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Full Length Research Paper

Phyto-agglutinin, total proteins and amino assimilating enzymatic activity of indigeneous chickpea (*Cicer arietinum* L.) cultivars

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Biochemical studies were carried out with *in vitro* micropropagated plantlets of two indigenous cultivars of chickpea (*Cicer arietinum* L.), KK-1 and Hassan-2K, where extract from shoots, leaves, roots, and reproductive organs were determined for human erythrocyte agglutination (by mixing the lectin containing extract 1:1 with a 2% erythrocyte suspension), protein profiling and the amino assimilating (glutamine synthetase) enzymatic activity. Both of KK-1 and Hassan-2K cultivars extract showed highly phyto-agglutination of human erythrocytes with reproductive organs and other tissues, which represents the presence of potent lectins (phyto-agglutinin). The amino assimilating enzymatic activity of green leaves of Hasan-2K was found higher (3.545 GS µmol.gfw^{-1}.min^{-1}) as compared to KK-1 (2.873 GS µmol.gfw^{-1}.min^{-1}). Protein content profile showed variation in bulk proteins of various tissues, higher (12.634 µg/gfw) and (2.987 µg/gfw) in reproductive organs of KK-1 and Hasan-2K, respectively.

Key words: Chickpea, glutamine synthetase, glyco-protein, lectin, phyto-agglutinin.

INTRODUCTION

Lectins are non-immunoglobulin-type carbohydrate recognition molecules that are involved in haemagglutination, lymphocyte transformation, inactivation of certain types of tumor cells and precipitation of certain polysaccharides and glycoprotein (Lis and Sharon, 1986; Goldstein and Hayes, 1978). These lectins bind to sugar moieties in cell walls or membranes and thereby change the physiology of the membrane to cause agglutination (hem agglutination) and other biochemical changes in the cell (Sullivan, 2008). Agglutination test for the detection of lectin is an established protocol (Lin et al., 1981; Yeasmin et al., 2001). These multivalent cell-agglutinating proteins are highly specific in binding with their carbohydrates moieties and due to this binding specificity; they are being used increasingly to probe the structure of carbohydrates on the surface of normal and malignant cells (Liener et al., 1986). Lectins are particularly abundant in the seeds of legumes. They constitute up to 10% of the soluble protein in the seed extracts (Talbot, 1978). The legume lectins, despite the high sequential and structural similarity of their subunits, show a remarkable range of sugar specificities (Hamelryck, 1998). Lectin attract many researcher for their unique biological activities and have been isolated from different plant species like *Phaseolus vulgaris* seeds (Itoh et al., 1980), *Viscum album* L. (Franz et al., 1981), *Lathyrus sativus* seeds (Kolerg and Sletten, 1982) *Vicia unijuga* leave (Yanagi et al., 1990), mulberry seeds (Yeasmin et al., 2001), *Calyslegia sepium* (Willy et al., 1997). Lectin has also been isolated from chickpea seed (Kolberg et al., 1983; Insaf et al., 2004).

In legumes, chickpea is one of the most important grain legumes used for human food and animal feed in developing countries and is a rich source of dietary protein (Singh, 1990). In Asia, India is the largest producer of chickpea. Pakistan ranks second in terms of acreage under its cultivation (Hassan and Khan, 1991).

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Chickpea is valued for its nutritive seeds with high protein content which vary between (20 to 26%). The protein is mainly located in the cotyledons and the embryonic axis, with small amount present in the testa. Chickpeas are a good source of zinc, folate, phosphorus, iron and certain water soluble vitamins. They are also very high in dietary fiber and thus are a healthy food source of carbohydrates for persons with insulin sensitivity or diabetes (Hulse, 1991). Chickpea seed has 3% fiber, 4.8 to 5.5% oil, 3% ash, 0.2% calcium, and 0.3% phosphorus, the carbohydrates content varies between 60 to 66% while the lipid content comprise a relatively small proportion of the overall composition and varies from 1 to 6% depending on the varieties (Hulse, 1991; Huisman and van der Poel, 1994).

Glutamine synthetase is the key enzyme involved in the assimilation of inorganic nitrogen in higher plants. It works together with glutamate synthetase (glutamine-oxoglutarate aminotransferase or GOGAT), GS/GOGAT pair provide primary port of entry for nitrogen in whole plant metabolism (Lee and Miflin, 1974; Keys et al., 1978; Miflin and Lee, 1980; Stewart et al., 1980). Inorganic nitrogen, in the form of ammonia, is assimilated via this glutamate synthase cycle into the organic nitrogen compounds glutamine and glutamate further used in amino acids, nucleic acids and chlorophyll synthesis (Sechley et al., 1992). In addition to its major role in primary nitrogen assimilation, a large amount of ammonia is released during photorespiration (Somerville and Ogren, 1980; Kendall et al., 1986). The nitrogen metabolism and assimilation is involved in providing the amino group of amino acid which directly reflects the strength and potency of Lectin (Glyco-protein).

The present study was carried out to determine the lectin, total protein and nitrogen assimilating enzymatic activity in different organ of indigenous cultivars of chickpea.

MATERIALS AND METHODS

Plant material

In vitro micro-propagated plantlets of two indigenous chickpea cultivars, Hassan-2K (taken from Nuclear Institute for Food and Agriculture Peshawar, Pakistan) and KK-1 (taken from Ahmad Wala Research Station Karak, Pakistan) were used for biochemical analysis. The varieties used were purely indigenous and have more protein content (20 to 26%) including glycoprotein.

Lectin extraction

Two grams of roots, shoots, leaves, inflorescence, and seeds were finely ground and stirred with 20 ml of 0.15 M NaCl. Suspensions were maintained for at least 3 h at room temperature or stored at 4°C for 1 to 2 days and then centrifuged at 10,000 g for 30 min at 10°C. After centrifugation the clear supernatants taken from suspension were used as clarified extract to determine the hem agglutinating activity.

Erythrocyte agglutination

The assay of erythrocyte agglutination was carried out in glass test tubes. The clarified extract prepared for the determination of hem agglutinating activity was serially diluted with 0.15 M NaCl and each dilution was tested for erythrocyte agglutination by mixing the extract 1:1 with a 2% erythrocyte suspension. The extent of hem agglutination was monitored visually after the tubes were allowed to stand at room temperature for 30 min. The results were recorded as hem agglutination titer (the reciprocal of the highest dilution given visible hem agglutination).

Detecting lectin molecules in protein fractions

A blood supply facility was established to obtain erythrocytes from humans for testing hem agglutinating activities in the isolated protein fractions. Hem agglutination of erythrocytes was carried out with human erythrocytes of different blood groups to test the potency of extracted lectins.

Determination of protein contents

Protein in the soluble fraction was determined by colorimetric method using Futura System Kit containing pyrogallol as the dye. The readings were obtained at 600 nm on a Smart Spec plus UV-Visible spectrophotometer using Bovine Serum as standard.

Enzyme extraction

Plant material was homogenized at in a mortal and pestle with two volumes of an extraction buffer containing 50 mm Tris-HCl, pH 7.6, 5 mm sodium Glu, 10 mm $MgSO_4$, 1 mm dithiothreitol, 600 mM sorbitol and 1% glycine betain. The homogenates were centrifuged at 10,000 g for 10 min. The extracts were assayed for glutamine synthetase activity by the transferase assay.

GS assay

The reaction started in a 2 ml reaction mixture with 100 mM Tris-acetate buffer, pH 6.4, containing 100 mM glutamine, 30 mM hydroxylamine, 30 mM sodium arsenate, 1.5 mM $MnCl_2$, 0.2 mM ADP and 200 µl enzyme extract. The reaction is stopped after 30 min by the addition of acidified ferric chloride (26 g of ferric chloride and 40 g of trichloric acetic acid in one litre of 1N HCl), and the GS activity is determined spectrophotometrically at 540 nm.

RESULTS AND DISCUSSION

Agglutination of human erythrocytes as an indicator of lectin presence

Phyto-agglutination of human erythrocytes with extracts from different organs of KK-1 and Hassan-2K cultivars were used as an indicator of the presence of sugar binding lectin (phyto-agglutinin) in different tissues which are presented in tables 1 and 2.

In KK-1, the extract from different organs used for hem agglutination of human blood showed that the strength of their agglutination reaction was dependent on the plant organ and the type of blood group tested. The amount of

Table 1. Agglutination of erythrocytes by extracts from different tissues of KK-1 and Hussan-2K.

Plant part	Blood group A		Blood group B		Blood group AB		Blood group O	
	KK-1	Hassan-2K	KK-1	Hassan-2K	KK-1	Hassan-2K	KK-1	Hassan-2K
Leaves	+++	++++	++	-	-	-	++	+++
Reproductive	+++++	+++++	-	++++	++++	++	+++	-
Shoot	++	-	++	++	-	-	-	++
Root	++	-	-	-	-	-	+	-

- = Nil, + = satisfactory, ++ = fair, +++ = good, ++++ = very good, and +++++ = excellent.

lectins could be predicted by the visual grading of agglutination. The most potent reaction was exhibited by the reproductive organ extract with blood group A, while its reaction with blood group AB is also strong and its reaction with blood group O showed a mild reaction (Table 1). Similar agglutination reaction of red blood cell has been reported in reproductive organs (seeds) of chickpea species by Justo et al. (2004). Kolberg et al. (1983) also isolated a potent lectin from seed extracts of *Cicer arietinum.* The extract from leaves of KK-1 variety exhibited a reasonable reaction with blood group A, B and O, while it shows no reaction with blood group AB. The hem agglutination test is an established protocol for the detection of lectins (Lin et al., 1981; Yeasmin et al., 2001). The shoot extract of KK-1 appear to contain lectins with a detectable agglutinating activity against blood group A and B. In the root extract, a weak agglutination reaction was observed with blood group A and O (Table 1). Kiran (1991) studied the property of lectins as agglutinin and to bind sugars in the blood on normal and cancer cervix patients.

Phyto-agglutinin potency in KK-1 can be seen after testing a 10 fold dilution of extracts as presented in Table 2, where a strong reaction with blood group A, a mild reaction with blood group AB and a weak reaction with blood group O was observed in the extract from reproductive organ, Barre et al. (1996) has reported the same results in monocot. Naeem et al. (2007) reported that a lectin present in seeds of *Clitoria ternatea* agglutinated trypsin-treated human B erythrocytes. In addition, extract from leaves and shoot showed a mild reaction with blood group A which only represent the presence of potent lectins in these organs (Table 2). Similarly, Damme et al. (1998) reported the carbohydrate-binding proteins (lectins) in leaves, roots and other organs of chick pea (*Cicer arietinum* L.). High level potent lectins were also detected through hem agglutination in reproductive organs, leaves, shoots and roots of mulberry species (Zahoor et al., 2009).

In Hassan-2K cultivar of chickpea, erythrocyte coagulation reaction by the extract is presented in Table 1. The reaction between reproductive organs extract and blood group A was the most potent, and this extract also exhibited a strong ability to coagulate blood group B and an observable coagulation of blood group AB. These results get sufficient validation from the findings of Esteban et al. (2002) reported that plant lectins are a group of glycoprotein with the ability to recognize and bind carbohydrate ligands. Similarly, Justo et al. (2004) reported that pa2 albumin in chickpea seeds is a lectin that causes red blood cell agglutination. The extract from the leaves of Hassan-2K showed a strong reaction with blood group A and O; in addition, the shoot of Hassan-2K exhibited a detectable agglutination of blood group B and O (Table 1). Castillo et al. (2007) achieved purification of the lectin from *Phaseolus acutifolius* var. escumite by agglutination of blood group O erythrocytes.

In Hassan-2K following a 10 fold dilution with 150 Mm NaCl, the diluted extract from reproductive organ showed a measurable reaction with blood group A and B, similarly, the reaction of leaf extract with blood group A and O remained detectable (Table 2). These results indicate the presence of several potent lectin proteins in various organs of chickpea. These results are in great harmony with the findings of Zahoor et al. (2009) which reported similar erythrocytes agglutination reaction with 10X dilution extract of mulberry species.

Protein content

Table 3 shows the protein content profile of different organs of the two chickpea expressed as µg/gfw. These variations in the bulk proteins of various tissues are related to the differences in water content, the fibrous nature of the tissue as well as the distribution of storage proteins, and soluble protein fractions. The total protein contents were found highest 12.634 and 2.987 µg/gfw in the reproductive organs of KK-1 and Hassan-2K respectively, Singh et al. (1980) obtained similar results regarding protein content in chickpea. Similarly, in leaves also higher protein content was detected in both cultivars, while the shoot of KK-1 and roots of both cultivars showed minimal protein contents (Table 3). Seed protein in chickpea and lentil was determined and isolated by Alsohaimy et al. (2007) and Kumar et al. (1980) characterized total protein content in *Cicer arietinum*. Total protein content were found higher in KK-1 which represent that lectin activities in different organs of indigenous chickpea cultivars do not follow the protein

Table 2. Agglutination of erythrocytes by extracts of KK-1 and Hussan-2K different tissues following 10X dilution.

Plant part	Blood group A		Blood group B		Blood group AB		Blood group O	
	KK-1	Hassan-2K	KK-1	Hassan-2K	KK-1	Hassan-2K	KK-1	Hassan-2K
Leaves	++	++	-	-	-	-	-	+
Reproductive	+++	+++	-	++	++	-	+	-
Shoot	+	-	-	-	-	-	-	-
Root	-	-	-	-	-	-	-	-

- = Nil, + = satisfactory, ++ = fair, +++ = good, ++++ = very good, and +++++ = excellent.

Table 3. Protein content (µg/gfw) of different organs in chickpea.

Plant part	Hassan-2K	KK-1
Leaves	2.560	4.298
Reproductive	2.987	12.634
Shoot	2.742	0.898
Root	1.609	0.652

Table 4. Glutamine synthetase (GS) activity in the leaf extracts of chickpea cultivars.

Cultivar	GS (µmol.gfw^{-1}.min-^{1})
KK-1	2.873
Hassan- 2K	3.545

content profiles of the plant where the tissue with low protein content showed high agglutinin activity and vice versa. Zahoor et al. (2009) recorded similar observation regarding hem agglutination and total protein content in mulberry species.

Glutamine synthetase (GS) assays

Glutamine synthetase is the key enzyme of nitrogen assimilation playing an exclusive role in ammonium incorporation for amino acid biosynthesis, and thus, its activity is directly implicated in amino acid pool for protein synthesis, which in turn would be associated with the synthesis of plant lectins. Zahoor et al. (2009) reported highly active glutamine synthetase enzyme in *Morus alba* and *Morus nigra* roots and shoots and described their relation with erythrocytes agglutination reaction for the presence of potent lectins in these tissues. The amino assimilating enzymatic activity in the photosynthetic leaf extract of KK-1 and Hassan-2K cultivars are presented in Table 4. Paul et al. (1976) also recorded similar results on relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Franqoise (1989) reported multiple forms of glutamine synthetase in nodules of tropical legumes. As per visible nodulation in the roots of both cultivars, the photosynthetic leaf extract also showed the presence of highly active glutamine synthetase (nitrogen-assimilating) enzyme, higher (3.545 GSµmol.gfw^{-1}.min^{-1}) in Hassan-2K as compared to KK-1 (2.877 GSµmol.gfw^{-1}.min^{-1}), shown in Table 4. Chopra et al. (2003) described the role of ammonium assismilating enzymes in lentil roots and nodules. Krieg et al. (1990) reported ammonia accumulation and glutamine synthetase activity in alfalfa (*Medicago sativa* L.)

Conclusion

KK-1 and Hassan-2K extract contained potent phyto-agglutinin in various tissues with highest contents in the reproductive organs. These highly potent lectins could be isolated and characterized according to their molecular weight, carbohydrates moieties, toxicity and antimicrobial activity and can be used for various biological activities, some of these activities for plant lectins are studied by Talbot (1978), Hamelryck (1998), Itoh et al. (1980), Kolberg et al. (1983) and Insaf et al. (2004).

The ammonium assimilating enzymatic activity of photosynthetic tissues was higher (3.545 GSµmol.gfw^{-1}.min^{-1}) in Hassan-2K. The total protein contents correlated with the degree of hem agglutination activity in case of both species which in turn indicates the presence of lectins as per protein contents in indigenous chickpea species.

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