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ISOLATION AND PROPERTIES OF *N*-ACETYLLACTOSAMINE-SPECIFIC LECTINS FROM NINE *ERYTHRINA* SPECIES

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Key Word Index—*Erythrina*; Leguminosae; isolation; characterization; lectins.

Abstract—Lectins from seeds of nine species of *Erythrina* have been purified by affinity chromatography on columns of lactose coupled to Sepharose and their properties compared with those of the lectin from *Erythrina cristagalli*. All lectins are glycoproteins of M_r *ca* 60 000 composed of two identical or nearly identical subunits. They contain between 3–10% carbohydrates comprised of *N*-acetylglucosamine, mannose, fucose and xylose. The amino acid composition of all *Erythrina* lectins is very similar. The *N*-terminal amino acid is valine, with the exception of the lectin from *E. flabelliformis* in which it is alanine. To the extent tested, identities or near identities have been found in the *N*-terminal sequences (up to 15 residues in some cases) of the lectins. Hapten inhibition experiments of agglutination have shown that the lectins are specific for *N*-acetyllactosamine, this disaccharide being 10–30 times more inhibitory than D-galactose and 10–20 times more than *N*-acetyl-D-galactosamine. All lectins agglutinate human erythrocytes equally well, irrespective of blood type, at minimal concentrations of 5–20 μg/ml. Six of the lectins are also very effective in agglutinating rabbit erythrocytes and are mitogenic for human peripheral blood lymphocytes, whereas three of them are considerably weaker hemagglutinins for rabbit erythrocytes, and two of these are also very weak mitogens. Our results, while demonstrating striking similarities in the molecular properties and sugar specificity of all *Erythrina* lectins studied, suggest the existence of differences at or close to the carbohydrate-binding site.

INTRODUCTION

The demonstration that the amino acid sequences of lectins from different legumes exhibit extensive homologies [1] has stimulated interest in the correlation between the taxonomy of plant species and the physicochemical properties of lectins derived from them (reviewed in refs [2–4]). We have focused our attention on the genus *Erythrina*, which belongs to the subtribe Phaseoleae.

Trees and shrubs of *Erythrina* are widely distributed throughout the tropics and subtropics. The genus consists of *ca* 110 species and is very distinct from all other genera of its family [5]. The presence of hemagglutinating activity in extracts from seeds of different species of *Erythrina* has been known for a long time. Thus, Mäkelä [6] has found such activity in seeds of *E. bogotiana*, *E. cristagalli*, *E. glauca*, *E. indica* and *E. vespertilis* and has shown that the extract of *E. indica* is specifically inhibited by galactose* and lactose. Bhatia and Boyd [7] demonstrated that the hemagglutinating activity of the extract of *E. suberosa* is inhibited by *N*-acetylgalactosamine as well. Additional studies extended the number of *Erythrina* species exhibiting hemagglutininating activity [8]. However, prior to 1980, no work was done on the lectins responsible for this activity. Since then, a number of *Erythrina* lectins have been purified and characterized to varying degrees (*E. indica* [9]; *E. indica*, *E. arborescens*, *E. subero* and *E. litosperma* [10]; *E. corallodendron* [11]; *E. variegata* [12]; and *E. edulis* [13]).

We have isolated and extensively characterised the lectin from *Erythrina cristagalli* (ECL)† [14] and examined in detail its carbohydrate specificity [15–16]. ECL is a dimeric glycoprotein of M_r 56 000, specific for *N*-acetyllactosamine; it is the first lectin demonstrated to be specific for this disaccharide which occurs frequently in glycoproteins. Here we report on the isolation in good yield and on the properties of lectins from nine other species of *Erythrina* of widely different origin, namely *E. caffra* (Cape Province, South Africa), *E. corallodendron* (West Indies), *E. flabelliformis* (North-west Mexico and Arizona, U.S.A.), *E. latissima* (tropical Africa), *E. lysistemon* (South Africa), *E. humeana* (South Africa), *E. perrieri* (Madagascar and Mauritius), *E. stricta* (Continental Asia) and *E. zeyheri* (South Africa). The nine lectins are compared with those of other *Erythrina* species previously described. Preliminary reports dealing with part of this work have been presented [17, 18].

RESULTS AND DISCUSSION

All nine lectins were purified by affinity chromatography on a column consisting of lactose coupled to divinylsulphone-activated Sepharose. This column was found to give better yields than that of immobilized

*All sugars are of the D configuration, except for fucose which is presumably L.

†The following abbreviated names for the *Erythrina* lectins are used: ECL—lectin from *E. cristagalli* (hitherto known as ECA); ECafL—*E. caffra*; ECorL—*E. corallodendron*; EFL—*E. flabelliformis*; EHL—*E. humeana*; ELatL—*E. latissima*; ELysL—*E. lysistemon*; EPL—*E. perrieri*; ESL—*E. stricta*; EZL—*E. zeyheri*.

galactose used for the isolation of ECL [14]. Each lectin was eluted from the affinity column with 0.2 M galactose as a sharp peak, as illustrated for *E. corallodendron* in Fig. 1. Yields were in the range of 60–120 mg from 60 g defatted meal, representing 50–80% of the hemagglutinating activity of the crude meal extract (Table 1). Upon gel filtration on a column of Sephadex G150, each lectin emerged as a single symmetrical peak at a volume close to that of ECL, indicating that all of them have M_rs *ca* 60000 (Fig. 2). The M_r values of several of the lectins were also determined by ultracentrifugation using the same partial specific volume as that calculated for ECL. This is justified in view of the very similar composition of the different lectins (see below). The values obtained were all close to 60000 (Table 1). On discontinuous sodium dodecylsulfate polyacrylamide gel electrophoresis (PAGE), each of the lectins of *E. corallodendron*, *E. lysistemon*, *E. caffra*, *E. zeyheri*, *E. stricta* and *E. humeana*, migrated as a single band of M_r close to 30000, demonstrating that they each consist of two identical subunits (Fig. 3). In the remaining lectins, two bands were discerned, a main band of apparent M_r close to 30000 and a fainter, somewhat slower one (Fig. 3); these lectins may thus consist of two subunits of slightly different M_r. The reason for the apparently unequal amounts of the two subunits is not clear. In no case was there any evidence for protein impurities in the isolated lectins. The molecular properties of the newly isolated lectins resemble those of the *Erythrina* lectins described previously [9, 10, 13, 14]. The only exception is a M_r of 110000–120000 reported for the lectin from *E. corallodendron* [11]. This value appears to be incorrect.

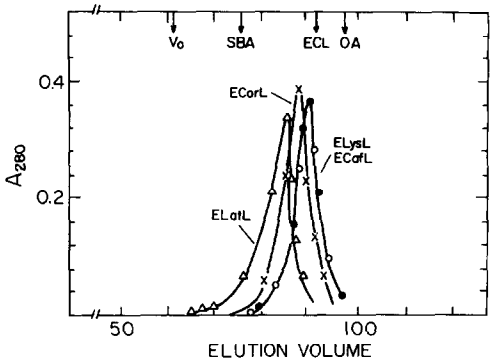

Fig. 2. Gel filtration of purified *Erythrina* lectins on Sephadex G 150. Composite figure of four independent runs. In each run one of the lectins (5 mg in 2 ml phosphate buffered saline, pH 7.4) was applied together with ^{125}I-ECL (10000 cpm) to a column (1.8 × 65 cm) of Sephadex G 150 superfine equilibrated with phosphate buffered saline, pH 7.4. Fractions of 1.2 ml were collected at a speed of 5 ml/hr. SBA—soybean agglutinin (M_r 120000); OA—ovalbumin (M_r 46000).

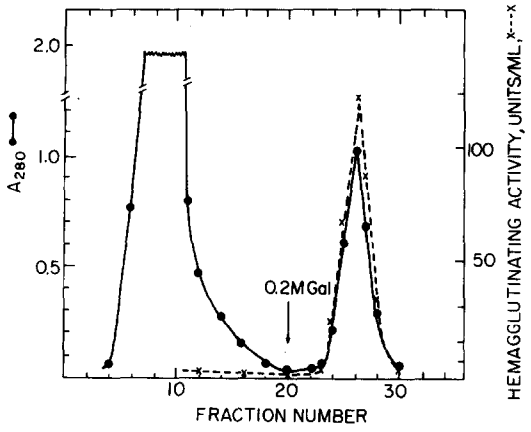

Fig. 1. Affinity chromatography of *E. corallodendron* on a column of lactose-derivatized Sepharose 4B. The dialysed ammonium sulphate precipitate obtained from 20 g defatted meal was applied to a 1.8 × 15 cm column equilibrated with phosphate buffered saline, pH 7.4. Elution was with 0.2 M galactose.

The *Erythrina* lectins are glycoproteins with between 3–10% neutral sugar (Table 1). After PAGE, they all give a positive reaction for carbohydrates with the Schiff reagent (Fig. 3). They contain fucose, xylose, mannose and *N*-acetylglucosamine in varying proportions (Table 1). The same monosaccharide components have been shown to be present in bromelain, the proteolytic enzyme from pineapple [19], and in a number of plant lectins [20]. No xylose has ever been found together with mannose and *N*-acetylglucosamine in the same carbohydrate units of

Table 1. Purification and molecular properties of the *Erythrina* lectins

	ECorL	ELatL	ELysL	ECafL	ECL*
Yield (mg)	75–100	60–70	100–120	80–100	100
Yield (%)	50–70	60–70	60–75	65–80	75
M_r (ultracentrifugation)	60246	61980	58840	59300	56800
Subunit M_r (SDS-PAGE)	28000	32000	28000	30000	27000 29000
Neutral sugars (phenol)	5.5%	9%	6.2%	5.6%	2.8%
Ratio of monosaccharides (GC)					
Fucose	1.0	1.0	1.0	1.0	1.0
Xylose	1.0	1.2	1.2	1.1	1.0
Mannose	3.3	3.0	3.0	3.8	3.5
Glucosamine	2.5	0.8	1.0	1.1	2.0

*Data from ref. [14].

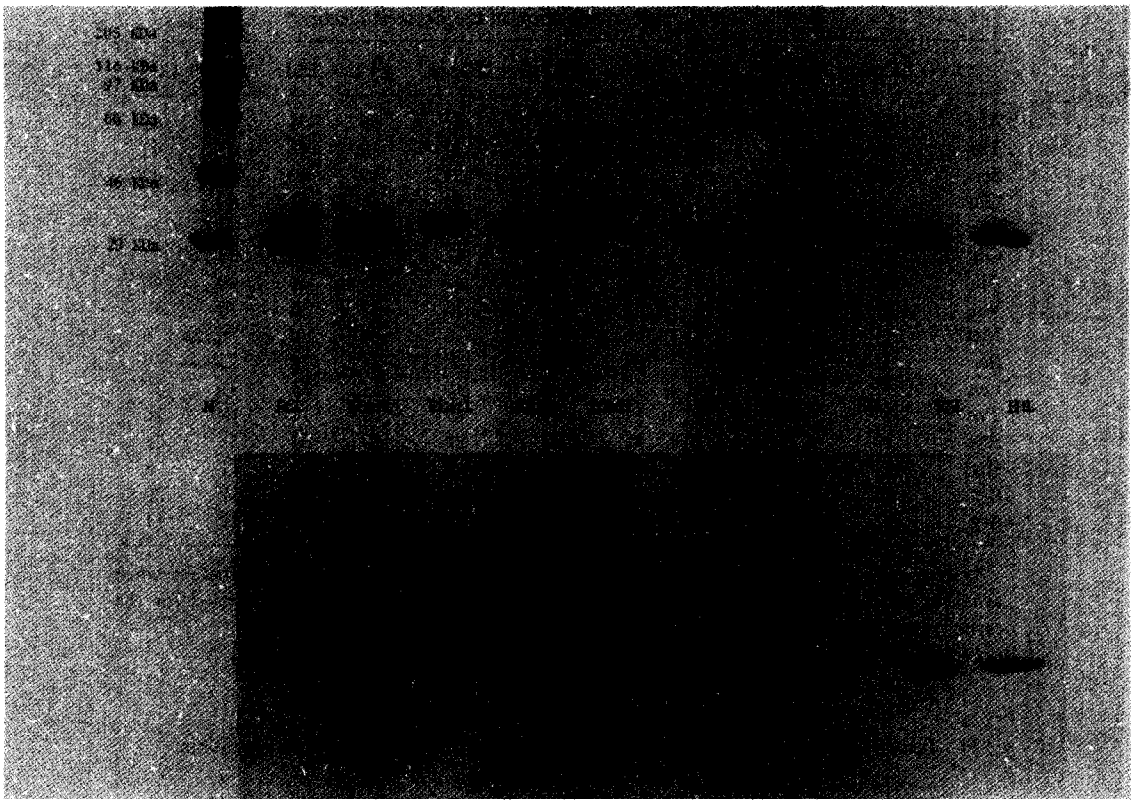

Fig. 3. Polyacrylamide gel electrophoresis of purified *Erythrina* lectins. The lectins were run on 10% gels in the presence of 0.4% sodium dodecyl sulphate. Upper gel was stained with Coomassie blue (proteins), lower gel with the periodate–Schiff reagent (carbohydrates). Lane M—M_r markers: myosin (M_r 205 000); β-galactosidase (M_r 116 000); phosphorylase B (M_r 97 400); bovine serum albumin (M_r 66 000); ovalbumin (M_r 46 000); carbonic anhydrase (M_r 29 000).

animal or yeast glycoproteins. This carbohydrate composition thus appears to be characteristic of a class of plant glycoproteins.

Only a few studies have been done on the structure of the carbohydrate chains of glycoproteins of this type. One such glycoprotein, the lectin from Tora bean, was found to contain the pentasaccharide core Manα6(Manα3)Manβ4GlcNAcβ4GlcNAc common to all *N*-glycoproteins, with xylose attached by an α1 → 2 linkage to the β-linked mannose [21]. The same position and linkage was reported for the xylose residue of bromelain [19, 22]; however, in this case the core oligosaccharide appears to lack the α1 → 3 linked mannose. A similar structure to that in bromelain has been proposed for the carbohydrate unit of the lectins from *Sophora japonica* and *Wistaria floribunda* [23]. The finding of *N*-linked carbohydrate chains with a core region different from the pentasaccharide mentioned above is very unusual and merits further studies. A particularly intriguing question is how and at what stage of biosynthesis is the xylose residue incorporated into the carbohydrate chain.

The amino acid composition of all nine *Erythrina* lectins tested (Table 2) is almost identical and very similar to that of ECL [14], as well as to that of the *E. indica* lectin [10]. It is characterized by a large content of acidic and hydroxy amino acids, small content of methionine and absence of cysteine. Such a composition is characteristic of many other legume lectins [3]. The *N*-terminal amino acid sequence (determined in seven of the lectins for up to 14–15 amino acids, and for 9–10 amino acids in three lectins) is nearly identical (Table 3). There are extensive homologies also with the *N*-terminal sequence of the lectin from *E. indica* [9] and of other legume lectins, two of which (soybean agglutinin and peanut agglutinin) are included in Table 3. It is worth noting that phenylalanine in positions 6 and 11 [and in the corresponding positions (128 and 133) of concanavalin A] has been found in all 26 legume lectins tested so far (not counting the *Erythrina* lectins), and serine in position 5 has been found in 23 of these [4, 24]. This is in line with the suggestion [1] that all Leguminosae lectins have evolved from the same genetic ancestor (see also refs [2, 25]).

Hapten inhibition experiments of hemagglutination show that the *Erythrina* lectins belong to the galactose/*N*-acetylgalactosamine specificity group and that they exhibit high preference for *N*-acetyllactosamine (Table 4), this disaccharide being 15–30 times more inhibitory than galactose and 10–20 times more than *N*-acetylgalactosamine; there are no large differences in the inhibitory activities of α- and β-galactosides, leading to the conclusion that none of the lectins possesses pronounced anomeric specificity. The much stronger inhibitory activity of the *p*-nitrophenyl-β-galactoside, as compared with that of the corresponding methyl derivative, in-

Table 2. Amino acid composition of the *Erythrina* lectins*

	ECafL	ECorL	EFL	EHL	ELatL	ELysL	EPL	ESL	EZL	ECL†
Ala	62	60	61	62	62	62	66	58	63	62
Thr	41	41	44	42	38	39	38	43	43	43
Ser	49	47	46	48	50	51	45	47	48	47
Glu	58	58	57	60	55	59	50	53	60	55
Pro	36	39	36	34	37	43	30	35	35	39
Gly	41	41	39	41	41	41	36	40	39	39
Ala	40	40	46	40	39	41	43	36	40	40
Val	34	33	30	32	33	32	33	35	30	42
Met	4	4	4	6	4	6	4	4	6	6
Ile	31	29	22	26	28	28	24	24	25	30
Leu	37	36	34	35	39	36	33	34	31	37
Tyr	17	18	18	17	17	19	18	17	18	20
Phe	29	28	32	31	29	29	30	34	31	28
His	10	9	11	9	10	9	10	12	11	8
Lys	20	19	21	19	17	20	20	18	17	20
Arg	12	11	12	12	12	11	11	14	12	11

*Given as residues/mole. Results are based on M_rs as determined in the ultracentrifuge when available (cf. Table 1), or on the approximate value of 60000; all M_rs were corrected for the carbohydrate content of the lectins.

†The composition of ECL is included for comparison; values taken from ref. [14].

Table 3. Amino terminal sequences of the *Erythrina* lectins and of peanut agglutinin and soybean agglutinin

ECL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Glu Pro Gly Asn
ECafL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Glu Pro Gly Asn
ECorL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Gly Pro Gly Asn
EFL	Ala Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Glu Pro Gly
EHL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Glu Pro Gly Asn
ELatL	Val Glu Thr Ile Ser Phe Ser Phe Ser
ELysL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu
EPL	Val Glu Thr Ile Ser Phe Ser Phe Ser Lys Phe Glu Ala Gly
ESL	Val Glu Thr Ile Ser Phe Ser Phe Ser
EZL	Val Glu Thr Ile Ser Phe Ser Phe Ser Glu Phe Glu Pro Gly Asn
PNA*	Ala Glu Thr Val Ser Phe Asn Phe Asn Ser Phe Ser Glu Gly Asn
SBA*	Ala Glu Thr Val Ser Phe Ser Trp Asn Lys Phe Val Pro Lys Glu

*Data from ref. [4]. PNA, peanut agglutinin; SBA, soybean agglutinin.

dicates the presence of a hydrophobic region in or near the binding site of the lectins, as found for other legume lectins [20]. Oligosaccharides with two and three branches (biantennary and triantennary, respectively) of the type found in many animal glycoproteins, containing two or three terminal *N*-acetyllactosamine units, respectively, are 2–3 times more active than the free disaccharide. The thermodynamic parameters for the binding of various saccharides to ECL imply that this lectin has an extended combining site, corresponding to a disaccharide [16]. The inhibition data described above suggest that the other *Erythrina* lectins also have extended binding sites.

The *Erythrina* lectins agglutinate human erythrocytes of all blood types equally well, with a slight preference for O-type cells, at minimal concentrations of 5–20 μg/ml (data not shown). Treatment of the cells with sialidase or trypsin increases their sensitivity to agglutination by all the lectins to the same extent (2–4-fold and up to 10-fold, respectively). None of the lectins agglutinates untreated or trypsin-treated sheep, goat and mouse erythrocytes even at 500 μg/ml, but all show agglutinating activity (at 10–30 μg/ml) with the sialidase treated cells. On the other hand, the 10 lectins examined in our laboratories show clear differences in their activity towards rabbit erythrocytes. Whereas most of them are almost as active with rabbit red blood cells as they are with human erythrocytes, those from *E. humeana*, *E. perrieri* and *E. zeyheri* are much weaker agglutinins when tested with the former cells, the minimal agglutinating concentration being 250–500 μg/ml. Agglutination of rabbit erythrocytes is not affected by treatment of the cells with sialidase, but is greatly (about 20-fold) increased by trypsinization. However, the differences in the activity of the various lectins are not abolished by the latter treatment.

Like ECL, most of the lectins described in this study are mitogenic for human peripheral blood lymphocytes but do not stimulate mouse thymocytes or splenocytes.

Table 4. Inhibition by various sugars of the hemagglutinating activity of the *Erythrina* lectins tested with human erythrocytes*

	Relative inhibitory activity									
	ECafL	ECorL	EFL	EHL	ELatL	ELysL	EPL	ESL	EZL	ECL
Galactose	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
N-Acetylgalactosamine	1.8	1.9	2.5	2.6	5.0	1.2	5.0	1.25	2.5	2.4
p-Nitrophenyl-α-galactoside	3.5	1.6	3.5	3.0	3.3	2.5	1.75	1.75	2.0	3.0
p-Nitrophenyl-β-galactoside	14.0	3.2	7.0	10.0	10.0	6.0	7.0	6.0	8.0	7.1
Methyl-α-galactoside	4.0	1.5	2.0	1.0	1.8	1.5	1.0	2.0	1.0	3.0
Methyl-β-galactoside	1.0	1.0	0.5	1.0	1.4	1.3	1.0	0.5	1.0	1.4
D-Fucose	0.4	0.35	0.23	0.23	0.5	0.45	0.44	0.44	0.23	0.8
Lactose	1.0	2.7	7.4	4.0	7.5	1.8	4	8	3	7.1
N-Acetyllactosamine	33	19	35	17	35	20	17	35	17	33
Galβ4GlcNAcβ2Manα$\searrow_{6}$ Manβ4GlcNAc Galβ4GlcNAcβ2Manα$\nearrow_{3}$	80	40	85	50	80	40	50	70	35	80
Galβ4GlcNAcβ2Manα$\searrow_{6}$ Manβ4GlcNAc Galβ4GlcNAcβ2Manα$\nearrow_{3}$ Galβ4GlcNAcβ$\nearrow_{4}$	100	45			100	45				100

*Four agglutinating doses of the lectin were routinely used. The inhibitory activity of galactose was arbitrarily set as 1. The minimal galactose concentrations required to give complete inhibition in a range of 3–4 experiments were: ECafL, 5–10 mM; ECorL, 4–14 mM; EFL, 5–10 mM; EHL, 7–14 mM; ELatL, 2–7 mM; ELysL, 3.3–5 mM; EPL, 7–14 mM; ESL, 3–7 mM; EZL, 7–14 mM; ECL, 7–14 mM.

Notable exceptions are the lectins from *E. humeana* and *E. zeyheri*, which are poor mitogens, if at all (data not shown). Interestingly, these are two of the three lectins that interact only weakly with the rabbit erythrocytes. Thus, in spite of the fact that all the *Erythrina* lectins exhibit the same specificity for mono- and disaccharides, they differ in their cell specificity, suggesting variations at or close to their carbohydrate-binding site.

As mentioned, the *Erythrina* lectins studied by us are very similar to those of other *Erythrina* species described in the literature [9–13] with respect to molecular properties and specificity for galactose/*N*-acetylgalactosamine. This similarity in lectins isolated from various species of a genus indigenous to well-defined, widely different, geographic locations spanning three continents indicates a high degree of conservation during evolution and argues for an important, although still unknown, function for these proteins.

EXPERIMENTAL

Materials. Divinylsulphone was a product of Polysciences, Warrington, PA. Sepharose 4B was from Pharmacia. Sialidase (neuraminidase from *Vibrio cholerae*, 1 U/ml) was purchased from Behringwerke, Marburg. *N*-Acetyllactosamine was a synthetic product kindly donated by Dr. A. Veyrières (University of Paris-Sud, Orsay), and the oligosaccharides Galβ4GlcNAcβ-2Manα6(Galβ4GlcNacβ2Manα3)Manβ4GlcNAc and Galβ4GlcNAcβ2Manα6[Galβ4GlcNAcβ2(Galβ4GlcNAcβ4)-Manα3]Manβ4GlcNAc were a gift of Dr. J. Montreuil and Dr. G. Spik (University of Lille). All other sugars were commercial products of highest purity available and all other reagents were of analytical grade. Slightly outdated human blood was obtained from Kaplan Hospital, Rehovot; rabbit, sheep and mouse blood was from animals supplied by the Animal Breeding Centre at the Weizmann Institute. Fresh human blood was provided by the Blood Bank, Tel Aviv-Yaffo.

Seeds of *E. corallodendron* were collected in Israel and those of *E. caffra*, *E. latissima* and *E. lysistimon* were collected in South Africa; seeds of *E. flabelliformis* were kindly provided by W. C. Sherbrook, Department of Ecology and Evolutionary Biology, University of Arizona, Tuscon, Arizona and those of *E. humeana*, *E. perrieri*, *E. stricta* and *E. zeyheri* by S. A. Lucas, Pacific Tropical Botanical Gardens, Hawaii.

Preparation of affinity column. Lactose-derivatized Sepharose 4B was prepared by the divinylsulphone method of ref. [26] as described in ref. [14].

Purification of the lectins was carried out essentially as described for *E. cristagalli* lectin [14]. Briefly, finely ground seeds defatted with petrol, were extracted with Pi–NaCl for 3–4 hr with stirring; the extract was clarified by centrifugation (13 000 rpm, 10 min) and the lectin precipitated by the addition of $(NH_4)_2SO_4$ to 60% satn. The ppt was collected, dialysed against H_2O, followed by Pi–NaCl, and the retentate applied to the column of lactose-derivatized Sepharose. Elution of the lectins was done with 0.2 M galactose.

Protein estimation. The method of ref. [27] was routinely used with BSA as standard. For the purified lectins, concn was estimated from A 280 nm, using the factor $A^{0.1\%}_{1\,cm} = 1.53$, as found for the *E. cristagalli* lectin [16].

Carbohydrate determination. Total neutral carbohydrate content was determined by the phenol–H_2SO_4 method [28] using mannose as reference sugar. Individual monosaccharides were determined by GC as TMSi derivatives after methanolysis of the lectin in the presence of mannitol as internal standard. A column of 3% SE-30 in the temp. range 140–200° at 0.5°/min was used [29].

Amino acid composition was determined on a Dionex D500 amino acid analyser. Methionine was determined as methionine sulphone after oxidation with performic acid [30]. *N*-Terminal amino acid sequences were determined by Edman degradation on a Beckman Model C Sequencer with Quadrol buffer. Identification of phenylthiohydantoin amino acids was done by HPLC on a Zorbax 5 μ (Dupont) column.

Gel electrophoresis in the presence of sodium dodecylsulphate was carried out on 10% polyacrylamide gels in the discontinuous buffer system of ref. [31]. The gels were stained for protein with Coomassie Brilliant Blue R 250 and for carbohydrates with the Schiff reagent [32].

Ultracentrifugation analysis. The M_r was calculated [33] from sedimentation equilibrium data obtained at 17 000 rpm, temp. of 24 ± 1°, and a protein concn of 0.35 mg/ml NaPi buffered saline, pH 7.4, and assuming the same partial sp. vol. (0.724) as that calculated by us for ECL [14] according to the lit. [34, 35].

The hemagglutinating activity of the lectin was assayed by the serial dilution method on microtiter plates, using 50 μl of lectin soln and 50 μl of a 4% suspension of erythrocytes ([36]; see also [3]). One unit of activity is defined as the smallest concn of lectin giving visible agglutination. The inhibitory activity of sugars was measured by mixing serial dilutions of the inhibitor with four hemagglutinating units of the lectin before addition of erythrocytes and determining the smallest concn giving full inhibition of agglutination.

Mitogenic activity. Human lymphocytes were isolated from freshly drawn peripheral blood by centrifugation on Ficoll-Hypaque [37]. Preparation of mouse thymocytes and splenocytes, treatment of the cells with neuraminidase, culture conditions and measurement of the stimulation of DNA synthesis, were carried out as described in ref. [38].

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