Tissue and Subcellular Distribution of the Lectin from *Datura stramonium* (Thorn Apple)

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Plants of *Datura stramonium* (thorn-apple) were dissected into their component tissues and examined for the presence of the *Datura* lectin. This lectin was easily detected in seeds and in various parts of the flowers of adult plants. Traces were also found in green (emerged) cotyledons and roots of seedlings. The specific lectin activity in seeds contained within the fruits increased as the seeds matured. Mature seeds were homogenized in sucrose and separated by differential centrifugation into four fractions, three of which were clearly of distinct composition. Most of the lectin activity sedimented with the low-speed (cell-wall/protein-body) pellet, but a similar specific activity was recovered from the other fractions. However, if EDTA was included in the homogenization medium, three or four times more lectin activity was recovered in the soluble fraction. Immunofluorescent staining of formaldehyde-fixed sections showed that the lectin was localized in the cytoplasm, with little associated with cell walls. The possible relevance of these results to the function of the lectin in plant cells is discussed.

Lectins are protein or glycoprotein agglutinins that appear to act after binding to glycoconjugates on the surface of cells that they agglutinate (Sharon & Lis, 1972). Despite extensive studies on the interactions of lectins with animal cells (Nicolson, 1974), little is known about the function(s) of such proteins in the plants from which they are isolated. The possibilities catalogued in recent reviews (Liener, 1976; Yeoman *et al.*, 1978) have at best circumstantial evidence to support them, and represent little advance over the ideas discussed by Boyd (1963) 16 years ago.

Clearly a helpful step towards understanding the physiological role of lectins would be to discover the distribution of these proteins both within the plant and within the cell. Most lectins have been detected in, and isolated from, seeds (a readily available source with a low water content, which makes them convenient to work with), but in some species lectin activity has also been found in other tissues. For example, extracts of root and stem of the common lentil plant (*Lens culinaris*) (Howard *et al.*, 1972), tubers of the potato (*Solanum tuberosum*) (Marinkovich, 1964), and the megaspore wall of the cycad *Cycas siamensis* (Pettitt, 1977), all exhibit lectin

activity. Additionally, there have been several reports of the subcellular location of certain lectins. Clarke *et al.* (1975) used an immunofluorescence method to demonstrate the presence of concanavalin A and phytohaemagglutinin in cytoplasmic organelles of cotyledons of the jack (*Canavalia ensiformis*) and kidney bean (*Phaseolus vulgaris*) respectively. Bowles & Kaus (1975) carried out a subcellular fractionation of mung-bean (*Phaseolus aureus*) hypocotyls and found lectin activity associated with all the membrane fractions obtained, including mitochondria and plasmalemma. Lectin activity has also been extracted from very pure preparations of inner mitochondrial membrane obtained from the endosperm of the castor bean (*Ricinus communis*) (Bowles *et al.*, 1976).

Here studies are described on the tissue distribution of the lectin from *Datura stramonium* (thorn-apple) (Kilpatrick *et al.*, 1978; Horejsi & Kocourek, 1978) and the subcellular location of this substance within the cotyledonary cells of the mature seed.

Materials and Methods

Purification of the *D. stramonium* lectin

Seeds of *D. stramonium* (obtained from Thompson and Morgan, Ipswich, Suffolk, U.K.) were ground in a seed mill, suspended in 15 vol. of 0.15 M-NaCl, and stirred at 4°C overnight. The supernatant obtained after centrifugation at 35000g for 30 min was cooled to 0°C and mixed with 3 vol. of acetone at 0°C. The resulting precipitate was extracted with 0.1 M-sodium

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acetate adjusted to pH 7.2 with acetic acid. The solution obtained contained all of the original lectin activity.

This crude lectin preparation was dialysed against phosphate-buffered saline (0.15M-NaCl/0.05M-sodium phosphate, pH 7.2), then mixed with glutaraldehyde-fixed erythrocytes (20% cells, v/v) and left with very gentle stirring for 30 min at room temperature. The fixed erythrocytes were prepared by suspending freshly obtained human blood-group-O cells in 3% (v/v) glutaraldehyde (Sigma Chemical Co., Poole, Dorset, U.K.) in phosphate-buffered saline (at a concentration of 20%, v/v) for 1 h at room temperature before washing the cells four times with phosphate-buffered saline.

The lectin was eluted from the erythrocytes by suspending the cells for 30 min at 20°C in 10mM-sodium acetate adjusted to pH 3.0 with acetic acid. The cells were then harvested (1000g; 10 min) and the supernatant was neutralized and dialysed against a large volume of phosphate-buffered saline.

It was calculated that approx. 1% of the lectin activity bound to the fixed erythrocytes was recovered by this means. The purified preparation exhibited two protein bands after electrophoresis in sodium dodecyl sulphate/polyacrylamide gels by the method of Laemmli (1970). A single protein band was obtained when sodium dodecyl sulphate was omitted from the system.

Preparation of anti-lectin serum

The purified lectin preparation (50 µg of protein/ml), suspended in Freund's Complete Adjuvant (1:1, v/v), was injected subcutaneously into rabbits at multiple sites (0.3 ml/site) at the base of the neck. Subsequent injections were given in Freund's Incomplete Adjuvant at 10-day intervals. The adjuvants were purchased from Difco Laboratories, West Molesey, Surrey, U.K. After 2 months the rabbits were bled from ear veins. The blood was allowed to clot overnight at 4°C and, after centrifugation at 1000g for 10 min, the serum was removed and stored at -20°C until used.

Subcellular fractionation

Seeds of *D. stramonium* (2 g dry wt.), obtained from mature fruits grown in a greenhouse, were divided into two groups, and each group was covered with water in a Petri dish and left at room temperature for 24–28 h. The imbibed seeds were blotted with filter paper and transferred to the cold-room. All subsequent operations were performed between 0 and 4°C. One group of seeds was homogenized with a mortar and pestle in 12 ml of 0.25M-sucrose. The other group was similarly homogenized but in 0.25M-sucrose containing 5mM-EDTA (disodium salt). Each homogenate was strained separately through Miracloth (Calbiochem, Bishop's Stortford,

Herts., U.K.), a small sample (about 1 ml) was retained for assays (starting homogenate) and 10 ml was centrifuged at 1000g for 5 min to obtain the wall pellet. The supernatant was then centrifuged at 13000g for 15 min to obtain the intermediate pellet. Both centrifugations took place in an MSE 18 High-Speed centrifuge with an 8×50 rotor. The supernatant resulting from the second centrifugation step was then centrifuged at 100000g for 1 h in an MSE Super-Speed 50 centrifuge in a 10×10 rotor. All the pellets were resuspended in approx. 10 ml of phosphate-buffered saline and, along with the starting homogenate and the supernatant from the final centrifugation (soluble fraction), were dialysed overnight against 5 litres of phosphate-buffered saline. Finally, each fraction was adjusted to 13 ml in phosphate-buffered saline and assayed for protein and lectin activity.

Immunofluorescent staining of sections

Seeds of *D. stramonium* obtained from mature fruits were allowed to swell for 24 h at room temperature in water. The seed coats were then removed with a razor blade and the coat-less 'seeds' left overnight at 4°C in a large excess of 2% (w/v) formaldehyde in 5mM-sodium phosphate, pH 7.2. The fixed 'seeds' were frozen by immersion in solid CO₂/ethanol and cut into 20 µm sections with a cryostat. The sections were placed in wells of agglutination trays containing phosphate-buffered saline. The phosphate-buffered saline was removed with a Pasteur pipette, and replaced either with a globulin fraction prepared from rabbit anti-*(Datura)* lectin serum or a corresponding globulin fraction prepared from non-immune rabbit serum. In each case the globulin fraction was obtained from the appropriate serum by precipitation with (NH₄)₂SO₄ added to 42% saturation, and was used at a protein concentration of 10 mg/ml. After 30 min at room temperature, the sections were washed every 10 min for 1 h and then treated with Fluorescein isothiocyanate-conjugated goat anti-(rabbit immunoglobulin G) solution (1 vol. of conjugate from Miles Laboratories in 9 vol. of phosphate-buffered saline). Finally, they were again washed frequently with phosphate-buffered saline and examined under visible and u.v. light with a Vickers Photoplan fluorescence microscope.

Assays

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma type V) as standard. Lectin activity was measured by observing the settling pattern of rabbit or human erythrocytes in agglutination trays, as previously described (Kilpatrick *et al.*, 1978). Activity was taken to be the reciprocal of the titre. Since the volume of lectin solution added to each well of the agglutination trays is 25 µl, a titre of 1/8, for example, would be

EXPLANATION OF PLATE I

Subcellular fractionation of D. stramonium seeds

(a) Section of intact seed through a cotyledon; magnification $\times 3000$. (b) Wall pellet; magnification $\times 4800$. (c) Intermediate fraction; magnification $\times 15600$. (d) Microsomal pellet; magnification $\times 36000$. All specimens were fixed with 3% glutaraldehyde in 0.1M-sodium cacodylate, pH 7.2, post-fixed with OsO_4 and stained with lead citrate. Details of the fractionation method are given in the Materials and Methods section. Key: P, protein body; L, lipid; cw, cell wall.

EXPLANATION OF PLATE 2

*Immunofluorescent staining of sections through the cotyledons of *D. stramonium* seeds*

(a) Specific and (b) control globulin staining viewed under u.v. light; (c) and (d) respectively show the same areas viewed under visible light. Details are given in the Materials and Methods section. All magnifications are $\times 200$.

8 units/25 μ l or 320 units/ml. Specific lectin activity is the activity in such units per mg of protein.

Results

Tissue distribution of the Datura lectin

Datura stramonium plants were dissected into various component tissues. Each tissue was homogenized in phosphate-buffered saline and tested both for haemagglutinating activity and for the ability to form a precipitation line after double diffusion on agar against antiserum to the purified lectin (Table 1).

By far the highest specific activity was found in the seed. Furthermore, mature seeds had a much higher specific activity than immature seeds. (By 'immature' seeds is meant the soft white seeds taken from newly formed fruits, and by 'mature' seeds is meant the hard desiccated black or brown seeds obtained from old plants in which the fruits have burst open.) Flowers in bloom were also a minor source of lectin, and when such flowers were further dissected, lectin was clearly demonstrated in petals, stamens and ovaries. Homogenates of stigma plus style also reacted with the antiserum, giving a very weak precipitation line.

Emerged (green) cotyledons and roots from seedlings also possessed traces of lectin, but none could be demonstrated in stems or leaves, whether the homogenates of these latter tissues were prepared from seedlings or from mature plants bearing fruits.

Subcellular distribution of the Datura lectin

Mature seeds of *D. stramonium* were homogenized in 0.25M-sucrose and separated by differential centrifugation into four fractions. Intact cells from the cotyledons of such seeds (Plate 1a) have a very

high concentration of protein bodies and lipid bodies or spherules. After homogenization, a fraction rich in protein bodies and cell-wall fragments (Plate 1b) can be obtained by gentle centrifugation. Further centrifugation (13000g, 15min) yields an 'intermediate' fraction containing many spherules plus some large membrane vesicles, some amorphous electron-dense material, which may be chromatin released from nuclei, and a few apparently dormant mitochondria, proplastids and other structures difficult to identify with certainty (Plate 1c). A final high-speed centrifugation (100000g, 1h) resulted in a microsomal pellet rich in membrane vesicles (Plate 1d), and a final supernatant, the soluble fraction.

Lectin (haemagglutinating) activity was recovered in all four fractions, and typical values are given in Table 2. The specific lectin activity does not vary greatly between the fractions, although the intermediate and microsomal-fraction values are marginally higher than in the starting homogenate.

A significant difference was obtained, however, when the seeds were homogenized in sucrose to which 5mM-EDTA (disodium salt) had been added. Typically, a 3-4-fold increase in the proportion of lectin activity recovered in the soluble fraction was obtained in the presence of EDTA. Although EDTA was responsible for an increase in protein in the soluble fraction of less than 5%, the proportion of lectin activity increased from 15-20% to 40-60%. Correspondingly, all three particulate fractions had lower proportions of lectin activity in the presence of EDTA.

Triton X-100 was added to each fraction to a final concentration of 0.1%, and the samples were extracted overnight before being assayed again for lectin

Table 1. *Tissue distribution of the Datura lectin*

The individual tissues were dissected from whole plants, homogenized in phosphate-buffered saline with a mortar and pestle, strained through a layer of Miracloth, and freed of large particles by centrifugation at 5000g for 15min. The supernatant from each homogenate was then adjusted to a protein concentration of 2mg/ml before being assayed for haemagglutinating activity. The ability of the homogenates at a variety of protein concentrations to form a precipitation line in double diffusion against antiserum to purified *Datura* lectin was also tested.

Source	Agglutination titre	Immunoprecipitation ?
Immature seeds	1/8	Yes
Mature seeds	1/128	Yes
Emerged cotyledons	1	Yes
Roots	1	Yes
Leaves	0	No
Stem	0	No
Whole flowers	1/2	Yes
Ovaries	1/2	Yes
Stigma + style	0	Yes
Anthers	1/2	Yes
Stamens minus anthers	1/2	Yes
Petals	1/4	Yes
Sepals	0	No

Table 2. *Subcellular distribution of the Datura lectin*

Values given in parentheses represent the activity recovered as a percentage of the starting homogenate. For experimental details, see the Materials and Methods section. A unit of activity is that amount of lectin producing a titre of 1 under standard assay conditions.

Fraction	(a) Sucrose only			(b) EDTA/sucrose		
	$10^{-3} \times$ Activity (units)	Protein (mg)	$10^{-3} \times$ Sp. activity	$10^{-3} \times$ Activity (units)	Protein (mg)	$10^{-3} \times$ Sp. activity
Homogenate	64.0	75.5	0.85	96.0	84	1.14
Wall	38.4 (60 %)	47.0 (62.3 %)	0.82	51.2 (53 %)	55 (65 %)	0.93
Intermediate	6.4 (10 %)	5.0 (6.6 %)	1.28	3.2 (3.3 %)	3.6 (4.3 %)	0.89
Microsomal	0.8 (1.25 %)	0.8 (1.1 %)	1.00	0.8 (0.8 %)	1.2 (1.4 %)	0.67
Soluble	10.0 (15.6 %)	12.25 (16.2 %)	0.82	40.0 (41.7 %)	14.8 (17.6 %)	2.7

activity. This treatment had no effect on the soluble fractions, but the lectin activity of the starting homogenates, wall fractions and intermediate fractions increased by 50–100 %. The greatest change was in the microsomal-pellet extract, the lectin activity of which increased about 3-fold.

Immunofluorescent localization of the Datura lectin

An alternative and complementary method of examining the subcellular distribution of the lectin is to use an immunofluorescence method. Sections of formaldehyde-fixed seed tissue were treated with rabbit anti-(*Datura* lectin) globulin followed by Fluorescein isothiocyanate-conjugated goat anti-(rabbit immunoglobulin G) and compared with a control in which non-immune rabbit serum globulin was used (Plate 2). Specific fluorescence appeared to be associated with the plasmalemma and throughout the cytoplasm. Little or none was associated with the cell wall. As a result of the extensive washing procedure used, the cytoplasmic contents of some cells were lost, leaving only the cell walls. This appears to confirm the lack of specific fluorescence in the wall.

Discussion

Plant tissues sometimes possess non-protein haemagglutinins (Mäkelä, 1957; Howard *et al.*, 1972) that might be mistaken for lectins if care is not taken to exclude that possibility. Therefore, in the present study, component tissues of *D. stramonium* were examined not only for haemagglutinating activity, but also for the ability to form a precipitation line after Ouchterlony immunodiffusion against anti-serum raised against the purified lectin.

By such means the lectin was detected in trace amounts in the emerged cotyledons and roots of *Datura* seedlings, but could not be detected in the

somatic tissues of the adult plant. Detectable lectin re-appeared with flower formation, and exhibited a marked increase in specific activity in seed tissue, which correlated with the development of the fruit. The appearance of lectin coincides temporally with the formation of protein bodies and spherules, suggesting that the lectin may be involved in some process connected either with the storage of food reserves or with the utilization of food reserves during germination.

After homogenization of seeds in sucrose, more than half of the lectin activity co-sedimented with protein bodies in the 5000g-min pellet. The specific activity of the lectin in that fraction, however, was not significantly different from that of the other three fractions. As three of the fractions were clearly of distinct composition, these results may be an indication that the lectin occurs in several different intracellular structures or in some material common to all fractions. When EDTA was added to the homogenization medium, most lectin activity was recovered in the soluble fraction, and that fraction also exhibited the highest specific activity under those conditions. It is possible that EDTA acts by preventing soluble lectin from binding specifically or non-specifically to particulate material during homogenization. It is known, however, that EDTA does not affect *Datura* lectin activity (Kilpatrick *et al.*, 1978), and also that EDTA is effective at removing 'extrinsic' or 'peripheral' proteins from membranes (Singer & Nicolson, 1972). It is therefore possible that the EDTA acts by releasing lectin loosely bound to intracellular membranes. The finding that further lectin could be extracted with Triton X-100 from all but the soluble fractions (obtained after homogenization with or without EDTA), and most notably with the microsomal fraction, suggests that at least some of the lectin is membrane-bound within the cell. Alternatively, the lectin could be trapped in

sealed membrane vesicles that are made leaky by the detergent.

The immunofluorescent staining of cotyledon sections provides information complementary to the subcellular-fractionation data. The lectin sedimenting with the 5000g-min pellet is most probably associated with protein bodies or cell-wall fragments, as those were the structures most enriched in that low-speed fraction. The immunofluorescent staining, however, clearly indicated insignificant lectin association with cell walls. Specific staining was found throughout the cytoplasm, and the plasmalemmas also were delineated. The staining pattern, however, was not uniform, possibly indicating association with particulate material, yet it did not appear to be confined to any single type of structure, organelle or inclusion. Previous studies in this laboratory with immunofluorescent staining of sections from the stem of adult *Datura stramonium* have shown a staining pattern similar to that obtained with cotyledons (Yeoman *et al.*, 1978). Presumably the inability to detect the lectin in preparations from stems by using Ouchterlony immunodiffusion against serum raised against the purified lectin is due to the great sensitivity of the immunofluorescence technique. Therefore it would appear that the lectin, albeit at a very low concentration, is also a component of mature somatic cells. It is possible that the lectin is a general membrane component, occurring in several or perhaps all intracellular membranes, but in varying abundance.

Clearly the precise function of a lectin cannot be determined simply by ascertaining its location within a cell or tissue. However, any suggested function must be consistent with where the lectin is located. In particular, it has been suggested that lectins might play a part in the recognition of self from non-self (Clarke *et al.*, 1975). If that is true, then it might be expected that such lectins would be located partly or largely in the cell wall and/or plasmalemma, and would presumably be found in most if not all of the plant. The location and developmental regulation of the *Datura* lectin do not rule out the possibility of a recognition function for this molecule, but are perhaps more consistent with the lectin as a membrane

protein in tissues, such as the seed, involved with the formation, maintenance or utilization of food reserves.

Lectins are a heterogeneous group of proteins that share a common and definitive property, the ability to bring about haemagglutination by binding to surface saccharides. Lectins from different sources are not necessarily related, nor need they have the same function. Furthermore, the *Datura* lectin belongs to the minority that are not from leguminous sources. It is therefore important to remember that the results reported here cannot with any confidence be used to predict the tissue or subcellular location of lectins from other species.

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