Isolation, Partial Purification, and Characterization of Lectins from the Roots of *Vigna unguiculata* (L.) Walp.

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ABSTRACT

A lectin was isolated from the roots of 7-day old *Vigna unguiculata* (L.) Walp. seedlings using size-exclusion chromatography. The root lectin revealed two subunits in SDS-PAGE and migrated as a single protein in a pH gradient isoelectrofocusing gel electrophoresis as well as in native gel electrophoresis. The root lectin exhibited no specificity to any of the erythrocytes tested. In addition, the agglutinating activity of the root lectin was markedly increased by enzymes. The hemeagglutinating activity of the root lectin was inhibited by the sugars N-acetyl-galactosamine and methyl-a-D-glucopyranoside.

INTRODUCTION

Many plants contain lectins, a class of proteins or glycoproteins of non-immune origin which specifically and noncovalently bind to carbohydrate moieties (Dazzo et al. 1978; Roberts and Etzler 1984; Damjanov 1987; Quinn and Etzler 1987; Barondes 1988; Sharon and Lis 1989). They have no enzymatic activity, may be soluble or membrane bound, and contain at least two binding sites (Sharon and Lis 1972; Bowles and Kauss 1975; Callow 1975; Bowles et al. 1979; Damjanov 1987; Sharon and Lis 1989). Aside from distinguishing normal from transformed cells, lectins are known to exhibit a host of other interesting chemical and biological properties (Lis and Sharon 1973). They agglutinate erythrocytes, with very high specificity in certain cases, precipitate polysaccharides or glycoproteins, and stimulate resting lymphocytes into actively growing and dividing blast-like cells (Lis and Sharon 1973; Callow 1975; Hapner and Robbins 1979; Pueppke 1979; Nag et al. 1981; Damjanov 1987). These properties of lectins provide investigators with an extremely useful tool with varied applications as specific reagents for the isolation and characterization of polysaccharides in solution or on cell surfaces (Lis and Sharon 1986), for structural studies of carbohydrate-containing polymers, for the identification and separation of cells, for probing the molecular architecture of cell surfaces and the changes therein by transformation, and for isolating specific membrane constituents (Lis and Sharon 1973; Liener 1976; Goldstein and Hayes 1978; Nag et al. 1981).

Lectins have been studied and isolated from both plants and animals, as well as from fungi, lichens, and bacteria (Liener 1976; Hapner and Robbins 1979; Damjanov 1987; Sharon and Lis 1989). In legumes and other plant species, lectins have been found mostly in seeds (Liener 1976; Pueppke 1979; Quinn and Etzler 1987). Lectins have also been detected in other vegetative tissues such as leaves, stems, barks, and roots (Callow 1975; Goldstein and Hayes 1978; Quinn and Etzler 1987).

Numerous research and studies on plant agglutinins have focused on seeds. Studies on leaf and stem lectins started much later. Root lectins, on the other hand, have been gaining much attention because of their cross-reactivity to seed lectins and their possible role in the root-Rhizobium symbiosis (Hapner and Robbins 1979; Gatehouse and Boulter 1980; Barondes 1981; Lis and Sharon 1986; Quinn and Etzler 1987; Sharon and Lis 1989).

This study focused on the isolation, partial purification, and characterization of a lectin from the roots of *Vigna unguiculata* (L.) Walp. Specifically, it sought to provide information on its hemeagglutinating ability, the molecular sizes of its subunits, isoelectric point, and carbohydrate-binding specificity.

Key words: lectins, *Vigna unguiculata*, lectin subunits, molecular weight, isoelectric point, hemeagglutinating activity, sugar-binding sites.

REVIEW OF RELATED LITERATURE

Relatively few lectins have been purified from plants. Only recently have much attention been given to the isolation, determination of content and properties of legume root lectins (Horejsi et al. 1978; Gatehouse and Boulter 1980; Quinn and Etzler 1987). As reported earlier, a lectin known as trifoliin has been isolated from the root of white clover (*Trifolium repens*) (Lis and Sharon 1986; Sharon and Lis 1989). This root lectin was purified and was found to be similar in immunological and electrophoretic properties to white clover seed lectin (Dazzo et al. 1978). Also, roots of soybean (*Glycine max*) have hemeagglutinating activity and contain a protein which reacted with antibodies directed against soybean seed lectin (Pueppke et al. 1978). Thus, it was then assumed that seed lectin was the same as root lectin (Gatehouse and Boulter 1980). However, more recent studies have revealed that this was not the case (Gatehouse and Boulter 1980; Quinn amd Etzler 1987).

The difference between root lectins and seed lectins was demonstrated through tests on the specificity of root lectins binding to *Rhizobia* conducted by Bohlhool and Schmidt (1974), Chen and Phillips (1976), Dazzo and Brill (1977), Law and Strijdom (1977), and Bhuvanesurai and Bauer (1978) as reviewed by Gatehouse and Boulter (1980). Root lectin from *Pisum sativum* (pea) gave a different banding pattern in isoelectric focusing as compared to its seed lectin (Gatehouse and Boulter 1980). In addition, the two lectins gave different carbohydrate specificities when assayed using sugar inhibition (Gatehouse and Boulter 1980). Quinn and Etzler (1987) pointed out differences in amino acid composition between the root and seed lectins from *Dolichos biflorus* (horsegram). They also discovered differences in the banding patterns of the two lectins in isoelectric focusing.

MATERIALS AND METHODS

Plant Material

Seeds of Vigna unguiculata L. (Walp.) secured from the Bureau of Plant Industry, Manila were imbibed for 24 h in distilled water in a round tray (diameter, 38 cm) and were covered with Glad Cling Wrap (First Brands Corp., Danbury, CT) to prevent evaporation and desiccation. The seedlings were suspended on a nylon net (0.3 cm x 0.3 cm) over half-strength Hoagland solution (Hoagland and Arnon 1950) in a dark-colored basin (32 cm x 32 cm x 12 cm). The seedlings were maintained for 7 days following the modified procedures of Quinn and Etzler (1987). The roots were excised at the root-shoot junction, washed three times with distilled water and stored in a freezer (4°C) for 24 h before processing.

Fractionation of Lectins

The roots were thawed to about 10° C and weighed. Thirty grams of roots were homogenized in 150 ml of cold 0.1 M phosphate buffered saline (PBS) (pH 7.2) (Gatehouse and Boulter 1980) using precooled Moulinex blender (MS-223). The homogenate was filtered through cheesecloth and was centrifuged (Sigma 2K-15) at 2000 rpm for 15 min at room temperature. The supernatant was pooled and 90 ml was transferred to a 250 ml Erlenmeyer flask and stirred inside a cold cabinet. During this time, 180 ml of saturated ammonium sulfate (SAS) (pH 7.5) (Deutscher 1990) was added dropwise to the supernatant. The solution was centrifuged (Sigma 4K-15) at 15,000 rpm for 1 h at 40°C. The pellet was washed with 70% SAS and finally resuspended in 0.1 M PBS (Matias, personal communication). It was then dialyzed against the same buffer (Gatehouse and Boulter 1980) with constant stirring for 5 d in a cold cabinet. The dialysate was centrifuged (Sigma 2K-15) at 2000 rpm for 20 min at 40° C (Gatehouse and Boulter 1980). The supernatant was concentrated to 3 ml with poly-ethyleneglycol (PEG 1,5000). This was passed separately through Sepharose 2B and CL-4B column (Matias, personal communication), previously washed and equilibrated with Tris-HCl buffer (Deutscher 1990). Two ml-fractions were collected in 30 test tubes. Absorbance of each fraction was read at 280 nm using a Beckman DU-65 spectrophotometer. Fractions containing protein were pooled and concentrated as above.

Total Protein Content

Total protein content was determined using the method of Bradford (1976). Absorbance at 595 nm was measured using a Beckman DU-65 spectrophotometer.

Gel Electrophoresis

Fractions making up prominent peaks were analyzed both by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and native gel electrophoresis by the method of Laemmli (1970), as modified by Matias (1991). Partially purified extracts were further electrofocused (LKB multiphor II) by the methods of Vesterberg (1975), as modified by Matias (1991), into a polyacrylamide gel with a pH gradient ranging from 3 to 9.5. High and low molecular weight standards, as well as isoelectrofocusing markers were loaded simultaneously with the test fractions. Protein

bands were visualized by silver staining (Laemmli 1970). The gels were then photographed and dried using a Biorad slab gel dryer. Rf values were estimated by obtaining the ratio of the distance travelled by the protein over the total distance travelled by the dye. Molecular weights were then approximated by comparing the Rf values of the sample with the values of the known standards.

Hemeagglutination Tests

The dialysate, as well as protein peaks 1 and 2 were assayed for hemeagglutinating activities by the method of Lis and Sharon (1973). Fresh human blood types A, B, and O and goose erythrocytes were used. Fresh blood was washed 3x with PBS by repeated centrifugation. A final concentration of 2% blood was prepared prior to hemeagglutination. Agglutination was performed in V-bottom shaped microtiter plates and incubated at 37°C for 1 h. Titer was expressed as the reciprocal of the highest dilution which gave a positive reaction. Human blood type B was further treated with 200 ug/ml neuraminidase, trypsin, chymotrypsin, and pronase to expose possible cryptic binding sites. Hemeagglutination was also performed in Eppendorf tubes. Control and agglutinated erythrocytes were observed using a phase contrast Carl Zeiss Axiovert 35M inverted microscope.

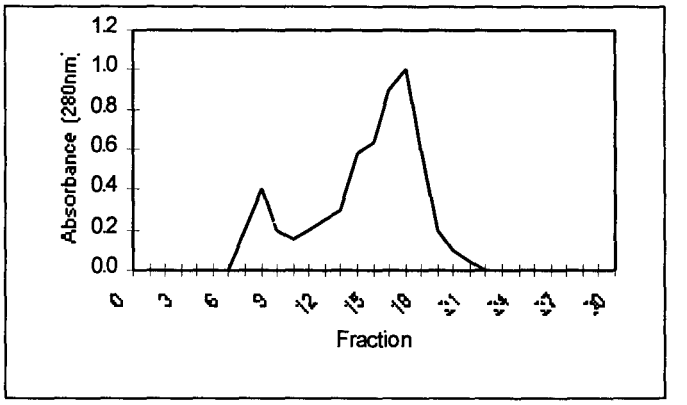
Sugar Inhibition Tests

Sugar inhibition of hemeagglutination was further performed on partially purified root extracts in V-bottom shaped microtiter plates. Sugars, namely, N-acetyl-D-galactosamine (Gal-NAc), methyl-a-D-mannopyranoside, and methyl-a-D-glucopyranoside, dissolved in 0.1 M in PBS were added to serial dilutions of the root extracts and were then incubated for 1 h at 37°C. To serve as the control, serial dilutions of the extracts in 0.1 M PBS were also prepared. Sugar inhibition was then performed using 2% neuraminidase-treated human type B erythrocyte and incubated for 3 h at 37°C.

RESULTS

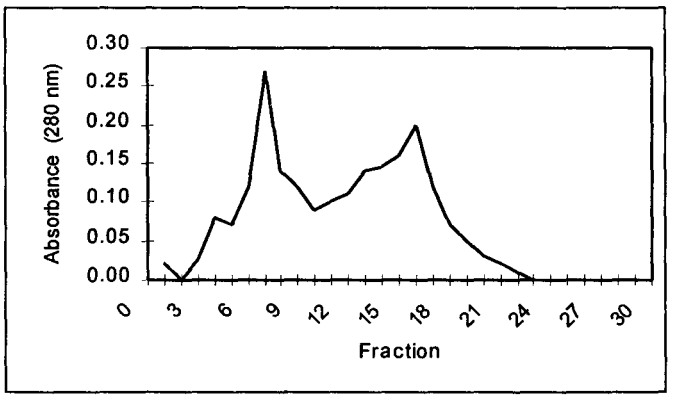
Size-Exclusion Gel Chromatography

The dialysate was partially purified by size exclusion chromatography by using Sepharose CL-4B and 2B matrices. The elution profiles for both revealed prominent protein peaks (Figs. 1 and 2). Chromatography in CL-4B was further repeated using a longer column. Similarly, two protein peaks were obtained (Fig. 1).



Bed volume: 120 ml Bed height: 60 cm
Flow rate: 1.3 ml/min

Fig. 1. Sepharose CL-4B elution profile of SAS precipitated *Vigna unguiculata* (L.) Walp. root extract. Two protein peaks (1 and 2) were obtained with elution using Tris-HCl buffer (pH 7.4).



Bed volume: 2 ml Bed height: 10 cm
Flow rate: 1 ml/min

Fig. 2. Sepharose 2B elution profile of SAS precipitated *Vigna unguiculata* (L.) Walp. root extract. Protein peaks 1 and 2 were obtained with elution using Tris-HCl buffer (pH 7.4).

Gel Electrophoresis

Protein peaks 1 and 2 from both matrices used migrated as a single band in an isoelectrofocusing gradient gel (Fig. 3). However, two distinct bands were observed in an SDS-PAGE gel (Fig. 4). Based on the high molecular weight marker used, the protein bands were estimated, with the aid of linear regression analysis, to have molecular radii of 70 and 67 kD. The protein extract that was not solubilized, on the other hand, migrated as a single band in a non-denaturing gel electrophoresis (Fig. 5). SDS-PAGE

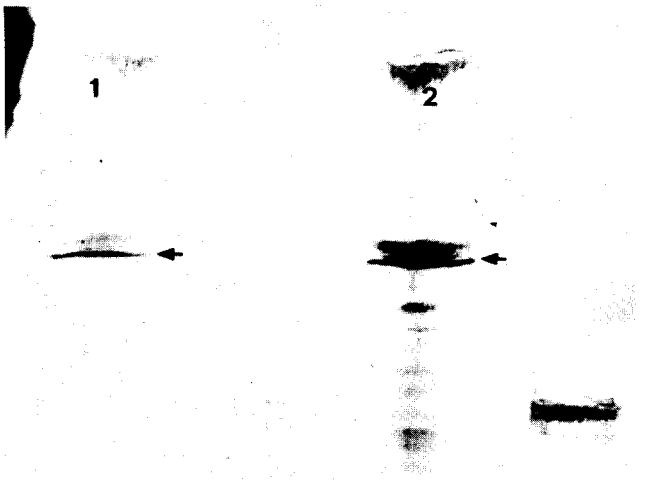


Fig. 3. Isoelectrofusing gel electrophoresis migration of the protein peaks of the root extract. Peaks 1 and 2 migrated as single bands (←) in their respective lanes (1 and 2).

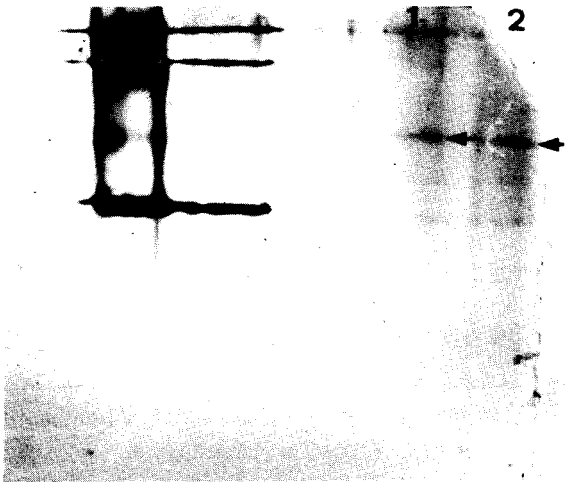


Fig. 5. Native gel electrophoresis banding pattern of the root extract protein peaks.

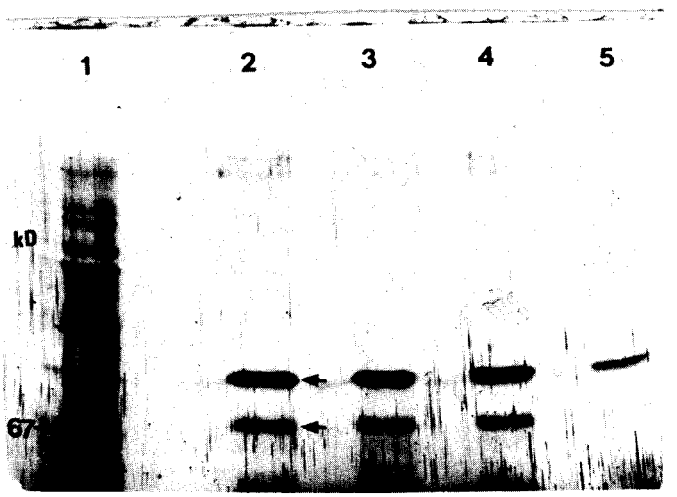


Fig. 4. SDS-PAGE banding pattern of the protein peaks of the root extract. Lane 1, high MW marker: lanes 2 and 3, peak 1: lanes 4 and 5, peak 2. Two protein subunits (←) consistently appeared for both peaks in a 10% acrylaminde gel.

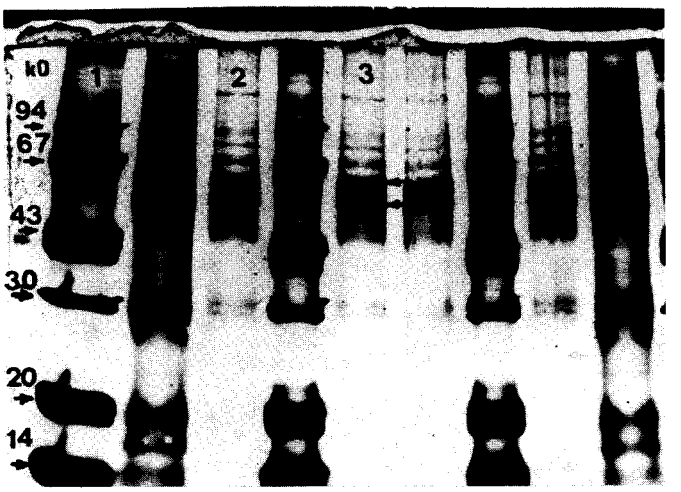


Fig. 6a. Vigna unguiculata (L.) Walp. root lectin banding pattern in SDS-PAGE electrophoresis. Lane 1, low MW marker: lanes 2 and 3, root lectin (←) purified by Sepharose 2B and CL-4B size-exclusion chromatography.

electrophoresis of the resulting pooled fractions from the longer CL-4B column again showed two distinct bands of Mr 68 and 73 kD (Fig. 6a). Molecular radii of the protein subunits of the root extract were calculated based on linear regression anlaysis of the lower molecular weight marker loaded alongside the protein sample (Fig. 6b). Consistently, a single band (pH 6.4) was again observed in a pH gradient isoelectrofocusing gel (Fig. 7a). Linear regression analysis, with the aid of the isoelectrofocusing marker, was again utilized in order to determine the isoelectric point of the root extract (Fig. 7b).

Hemeagglutinating Experiments

Table 1 reflects the hemeagglutination test results which ranged from weakly to strongly agglutinated erythrocytes. The first peak derived from the Sepharose 2B column weakly agglutinated (titer=1) both goose and human erythrocytes of types A, B, and O while the second peak moderately agglutinated (titer=2) both blood types. The dialysate however strongly agglutinated (titer= 8) all blood types tested (Table 1).

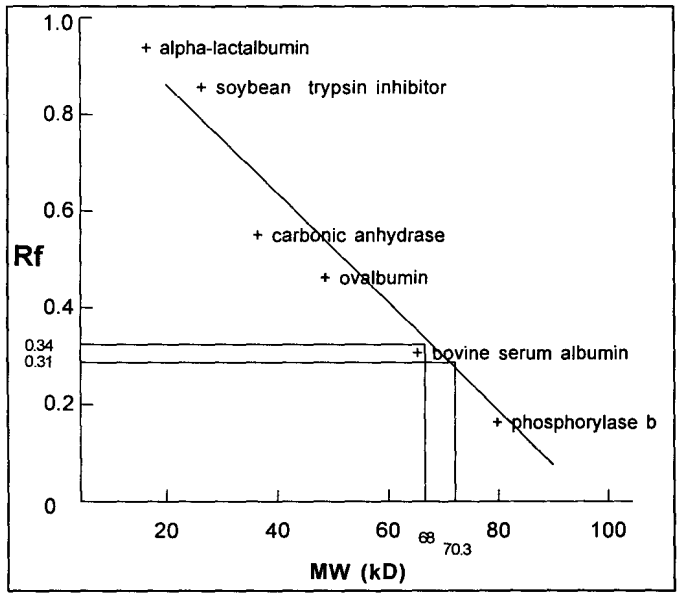


Fig. 6b. Molecular weight determination of the root lectin. Linear regression analysis was used to determine the native and subunit molecular weights of root lectin.

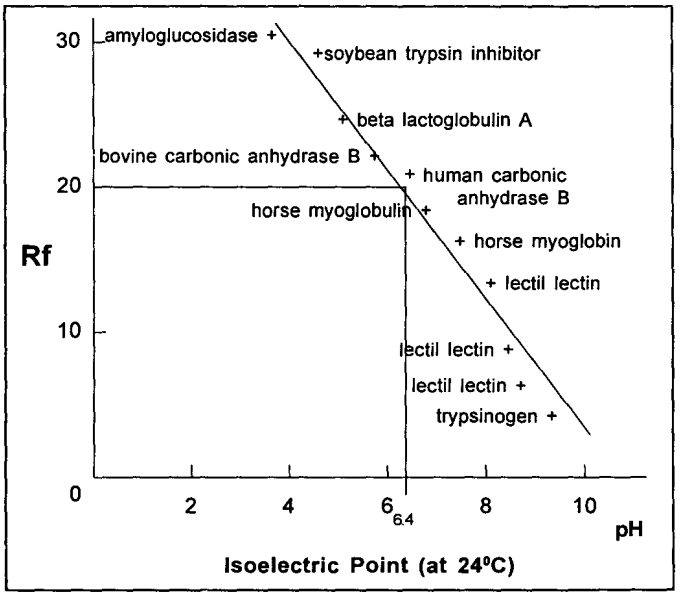


Fig. 7b. Isoelectric point determination of the root lectin. Linear regression was used to determine the root lectin isoelectric point.

Fig. 7a: Isoelectrofusing gel pattern of the root lectin. The root lectin migrated as a single band (➔) in a thin-layer isoelectrofusing gel. Lane 1: root lectin; lane M: IEF marker.

Table 1. Agglutinating activity of CL-4B fractionated root extract against different types of erythrocytes.

Extract vs. Erythrocyte	Dilution sample : PBS							
	1	2	4	8	16	32	64	ctrl
	1:0	1:1	1:2	1:4	1:8	1:16	1:32	0:1
crude vs. human O	+	+	+	+	-	-	-	-
peak 1 vs. human A	+	-	-	-	-	-	-	-
human B	+	-	-	-	-	-	-	-
human O	+	-	-	-	-	-	-	-
goose	+	-	-	-	-	-	-	-
peak 2 vs. human A	+	+	-	-	-	-	-	-
human B	+	+	-	-	-	-	-	-
human O	+	+	-	-	-	-	-	-
goose	+	+	-	-	-	-	-	-

+, agglutination; -, no agglutination; ctrl, control

The two protein peaks from the Sepharose CL-4B were pooled, concentrated, and used to agglutinate fresh and enzyme-treated (neuraminidase-, trypsin-, chymotrypsin- and pronase e-treated) human type B erythrocytes. Table 2 reflects the degree of agglutination. The pooled fractions weakly agglutinated both fresh and neuraminidase-treated blood cells (Fig. 8b), moderately agglutinated both trypsin- and chymotrypsin-treated cells (Fig. 8c) and strongly agglutinated (titer = 4) pronase-treated erythrocytes (Fig. 8d).

Sugars (N-acetyl-D-galactosamine (Gal-NAc) and methyl-a-D-gluco-pyranoside) were most effective in inhibiting the hemeagglutinating activity of the root lectin. Both sugars were able to inhibit agglutination in all dilutions while mannopyranoside was able to decrease the titer from 4 to 2 (Table 3).

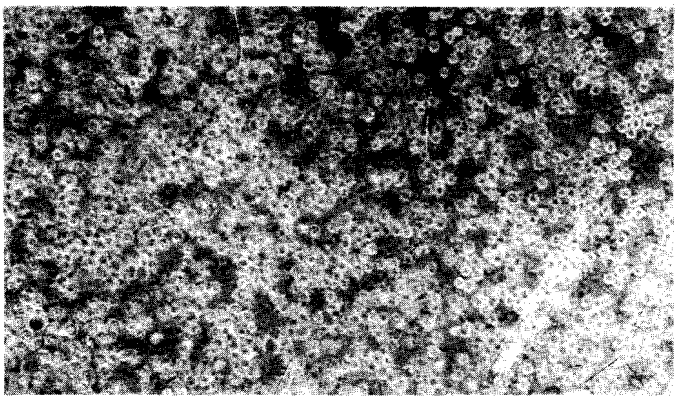


Fig. 8a. Fresh human type B erythrocytes showing no agglutination. (100x)

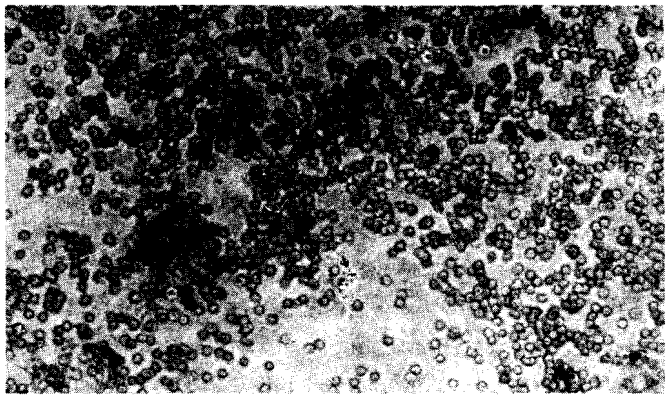


Fig. 8c. Chymotrypsin-treated human type B erythrocytes showing moderate agglutination. (430x)

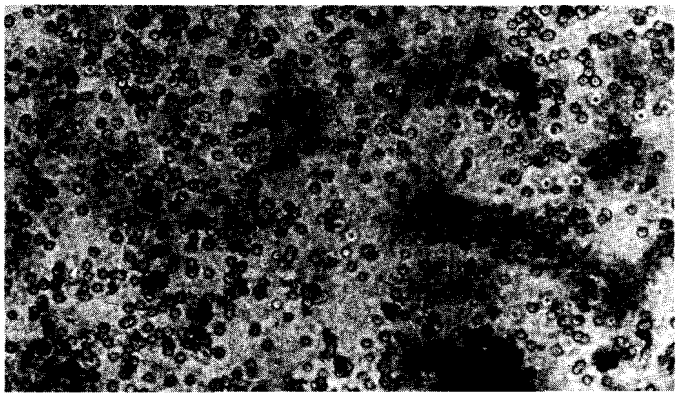


Fig. 8b. Neuraminidase-treated human type B erythrocytes showing weak agglutination. (430x)

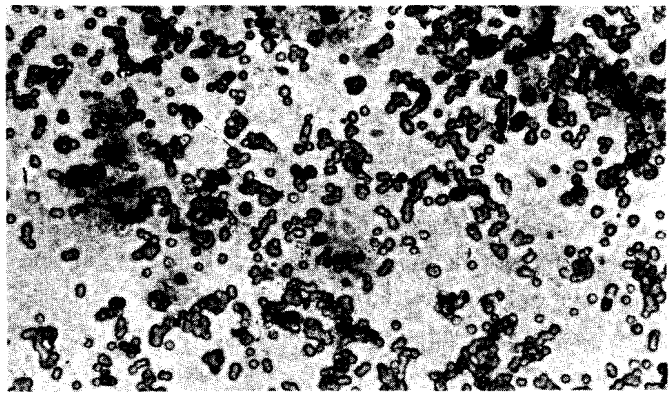


Fig. 8d. Pronase-treated human type B erythrocytes showing strong agglutination. (430x)

Table 2. Agglutinating activity of CL-4B extract against different types of enzyme-treated type B human erythrocytes.

Enzyme	Dilution sample : PBS	
	1 1:0	2 1:1
untreated	+	-
neuraminidase	+	-
trypsin	++	-
chymotrypsin	+++	++

+, weak agglutination; ++, moderate agglutination;
+++, strong agglutination; -, no agglutination

Table 3. Inhibition of hemeagglutinating activity of the root lectin by different sugars.

Sugar	Dilution sample : PBS						
	2	4	8	16	32	64	ctrl
	1:1	1:2	1:4	1:8	1:16	1:32	
no sugar	+	+	-	-	-	-	-
Gal-NAc	-	-	-	-	-	-	-
mannopyranoside	+	-	-	-	-	-	-
glucopyranoside	-	-	-	-	-	-	-