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Characterization of the Lectin Purified from *Canavalia ensiformis* Shoots

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Abstract Lectin is a cell-agglutinating and carbohydrate-binding protein present in many plants. The lectin of *Canavalia ensiformis* shoot with specific affinity for D-glucose was purified by affinity chromatography using Sephadex G-100, and some of its biochemical characterizations were studied. Lectin was purified 8.87-fold and exhibited final specific activity of 225.74 units/mg protein with a 2.3% yield. SDS-PAGE analysis demonstrated that the purified shoot lectin exists as a tetramer of 102 kD, composed of two subunits with molecular weight of 29 and 22 kD. The purified lectin was observed to agglutinate rabbit blood cell. The optimal temperature for the activity of this lectin was 40°C, and this lectin was relatively stable to heat with the highest activity at 50~60°C. The maximal activity was observed at pH 7.2.

Keywords: *Canavalia ensiformis* shoot, hemagglutination activity, lectin, pH, thermal stability

INTRODUCTION

Lectins are cell-agglutinating and carbohydrate-binding proteins and bind to cell surface glycoconjugates. They are present in many plants, particularly legume seeds, and also in bacteria, slime molds, invertebrates, and animals [1], and are classified into several groups based on their structures [2]. Because of specific binding, they have the capability to serve as recognition molecules within a cell, and between cells or organisms [3]. Binding characteristics of lectins are affected by many other factors, including the presence of ions, ionic strength, and pH [4].

The biological properties of lectins include control of deposition of storage materials, mediation of cell-cell surface interactions, and control of cell differentiation [5]. One of the possible physiological, biochemical, and molecular roles of lectins in plants is their involvement in defense mechanism [6]. Lectins have also emerged as an important class of proteins possessing a wide variety of biochemical applications like their use in bioseparation, symbiosis, transport, storage of carbohydrate, and reversible immobilization [7].

Lectins are found in many different species, organs, and tissues of plants. Seeds are the richest source of lectin, but these agglutinins are also quite often abundant in vegetative organs such as leaves, petioles, rhizomes, bark, and stems [8]. A cell wall lectin has been reported to be present in mung bean hypocotyls [9] and in stems and leaves of horse bean [10]. These lectins vary in molecular weight, blood group and carbohydrate specificity, amino acid composition, metal ion requirement, and three-dimensional structure [11]. Most plant lectins are able to withstand heat, are stable over a wide pH range, and are resistant to animal and insect proteases [6].

The synthesis of lectin is reported to be under developmental regulation and accompanied by an increase in the levels of endogenous and exogenous auxin and cytokinin [12]. Lectins synthesized in plant are transported into the secretory system through the endoplasmic reticulum and subsequently accumulated either in vacuoles or in the cell walls and intercellular spaces at relatively high levels. On the other hand, lectins are broken down to provide amino acids for the seed germination and growth of seedling [13].

In recent studies carried out in authors' laboratory, the purification of lectin from seeds of *Canavalia ensiformis* L. and some of their properties have been reported [14]. To our knowledge, the present study represents detailed purification of the lectin from shoot of *Canavalia ensiformis* L. by affinity chromatography using Sephadex G-100 and its biochemical characterization.

MATERIALS AND METHODS

Germination of Seeds

Seeds of *Canavalia ensiformis* L. were obtained from Bean and World Company in Jinchun, Chungbuk, and stored at 4°C until used. After imbibition for 24 h, seeds were germinated in a mixture of vermiculite, perlite and peat moss (4:1:1) in growth chamber at 26°C under dark condition with a relative humidity of 60% [15,16], subsequently the shoots and roots were cut from 4-week-old seedlings for our experiments.

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Purification of Lectin

Lectins were purified from shoots and roots of *Canavalia ensiformis* L. following a modification of the method of Gupta and Srivastava [17]. Samples were ground to a fine power with a pre-cooled mortar and pestle in liquid nitrogen and stirred overnight in three volumes of 0.01 M sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS) (wt/vol). The extracts were centrifuged at 40,000 × *g* for 30 min. The supernatant was collected, and pellets were resuspended in small volume of PBS, stirred for 6 h and recentrifuged. The 25~50% $(NH_4)_2SO_4$ fractions were collected by centrifugation at 160,000 × *g* for 30 min. Pellets resuspended in 10 mL PBS were dialyzed against double-distilled water for 48 h and then against PBS for 24 h by replacing PBS with the fresh solution at 12-h intervals.

A Sephadex G-100 column (1.6 × 40 cm) was equilibrated with PBS before loading the sample. Once loaded, the column was washed with the same buffer until the absorbance at 280 nm read zero. The bound lectin was eluted with 0.1 M D-glucose (wt/vol) and collected at a rate of 0.2 mL/min. Each fraction was repeatedly tested for hemagglutination activity. The 3-mL fractions with the highest activity were pooled and dialyzed against double-distilled water by replacing the external medium at 12-h intervals. The dialysates were kept at 0°C until used.

Protein assay was carried out by the Bradford [18] method using bovine serum albumin as standard. Absorbance at 595 nm was determined by an ELISA microplate reader (Bio-Rad Model 3550-UV).

All purification processings were done at 4°C except as indicated.

Hemagglutination Activity

Hemagglutination activity was determined by a 2-fold serial dilution method using the microtitrator of Takatsy [19]. Rabbit erythrocyte was prepared by taking 2% cell suspension in 0.01 M PBS (pH 7.2) containing 0.15 M NaCl. Each of the samples (0.025 mL) was serially diluted in PBS (0.025 mL), and a 2% suspension of erythrocyte (0.025 mL) was added to each well of a microtiter plate, and agglutination was monitored after incubating the reaction mixture at 37°C for 1 h. The degree of agglutination was assessed visually and through optical microscope. The reciprocal of the highest dilution of the lectin showing complete agglutination was taken as the hemagglutination titer.

SDS-PAGE and Molecular Weight

SDS-PAGE was performed in 18% polyacrylamide gel at room temperature by the method of Laemmli [20]. The protein samples were denatured in a boiling water for 10 min before loading. The bands were identified by staining with Coomassie brilliant blue R-250 [21], and by subsequent destaining in 7.5% acetic acid.

The molecular weight was determined using the method of Weber and Osborn [22]. The molecular weight markers were rabbit muscle phosphorylase b (97 kD), bovine serum albumin (66 kD), chicken egg white ovalbumin (45 kD), bovine erythrocyte carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20.1 kD).

Effect of Temperature on Lectin Activity

The effect of temperature on lectin activity was studied by varying the incubation temperature from 10 to 70°C. The dialysates containing purified lectin were incubated for 60 min at 10~70°C, respectively, prior to determination of hemagglutination activity using a 2% rabbit erythrocyte suspension.

Thermal Stability

The purified lectin was incubated at different temperatures from 50 to 90°C for 10 min. After having been cooled immediately in ice water bath, the hemagglutination activity was determined by a serial 2-fold dilution method at 40°C.

Effect of pH on Lectin Activity

The effect of pH on lectin activity was studied in pH range of 2.0~10.0. The purified lectin was preincubated in buffers with different pH for 4 h at 4°C, and the hemagglutination activity was determined by a serial 2-fold dilution method. The different buffers employed were 0.025 M glycine-HCl buffer (pH 2.2), 0.2 M acetate buffer (pH 3.2, 4.2), 0.01 M phosphate buffer (pH 6.2, 7.2), 0.2 M Tris-HCl buffer (pH 8.0, 9.1), and 0.2 M carbonate-bicarbonate buffer (pH 10.0).

RESULTS AND DISCUSSION

Previous study on *Canavalia ensiformis* seed lectin showed that the purified lectin could agglutinate only rabbit erythrocyte [13]. These results were in agreement with those obtained from lectin of *Neoregelia flandria* leaves [23] and *Polygonatum multiflorum* [24], which showed that trypsinized rabbit erythrocyte were agglutinated but human erythrocytes were not. Therefore, in the present work, we determined to study the hemagglutination activity of lectin by a 2-fold serial dilution method using the rabbit erythrocyte. Lectins from some plants agglutinate only specific types of erythrocytes. Shahnaz *et al.* [25] demonstrated the specificity of a rabbit antibody directed against seed soybean agglutinin. Higuchi and Iwai [26] reported that winged bean seed lectin agglutinate trypsinized and untreated rabbit erythrocytes and human erythrocytes of the ABO system, except type O blood. However, the seed lectin of *Artocarpus incise* L. [27] and *Dioclea reflexa* [28] were reported to agglutinate all human erythrocytes. A lectin from tulip bulbs was found to agglutinate only mouse and rat erythrocytes [29].

Purification of Lectin

Lectin from shoot and root of *Canavalia ensiformis* L.

Table 1. Purification of lectin from shoot of *Canavalia ensiformis* L. seedling

Purification step	Total protein (mg)	HA* (units)	Specific activity (units/mg)	Purification (fold)	Recovery (%)
Crude extract	522.4	13,295	25.45	1	100
$(NH_4)_2SO_4$	304.2	9,216	30.30	1.19	69.3
Sephadex G-100	1.36	307	225.74	8.87	2.3

*HA: hemagglutination activity

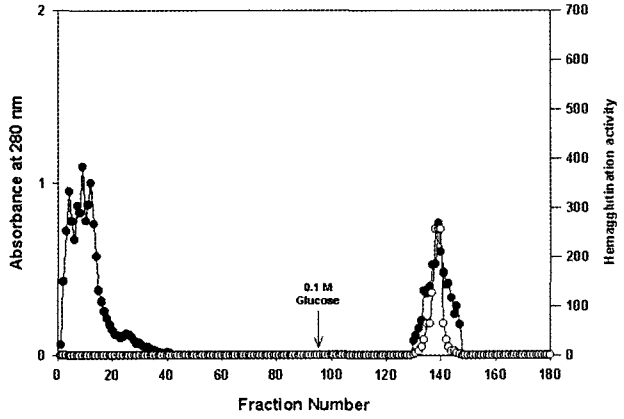

Fig. 1. Elution profile for protein (●) and hemagglutination activity of lectin (○) purified from shoot of *Canavalia ensiformis* L. by affinity chromatography on Sephadex G-100. The bound lectin was eluted with 0.1 M D-glucose. Allow indicates the start of elution with 0.1 M D-glucose. Hemagglutination activity was determined using the rabbit erythrocyte.

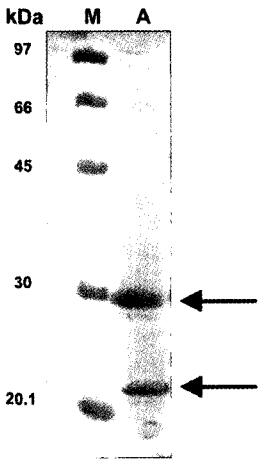

Fig. 2. 18% SDS-PAGE of lectin purified from shoot of *Canavalia ensiformis* L. The gels were run at 50 mA for 11 h and stained with Coomassie brilliant blue R-250, and then destained by 7.5% acetic acid. Arrows indicate lectin band purified by affinity chromatography on Sephadex G-100. Lane: M, molecular weight markers; A, purified lectin. The molecular weight markers were rabbit muscle phosphorylase b (97 kD), bovine serum albumin (66 kD), chicken egg white ovalbumin (45 kD), bovine erythrocyte carbonic anhydrase (30 kD), and soybean trypsin inhibitor (20.1 kD).

was purified through 0.15 M NaCl extraction, ammonium sulfate precipitation, and Sephadex G-100 affinity chromatography. The degree and yield of purification of lectin present in shoot are summarized in Table 1. The purification of crude extract that was affected by the ammonium sulfate precipitation showed that most of activity was preserved in the precipitate. 13,295 units of hemagglutination activity in crude extract step decreased to 9,216 units with 69.3% recovery in ammonium sulfate step. The specific activity increased from 25.45 units/mg in crude extract to 225.74 units/mg in Sephadex G-100. The final affinity chromatography step resulted in 8.87 folds purification with 2.3% of recovery yield.

Elution profile for protein and hemagglutination activity of shoot lectin by affinity chromatography on Sephadex G-100 is shown in Fig. 1. The bound lectin was eluted with 0.1 M D-glucose. A sharp distinctive peak of the highest activity, which fits with only one protein peak, was obtained. This peak fraction was used for our further studies.

Hemagglutination activity of lectin was detected in shoot, but not in root. This result provides evidence that lectin is not simply a reserve substance but plays an important role in the growth and development of shoots [30]. Therefore, because of its presence in shoot only, we studied its biochemical characterization in the shoot. Similarly, Raikhel *et al.* [31] reported that the lectin was detected in the shoot of wheat seedlings. The hemagglutination activity could not be detected in the root of *Zizyphus mauritiana* [17]. However, in contrast to our result, there are some reports on the presence of hemagglutination activity in the lectins isolated from roots. Grade *et al.* [32] isolated and characterized a lectin from soybean seeds and roots. As reported by Kato *et al.* [33] and Dazzo and Truchet [34], lectin was present in young roots of pea and clover, and was most abundant in the region of developing root hairs. Lectin was also reported to be present in root of adult wheat plants [35]. The distribution of lectin in root of winged bean [36] and soybean [25] has been measured by hemagglutination activity.

Molecular Weight

Typically, lectins are glycoproteins consisting of subunits with molecular mass in a range of 25 to 35 kD arranged as dimers or tetramers [37]. The final preparation by affinity chromatography was examined by SDS-PAGE, which showed the presence of double protein band as shown in Fig. 2. The molecular weight of the lectin purified from shoot was calculated to be 29 and 22 kD by

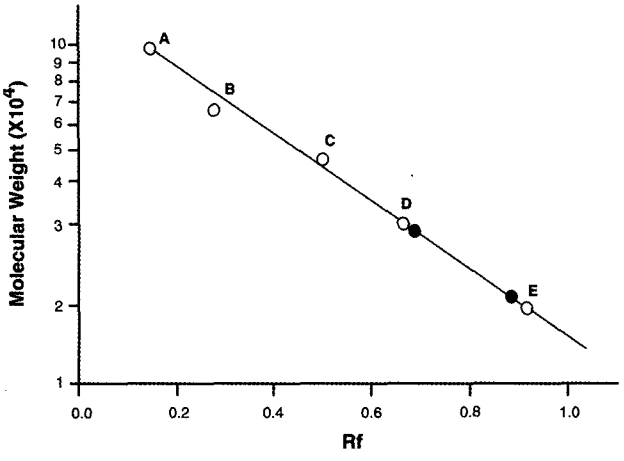

Fig. 3. Determination of molecular weight of lectin purified from shoot of *Canavalia ensiformis* L. by SDS-PAGE. Closed black circles (●) indicate lectin purified by affinity chromatography on Sephadex G-100. The molecular weight markers (○) were rabbit muscle phosphorylase b (A, 97 kD), bovine serum albumin (B, 66 kD), chicken egg white ovalbumin (C, 45 kD), bovine erythrocyte carbonic anhydrase (D, 30 kD), and soybean trypsin inhibitor (E, 20.1 kD).

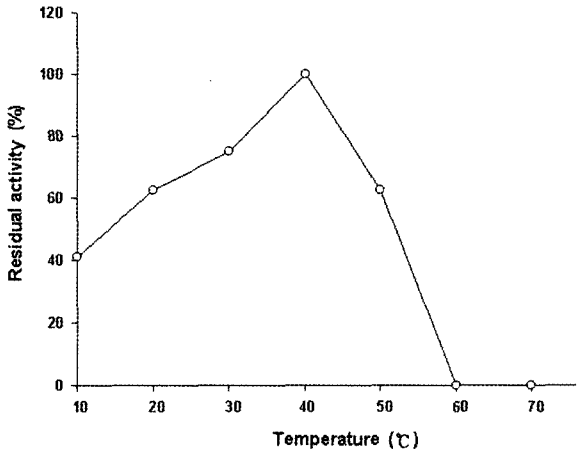

Fig. 4. Effect of temperature on the activity of lectin purified from shoot of *Canavalia ensiformis* L. The lectin activity was tested by incubation at 10~70°C, respectively.

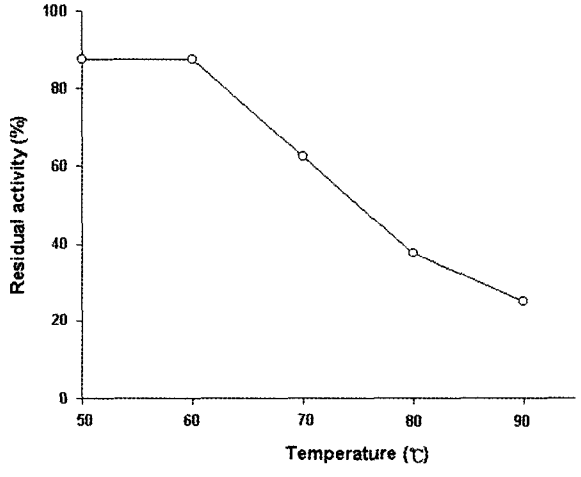

Fig. 5. The thermal stability of lectin purified from shoot of *Canavalia ensiformis* L. Purified lectin was preheated at 50~90°C for 10 min, respectively.

comparing the relative mobilities with those of the standard proteins (Fig. 3). The pattern of polypeptides in shoot was greatly was consistent with that in seed [14].

However, in contrast to our results, Grade *et al.* [32] reported that seed lectin and the root-specific lectin of soybean had molecular weight of 28 to 30 kD and 32 to 33 kD, respectively. The lectin of *Dioclea altissima* seed [38] and *Talisia esculenta* seed [39] had three protein bands with molecular weight of 26, 15, 9 kD and two bands with 20, 40 kD, respectively. Analysis of nectar from leek flowers by SDS-PAGE revealed the presence of two major polypeptide bands of 50 and 13 kD [40]. The molecular weight of lectin from winged bean seed was 53 kD as assessed by gel filtration, and SDS-PAGE showed a single component of 27 kD, suggesting that the lectin was a dimer [26].

Two polypeptides, with molecular weight of 32 and 30 kD, and 34.5 and 29 kD, have been reported during the electrophoretical detection of lectin from leaves of Korean *Viscum album* [41] and European *Viscum album* [42], respectively.

In the present study, only a single band of 102 kD was detected by native PAGE analysis (data not shown). Therefore, we confirmed that shoot lectin was a tetramer with molecular weight of 102 kD composed of two subunits with 29 and 22 kD. These results are in agreement with those for seed lectin, which revealed a tetrameric structure [14]. Unlike, the native molecular weight of *Trichosanthes anguina* seed [43] and *Kalanchoe crenata* leaves lectin [44] was 45 kD and 44 kD as monomer, respectively. Also, the molecular weight of 44 kD as monomer in the native lectin was similar to those of *Tetracarpidium conophorum* [45], *Dioclea reflexa* [46], and *Artocarpus incise* [27]. These different results suggest that the molecular weight of lectin differs greatly depending on species and parts of plants.

Effect of Temperature on Lectin Activity

The effect of temperature on the purified shoot lectin was examined in the range from 10 to 70°C. The hemagglutination activity of lectin in PBS (pH 7.2) was stable at 20~50°C, and the highest activity was recorded at 40°C. Above 50°C the activity was lowered rapidly and was completely lost at 60°C (Fig. 4). Although the pattern of activity in a stable range (20~50°C) was different, similar result was observed in a study conducted on *Canavalia ensiformis* seed lectin [13]. However, in contrast to our result, the lectin of *Dioclea altissima* seed was submitted at temperatures between 78 and 80°C [38]. Gupta and Srivastava [17] reported that the activity of lectin from

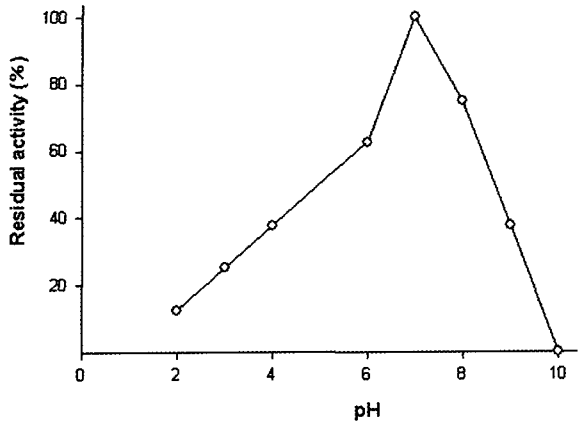

Fig. 6. The pH stability of lectin purified from shoot of *Canavalia ensiformis* L. Purified lectin was incubated at different pH for 4 h at 4°C.

seeds of *Zizyphus mauritiana* was completely lost at 80~100°C.

Thermal Stability

The heat stability of the purified shoot lectin was examined by heating at various temperatures for 10 min. The highest activity of lectin was in the range from 50 to 60°C. Residual activity greater than 50% was only achieved below 70°C, but above 80°C the activity was decreased rapidly to 20% (Fig. 5). Therefore, in the present study it was confirmed that shoot lectin is a relatively heat stable protein. This result was in agreement with previous study on *Canavalia ensiformis* seed lectin [14]. The bark lectin of the rubber tree was also heat-stable up to 60°C [47]. The activity of *Viscum album* lectin was unchanged by warming to 50°C for a short time, but heating to about 60°C destroyed the agglutinating activity [48]. The hemagglutinating activity of the basic lectin from winged bean seed was stable at 70°C [26]. The heat stability of *Ptilota filicina* lectin [49] and *Kalanchoe crenata* leaves lectin [44] was up to 50 and 90°C, respectively.

Effect of pH on Lectin Activity

The activity of lectin depends on pH also. A higher activity was recorded at pH 7.2, whereas the activity below pH 4 and above 9 was substantially reduced to half of its activity. However, the activity was completely lost at pH 10 (Fig. 6). It is possible that changes in the ionization state with an increase in pH may lead to a weaker binding of the metal ions, which are apparently required for the maintenance of the structure that is required for maximal activity. It is also possible that the increase in OH (ions can bring about a change in the ionization state of the lectin, thereby affecting the binding forces between the lectin and the erythrocyte membrane, which can eventually led to a loss of activity [44]. A similar pattern of pH value was also observed in the case of *Canavalia ensiformis* seed [14]. The maximum activity at pH 7.2 corresponded to that found in *Parkia javanica* bean lectin [50]. Allen and Johnson [51] found that the hemagglutinating activity of *Sophora japonica* lectin was optimum at pH 8.5 and above. The basic lectin purified from winged bean was stable in the pH range from 1.8 to 10.0 [26]. The lectin of *Ptilota filicina* was stable in the range from 4 to 9, retaining 50% of activity at pH 3 and 10, and 25% of its activity at pH 11 and 12 [49]. When incubated at different pH, *Zizyphus mauritiana* lectin was structurally as well as functionally stable only between pH 7.0~7.5. It lost most of its activity below pH 6.0 and above pH 8.0 [17]. Although the optimal pH range was much more restricted, the pH sensitivity of peanut root lectin also lied in the same range [52].

CONCLUSION

In continuation of the study on identification of lectins in *Canavalia ensiformis* L., we have purified the lectin from shoot of *Canavalia ensiformis* L., and studied its partial biochemical characterization. Based on these studies collectively, it is strongly suggest that not only lectins from the two sources (seed and shoot) exhibited similar properties to the seed lectin, but also shoot lectin was originated from the seed stored lectin.

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