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Original Article

Characterization of novel enzymic hemagglutinin isolated from Dregea *volubilis*

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ABSTRACT

A hemagglutinin was isolated from the fruit coats of the plant *Dregea volubils* to purity. The purified protein exhibited similar hemagglutination pattern with all the blood groups. The hemagglutinin could agglutinate erythrocytes of other animals also except bullock. Molecular weight of the purified hemagglutinin was similar on SDS PAGE and sephadex G-75 gel filtration also. The hemagglutinating activity of the purified fraction was inhibited by α-D-glucose and α-D-mannose. Optimum pH was found to be 5.0 and optimum temperature stability was recorded to be at 45ºC, respectively. However, the hemagglutinin was stable from pH 4.0 to 7.0 and from temperature 5.0 to 55ºC, only. EDTA and 1, 10 phenanthrolein scavenged the metallic ion from the purified agglutinin, but Mg^{++} was found to restore the activity to normal. The purified fraction exhibited α and β-glucosidase activity. Trypsin treatment of the purified fraction reduced the hemagglutinating and enzyme activity to nearly half. The purified fraction was found to be glycoprotein in nature and the carbohydrate moiety was detected to be GluNAc by TLC.

Key words: Dregea, Hemagglutinin, Phytoagglutinin, Plant protein, Plant enzyme

INTRODUCTION

Phytohemagglutinins are a class of proteins which are of non-immune in origin showing affinity towards the specific carbohydrates. Different hemagglutinins bind with different carbohydrates ranging from monosaccharide to polysaccharides and from free carbohydrates to bound one, even on cell membranes. Due to this ability the hemagglutinins agglutinate the erythrocytes, which are normally used as identifying characteristics of plant hemagglutinins. Due to the carbohydrate binding property these proteins are thought to transport carbohydrates, plant hormones and glycoproteins from one place to other and might be involved in growth and development of plants. Phytohemagglutinins are reported to bind with some pathogenic and nonpathogenic bacteria and therefore, are involved in plant defense also, protecting the plants from diseases and attack by insects and predators [1-3]. Phytohemagglutinins due to their bacteria binding property are associated with root nodulation and nitrogen fixation by the plant root nodules. Thus, phytohemagglutins are involved in overall metabolic activity of the plant [4]. Till this date more than 500 phytohemagglutinins have been isolated and characterized. Some important hemagglutinins isolated from plants include *Concanavalin* A [5], wheat germ agglutinin [6], A. *integra* lectin [7], *Bauhinea purpuria* lectin [8] and *Phaseolus vulgaris* lectin [9].

MATERIALS AND METHODS

Source of hemagglutinin: Fruit coats of the mature fruits of the wild plant *Dregea volubilis* were used. The plant was identified by the taxonomists of the region.

Chemicals: BSA, M.W. markers, papain were obtained from Sigma Chem. Co. USA. Folin Ciocalteau reagent was purchased from Qualigens, Mumbai. Defatted starch, epichlorohydrin, ammonium sulphate were obtained from E. Merck, Germany. DEAE cellulose was purchased from Hi Media, Mumbai. Other chemicals were of analytical reagent grade. Blood from the healthy human donors was obtained from the clinical laboratory of University Department of Biochemistry, Nagpur.

Extraction of hemagglutinin: Fresh green growing fruits were collected from the plant for extraction of hemagglutinin. The fruits were hand peeled and the fruit coats were washed with

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distilled water two times to remove the adhered dust and crushed into the grinder to get 30% extract. The extraction medium was sodium phosphate buffer saline referred to as PBS1 (20 mM sodium phosphate buffer, pH 7.0 containing 100 mM sodium chloride). The extract was kept on the shaker (100 rpm) for 1 h for complete solubilization of proteins in to the extraction medium. The contents were filtered through muslin cloth and the filtrate was subjected to centrifugation (5000 rpm for 20 min, C24 Remi). The supernatant was designated as crude fraction and used for further purification of hemagglutinin.

Ammonium sulphate precipitation: Proteins in the crude fraction were precipitated by ammonium sulphate by adding the salt very slowly to the crude extract. The mixture was kept in refrigerator for 2 h for complete solubilization of salt and precipitation of proteins. The precipitate was collected by centrifugation at 9886 *g* for 20 min (C24 Remi.). The precipitate was solubilized in small volume of PBS1 and dialyzed overnight against the same [10].

DEAE cellulose chromatography: Ammonium sulphate precipitate was further subjected to ion exchange chromatography on DEAE cellulose column (30×2.5 cm). The bound proteins with DEAE cellulose were eluted with sodium chloride gradient ranging from 100 to 500mM. Fractions were collected at a flow rate of 12 ml/h in LKB-Pharma fraction collector and read at 280 nm (Gilford spectrophotometer, Germany). Fractions for individual peaks were checked for hemagglutination activity units (HAU) with papain treated erythrocytes of blood group A by the method described elsewhere (Deshpande and Patil, 2002) [11].

Affinity Chromatography: Affinity chromatography on cross linked starch (entrapped in agarose) column was performed as described by Sawhney *et al*. (1988) [12].

Simple and SDSPAGE: Homogeneity of the fractions was detected by running the proteins after affinity chromatography on simple PAGE (Davis, 1964) [13] and SDS PAGE (Laemmli, 1970) [14].

Preparation of erythrocytes of blood group A: Heparinized blood of group A was used for preparation of papain treated erythrocytes for hemagglutination assay (Deshpande and Patil, 2002) [11].

Determination of properties of purified hemagglutinins:

Determination of carbohydrate specificity: The purified hemagglutinin in PBS1 was mixed in equal volume with various pentoses, hexoses, di and trisaccharides and incubated at 45ºC for 1 h. Hemagglutinating activity of the carbohydrate treated protein was determined by the method of Deshpande and Patil (2002) [11].

Phenol-sulphuric acid method: This was performed with purified protein to identify the glycoprotein nature of the purified protein (Dubois *et al*. 1956) [15].

Measurement of protein contents: Proteins in all the fractions were measured by the method of Lowry *et al*., (1951) using fat free BSA as standard protein. Absorbance of fractions was also measured at 280 nm whenever necessary (Gilford spectrophotometer, Germany) [16].

Effect of pH on activity and stability: Purified protein was mixed with buffers of various pH ranging from pH 3.0 to 10.0. Effect of pH on activity and stability was determined by the method of Suseelan *et al*. (1997) [17].

Effect of temperature on activity and stability: This was also determined by the method of Suseelan *et al*. (1997) [17].

Effect of EDTA and 1, 10 phenanthrolein: Scavenging the metal ions from the purified fraction of protein was performed by incubating the purified fractions with EDTA and 1, 10 phenanthrolein at 45ºC for 1 h. The metal ion chelator was removed and the activity was tested as described by Deshpande and Patil (2002) [11].

Effect of metal ions on activity: This was performed as described by Deshpande and Patil (2002) [11]. Various chloride salts of metals were mixed with metal ion chelator treated purified protein and tested for hemagglutination activity.

Agglutination of human and other animal erythrocytes: This was performed as suggested by Deshpande and Patil (2002) [11].

Thin layer chromatography of the purified fraction: Major carbohydrate content of the purified fraction of *Dregea volubilis* was determined as described by Upadhyay *et al*. (19 97) [18].

Molecular weight determination: Molecular weight of the purified homogeneous fraction was determined on SDS PAGE (Laemmli, 1970) [19] and by gel filtration chromatography on sephadex G-75 using BSA- 66kD, Pepsin- 34.7 kD, Trypsinogen- 24 KD and lysozyme – 14.3 (Andrews; 1964) [20].

Enzyme activity: α and β glucosidase activity of purified fraction was determined by method described by Herr (1979) using p-nitrophenyl-α-D-glucopyranoside and p- nitrophenyl- β-D-glucopyranoside as substrates. P-nitrophenol was used as standard. One unit of enzyme activity was defined as the amount of enzyme that liberates one micromole of p-nitrophenol per min under experimental conditions [21].

Effect of trypsin on hemagglutination and enzyme activity: Purified protein was mixed with 1% trypsin in equal volume at pH 7.5 in Tris buffer and incubated for 2 h at 37ºC. Trypsin treated fraction was used for hemagglutination and enzyme activity as described by Deshpande and Patil (2002) [11].
Estimation of proteins in terms of amino acids: Proteins in terms of amino acids were measured by the method described by Spice (1959) using purified tyrosine as standard [22].

Estimation of Tryptophan in purified fraction: Tryptophan content in purified fraction was measured by the method described by Spande and Witkop (1967) using pure tryptophan as standard amino acid [23].

Statistical analysis: All results were statistically analyzed by the method described by Walpole (1982). P value was set at < 0.05 [24].

RESULTS AND DISCUSSIONS

Hemagglutinin from the fruit coats of *D. volubilis* was purified to homogeneity by salt precipitation, ion exchange (Fig. 1) and affinity chromatography as described above. Table 1 shows the purification scheme of the hemagglutinin showing 91 fold purification with 31% yield. This type of result has been obtained by Deshpande & Patil (2002) with lectins isolated from leaves of the same plant [11]. The hemagglutinating activity of the purified fraction from fruit coat is inhibited by α-D-glucose and α-D-mannose indicating the nature of hemagglutinin to be glucose specific (Table 4). Similar results were also shown by WGA [6], rice lectin [25], and Con A [5] which are also α-D-glucose and α-D-mannose specific hemagglutinins. .*D. volubilis* fruit coat purified hemagglutinin was active over a broad range of pH ranging from 4.0 to 7.0 showing same hemagglutination pattern. Similar property was exhibited by *V. mungo* lectin as reported by Suseelan *et al*. (1997) [17]. Like all other proteins exposure above a particular temperature destroys the activity of hemagglutinins. *D. volubilis* fruit coat hemagglutinin retained activity from 5.0 to 55ºC. Nearly 50% activity was lost by the same protein at 65ºC within 10 min. Similar effect of temperature was observed with *P. mungo* lectin which was sensitive to temperature just above 40ºC only (Sivkumar and Rao,1988) [26] (Table 6). Treatment of purified hemagglutinin with metal ion scavengers lost the hemagglutination activity. Out of many metal ions tested for restoration of activity only Mg^{++} ions were found to restore the activity of purified hemagglutinin of *D. volubilis* (Table 2). This type of Mg^{++} ion dependence was also observed with Con A [27] and *D. lablab* lectin [28]. *D. volubilis* fruit coat hemagglutinin agglutinated papain treated erythrocytes of all the blood groups with same efficiency indicating the hemagglutinin to be nonspecific to any blood group, but the untreated erythrocytes of any blood group were not agglutinated. This may be due to the masking of reactive receptors on the untreated erythrocyte membrane, which are exposed due to the proteolytic treatment by papain Other known blood group nonspecific hemagglutinin are WGA [6], soybean lectin [29]. The purified fraction also agglutinated erythrocytes of other animals except bullock which may be lacking the reactive receptor on the erythrocytes (Table 5). Other agglutinins agglutinating animal erythrocytes are ricin, abrin, and crotin, etc. (Gold and Balding, 1975) [30]. The major carbohydrate content of *D. volubilis* fruit coat hemagglutinin contained GluNAc (N acetyl Glucosamine) as indicated by TLC. Similar glucosamine moiety was also found to be the part of *B. pupurea* and *Ulex I* lectin (Gold and Balding, 1975) [30]. Glycoprotein nature of the *D. volubilis* fruit coat hemagglutinin was confirmed by measurement of protein in terms of amino acid where 100 µg proteins were found to contain 96 µg amino acid and 11.5 µg tryptophan. Similar types of results were also reported for leaf lectin of *D. volubilis* as that for fruit coat hemagglutinin (Deshpande and Patil, 2002) [11] (Table 2).

Affinity chromatography of purified fraction on native PAGE was resolved in to a single band. Molecular weight of *D. volubilis* fruit coat hemagglutininas determined by SDS PAGE was 25.120 kD which was found to be similar to that of sephadex G 75 gel filtration also (Fig. 2). Similar results for other lectins were observed for WGA with molecular weight to be 25.5 kD (Nagata and Berger, 1972) [31]. *D. volubilis* fruit coat hemagglutinin expressed α and β glucosidase activities. Other lectins such as ricin, abrin and

mistletoe lectin were also reported to possess enzyme activity (Peumans *et al*., 2000) [32]. *D.volubilis* fruit coat hemagglutinin when treated with trypsin shows remarkable decrease in hemagglutanation units and α and β glucosidase activities indicating the presence of two different sites for these activities on the same protein (Table 6).

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Table 1: Purification scheme of fruit coat hemagglutinin of *D. volubils*

Fraction	**HAU/g fruit coat**	**Protein mg/g**	**Specific activity**	**% yield**	**Purification (fold)**
Crude fraction	100570	148.3	678	100	1
0-90% A S P fraction*	50793	7.3	6957	51	10
DEAE cellulose fraction	47890	5.5	8707	47.6	12.8
Affinity chromatography fraction	30781	0.5	61562	31	91

A.S.P. Fraction= Ammonium sulphate precipitated fraction

Table 2: Properties of the *D. volubilis* fruit coat hemagglutinin

Property	**Observation**
Optimum pH	5.0
pH dependence	4.0 to 7.0
Optimum temperature	45ºC
Temperature stability	5 to 55ºC
Inhibitory carbohydrate	α-D-glucose and α-D-mannose
Nature of hemagglutinin	Glycoprotein
Identified carbohydrate moiety	GluNAC
Molecular weight by SDS PAGE	25.12kD
Molecular weight by sephadex G 75	25.12 kD
α-glucosidase	10 U/mL
β-glucosidase	95 U/mL
Metal ion scavenger effect	Inhibitory
Metal ion dependency	Mg^{++}
Effect of trypsin on agglutination	Decrease in agglutination activity
Effect of trypsin on enzyme activities	Decrease in α & β glucosidase activity
100µg hemagglutinin	96 µg amino acid &11.5 µg tryptophan

Table 3: Effect of metal ion chelators and metal ions on activity of *D. volubilis* fruitcoat hemagglutinin

Compound	Inhibition [I] or Activation [A]
EDTA	I
1,10 phenathrolein	I
Mg^{++}	A
Ag^{++}	I
Ba^{++}	I
Ca^{++}	I
Fe^{++}	I
Hg^{++}	I
Mn^{++}	I
Sn^{++}	I

A: Activation; I: Inhibition

Table 4: Inhibition of agglutination of *D. volubilis* fruit coat hemagglutinin by carbohydrates

Carbohydrate	**Inhibitory Concentration (mM)**
α-D-glucose	200
α-D-mannose	200
N-acetyl-D-glucosamine	225
Glucosamine hydrochloride	250
α-methyl-D-glucoside	250
3-O-methyl –D-glucose	250

Table: 5 Agglutination of erythrocytes of various animals by *D. volubilis* fruitcoat hemagglutinin

Animal species	**Untreated erythrocytes**	**Papain treated erythrocytes**
Buffalo	+	+
Bullock	--	--
Chick	+	+
Dog	+	+
Goat	+	+
Guinea pig	+	+
Mice	+	+
Owl	+	+
Rabbit	+	+
Rat	+	+

+ : Agglutination; -- : No agglutination

Table 6:Hemagglutination units and enzyme activities of *D. volubilis* fruitcoat hemagglutinin in presence and absence of trypsin

Activity	**In absence of trypsin**	**In presence of trypsin**
Hemagglutination (human A group RBCs)	30781 HAU/g	15390 HAU/g
α- glucosidase	10 U/mL	5 HAU/mL
β-glucosidase	95 U/mL	46 HAU/mL

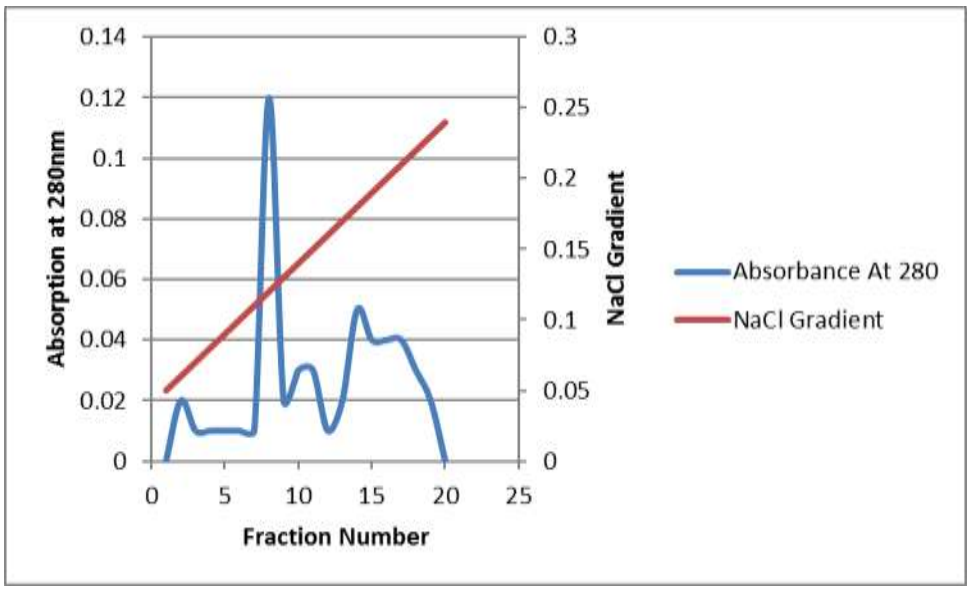

Fig. 1 DEAE cellulose chromatography of *D. volubilis* hemagglutinin

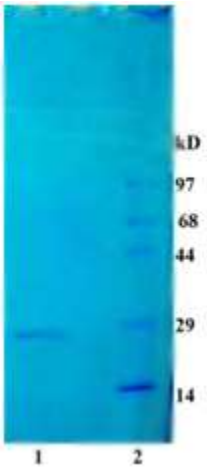

Fig.2. SDS PAGE of *D. volubilis*

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