Purification and Properties of the Hemagglutinin from *Maclura pomifera* Seeds†

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ABSTRACT: *Maclura pomifera* seeds contain a protein which agglutinates human erythrocytes at concentrations as low as 4 ng/mL. This property is related to its ability to bind with high specificity various α-D-galactopyranosides. The agglutinin, which was purified by affinity adsorption, exhibits one band on immunoelectrophoresis and displays one peak during ultracentrifugation, isoelectric focusing, and gel permeation chromatography. The active protein has a molecular weight of 40 000–43 000 and contains two dissimilar polypeptide chains of 12 000 and 10 000, respectively.

With the realization that lectins exhibit a host of interesting biological and chemical properties, research on these proteins has become of considerable importance. One such protein is the hemagglutinin of the seeds of *Maclura pomifera* (Osage Orange tree). Aqueous extracts of the seeds from this tree were shown to possess agglutinating activity toward dog, sheep, horse, and human erythrocytes (Jones et al., 1967a), as well as leukoagglutinating activity with human cells (Jones et al., 1967b). In the later study, inhibition of the agglutinating activity of the extract established the relatively strong inhibitory properties of melibiose, stachyose, and *N*-acetyl-D-galactosamine. Recently, studies on the *M. pomifera* lectin have been stimulated by a publication suggesting that the lectin is a T-lymphocyte binding reagent and lymphocyte mitogen (Jones and Feldman, 1973). Preliminary experiments have indicated that the lymphocyte receptors differ from those for concanavalin A and on erythrocytes differ from those receptors for the *Sophora japonica* and *Wistaria floribunda* lectins as well as concanavalin A (Poretz, 1973a).

Recently, Ulevitch et al. (1974) briefly reported the purification of the lectin employing an affinity adsorbant. We now wish to report the practical and specific purification of this hemagglutinin and describe its chemical characteristics.

Experimental Procedures

Immunochemical Procedures. Antisera were produced by footpad (1.0 mL) and intraperitoneal (1.0 mL) immunization of New Zealand White rabbits using either fraction F-1 (1.5 mg/mL) or fraction F-5 (1.0 mg/mL) (see purification section) emulsified with an equal volume of Freund's complete adjuvant. Intramuscular booster injections with an emulsion of either fraction F-1 or F-5 and Freund's incomplete adjuvant were given at 2-week intervals. The animals were bled by cardiac puncture after the sixth week.

Agglutination titrations of various solutions with human type A, B, and O erythrocytes were conducted as previously described (Poretz, 1973b). Saccharide inhibition of hemagglutination was performed in a standard manner with microtitration equipment (Cooke Engineering, Alexandria, Va.). Appropriate sugar solutions were twofold serially diluted in PBS[1] buffer. Equal volumes of a 2% erythrocyte suspension and of a solution containing two complete hemagglutinating doses of agglutinin were added to each well. The degree of agglutination was scored in a manner identical with the scoring of the hemagglutination titers.

The procedure of Grabar and Williams (1953) was followed for immunoelectrophoresis utilizing commercial precoated 1% agarose plates containing 0.05 M sodium barbital buffer (pH 8.6) (Analytical Chemists, Palo Alto, Calif.). Electrophoresis was conducted at 100 V for 35 min and developed with the appropriate antiserum at 20 °C.

Purification of the Lectin. One hundred grams of freshly ground *M. pomifera* seeds (F. W. Schumacher, Sandwich, Mass.) was suspended by stirring in 1 L of PBS (0.01 M sodium phosphate buffer (pH 6.8) containing 0.12 M NaCl) at 4 °C. After 24 h, the suspension was filtered through cheesecloth, the residue washed with 100 mL of PBS and the combined filtrates were decanted and retained (fraction F-1).

To each stirred volume of fraction F-1 was slowly added at 4 °C, 1.1 volumes of 95% ethanol. After stirring for 2 h, the suspension was centrifuged at 15 000*g* and 4 °C for 45 min. Following removal of the supernatant (fraction F-2), the precipitate was suspended in 150 mL of PBS and dialyzed against 3 × 4 L of the same buffer. This was centrifuged at 25 000*g* for 1 h at 4 °C and the resulting supernatant (fraction F-3) was filtered through a glass wool plug.

Fraction F-3 was mixed with 2 g of polyleucyl hog gastric mucin (Kaplan and Kabat, 1966; Poretz, 1973b) or 20 mL of packed *p*-aminophenyl α-D-galactopyranoside–Sepharose conjugate (Cuatrecasas, 1970) and after stirring for 30 min at 4 °C the suspension was centrifuged at 400*g* and 4 °C for 15 min producing a supernatant (fraction F-4). The resulting sediment was repeatedly washed by resuspension in 100 mL of cold PBS and centrifugation. Following the last wash (the supernatant exhibited an OD_{280} of 0.05 unit or less), the absorbed lectin was eluted from the adsorbant at room temperature with 100 mL of 0.23 M borate buffer (pH 10.0). The supernatant produced upon centrifugation was neutralized with 0.2 M HCl and dialyzed at 4 °C against PBS.

Protein Analyses. The protein content of various solutions was quantified by the colorimetric procedure of Mage and Dray (1965), or by absorption of light at 280 nm.

Carbohydrate Analyses. The total neutral carbohydrate content of protein solutions was obtained by the anthrone procedure (Spiro, 1973) and the phenol–sulfuric acid method

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[1] Abbreviations used: PBS, phosphate-buffered saline; Pth, phenylthiohydantoin.

TABLE I: Characteristics of Fractions of the *M. pomifera* Lectin Purification with Polyleucyl Hog Gastric Mucin.

Fraction	Vol (mL)	Protein (μg/mL)	Total protein (mg)	Hemagglutination: A erythrocytes Titer	A erythrocytes Specific titer[a]	B erythrocytes Titer	B erythrocytes Specific titer[a]	O erythrocytes Titer	O erythrocytes Specific titer[a]
F-1	1000.0	625	625.0	64	102	64	102	64	102
F-2	2000.0	193	386.0	NA[b]	NA	NA	NA	NA	NA
F-3	190.0	560	106.0	250	446	500	892	250	446
F-4	190.0	326	62.0	NA	NA	NA	NA	NA	NA
F-5	30.6	470	14.4	64 000	136 470	128 000	272 340	128 000	272 340

[a] Titer/protein concentration. [b] No activity.

(Dubois et al., 1956) using D-galactose as a reference standard.

Amino Acid Analysis. Untreated or performic acid oxidized (Hirs, 1967) proteins were dissolved in deionized water, mixed with an equal volume of 12 N HCl, and heated in a sealed evacuated tube at 110 °C for 24, 48, and 78 h. The hydrolyzed proteins were analyzed according to the method of Spackman et al. (1958) employing a modified Beckman automatic amino acid analyzer. Tryptophan was determined spectrophotometrically (Beaven and Holiday, 1952).

Glycoside Hydrolase Activity. The presence of α- and β-D-galactosidase, α-D-glucosidase, α-D-mannosidase, and *N*-acetyl-β-D-galactosaminidase activities were determined at pH 4.1 and pH 7.0 using the appropriate *p*-nitrophenyl glycosides as substrates. The assays were conducted as described by Lederberg (1950) and the liberated *p*-nitrophenol was determined spectrophotometrically at 420 nm using *p*-nitrophenol as a standard.

Polyacrylamide Gel Electrophoresis. Electrophoresis in 7.5% polyacrylamide gels at pH 8.9 was conducted as described by Davis (1964) except the samples, containing 20% sucrose, were layered on top of the separation gels. Disc electrophoresis at pH 4.1 was conducted by the procedure of Reisfeld et al. (1962). Electrophoresis in the presence of sodium lauryl sulfate through 15% polyacrylamide gels was performed by the method of Laemmli and Favre (1973).

Analytical Ultracentrifugation. Sedimentation velocity centrifugation was conducted with a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and using an AN-D rotor with standard aluminum cells. Photographs were taken before and after the rotor attained a running speed at 4 °C of 59 780 rpm.

Analysis of the N-Terminal Amino Acid. The method of Edman (1956) as modified by Wong (1969) was followed for the preparation of the Pth-amino acid. The derivatized amino acid was identified by thin-layer chromatography on silica gel plates (Blomback et al., 1966) and high performance liquid chromatography using a silicic acid column (10 cm × 0.5 cm) (Graffeo et al., 1973) and commercial preparations of Pth-amino acids as reference compounds. In addition, the N-terminal amino acid was identified by hydrolysis of the Pth-amino acid (Smithies et al., 1971) and analysis of the liberated amino acid by automatic amino acid analysis.

Analytical Gel Permeation Chromatography. Molecular weight determinations were performed in PBS (pH 6.8) using calibrated gel filtration columns (0.9 × 80.0 cm) of Sephadex G-200 and Bio-Gel P-150 in a manner as described by Andrews (1965). Gel permeation chromatography in the presence of denaturing solutes was conducted using a column (0.9 × 60.0 cm) of Bio-Gel P-150 equilibrated with PBS (pH 6.8) containing 0.1% sodium lauryl sulfate and 0.1 M 2-mercaptoethanol or with a column (0.9 × 80.0 cm) of Sephadex G-150 equilibrated with 6 M guanidinium chloride (Fish et al., 1970).

TABLE II: Glycoside Hydrolase Activity of Fractions from the Purification of the *M. pomifera* Hemagglutinin.

	Activity of fraction[a] F-1	F-3	F-5[b]
α-D-Galactosidase	50.7	27.6	0.0
β-D-Galactosidase	55.3	552.6	0.0
α-D-Mannosidase	12.3	127.8	0.0
N-Acetyl-β-D-galactosaminidase	21.5	145.0	0.0
α-D-Glucosidase	60.5	492.2	0.0

[a] Expressed as nmol of substrate hydrolyzed per min per mg of protein at pH 4.1. [b] Identical results were obtained at pH 7.0.

Elution of the lectin and appropriate standard proteins from the Bio-Gel P-150 column was monitored by adsorption of light at 280 nm and for the Sephadex G-150 column, turbidimetrically at 435 nm following the addition of an equal volume of 5% Cl_3CCOOH to each fraction (Fish et al., 1970), or by the optical density of fractions at 280 nm following dialysis of each fraction.

Results

Table I shows the characteristics of the various fractions obtained during the purification of the *M. pomifera* lectin. Each 100 g of pulverized seeds yields 625 mg of protein, containing 14 mg of *M. pomifera* lectin. Ethanol precipitation (fraction F-3) results in a 5-fold purification of the lectin with no loss of activity. Fraction F-2, the supernatant of the ethanol fractionation following extensive dialysis and 10-fold concentration as compared with the original volume of fraction F-1, lacks detectable hemagglutinating activity. Specific adsorption of the lectin from fraction F-3 with polyleucyl hog gastric mucin results in a supernatant (fraction F-4) containing no demonstrable hemagglutinating activity. Elution with borate buffer produces 14 mg of lectin with a 2500-fold increase in specific activity as compared with fraction F-1 and which caused agglutination of human type A, B, and O erythrocytes at a concentration of 4 ng/mL.

Table II demonstrates that, though high levels of glycoside hydrolase activities are evident in fractions F-1 and F-3, the purified hemagglutinin lacks any detectable α- and β-D-galactosidase, α-D-mannosidase, *N*-acetyl-β-D-galactosaminidase, and α-D-glucosidase activities at either pH 4.1 or pH 7.0.

Evidence for chemical homogeneity is provided by the isoelectric focusing profile of the purified lectin in Ampholine gradients of pH 3-10 and 3-6 as shown in Figures 1A and 1B, respectively. A single symmetrical protein peak is evident in each gradient with an observed isoelectric point at pH 4.75.

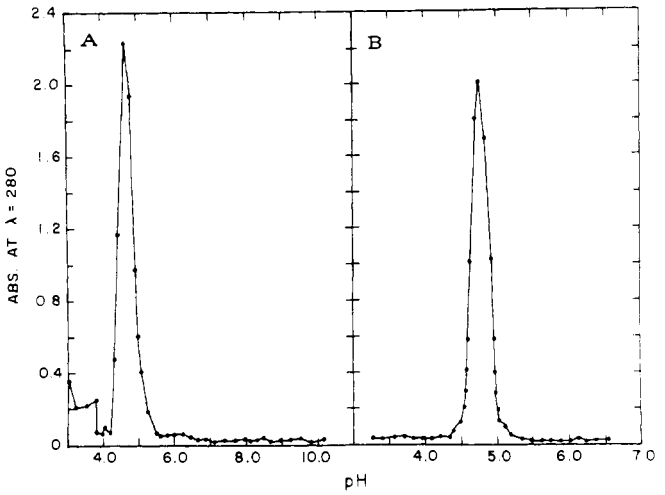

FIGURE 1: Isoelectric focusing in sucrose gradients of affinity purified *M. pomifera* lectin at (a) pH 3-10; (b) pH 3-6.

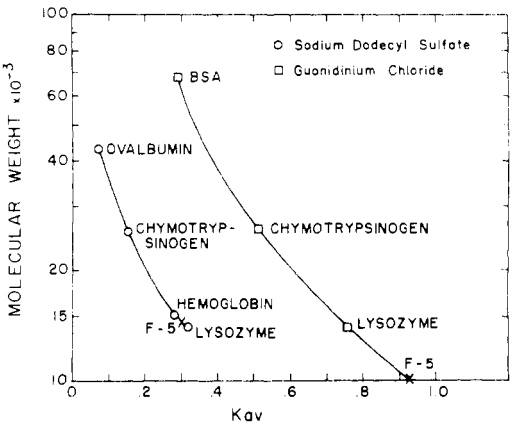

FIGURE 2: Molecular weight by gel filtration of denatured *M. pomifera* lectin.

TABLE III: Composition and Physicochemical Characteristics of the *M. pomifera* Lectin.

Residue	Residues/40 000 g	Residue	Residues/40 000 g
Lys	23.7	Ala	8.8
His	2.3	Val	25.7
Arg	8.9	Met	1.7
Asp	33.6	Ile	23.5
Thr	27.0	Leu	18.9
Ser	24.6	Tyr	20.1
Glu	27.4	Phe	21.5
Pro	15.1	Trp	3.6
Gly	47.6	Cys-SO_3H	Trace
	$E_{1\%}^{1cm}$ (280 nm):	15.7	
	pI:	4.75	
	$s_{20,w}$ (S):	4.11	
	Mol wt:	40 000	

Virtually all of the protein focuses within 0.05 pH unit. Polyacrylamide gel electrophoresis at both pH 4.3 and 8.6 results in each case, with a diffuse single component when stained with amido black dye. Similarly, immunoelectrophoresis of the purified lectin produces only one diffuse precipitin band when developed with antisera raised against fraction F-1 or F-5. In contrast, the fraction F-1 yields at least six components when developed with anti-fraction F-1 sera.

The homogeneous behavior of fraction F-5 is demonstrated by the elution of a single symmetrical peak from either a column of Sephadex G-200 or Bio-Gel P-150. The elution volume of the lectin corresponds to a molecular weight of 40 000 in both the Sephadex G-200 column calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme and the Bio-Gel P-150 column standardized with asparaginase, bovine serum albumin, and chymotrypsinogen. The absence of any carbohydrate specific interactions between the Sephadex gel and the lectin was confirmed by observing a similar apparent molecular weight and elution pattern for the lectin when gel filtration was conducted through a calibrated Sephadex G-200 column equilibrated with 0.2 M D-galactose, a known ligand for this lectin. Velocity sedimentation patterns of the protein when subjected to ultracentrifugation further verify the molecular weight homogeneity of the protein. The single symmetrical peak obtained at protein concentrations of either 5 or 10 mg/mL indicates a single molecular weight species with a $s_{20,w}$ of 4.1 S.

Table III presents the amino acid composition of the purified lectin. This protein is relatively rich in acidic and hydroxy amino acyl residues and contains small amounts of basic amino acyl residues, consistent with the acidic isoelectric point observed in isoelectric focusing. Very small amounts of methionine and essentially no cysteic acid were detected in the hydrolysates of the native and performic acid oxidized proteins.

Though two polypeptide chains were observed in sodium dodecyl sulfate-acrylamide gel electrophoresis (see later), glycine was the only amino acid identified at the N termini by use of the Edman procedure.

Analysis of the lectin with anthrone reagent yielded a carbohydrate content of 0.8% w/w and with the phenol-sulfuric acid procedure, 1% w/w in terms of D-galactose. This represents a maximum of approximately two residues of neutral hexose/40 000 g.

The dissociability of the purified *M. pomifera* lectin was examined in three systems: sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration through Bio-Gel P-150 in the presence of 0.1% sodium dodecyl sulfate, and 0.1 M β-mercaptoethanol and gel filtration through Sephadex G-150 equilibrated with 6 M guanidinium chloride. When polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on slab gels of 15% acrylamide by the Laemmli and Favre (1973) method, two Coomassie blue staining components were detected with molecular weights of approximately 12 000 and 10 000. Gel filtration of the *M. pomifera* lectin resulted in elution of a single peak in each case, with volumes corresponding to 14 000 (Bio-Gel P-150/sodium dodecyl sulfate) and 10 000 (Sephadex G-150/guanidinum chloride) (see Figure 2). In agreement with the low molecular weight estimate, the eluted lectin from the guanidinium chloride column could not be precipitated with 7% Cl_3CCOOH, a procedure successfully used for the turbidimetric detection of the standard proteins used in this experiment.

The specificity of the interaction between the purified lectin and simple saccharides was explored by examining the ability of sugars to inhibit lectin mediated hemagglutination of human cells. It is evident from Table IV that the lectin displays a high specificity for α-D-galactosyl residues. Thus, methyl α-D-galactopyranoside is greater than 5000 times more potent as an inhibitor of hemagglutination than methyl β-D-galactopyranoside; and similarly, melibiose (gal_p 1-α-6 glc_p) is at least 300 times more effective than lactose (gal_p 1-β-4 glc_p). It is also apparent that the 2-deoxy-2-acetamido derivative of D-ga-

lactose requires one-fourth the concentration of the parent compound to cause inhibition of hemagglutination. However, methyl α-D-galactopyranoside is 2500 times more potent than its 4-epimer, methyl α-D-glucopyranoside; but surprisingly, it is only 300 times more active as an inhibitor of the lectin than methyl α-D-mannopyranoside, a sugar which differs from it at both the C-2 and C-4 positions.

TABLE IV: Inhibition by Carbohydrates of *M. pomifera* Lectin Mediated Agglutination of Erythrocytes.[a]

Carbohydrate	Minimum concn (mM) required for complete inhibition of agglutination
Methyl α-D-galactopyranoside	0.10
Methyl β-D-galactopyranoside	>500
Melibiose (Gal $\xrightarrow{\alpha\text{-}1,6}$ Glc)	1.5
Lactose (Gal $\xrightarrow{\beta\text{-}1,4}$ Glc)	>500
Maltose (Glu $\xrightarrow{\alpha\text{-}1,4}$ Glc)	>500
D-Galactose	12
N-Acetyl-D-galactosamine	3.0
Methyl α-D-mannopyranoside	30
Methyl α-D-glucopyranoside	250

[a] Type A human erythrocytes. Gal = D-galactopyranose. Glc = D-glucopyranose.

Discussion

The T-lymphocyte binding specificity and lymphocyte mitogenic behavior of the *M.* pomifera lectin (Jones and Feldman, 1973) make it a valuable reagent for the study of cell surface carbohydrates and the characteristics of cells of the immune system. We therefore have developed a practical purification and explored the chemical and carbohydrate binding specificity of this lectin.

The use of polyleucyl hog gastric mucin and glycoside-Sepharose conjugates for the adsorption of lectins has proved successful in a number of instances (see Sharon and Lis, 1972). We find complete adsorption of *M. pomifera* induced hemagglutinating activity with polyleucyl hog gastric mucin and *p*-aminophenyl α-D-galactopyranoside-Sepharose conjugate. Addition of borate buffer to the adsorbant-lectin complex releases 14 mg of agglutinin per 100 g of seeds. The desorbed lectin displays a total activity considerably greater than that of the crude extract, suggesting the presence of a nondialyzable inhibitor in the crude extract.

The final preparation which appears homogeneous by liquid isoelectric focusing and by immunochemical criteria is void of any detectable glycosidase activity. The absence of glycoside hydrolase activities, which are present in the fraction prior to specific adsorption, further demonstrates the specificity of the adsorption procedure, which is followed by the relatively nonspecific desorbing condition of borate buffer. The purified lectin has an apparent molecular weight of 40 000, obtained by analytical gel filtration, which is consistent with a sedimentation coefficient of 4.1 S assuming that the lectin behaves as a globular sphere. This molecular weight is in sharp contrast with the 80 000–100 000 value reported by Ulevitch et al. (1974). This discrepancy may be explained in part by our observation that the *M. pomifera* lectin tends to aggregate and behave as an 80 000 unit when in the presence of sodium dodecyl sulfate under mild denaturing conditions. However, we have found that boiling the sample in 1% sodium dodecyl sulfate for 1 min eliminates the high molecular weight components and produces two Coomassie blue staining bands of 12 000 and 10 000, respectively. Our results are consistent with the unusual stability of the lectin as originally reported by Jones et al. (1967a,b) who noted that the hemagglutinating activity of the seed extract was stable to temperatures as high as 75 °C. The appearance of relatively small subunits upon denaturation of the lectin was confirmed by gel filtration in the presence of either sodium dodecyl sulfate or 6 M guanidinium chloride. In each case, however, the column was unable to resolve the presence of both types of small subunits.

The amino acid analysis of the agglutinin is similar to a number of other purified plant lectins, characterized by high levels of hydroxy amino acids and small amounts of sulfur containing amino acids. In particular, the *M. pomifera* lectin appears to lack cysteine, as does concanavalin A (Agrawal and Goldstein, 1968).

This finding in conjunction with the subunit nature of the lectin indicates that the protein exists as a 40 000–44 000 structure composed of two pairs of dissimilar polypeptide chains stabilized by non-covalent interactions. It is noteworthy that the high thermal stability and resistance to denaturation by detergents of the lectin as well as the high percentage of apolar amino acids found in this protein are consistent with a structure stabilized by apolar interchain interactions.

The lectin is capable of agglutinating human type A, B, and O erythrocytes equally well, requiring a minimum concentration of of 4 ng/mL. This activity is relatively high compared with the minimum concentration of other lectins required to agglutinate human and animal erythrocytes (being in the order of μg/mL levels). The lectin induced hemagglutination appears related to the ability of the protein to bind specifically α-linked D-galactopyranosyl and 2-acetamido-2-deoxy-D-galactopyranosyl residues. However, the erythrocyte receptors binding the lectin must be structures other than or in addition to the blood group type A (terminal 2-acetamido-2-deoxy-α-D-galactopyranosyl moiety) or B (terminal α-D-galactopyranosyl moiety) determinants, in that type O erythrocytes and animal cells lacking these blood group receptors (Sprenger et al., 1970) are agglutinated by the lectin.

Though our data demonstrate that the *M. pomifera* lectin is capable of binding the nonreducing terminal D-galactopyranosyl unit of dissaccharides, the question is still open as to the ability of the protein to interact with internal α-D-galactopyranosyl or *N*-acetyl-α-D-galactosaminyl residues. Chuba and Kuhns (1973) reported the relatively good inhibitory activity of crude extracts of *M. pomifera* seeds by the anti-freeze glycoprotein. This protein possesses a large number of threonyl residues substituted by 3 (or 4)-*O*-β-galactopyranosyl-*N*-acetyl-α-D-galactosaminyl residues. Similarly, Sprenger et al. (1970) noted that 3-*O*-β-D-galactopyranosyl-2-acetamido-2-deoxy-D-galactose inhibited the *M. pomifera* induced agglutination of type O erythrocytes 25% as well as *N*-acetyl-D-galactosamine. These data suggest that 3-*O*-glycosyl substituted *N*-acetyl-D-galactosaminyl structures may be capable of reacting with this lectin. Furthermore, our observations as well as that of Chuba and Kuhns (1973) that methyl α-D-mannopyranoside is a considerably better inhibitor of the lectin than methyl α-D-glucopyranoside is difficult to correlate with a single type of binding site complementary to *N*-acetyl-D-galactosamine-like structures. It is quite clear now that the protein can be purified, that unequivocal studies must

be performed to completely describe the binding specificities of this lectin.

During the course of these studies we became aware that I. J. Goldstein and C. E. Hayes were isolating the *M. pomifera* agglutinin using a melibionate-Bio-Gel affinity adsorbant (I. J. Goldstein, personal communication). We have since determined that their preparation displays a specific hemagglutinating activity toward A, B, and O human erythrocyte and a polyacrylamide gel electrophoresis pattern identical with that of fraction F-5 reported in this paper.

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