A Lectin from the Exudate of the Fruit of the Vegetable Marrow (*Cucurbita pepo*) that has a Specificity for β -1,4-Linked *N*-Acetylglucosamine Oligosaccharides

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(Received 21 March 1979)

Lectins are present in the exudate (presumably from the phloem) of the fruits of three species of the Cucurbitaceae, namely vegetable marrow (*Cucurbita pepo*), melon (*Cucumis melo*) and cucumber (*Cucumis sativus*). They are all strongly inhibited in their activities by chitin oligosaccharides, but only weakly by *N*-acetylglucosamine. Glycopeptides from soya-bean agglutinin and fetuin are also strong inhibitors of *Cucurbita pepo* lectin, indicating that it interacts with internal *N*-acetylglucosamine residues. The lectin from *Cucurbita pepo* fruit was purified by affinity chromatography by using chitin oligosaccharides covalently attached to Sepharose. The lectin is not a glycoprotein, and it consists of a single polypeptide chain of about 20000 mol.wt. It is a major protein (18% of the total) of the phloem exudate and it is postulated that it may have an anti-parasitic function.

Lectins are proteins or glycoproteins that are capable of binding monosaccharides, oligosaccharides and glycoproteins with an enzyme-like specificity and of agglutinating cells by forming cross-bridges between membrane glycoproteins. Although the commonest source of plant lectins has been the seeds of legumes, lectins are also found in plants of other families, and not only in seeds but also in roots, leaves and tubers [see reviews by Lis & Sharon (1973) and Goldstein & Hayes (1978)].

Sabnis & Hart (1978) reported that one of the well-characterized major proteins of the phloem exudate of the pumpkin (*Cucurbita maxima*) and of other members of the Cucurbitaceae was a lectin. Although this protein agglutinated human erythrocytes, none of the saccharides that were tested by those authors inhibited the reaction. In the present paper I report that the lectin from the marrow (*Cucurbita pepo*)-fruit exudate and from related species is specifically inhibited by *NN'*-diacetylchitobiose and by glycopeptides that contain this disaccharide, and is therefore similar in specificity to the lectins from wheat-germ (Allen *et al.*, 1973), the potato tuber (Allen & Neuberger, 1973) and thorn-apple (*Datura stramonium*) (Pardoe *et al.*, 1970; Kilpatrick & Yeoman, 1978).

Experimental

Materials

Sepharose was a product of Pharmacia, Uppsala, Sweden. Proteins used as standards for molecular-weight determinations were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. or

from the sources indicated previously (Allen *et al.*, 1976). The methods of synthesis of the derivatives of *N*-acetylglucosamine are given elsewhere (Allen *et al.*, 1973). Chitin oligosaccharides, which are β -1,4-linked polymers of *N*-acetylglucosamine [(GlcNAc)_{2–4}], and the bacterial cell-wall disaccharide, which has a β -1,4 link between *N*-acetylglucosamine and *N*-acetylmuramic acid (GlcNAc–MurNAc) were gifts of Dr. R. C. Davies of this laboratory. Other sugars were bought from Sigma or from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Vegetable marrows (*Cucurbita pepo*), cucumbers (*Cucumis sativus*) and melons (*Cucumis melo*) were bought from local greengrocers. Fetuin was a product of Sigma, soya-bean agglutinin was prepared from soya-bean meal by affinity chromatography on galactosamine–CH–Sepharose (an *N*-hexanoylgalactosamine–Sepharose conjugate) (Allen & Neuberger, 1975a). Glycopeptides were prepared from these glycoproteins by Pronase digestion under the conditions described by Spiro & Bhoyroo (1974) and purified by chromatography on Bio-Gel P-2. They were analysed after hydrolysis in toluene-*p*-sulphonic acid or methanolysis (see under 'Methods').

Methods

Amino acid and amino sugar analyses. A Locarte mini analyser fitted with an automatic-loading attachment was used. The standard system of Mayes *et al.* (1973) was used for the separation of the amino acids (apart from those mentioned below) and amino sugars; this involved successive elution of the 25 cm column of the analyser with sodium citrate buffers

at pH 3.35, 4.40 and 6.65. Tryptophan was separated from the other amino acids by the programme of Liu (1972), which involved elution with sodium citrate buffers at pH 4.25 and 5.28. The absence of hydroxyproline from the hydrolysates was demonstrated by elution of the 25 cm column with a sodium citrate buffer at pH 2.80. Further details of the analytical systems are given elsewhere (Allen *et al.*, 1976).

Samples of protein were hydrolysed for 24, 48 and 72 h in 3 M-toluene-*p*-sulphonic acid at 110°C under N₂ and correction factors obtained for the destruction of serine and threonine residues. The content of glucosamine and galactosamine in the protein was determined in samples that had been hydrolysed at 100°C for 24 h under N₂; this method gives almost quantitative recovery of amino sugars from glycoproteins (Allen & Neuberger, 1975b). Tryptophan was determined on the analyser after hydrolysis in 4 M-methanesulphonic acid [containing 0.2% of 3-(2-aminoethyl)indole] *in vacuo*, at 115°C for 24 h (Simpson *et al.*, 1976).

Half-cystine values were obtained from the analysis of cysteic acid residues resulting from the performic acid oxidation (Hirs, 1956) and subsequent hydrolysis of the protein for 24 h at 110°C in constant-boiling HCl.

Neutral-sugar analyses. These compounds were measured by g.l.c. after methanolysis and trimethylsilylation of the protein samples (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 and amino sugars) to samples taken from the same stock solution of protein.

Assay of agglutination. Full details of the procedure are given elsewhere (Allen *et al.*, 1973, 1976). A serial dilution of the lectin was made in 100 µl of phosphate-buffered saline (7.20 g of NaCl, 1.48 g of Na₂HPO₄ and 0.43 g of KH₂PO₄ per litre; final pH 7.2) and 200 µl of a 1.5% suspension of rabbit erythrocytes was added. The tubes were shaken at intervals and after 2 h the degree of agglutination was assessed visually on the serological scale (0 to ++++). The amount of lectin required to produce half-maximal (++) agglutination was taken as 1 unit. With this method an activity of about 4000 units/mg was obtained for pure marrow lectin. This method is only semi-quantitative and should be regarded as being liable to an error of ±20%. To determine the 50%-inhibition values for various sugars, the serial-dilution method of Lis *et al.* (1970) was used.

Preparation of the affinity support. Details of the preparation of the NN'N''-triacylchitotriose-

Sepharose affinity support are given elsewhere (Desai & Allen, 1979).

Polyacrylamide-gel electrophoresis. The method of Weber *et al.* (1972) was used, which involved electrophoresis in columns (7 cm × 0.5 cm) of 7.5% (w/v) polyacrylamide gel in 0.1 M-sodium phosphate buffer, pH 7.2, containing 0.1% sodium dodecyl sulphate for 6 h at 5 mA/gel. Before electrophoresis the samples were dissolved in a solution of 0.01 M-sodium phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulphate and 1% 2-mercaptoethanol and heated in a boiling-water bath for 2 min. The proteins were stained with Coomassie Brilliant Blue. Cytochrome *c* (mol.wt. 12400), α-chymotrypsinogen (25700), ovalbumin (45000), bovine serum albumin (68000) and phosphorylase (90500) were used as molecular-weight standards.

Results

Purification of marrow lectin

Weber *et al.* (1974) showed that the fruit of pumpkin yielded greater quantities of phloem exudate than did stems; their technique was therefore used for obtaining the exudate from marrow fruit. Ten vegetable marrows (*Cucurbita pepo*) of weight range 700–1500 g were washed and dried. Longitudinal cuts, 2–5 mm deep, were made in the skin of the fruit with a scalpel, and the exudate was quickly sucked into a Pasteur pipette and immediately transferred to about 15 ml of ice-cold phosphate-buffered saline that contained 0.01 M-dithiothreitol or dithioerythritol and had been previously saturated with N₂. About 2–3 ml of exudate (173 mg of protein) was extracted by this method. (It is essential that the transfer and mixing are done rapidly, as the phloem exudate forms a gel within a few minutes of being exposed to air.) Solid (NH₄)₂SO₄ (39.8 g/100 ml) was added to this extract to give 65% saturation at 4°C. The precipitate was allowed to settle at this temperature overnight and then collected by centrifugation at 9000 g for 20 min. This precipitate was then dialysed extensively against phosphate-buffered saline containing 0.01 M-dithiothreitol saturated with N₂, and then re-centrifuged at 9000 g for 20 min.

Affinity chromatography. The supernatant from the previous step (containing 109 mg of protein) was applied to a column (1 cm × 7 cm) of NN'N''-triacylchitotriose-Sepharose that had been equilibrated with the N₂-saturated phosphate-buffered saline/0.01 M-dithiothreitol buffer and washed with 80 ml of the same buffer. Fractions (10 ml) were collected and assayed for agglutinating activity and protein by u.v. absorption. Although about 80% of the protein was eluted with this buffer, the lectin was retained by the column. The column was then washed with 1000 ml of 1 M-NaCl and 250 ml of water

Table 1. *Analysis of marrow lectin*

The values were calculated as molar proportions by assuming that the mol.wt. of the protein subunit is 20000. The values for the nearest integer were derived from an average of the three hydrolyses in 3M-toluene-*p*-sulphonic acid at 110°C, except for serine, which was extrapolated to zero time, tyrosine and methionine, for which the 24h values were taken, valine and isoleucine, for which the 72h values were taken, and cysteine and tryptophan (see the footnotes). For further details, see under 'Methods'.

Time of hydrolysis ...	Amino acid content (mol/mol)			
	24h	48h	72h	Nearest integer
Asp	18.6	18.3	17.9	18
Thr	0.1	0.0	0.2	0
Ser	11.9	11.2	11.0	12
Glu	19.5	19.1	19.1	19
Pro	4.2	4.1	4.6	4
Gly	19.7	19.7	20.0	20
Ala	12.2	12.2	12.0	12
½Cys*	4.7*	—	—	5
Val	13.9	14.8	14.9	15
Met	1.7	1.4	1.2	2
Ile	10.8	11.9	11.8	12
Leu	19.9	19.2	18.7	19
Tyr	5.8	5.4	3.8	6
Phe	7.1	7.3	7.0	7
His	4.7	3.9	4.0	4
Lys	16.3	15.5	15.8	16
Arg	6.9	7.4	6.7	7
Trp†	3.9†	—	—	4
Total				182

* Determined separately as cysteic acid after hydrolysis of a performic acid-oxidized sample (Hirs, 1956).

† Determined from a sample hydrolysed at 115°C for 24h in 4M-methanesulphonic acid containing 0.2% of 3-(2-aminoethyl)indole (Simpson *et al.*, 1976).

Table 2. *Inhibitory effect of various saccharides on the agglutinating activity of marrow lectin*

For conditions of the assay see under 'Methods'. Oligosaccharides contained β -1,4 linkages. The molarity of the glycopeptides is expressed in terms of the core GlcNAc β 1-4GlcNAc content.

Compound	Concn. needed for 50% inhibition (M)
GlcNAc	500
Me- α -D-GlcNAc	500
Me- β -D-GlcNAc	500
(GlcNAc) ₂	0.1
(GlcNAc) ₃	0.1
(GlcNAc) ₄	0.1
GlcNAc-MurNAc	10
(Glc) ₂	>300
Fetuin glycopeptide	0.1
Soya-bean-agglutinin glycopeptide	0.01

without fractions being collected. The lectin was finally eluted from the column with 0.1M-acetic acid, 5ml fractions being collected. The fractions containing the active protein (32mg) were pooled, dialysed against 0.01M-2-mercaptoethanol, concentrated by ultrafiltration and frozen for storage. The recovery of lectin activity was almost quantitative.

Gel filtration in 6M-guanidine hydrochloride. Sepharose 4B was equilibrated with a solution of 6M-guanidine hydrochloride containing 0.1M-Tris/HCl buffer, pH8.6, packed in a column (60cm $\times$ 1.6cm) and eluted at room temperature (20–25°C). The column was calibrated with both native and *S*-carboxymethylated (Konigsberg, 1972) derivatives of cytochrome *c*, α -chymotrypsinogen, ovalbumin, bovine serum albumin, immunoglobulin G and human Tamm-Horsfall glycoprotein. When plotted, both curves showed the linear relationship between the logarithm of the molecular weight and elution volume reported by Mann & Fish (1972). For both the native and the *S*-carboxymethylated marrow lectin, single symmetrical peaks were obtained. Values for the mol.wts. of 29000 ($\pm$ 3500) for the native lectin and 26000 ($\pm$ 3000) for the *S*-carboxymethylated marrow lectin were derived from the relevant calibration curves.

Polyacrylamide-gel electrophoresis. A molecular weight for the reduced protein of 20000 ($\pm$ 1000) was obtained in comparison with marker proteins in 7.5% polyacrylamide gel in the presence of sodium dodecyl sulphate. Only one band was apparent on staining with Coomassie Blue.

Composition of the lectin

The composition of marrow lectin is given in Table 1; the very low content of threonine is noteworthy, and hydroxyproline is absent. The analyses are calculated from the assumption that the subunit mol.wt. is 20000. No trace of glucosamine or galactosamine was found during amino acid analyses, nor were the neutral sugars arabinose, xylose, fucose, galactose or mannose detectable by g.l.c.; the amount of any one of these sugars was assessed as being below 0.02mol/mol of protein.

Table 3. *Inhibitory effects of saccharides on the agglutinating activity of phloem exudates of cucumber (Cucumis sativus) and melon (Cucumis melo)*

For conditions of assay, see Table 2 and the text.

Source of phloem exudate	Inhibitor	Concn. needed for 50% inhibition (mM)
<i>Cucumis sativus</i>	GlcNAc	>500
<i>Cucumis sativus</i>	(GlcNAc) ₂	2
<i>Cucumis melo</i>	GlcNAc	500
<i>Cucumis melo</i>	(GlcNAc) ₂	0.1

Glucose (1–2 mol/mol) was detected by g.l.c. in the preparations, but the presence of this ubiquitous contaminant does not affect the conclusion that marrow lectin is not a glycoprotein.

Inhibition of agglutination by sugars

The results in Table 2 show that marrow lectin activity is inhibited by oligosaccharides or glycopeptides that contain two β -1,4-linked *N*-acetylglucosamine residues. There is weak inhibition by *N*-acetylglucosamine and its methyl glycosides; in molar terms they are about 5000 times less inhibitory than *NN'*-diacetylchitobiose. Cellobiose, the β -1,4-linked disaccharide of glucose, is not inhibitory. It is also noteworthy that the disaccharide from the bacterial cell wall (GlcNAc–MurNAc) is 100 times less inhibitory than the chitin disaccharide. A very slight inhibition was produced by 500 mM-*N*-acetylgalactosamine and -*N*-acetylmannosamine, and there was no inhibition by 500 mM-glucose, -galactose, -mannose or -fucose, which confirms the results of Sabnis & Hart (1978).

Assay of lectin activity in related species (Table 3)

Lectin activity was also detected in the phloem exudate of the fruit of two other members of the Cucurbitaceae, as reported by Sabnis & Hart (1978). The lectins from *Cucumis melo* and *Cucumis sativus* were both strongly inhibited in their activities by *NN'*-diacetylchitobiose, and the lectin from *Cucumis melo* is also weakly inhibited by *N*-acetylglucosamine.

Discussion

There is no doubt from the observations of Sabnis & Hart (1978) and the confirmation that I report in the present paper that the phloem exudates of at least three species of the Cucurbitaceae contain lectins. The lectins of *Cucurbita maxima* (Sabnis & Hart, 1978) and *Cucurbita pepo* (the present paper) are identified with the components of 20000–22000 mol.wt. known as 'fraction 2' of the phloem exudate. This fraction is a major component of the exudate, representing 15–25% of the total protein, and has been studied in a number of laboratories (Walker, 1972; Beyenbach *et al.*, 1974; Weber *et al.*, 1974; Sabnis & Hart, 1976, 1978).

For the preparation of *Cucurbita* lectin I have used the marrow fruit, as recommended by Weber *et al.* (1974), since yields are quite high and the same fruit can be 'milked' repeatedly at intervals of a few days. Sabnis & Hart (1978) purified the lectin by conventional ion-exchange chromatography; I have found that an *NN''*-triacetylchitotriose-Sepharose column that has previously been used in this laboratory for the purification of potato lectin (Desai & Allen, 1979) is very effective for the purification of marrow lectin (although an affinity support

with a single glucosamine attached to CH-Sepharose would not bind this lectin). By this method a yield of 18% in terms of total protein of the phloem exudate was obtained. From the specificities given in Table 3 it would seem likely that the melon lectin, but possibly not the cucumber lectin, could be purified by using this system.

The amino acid analysis shows a general but not complete similarity to that published by Weber *et al.* (1974) for the pumpkin (*Cucurbita maxima*) fraction 2, aspartic acid and glutamic acid or their amides, glycine, leucine and lysine being the most abundant amino acids, and cyst(e)ine is present. In contrast with *Cucurbita maxima*, this preparation of *Cucurbita pepo* fraction 2 lacks threonine. Weber *et al.* (1974) reported that the phloem proteins of *Cucurbita maxima* do not contain sugars; from the analyses reported in this paper there is no doubt that *Cucurbita pepo* lectin is not a glycoprotein. In this respect it is similar to concanavalin A (Agrawal & Goldstein, 1968), wheat-germ agglutinin (Allen *et al.*, 1973), peanut lectin (Lotan *et al.*, 1975), pea lectin (Entlicher *et al.*, 1970) and lentil lectin (M. J. Crumpton & A. K. Allen, unpublished work reported in Allen *et al.*, 1976). It seems likely that marrow lectin is composed of a single polypeptide of about 20000 mol.wt. If it is associated into oligomers in the native state, as are most lectins, the linkages would be non-covalent, because, on chromatography in 6M-guanidine hydrochloride, the native and *S*-carboxymethylated lectins have similar molecular weights.

The lectins from *Cucurbita pepo*, *Cucumis sativus* and *Cucumis melo* are all specifically inhibited by *NN'*-diacetylchitobiose, the β -1,4-linked disaccharide of *N*-acetylglucosamine. *N*-Acetylglucosamine and its methyl glucosides are very weak inhibitors, being 5000 times less inhibitory than the disaccharide. When *Cucurbita pepo* lectin was studied further it was apparent that tri- and tetra-saccharides are not notably better inhibitors than the disaccharide. This is in contrast with wheat-germ agglutinin, for which the best inhibitor is the tri-*N*-acetylchitotriose (Allen *et al.*, 1973), and potato lectin, which is most strongly inhibited by the tetrasaccharide (Allen & Neuberger, 1973). *Cucurbita* lectin does resemble these lectins in that cellobiose (Glc β 1–4Glc) is not an inhibitor, and the bacterial cell-wall disaccharide (GlcNAc β 1–4MurNAc) is a moderately potent inhibitor. It differs from the lectins from *Cytisus sessilifolius* (broom) and *Laburnum alpinum* (laburnum), which are inhibited by both *NN'*-diacetylchitobiose and cellobiose (Watkins & Morgan, 1962).

It is also apparent that marrow lectin resembles wheat-germ agglutinin (Goldstein *et al.*, 1975) in interacting with internal disaccharides, as the glycopeptides from soya-bean agglutinin and fetuin are

both strong inhibitors. Both these glycopeptides have been shown to have a core structure of Man β 1-4GlcNAc β 1-4GlcNAc β Asn (Spiro, 1973; Lis & Sharon, 1978). In neither compound are *N*-acetylglucosamine residues terminal, and in soya-bean agglutinin the *N*-acetylglucosamine residues are confined to the core disaccharide, which is linked to asparagine (Lis & Sharon, 1978). The glycopeptide from soya-bean agglutinin is a 10-fold better inhibitor of marrow lectin than fetuin glycopeptide or the oligosaccharides, and it is possible that there is some interaction with either the β -linked mannose or the asparagine residue.

Possible roles for plant lectins have been discussed in reviews by Callow (1977) and Sequeira (1978). When reporting the presence of a lectin in *Cucurbita* phloem exudate, Sabnis & Hart (1978) discussed the possible biological advantage of having a lectin at such concentrations (15–25% of the total protein) in the phloem sieve tube. They considered that involvement in a sugar-transporting system was unlikely, and this hypothesis now becomes more unlikely with the knowledge that the specificity is directed towards a disaccharide that is not present in plant extracts in an unconjugated form. An alternative suggestion was that the fraction-2 protein (lectin) is linked by disulphide bonds to the fraction-1 protein (in filamentous form) and that the lectin could then anchor the filaments to glycoproteins of the sieve-tube plasma membrane. Some support may be given to this hypothesis by the observation presented here that the most tightly bound inhibitor is a glycopeptide from a plant glycoprotein (soya-bean agglutinin).

Marrow lectin binds to chitin oligosaccharides, and chitin is a common component of fungal cell walls. It also interacts with a disaccharide of murein, the polysaccharide of bacterial cell walls. Wheat-germ agglutinin, which has a similar specificity, has been shown by Mirelman *et al.* (1975) and Barkai-Golan *et al.* (1978) to inhibit the growth of fungal hyphae, and Sequeira & Graham (1977) have shown an interaction between the lipopolysaccharide of *Pseudomonas solanacearum* and potato lectin. It has been suggested that interactions such as these are involved in the defence of the plant against infection, and it would therefore seem possible that the lectins that are present in such large amounts in the phloem of the Cucurbitaceae have an anti-parasitic function.

I thank the Agricultural Research Council for financial support.

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