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The Purification of the Glycoprotein Lectin from the Broad Bean (*Vicia faba*) and a Comparison of its Properties with Lectins of Similar Specificity

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1. The lectin from the broad bean (*Vicia faba*) was purified by affinity chromatography by using 3-*O*-methylglucosamine covalently attached through the amino group to CH-Sepharose (an ω-hexanoic acid derivative of agarose). Its composition and the nature of its subunits were compared with concanavalin A and the lectins from pea and lentil. 2. Unlike the other three lectins, broad-bean lectin is a glycoprotein; a glycopeptide containing glucosamine and mannose was isolated from a proteolytic digest. 3. The mol.wt. is about 47 500; the glycoprotein consists of two apparently identical subunits, held together by non-covalent forces. Fragments of the subunits, similar to those found in concanavalin A and soya-bean agglutinin, were found in active preparations. 4. Broad-bean lectin was compared with concanavalin A and the lectins from pea and lentil in an investigation of the inhibition of their action by a number of monosaccharides, methyl ethers of monosaccharides, disaccharides and glycopeptides. The most striking differences concern 3-*O*-substituted monosaccharides, which are strong inhibitors of the action of broad-bean, pea and lentil lectins but not of the action of concanavalin A. There is, however, no strong inhibition of the action of these lectins by 3-*O*-linked disaccharides.

Lectins are carbohydrate-binding proteins or glycoproteins that occur in many plants, the most widely used source being the seeds of leguminous plants; the definition is now generally extended to include animal protein with similar activities (see reviews by Sharon & Lis, 1972; Lis & Sharon, 1973; Nicholson, 1974). They are usually recognized by their ability to agglutinate erythrocytes; many also preferentially agglutinate transformed cells and are mitogenic for lymphocytes. Lectins bind specifically to particular saccharide groups and when coupled to insoluble supports they are used for the purification, by affinity chromatography, of cell-membrane and other glycoproteins (Allan *et al.*, 1972; Hayman & Crumpton, 1972; Adair & Kornfeld, 1974).

It is becoming apparent that lectins, which have a similar specificity towards monosaccharides, may differ in their affinity for particular oligosaccharides or glycopeptides. For example, the lectins from jack bean (concanavalin A) and lentil, which are both specifically inhibited by mannose and glucose derivatives, are inhibited to different degrees by glycopeptides from ovalbumin or transferrin (Young & Leon, 1974). Since both lectins are used for affinity chromatography and have been shown to bind different glycoproteins from erythrocyte membranes (Findlay, 1974; Robinson *et al.*, 1975), we decided to compare their specificities with those of the lectins from pea (Entlicher *et al.*, 1970) and broad bean (Tomita *et al.*, 1970), which have also been reported to be specific for glucose and mannose.

Although the lectin from the pea has been purified (Entlicher *et al.*, 1970) and the subunit structure determined, (Mařík *et al.*, 1974; Trowbridge, 1974), the lectin from the broad bean had not, at the beginning of this work, been purified. We have now purified this lectin by affinity chromatography using 3-*O*-methylglucosamine attached to CH-Sepharose (an ω-hexanoic acid derivative of agarose). Unlike the three lectins mentioned above, it is a glycoprotein. In specificity it resembles pea lectin more than lentil lectin, and all three lectins differ from concanavalin A in a number of respects. The purification of the lectin from the broad bean (also known as the fava bean) has been reported by Wang *et al.* (1974), who showed that it can be crystallized in a form suitable for high-resolution crystallographic structural analysis.

Experimental

Materials

Sephadex G-100, Sepharose 4B and CH-Sepharose 4B were from Pharmacia, Uppsala, Sweden, and Bio-Gel P-2 and P-100 from Bio-Rad Laboratories,

Richmond, CA, U.S.A. Cytochrome *c* (horse heart), lysozyme (hen's-egg-white), α-chymotrypsinogen, bovine serum albumin, ovalbumin (hen), transferrin (human) and the non-specific proteinase from *Streptomyces griseus* (also known as Pronase) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Rabbit IgG (immunoglobulin G) was a gift from Dr. R. D. Marshall, Department of Chemical Pathology, St. Mary's Hospital Medical School, London W.2, U.K.

The methods of synthesis or sources of the derivatives of *N*-acetylglucosamine have been given elsewhere (Allen *et al.*, 1973). Other sugars were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., except for trehalose, which was from Sigma, and the gifts of nigerose from Dr. J. J. Marshall, Department of Biochemistry, University of Miami, and of isomaltose from Dr. T. N. Palmer of this Department. Laminaribiose was prepared by hydrolysis of laminaran (a gift from Dr. E. Percival, Department of Chemistry, Royal Holloway College, Egham, Surrey, U.K.) with 1M-oxalic acid for 6h at 95°C followed by purification by paper chromatography. Disaccharides were checked for purity by g.l.c. Other chemicals used were of analytical grade or of the highest purity available.

Methods

Preparation of lectins. The purification of the broad-bean (*Vicia faba*) lectin is described in the Results section. Concanavalin A, the lectin from the jack bean (*Canavalia ensiformis*), was from Pharmacia.

Lentil (*Lens culinaris*) lectin was a gift from Dr. M. J. Crumpton, National Institute for Medical Research, Mill Hill, London N.W.7, U.K. It had been prepared by affinity chromatography on Sephadex G-75 (Hayman & Crumpton, 1972). Analysis of this sample by g.l.c. showed that it contained only 0.2 mol of glucose/mol of subunit and that no other sugar was present (M. J. Crumpton & A. K. Allen, unpublished work). This shows that lentil lectin is not a glycoprotein, which is contrary to previous reports (Howard *et al.*, 1971). Lentil lectin was also prepared by affinity chromatography on the 3-*O*-methylglucosamine–CH-Sepharose column used for the purification of the broad-bean lectin (see below).

Pea (*Pisum sativum*) lectin was prepared from dried peas (Whitworths yellow split peas; Whitworths Holdings Ltd., Wellingborough, Northants., U.K.) by a modification (Trowbridge, 1974) of the method of Entlicher *et al.* (1970), which involved absorption on Sephadex G-100 and elution of the bound lectin with 0.2M-glucose. Pea lectin was also prepared by affinity chromatography on the 3-*O*-methylglucosamine–CH-Sepharose column used for the purification of the broad-bean lectin (see below). The amino acid analysis was in good agreement with those published by Mařík *et al.* (1974) and Trowbridge (1974); analysis for sugars by g.l.c. showed that less than 1 mol of glucose/mol of subunit was present and that other sugars were absent, thus confirming the observation (Entlicher *et al.*, 1970; Trowbridge, 1974) that the pea lectin is not a glycoprotein.

Soya-bean agglutinin was prepared from soya-bean (*Glycine max*) meal by affinity chromatography on a column of galactosamine–CH-Sepharose (Allen & Neuberger, 1975*a*).

Amino acid and amino sugar analyses. Analyses were done on a Locarte Mini analyser fitted with an automatic-loading attachment. The standard system for analyses was that of Mayes *et al.* (1973), which involved successive elution of the column (25cm × 0.9cm) with sodium citrate buffers, pH3.25 (0.2M) for 33min, pH4.35 (0.2M) for 110min and pH6.65 (1.0M) for 160min, at a flow rate of 30ml/h. The temperature was set to 50°C for the first buffer, and to 60°C thereafter. Although this system separated glucosamine and galactosamine from the amino acids, for quantitative determination of glycoprotein hydrolysates the amino sugars were separated fully from any traces of breakdown products of tryptophan by elution on the column with pH4.25 buffer for 200min at 50°C (Allen & Neuberger, 1975*b*). The absence of hydroxyproline from the hydrolysates was demonstrated by elution with sodium citrate buffer, pH2.80 (0.2M), as described previously (Allen & Neuberger, 1973).

Samples of protein were hydrolysed for 24, 48 and 72h in 3M-toluene-*p*-sulphonic acid at 110°C *in vacuo* and correction factors were obtained for the destruction of serine and threonine residues. Cysteine values were obtained from the analysis of cysteic acid residues resulting from performic acid oxidation (Hirs, 1956) and subsequent hydrolysis of the protein for 24h at 110°C in constant-boiling HCl. Tryptophan was determined spectrophotometrically in 6M-guanidine hydrochloride (Edelhoch, 1967). Glucosamine and galactosamine were determined on the analyser on a sample of protein that had been hydrolysed for 24h at 100°C in 3M-toluene-*p*-sulphonic acid. When this procedure was carried out with well-characterized glycoproteins the recovery of amino sugars was over 95% (Allen & Neuberger, 1975*b*).

Neutral-sugar analyses. These compounds were measured by g.l.c. after methanolysis and trimethylsilylation of the glycoprotein (Chambers & Clamp, 1971), with a Perkin–Elmer F.11 gas chromatograph fitted with dual columns. Sugar analyses were related to the amino acid analyses by adding internal standards (mannitol for neutral sugars and *p*-fluorophenylalanine for amino acids) to samples taken from the same stock solution of glycoprotein or glycopeptide.

Preparation of peptide 'maps'. The procedure followed was that of Bennett (1967). Protein samples

(1–5mg) were dissolved in 2ml of 0.05M-NH_4HCO_3, trypsin (diphenylcarbamoyl chloride-treated) was added (1%, w/w) and the samples were incubated at 37°C for 5h. After concentration by freeze-drying the digests were dissolved in water and portions (equivalent to 0.5–1mg of protein) were applied to Whatman 3MM paper. The papers were subjected to chromatography for 18h in butan-1-ol/acetic acid/water (4:1:1, by vol.) in the first dimension and to electrophoresis for 60min (2.5kV, 40V/cm) in the second dimension with a buffer, pH3.6, containing pyridine/acetic acid/water (1:10:289, by vol.). The peptides were located either by dipping the paper in 0.2% ninhydrin in acetone or by use of the Sakaguchi (Bennett, 1967) reagent.

End-group determinations. The dansyl method of Gray (1972) was used, the derivatives being identified by chromatography on polyamide layers (see Gray, 1972).

Alkali treatment of the glycoprotein. The lectin (5mg) was dissolved in 1.0ml of 0.5M-NaOH and dialysed against 0.5M-NaOH at 4°C for 2 days, followed by dialysis against several changes of water. The lectin was then analysed for amino acids and sugars.

Proteolytic digestion of the lectin and isolation of the glycopeptide. The conditions for digestion of *V. faba* lectin were the same as those used by Spiro & Bhoyroo (1974) for the digestion of fetuin. The lectin (25mg/ml) was digested at 37°C with the proteinase from *S. griseus* (Sigma) in 0.15M-Tris/acetate buffer (pH7.8) containing 1.5mM-$CaCl_2$ in the presence of toluene for a total of 96h. Initially, proteinase equal to 1% of the weight of the lectin was added, followed at 24h and 48h by amounts equal to 0.05% of the weight of the lectin. The digest was then centrifuged at 600*g* for 2min; the supernatant was concentrated by rotary evaporation and applied to a column (60cm×2cm) of Bio-Gel P2 equilibrated with water. Fractions (3ml) were collected and portions were assayed for neutral sugar by the phenol–H_2SO_4 method (Dubois *et al.*, 1956), and the fractions that contained carbohydrate were concentrated. A sample of this fraction was hydrolysed in toluene-*p*-sulphonic acid and analysed for its amino acid and amino sugar content. Since this first analysis showed that the glycopeptide contained a number of amino acids the cycle of digestion and gel chromatography was repeated twice more until the amino acid analysis was constant. Glycopeptides from ovalbumin and human transferrin were isolated in a similar manner.

Polyacrylamide-gel disc electrophoresis. The method of Weber *et al.* (1972) was used, which involved electrophoresis in columns (7cm×0.5cm) of 10% (w/v) polyacrylamide gel in 0.1M-sodium phosphate buffer (pH7.2) containing 0.1% sodium dodecyl sulphate for 6h at 5mA/gel. Before electrophoresis the samples were dissolved in a solution of 0.01M-sodium phosphate buffer (pH7.0) containing 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol and heated in a boiling-water bath for 2min. The proteins were stained with Coomassie Brilliant Blue. Cytochrome *c* (12400), lysozyme (14600), α-chymotrypsinogen (25700), soya-bean agglutinin (30000), ovalbumin (45000) and bovine serum albumin (68000) were used as standards for molecular weight.

Assay of agglutination. Full details of the procedure are given elsewhere (Allen *et al.*, 1973). A serial dilution of the lectin was made in 100μl of phosphate-buffered saline (7.20g of NaCl, 1.48g of Na_2HPO_4, 0.43g of KH_2PO_4 per litre; final pH7.2) and 200μl of a 1.5% suspension of rabbit erythrocytes were added. The tubes were shaken at 15min intervals and after 2h the degree of agglutination was assessed on the serological scale (0 to ++++). The amount of lectin required to cause half-maximal (++) agglutination of the cells was taken as 1 unit. The assay is only semi-quantitative and should be regarded as being liable to an error of ±20%. With this method an activity of 2000 units/mg was obtained for pure *V. faba* lectin; with the same assay pea lectin had an activity of 2500 units/mg, lentil lectin 1250 units/mg and concanavalin A 1250 units/mg.

Although guinea-pig erythrocytes were about as sensitive to *V. faba* lectin as were rabbit erythrocytes, human erythrocytes (group A or O) were 30–50 times less sensitive, and sheep erythrocytes were not agglutinated by 500 times the quantity of lectin needed to agglutinate the rabbit cells.

To determine the 50%-inhibition values for various sugars in this system, the method of Lis *et al.* (1970) was used, except that the degree of agglutination was determined on the visual serological scale rather than spectrophotometrically.

Preparation of the 3-O-methyl-N-hexanoylglucosamine–Sepharose conjugate. 3-*O*-Methylglucosamine was prepared by the method of Neuberger (1941). This involved reaction of *N*-acetylglucosamine with benzaldehyde to block the 4- and 6-hydroxyl groups, followed by methylation with dimethyl sulphate to introduce a 3-*O*-methyl group. Hydrolysis of this product with 3M-HCl at 100°C for 4h gave 3-*O*-methylglucosamine, which was purified by crystallization from a water/acetone mixture as the hydrochloride.

The procedure for coupling 3-*O*-methylglucosamine to CH-Sepharose was similar to that used previously to couple galactosamine and glucosamine (Allen & Neuberger, 1975*a*). CH-Sepharose 4B (10g) was swollen in 0.5M-NaCl and washed three times with the same solution by centrifugation at 600*g* for 1min; the NaCl was then removed by washing with water. The gel was suspended in water to make a slurry of 100ml and stirred at 20–25°C. 3-*O*-Methylglucosamine hydrochloride (500mg) was added and the pH was adjusted to 5.6 with 0.1M-

NaOH. Solid 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide hydrochloride (250mg) was added over a period of 10min; the pH rose slightly to 5.8 and then remained constant. The reaction mixture was stirred for 1h and then left at 20–25°C for 20h without stirring. The gel was then washed successively with 1M-NaCl, 1M-NaCl containing 0.1M-Tris/HCl (pH8.6), 1M-NaCl containing 0.05M-sodium formate buffer (pH3.0), 1M-NaCl containing 0.1M-Tris/HCl buffer (pH8.6), water and phosphate-buffered saline. The gel (about 40ml) was then ready for use; if not immediately required it was stored in 2M-NaCl.

Results

Purification of V. faba lectin

The purification scheme is shown in Table 1. A 160g batch of testa-free broad-bean seeds (variety: Bunyard exhibition long pod) were ground in a mechanical grinder, and the resulting powder was defatted with light petroleum (b.p. 60–80°C) and dried in an air-stream overnight. The product (155g) was stirred with 450ml of 0.15M-NaCl at 4°C for 8h, then centrifuged at 17000*g* for 60min, and the supernatant was retained. The deposit was resuspended in 0.15M-NaCl and the procedure was repeated three times. Solid $(NH_4)_2SO_4$ (39.8g/100ml) was added to the combined supernatants to give 65% saturation at 4°C. The precipitate was allowed to settle overnight and then collected by centrifugation at 9000*g* for 20min. The precipitate was resuspended in a minimum volume of 1M-NaCl, extensively dialysed against the same solution, and the solution was finally centrifuged at 9000*g* for 20min.

Affinity chromatography. The supernatant from the previous step was applied directly to a column (13cm×2cm) of 3-*O*-methyl-*N*-hexanoylglucosamine–Sepharose equilibrated with 1M-NaCl and eluted with 1M-NaCl. Fractions (25ml) were collected, and although over 99% of the protein was eluted with this solution, the lectin was retained. The column was washed with about 1000ml of 1M-NaCl until the E_{280} of the eluate was below 0.015. Smaller fractions (5ml) were then collected and the lectin was eluted with 1M-NaCl containing 0.1M-methyl α-glucoside. Those fractions that contained the active protein were pooled, dialysed against 1M-NaCl, concentrated by ultrafiltration and stored at 4°C in 1M-NaCl. Freeze-drying of this lectin was avoided as preparations that had been so treated were found to be incompletely soluble in NaCl solutions.

As reported under 'Methods', the 3-*O*-methyl-*N*-hexanoylglucosamine–Sepharose column was also capable of binding the pea and lentil lectins; it did not, however, bind concanavalin A. None of the four lectins was bound by an *N*-hexanoylglucosamine–Sepharose column, which was capable of binding wheat-germ agglutinin (Allen & Neuberger, 1975*a*). These differences in binding to the columns are in agreement with the specificity of the lectins (see Table 5).

In common with the other three lectins mentioned above, *V. faba* lectin was capable of binding to Sephadex G-100 (but not G-50). However, its affinity for this material was not very great, and a large column (60cm×3cm) was required for its purification, the operation of which was much more time-consuming than that of the smaller (13cm×2cm) 3-*O*-methyl-*N*-hexanoylglucosamine–Sepharose column.

Composition of the lectin

By the use of internal standards for amino acid and carbohydrate analysis (see under 'Methods') we concluded that *V. faba* lectin was a glycoprotein containing about 3% (w/w) of carbohydrate. The analyses (Table 2) are calculated from the assumption that the mol.wt. of the subunit of the glycoprotein is 24000 (see below). The analyses showed that cysteine, methionine and hydroxyproline were absent. The sugar analyses did not change after treatment of the glycoprotein with 0.5M-NaOH at 4°C (see under 'Methods'). Glucose was found in the preparations in variable quantities (between 0.2 and 2.1mol/mol) and is therefore presumably not covalently bound. Galactose and galactosamine were not detected.

Table 1. *Purification of V. faba lectin*

This procedure starts from 155g of defatted bean meal. Owing to the inaccuracy of the haemagglutination assay, the activities are liable to an error of ±20%. In the first two steps protein was determined by the method of Warburg & Christian (1941) and for the purified material an E_{280}^{1cm} of 1.28 was used.

Procedure	10^{-3}×Total activity (agglutinating units)	Total protein content (g)	Specific activity (units/mg)	Overall yield (%)
0.15M-NaCl extract	1020	63	16	100
$(NH_4)_2SO_4$ precipitate after dialysis and centrifugation	490	7.3	67	48
Active fraction off affinity column	460	0.164	2800	45

Table 2. *Analysis of V. faba lectin*

The values were calculated as molar proportions by assuming that the mol.wt. of the glycoprotein subunit is 24000. The values for the nearest integer were derived from an average of the three hydrolyses in 3 M-toluene-*p*-sulphonic acid at 110°C, except for threonine and serine, which were extrapolated to zero time, valine and isoleucine, for which the 72h values were taken, and cysteine and tryptophan (see footnotes). For further details see under 'Methods'.

	Amino acid content (mol/mol)			Nearest integer
Time of hydrolysis ...	24h	48h	72h	
Asp	23.6	23.3	23.3	23
Thr	20.9	20.5	20.5	21
Ser	13.7	12.7	12.6	14
Glu	17.1	15.6	16.0	16
Pro	11.1	12.0	11.0	11
Gly	17.2	17.2	17.2	17
Ala	14.2	14.2	14.7	14
Cys	0.0	0.0	0.0	0*
Val	13.5	14.5	17.3	17
Met	0.0	0.0	0.0	0
Ile	10.5	11.0	11.5	12
Leu	14.0	13.9	14.2	14
Tyr	7.7	7.0	6.1	7
Phe	12.4	12.5	13.0	13
His	3.6	3.5	3.7	4
Lys	10.1	10.5	11.0	11
Arg	4.1	4.1	4.5	4
Trp	—	—	—	9†
GlcN	—	—	—	1.6‡
Man	—	—	—	2.9§

* Determined separately as cysteic acid after hydrolysis of a performic acid-oxidized sample (Hirs, 1956).
† Determined by a spectrophotometric method (Edelhoch, 1967).
‡ Determined from a sample hydrolysed at 100°C for 24h; actual value (Allen & Neuberger, 1975*b*).
§ Analysed by g.l.c.; actual value (Chambers & Clamp, 1971).

Table 3. *Composition of the glycopeptide isolated from V. faba lectin after proteolytic digestion*

The values are calculated as molar proportions based on Asx = 1.0. The relative amounts of other amino acids were less than 0.02. Amino acids and glucosamine were measured with an amino acid analyser and neutral sugars by g.l.c.

Asx	1.00	Gly	0.18
Thr	0.06	Ala	0.76
Ser	0.11	GlcN	1.44
Glu	0.17	Man	1.64
Pro	0.10		

Isolation of a glycopeptide

The product of the repeated proteolytic digestion of *V. faba* lectin (see under 'Methods') was applied to a column of Bio-Gel P-2, eluted with water and analysed for amino acids, amino sugars and neutral sugars. The composition of the glycopeptide is given in Table 3. The values for glucosamine and mannose in Tables 2 and 3 do not approximate to whole integers, which suggests that, in common with many glycoproteins (Beeley, 1974), *V. faba* lectin is heterogeneous with respect to its carbohydrate chain.

Molecular-weight determinations

Gel filtration. A column (75 cm × 3 cm) of the polyacrylamide support Bio-Gel P-100 equilibrated with 1 M-NaCl was calibrated by applying proteins of known molecular weight. A linear relationship was obtained between the elution volume and the logarithm of the molecular weight for the following proteins: cytochrome *c* (12400); chymotrypsinogen (25700); ovalbumin (45000) and its dimer; bovine serum albumin (68000). *V. faba* lectin was eluted as a symmetrical peak in a position expected for a globular protein of mol.wt. 47500±1500.

Gel filtration in 6 M-guanidine hydrochloride. Sepharose 4B was equilibrated with a solution of 6M-guanidine hydrochloride containing 0.1 M-Tris/HCl buffer (pH 8.6), packed in a column (60 cm × 2 cm) and eluted at room temperature (20–25°C). The column was calibrated with fully reduced and *S*-carboxymethylated derivatives of the proteins used for the Bio-Gel P-100 column. In addition, fully reduced and *S*-carboxymethylated rabbit IgG light (mol.wt.

23500) and heavy (mol.wt. 50000) chains were chromatographed. These derivatives showed a linear relationship between the logarithms of the molecular weight and the elution volume, as reported by Mann & Fish (1972). The lectin from *V. faba* was separated into three fractions in this system (Fig. 1). The proportions of these fractions varied in different preparations, and it was found that the first fraction (of mol.wt. 24500±1000) had almost the same amino acid and amino sugar composition as the whole lectin (Table 4). The material in the first fraction had the same *N*-terminal amino acid (leucine) as the whole lectin.

The peptide 'maps' of the whole lectin and the material in the first fraction were similar; both had about 16 peptides that reacted with ninhydrin and five that reacted with the Sakaguchi reagent. The peptide 'maps' of the material from the second fraction showed 11 ninhydrin-reacting peptides (this is twice the number expected from the sum of arginine and lysine residues and indicates that there are probably two polypeptides in this fraction) and two reacting with Sakaguchi reagent. The third fraction produced only three tryptic peptides, none of which reacted with the Sakaguchi reagent. All the tryptic peptides that were present in the peptide 'maps' of the second and third fractions appeared to be present in the peptide 'maps' of the whole lectin and the first fraction. Owing to its small size some of the material in the third fraction may have been lost on dialysis.

Polyacrylamide-gel electrophoresis. A mol.wt. of 22500 (±1500), by comparison with marker proteins, was obtained for the subunit of *V. faba* lectin in 10% polyacrylamide gel in the presence of sodium dodecyl sulphate (see the Experimental section). Fragments of under 10000 mol.wt., such as were detected by gel filtration in 6M-guanidine hydrochloride, would not have been detected in this system.

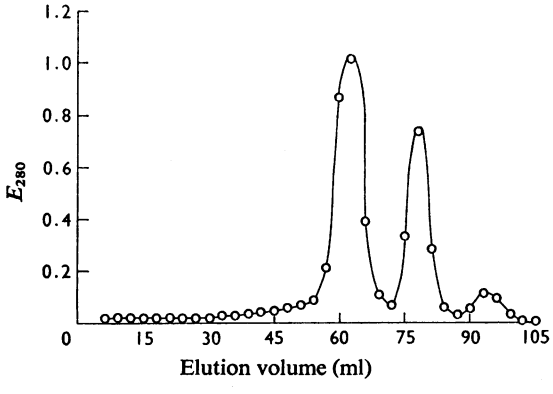

Fig. 1. *Elution profile of V. faba lectin from Sepharose-4B with 6M-guanidine hydrochloride*

Freeze-dried lectin (10mg) was dissolved in 6M-guanidine hydrochloride containing 0.1M-Tris/HCl (pH8.6) and chromatographed on the column with the same buffer. The first peak had the same composition as the original lectin (see Table 4).

Table 4. *Composition of the fractions obtained by chromatography of V. faba lectin in guanidine hydrochloride*

The values show the amino acid and amino sugar composition, after 24h hydrolysis in 3M-toluene-*p*-sulphonic acid at 110°C, of the three fractions obtained by chromatography of the lectin in guanidine hydrochloride (see Fig. 1). The composition of a hydrolysate of the whole lectin is given for comparison and is expressed as mol/mol of 24000-mol.wt. subunit. The values for the compositions of all the hydrolysates are adjusted to the same value for serine to aid comparison. The values are not corrected for destruction or slow release of amino acids.

	Whole lectin	Fractions from Sepharose column		
		First	Second	Third
Asp	23.6	25.2	6.6	7.6
Thr	20.9	19.2	10.8	3.4
Ser	13.7	13.6	13.6	13.7
Glu	17.1	14.0	13.7	11.2
Pro	11.1	9.7	6.0	2.1
Gly	17.2	17.2	13.7	14.7
Ala	14.2	13.8	7.2	5.9
Cys	0.0	0.0	0.0	0.0
Val	13.5	11.1	6.5	5.6
Met	0.0	0.0	0.0	0.0
Ile	10.5	11.4	2.7	1.1
Leu	14.0	10.4	8.3	1.8
Tyr	7.7	6.7	3.2	0.5
Phe	12.4	12.4	3.7	0.8
His	3.6	2.7	1.7	0.8
Lys	10.1	9.8	2.3	0.9
Arg	4.1	3.3	2.3	0.0
GlcN	1.6	1.6	0.0	0.0

Inhibition of agglutination by sugars (*Table* 5)

Our observations confirm those of Tomita *et al.* (1970), that *V. faba* lectin is specific for α-linked derivatives of glucose and mannose. We have compared the inhibitory action of many of the sugars on the four lectins that comprise this group and have found that the specificity of *V. faba* lectin resembles that of *L. culinaris* and *P. sativum* more closely than the lectin from *C. ensiformis*.

Discussion

The lectin from *V. faba*, together with those from *C. ensiformis*, *P. sativum* and *L. culinaris*, form a class of proteins from leguminous plants that specifically bind α-glucose and α-mannose derivatives. The amino acid composition of *V. faba* lectin is similar to that of the other lectins notably in that cyst(e)ine and

Table 5. *Comparison of the inhibitory effect of various saccharides on the agglutinating activity of four glucose/mannose-specific lectins*

For conditions of assay see the Experimental section.

Inhibitor	Concentration (mM) needed to produce 50% inhibition of agglutination			
	Concanavalin A	*L. culinaris* lectin	*P. sativum* lectin	*V. faba* lectin
D-Man	9	50	25	6.3
D-Glc	25	50	25	25
3-*O*-Methyl-D-Glc	>200	12.5	1.6	1.6
3-*O*-Benzyl-D-Glc	67	33	4.2	2.1
Methyl α-D-Glc	6.3	33	12.5	25
Methyl 2,3-di-*O*-methyl-α-D-Glc	16	2.1	0.5	0.5
Methyl α-D-GlcNAc	6.3	50	25	25
Methyl 3-*O*-methyl-α-D-GlcNAc	200	4.6	1.6	2.3
Methyl 4-*O*-methyl-α-D-GlcNAc	100	100	>200	>200
Methyl 6-*O*-methyl-α-D-GlcNAc	200	200	>200	>200
Methyl β-D-GlcNAc	>200	>200	200	>200
Methyl 3-*O*-methyl-β-D-GlcNAc	>200	50	25	50
Glcα1→αGlc (trehalose)	3.1	50	12.5	12.5
Glcα1→2Glc (kojibiose)	2.1	8.3	2.1	4.2
Glcα1→3Glc (nigerose)	8.3	100	67	33
Glcα1→4Glc (maltose)	3.1	50	12.5	12.5
Glcα1→6Glc (isomaltose)	1.2	19.5	4.9	9.8
Glcβ1→2Glc (sophorose)	4.2	50	17	17
Glcβ1→3Glc (laminaribiose)	50	67	67	33
Glcβ1→4Glc (cellobiose)	>200	>200	200	200
Glcβ1→6Glc (gentiobiose)	100	200	100	100
Ovalbumin glycopeptide*	0.02	>3.3	2.0	>3.3
Transferrin glycopeptide*	0.26	0.14	0.53	0.26

* Molarity expressed in terms of mannose content.

methionine are absent. One significant difference is that, whereas *V. faba* lectin is a glycoprotein, the lectins from *C. ensiformis* (Agrawal & Goldstein, 1968), *P. sativum* (Entlicher *et al.*, 1970) and *L. culinaris* (M. J. Crumpton & A. K. Allen, unpublished work) have been shown to contain no covalently bound carbohydrate.

Glycoprotein linkage

We have concluded that the carbohydrate is linked to an asparagine residue of the polypeptide chain by the following reasoning. Of the protein amino acids that have so far been found in glycoprotein linkages, namely asparagine, serine, threonine, cysteine, hydroxyproline and hydroxylysine (see Marshall, 1972), only asparagine, serine and threonine are present in *V. faba* lectin. *O*-Glycosidic linkages that involve the hydroxyl groups of serine and threonine are known in general to undergo β-elimination in the presence of alkali (see review by Marshall & Neuberger, 1970). The conditions that we used for alkaline treatment (see under 'Methods') would have caused the hydrolysis of any such bonds; since there was no decrease in the carbohydrate content of the lectin after this treatment, it is unlikely that serine or threonine is involved in glycosidic linkages. A glycopeptide has been isolated from a Pronase digest of *V. faba* lectin and found to contain, on hydrolysis, aspartic acid (presumably arising from the hydrolysis of asparagine) and alanine as the only amino acids in near-integral amounts. Since peptide-bound alanine is not capable of forming linkages with sugars it is reasonable to conclude that the oligosaccharide is linked to asparagine. Asparagine has so far been found linked only to *N*-acetylglucosamine, and since the analysis of the glycopeptide indicates a molar ratio of asparagine to *N*-acetylglucosamine of approx. 1:1.6 it is reasonable to conclude that the oligosaccharide portion is linked to the protein chain via a linkage between asparagine and *N*-acetylglucosamine.

Subunit structure

From the elution volume of *V. faba* lectin from a column of Bio-Gel P-100 we concluded that the mol.wt. of the whole molecule is approx. 47500, which is in reasonable agreement with the value of 51000 obtained by Wang *et al.* (1974) by sedimentation equilibrium.

From polyacrylamide-gel-electrophoresis patterns Wang *et al.* (1974) concluded that *V. faba* lectin

consisted of dissimilar subunits of 18000, 9000 and possibly 16000 mol.wt. They suggested that the whole molecule could be composed of a tetramer of two 18000- and two 9000-mol.wt. polypeptide chains, a trimer of 18000-mol.wt. chains, or that the minor components were contaminants or fragments of the major polypeptide chain. We have shown by chromatography on Sepharose 4B in guanidine hydrochloride that in our preparations of *V. faba* lectin there are at least three components. The first fraction is eluted from the guanidine hydrochloride-containing column in the volume expected for a protein of mol.wt. 24500, which is in agreement with the subunit mol.wt. determined by polyacrylamide-gel disc electrophoresis in sodium dodecyl sulphate (22500). The polypeptide in the first fraction has the same *N*-terminal amino acid and produces the same number of peptides on tryptic digestion as the whole lectin. The lower-molecular-weight fragments in the second and third fractions produce fewer peptides on tryptic digestion. We therefore suggest that the first fraction eluted from the guanidine hydrochloride-containing column is a true subunit and that the other peaks (about 35% of the total protein) are derived from the subunit (possibly by proteolysis), but in the absence of dissociating agents are joined together by non-covalent forces. This would be similar to the model given by Wang *et al.* (1971) for concanavalin A, in which 40% of the total fully active protein was found to consist of fragments. In highly purified soya-bean agglutinin preparations Lotan *et al.* (1975) have found, by sodium dodecyl sulphate chromatography, low-molecular-weight polypeptide fragments amounting to about 10% of the total protein and have suggested that they are the result of proteolytic cleavage of the intact subunit.

We therefore propose that *V. faba* lectin is composed of two subunits of about 24000 mol.wt. It is likely that the subunits are at least similar, as the number of peptides produced on tryptic digestion is in agreement with that expected from the sum of the lysine and arginine residues for a mol.wt. of 24000 rather than 48000. If we follow the model of concanavalin A (Wang *et al.*, 1971), the subunits will not all be uninterrupted peptide chains, but they will form an active complex of mol.wt. 48000 held together by non-covalent forces.

Specificity of the lectins

A comparison of the inhibitory activity of various sugars on the four lectins shown in Table 5 indicates that concanavalin A differs from the other three lectins in its response to substituted monosaccharide derivatives. Whereas all four lectins are inhibited by α-methyl glycosides and this inhibitory activity is abolished by substitution of the hydroxyl groups at C-4 or C-6, they differ in their response to 3-*O*-substituted monosaccharides. As previously reported (Poretz & Goldstein, 1970), concanavalin A is not inhibited by 3-*O*-substituted sugars, but we have found that *V. faba* lectin, in common with the lectins from *L. culinaris* and *P. sativum*, is at least 10 times more strongly inhibited by 3-*O*-substituted monosaccharides than by the parent sugars. Similar results have been reported for the inhibition of the interaction of the pea lectin with a phosphomannan by 3-*O*-substituted glucose derivatives (Van Wauwe *et al.*, 1975) and also the lentil lectin (J. P. Van Wauwe & F. G. Loontiens, personal communication). The best inhibitor of all the monosaccharide derivatives for the three lectins is methyl 2,3-di-*O*-methyl-α-D-glucoside, which is 25–50 times as inhibitory for *V. faba* and *P. sativum* lectins as is methyl α-D-glucoside. The interaction between the lectins and the 3-*O*-methyl group seems to be strong enough to overcome the steric hindrance of the β-anomer, since methyl 3-*O*-methyl-β-D-*N*-acetylglucosaminide is an inhibitor of the three lectins. Despite the fact that the 3-*O*-methyl and 3-*O*-benzyl sugars are powerful inhibitors of the lectins, the 3-*O*-linked disaccharides of glucose, nigerose and laminaribiose are fairly poor inhibitors, which is also in agreement with the work of Van Wauwe *et al.* (1975) on the pea lectin. From these results we would suggest that there is a hydrophobic area of the binding site that interacts with the methyl group of the 3-*O*-methyl derivatives and the methylene (or benzyl) group of 3-*O*-benzylglucose. The equivalent carbon atom in the 3-*O*-linked disaccharides is C-1 of the pyranose ring of the substituent sugars, which is less hydrophobic than an *O*-methyl group. Moreover, the positions that the two pyranose rings can take up relative to each other are very limited, due to steric hindrance, which may adversely affect the interaction of the inhibitors with the binding site of the lectin. There may, however, be some interaction, since laminaribiose is a better inhibitor of the agglutinating activity of the lectin than are cellobiose and gentiobiose. These results demonstrate that one cannot necessarily extrapolate from results obtained with monosaccharides to conclusions about the specificity of lectins for oligosaccharides. The affinity of the lectins for derivatives of 3-*O*-methylglucose, although probably not of significance for the binding of glycoproteins, does have possible applications. We have found that an affinity column made by linking 3-*O*-methylglucosamine to CH-Sepharose is a much more effective affinity absorbent for the purification of the lectins of *V. faba*, *P. sativum* and *L. culinaris* than is Sephadex. Also, if these lectins are attached to insoluble supports and used for the affinity chromatography of glycoproteins or polysaccharides, 3-*O*-substituted sugars should be useful reagents for the elution of firmly bound glycoproteins.

It is apparent that the four lectins examined in this work differ in their specificities towards mono-

saccharides, disaccharides and glycopeptides (Table 5). The most obvious difference is between concanavalin A and the other three lectins, not only in the inhibitory effect of 3-*O*-substituted sugars, but also in the effect of disaccharides and, particularly, the glycopeptides of ovalbumin and transferrin. There are also differences between the *L. culinaris* lectin and the lectins from *V. faba* and *P. sativum* in that the former is less strongly inhibited by monosaccharides and disaccharides but more strongly inhibited by the transferrin glycopeptide than are the other two lectins. It may be of significance that *Vicia*, *Pisum* and *Lens* are regarded as closely related genera and are classified together in the tribe Vicieae (subfamily Lotoideae) of the family Leguminosae, whereas *Canavalia* is placed in the tribe Diocleae of the same subfamily (Toms & Western, 1971).

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