Purification and Partial Characterization of a Lectin from the Fresh Leaves of *Kalanchoe crenata* (Andr.) Haw

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A haemagglutinating protein from the saline extracts of *Kalanchoe crenata* leaves, which agglutinate all human blood types, was purified to homogeneity by ion-exchange chromatography on a DEAE-Cellulose column followed by gel filtration on a Sephadex G-100 column. The purified protein showed one band, both in non-denaturing PAGE and SDS-PAGE. The M_r that was determined by SDS-PAGE was 44,000 Da and that estimated from gel filtration was 47,000. Treatment of the haemagglutinating protein with 5 mM EDTA diminished the haemagglutinating activity to 50% of the original level. The addition of divalent cations, 10 mM Mg^{2+}, 10 mM Mn^{2+}, or 10 mM Ba^{2+}, totally restored and enhanced the activity. The protein showed maximum activity over the 3-7 pH range and was heat-resistant. It was also a glycoprotein containing about 1.5% carbohydrate.

Introduction

Lectins are carbohydrate-binding proteins that are widely distributed in the plant kingdom. This class of proteins has been isolated from many plants and animals. Their characteristics have also been extensively exploited in many aspects of biochemistry and biomedicine (Pusztai, 1993). In plants, leguminous seeds are a particularly rich source of lectins, while only a few have been isolated from other families (Dong *et al.*, 1993; Cavada *et al.*, 1998).

The main properties of lectins are based on their ability to interact with carbohydrates and thus combine with glycocomponents on the cell surface, leading to their biological properties (Goldstein and Poretz, 1986). Lectins are able to tightly bind to and cause the precipitation of specific polysaccharides and glycoproteins because they are polyvalent (i.e. each lectin molecule has at least two carbohydrate binding sites to allow cross-linking between the cells or between the sugars containing the macromolecules). These lectins vary, however, in molecular size, amino acid composition, metal ion requirements, and three-dimensional structure (Sharon, 1993).

Most of the lectins that were examined contained metal ions, Mn^{2+} and/or Ca^{2+}. In some cases, evidence has been presented for the requirement of the metal ions for activity. Interaction with carbohydrates by these molecules requires tightly bound Ca^{2+} and Mn^{2+} (or another transition metal ion). These metal ions are located close to the carbohydrate-binding site and are identically positioned. Thus, metal ions serve to maintain the integrity of the subunits of the lectins, and in addition, help to position amino acid residues for carbohydrate binding (Lis and Sharon, 1989).

The isolation and characterization of non-leguminous lectins is of importance in order to study the minor structural differences that can lead to dramatic differences in biological properties and also to investigate the ubiquitous nature of the lectins. The isolation and partial characterization of a lectin from the leaves of *Kalanchoe crenata* is described here. The plant is from the Saxifragaceae family and Crassulaceae tribe that are widely found in temperate and tropical regions. In southwestern Nigeria, the juice from the leaves is traditionally used to treat a wide range of ailments. These include earaches, headaches, general debilities, convulsions, stiff joints, and rheumatism. The juice, in addition, is also employed as a general antidote to poison (Dalziel, 1955).

Materials and Methods

K. crenata leaves were obtained from the surroundings of the Biological Sciences Building of the Obafemi Awolowo University, Ile-Ife, and were taxonomically identified in the Herbarium of the Department of Botany of the University. Human blood cells from the

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Table 1. Summary of purification procedures

Fraction	Protein (mg)	Total activity	Specific activity	Yield (%)
Crude extract	4839	16.4	3.38	100
DEAE-Cellulose	109.3	0.5	4.69	3.13
Sephadex G-100	7.33	0.06	8.73	0.3

ABO system were obtained from healthy donors in the Department of Biochemistry, Obafemi Awolowo University, Ile-Ife. Acrylamide, Methylenebisacrylamide, DEAE-Cellulose, and all of the sugars were products of Sigma (St. Louis, USA). The Sephadex G-100 and molecular weight markers were from Pharmacia (Uppsala, Sweden). All of the other reagents were of analytical grade.

Lectin extraction *K. crenata* leaves were washed and sliced into small pieces. Then 100 g of the sliced leaves were homogenized in a blender with 150 ml of phosphate buffered saline (PBS), pH 7.2, with and without ε-amino-n-caproic acid. The solution was collected, filtered, and stored at −20ºC.

Protein concentration The protein concentration was determined by the method described by Gornall *et al.* (1949) using bovine serum albumin (BSA) as the standard.

Haemagglutinating activity Agglutination of the red blood cells by the crude extract and various fractions that were obtained during purification was estimated as described before by Bing *et al.* (1967). In a U-shaped microtitre plate arranged in rows of wells, 1 : 2 dilutions of the sample in PBS buffer were mixed with 50 μl of 2% suspension of erythrocytes. The plate was left undisturbed for 30 min to 1 h at room temperature in order to allow for agglutination of the erythrocytes to take place. The titre of the lectin was expressed as the reciprocal of the highest dilution showing visible agglutination of erythrocytes. Specific activity was expressed as haemagglutination units (HU)/mg or as the minimum concentration of protein (μg/ml) still giving activity (Table 1).

Blood group specificity The blood group specificity of the extract was established using erythrocytes from different blood groups of the ABO system.

Sugar specificity The sugar specificity of the lectin was investigated by the ability of a series of simple sugars to inhibit the agglutination of human erythrocytes. Two-fold dilutions of sugar (0.2 M initial concentration) solutions in PBS were mixed with 0.1 ml of the lectin solution (3.2 mg/ml) and allowed to react for 30 min at room temperature. An erythrocyte suspension (0.05 ml) was then added and the mixture left for another 30 min. The haemagglutinating titres that were obtained were compared with a non-sugar containing blank. In this study, the following sugars were used: D-glucose, D-galactose, D-melibiose, cellobiose, D-arabinose, Lactose, Trehalose, L-sorbose, D-xylose, D-glucosamine, 2-deoxy-D-glucose, and α-methyl-D-glucopyranoside.

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis in the absence and presence of SDS was performed on 10% gels on either the rod or slab apparatus in a Tris-glycine buffer, pH 8.9, according to the Pharmacia manual. The proteins were stained with Coomassie Brilliant Blue R, while the presence of covalently bound sugar in the lectin was detected by staining the gels with Periodic Acid Schiff reagent (PAS staining), as described in the Pharmacia Manual (Polyacrylamide Gel Electrophoresis Laboratory Techniques, revised edition, 1983).

Molecular weight determination The apparent molecular weight of the lectin was estimated under non-denaturing conditions by gel filtration on a Sephadex G-100 column (2.5 × 100 cm) using the following protein markers: α-chymotrypsinogen A (M_r 25,000, 3 mg/ml), Bovine serum albumin (M_r 66,000, 3 mg/ml), Ovine albumin (M_r 45,000, 3 mg/ml), and Thermolysin (M_r 37,500, 3 mg/ml). One ml of each standard protein was applied to the column and ran separately using a 10 mM phosphate buffer, pH 7.0 as eluant at a flow rate of 8.4 ml/h. Fractions of 2 ml were collected and the elution was monitored for each protein at 280 nm. The void volume (V_o) of the column was determined using Blue Dextran (its elution being monitored at 620 nm).

Estimation of the subunit molecular weight of the purified lectin was determined by SDS-polyacrylamide gel electrophoresis using the following protein markers: carbonic anhydrase (29,000), α-lactalbumin (14,000), glyceraldehyde-5-phosphate dehydrogenase (36,000), egg albumin (45,000), and bovine serum albumin (66,000).

Effect of EDTA and divalent cations The effect of EDTA and divalent cations on the haemagglutinating activity of the protein was carried out according to the method of Sampaio *et al.* (1998). Two-fold serial dilutions of lectin that were prepared in 0.2 M PBS alone and 0.2 M PBS containing 5 mM EDTA were carried out. Human erythrocytes (4%) in 0.2 M PBS with 5 mM EDTA were used as the control. Equal volumes (50 μl) of 10 mM $MgSO_4$, $MnCl_2$, or $BaCl_2$ were later added to the haemagglutination assay that was performed in the presence of EDTA in order to evaluate their capacity to restore haemagglutination.

Effect of temperature on haemagglutinating activity The effect of temperature on the haemagglutinating activity was monitored, as described by Sampaio *et al.* (1998). Aliquots of lectin were incubated at different temperatures (30-90ºC). The heated solution was rapidly cooled in ice and assayed for agglutinating activity. Agglutinating activity of the control that was kept at 20ºC for 30 min was used as a reference.

Effect of pH on haemagglutinating activity The effect of pH on the haemagglutinating activity was determined by carrying out the haemagglutinating assay of the lectin using the following buffers at different pH values; 0.2 M glycine-NaOH buffer, pH 10-12; 0.2 M citrate-phosphate buffer, pH 3-7, and 0.2 M borate buffer, pH 7.5-
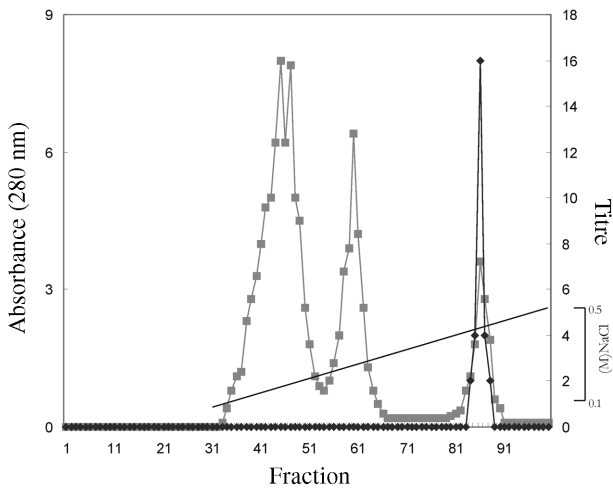
Fig. 1. DEAE-cellulose ion exchange chromatography of *K. crenata* extract. Eluant was PBS, pH 7.2, followed by a linear 0.1-0.5 M NaCl gradient. Column size was 1.5×35 cm; Flow rate was 24 ml/h while the fraction size was 2 ml. (■) Absorbance at 280 nm, (◆) Agglutination titre, (—) NaCl gradient.

9.5. The control values were the agglutination titre of the lectin in PBS, pH 7.2.

Results and Discussion

Isolation of the lectin   The isolation and purification of the lectin from the crude extract of the leaves was obtained by a two-step procedure. This involved an initial ion-exchange chromatography of the crude extract on DEAE-cellulose, carried out according to the method of Knight (1967) using a linear 0.1-0.5 M NaCl gradient (Fig. 1). The protein fractions, with the activity combined, constituted no more than 10% of the extractable soluble leaf proteins. In the second step, the fractions with the haemagglutinating activity from the first step were subjected to gel filtration on a Sephadex G-100 column (Fig. 2). The single peak that was obtained from the gel filtration contained all of the haemagglutinating activity and was a homogenous preparation, as indicated by the polyacrylamide gel electrophoresis (figure not shown). Table 1 summarizes the purification scheme for *K. crenata* lectin.

Properties of *K. crenata* lectin   The phosphate-buffered saline (PBS) extract of the *K. crenata* leaves agglutinate the human red blood cells of the ABO system at approximately the same dilution (non-specifically and fairly strongly). The *K. crenata* lectin may then be classified into the category of lectins that agglutinate erythrocytes of all human blood groups alike, which are usually referred to as non-specific lectins (Watkins *et al.*, 1981). Moreira and co-workers (1998) found that the lectin from *Artocarpus incisa* L. agglutinates human erythrocytes of the ABO system. Also, PBS extracts of the seeds of *Dioclea reflexa* non-specifically agglutinate the
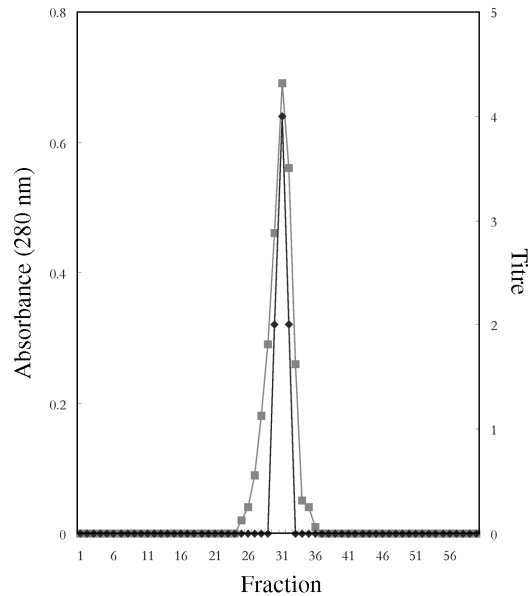
Fig. 2. Gel filtration of the active fractions from the DEAE-cellulose column on Sephadex G-100 column. Eluant was PBS, pH 7.2. The flow rate was 20 ml/h and the fraction size was 2 ml. (■) Absorbance at 280 nm; (◆) Agglutination titre.

erythrocytes of human blood groups (Kuku *et al.*, 2000), among all others.

Of the twelve sugars that were tested for their inhibitory activity, only melibiose, 6-α-D-galactosyl-D-glucose, showed inhibitory activity (Table 2). Therefore, it is relevant to observe that melibiose has been employed as a ligand in agarose-based affinity matrices for the purification of both *Bandeiraea simplicifolia* (BS-1) lectin (Hayes and Goldstein, 1974) and *Arachis hypogea* agglutinin (Lotan *et.al.,* 1974) The latter lectin, as well as *Agaricus bisporus* lectin, shows specificity for the disaccharide β-D-gal $(1 \rightarrow 3)$-D-galNAc (Presant and Kornfeld, 1972). There is, however, the enigmatic enhancement of the haemagglutinating activity by both arabinose and the disaccharide, cellobiose.

The incubation of *K. crenata* lectin with 5 mM EDTA significantly decreased the haemagglutinating activity. The addition of divalent cations (such as Mg^{2+}, Mn^{2+}, or Ba^{2+} to inhibited lectin aliquots) completely restored the activity (Table 3). Although no direct determination of the presence, identity, and amount of divalent metal ion in the native protein has been carried out, these results suggest that the lectin requires divalent ions for activity.

Molecular weight   The M_r of purified *K. crenata* lectin that was determined by gel filtration was 47,000 Da. SDS-PAGE showed a protein band with M_r 44,000 Da. It would appear, therefore, that the native protein is monomeric in a manner that is similar to that observed for the lectins from *A. bisporus* (Presant and Kornfeld, 1972) and *Tetracarpidium conophorum* (Togun *et al.*, 1994) and *Dioclea reflexa* (Adeyemi *et al.*, 1994). The molecular weight of the native

Table 2. Sugar inhibition of haemagglutination by *Kalanchoe crenata* extract

Sugar	Titre	Minimum inhibitory Concentration
PBS control	2^{12}	
D-Glucose	2^{12}	No inhibition
D-Galactose	2^{12}	No inhibition
D-Melibiose	2^{2}	100 mM
Cellobiose	2^{16}	Enhancement
Arabinose	2^{15}	Enhancement
D-Lactose	2^{12}	No inhibition
Trehalose	2^{12}	No inhibition
L(-) Sorbose	2^{12}	No inhibition
Xylose	2^{12}	No inhibition
D(+) Glucosamine-hydrochloride	2^{12}	No inhibition
2-deoxy-D-Glucose	2^{12}	No inhibition
α-methyl-D-glucopyranoside	2^{12}	No inhibition

Each experiment consisted of 100 μl of serially diluted lectin in U-shaped microtitre wells. To each well was added 50 μl of 0.2 M sugar solution in PBS and 50 μl of 4% suspension of type B red blood cells.

lectin is similar to those of *Trichosanthes anguina* seeds, 45,000 Da (Anuradha and Bhide, 1999) *Artocarpus integrifolia,* 46,000 Da, and *Maclura pomifera*, 44,000 Da (Moreira *et al.*, 1998).

Carbohydrate content A sugar analysis revealed that *K. crenata* lectin is a glycoprotein since it stained purplish pink with Schiffs reagent after PAGE. An application of the modified anthrone reaction method of Jermyn (1975) further showed that there are approximately three molecules of sugar residues that are covalently bound to a molecule of the protein.

Molecular stability The lectin is remarkably stable in the pH range of 2-7.5. The activity, however, falls off fairly rapidly thereafter, with essentially all activity lost after pH 9.2 (Fig. 3). It is possible that changes in the ionisation 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 (*vide supra*). The pH dependence, which is observed in virtually all enzyme reactions, is a consequence of the protein composition. Numerous ionisable groups at the surface of the protein molecule and the active center are capable of reacting with H^+ or OH^-. Any pH change is therefore associated with a change in the ionization state of the molecule, which in turn, determines the binding forces between enzyme and substrate (Adolph and Lorenz, 1982). It is also possible that the increase in OH^- ions caused a change in the ionization state of the lectin, thereby affecting the binding forces between the lectin and the erythrocyte membrane that eventually led to a

Table 3. Effect of EDTA and divalent cations on haemagglutinating activity

Experiment	Titre
PBS control	2^{6}
PBS containing 5 mM EDTA	2^{3}
PBS+5 mM EDTA+10 mM MgSO$_4$	2^{8}
PBS+5 mM EDTA+10 mM MnSO$_4$	2^{7}
PBS+5 mM EDTA+10 mM BaCl$_2$	2^{8}
PBS+5 mM EDTA+10 mM MnCl$_2$	2^{7}

Each experiment consisted of (i) 0.1 ml of lectin serially diluted with PBS and 50 μl of 4% suspension of type O red blood cells, (ii) 0.1 ml of lectin serially diluted with PBS containing 5 mM EDTA and 50 μl of 4% suspension of type O red blood cells, and (iii) 0.1 ml of lectin serially diluted with PBS containing 5 mM EDTA, 10 mM of divalent cation, and 50 μl of 4% suspension of type O red blood cells.
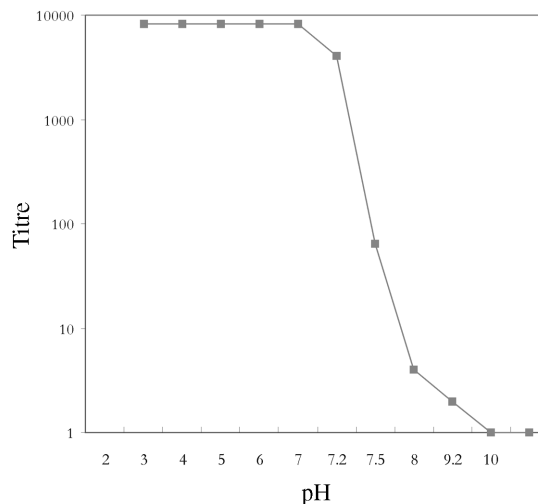
Fig. 3. Effect of pH on haemagglutinating activity. The buffers used were 0.2 M citrate-phosphate pH 3-7.2, 0.2 M borate pH 7.5-9.5, 0.2 M glycine-NaOH pH 10-12.

loss of activity. The lectin of *P. filicina* was also stable in the pH range of 4-9, retaining 50% of its activity at pH 3 and 10, and 25% of its activity at pH 11-12 (Sampaio *et al.*, 1998). The lectin from *Parkia javanica* beans also depended on pH. The optimal pH value was 7. It was stable in the pH range of 7-10. Also, more acidic or basic pHs decreased both the stability and activity (Utarabhand and Akkayanont, 1995).

In addition, *K. crenata* lectin is heat-stable up to 90°C. As reported, the haemagglutinating activity of *Hevea brasiliensis* lectin (Wittsuwannakul *et al.*, 1998) was heat-stable up to 60°C and *P. filicina* up to 50°C (Sampaio *et al.*, 1998).

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