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Full Length Research Paper

Purification of a mannose/glucose-specific lectin with antifungal activity from pepper seeds (*Capsicum annuum*)

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A lectin was isolated and purified from the pepper seeds *Capsicum annuum.* The purification procedure involved anionic exchange chromatography on DEAE – Cellulose and QAE – Sephadex columns followed by gel filtration on Sephadex G–100. The heamagglutinating activity of the lectin towards human erythrocytes was sensitive to inhibition by D-mannose and D-glucose; and enhancement by $CaCl_2$ and $MgCl_2$. The lectin activity was enhanced at very high acidic pH, inhibited at high basic pH but stable at physiological pH range of 6- 8. The lectin was heat stable up to 30°C. SDS-PAGE of the purified lectin revealed the presence of single band of 25 kDa. The lectin was capable of inhibiting the germination of spores and hyphal growth in the fungi *Aspergillus niger, Aspergillus flavus, Fuscarium solani* and *Fuscarium graminearum.*

Key words: Lectin, pepper, seeds, antifungal, glucose, mannose.

INTRODUCTION

Lectins are proteins that bind mono- and oligosaccharides specifically and reversibly but are devoid of catalytic activity (i.e. are not enzymes) and, in contrast to antibodies, are not products of an immune response (Sharon and Lis, 1989; Rudiger and Gabius, 2001). They are widely distributed in nature and can be found in almost all living organisms including plants, animals (vertebrates and invertebrates), algae, fungi, microorganisms and viruses (Goldstein, 1978; Loris, 2002). Most lectins that have been extensively studied are from plants and have been isolated from several parts such as the seeds, barks, leaves, pollen grains, roots, shoots etc. Over 500 different plant lectins have been isolated and characterrized and these lectins form a heterogeneous group of proteins due to the differences in structure, specificity and biological activities. Although their exact biological roles remain elusive, in many instances, lectins from plants and animals have been extensively exploited as biochemical tools in biotechnology and biomedical research (Van Driessche, 1988). In the last decade, substantial progress has been made on the understanding of the roles of plant lectins; until recently, the role of most lectins was associated with their binding to foreign glycans in either recognition and/or defense-related phenomena (Petersen *et al*., 2006; Sahly et al., 2008). Biochemical and molecular studies of numerous lectins demonstrated that only a limited number of carbohydrate-binding motifs evolved in plants and since the specificity of these binding motifs is primarily directed against foreign glycans, it is generally accepted now that many plant lectins are involved in the recognition and binding of glycans from foreign organisms, and accordingly play a role in plant defense (Pneumans and van Damme, 1995; Chen, 2008). Animal and insect studies with purified lectins and experiments with transgenic plants suggested that at least some lectins enhance the plant's resistance against herbivorous animals or phytophagous invertebrates (Carlini and Grossidesa, 2002). The ability of lectins to recognize specific carbohydrates makes them valuable tools for taxonomic studies and the isolation and purification of glycoconjugates (Van Leuven et al., 1993). Lectins extracted from plants may also function as lymphocyte polyclonal mitogens by binding to glycoconjugates on cell surface and thereby activating a series of events that result in cellular activation and proliferation (Sansford and Harris-H,

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1990; Maennel et al., 1991; Singh et al., 2006; Huang et al., 2008). The biological importance of lectins has increased due to their potential for the purification, selection and cloning of subpopulations of cells involved in normal or pathological immune response (Cunnick et al., 1990; Tietz et al., 1991).

Over the past few years, evidence has accumulated to support the idea that when plants are stimulated by specific biotic or abiotic stimuli they respond through the expression of cytoplasmic and/or nuclear plant lectins. The location and the regulation of the expression of these lectins indicate that lectins are involved in specific endogenous protein–carbohydrate interactions. These novel findings led to the challenging idea that lectins might be involved in cellular regulation and signaling.

Although capsicum may cause heartburn for many individuals, it's most common oral use is to treat digestive complaints such as colic, gas, indigestion, and poor appetite. Chemicals in capsicum have been shown to increase not only the amount of acid the stomach produces, but also blood flow in the lining of the stomach and intestines. All these effects may improve digestion, but they may also irritate the stomach. The dried fruit is a powerful local stimulant with no narcotic effect. It has proved efficacious in dilating blood vessels and thus relieving chronic congestion of people addicted to drink. The pepper *Capsicum annuum* contains other active ingredients, including oily substances called oleoresins. One of the main oleoresins, capsaicin, is used topically as a counter-irritant, renowned for its extensive application in neuroscience research (Szolcsanyi and Bartho, 2001). Tropical capsaicin has been used to treat arthritis pain and it may also be useful for relieving pain from fibromyalgia and shingles. Some scientific evidence also support its topical use for itching associated with conditions such as psoriasis, but this use is less common (Mason et al., 2004; Kamo et al., 2008).

The fruit, raw or cooked is very hot and normally used as flavouring. The fruit can be dried and ground into a powder for use as flavoring. The seed can be dried, ground and used as pepper while the leaves can be cooked as a potherb.

This study aims at extracting and purifying a lectin from the seeds of dry pepper, *Capsicum annuum,* characterize the protein as well as investigate its antifungal activities.

MATERIALS AND METHODS

Crude extraction

Dried short pepper *Capsicum annuum* was purchased from a local market. The seeds were removed, weighed, and soaked in distilled water for 5 days before homogenization in distilled water, adjusted to pH 9. The homogenate was filtered through cheesecloth and allowed to stand at 4°C overnight. The suspension was centrifuged at 10,000 x g for 30 min and the resulting supernatant which constitutes the crude extract was lyophilized.

Protein concentration

The lyophilized powder was dissolved in 50 mM NH_4HCO_3 pH 9.2 (crude extract) and the protein concentration was determined by the method described by Gornall et al., 1949) using bovine serum albumin (BSA) as standard.

Purification of lectin

10 ml of the extract was applied to a column of DEAE- Cellulose (1.5 x 20 cm). After elution of unadsorbed proteins, the column was eluted sequentially with 200 mM, 300 mM and 500 mM NH_4HCO_3, pH 9.2. The fractions were tested for heamagglutinating activity and the active fractions were pooled and applied to a QAE -Sephadex column (1.5 x 20 cm) previously equilibrated with 10 mM Tris-HCl buffer, pH 7.2. After washing the column with the Tris-HCl buffer, the column was eluted with a linear concentration (0 - 0.1 M) gradient of NaCl in the Tris-HCl buffer. The fractions with hemagglutinating activity were pooled and dialysed and lyophilized and further purified on a Sephadex G-100 column previously equilibrated with 50 mM NH_4HCO_3, pH 9.2. The active peak represented the purified lectin.

Assay for lectin (hemagglutinating) activity

Agglutination of the red blood cells by the crude extract and the various fractions that were obtained during purification was estimated as described by Bing *et al. (*1967).

A serial two-fold dilution of the lectin solution in U-shaped microtitre plates (100 µl) was mixed with 50 µl of a 2% suspension of human erythrocytes in phosphate buffered saline, pH 7.2 at room temperature (the erythrocytes of human blood group A,B and O were fixed with 1% glutaraldehyde). The plate was left undisturbed for 1 h at room temperature in order to allow for agglutination of the erythrocytes to take place. The hemagglutination titre of the lectin expressed as the reciprocal of the highest dilution exhibiting visible agglutination of erythrocytes was reckoned as one hemagglutinating unit. Specific activity is the number of hemagglutination units per mg protein (Wang et al., 2000).

Blood group specificity

The blood group specificity of the lectin was established using red blood cells of different blood groups of the ABO system.

Test of hemagglutination inhibition by various carbohydrates

The hemagglutination inhibition tests to investigate inhibition of lectin-induced hemagglutinations by various carbohydrates were performed in a manner analogous to the hemagglutination test. Serial two-fold dilutions of sugar samples were prepared in phosphate buffer saline (0.2 M initial concentration). All the dilutions were mixed with an equal volume (50 µl) of the lectin solution of known hemagglutination units. The mixture was allowed to stand for 1 hr at room temperature and then mixed with 50 µl of a 2 % human erythrocyte suspension. The hemagglutination titres obtained were compared with a non-sugar containing blank. In this study, the sugars used were: glucose, mannose, cellobiose, galactose, maltose, arabinose, fructose, sucrose, lactose, raffinose, sorbitol, dulcitol and glucosamine-HCl. The minimum concentration of the sugar in the final reaction mixture which completely inhibited hemagglutination units of the lectin sample were calculated (Wang et al., 2000).

Estimation of subunit and native molecular weight

The purified lectin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for molecular weight determination in accordance with the procedure of Laemmli and Favre

(1970) using the following protein markers: α–lactabulmin(14,000), carbonic anhydrase (29,000) glyceraldehyde – 5-phosphate dehydrogenase (36,000), egg albumin (45,000) and bovine serum albumin (66,000).The protein bands 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 of Laboratory Techniques, revised edition (1983).

The native molecular weight was estimated under non-denaturing conditions by gel filtration on Bio gel P-300 column(1.5 x 100 cm) which had been calibrated with molecular weight markers: α–chymotrypsinogen (25,000, 3 mg/ml), thermolysin (37,500, 3mg/ml) ovine albumin (45,000, 3 mg/ml), and bovine serum albumin (66,000, 3mg/ml). One ml of each standard was applied to the column and ran separately using a 10 mM phosphate buffer, pH 7.2 as eluant at a flow rate of 10 ml/h. Fractions of 2.5 ml were collected and the elution was monitored for each protein at 280 nm. The void volume (V_0) of the column was determined using Ferritin (elution monitored at 620 nm).

Effect of temperature on hemagglutinating activity

The effect of temperature on the agglutinating activity of the lectin from *C. annuum* was determined by carrying out assay at different temperatures according to the method described by Patrick *et al.,* (2007). The purified lectin was incubated in a water bath for 30 min at various temperatutres: 10, 20, 30,40,50,60,70,80,90 and 100°C, and then cooled to 20°C. Hemagglutination assay was carried out as previously described.

Effect of pH on hemagglutinating activity

The effect of pH on the activity of the lectin from *C. annuum* was determined by incubating the lectin in the following buffers at different pH values: 0.2 M NaOAc buffer, pH 3 - 6, 0.2 M Tris-HCl buffer, pH 7 - 8, and 0.2 M Glycine NaOH buffer, pH 10 - 12, and assaying for hemagglutinating activity. The control values were the agglutination titre of the lectin in PBS, pH 7.2.

Effect of EDTA and divalent cations

The purified lectin was analysed for metal binding site by demetallizing with EDTA and then incubated in a water bath at 20°C for 30 min in the presence of chlorides of various metals such as KCl, NaCl, $CaCl_2$, $MgCl_2$, $MnCl_2$, $FeCl_2$, $FeCl_3$ and NH_4Cl, all at 25 mM, followed by hemagglutination assay.

The ouchterlony double diffusion experiment

1.5% (w/v) agar solution in PBS containing 0.01% (w/v) sodium azide was prepared. The solution was slowly heated until the agar had completely dissolved and poured into clean Petri dishes. A well was made at the centre of each Petri dish and eight other wells equidistant from the centre were made around it. 100 µl of the samples were dispensed at the centre wells and into the surrounding wells 100 µl of 10 mg/ml of dextrin, 100 mg/ml of dextrin, 10 mg/ml of inulin, 100 mg/ml of insulin, 10 mg/ml of glycogen, 100 mg/ml of glycogen and polysaccharide extract.

Assay for antifungal activity

The assay of the lectin for antifungal activity toward fungal species was carried out in 100 x 15 mm Petri plates containing 10 ml of potato dextrose agar (PDA) (Wang et al., 2004). At the centre of the plate was inoculated the tested fungal mycelia. After the mycelia colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed at a distance of 0.5 cm away from the rim of the mycelia colony. An aliquot (15 µl) of the lectin was added to a disk while 15 µl of the buffer served as control. The plates were incubated at 25°C for 72 h until mycelia growth had enveloped peripheral disks containing the control and had formed crescents of inhibition around disks containing samples with antifungal activity. The zone sizes around each disk were measured. The organisms used for this test were *A. niger, Aspergillus flavus and Fuscarium spp.*

RESULTS

The phosphate buffered saline extract from *C. annuum* seeds agglutinated non-specifically the human red cells of the A, B and O blood groups. The soluble protein content and the specific activity of the crude lectin extract were 76 (mg/ml) and 2048 (HU/mg protein) respectively. For the purified lectin, the values were 6.5 (mg/ml) and 512 (HU/mg protein) respectively.

The hapten inhibition studies to define the sugar specificities of the crude extract (the phosphate buffered saline extract) of *C. annuum* seeds showed that Glucosamine-HCl, maltose, fructose, sucrose, arabinose, and cellobiose had no effect on the hemagglutinating activity. , dulcitol and sorbitol enhanced the activity of the lectin while galactose, lactose, rhamnose and raffinose slightly inhibited the activity of the lectin. The activity of the lectin was completely inhibited by mannose and glucose with minimum inhibitory concentration of 12.5 mM and 6.25 mM respectively (Table 1).

The elution profile of the ion exchange chromatography of the crude extract on DEAE-Cellulose column is as shown in Figure 1. Three protein peaks were obtained only one of which exhibited haemagglutination activity (D2), this was eluted with 200 mM NH_4HCO_3, pH 9.2. Peak D2 on QAE-Sephadex column was resolved into four protein peaks, with only Peak Q2 exhibiting haemagglutinating activity (Figure.2). Gel filtration of Peak Q2 on Sephadex G-100 column resulted in two peaks. CAL 1 and CAL 2. Lectin activity resided in CAL2 and constituted the homogenous preparation of the pepper seed lectin (Figure 3) which gave a distinct band in SDS- PAGE (data not shown).

The purified lectin sample was found to be heat stable up to 30°C, at incubation temperature of 30 to 40°C, a small decrease in the hemagglutinating activity of the lectin was observed whereas at 50 - 60°C, 50% of the activity of the lectin remained while at 65°C, the activity was completely lost (Table 2). The lectin activity was found to be stable at pH range of 6 - 8. At acidic pH, the activity of the lectin became indiscernible whereas at alkaline pH, the activity declined Table 3. The lectin activity was enhanced by both $CaCl_2$ and $MgCl_2$.

The PAS reaction revealed that the lectin was non glycosylated. The lectin when examined for possible interactions with some polysaccharides by Ouchterlony double diffusion experiment formed precipitin band with dextrin.

The lectin exhibited a strong inhibitory effect on growth

Table1. Sugar inhibition study of hemagglutinating activity.

(sugar in mmol/l)	200	100	50	25	12.5	6.25	3.12	1.56	0.78	PBS
Lactose	-	+	+	+	+	+	+	+	+	+
Glucose	-	-	-	-	-	-	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+
Glucosamine-HCl	+	+	+	+	+	+	+	+	+	+
Raffinose	-	+	+	+	+	+	+	+	+	+
Mannose	-	-	-	-	-	+	+	+	+	+
Dulcitol	+	+	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+	+	+
Galactose	-	-	+	+	+	+	+	+	+	+

Note: +, hemagglutinating activity; -, no hemagglutinating activity; PBS, phosphate-buffered saline.

Table 2. Effect of temperature on hemagglutinating activity of CAL.

Temperature (°C)	20	30	40	50	60	65	70	80	90
Hemagglutinating activity (number of units)	64	64	32	8	8	0	0	0	0

Table 3. Effect of pH on hemagglutinating activity of CAL.

pH	3.0	4.0	5.0	6.0	7.0	8.0	10.0	11.0	12.0
Hemagglutinating activity (number of units)	-	-	32	64	64	64	32	2	2

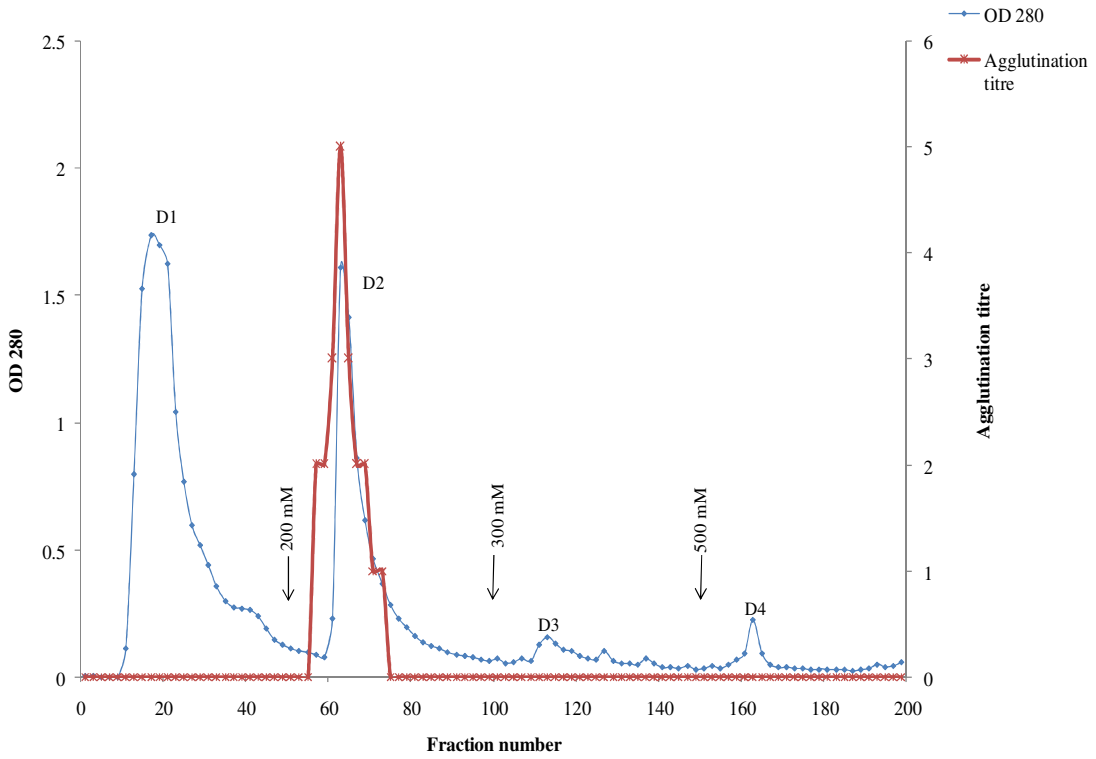

Figure 1. Ion exchange chromatography of *Capsicum annuum* extract on DEAE-Cellulose column.

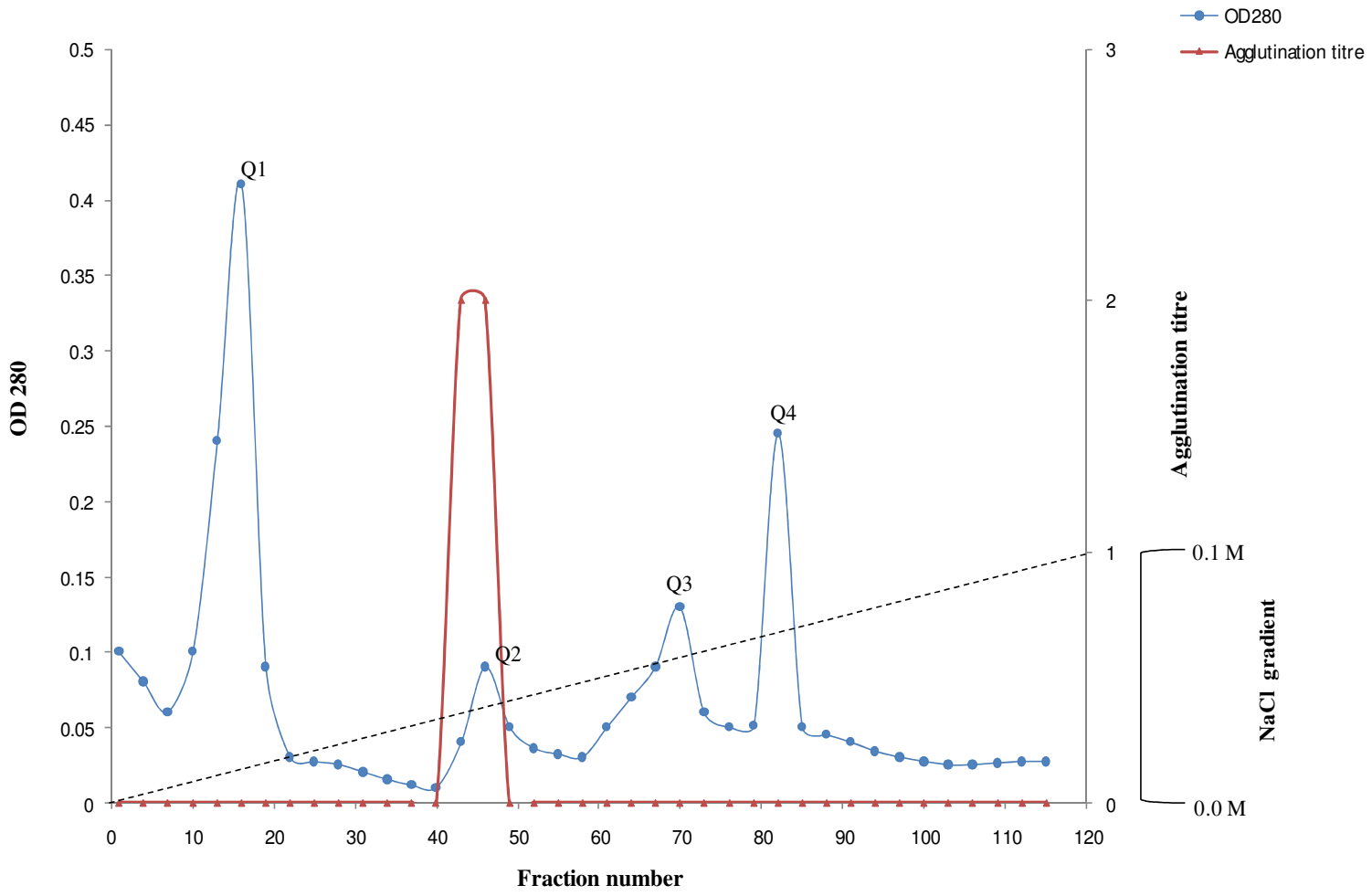

Figure 2: Ion exchange chromatography of Fraction D2 on QAE-Sephadex column.

Column size	1.5 x 20cm
Elution buffer	10mM Tris-HCl buffer pH 7.2
Volume of extract layered	5ml (46mg protein)
Fraction volume	2ml
Flow rate	24ml/h

Protein was eluted with gradient solution of 0 – 0.1M NaCl in Tris-HCl buffer.

Agglutination titre

Absorbance at 280 nm

and spore germination in the fungi, *A. flavus* and *Fusca-rium graminearum* while its effect on the other fungal species was insignificant. Inhibition of hyphal growth was 55 ± 5% for *A .flavus* and 70 ± 5% for *F. graminearum* was 70 ± 5% at a dose of 1 mg/ml lectin. The widths of inhibition zone in the spore germination test in the two fungi at 200 µg lectin/ml were 3 ± 0.4 and 5 ± 0.4 mm respectively. (Mean ± SD, n = 3)

DISCUSSION

The data presented from this study showed that the extract of the seeds of *C. annuum* contained a measurable amount of haemagglutinating protein (lectin). The lectin from *C. annuum* agglutinated red blood cells non-specifically which is typical of many lectins.

The thermostability and pH stability characteristics of lectins are known to differ from lectin to lectin (Patrick and Ngai, 2007) the haemagglutinating activity of this pepper seed lectin is thermostable and pH sensitive. This investigation showed that *C. annuum* was stable in the pH range 6 - 8. Lectins are known to be heat labile and their activity can be decreased by heat treatment (Leiner, 1994). The haemagglutinating activity of lectin from *C. annuum* was heat stable up to 30°C, beyond which the activity started declining, similar observation has been re-

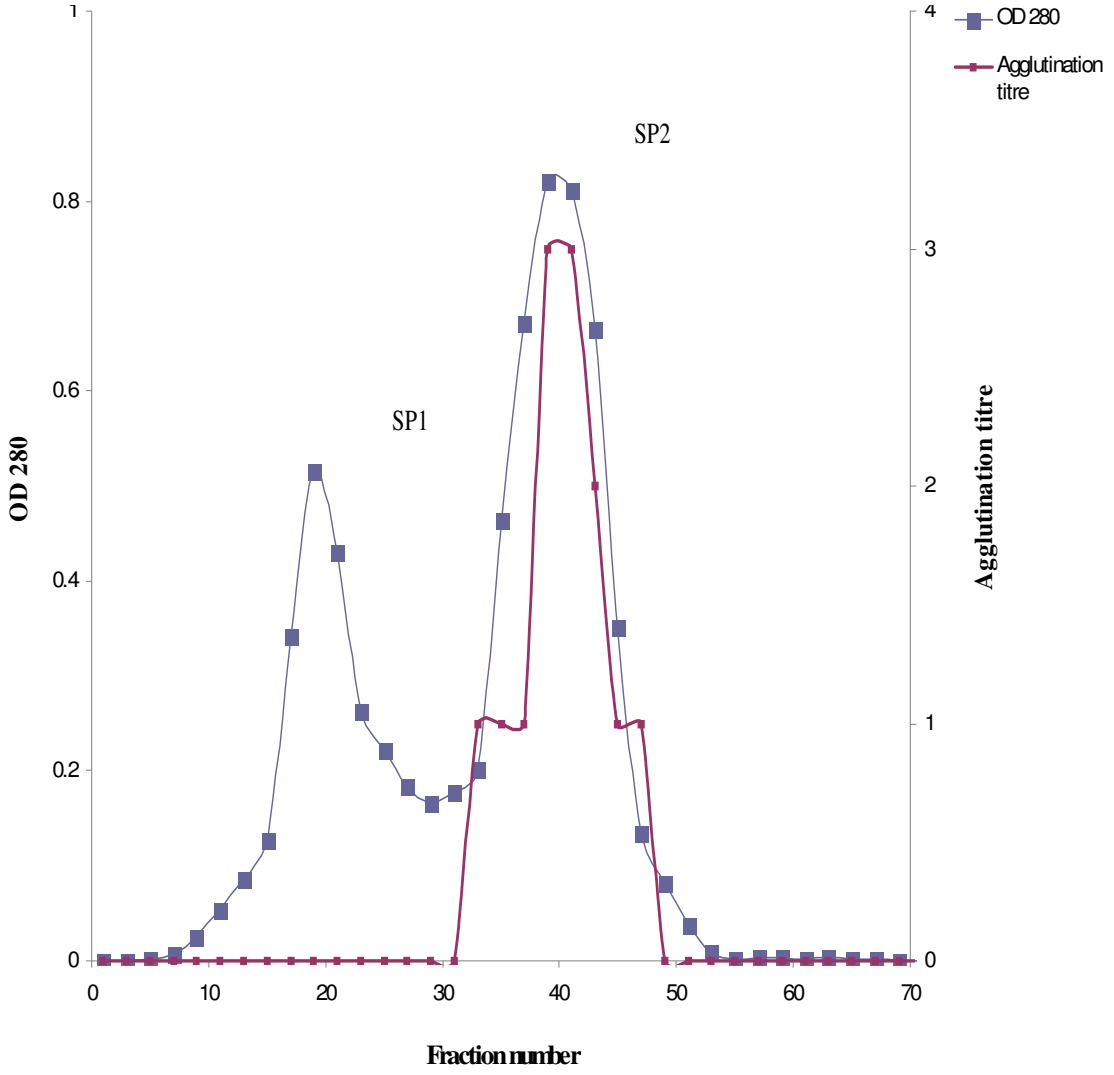

Figure 3: Gel Filtration of Fraction Q2 on Sephadex G-100

Column size	2.5 x 40cm
Elution buffer	50 mM NH_4HCO_3, pH 9.2
Volume of extract layered	10ml (46mg protein)
Fraction volume	2ml
Flow rate	24ml/hr

Agglutination titre

Absorbance at 280 nm

ported for many lectins. Ca^{2+} and Mg^{2+} showed ability to potentiate the activity of the pepper seed lectin which is consistent with the observation with Con A. Polyacylamide gel electrophoresis revealed that the purified lectin was homogenous and has a monomeric structure consisting of a single polypeptide chain, the molecular mass of the seed lectin is similar to that of Con A subunit. A few numbers of lectins with a single polypeptide chain have been reported; for instance, Utarabhand and Akkayanont (1995) reported that *Parkia speciosa* lectin has a single polypeptide chain, also, the purified lectin from mycelia and fruiting bodies of *Ganoderma lucidum* appeared as a single band on SDS-PAGE (Kawagishi et al., 1997).

The Ouchterlony double-diffusion experiment revealed that there was interaction between the lectin and Dextrin among the polysaccharides tested; failure to form precipitin line with other polysaccharides could however be due to many factors such as the concentration of the protein as well as the molecular size of the polysaccharides. This could also be explained based on the theory that a

lectin may either fail to precipitate a polysaccharide or form precipitin bands in agar gel because the lectin may not be specific for that polysaccharide.

There are only a few lectins known to possess antifungal activity such as the lectins from the seeds of *Phaseolus vulgaris* and *Pisum sativum*, from the pepper seed *Capsicum frutescens* and from the mushroom *Astragalus mongholicus* (Ye et al., 2001; Yan et al., 2005; Ngai and Ng, 2007and Sitohy et al., 2007). The lectin from *C. annuum* like the one from *Capsicum frutescens* (Ngai and Ng, 2007) possess antifungal and sugar binding characteristics that make them potentially exploitable and interesting lectins.

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