International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2013): 4.438

A Novel Hemagglutinin from Seeds of *Dregea volubilis*

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Abstract: ***A novel hemagglutinating protein was isolated from seeds of wild medicinal plant Dregea volubilis. The hemagglutinin was purified by conventional purification procedure followed by affinity chromatography on cross linked starch entrapped in agarose beads. The homogeneous preparation on simple PAGE and SDSPAGE expressed little low molecular weight. Mr detected on SDS PAGE was found to be 16 kD and on sephadex G75 it was recorded as 15.48 kD. Optimum pH for agglutinating activity was between pH 4 to7. Optimum temperature for activity was 45^{o} C but the protein was stable from 5 to 55^{o} C. Activity of purified hemagglutinin of D. volubilis seeds was activated by Mg^{++}. The hemagglutinin was identified as glycoprotein in nature with enzymic activity and antiproliferting property towards CPA47 cells.***

Keywords: Dregea, Hemagglutinin, Seed protein, Phytoagglutinin, Plant protein

1. Introduction

Discovery of hemagglutinins dates back to nearly hundred years ago. Hemagglutinins have the specific property to agglutinate erythrocytes or precipitate glycoconjugates (Goldstein, *et al*., 1980) [1]. Peumans and Van Damme [1995] have shown that these proteins possess at least one non catalytic domain that binds reversibly to specific carbohydrates [2]. Hemagglutinins are widely distributed in plants, animals and microorganisms (Goldstein and Heys, 1978; Dey *et al*., 1982a) [3, 4]. Hemagglutinins present on the roots of leguminous plants play important role in symbiosis and act as mediator between the roots and symbiotic bacteria (Bohlool, 1974) [5]. Wide varieties of seeds have been investigated for presence of hemagglutinins (Liener, 1976; Pusztai and Watt, 1974; Osborn, *et al*., 1985) [6-8]. Many hemagglutinins have been shown to be bifunctional in nature as they possess the property of both, agglutination and enzyme activity (Hankins and Shannon, 1978; Dey, 1984; and Dey *et al*., 1982b) [9-11]. This paper describes the methods used for purification of a novel hemagglutinin isolated from seeds of wild plant *Dregea volubilis* and determination of some of the important physicochemical properties.

2. Materials and methods

Materials

Mature dry seeds were collected from fruits of *Dregea volubilis* plant growing in local region. Seeds were dried under the controlled condition, removed any dirt or other fruit remnants by straining through the strainer and stored in brown air tight glass bottles until use. *D. volubils* was identified by the taxonomists of University Department of Botany, RTM Nagpur University, Nagpur. Voucher specimens were preserved in the herbarium.

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.

Methods

Seeds were rinsed twice in double distilled water to remove any dirt and crushed in 20 mM sodium phosphate buffer, pH 7.0 containing 100 mM sodium chloride (PBS1) using motor driven mixer at low speed to avoid frothing. The slurry was kept on shaker at 100 rpm for 2h for complete extraction of proteins. The slurry was then passed through muslin cloth to remove the debris. The filtrate was centrifuged at 5000 rpm at cold condition for 20 min (Remi C24) to remove remaining debris before the supernatant was subjected to further purification procedure (Deshpande and Patil, 2002) [12].

Preparation of erythrocytes

Fresh blood of all the blood groups was collected from healthy donors in heparinized tubes from the out patients visiting the clinical biochemistry laboratory of University Department of Biochemistry, R.T.M. Nagpur University, Nagpur, and used for preparation of papain treated erythrocytes as suggested earlier. Papain treated erythrocytes were used fresh for accurate results (Deshpande and Patil, 2002) [12].

Purification of hemagglutinin

Suitable aliquots of crude extract were subjected to ammonium sulphate precipitation by the method of Patil and Shastri [1985] [13]. The 0-90% precipitate after dialysis was loaded on to DEAE cellulose column by the method described by Deshpande and Patil (2002) [12]. Further purification of the hemagglutinin was performed by affinity chromatography on cross linked starch entrapped in agarose beads as per the method of Sawhney *et al*., [1988] [14]. All purification procedures were carried out at 4^{o}C unless otherwise mentioned.

Homogeneity of the Sample

This was tested by simple PAGE followed by SDS PAGE as described by Laemmli [1970] using Tris-glycine buffer, pH 8.3 with a constant current of 15 mA for 90 min. Gels were stained by Coomasssie brilliant blue for 45 min and destained in 7.5 % acetic acid at 37^0C. Destaining was repeated at every 30 min interval till the color was removed from the gels. [15]. Homogeneous purified *Dregea volubilis* seed hemagglutinin was designated as DVSH.

Molecular weight determination: Molecular weight of the DVSH was determined on SDS PAGE and by gel filtration chromatography on sephadex G-75 using BSA- 66kD, Pepsin- 34.7 kD, Trypsinogen- 24 KD and Lysozyme – 14.3 (Patil and Shastri, 1985) [13].

Protein estimation: Protein estimation was performed by the method of Lowry *et al.*, [1951] using fat free BSA as standard protein using Gilford spectrophotometer (Germany) [16].

Carbohydrate estimation: This was performed by the method of Dubois *et al.* [1956] using D- glucose as standard [17].

Agglutination inhibition assay: This was performed as suggested by Kurokawa *et al.* [1976] [18]. Various pentoses, hexoses, di and tri saccharides along with glucose derivatives were used for agglutination inhibition assay. Inhibitory concentration of carbohydrate was determined as the concentration that prevents the RBC to agglutinate by DVSH.

pH stability: pH dependence of DVSH was determined by incubating DVSH with buffers ranging from pH 1.0 to 10 as per the method of Suseelan *et a*l. [1997] with suitable controls [19].

Temperature stability: DVSH was mixed in PBS2 (20 mM sodium phosphate buffer pH 7.0 containing 0.9% sodium chloride) for determination of effect of temperature on hemagglutination activity. Tubes containing equal quantity of PBS2 and DVSH containing 4.5 µg protein were exposed to the temperature ranging from 5, 15, 25, 35, 45, 55, 65, 75, 85, and 95^o C. Suitable controls were run simultaneously and the activity was compared with the expression of HAU by DVSH at 37^{o}C [Suseelan *et al*. 1997][19].

Metal ion dependency of DVSH: This was determined by the method of Kawagishi *et al*. [1990] by chelating the metal ions initially with EDTA and then incubating the demetalized DVSH with various metal ions as described elsewhere[20].

Blood group specificity of DVSH: This was detected as described by Deshpande and Patil [2002], using 2 % suspension of papain treated erythrocytes of all the blood groups [12].

Agglutination of erythrocytes of different animals: DVSH was checked for the agglutination with erythrocytes of buffalo, bullock, chick, dog, goat, guinea pig, mice, rabbit, rat and owl [Deshpande and Patil, 2002] [12].

Determination of α and β glucosidase activity: α and β glucosidase activity of DVSH 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 that liberates one micromole of p - nitrophenol per min under experimental conditions [21].

Thin layer chromatography of DVSH: Major carbohydrate content of DVSH was determined by TLC [Upadhyay *et al*. 1997] using various carbohydrate as standards. R_f values were determined by the formula: R_f = The distance travelled by the solute/ The distance travelled the solvent [22].

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 [23].

Estimation of Tryptophan in DVSH: Tryptophan content in DVSH was measured by the method of Spande and Witkop [1967] using pure tryptophan as standard amino acid [24].

Effect of trypsin: DVSH was treated with 1% trypsin for 2h at 37^{o}C. Agglutinating assay of trypsin treated DVSH was performed as described by Deshpande and Patil, 2002 [12].

Cell proliferation assay: Cytotoxic activity of DVSH was performed by using CPA47 cells by colorimetric measurement of MTT reduction [25]. Various dilutions of DVSH fraction (25, 50, 100 µg/ml) were added to culture medium and assessed against growth CPA47 cells. Since reduction of MTT can occur only by metabolically active cells, the level of activity thus can be considered as a measure of viability of cells [26].

Statistical analysis: All results were statistically analyzed by the method described by Walpole [1982] [27]. P value was set at < 0.05.

3. Results and Discussion

Dregea volubilis seed hemagglutinin was purified to homogeneity with good yield as presented in Table 1 and Fig. 1 & 2. The hemagglutinin was purified to nearly 92 fold with good yield of 33% by conventional purification procedures. Similar types of results were also reported by Deshpande & Patil for the lectin isolated from leaves of the same the plant [12]. The hemagglutinating activity of DVSH was inhibited by α-D-glucose and α-D-mannose indicating the nature of hemagglutinin to be glucose specific (Table 2). Similar results were also shown by WGA [28], rice lectin [29], and Con A [30] which are also α-D-glucose and α-D-mannose specific hemagglutinins.

SDSPAGE shows the protein to be monomeric and glycoprotein in nature. The optimum pH for the agglutination was found to be in between 4 to 7.

Temperature stability shows that the purified hemagglutinin is stable from 5°C to 55°C with the temperature optimum at 45°C. The same nature of hemagglutinin with leaf lectin was observed by Deshpande and Patil, 2002 [12]. The hemagglutinin of seeds of *D. volubils* agglutinates erythrocytes of all the human blood group erythrocytes with same efficiency. Similarly, the hemagglutinin agglutinates the erythrocytes of all the animals except bullock indicating that probably the binding receptors are absent on the erythrocytes of bullock (Table 3). The hemagglutinin is shown to be glycoprotein in nature where the GluNAc probably is the carbohydrate moiety present with the protein. Glycoprotein types of hemagglutinin are also reported for other plants as mentioned by other authors. Enzyme type of hemagglutinins are not widely observed, however, few purified hemagglutinins have been reported to have both agglutination and enzyme activities (Deshpande and Patil, 2002) [12]. DVSH shows both types of α- and β-glucosidase activities. The amino acid content is 94 µg in 100 µg protein in which 11.2µg tryptophan content was determined. DVSH 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 4).

DVSH lost both enzymic and hemagglutinating activity on treatment with metal ion scavengers, but activity was restored to its original in presence of Mg^{++}. Similar type results were also observed by Deshpande and Patil with leaf lectin of *D. volubilis* [12] (Table 5).

Fig. 3 shows the antiproliferative activity of DVSH. As the concentration increases the proliferation of CPA47 cells decreases (IC_{50} =67 µg/ml, R^2 =0.94) showing that DVSH has effect at the cell cycle level. Similar type of cytotoxic activity was also recorded with Con A [30] and other lectins as mentioned by Deshpande and Patil, 2002 [12].

Thus, the DVSH is unique in properties. It is a glycoprotein hemagglutinin containing N acetyl glucosamine as a carbohydrate moiety. It shows enzymic activity along with antiproliferating properties also, making this protein to be really unique and novel.

4. Acknowledgement

Authors would like to thank The Head, University Department of Biochemistry, R.T.M. Nagpur University, Nagpur, for laboratory facilities and encouragement.

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Table 1: Purification of *Dregea volubils* seed hemagglutinin

Fraction	*HAU/g seeds*	*Protein (Mg/g seed)*	*Specific activity*	*% yield*	*Purification fold*
Crude fraction	70150	137	512	100	1
0-90% ASP	36537	6.6	5573	52	10.7
DEAE Cellulose fraction	30500	4.3	7093	43.4	13.8
Affinity fraction	23578	0.5	47155	33.6	92

ASP = Ammonium sulphate precipitate

Table 2: Inhibition of agglutination of *D. volubilis* seed hemagglutinin by various carbohydrates

Carbohydrate	**Inhibitory** Concentration (mM)
α-D-glucose	200
α-D-Mannose	200
N-acetyl-D-mannosamine	225
Glucosamine hydrochloride	250
α-Methyl-D-glucoside	250
3-O-methyl-D-glucose	250

Table 3: Agglutination of erythrocytes of various animals by seed hemagglutinin of *D. volubilis*

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

+ = agglutination; -- = No agglutination

Table 4: Properties of the *D. volubilis* seed hemagglutinin

Property	**Observation**
Optimum pH	4 to 7
pH dependence	4 to 7
Optimum temperature	45°C
Temperature stability	5 to 45°C
Inhibitory carbohydrate	α-D-glucose and α-D-mannose
Nature of hemagglutinin	Glycoprotein
Identified carbohydrate moiety	GluNAC
Molecular weight by SDS PAGE	16 kD
Molecular weight by Sephadex G 75	15.484 kD
α-glucosidase	20 U/mL
β-glucosidase	100 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 5: Effect of metal ion chelators and metal ions on activity of *D. volubilis* seed hemagglutinin

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

A = activation; I = inhibition

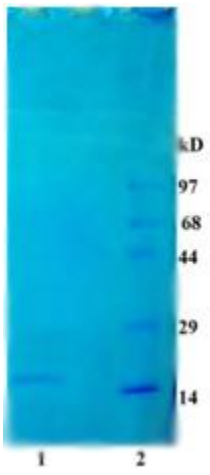

Figure 1: SDSPAGE of *Dregea volubilis* seed hemaglutinin

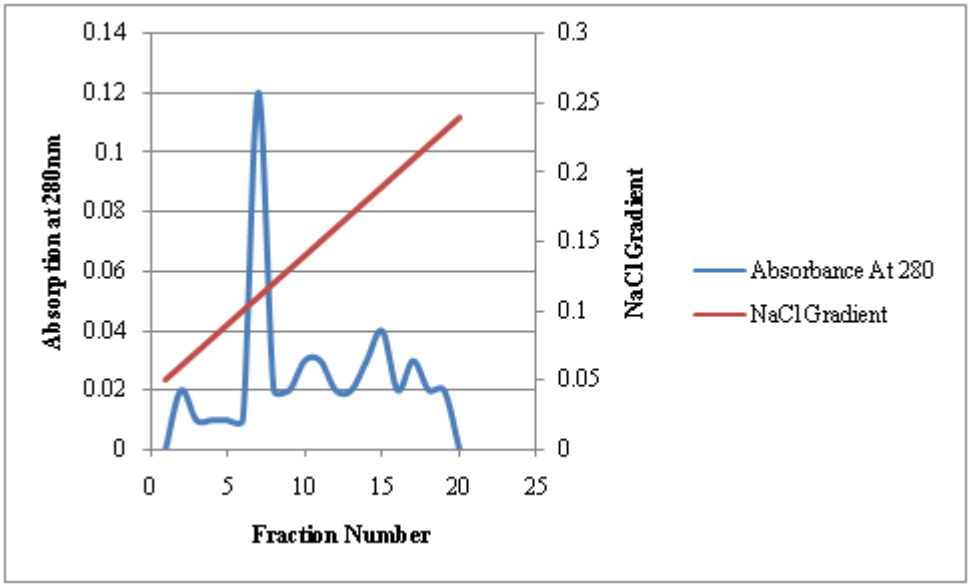

Figure 2: DEAE cellulose chromatography of *D. volubilis* seed hemaglutinin showing elution pattern.

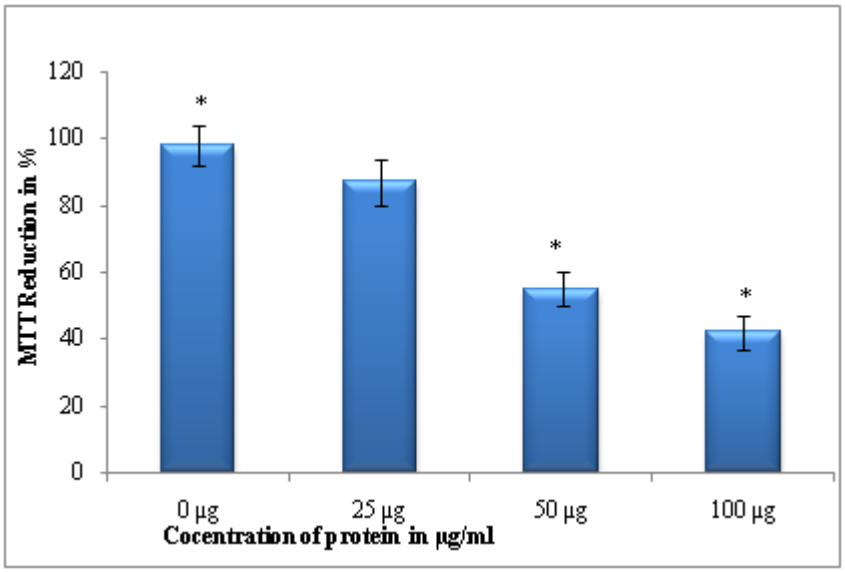

Figure 3: Antiprolifertive activity of hemagglutinin of *D. volubilis* seeds with CPA47