

Isolation and properties of a lectin from the seeds of *Mimosa invisa* L.

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Abstract. A lectin has been purified from the seeds of *Mimosa invisa* L. by gel filtration and preparative Polyacrylamide gel electrophoresis. The purified lectin was homogeneous as judged by analytical Polyacrylamide gel electrophoresis, immunodiffusion and Immunoelectrophoresis. The apparent molecular weight is 100,000; the protein is a tetramer with two types of subunits (molecular weight 35,000 and 15,000). The lectin is a glycoprotein with approximately 21% carbohydrate and interacts with Sephadex and concanavalin A-Sepharose. It agglutinates erythrocytes non-specifically, does not agglutinate leucocytes and is not mitogenic, agglutinates *Mimosa*-nodulating *Rhizobium* and is a panagglutinin; the agglutination is not inhibited by several simple sugars. It is thermo-stable and has no metal ions.

Keywords. Lectin; *Mimosa invisa*; *Rhizobium*.

Introduction

Lectins are sugar binding proteins or glycoproteins of non-immune origin which agglutinate cells or precipitate glycoconjugates (Goldstein *et al.*, 1980). Though lectins were first isolated from higher plants, they have been observed to be present in many classes of the plant and animal kingdoms, including microorganisms. Lectin research was stimulated because of the specific erythroagglutinating properties, mitogenic activity and transformed cell-agglutinating properties of lectins. Lectins are currently used as tools in elucidating membrane structure and cell transformation (Lis and Sharon, 1981). Legume lectins are implicated in recognition of specific nodulating rhizobia (Dazzo and Truchet, 1983; Bauer, 1981). It was shown that a lectin from a particular legume binds only to the corresponding rhizobial species and not to rhizobia infecting other legumes. This specific interaction has not been studied in Mimosoideae. The majority of the legume lectins studied belong to the tribe papilionoideae. This report describes the isolation and partial characterization of a *Rhizobium*-binding lectin from the seeds of *Mimosa invisa* L., a tropical legume of Mimosoideae.

Materials and methods

Seeds of *M. invisa* were collected from the Central Plantation Crops Research Institute, Hirehalli, Karnataka. Rhizobia used for agglutination studies were isolated

Abbreviations used: PBS, Phosphate buffered saline; PAGE, Polyacrylamide gel electrophoresis; ConA, concanavalin A; SDS, sodium dodecyl sulphate; *M_r*, molecular weight; BSA, bovine serum albumin; MI lectin, *M. invisa* lectin; PVP, polyvinylpyrrolidone.

in the laboratory from nodules of *M. invisa*. Sephadex gels, Standard sugars, arabinogalactan, xylan, mannan, standard lectins, marker proteins, were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Bio-gels were obtained from Bio-Rad Laboratories, Richmond, California, USA. Other chemicals used were of analytical reagent grade.

Isolation of lectin

Dehusked *M. invisa* seeds were finely ground and defatted by extraction with hexane (1:2, w/v) in a liquid nitrogen/isopropanol bath. The extract was filtered through Whatman No. 1 filter paper and air dried. Seed powder (1 g) was extracted by grinding in a mortar and pestle with 10 ml of 10 mM potassium phosphate buffer, pH 7.2, containing 150 mM NaCl (phosphate buffered saline, PBS), 1 mM MgSO₄, 50 mM sodium ascorbate and 0.3 g acid washed polyvinylpyrrolidone (PVP) (Dazzo *et al.*, 1978). All the steps were performed at room temperature. The extract was filtered through cheesecloth, and the filtrate centrifuged for 1 h at 27,000 g at 4°C. The supernatant was treated with ammonium sulphate (100% saturation) to recover total protein. The protein was further subjected to ammonium sulphate precipitation (45% saturation), and the precipitate was dissolved in PBS (10 mM potassium phosphate, pH 7.2, 150 mM NaCl) and desalted using a Sephadex G-25 column. Protein fractions were collected, concentrated by lyophilization and applied to a column of Bio-gel P-100. Fractions containing the lectin were pooled and electrophoresed through 5% Polyacrylamide slab gels. Part of the gel was stained with Coomassie brilliant blue G, and the lectin band was cut, eluted with PBS and the preparative Polyacrylamide gel electrophoresis (PAGE) step repeated till a homogeneous preparation was obtained.

Affinity chromatography on Concanavalin A-Sepharose

Concanavalin A (ConA)-Sepharose was prepared as described by Etten and Saini (1977). Elution of the bound lectin was tried with 0.1 M glucose, 0.1 M α -methyl-D-glucoside in 1 M NaCl, 0.1 M α -methyl-D-mannoside + 50% (v/v) ethylene glycol, acetic acid-sodium acetate buffer, pH 3.6 with 1 M NaCl and 1 mM each of CaCl₂, MnCl₂ and MgCl₂, and detergent buffer (1.4% cetyltrimethylammonium bromide/1 M NaCl in 50 mM acetic acid-sodium acetate buffer). Sugar concentrations upto 2 M did not elute the bound lectin.

Analytical methods

Analytical PAGE was performed according to Davis (1964). Electrophoresis was done using 7.5% gels at pH 8.6 for 4 h at a current density of 4 mA per tube (65 × 6 mm). Gels were stained with Coomassie brilliant blue R and destained with 7% (v/v) acetic acid in 50% (v/v) methanol. PAGE in the presence of sodium dodecyl sulphate (SDS) was performed as described by Laemmli (1970) in 8% gels. The approximate subunit molecular weight (M_r) values were obtained from the electrophoretic mobilities by comparison with those of cytochrome C (M_r 13,000), ovalbumin (M_r 45,000) and bovine serum albumin (BSA) (M_r 68,000). M_r

determination on Bio-gel P-300 was done according to the method described by Whitaker (1963) using BSA (M_r 68,000), ConA (M_r 105,000) and catalase (M_r 232,000) as markers. Glycoprotein staining was done with the periodic acid-Schiff stain (Segrest and Jackson, 1972).

Antibody to *M. invisa* lectin (MI lectin) was raised in rabbits. Two hundred μg of lectin in PBS emulsified in Freund's complete adjuvant was injected into rabbits intradermally once a week. After 3 weeks, a booster injection of 400 μg of protein in PBS was given and 6 days later, the animals were bled through the marginal ear vein and serum prepared. The immunoglobulin G fraction was prepared by precipitating it from the serum at 33% saturation of ammonium sulphate and negative adsorption on DEAE cellulose. Immunodiffusion was carried out according to the method of Ouchterlony (1962). Immunoelectrophoresis was performed as described by Clausen (1970).

Erythroagglutination was done according to the procedure of Kabat (1961). Leucoagglutination was tested by observing lectin-treated sheep leucocytes microscopically. Leucocytes were separated as described by Kampine *et al.* (1966). To check mitogenic activity, lymphocytes were cultured as described by Lubs *et al.* (1973). The slides were stained by the Giemsa stain (Seabright, 1971). Rhizobial agglutination was tested according to Dazzo and Brill (1977). A cell concentration of 6×10^9 cells/ml prepared from two day-old (mid-log phase) broth (yeast extract glucose medium) grown cultures were used for assay. The agglutination mixture was supplemented with 1 mg/ml of PVP 360 where indicated. The rhizobia used for agglutination experiments were isolated from the nodules of *M. invisa*. Sugar inhibition test was done by preincubating the lectin (4 hemagglutination units) with sugar for 30 min followed by incubation of the mixture with erythrocytes or rhizobia (9×10^9 cells/ml).

Demetallization of the lectin was done according to Paulova *et al.* (1971). Lectin in PBS was dialysed against 0.1 M EDTA (disodium salt) for 48 h and then against 1 M acetic acid for 48 h and against water for 50 h. The temperature stability of the lectin was tested by incubating the lectin solution in PBS (2 mg/ml) in a water bath at different temperatures for 5–20 min.

The effect of exposure of lectin to different hydrogen ion concentrations was studied by dialyzing the lectin against (i) 0.2 M KCl-HCl buffer, pH 2.0; (ii) 0.2 M acetic acid-sodium acetate buffer, pH 4; (iii) 0.2 M potassium phosphate buffer, pH 7.4; (iv) 0.2 M Tris-HCl buffer, pH 8 and (v) 0.2 M sodium carbonate-bicarbonate-buffer, pH 10. All buffers had 0.15 M NaCl. The lectin was assayed for activity with rhizobial cells after dialysis against 3 changes of PBS for 24 h.

Total neutral sugar was determined by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) with D-glucose as the reference sugar. Protein was estimated by the method of Lowry *et al.* (1951) with BSA as standard.

Results

Purification of MI lectin

The lectin was purified by ammonium sulphate precipitation, gel filtration and preparative PAGE. Dialysis of the crude seed extract resulted in precipitation of the protein. Hence, desalting in the initial states of purification was done on a Sephadex

G-25 column. However, at later stages of purification, dialysis did not aggregate the lectin. Gel filtration on Bio-gel P-100 yielded two protein peaks (figure 1), of which the first peak had agglutinating activity. The yield of the purified lectin was 50 mg per kg of seeds. A 72 fold increase in the specific activity (table 1) was achieved. The purified lectin is homogeneous as judged by analytical PAGE (figure 2), immunodiffusion of crude seed extract and antiserum prepared against purified lectin and Immunoelectrophoresis (data not shown). On Polyacrylamide gels, the lectin band was slightly diffuse. The lectin is a glycoprotein with approximately 21 % (w/w) carbohydrate.

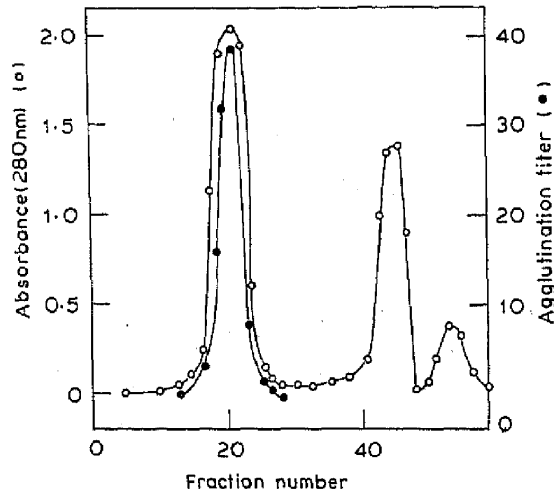


Figure 1. Gel filtration of MI lectin on Bio-gel P-100. The column (1.5 × 50 cm) was equilibrated with PBS and 2 ml fractions were collected. Agglutination activity was assayed using sheep erythrocytes and rhizobia as described in methods. Rhizobial agglutination is represented in the graph.

Table 1. Purification of MI lectin from *M. invisa* seeds.

	Volume (ml)	Total protein (mg)	Total activity (units)*	Specific activity (units/mg protein)	Recovery (%)	Fold-purification
Crude extract (as 100% ammonium sulphate precipitate	50	80	160	2	100	1
0-45% ammonium sulphate precipitate		26	156	6	95	3
Biogel P-100		7	150	22	92	11
Preparative PAGE		1	145	145	90	72

*Two-day old rhizobia were used for the assay. One unit of agglutination is the smallest amount of lectin which produces visible agglutination of cells under the specified conditions.

Interaction with Sephadex

Sephadex gels could not be used for purification since the agglutinin was retarded on Sephadex G-100, G-150 and G-200 though firm binding to the beads was not

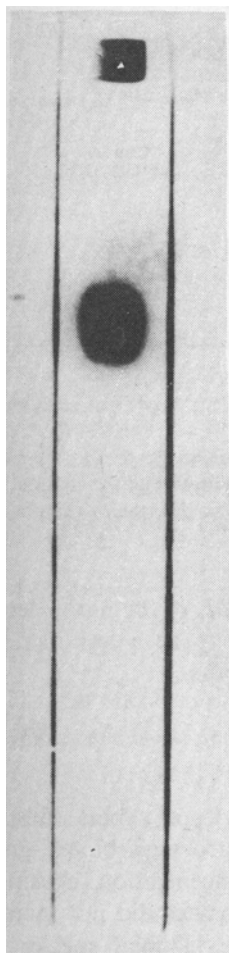


Figure 2. Analytical PAGE of MI lectin.

observed. Sephadex G-25 did not retain the lectin. Such interaction was not observed on Biogels, Sepharose or Sephacryl.

Affinity chromatography on ConA-Sepharose

Elution of the ConA-Sepharose column with 0.1 M α -methyl-D-glucoside resulted in the elution of less than 5% of the bound protein. Because of the low efficiency of the column, ConA-Sepharose could not be employed for routine purifications.

M_r of MI lectin

M_r determination by gel filtration on a calibrated Bio-gel P-300 column gave an approximate value of 100,000 (figure 3). Electrophoresis in the presence of SDS and 2-mercaptoethanol showed two bands corresponding to M_r of 35,000 and 15,000

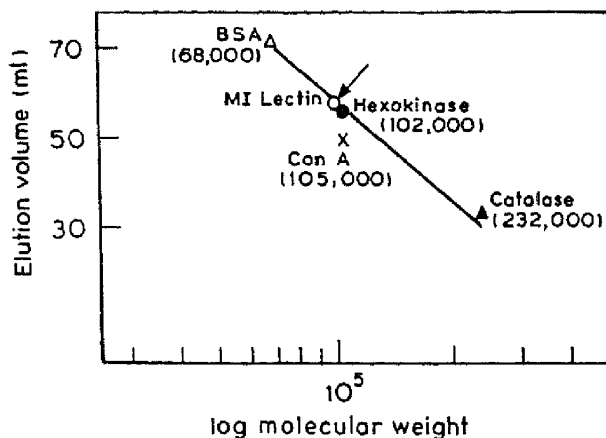


Figure 3. M_r determination by gel filtration on Bio-gel P-300. The column was equilibrated with PBS. Calibration of the column was done with BSA, ConA, catalase and hexokinase. Void volume was determined using blue dextran.

(data not shown). The apparent M_r of the native lectin as judged by these criteria is 100,000. MI lectin thus appears to be a tetrameric molecule consisting of 2α (M_r 35,000) and 2β (M_r 15,000) subunits.

Agglutination properties

MI lectin agglutinated erythrocytes of rabbit, rhesus monkey, guinea pig, hamster, goat and sheep. Human erythrocytes blood group, A, B, AB and O were agglutinated; the minimum concentration causing agglutination was $10 \mu\text{g/ml}$. Trypsin treatment of the erythrocytes did not increase the agglutination titre. The agglutinability of the following rhizobial species was tested: *Rhizobium meliloti*, *R. japonicum*, *R. leguminosarum*, *Rhizobium* sp 32 HI, *R. phaseoli*, *Rhizobium* spp. nodulating *Dolichos*, and *Rhizobium* sp. nodulating *M. invisa*. There was agglutination only of *Rhizobium* sp. nodulating *M. invisa*, demonstrating the specificity of interaction. Rhizobia isolated from the nodules of *M. invisa* were agglutinated by the lectin. This agglutination was improved by the addition of PVP (1 mg/ml). Addition of BSA to the agglutination mixture did not alter the titre.

Sheep leucocytes were not agglutinated by the lectin.

Treatments to remove any metal present in the lectin did not result in loss of activity. Addition of several metal ions to the agglutination mixture to give a final molarity of 0.01–0.15 M did not enhance erythroagglutinating activity of the lectin.

Sugar specificity

Monosaccharides glucose, galactose, mannose, ribose, arabinose, fructose, sorbose, xylose, D(+) fucose and L(–) fucose, disaccharides maltose, lactose, cellobiose, sucrose, trehalose, turanose and melibiose, trisaccharides raffinose and melezitose and several monosaccharide derivatives were tested at 1, 5, 10, 50 and 100 mM concentrations (and for some sugars up to 1 M) for their ability to inhibit the agglutination

of *Rhizobium* spp. by purified *M. invis*a seed lectin. There was no inhibition of agglutination at any concentration of the compounds tested. However, the lectin binds specifically to isolated polysaccharide from the specific nodulating *Rhizobium* sp. (data to be published elsewhere).

pH optimum

MI lectin had maximum activity in buffers of pH 7–8. At pH 2, the protein was precipitated; at pH 10 the activity was reduced to almost 50%.

Temperature stability

MI lectin was active even after incubation at 97°C for 5 min; when incubation was for 40 min, the lectin retained full activity after incubation at 70°C but the activity was lost after incubation at 80°C. Activity of the lectin was tested by rhizobial agglutination. Figure 4 shows the time dependent inactivation of the lectin at 80°C.

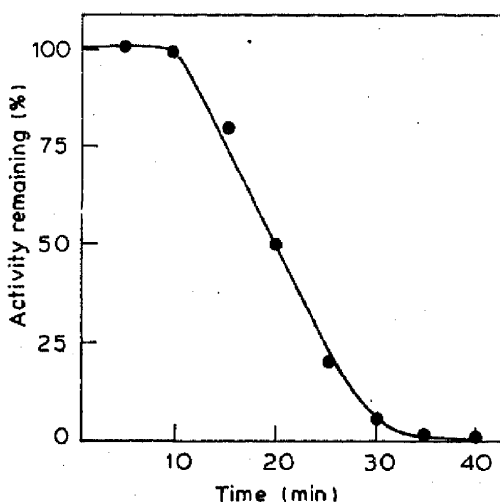


Figure 4. Thermal denaturation of MI lectin at 80°C. The lectin solution (2 mg/ml PBS) was incubated at 80°C. Samples were removed at different times, cooled in ice and centrifuged, and the supernatant assayed.

Interaction of MI lectin with other lectins

Interaction of MI lectin with phytohemagglutinin, ConA, soyabean lectin, peanut agglutinin, pea lectin and pokeweed mitogen was checked by double diffusion in agar plates. Precipitin-like bands were seen only with ConA. The concentration of all lectins was 250 µg/50 µl in PBS.

Interaction with polysaccharides

Double diffusion of MI lectin with mannan, arabinan, galactan, xylan and arabinogalactan was studied in agar plates. At 1 mg/ml polysaccharide

concentration and 3 mg/ml lectin concentration, there were no bands between MI lectin and any of the polysaccharides. When the polysaccharide concentration was increased to 5 mg/ml and the lectin concentration to 8 mg/ml, there were diffuse bands between the lectin and galactan (data not shown).

Discussion

M. invisa seeds contain a lectin which non-specifically agglutinates human erythrocytes. In crude form, dialysis of the lectin preparation causes aggregation of the protein. Similar aggregating behaviour was reported by Takahashi *et al.* (1980) for high concentrations of Torabean lectin. However, the pure MI lectin did not aggregate upon dialysis at concentrations as high as 10 mg/ml.

The lectin bound irreversibly to ConA-Sepharose and displacement of the bound protein was difficult. Hydrophobic interactions may be responsible for the poor elution with α -methyl-D-glucoside. Secondary non-specific polar interactions along with specific ConA-glycoprotein interactions may be playing a role in the binding of lectin to the affinity matrix (Davy *et al.*, 1974).

The lectin showed affinity towards Sephadex G-100 and G-200 but was non-reactive towards G-25; this behaviour is similar to that of *Lens culinaris* lectin (Howard and Sage, 1969). Sephadex could not be used either for gel filtration or for affinity chromatography since the lectin was retarded on Sephadex and was not tightly bound to the matrix.

The lectin has a M_r of 100,000 and is composed of 4 subunits (two of M_r 35,000 and two of 15,000). The lectin has a rather high percentage of carbohydrate, and is specific to rhizobial polysaccharides. The other known *Rhizobium*-binding lectins have different M_r , subunit structure and sugar specificities. For example, pea lectin has a M_r of 49,000, 4 subunits of M_r 7,000 and 18,000, 4.7% carbohydrate and D-mannose specific (Sharon and Lis 1972; Goldstein and Hayes, 1978; Trowbridge, 1973). Soyabean lectin has a M_r of 120,000 with a subunit M_r of 30,000 and 5% carbohydrate. It is specific to D-GalNAc and requires metal ions for binding (Goldstein and Hayes, 1978). The M_r of MI lectin determined by gel filtration and SDS-PAGE could be more than the true M_r since the lectin has a high percentage of carbohydrate (Andrews, 1965; Segrest and Jackson, 1972).

Unlike several lectins, MI lectin did not show any group specificity. The lectin can be classified as a 'panagglutinin' since the agglutination titre improved considerably when PVP was added to the agglutination mixture. Simple mono- and disaccharides did not inhibit agglutination of erythrocytes and rhizobia by MI lectin. Several lectins do not show inhibition of agglutination by simple sugars and may require complex carbohydrate receptors for binding, as in the case of *Phaseolus vulgaris* lectin (Irimura *et al.*, 1975), *Vicia graminea* lectin (Codington *et al.*, 1975), *Robinia pseudoacacia* lectin (Lemonnier *et al.*, 1972) and *Rana japonica* lectin (Sakakibara *et al.*, 1979). The carbohydrate receptor for MI lectin appears to be a capsular polysaccharide isolated from the nodulating *Rhizobium* (to be published elsewhere).

Recently, legume lectins have been recognised to have a role in specific attachment of soil rhizobia (Bohlool and Schmidt, 1974). MI lectin agglutinates the specific nodulating *Rhizobium* but not several other *Rhizobium* species.

To summarize, the lectin from *M. invisa* seeds is an erythroagglutinating, *Rhizobium*-binding protein with complex sugar binding properties.

References

- Andrews, P. (1965) *Biochem. J.*, **96**, 595.
- Bauer, W. D. (1981) *Annu. Rev. Plant Physiol.*, **32**, 407.
- Bohlool, B. B. and Schmidt, E. L. (1974) *Science*, **185**, 269.
- Clausen, J. (1970) in *The Laboratory Techniques in Biochemistry and Molecular Biology* (eds T. S. Work and E. Work) (Amsterdam, London: North Holland Publishing Company) Vol. 1, Part III, p. 454.
- Codington, J. F., Cooper, A. G., Brown, M. C. and Jeanloz, R. W. (1975) *Biochemistry*, **14**, 855.
- Davey, M. W., Huang, J. W., Suklowski, E. and Carter, W. A. (1974) *J. Biol. Chem.*, **249**, 6354.
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.*, **121**, 404.
- Dazzo, F. B. and Brill, W. J. (1977) *Appl. Environ. Microbiol.*, **33**, 132.
- Dazzo, F. B. and Truchet, G. L. (1983) *J. Membr. Biol.*, **73**, 1.
- Dazzo, F. B., Yanke, W. E. and Brill, W. J. (1978) *Biochem. Biophys. Acta*, **536**, 276.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Renbers, P. A. and Smith, F. (1956) *Anal. Chem.*, **28**, 350.
- Etten, R. L. V. and Saini, M. S. (1977) *Biochim. Biophys. Acta*, **484**, 487.
- Goldstein, I. J., Hughes, R. C., Monsigny, M., Osawa, T. and Sharon, N. (1980) *Nature (London)*, **285**, 66.
- Goldstein, I. J. and Hayes, C. E. (1978) *Adv. Carbohydr. Chem. Biochem.*, **35**, 127.
- Howard, I. K. and Sage, H. J. (1969) *Biochemistry*, **8**, 2436.
- Irimura, T., Kawaguchi, T., Terao, T. and Osawa, T. (1975) *Carbohydr. Res.*, **39**, 317.
- Kabat, E. A., (1961) *Experimental Immunochemistry*, 2nd edition (Springfield, Illinois: Charles C. Thomas).
- Kampine, J. P., Bradley, R. O., Kanfer, J. N., Feld, M. and Shapiro, D. (1966) *Science*, **155**, 86.
- Laemmli, U. K. (1970) *Nature (London)*, **227**, 680.
- Lemonnier, M., Goussault, Y. and Bourrillon, R. (1972) *Carbohydr. Res.*, **24**, 323.
- Lis, H. and Sharon, N. (1981) in *The Biochemistry of Plants, a Comprehensive Treatise* (eds P. K. Stumph and E. E. Conn) (New York: Academic Press) vol. 6, p. 372.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265.
- Lubs, H. A., McKenzie, W. H., Patil, S. R. and Merrick, S. (1973) *Methods Cell Biol.*, **6**, 345.
- Ouchterlony, O. (1962) *Prog. Allergy*, **6**, 30.
- Paulova, M., Entlicher, G., Ticha, M., Kostir, J. V. and Kocourek, J. (1971) *Biochim. Biophys. Acta*, **237**, 513.
- Sakakibara, F., Kawaguchi, H., Takayanagi, G. and Ise, H. (1979) *Cancer Res.*, **39**, 1347.
- Seabright, M. (1971) *Lancet*, **2**, 971.
- Segrest, J. P. and Jackson, R. L. (1972) *Methods Enzymol.*, **B28**, 54.
- Sharon, N. and Lis, H. (1972) *Science*, **177**, 949.
- Takahashi, T., Itoh, M. and Shimabayashi, Y. (1980) *Agric. Biol. Chem.*, **44**, 1655.
- Trowbridge, I. S. (1973) *Proc. Natl. Acad. Sci. USA*, **70**, 3650.
- Whitaker, J. R. (1963) *Anal. Chem.*, **35**, 1950.