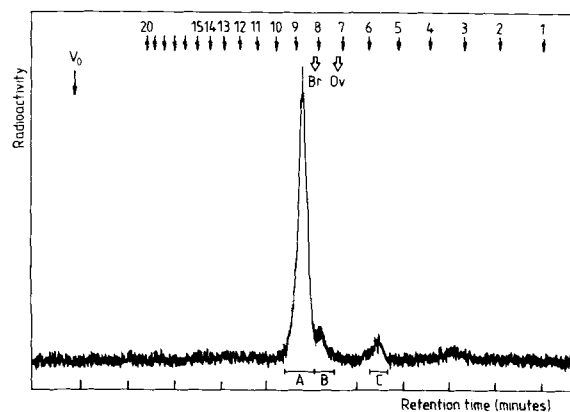


Fig. 2. Gel filtration of *E. cristagalli* lectin neutral oligosaccharides. Neutral oligosaccharides from pyridine electrophoresis were separated by high-resolution gel filtration on Bio-Gel P-4 as described in Experimental Procedures. The solid arrows indicate the elution position of glucose and isomalto-oligosaccharides containing the corresponding number of glucose units. The open arrows indicate the elution position of $\text{Man}\alpha 6(\text{Xyl}\beta 2)\text{Man}\beta 4\text{GlcNAc}\beta 4(\text{Fuc}\alpha 3)\text{GlcNAcol}$ (Br) from pineapple stem bromelain and $\text{Man}\alpha 3(\text{Man}\alpha 6)\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAcol}$ (Ov) from hen egg albumin, respectively. The time axis is marked at 100-min intervals and the bars indicate the areas that were pooled

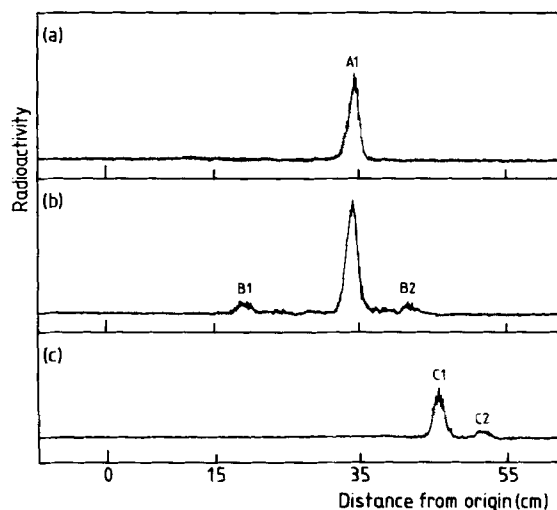


Fig. 3. Borate electrophoresis of *E. cristagalli* lectin neutral oligosaccharides. Pools from Bio-Gel P-4 were subjected to high-voltage paper electrophoresis in 15 mM sodium borate buffer pH 9.5 as described in Experimental Procedures. (a) Pool A; (b) Pool B; (c) Pool C. After separation, purified oligosaccharides A1, B1, B2, C1 and C2 were rechromatographed on Bio-Gel P-4 to determine precisely their elution position (in Glc units): oligosaccharide A1 ran at 8.8, B1 at 8.1, B2 at 8.0, C1 and C2 both at 5.8. These elution positions were calculated by cubic spline interpolation between the internal standard isomalto-oligosaccharide elution positions

above data suggest that B1 is the afucosyl form of A1. This is consistent with the difference in size as determined by Bio-Gel P-4. Oligosaccharide B2 has a similar composition to A1 except for the low glucosamine value, which suggests that one of these residues may be absent in the former component.

Carbohydrate analysis of the charged moieties Ac-1 and Ac-2 isolated by paper electrophoresis indicates that they contain xylose as the major component as well as arabinose, mannose, galactose, glucose and *N*-acetylglucosamine. The negative charge was not removed by treatment with sialidase,

Table 1. *Compositional analysis of neutral oligosaccharides from Erythrina cristagalli lectin*

Values are expressed as molar ratios relative to Man = 3.0. Data for the intact lectin are taken from [2]. Values for A1 were determined by GLC of the per-*O*-acetylated alditols, those for B1 and B2 by GLC of the per-*O*-trimethylsilyl methyl glycosides, those for C1 and C2 by the radio-electrophoretic method [28]

Monosaccharide	Intact lectin	Oligosaccharide				
		A1	B1	B2	C1	C2
Fuc	0.8	0.7	0.0	0.4	0.0	0.0
Xyl	0.8	0.9	1.3	1.1	1.0	1.0
Man	3.0	3.0	3.0	3.0	3.0	3.0
GlcNAc	1.6	2.1	1.9	0.7	1.0	0.0
ManNAc	—	—	—	—	0.0	1.0
Relative amount (%)	—	89.7	2.3	2.1	4.0	1.9

Table 2. *Methylation analysis of oligosaccharide A1*

Molar ratios are expressed relative to 4-*O*-methyl mannitol = 1.0

Methylated alditol acetate	Molar ratio on		Deduced position in oligosaccharide
	OV17	SP1000	
Xylitol			
2,3,4-tri- <i>O</i> -Me	— ^a	0.2 ^b	non-reducing terminal
Fucitol			
2,3,4-tri- <i>O</i> -Me	0.5 ^b	0.6 ^b	non-reducing terminal
Mannitol			
2,3,4,6-tetra- <i>O</i> -Me	1.9	2.4	non-reducing terminal
4- <i>O</i> -Me	1.0	1.0	internal: 2,3,6-trisubstituted
<i>N</i> -Acetylglucosaminitol			
3,6-di- <i>O</i> -Me	1.0	— ^c	internal: 4-substituted
1,5,6-tri- <i>O</i> -Me	1.1	1.2	reducing terminal: 3,4-disubstituted

^a 2,3,4-Tri-*O*-methyl xylitol could not be quantified on the OV-17 column due to co-elution with a reagent peak under the conditions given in Experimental Procedures.

^b Less than integral values are seen for these residues due to destruction during hydrolysis. No other partially methylated derivatives of xylose or fucose were seen in the analysis.

^c 3,6-Di-*O*-methyl *N*-acetylglucosaminitol was not recovered from the SP-1000 column.

alkaline phosphatase or chemical desulphation by solvolysis or methanolic HCl (data not shown). Borate electrophoresis of an acetylated acid hydrolysate of Ac-2 showed that the radioactive component retained the negative charge. Although the fractions Ac-1 and Ac-2 together comprise approximately 20% of the total radioactivity labelled material, they were not further investigated in this study.

Methylation analysis of oligosaccharide A1

From Table 2 it can be seen that this oligosaccharide has several distinct structural features. Firstly, there were four non-reducing termini in this heptasaccharide, two were mannose and one each of fucose and xylose. Secondly, the occurrence of 4-*O*-methylmannitolpenta-acetate (shown to be exclusively this isomer by the use of NaB²H₄ for the reduction) indicated that a single mannose residue was substituted

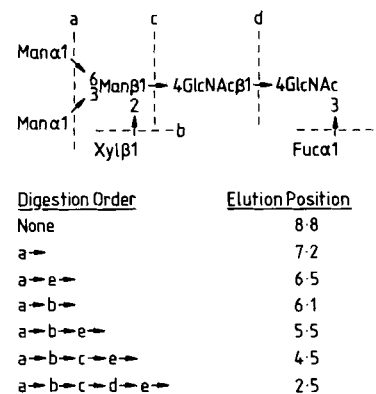


Fig. 4. *Sequential exoglycosidase treatment of E. cristagalli lectin oligosaccharide A1*. Oligosaccharide A1 was enzymatically treated with: (a) jack bean α -mannosidase; (b) almond or *Charonia lampas* β -xylosidase; (c) *Achatina fulica* β -mannosidase; (d) jack bean β -hexosaminidase; (e) *Charonia lampas* α -fucosidase. In cases where more than one enzymatic treatment is indicated, the reactions were not performed simultaneously but sequentially. The elution positions on Bio-Gel P-4 after enzyme treatment were determined as described in Fig. 2 and are indicated above (in Glc units). In all cases the indicated product of enzymatic digestion accounted for > 95% of the recovered radioactivity

at positions 2, 3 and 6. Thirdly, the reducing terminal *N*-acetylglucosamine was substituted at both positions 3 and 4.

Many of these features have been reported in other xylose- and fucose-containing *N*-linked oligosaccharides [17, 20–23, 37]. However, the 3,4-disubstituted reducing terminal *N*-acetylglucosamine residue (first reported by Fukuda et al. [38]) appears to be unique to plant oligosaccharides of this type. In *Helix pomatia* α -haemocyanin the *N*-acetylglucosaminitol is 4,6-disubstituted [23].

Exoglycosidase digestion of oligosaccharide A1

The terminal mannose residues of oligosaccharide A1 were not removed by treatment with *Aspergillus saitoi* α (1→2)-mannosidase indicating that neither of these residues were α -linked to the 2-position of the 2,3,6-tri-*O*-substituted mannose residue [24]. They were, however, removed by jack bean α -mannosidase (Fig. 4, reaction a). The terminal fucose residue was susceptible to digestion with *Charonia lampas* α -fucosidase (Fig. 4, reaction e) but not to bovine epididymal α -fucosidase. Surprisingly, the terminal xylose residue was resistant to digestion with either almond or *C. lampas* β -xylosidases in the complete oligosaccharide but could be digested with these glycosidases following jack bean α -mannosidase treatment. The product of these digestions was susceptible to *Achatina fulica* β -mannosidase (Fig. 4, reaction c) and, subsequent to this treatment, to jack bean β -hexosaminidase (Fig. 4, reaction d). By comparing the initial and final elution positions (Fig. 4) and the results of the methylation analysis (Table 2), the fragmentation which resulted from the sequential exoglycosidase digestion is consistent with the structure given in Fig. 4 when the specificities of the individual exoglycosidases were taken into account.

¹H-NMR spectroscopy of oligosaccharide A1

To resolve any ambiguity in the structure of oligosaccharide A1, additional evidence was obtained from 500-MHz ¹H-

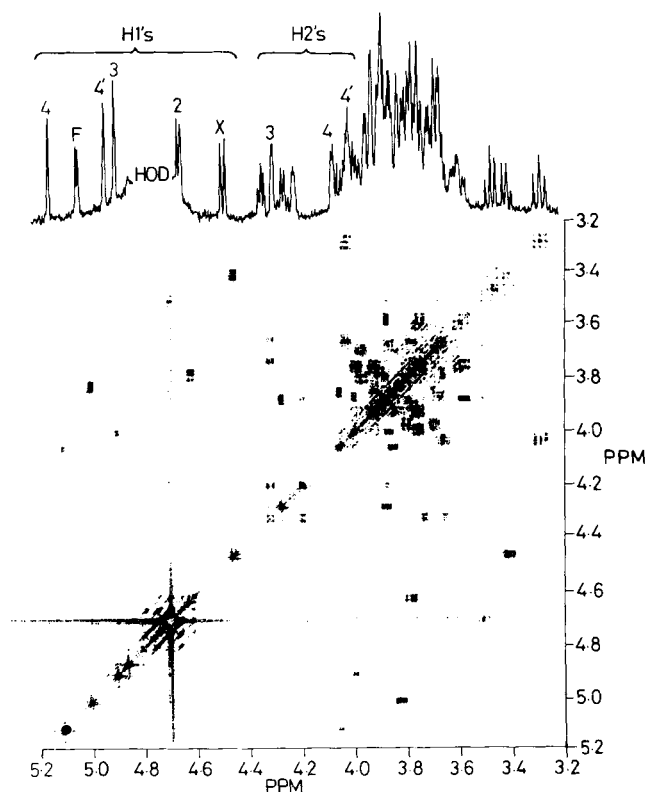


Fig. 5. Contour plot of ^1H - ^1H correlated NMR spectrum of oligosaccharide A1. The off-diagonal peaks correlate J -coupled protons which lie along the main diagonal. The one-dimensional spectrum is shown above with the assignment of the individual proton resonances referred to in the text. The strong resonance at around 4.7 ppm is of residual HO^2H

NMR spectroscopy. The ^1H - ^1H phase-sensitive correlated (COSY) spectrum together with the one-dimensional spectrum are shown in Fig. 5. The chemical shifts of the 'reporter resonances' derived from Man-3 ($\text{H1} = 4.87$ ppm, $\text{H2} = 4.27$ ppm), Man-4 ($\text{H1} = 5.12$ ppm, $\text{H2} = 4.03$ ppm) and Man-4' ($\text{H1} = 4.91$ ppm, $\text{H2} = 3.98$ ppm) are similar to those reported previously for the xylose-containing oligosaccharides of *Helix pomatia* α -haemocyanin [23] and sycamore laccase [21]. The H1 (4.45 ppm), H2 (3.38 ppm), H3 (3.44 ppm) and H5_{ax} (3.25 ppm), signals of xylose in oligosaccharide A1 are also very similar to those reported [21, 23, 37]. These data confirm that the partial core structure of oligosaccharide A1 is the hexasaccharide $\text{Man}\alpha 3(\text{Man}\alpha 6)(\text{Xyl}\beta 2)\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc}$ ol. Additional resonances were observed in the present study at 5.02 ppm, 4.23 ppm and 4.19 ppm. The first resonance is a doublet with a splitting of 3.8 Hz and is characteristic of α -L-fucose H1. The second resonance can be assigned to fucose H5. These resonances together with the characteristic doublet of fucose methyl at approximately 1.2 ppm ($J = 6.7$ Hz, data not shown) indicate that the core of the hexasaccharide is substituted with an α -fucose residue. However, the chemical shifts of fucose H1 (5.02 ppm) and H5 (4.23 ppm) are different from those of an $\alpha(1\rightarrow 6)$ linkage to GlcNAc-1ol which are at 4.90 ppm (H1) and 4.08 ppm (H5) [23]. The resonance at 4.19 ppm bears a resemblance to a mannose H2 proton but the connectivities in the COSY plot indicate that this resonance is coupled to GlcNAc-1ol H2 and can therefore be assigned to GlcNAc-1ol H3. This together with the 'anomalous' shifts of the fucose

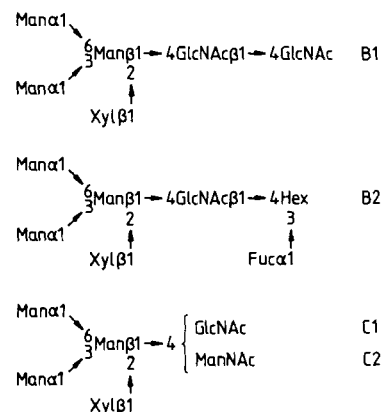


Fig. 6. The structures of neutral oligosaccharides B1, B2, C1 and C2. Hex in B2 refers to an unidentified hexose residue

H1 and H5 strongly suggests that GlcNAc-1ol is substituted by fucose in an $\alpha(1\rightarrow 3)$ linkage. Our data for fucosyl $\alpha(1\rightarrow 3)$ -substituted GlcNAc are in agreement with those reported recently by Van Kuik et al. [37] for $\text{Man}\alpha 6(\text{Xyl}\beta 2)\text{Man}\beta 4\text{GlcNAc}\beta 4(\text{Fuca}3)\text{GlcNAc}$ ol with one exception. These authors assign the two coupled resonances at 4.19 ppm and 4.31 ppm to GlcNAc H2 and H3 respectively. The data obtained by us would indicate that the resonance positions of GlcNAc H2 and H3 should be reversed. This is based on the characteristic cross-peak pattern observed in COSY spectra of oligosaccharides bearing GlcNAc. In these structures, the H2 of GlcNAc is coupled to three other protons, H1, H1' and H3, and the resonance at 4.31 ppm in Fig. 5 conforms to this pattern.

The structure of oligosaccharides B1, B2, C1 and C2

The quantities of oligosaccharides B1, B2, C1 and C2 were insufficient for full chemical analysis of structure. Methylation analysis, however, indicated that each oligosaccharide contained terminal mannose and xylose residues and 2,3,6-trisubstituted mannose. Exoglycosidase sequencing showed that all oligosaccharides behaved in the same manner as oligosaccharide A1 with respect to their susceptibility to α -mannosidase and β -xylosidase digestion. These results suggest that the partial structure $\text{Man}\alpha 3(\text{Man}\alpha 6)(\text{Xyl}\beta 2)\text{Man}$ found in A1 is common to B1, B2, C1 and C2. Also the fucose residue in B2 was only susceptible to *C. lampas* α -fucosidase suggesting by analogy with A1 that it is $\alpha(1\rightarrow 3)$ -linked. The proposed structures for oligosaccharides B1, B2, C1 and C2 given in Fig. 6 are consistent with the above data, the monosaccharide composition (Table 1) and reducing terminal monosaccharide analysis.

Intragenus comparison of lectin oligosaccharides

The purified lectins from *E. latissima*, *E. caffra*, *E. lysistemon* and *E. corallodendron* were treated with hydrazine, reduced with NaB^3H_4 and subjected to paper electrophoresis at pH 5.4 (data not shown). The lectin from *E. cristagalli* was included as control. Only the latter contained any significant amounts of negatively charged material. Bio-Gel P-4 analysis of the neutral oligosaccharides from the lectin of each species, gave similar patterns (Fig. 7a-e). In each case, the majority of radioactive material (63–79%) was eluted at 8.8 Glc units. A, B and C pools were made from each column experiment

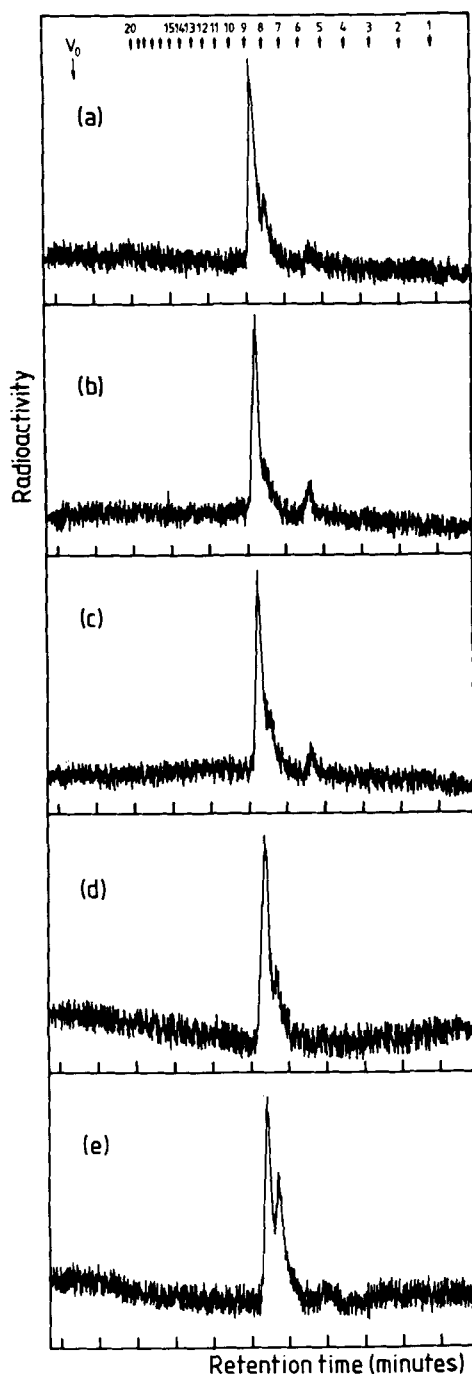


Fig. 7. Elution profiles of the neutral oligosaccharides from the lectins of five *Erythrina* species on Bio-Gel P-4. The lectins obtained from (a) *E. cristagalli*, (b) *E. lysistemon*, (c) *E. corallodendron*, (d) *E. caffra* and (e) *E. latissima* were subjected to hydrazinolysis and the isolated oligosaccharides were reduced, purified and separated on Bio-Gel P-4 as described in Experimental Procedures. For details of the annotation see Fig. 2

and subjected to borate paper electrophoresis. The electrophoresis profiles for *E. cristagalli*, *E. caffra*, *E. lysistemon* and *E. corallodendron* were similar although the proportions of A1, B1, B2, C1 and C2 varied (Fig. 8a–d). The profile for *E. latissima* lectin (Fig. 8e) was notably different from the others. Peak 'B2' from this species could not be aligned with those from the other species, although it was definitely derived from

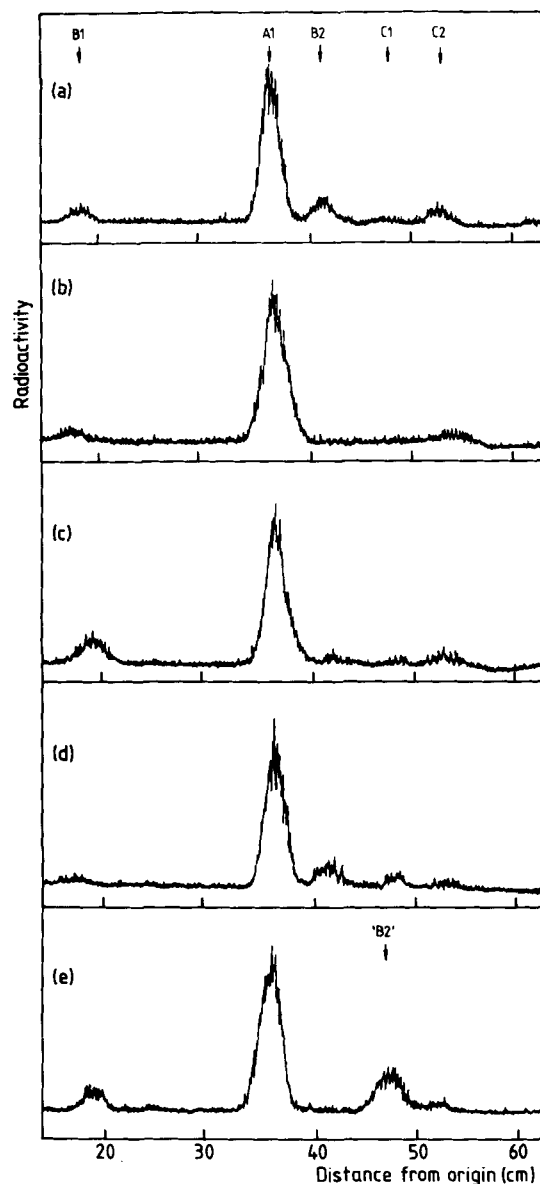


Fig. 8. Separation of the neutral oligosaccharides from the lectins of five *Erythrina* species by high-voltage paper electrophoresis. The neutral oligosaccharides from the lectin of each species (see Fig. 6) were electrophoresed on paper in 15 mM borate buffer pH 9.5 as described in Experimental Procedures. Assignment of the radioactive peaks as A1, B1, B2, C1 or C2 were made by comparison with the respective oligosaccharides from *E. cristagalli* lectin

the B pool. This and the other species-to-species differences are currently under investigation.

Analysis of the major glycopeptide from *E. corallodendron* lectin

Aspartic acid was the most abundant amino acid in an acid hydrolysate of the glycopeptide. Proline (0.4 mol/mol aspartic acid), glutamic acid (0.2 mol/mol) and serine (0.2 mol/mol) were the next most abundant residues and fucose, xylose, mannose and *N*-acetylglucosamine were present in the molar ratios 1.5:1.1:3.0:1.8 (relative to mannose = 3.0). ^1H -NMR spectroscopy at 500 MHz of the HPLC-purified material (data not shown) gave chemical shifts of the reporter resonances derived from Man-3 ($\text{H1} = 4.85 \text{ ppm}$,

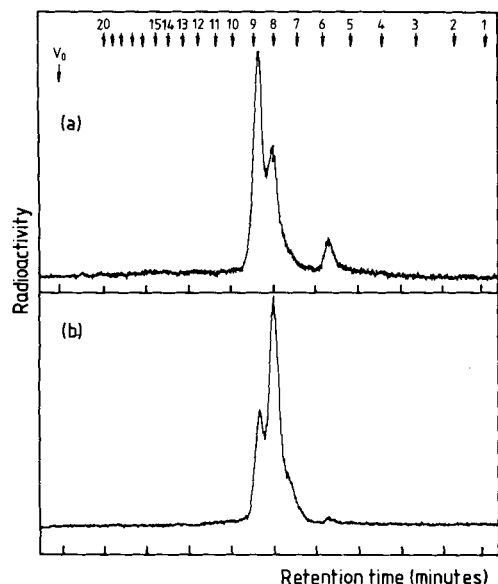


Fig. 9. Elution profiles on the neutral oligosaccharides of the lectins from *Sophora japonica* and *Lonchocarpus capassa* on Bio-Gel P-4. (a) *S. japonica* lectin and (b) *L. capassa* lectin were subjected to hydrazinolysis and the isolated oligosaccharides were reduced, purified and separated on Bio-Gel P-4 as described in Experimental Procedures. For details of the annotation see Fig. 2

H2 = 4.27 ppm), Man-4 (H1 = 5.13 ppm, H2 = 4.03 ppm), Man-4' (H1 = 4.91 ppm, H2 = 3.98 ppm) and xylose (H1 = 4.46 ppm, H2 = 3.38 ppm, H3 = 3.45 ppm, H5_{ax} = 3.26 ppm) that were almost identical to those obtained for oligosaccharide A1. As found by Van Kuik et al. [37], the chemical shift values for H1 (5.13 ppm), H5 ($\approx$ 4.7 ppm, under HO²H peak) and methyl (1.28 ppm) protons of the fucose residue are at more downfield positions in the glycopeptide than in the oligosaccharide-alditol.

The compositional data and ¹H-NMR spectroscopy indicate that the structure of the carbohydrate moiety of the glycopeptide is Man α 3(Man α 6)(Xyl β 2)Man β 4GlcNAc β 4-(Fuc α 3)GlcNAc β Asn.

Intergenous comparison of lectin oligosaccharides

Oligosaccharides isolated from hydrazinolysates of the lectins from *Sophora japonica* and *Lonchocarpus capassa* were reduced with NaB³H₄ and electrophoresed at pH 5.4 (data not shown). In both samples > 95% of the recovered radioactivity remained at the point of application and was therefore not charged. Bio-Gel P-4 chromatography of the neutral oligosaccharides from *S. japonica* lectin gave a similar pattern (Fig. 9a) to that seen for the lectins from the five species of *Erythrina* (Fig. 7). The major component was eluted at 8.8 Glc units (57.3%) with lesser amounts of material at 8.0 (33.6%) and 5.8 (9.1%). The elution profile of the oligosaccharides from *L. capassa* lectin (Fig. 9b) showed the same components but in different proportions. The major peak was at 8.0 Glc units (62.7%) with less material being eluted at 8.8 (26.5%) and trace radioactivity at 5.8 (1.9%). An additional component appeared as a shoulder to the main peak and was eluted at 7.3 Glc units. Further investigations of the oligosaccharide moieties of these two lectins are in progress to establish whether they are the same as those of the *Erythrina* lectins.

DISCUSSION

The structure of the major oligosaccharide moiety of *Erythrina cristagalli* lectin determined in this study shows the twin characteristics of a newly established family of N-linked glycans. These characteristics are the substitution of the common pentasaccharide core of N-glycoproteins [Man α 3(Man α 6)Man β 4GlcNAc β 4GlcNAc] by (a) a xylose residue linked β 1 $\rightarrow$ 2 to the β -mannosyl residue and (b) a fucose residue linked α 1 $\rightarrow$ 3 to the reducing terminal N-acetylglucosamine residue.

Using the hydrazinolysis conditions reported here, five structures could be isolated from *E. cristagalli* lectin (see Figs 2 and 3) with oligosaccharide A1 comprising approximately 90% of the mixture. The smaller oligosaccharides, B1, B2, C1 and C2, are structurally related to A1 and could possibly be derived from it.

The applicability of the hydrazinolysis technique to the examination of this class of oligosaccharide has recently been questioned. Van Kuik et al. [37] state that during their isolation of bromelain oligosaccharides the hydrazinolysis conditions of Takasaki et al. [39] resulted in the loss of the α (1 $\rightarrow$ 3)-linked fucose residue and degradation of the reducing terminal N-acetylglucosamine residue. Similarly Hase et al. [22] reported that approximately 30% of the recovered oligosaccharides from *Caesalpinia pulcherrima* protease inhibitor were degraded using hydrazinolysis. Reducing-terminal N-acetylhexosamine residues substituted at the 3-position are notoriously alkali-labile, as demonstrated by the 'peeling' reaction during β -elimination of O-linked oligosaccharides (reviewed in [40]). This peeling can be prevented during β -elimination by the addition of sodium borohydride such that cleavage is accompanied by simultaneous reduction [40]. We have found that rigorous exclusion of water from all stages of the hydrazinolysis procedure is necessary to minimize degradation. That oligosaccharide A1 was isolated in good yield by hydrazinolysis shows that the procedure used in this laboratory (see Experimental Procedures) ensures sufficiently anhydrous conditions such that the technique can be successfully applied to these labile structures. Indeed our conditions result in the release and recovery of sialylated Gal β 3GalNAc units from O-glycoproteins. For this reason it was important to confirm that the oligosaccharides released from *E. cristagalli* lectin were asparagine-linked and therefore glycopeptides were isolated. Chemical analysis of the major glycopeptide and ¹H-NMR spectra indicate that the reducing terminal N-acetylglucosamine of an oligosaccharide identical to A1 is indeed N-linked to asparagine.

Re-evaluation of the conditions for the reduction step in labelling these oligosaccharides was also necessary as we found that the published procedure (unbuffered 0.05 M NaOH/dimethylformamide mixture pH > 12.0 [39]) resulted in significant epimerisation at carbon-2 and loss of unfucosylated reducing terminal N-acetylglucosamine residues. Under the reduction conditions employed in this study, control experiments showed that unsubstituted N,N'-diacetylchitobiosyl reducing termini do not undergo epimerisation (Amatayakul, S., Dwek, R. A., and Rademacher, T. W., unpublished data) as is also demonstrated by the lack of epimerisation of the reducing-terminal N-acetylglucosamine of oligosaccharide B1. However, oligosaccharide C2 is the N-acetylmannosaminitol epimer of C1. This epimerisation probably occurred during the reduction of the R-Man β 4-GlcNAc terminus. The tendency to epimerisation, during reduction, of the terminal N-acetylglucosamine residue of

oligosaccharides released by endo- β -*N*-acetylglucosaminidase has previously been noted [41]. Lowering of the pH of reduction from 12.0 to 9.8 was found to be necessary to prevent this epimerisation.

Although the chemical identity of the reducing-terminal residue of B2 could not be unambiguously determined, it is substituted by fucose in a fashion similar to that of A1, as determined by sequential enzyme digestion. Therefore the difference in hydrodynamic volume observed between oligosaccharides A1 and B2 suggests a reducing terminal residue other than *N*-acetylglucosamine. We have observed similar compounds in the hydrazinolysis products of certain other glycoproteins and these have been identified as glucitol. At present we can not describe a chemical mechanism which would generate glucose from *N*-acetylglucosamine. We note however, that asparagine-linked glucose residues have been reported in oligosaccharides from *Halobacterium halobium* [42].

The hydrazinolysis technique results in non-quantitative recovery of reduced radioactive oligosaccharides. This is due to the incomplete removal of an acetohydrazide group attached to carbon-1 of the reducing terminal *N*-acetylglucosamine where the substitution pattern of this residue determines the lability of the leaving group. We have found that oligosaccharides substituted in this way at carbon-1 are not reduced by sodium borohydride and therefore do not contribute to the calculated radioactive recovery, although the dry weight yields are near theoretical values. Other authors suggest the use of acid to cleave the acetohydrazide group [43, 44] but this method is not applicable when acid-labile substitutions are present. We have recently found that copper(II) acetate can be successfully used to remove the acetohydrazide group resulting in quantitative recovery of oligosaccharides without affecting acid-labile groups such as sialic acids or fucose (Manger, I. D., Dwek, R. A., and Rademacher, T. W., unpublished data). Analysis of the oligosaccharide moieties of *E. cristagalli* lectin after hydrazinolysis and copper(II) acetate treatment gave identical results to those described above (data not shown); a comparison of the composition of the intact glycoprotein with the oligosaccharide mixture isolated after hydrazinolysis indicated that no selective loss of any sugar occurred, i.e. the recovery of fucose was at least 90%.

Bio-Gel P-4 chromatography and borate paper electrophoresis of the oligosaccharide of the lectins from five species of *Erythrina* (Figs 7 and 8) show similar glycosylation patterns. This suggests that within the genus these structures are conserved. Further analysis is necessary to confirm the structural identity of the oligosaccharide components of these lectins. The observed similarities in the oligosaccharide heterogeneity patterns of the *Erythrina* lectins could be a reflection of their extensive amino acid sequence homology [1, 2]. However, essentially the same Bio-Gel P-4 pattern (Fig. 9a) is seen for the lectin from *Sophora japonica*, a member of a different tribe of the Leguminosae. That these similarities in oligosaccharide 'fingerprints' are indicators of structural similarity is seen in both the present report and that of Fournet et al. [45]. We and these authors, independently, isolated glycopeptides from the lectins of *E. corallodendron* and *S. japonica*, respectively. In each case the carbohydrate moiety of the major glycopeptide is identical to oligosaccharide A1 of *E. cristagalli* lectin. The lectin of another unrelated legume, *Lonchocarpus capassa*, also contained the same oligosaccharide components, as determined by elution position on Bio-Gel P-4 (Fig. 9b), but in different proportions to *E. cristagalli*

or *S. japonica* lectins. This demonstrates that it is not the presence or absence of a particular oligosaccharide that differentiates between similar glycoproteins but the relative levels of the structures present.

The structure of oligosaccharide A1 raises interesting questions related to its biosynthesis. The acceptor specificity of the enzyme forming the $\alpha(1\rightarrow3)$ fucose linkage has been partly characterised but that forming the $\beta(1\rightarrow2)$ xylose linkage is still unknown. The mammalian $\alpha(1\rightarrow6)$ fucosyltransferase requires a terminal $\beta(1\rightarrow2)$ -linked *N*-acetylglucosamine attached to the mannose $\alpha(1\rightarrow3)$ -linked to the core β -mannose [46]. The formation of this structure requires the prior removal of all the $\alpha(1\rightarrow2)$ -linked mannose residues from $\text{Man}_9\text{GlcNAc}_2$ [47]. Substitution of the $\alpha(1\rightarrow3)$ arm by *N*-acetylglucosamine is also required for the subsequent removal of the $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow3)$ -linked mannose residues of the $\alpha(1\rightarrow6)$ arm in $\text{Man}_5\text{GlcNAc}_2$ [48]. Metabolic labelling suggests that the fucosyltransferase substrate specificity and the initial processing events in plants are similar to those in mammals [16, 49–51]. Preliminary characterisation of the glycopeptide acceptor specificities of, and the product generated by, the *N*-acetylglucosaminyl and fucosyl transferases from *Phaseolus vulgaris* cotyledons also support these conclusions [52]. These findings indicate that the carbohydrate structure proposed for Tora bean lectin [17] is a composite of oligomannose and xylose/fucose-type oligosaccharides. The co-existence of these oligosaccharide types on a single polypeptide has clearly been shown for the lectin from another cultivar (Greensleeves) of *P. vulgaris* [16, 53, 54]. Xylose is a poor biosynthetic precursor and incorporation of the radio-labelled sugar into plant glycoproteins is very low, therefore other techniques must be employed to elucidate the substrate specificity of the $\beta(1\rightarrow2)$ xylosyltransferase.

The xylose residue in plant glycoproteins may be considered in some ways analogous to the 'bisecting' *N*-acetylglucosamine residue in complex-type oligosaccharides of mammalian glycoproteins. Both of these residues are attached to a highly substituted branch point in the molecule. It will be interesting to see whether similar changes in the disposition of the core α -mannose residues are associated with the addition of xylose as has been seen with the 'bisecting' *N*-acetylglucosamine [55].

Finally the ability to form both xylosyl $\beta(1\rightarrow2)$ and fucosyl $\alpha(1\rightarrow3)$ linkages to the common pentasaccharide core of *N*-linked oligosaccharides appears to be limited to plants. *Helix pomatia* α -haemocyanin, although containing $\beta(1\rightarrow2)$ -linked xylose, has the animal-type $\alpha(1\rightarrow6)$ -fucosyl linkage [23]. The distribution of this class of oligosaccharides is demonstrated by its occurrence in such geographically and genetically distant species as pineapple [10, 37], sycamore [21] and now *Erythrina cristagalli*. This suggests an important role for these structures in plants.

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