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# ISOLATION AND CHARACTERIZATION OF *DIOCLEA ALTISSIMA* VAR. *MEGACARPA* SEED LECTIN

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**Key Word Index**—*Dioclea altissima*; Leguminosae; lectin; D-mannose(D-glucose)-binding.

**Abstract**—A lectin was isolated from *Dioclea altissima* var. *megacarpa* seeds by affinity chromatography on Sephadex G-50 column. The lectin showed carbohydrate specificity for D-mannose/D-glucose, and divalent metal cations ($Ca^{2+}$ and $Mn^{2+}$) are required for full activity. It contained no covalently bound carbohydrate and had an amino acid composition characterised by large contents of aspartic acid and serine. The activation energy of the heat denaturing process was 106.9 kJ $mol^{-1}$. The lectin presented three protein bands in SDS-PAGE, with $M_r$ of 26 000, 15 000 and 9000, respectively, and showed immunological identity and the same N-terminal sequence as other lectins already isolated from the sub-tribe *Diocleinae*. © 1997 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Lectins are widely distributed in the Plant Kingdom, and leguminous seeds are a particularly rich source. Many lectins from different genera of the sub-tribe *Diocleinae*, tribe *Phaseoleae*, have already been isolated [1–8]. The main characteristics of this class of proteins are their ability to interact specifically with carbohydrates and thus combine with glyco-components of the cell surface, leading to several important biological properties. Although the most rational classification of the lectins is based on their respective specificity for monosaccharides [9, 10], the individual members of the groups may show appreciable differences in oligosaccharide specificity [11]. Thus, even lectins belonging to the same sugar-binding specificity [D-mannose (D-glucose)], as those from seeds of *Pisum sativum*, *Lens culinaris*, *Vicia faba*, *Canavalia ensiformis*, *Canavalia brasiliensis*, *Cratylia floribunda*, *Dioclea grandiflora*, *Dioclea guianensis*, *Dioclea virgata*, *Dioclea violacea* and *Dioclea lehmanni*, show differences in their interactions with cell membranes and glycoconjugates (for references see [12–16]), although these differences are less significant when the lectins are within the same genera. These differences include mitogenic stimulation and gamma interferon production on human lymphocytes [17], macrophage stimulation and leukocyte accumulation [18], edematogenic effects [19] and histamine release [20], besides toxicity to some insects [21].

## RESULTS AND DISCUSSION

### *The plant*

*Dioclea altissima* (Velloso) Rock emend Maxwell var. *megacarpa*, is a pantropical leguminous vine, occurring in forest underground on unflooded land, in the southern islands of the West Indies, Trinidad, Costa Rica, Panamá and the States of Amazonas, Ceará and Piaui in Brazil. *D. altissima* belongs to the family Leguminosae, sub-family Papilionoideae, tribe *Phaseoleae*, sub-tribe *Diocleinae*, sub-genus *Pachylobium*. The common name, besides the usual 'mucunã', in Brazil, is 'ojo de buey' in Panamá [22]. The seeds of *Dioclea altissima*, show many similarities with other representatives of the sub-genus *Pachylobium* [23] and have a high content of protein, a characteristic property of legumes. The dehulled seed flour contains 23.5% of protein (3.76% of $N_2$) in dry basis. The seeds are also used in Ceará for meal.

### *Isolation of the lectin*

The purification of *Dioclea altissima* was obtained by a two-step methodology. Thus, the saline crude extract was fractionated by the addition of different amounts of solid ammonium sulphate in the cold (5°)

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Table 1. Overall recovery of protein and haemagglutinating activity from *Dioclea altissima* seeds at various stages of fractionation and purification. The flour was extracted with 0.15 M NaCl, centrifuged and the clear supernatant fractionated by treatment with ammonium sulphate to saturation levels as indicated. The lectin was purified by affinity chromatography on Sephadex G-50. All fractions were recovered after dialysis and freeze drying and tested with rabbit erythrocytes

| Fraction | Amount (g) | H.U.[a] ($mg^{-1}$) | Minimum dose[b] ($\mu g\ ml^{-1}$) | Total H.U. ($\times 10^{-3}$) | Extent of purification (times) |
|---|---|---|---|---|---|
| Seed meal | 100 | 8 | 125 | 800 | 1 |
| Crude extract | 9.88 | 80 | 12.5 | 80 | 10 |
| Ppt 0–50% saturation $F_{0\text{-}50}$ | 1.83 | 100 | 10.0 | 18.3 | 12 |
| Ppt 50–70% saturation $F_{50\text{-}70}$ | 2.97 | 256 | 3.9 | 760 | 32 |
| Ppt 70–90% | 1.60 | 10 | 100 | 16 | 1.2 |
| $P_{III}$ (Sephadex G-50) | 1.42 | 768 | 1.3 | 1090 | 96 |

[a] Haemagglutinating unit.
[b] Minimum dose capable to agglutinate a 2% erythrocyte suspension.

overnight. After centrifugation at 15000 *g*, 20 min at 7°, the precipitates were redissolved in and dialysed exhaustively against water and recovered by freeze-drying. This material was redissolved in 0.15 M NaCl containing 5 mM $Ca^{2+}$ and 5 mM $Mn^{2+}$ and assayed for protein and haemagglutinating activity (Table 1). Most of the activity was precipitated between 50 and 70% of saturation of ammonium sulphate (Table 1). This fraction (F50/70) was redissolved in 0.15 M NaCl containing 5 mM $Ca^{2+}$ and $Mn^{2+}$ and applied to a Sephadex G-50 column equilibrated with the same solvent. The column was first eluted with the equilibration solution followed by 0.1 M glucose dissolved in the equilibration solution (Fig. 1). All the haemagglutinating activity emerged with the protein glucose ($P_{III}$), corresponding to 1.42% of the seed dry weight.

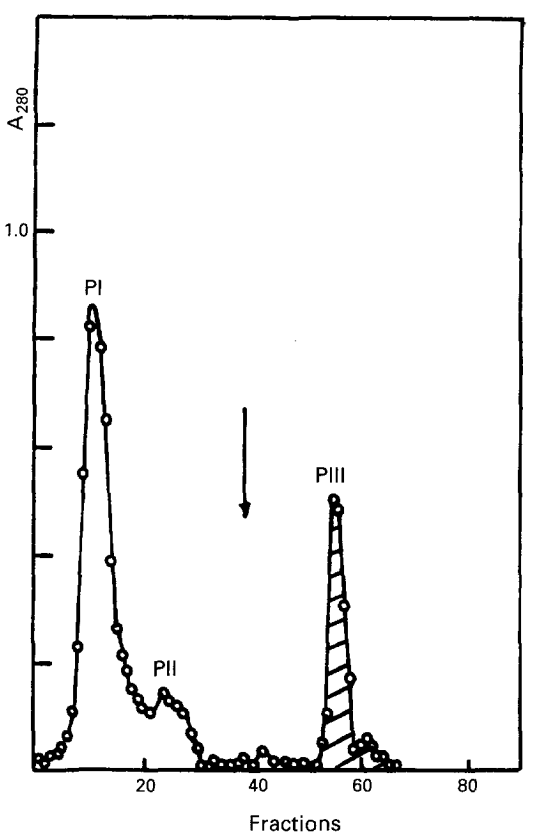


Fig. 1. Purification of the lectin from *Dioclea altissima* var. *megacarpa*, by affinity chromatography on Sephadex G-50 column. The column was equilibrated and first eluted with 0.15 M NaCl containing 5 mM $CaCl_2$ and $MnCl_2$ to remove the unbound proteins ($P_I$ and $P_{II}$). The lectin ($P_{III}$) was recovered with 0.1 M glucose in the equilibrium solution.

*Molecular properties of the lectin*

Gel filtration of the affinity purified lectin ($P_{III}$) on a Superose 12 R column, at the pH range from 2.3 up to 8.3, showed several patterns. While only one peak was found both at pH 2.6 ($M_r$ 26000 monomer) and pH 7.0 ($M_r$ 50000 dimer), at pH 8.3 two peaks of $M_r$ 50000 (dimer, 73%) and 100000 or higher (tetramer, 27%), respectively, emerged. Although for the other lectins isolated from the *Diocleinae*, the quaternary structure was also dependent on the pH, the proportion of tetramer/dimer was quite different near neutrality. The lectin showed an extinction coefficient of 12.0, which is slightly higher than those found for ConA (11.4), *D. guianensis* lectin (11.5), and equal to *D. grandiflora* (12.0).

Polyacrylamide gel electrophoresis of the lectin, treated with SDS and 2-mercaptoethanol gave three protein bands with $M_r$ values of 26, 14 and 9000 (Fig. 2). Dissociation in SDS was independent of the presence of 2-mercaptoethanol. Although qualitatively similar to other *Diocleinae* lectins, the proportion of the fragmented sub-units to the intact one was quite different [23, 24]. When this result is compared with the gel filtration, the existence of the dimer/tetramer pattern is more clearly understood.

The purified lectin also gave several components of distinct isoelectric points between 8.6 and 9.0 (data not shown). Furthermore, when the lectin from *D. altissima* was compared with the *Dioclea grandiflora*

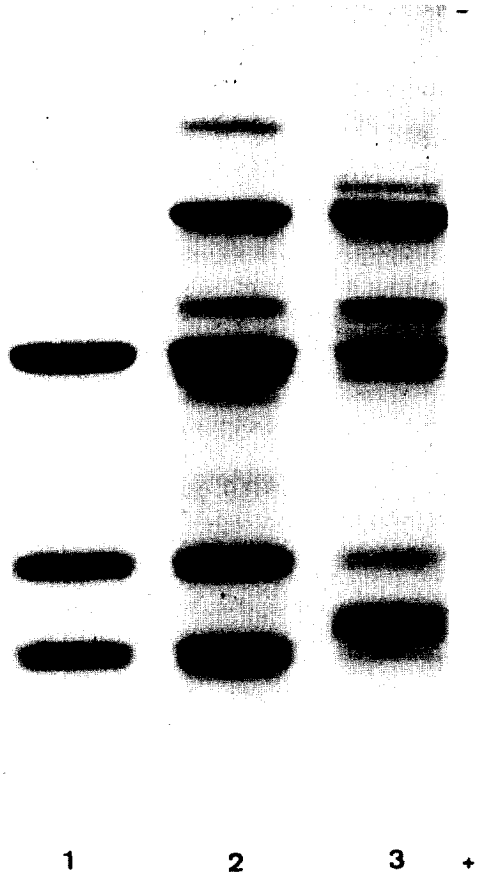


Fig. 2. SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol of the purified *Dioclea altissima* lectin. Lane 1: *Dioclea altissima* lectin, lane 2: fraction F50/70, lane 3: saline crude extract.

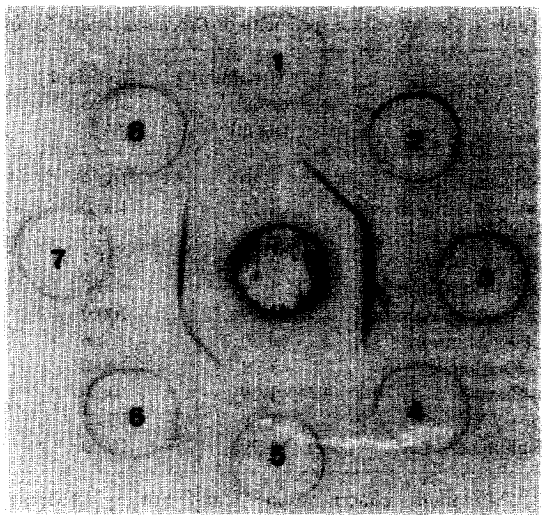


Fig. 3. Ouchterlony double immunodiffusion of the *Dioclea* seed lectin. 1,5—*D. altissima* unbound fraction; 2,6—*D. altissima* retained fraction (lectin); 3,7—*D. grandiflora* retained fraction; 4,8—*D. grandiflora* unbound fraction. Centre well—IgG against *D. altissima* lectin.

lectin by Ouchterlony double immunodiffusion using *D. altissima* lectin-antisera (Fig. 3), a complete immunological identity was found.

The lectin, when dissolved in 0.05 M Tris–acetate pH 8.3 buffer, containing 0.15 M NaCl, had an absorption value at 280 nm and 1 cm cell ($A_{1\,cm,\,1\%}$) of 12.0. We were not able to detect any sugar using the phenol-sulphuric acid method, indicating that the *D. altissima* lectin, similar to other lectins from the *Diocleinae* subtribe, is not a glycoprotein [2, 4–8, 12].

The amino acid composition was characterised by a high content of aspartic acid and serine and showed many similarities with the composition of other *Diocleinae* lectins so far isolated. The N-terminal amino acid sequence of the first seven residues of *D. altissima* lectin (Ala, Asp, Thr, Ile/Leu, Val, Ala, Val) was identical to the N-terminal sequence of all the *Diocleinae* lectins so far investigated [25].

The haemagglutinating activity of the pure lectin decreased appreciably after demetalization by prolonged dialysis against 0.2 M EDTA, followed by dialysis against 0.15 M NaCl. Full activity was restored by addition of both $Ca^{2+}$ and $Mn^{2+}$. The optimum concentration for maximum haemagglutinating activity appeared to be between 3 and 5 mM for both cations.

When the *D. altissima* lectin was dissolved in 0.1 M Na-borate, pH 8.0 buffer, and submitted to heat treatment at temperatures between 78 and 80°, the activation free energy of the denaturing process ($\Delta G'$) was estimated to be 106.9 kJ $mol^{-1}$.

The haemagglutinating activity was inhibited in the presence of a number of simple sugars tested. In preliminary inhibition studies carried out in 1 M sugar with mannose the activity dropped to 2.0%, with fructose to 3.7% and with glucose to 32.6%. Thus this lectin can be classified in the general group of D-mannose (D-glucose)-binding lectins [10] and can be named, following Peumans proposal [16], as LC.P.Dio.alt.1.12.1.

## EXPERIMENTAL

*Materials. Dioclea altissima* (Velloso) Rock emend. Maxwell var. *megacarpa* seeds were collected in the State of Ceará (Brazil). Human blood cells from the ABO system were obtained from healthy donors in the Centro de Hemoterapia do Ceará (HEMOCE). Rabbit blood cells were obtained by puncture of the marginal ear's vein of healthy animals. Acrylamide and methylene bisacrylamide were product of Sigma and Sephadex G-50 was from Pharmacia.

*Haemagglutination activity*. Clumping of red blood cells by the various frs obtained during purification was estimated as described before [26], in small glass tubes where, to a series of 1:2 dilutions in 0.15 M NaCl containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$ (0.25 ml), were added 0.25 ml of a 2% suspension of erythrocytes. The degree of agglutination was monitored visually after the tubes had been left at 37° for 30 min

Table 2. Carbohydrate specificity of *Dioclea altissima* lectin

| | Minimum dose* ($\mu$g ml$^{-1}$) | Residual activity† (%) |
|---|---|---|
| No sugar | 1.30 | 100.0 |
| D(+)-Arabinose | 1.29 | 100.0 |
| D(−)-Fructose | 34.70 | 2.9 |
| D(+)-Glucose | 3.99 | 25.1 |
| D(+)-Galactose | 1.29 | 100.0 |
| D(+)-Glucosamine | 1.29 | 100.0 |
| D(+)-Mannose | 64.70 | 1.6 |
| L-Rhamnose | 1.29 | 100.0 |
| L-Fucose | 1.29 | 100.0 |
| D(+)-Xylose | 1.29 | 100.0 |
| Raffinose | 1.29 | 100.0 |

*Minimum concentration still showing haemagglutinating activity in presence of 0.1 M sugar.

†Percent residual activity of 11 mg ml$^{-1}$ solution of the lectin in presence of 0.1 M sugar.

Table 3. Amino acid composition of *Dioclea altissima* lectin. The values are given in residues per 100 residues

| Amino acid | Residues/100 residues |
|---|---|
| Asx | 13.1 |
| Thr | 7.6 |
| Ser | 14.0 |
| Glx | 5.5 |
| Pro | 4.7 |
| Gly | 7.3 |
| Ala | 7.6 |
| Cys | 0.0 |
| Val | 6.8 |
| Met | 0.4 |
| Ile | 5.9 |
| Leu | 7.6 |
| Tyr | 3.0 |
| Phe | 4.7 |
| Lys | 5.1 |
| His | 1.7 |
| Arg | 3.0 |
| Trp | 2.1 |

and subsequently left at room temp. for a further 30 min. One H.U. was defined as the reciprocal of the highest dilution still giving a visible agglutination. Sp. act. was expressed as haemagglutination units (H.U.) mg$^{-1}$ or as the minimum concn of protein ($\mu$g ml$^{-1}$) giving activity.

*Protein concn.* This was determined by the method described of ref. [27], using BSA as standard. Readings at 280 nm were also used to determine protein content of the column eluates.

*Total nitrogen* was determined by the method of ref. [28]. The protein content was determined using a conversion factor of 6.25.

*The neutral sugar* was estimated by the phenol–sulphuric acid method of ref. [29] using glucose as the standard.

*Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).* This was carried out on vertical 2 mm gel slabs of 17.6% polyacrylamide separation gel and 4% stacking gel [30]. Samples were dissolved in 0.01 M Na-Pi pH 7.0, 2% SDS buffer with 1% 2-mercaptoethanol and incubated at 100° for 15 min. A few crystals of sucrose were dissolved in the samples which were then applied to the gel. The electrophoresis was conducted at a constant current of 13 mA for 4 hr. The protein bands were visualised by staining the gel with Coomassie Brilliant Blue R-250. Estimation of $M_r$ of the lectin sub-unit and fragments were made using proteins of known $M_r$ as standards.

*Lectin extraction.* Dehulled *D. altissima* seeds were finely ground and stirred with 0.1 M glycine–HCl pH 2.6, 0.1 M NaOAc pH 4.0, 0.1 M Tris–HCl pH 6.0, 0.1 M Na-borate pH 8.0 and 0.1 M Na-borate pH 10.0 buffers, all containing 0.15 M NaCl. The suspensions were left at room temp. for 3 hr and spun at 10000 *g*, 20 min, 7°. The clear supernatants were used for determining the protein content and haemagglutinating activity.

$(NH_4)_2SO_4$ *fractionation.* Solid $(NH_4)_2SO_4$ was added in small portions to the saline crude extract in order to obtain the desired satn. The mixt. was kept overnight at room temp. The ppt was collected by centrifuging the suspension at 15000 *g*, 20 min at 7° and dissolved with $H_2O$. After exhaustive dialysis against $H_2O$, the fr. was freeze-dried.

*Affinity chromatography* was done on a Sephadex G-50 column equilibrated with 0.15 M NaCl containing 5 mM $CaCl_2$ and 5 mM $MnCl_2$. The sample ($F_{50/70}$) was dissolved in the same soln. After removing the unbound material, the lectin was desorbed from the column with 0.1 M glucose in the equilibrium soln.

*Sugar specificity.* The carbohydrate-binding specificity of the lectin was estimated by the ability of a series of simple sugars to inhibit the haemagglutination of rabbit erythrocytes. Two-fold dilutions of sugars were done by addition of 0.50 $\mu$l of a 1.0 M sugar soln to the first hole of a microtiter plate (containing 0.50 $\mu$l of 0.15 M NaCl). The dilutions were done in the direction 1–12. After dilution of sugar, similar two-fold dilutions of the lectin were obtained by addition of 0.50 $\mu$l of a 1 mg ml$^{-1}$ soln at the first hole (A) and the dilutions done in the direction A–H. In this manner, we can obtain several proportions sugar/lectin. The lowest proportion sugar/lectin still giving full inhibition was obtained, and the minimum concn of the lectin, still showing haemagglutinating activity in the presence of 0.1 M sugar was calculated.

*Effect of EDTA and* $Ca^{2+}$ *and* $Mn^{2+}$. The purified lectin (2 mg) was dissolved in and dialysed exhaustively (48 hr) against 0.2 M EDTA, followed by dialysis against 0.15 M NaCl (24 hr). The recovery of the haemagglutinating activity was done by adding $CaCl_2$ and $MnCl_2$.

*Heat stability.* The heat stability of the haemag-

glutinating activity was determined treating the lectin at different temps during different times and the residual activity determined. The activation energy of the denaturing process was determined using the expression of Arrhenius [31].

*Immunodiffusion.* Ouchterlony radial double immunodiffusion were carried out according to ref. [32], using 1% agarose gels prepd with 0.15 M NaCl, containing 0.02% $NaN_3$ and 0.1 M glucose. The lectins (20 $\mu$g) were applied in wells in a circular distribution, around a central well containing the IgG (20$\mu$g) anti *Dioclea altissima* lectins (20 $\mu$g). The samples were allowed to diffuse for 48 hr at room temp., and the pptd arcs examined by colouring the 0.15 M NaCl washed gel plates with 0.005% Coomassie Brilliant Blue R-250.

*Amino acid analyses* were performed after hydrolysis of lectin samples (in sealed glass tubes under $N_2$) for 24, 48 and 72 hr with 6 M HCl at 110°. After hydrolysis, HCl was removed by evaporation and the residue was analysed in a LKB 4151 Alpha Plus amino acid analyser.

*The N-terminal sequence* of the *D. altissima* lectin was carried out by the manual DABITC-PITC method [33, 34].

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## REFERENCES

1. Hague, D. R., *Plant Physiology*, 1975, **55**, 636.
2. Moreira, R. A., Barros, A. C. H., Stewart, J. C. and Pusztai, A., *Planta*, 1983, **158**, 63.
3. Moreira, R. A. and Cavada, B. S., *Biology of Plants*, 1984, **26**, 113.
4. Oliveira, J. T. A., Cavada, B. S. and Moreira, R. A., *Revue of Brasilian Botany*, 1911, **14**, 63.
5. Vasconcelos, I. M., Cavada, B. S., Moreira, R. A. and Oliveira, J. T. A., *Journal of Biochemistry*, 1991, **15**, 137.
6. Moreira, R. A., Cordeiro, E. F., Ramos, M. V., Grangeiro, T. B., Martins, J. L., Oliveira, J. T. A. and Cavada, B. S., *Revue Brasilian Fisiologica Vegetale*, 1996, **8**, 37.
7. Cavada, B. S., Grangeiro, T. B., Ramos, M. V., Cordeiro, E. F., Oliveira, J. T. A. and Moreira, R. A., *Revue Brasilian Fisiologica Vegetale*, 1996, **8**, 31.
8. Cavada, B. S., Ramos, M. V., Cordeiro, E. F., Grangeiro, T. B., Oliveira, J. T. A., Carvalho, A. F. F. U. and Moreira, R. A., *Revue Brasilian Fisiologica Vegetale*, 1996, **8**, 37.
9. Makela, D., *Annals of Medical and Experimental Biology Fenn*, 1957, **35**, S11, 1.
10. Goldstein I. J. and Hayes, C. E., *Advances in Carbohydrate Chemistry and Biochemistry*, 1978, **35**, 127.
11. Ayouba, A., Roques, D., Cavada, B. S., Oliveira, J. T. A., Moreira, R. A. and Rougé, P., In *Lectins, Biology-Biochemistry, Clinical Biochemistry*, Vol. 9, ed. J. Basu, M. Kundu and P. Chakrabarti. Wiley, India, 1993.
12. Goldstein, I. J and Poretz, R. D., In *The Lectins. Properties, Functions, And Applications In Biology And Medicine*, ed. I. E. Liener, N. Sharon and I. J. Goldstein. Academic Press, Orlando, 1986, p. 33.
13. Lis, H. and Sharon, N., *Annual Review of Biochemistry*, **55**, 35.
14. Moreira, R. A., Ainouz, I. L., Oliveira, J. T. A. and Cavada, B. S., *Memoirs of the Institute of Oswaldo Cruz*, 1991, **86** (SII), 212.
15. Pusztai, A., In *Plant Lectins*. Cambridge University Press, Cambridge, p. 2.
16. Peumans, W. J., and Van Damme, E. J. M., In *Lectins, Biology-Biochemistry, Clinical Biochemistry*, Vol. 10, ed. E. Van Driesche, J. Fischer, S. Beeckman and T. C. Bog-Hansen. TEXTOP, Hellerup, Denmark, 1994.
17. Barral-Netto, M., Santos, S. B., Moreira, R. A., Oliveira, J. T. A., Barral, A., Moreira, L. I. M., Santos, C. F. and Cavada, B. S., *Immunology Investigations*, 1992, **21**, 297.
18. Rodriguez, D., Cavada, B. S., Oliveira, J. T. A., Moreira, R. A. and Russo, M., *Brasilian Journal of Medical and Biological Research*, 1992, **25**, 823.
19. Bento, C. A. M., Cavada, B. S., Oliveira, J. T. A., Moreira, R. A. and Barja-Fidalgo, C., *Agents Actions*, 1993, **38**, 48.
20. Gomes, J. C., Cavada, B. S., Moreira, R. A. and Oliveira, J. T. A., *Agents Actions*, 1994, **41**, 132.
21. Gatehouse, A. M. R., Powell, K. S., Peumans, W. J., Van Damme, E. J. M. and Gatehouse, J. A., in *Lectins, Biomedical Perspectives*, ed. A. Pusztai and S. Bardocz. Taylor and Francis, London, 1995.
22. Maxwell, R. H., The Genus *Dioclea* (Fabaceae) in the New World. Ph.D. Thesis, Department of Biology, Southern Illinois University, 1969, p. 430.
23. Moreira, R. A., Cordeiro, E. F., Cavada, B. S., Nunes, E. P., Fernandes, A. G. and Oliveira, J. T. A., *Revue Brasilian Fisiologica Vegetale*, 1993, **5**, 127.
24. Moreira, R. A., Cordeiro, E. F., Cavada, B. S., Nunes, E. P., Fernandes, A. G. and Oliveira, J. T. A., *Revue Brasilian Fisiologica Vegetale*, 1995, **7**, 7.
25. Cavada, B. S., Moreira, R. A., Oliveira, J. T. A. and Grangeiro, T. B., *Revue Brasilian Fisiologica Vegetale*, 1993, **5**, 193.
26. Moreira, R. A. and Perrone, J. C., *Physiology*, 1977, **59**, 783.
27. Bradford, M. M., *Analytical Biochemistry*, 1976, **72**, 248.

28. Baethgen, W. E. and Alley, M. M., *Science and Plant Analysis*, 1969, **20**, 961.
29. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F., *Analytical Chemistry*, 1956, **28**, 350.
30. Laemmli, U. K., *Nature*, 1970, **227**, 680.
31. Dawes, E. A. *Quantitative Problems in Biochemistry*. Churchill Livingstone, Edinburgh, 1972, p. 470.
32. Clausen, J., in *Laboratory Techniques in Biochemistry and Molecular Biology*, ed. T. S. Work and E. Work. North-Holland Publishing, Amsterdam 1969, p. 399.
33. Chang, J. Y., In *Methods In Enzymology*, ed. C. H. W. Hirs and S. N. Timasheff. Academic Press, New York, 1983, p. 455.
34. Chang, J. Y., Brauer, D and Wittmann-Liebold, B., *FEBS Letters*, 1978, **93**, 205.