Purification and Characterization of Two Lectins from *Aloe arborescens* Mill

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Two lectins have been isolated from leaves of *Aloe arborescens* Mill by salt precipitation, pH-dependent fractionation and gel filtration. One lectin (P-2) has a molecular weight of approximately 18,000, consists of two subunits ($\alpha\beta$) and contains more than 18% by weight of neutral carbohydrate. The smaller subunit (α) has a molecular weight of approximately 7,500 and the larger subunit (β) a molecular weight of approximately 10,500. The other lectin (S-1) has a molecular weight of approximately 24,000, consists of two subunits (γ_2) with a molecular weight of approximately 12,000 and contains more than 50% by weight of neutral carbohydrate. An interesting feature of the amino acid compositions of these lectins is the high proportion of acidic amino acids, such as aspartic acid and glutamic acid, and the low proportion of methionine and histidine.

S-1 has a strong hemagglutinating activity. On the other hand, P-2 has not only hemagglutinating activity but also mitogenic activity on lymphocytes, precipitate-forming reactivity with serum proteins, one of which is α_1-macroglobulin, and complement C3 activating activity *via* the alternate pathway.

It has long been known that extracts of certain plant seeds agglutinate human and other mammalian erythrocytes (*1–4*). Among these, several lectins can stimulate peripheral blood lymphocytes to undergo mitosis regardless of the antigenic specificity of their cell surface receptors (*5–7*). It has also been elucidated that lectins are proteins which bind in a specific manner to sugar residues (*3, 5, 8, 9*). In the last few years they have become powerful tools in biological research, especially in studies of membrane structure and cell transformation (*5, 6, 8, 10*).

We attempted to isolate the lectin from *Aloe arborescens* Mill (aloe), which has been used in folk medicine for centuries all over the world. This paper presents data on the purification and chemical and physical properties of two lectins extracted from aloe. We also report on their various biological activities, such as hemagglutinating activity, mitogenic activity against lymphocytes,

reactivity with serum proteins of various animals and activation of serum complement components.

MATERIALS AND METHODS

Purification of Lectins—Aloe leaves were crushed in a commercial juicer (Fuji Electric Co., Japan) and the juice was collected. After removal of coarse materials by centrifugation at 10,000 rpm, solid $(NH_4)_2SO_4$ was added to give 40% saturation. After further centrifugation at 10,000 rpm, the precipitate was dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.5) and then centrifuged at 10,000 rpm. Acetic acid (1 M) was added to the supernatant to give a pH of 4.4. After centrifugation at 10,000 rpm, the supernatant and the precipitate were separated.

The precipitate was dissolved in 0.05 M phosphate buffer (pH 8.0) and was then applied to a Sephadex G-200 column (1.5×25 cm) equilibrated with the same buffer. Elution was carried out with the same buffer at a flow rate of 2 ml per h at 4°C. A portion of each fraction was used for detection of hemagglutinating and mitogenic activities, and active fractions (from 27.3 ml to 44.2 ml eluent) were pooled, condensed with a Spectrapor membrane tube (Spectrum Medical Industries, Inc., Calif., U.S.A.), and re-applied to the same column. Elution was carried out with the same buffer at the same flow rate.

The supernatant was lyophilized and dissolved in 0.05 M phosphate buffer (pH 8.0) at a suitable concentration, and then applied to a Sephadex G-100 column (2.0×30 cm) equilibrated with the same buffer. Elution was carried out with the same buffer at a flow rate of 5 ml per h at 4°C.

Disc Electrophoresis—Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (*11*). Experiments were performed with various samples, some of which had been pre-treated with 2-mercaptoethanol. For estimation of molecular weight, the method of Segrest and Jackson (*12*) was followed, using various concentrations of gel from 5% to 20%. As molecular weight standards, bovine serum albumin (BSA), ovalbumin, chymotrypsinogen A, and cytochrome *c* were used. The gels were normally stained for protein with Coomassie brilliant blue and destained with a mixture of 10% acetic acid and 10% isopropanol.

Amino Acid and Sugar Analysis—Samples were hydrolyzed in 6 N HCl in evacuated, sealed tubes at 110°C for 20 h. The amino acid content of the hydrolysates was determined in a Hitachi amino acid analyzer (KLA-3B) according to the method of Spackman *et al.* (*13*). Cysteine and cystine were identified as *S*-sulfocysteine by the method of Liu *et al.* (*14*). Tryptophan was not determined. The total amount of neutral sugars was determined by the orcinol-H_2SO_4 procedure (*15*).

Preparation of Human Lymphocytes—Normal human venous blood was withdrawn into syringes previously treated with heparin. Purified lymphocytes were obtained by the method of Kawaguchi *et al.* (*16*). The cells were then washed with 0.14 M NaCl–0.01 M phosphate buffer (pH 7) and used for examination of mitogenic activity.

Hemagglutination Titration—Hemagglutination titration was carried out with microliter equipment (Cooke Lab. Products, Virginia, U.S.A.). Human erythrocytes were treated with 0.1% trypsin for 1 h at 37°C, washed three times with phosphate-buffered saline and a 2% suspension was used for assay. For the assay, 25 μl aliquots of 2-fold serial dilutions of lectin solution were mixed with an equal volume of erythrocyte suspension and left for 1 h at room temperature. The degree of agglutination was evaluated macroscopically and the liter value was defined as liter/mg protein.

Assay of Mitogenic Activity—Morphological examination of lymphocyte transformation was carried out by determining the percentage of transformed cells in a Giemsa-stained preparation, counting approximately 1,000 cells per mitogen sample.

Assay of [^{3}H]thymidine incorporation was performed by adding [methyl-^{3}H]thymidine (2.0 μCi, The Radiochemical Centre, England) to each tube (1×10^6 lymphocytes/ml, 2 ml) after incubation with the mitogen sample for 48 h. Twenty-four hours after the addition of [methyl-^{3}H]thymidine, the cells were collected and washed three times with cold phosphate-buffered saline (pH 7.2). After adding 1.5 ml of cold 5% TCA and 2 ml of cold methanol to the cell pellet, one drop of 3% bovine serum albumin (BSA) was added as a carrier protein. The precipitate was

washed on a membrane (GF/C 2.5 cm, Whatman) in a "Manifold" multiple sample collector (Millipore). To the dried membranes, 0.3 ml of solubilizer (Soluene TM 100, Packard) was added and the mixture was incubated at 37°C for 2 h, then 5 ml of scintillation fluid (5 g PPO and 0.1 g POPOP in 1,000 ml of toluene) was added. The radioactivity was counted in a Beckman LS-200B liquid scintillation counter.

Immunochemical Method—Immunodiffusion was performed by the Ouchterlony method (*17*), using 1.0% Agarose II (Dojin-do Laboratories, Japan) in 0.05 M phosphate buffer, pH 6.5. Immunoelectrophoresis was performed according to the technique of Hirschfeld (*18*), using 1.4% Agarose II in 0.025 M veronal buffer, pH 8.6.

RESULTS

Purification—Typical yields of protein, hemagglutinating and mitogenic activity in each fraction during the purification process are summarized in Table I.

Extraction: The juice from 1,000 g of *Aloe arborescens* Mill leaves was filtered through gauze then centrifuged, and the clear supernatant fluid ("crude extract") was subjected to ammonium sulfate fractionation. The fractions precipitated by 0–40%, 40–70%, and 70–100% saturation with ammonium sulfate were dissolved in 0.05 M carbonate-bicarbonate buffer (pH 9.5) and dialyzed overnight against the same buffer. Almost 80% of the hemagglutinating activity was recovered in the fraction precipitated at 0–40% saturation. However, since this fraction was not completely dissolved in the above buffer, the insoluble material was removed by centrifugation and the supernatant (AS 0-40), which possessed approximately all the hemagglutinating activity in the fraction, was used for the next step.

Fractionation by pH: A small amount of 1 M acetic acid was added to the AS-40 solution to give a pH of 4.4. Following the change in pH, the clear AS-40 solution become cloudy. The supernatant (acidic sup) and the precipitate (acidic ppt) were separated by centrifugation. Higher hemagglutinating activity was detected in the acidic sup than in the acidic ppt. Mitogenic activity was detected only in the acidic ppt.

Sephadex G-200 and G-100 gel filtration: The acidic ppt was lyophilized, dissolved in 0.05 M phosphate buffer (pH 8.0), and applied to a Sephadex G-200 column. Three fractions, P-1 in the void volume, P-2 and P-3, were obtained as shown in Fig. 1. Hemagglutinating and mitogenic activities were detected only in P-2; these activities were absent in P-1 and P-3. P-2 was re-chromatographed on a Sephadex G-200 and further purified fractions were obtained as shown in Fig. 2. The acidic sup was lyophilized, dissolved in 0.05 M phosphate buffer (pH 8.0), and applied to a Sephadex G-100 column. Two fractions, S-1 and S-2, were obtained as shown in Fig. 3. Hemagglutinating activity was detected in S-1 but not in S-2.

Chemical and Physical Properties—Chemical

TABLE I. Summary of purification of lectins from 1,000 g of aloe leaves. HA, Hemagglutination; n.d., not determined.

Fraction	Total protein (mg)	HA activity[a] (μg/ml)	HA titer (per mg protein)	HA activity recovered (%)	Mitogenic activity[b] (μg/ml)
Juice	n.d.	500	2		n.d.
AS-0-40	320.0	250	4	100	n.d.
Acidic ppt	47.4	250	4	14.8	30.0
P-1	1.05	>1.000	0	0	>100
P-2	2.6	62.5	16	3.3	5.0
Acidic sup	18.4	125	8	11.5	>100
S-1	1.5	15.6	64	7.4	>100

[a] Minimum hemagglutinating dose against human erythrocytes. [b] Mitogenic dose to give 20,000 cpm of [methyl-^{3}H]thymidine incorporation against 1×10^4 lymphocytes.

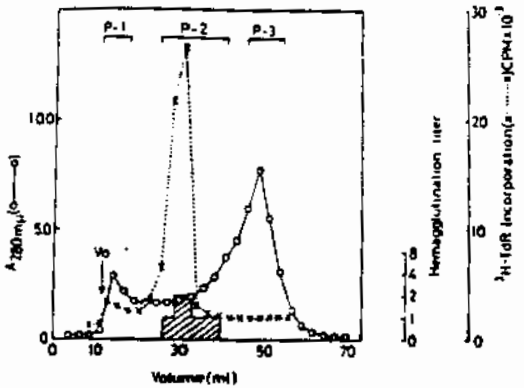

Fig. 1. Sephadex G-200 gel filtration of the acidic ppt. Acidic ppt (20 mg) was dissolved in 0.05 M phosphate buffer (pH 8.0) and applied to a column (1.5 × 25 cm) equilibrated with the same buffer. Elution was carried out with the same buffer and fractions of 1.3 ml were collected at a flow rate of 2 ml/h at 4°C. Hemagglutinating activity is denoted by shading. The elution volume of Blue Dextran is indicated by a vertical arrow (V_o).

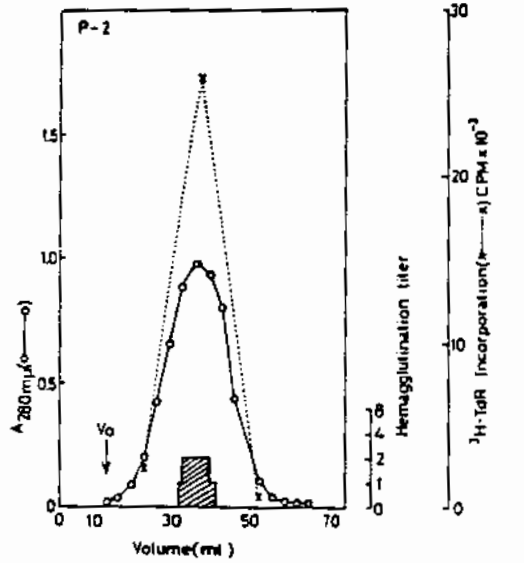

Fig. 2. Further purification of P-2 by Sephadex G-200 gel filtration. Pooled and concentrated P-2 fraction (1.2 mg) obtained from the 1st step of gel filtration (fractions from 27.3 to 44.2 ml in Fig. 1) was applied to a Sephadex G-200 column (1.5 × 25 cm) equilibrated with 0.05 M phosphate buffer (pH 8.0). Fractions of 1.3 ml were collected at a flow rate of 2 ml/h at 4°C. Hemagglutinating activity is denoted by shading. V_o: Void volume.

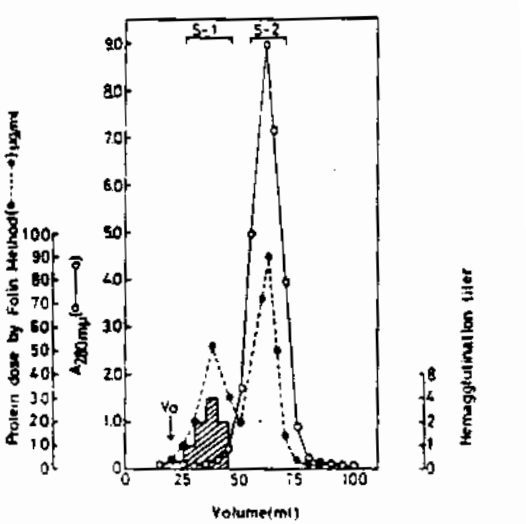

Fig. 3. Sephadex G-100 gel filtration of the acidic sup. Acidic sup (2.4 mg) was dissolved in 4 ml of 0.05 M phosphate buffer (pH 8.0) and applied to a column (2.0 × 30 cm) equilibrated with the same buffer. Elution was carried out with the same buffer and fractions of 2.5 ml were collected at a flow rate of 5 ml/h at 4°C. Hemagglutinating activity is denoted by shading. V_o: Void volume.

analysis: Both P-2 and S-1 were positive in the following qualitative protein and carbohydrate reactions; ninhydrin reaction, xanthprotein reaction, Molisch-Udranszky reaction, anthrone reaction, Fehling reaction, Elson-Morgan reaction, and periodic acid-Schiff reaction. Quantitive analysis showed that P-2 and S-1 contained 18.3% and more than 50% neutral carbohydrate, respectively, suggesting that both P-2 and S-1 were glycoproteins.

SDS-polyacrylamide gel electrophoresis: SDS-polyacrylamide gel electrophoresis in 10% gel of P-1, P-2, and S-1 without 2-mercaptoethanol treatment gave a single band for each fraction, as shown in Fig. 4a, b, and d. P-1 gave an identical single band with or without 2-mercaptoethanol treatment. P-2, after 2-mercaptoethanol treatment, gave two discrete bands, a smaller peptide band (α) and a larger peptide band (β) (Fig. 4c). S-1 also gave a single band (γ) which had a lower molecular weight than untreated S-1 (Fig. 4e). From these results it was concluded that the α and β peptides were subunits of P-2 and the γ peptide was a subunit of S-1.

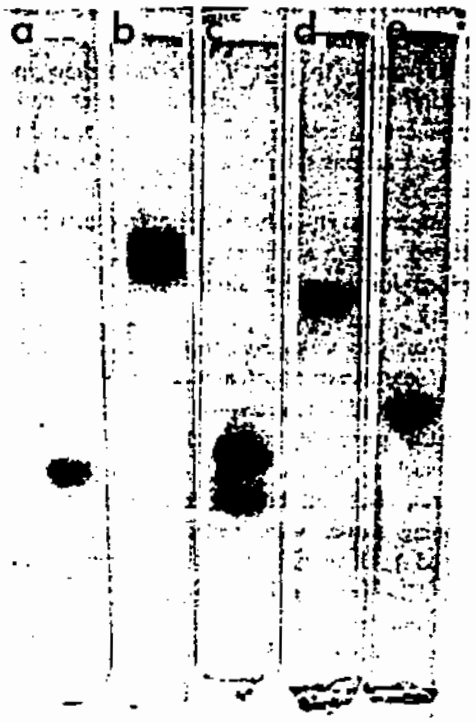

Fig. 4. SDS-polyacrylamide gel electrophoresis of P-1, P-2, and S-1. SDS-polyacrylamide gel electrophoresis was carried out with 10% gel in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS. Samples were heated at 100°C for 5 min in 0.01 M sodium phosphate buffer, pH 7.2, containing 1% SDS, 25% glycerol, and 0.001% bromphenol blue with and without 5% 2-mercaptoethanol. a, P-1 with and without 2-mercaptoethanol treatment (the same result was obtained in each case); b, P-2 without 2-mercaptoethanol treatment; c, P-2 with 2-mercaptoethanol treatment; d, S-1 without 2-mercaptoethanol treatment; e, S-1 with 2-mercaptoethanol treatment.

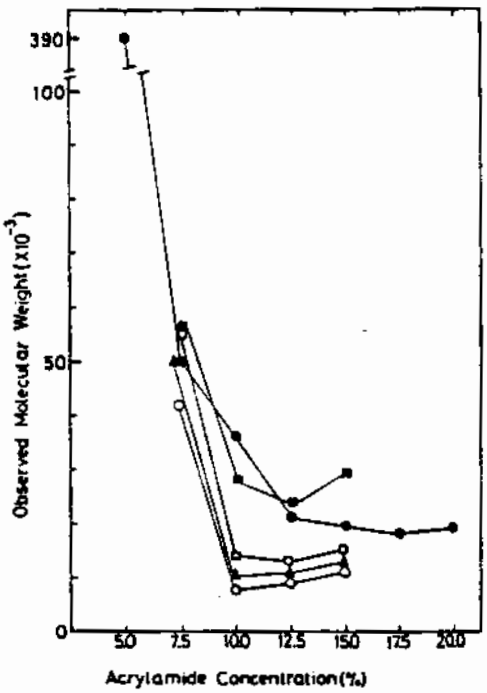

Fig. 5. Estimation of the molecular weights of P-1, P-2, S-1, and their subunits (α, β, and γ) by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out as described in Fig. 4. The mobility of proteins was measured and the relationship between the logarithm of the molecular weights of various marker proteins and their mobilities was plotted. Molecular weight markers (molecular weights shown in parentheses) used were: cytochrome *c* (12,500), chymotrypsinogen A (25,000), ovalbumin (45,000), bovine serum albumin (67,000). α, ○; β, ▲; γ, □; P-2, ●; S-1, ■.

Molecular weight estimation: To obtain the molecular weights of P-2 and S-1, the method of Segrest and Jackson (*12*) was adopted using various concentrations of gel, such as 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, and 20.0%. The results are shown in Fig. 5. An asymptotic minimal molecular weight could be estimated from each curve in Fig. 5. The following values were obtained: 18,000 for P-2 in 17.5% gel, 24,000 for S-1 in 12.5% gel, 7,500 for α in 10% gel, 10,500 for β in 10% gel, and 12,000 for γ in 12.5% gel. These values are more than the real values since we did not correct for total carbohydrate in the glycoprotein molecules.

Amino acid composition: The amino acid compositions of P-2 and S-1 are shown in Table II. In this experiment tryptophan was not determined. Since S-1 contains more than 50% neutral carbohydrate on a weight basis, only the molar ratios were determined in amino acid analysis of S-1. The most notable feature of the amino acid compositions of these proteins is the high proportion of acidic amino acids, such as aspartic acid and glutamic acid, and the low proportion of methionine and histidine.

Biological Properties—Hemagglutinating activity: P-2 and S-1 agglutinated erythrocytes of various species such as human, sheep, and rabbit,

TABLE II. Amino acid compositions of *Aloe arborescens* lectins.

Amino Acid	P-2 (mol/mol of protein[a])	S-1 (molar ratio of amino acid)
Aspartic acid	27.50 (14)[b]	5.00 (5)
Threonine	17.64 (9)	2.59 (3)
Serine	19.76 (10)	2.63 (3)
Glutamic acid	33.36 (17)	4.02 (4)
Proline	15.60 (8)	2.33 (2)
Glycine	30.68 (15)	4.15 (4)
Alanine	19.96 (10)	3.96 (4)
Half-cystine	7.94 (4)	2.15 (2)
Valine	18.16 (9)	2.64 (3)
Methionine	2.48 (1)	0.40 (0)
Isoleucine	14.64 (7)	1.80 (2)
Leucine	25.20 (13)	4.23 (4)
Tyrosine	9.80 (5)	1.60 (2)
Phenylalanine	6.12 (3)	1.06 (1)
Lysine	7.12 (4)	1.06 (1)
Histidine	5.68 (3)	0.69 (1)
Arginine	9.04 (5)	1.07 (1)

[a] Molecular weight of P-2 was taken as 18,000.
[b] Number of residues to the nearest integer.

and did not show A-B-O blood group specificity in hemagglutination tests in the human system. Treatment of human erythrocytes with 0.1% trypsin increased the agglutination by these two purified fractions approximately 5-fold compared with non-treated erythrocytes. The minimum hemagglutinating doses of the purified fractions on trypsin-treated human erythrocytes are shown in Table I. S-1 exhibited strong hemagglutinating activity, and P-2 showed approximately 1/4 of the activity of S-1.

Mitogenic activity: Seventy-two hours after the culture of human lymphocytes with purified P-2 or acidic ppt, large lymphocytes with morphologically transformed shapes were abundant compared to culture systems with purified P-1 or S-1 and without mitogen. When approximately 1,000 cells were counted, about 70% of the lymphocytes were found to be transformed after culture for 72 h. This degree of transformation is almost the same as that caused by phytohemagglutinin-W (PHA-W, Wellcome Co., U.S.A.) which was tested as a positive control.

In order to identify [^{3}H]thymidine incorporation as a function of the mitogen activity, lymphocytes were treated with various quantities of aloe fractions such as acidic ppt, P-1, P-2, S-1, S-2.

As shown in Table I, mitogenic activity was detected in both acidic ppt and P-2. Among the purified fractions P-2 alone displayed mitogenic activity.

Reactivity with serum proteins: The reactivity of the crude extract of aloe against various sera in the gel diffusion test (Ouchterlony test) was reported previously (*19*). Sixteen specimens of sera, such as human, rabbit, goat, dog, cat, horse, pig, rat, fetal rat, bovine, fetal calf, snake, and snapping turtle, were tested, and all of them showed positive reactivity. The extract of aloe reacted not only with mammalian sera but also with sera from fish and amphibia, as well as reptilia. Moreover, more than two precipitin lines were detected in almost all sera tested.

Among purified fractions P-1, P-2, S-1, and S-2, only P-2 reacted with human serum proteins, and two precipitin lines were detected (Fig. 6a). Through immunoelectrophoresis using human serum, α_1-macroglobulin was found to be one of the serum proteins that reacted with P-2 (Fig. 6b).

Activation of serum complement components by P-2: When the C3 component or the C3 proactivator is activated, there is a change in electrophoretic mobility; C3 component (β_1C) moves to the β region while activated C3 component (β_1A) shifts to the α region. C3 proactivator is seen in the β region in agarose electrophoresis but moves to the γ region when it is activated. Figures 6c and 6d show the immunoelectrophoretic mobility of the C3 component and the C3 proactivator of human serum incubated with P-2 at 37°C for 1 h. In the case of human serum treated with P-2, an immunoprecipitin line against rabbit anit-human C3 (β_1C/β_1A) was seen in the β and α regions, whereas in the non-treated human serum the immunoprecipitin line was found only in the β region. A precipitin line of human serum treated with P-2 against rabbit anti-human C3 proactivator was seen in the β and γ regions, whereas the precipitin line of non-treated human serum was found only in the β region.

DISCUSSION

We have described the preparation, purification, chemical and physical properties, and the biological activities of two lectins from *Aloe arborescens* Mill leaves. Not only hemagglutinating activity but also various other biological activities were observed in crude extracts. Purification was carried out using the biological activities, especially hemagglutinating activity, as markers. Two biologically different lectins (P-2 and S-1) were finally separated by ammonium sulfate fractionation, pH-dependent precipitation, and Sephadex-G gel filtration. The purity of each of these two lectins was indicated by the observation of a single band in SDS-polyacrylamide gel electrophoresis. By using the SDS-polyacrylamide gel electrophoresis system, the approximate molecular weights were estimated to be 18,000 for P-2 and 24,000 for S-1. It is proposed that two subunits of P-2, α and β, are linked by a disulfide bound, and that the S-1 molecule is composed of two subunits, each having a molecular weight of approximately 12,000, and linked by a disulfide bond. A more detailed investigation of the chemical properties of P-2 and S-1 is in progress.

A marked difference in biological activities exists between P-2 and S-1. As shown in Table I, S-1 has a stronger hemagglutinating activity than P-2, but no other biological activities.

The observation that glycoproteins inhibit the mitogenic response of both lentil and *Phaseolus vulgaris* (*25*) lectins suggests that both groups of lectins bind to a common structure on the lymphocyte cell surface. It seems likely that a necessary requirement for a lectin to induce mitogenesis is a sugar-binding specificity which enables the lectin to bind to specific glycoproteins on the cell surface of the lymphocyte. Therefore, determination of the sugar-binding specificity of the two lectins, especially that of the mitogenic lectin P-2, is important for their further characterization.

The reactivity of P-2 with serum proteins resembles those of Con A (*30*, *31*) and the lectin from *Wistria floribunda* (*32*). The reaction of P-2 with serum proteins indicates involvement in the binding reaction, as shown by precipitin lines in the immunodiffusion plate (Fig. 6b). As reported by Fujita *et al.* (*19*), two human serum proteins that reacted with crude extract of aloe were identified as α_2-macroglobulin and α_1-antitrypsin by means of an immunodiffusion system. A serum protein other than α_2-macroglobulin that reacted with P-2 in our system is now under investigation.

Activation of complement components of human serum *via* the alternate pathway was observed. It has been reported that the activated C3 component may stimulate B-lymphocytes and enhance the production of antibodies against sheep red blood cells (*26–28*). This biological characteristic, complement activation, has not previously been reported in other lectins, although antitumor substances from bacteria (*29*, *30*) and an antitumor polysaccharide (*31*) can activate the complement system.

Thus, we have isolated two lectins from leaves of aloe, belonging to *Liliaceae*. Most other lectins previously reported were isolated from the seeds of plants. Aloe has been known as a folk medicine for centuries, and in the last few decades its clinical application in skin injury and burn cases has been reported (*32–35*). To determine whether the various biological activities of P-2 purified from aloe leaves are correlated with these clinical effects further characterization of P-2 will be necessary.

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