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PURIFICATION AND CHARACTERIZATION OF *Tridax procumbens* CALYX LECTIN

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ABSTRACT

The calyx of the *Tridax procumbens L.* was found to contain galactose specific lectin. The lectin was purified to homogeneity by affinity chromatography and was found to have molecular weight of 23 kD. The lectin agglutinated papain treated erythrocytes of human blood group 'A' 'B' and 'O', rabbit and other animals. The lectin was stable between pH 4 to 12 for agglutination. The lectin did not require any metal ion for agglutination, but inhibited by Hg^{++} ions. The agglutination was inhibited by α - D galactose (0.1M) and galactose derivatives. It was found to exhibit α and β - galactosidase activities. The result showed clearly the presence of lectin and galactosidase activities by the same molecule indicating the protein to be bifunctional in nature.

Key words: Lectin, *Tridax procumbens*, galactosidase and calyx.

INTRODUCTION

Lectins are known to be the proteins of nonimmune origin. They have the ability to agglutinate cells or precipitate glycoproteins. Lectins are widely distributed and are found in plants, microorganisms and animals[1,2] Legume lectins have been broadly studied by various researchers, but very few reports are available on herbal lectins. Lectins are also found to be associated with enzymatic activity and agglutination activity[3]. Peumans and Van Damme (1995) worked on lectins and proposed that ''A lectin should posses atleast one non-catalytic domain that binds reversibly to specific carbohydrate", showing a bifunctional nature of lectin[4]. Lectins have the ability to recognize specific *rhizobium* species and induce the root nodulation[5]. In the present investigation isolation and characterization of *T. procumbens calyx lectin* has been presented. Most purified lectins are used as the reagents for characterization, isolation and analysis of simple carbohydrates or glycoconjugates[6]. Lectins are also used in various biomedical reasearch and even in targeted drug delivery system[7].

MATERIAL AND METHODS

Calyx of the plant *Tridax procumbens* L, a wild medicinal plant of the family compositeae, was used as the source of lectin[8]. Papain, BSA, guar - gum, D - glucose, D - galactose, sucrose, raffinose, N - Acetyl - D - glucosamine, D - glucosamine hydrochloride, α - pNPG, β - oNPG, Acrylamide, Bis - acrylamide, β - mercaptoethanol, coomassie brilliant blue (R 250), were obtained from Sigma chemicals, St Louis, M.O., U.S.A. Other chemicals were of analytical grade.

Preparation of crude extract

Calyx of 45 days old plants (100 g) grown in the garden of University Department of Biochemistry, Nagpur University, Nagpur, India[8], were collected, washed four times under the tap water and two times with distilled water. After soaking between the folds of filter paper, they were homogenized in 1L of 1M NaCl and kept on shaker at 4°C for 2 hours, to ensure proper extraction of the lectin. The extract was passed through two layers of cheese cloth and the filtrate was centrifuged at 12000 rpm (9668g) at 4°C for 30 min. The supernatant obtained, designated as crude extract, was used for isolation of lectin.

Ammonium sulphate precipitation

The lectins were precipitated from the clarified crude extract solution by adding ammonium sulphate at 4°C. The precipitate obtained between 20 to 60% saturation was collected by centrifugation at 12000 rpm (9668g) for 30 min. The precipitate was dissolved in extraction solution and dialyzed against the same till the solution was free from carbohydrates[9]. The dialyzed solution was designated as ammonium sulphate fraction (ASF).

Affinity chromatography

The ASF was subjected to affinity chromatography on cross linked guar gum column previously equilibrated with 1M NaCl[10]. The unbound proteins were washed thoroughly with 1M NaCl till the eluate showed no absorbance at 280 nm. The bound proteins were eluted from the column by the extraction solution containing 0.1M galactose. The flow rate was adjusted to 5ml/10min using fraction collector and peristaltic pump of LKB – Pharmacia (Sweden). Fractions of 5 ml each were collected and checked for proteins at 280 nm and subjected to dialysis against 1M NaCl till the galactose was removed and checked for agglutination activity[11]". The proteins in the fraction showing agglutination were precipitated to 60% saturation by ammonium sulphate. The precipitate was solubilised and dialyzed against 1M NaCl and was used for detection of the purity on SDS – PAGE. The purified fraction was designated as TPL – C (*Tridax procumbens Lectin* from *Calyx*).

Polyacrylamide Gel Electrophoresis

The homogeneity of TPL – C was tested on SDS – PAGE. Molecular weight of the purified lectin was determined by the method of Weber and Osborn (1969). Acrylamide concentration for the running separator gel was 7.5% and that of the stacking gel was 2.5%, marker proteins such as Carbonic anhydrase 29kD, Trypsin 23kD, Myoglobin 17.2kD, Cytochrome C – 12.3kD, were used as standard proteins. After electrophoresis the gels were stained with 1% coomassie brilliant blue (R – 250) prepared in destaining solution containing 7% acetic acid. The gels were destained in 7% acetic acid[12,13].

Protein Estimation

Proteins were estimated by the method of Lowry *et al.,* (1951). Bovine serum albumin was used as standard protein[14].

Colorimetric determination of carbohydrates in TPL - C was carried out by the method of Dubois (1956) using α- D - glucose as standard.

Agglutination assay

Agglutination assay was carried out by the method of Deshpande & Patil (2001), using 2% suspension of papain treated human and rabbit erythrocytes. The haemagglutination activity was determined by using serial dilution in 96 u well plate. Agglutination was observed visually and also examined microscopically. The reciprocal of the last dilution showed detectable agglutination was taken as titer strength of the lectin and was expressed as haemagglutination units (HAU). Specific activity (SA) was expressed as haemagglutination units per mg proteins[17].

Agglutination Inhibition Assay

Agglutination inhibition assay were carried out using the method described by Kurokawa *et. al.,* (1976) various hexoses, pentoses, disaccharides, oligosaccharides and glycoproteins etc. were used for agglutination inhibition assay. Agglutination in the presence of carbohydrates was carried out by the method described above. The minimum inhibitory concentration was taken as one which did not agglutinate the erythrocytes.

pH stability

pH stability was determined by exposing the lectin to the buffer from pH 1 to 13 , for pH 1 - 0.1.N HCl, for the pH 2 and 3 - 0.2M glycine - HCl buffer, for pH 4 and 5 - 0.2M sodium acetate buffer, for pH 6 and 7, 0.2M sodium phosphate buffer, for pH 8 - 0.2M Tris - HCl buffer for pH 9 - 0.2M glycine - NaOH buffer, for pH 10, to 13 - 0.2M carbonate - bicarbonate buffer were used. 100 µl lectin solution and 100 µl buffer solution were incubated for 1 hour at 37°C[19]. Agglutination assay was carried out by the method as described earlier.

Table - 1: Purification chart of *T. procumbens calyx lectin* (TPL-C)

Purification	Vol (ml)	Proteins (mg/ml)	HAU/ml 'A'	HAU/ml 'B'	HAU/ml 'O'	S A 'A'	S A 'B'	S A 'O'	Purification fold w.r.t. 'O' erythrocytes	Yield %
Crude Solution	1000	8.2	80	40	640	9.75	4.87	78.5	1	100
Ammonium sulphate fraction	25	3.2	320	160	2560	100	50.7	806	10.25	66.6
Affinity chromatography fraction	15	0.30	640	320	5120	2134	1066	17067	21.33	66.7

HAU – Haemagglutination Unit; SA – Specific activity

Table - 2: Agglutination of human and animal erythrocytes by *T. procumbens calyx lectin* (TPL-C)

Erythrocytes	No. of samples	Agglutination of Papain Treated	Erythrocytes Non Treated
Human 'O'	100	++++	-
Human 'A'	100	++	-
Human 'B'	100	+	-
Rabbit	5	++++	-
Hen	5	+	-
Cock	+	+	-
Goat	5	+	-
Dog	5	+	-
Buffalo	3	+	-
Bullock	2	+	-
Cow	2	+	-
Guinea pig	2	+	-
Sheep	2	+	-

TPL-C - *T. procumbens Calyx Lectin,*
Agglutination (+), No Agglutination (-)

Effect of temperature and thermal inactivation

Effect of temperature and thermal inactivation on agglutination activity of lectin solution was checked by adding 30 ml lectin solution to 1 ml 0.006 M sodium phosphate buffer, pH 7, and incubated at 30, 40, 60 and 80°C for 1 hour and agglutination assay was carried out at 37°C after cooling. Effect of temperature on stability was checked by heating 250 µl lectin solution and 250 µl, 25mM sodium phosphate buffer pH 7 at 37°C in a multi-block heater. Aliquots were withdrawn and assayed for agglutination after 20, 40, 60, 80 and 100 min[19].

Effect of metal ions on agglutination activity

This test was carried out by the method of Kawagishi *et al.* (1940). 100 µl lectin solution was added to 400 µl of 10 mM EDTA and incubated at 4°C for 1 hour. The mixture was dialysed against 25 mM sodium phosphate buffer pH 7 for 20 hours.

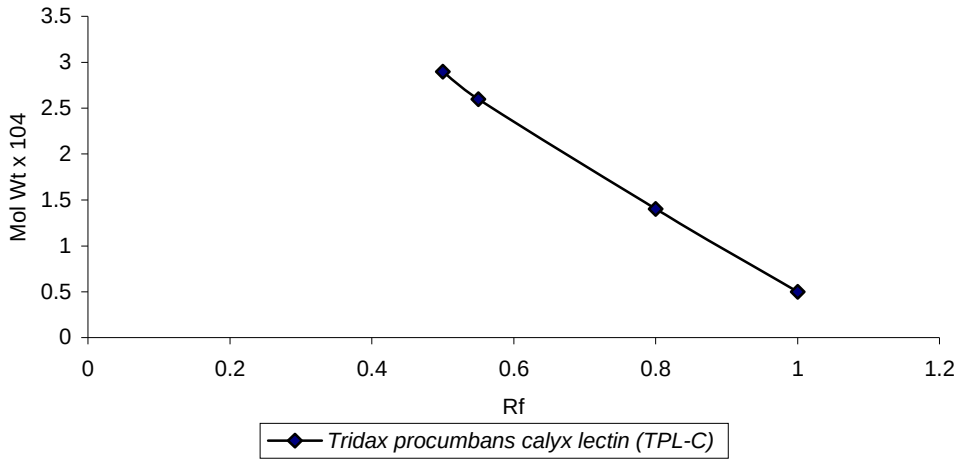

Fig. - 1: Electrophoretic mobilities of standard proteins and *T. Procumbens* calyx lectin (TPL-C). Acrylamide concentration. 7.5%, standard proteins a) carbonic anhydrase, b - Trypsin and T.procumbans calyx lectin (TPL-C (c) Myoglobin, (d) Cytochrome C,

Effect of metal ions on agglutination activity was checked by adding 50 µl of 0.1.M metal ion solution to 50 µl of demetalised lectin solution and incubated at 37°C for 1 hour. Agglutination assay was carried out as described earlier. Suitable controls were run simultaneously.

α and β galactosidase activity

The method of Murray *et al.,* (1983) was used to determine α and β galactosidase activity using 3mM α - pNPG and β - oNGP as the substrate. The assay mixture contained 20 to 100 µl of enzyme solution and 3mM substrate prepared in 0.1M sodium acetate buffer, pH 4.7. The reaction mixture was incubated at 40°C for 40 min. The reaction was stopped by adding 0.2M Na-Carbonate. The liberated nitrophenol was measured at 400 nm. Lectin was also inhibited by α - D - galactose and checked for galactosidase activity by the method of Deshpande and Patil (2003).

Effect of trypsin

100 ml lectin solution was mixed with 100 ml of 1% trypsin and incubated at 37°c for 1 hour. The tubes were placed in boiling water bath for 30 min for inactivation of trypsin. Agglutination activity and α and β galactosidase activities were measured as described earlier running suitable control and trypsin standards, Deshpande and Patil (2003).

RESULTS

The lectin agglutinated papain treated human and rabbit erythrocytes and was inhibited by α - D galactose indicating that the lectin is galactose specific, which could be purified in good yield by affinity chromatography on cross linked guar - gum. The purified lectin on SDS - PAGE exhibited little low molecular weight of 23kD as shown in Fig. - 1 and 2. Fig. -1 indicates a calibration curve of the standard proteins for determination of molecular weight of TPL - C. Electrophoresis was carried out by the method of Weber and Osborn (1969). The proteins were run in electrophoretic field for 3 hours in presence of β - mercaptoethanol. Molecular weight markers were used as standard proteins.

TPL - C showed a single band (Fig. - 2). The band appears within the bands of the standard molecular weight marker bands corresponding to the molecular weight of the protein to be 23kD indicating the homogeneity of the preparation of TPL - C.

Physical and chemical properties: Agglutination

The TPL - C agglutinated erythrocytes of blood group 'A', B and 'O' and animal erythrocytes. Agglutination of erythrocytes of group 'O' was predominant than that of blood group 'A' and 'B'. Agglutination was enhanced when the erythrocytes

Table - 3: Inhibition of agglutination with various sugars by *T. procumbens* calyx lectin (TPL – C)

Sugars	Minimum concentration required to Inhibit the hemagglutination (mM)
α-D Glucose	NI
D Fructose	NI
D Mannose	NI
D Maltose	NI
D Galactose	100
D Mannitol	NI
D Arabinose	NI
D Xylose	NI
Sorbitol	NI
D Ribose	NI
Sucrose	NI
Lactose	100
Raffinose	250
D Glucosamine hydrochloride	NI
N acetyl glucosamine	NI
p-nitrophenyl α- D galactopyranoside	3
o-nitrophenyl β- D galactopyranoside	3

NI - No Inhibition

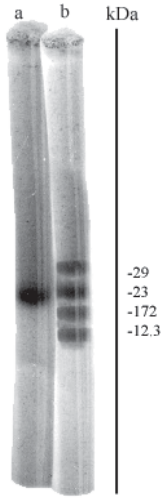

Fig. - 2 : SDS-PAGE of *T. procumbens calyx lectin* (TPL-C) (a) and Standard proteins (b)

were treated with papain for proteolytic enzyme treatment (Table -2).

Inhibition of agglutination with sugars

The lectin activity of TPL - C was inhibited by α - D - galactose and D - galactose derivatives (Table - 3), D galactose, lactose could inhibit agglutination activity at 100 mM, α - pNPG and β - oNPG, at 3mM and raffinose at 250 mM. The other sugars like α - D glucose, D - mannose, D fructose, D maltose, D mannitol, D - arabinose, D - xylose, D - sorbitol, D - ribose, sucrose and glucosamine derivatives did not inhibit it.

pH stability

Effect of pH on activity of TPL -C was checked. The optimum pH for agglutination activity was pH 7, while it was observed that the lectin activity was lost at a pH below 4 and pH above 12 as shown in the Fig. -3.

Thermostability and thermal inactivation

TPL - C was found to contain 100% agglutination activity when the lectin was heated from 37°C to 80°C for 1 hour (Fig. - 4) and from 20 to 100 min at 37°C (Fig. - 5) indicating the lectin to be thermostable.

Effect of metal ions (0.1mM)

Table 4 shows the effect of metal ions on agglutination by TPL - C when the demetalized TPL - C was treated with metal ions and checked for agglutination, it was observed that in the presence of heavy metal ions like Hg^{++}, the lectin was unable to agglutinate the erythrocytes.

α and β galactosidase activity

The TPL - C was found to exhibit α and β galactosidase activity along with agglutination activity with the substrates α - pNPG and β - oNPG. The α galactosidase activity was major as compared to the β galactosidase activity.

Michaelis constant

The Michaelis constant (Km) for α - galactosidase for α - pNPG was 0.30 mM for

Table - 4: Effect of metal ions (0.1M) on agglutination by *T. procumbens calyx lectin* (TPL – C)

Metal ions (1mM)	Agglutination of Erythrocytes
Mn^{++}	+
Mg^{++}	+
Ca^{++}	+
Hg^{++}	+
Al^{+++}	+
Fe^{+++}	+
Co^{++}	+
Zn^{++}	+

Agglutination (+), No Agglutination (-)

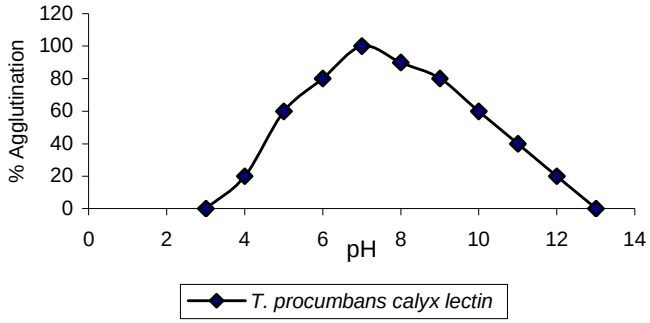

Fig. - 3 : Effect of pH on agglutination by *T. procumbens* calyx lectin (TPL - C)

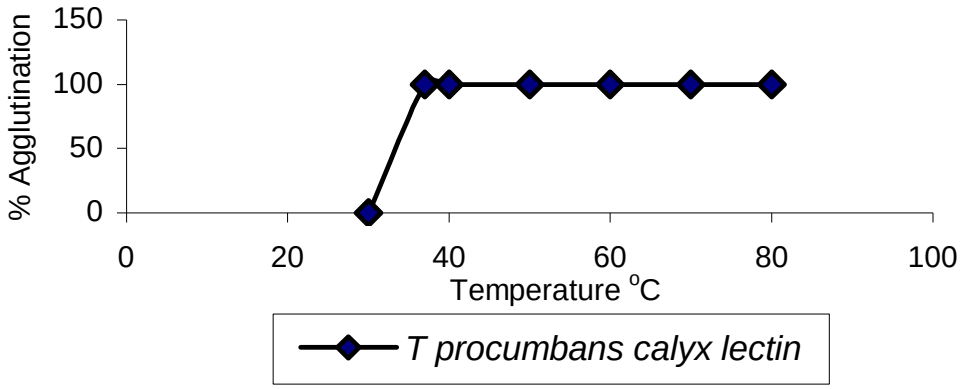

Fig. - 4: Effect of temperature on agglutination by *T. procumbens* calyx lectin (TPL-C)

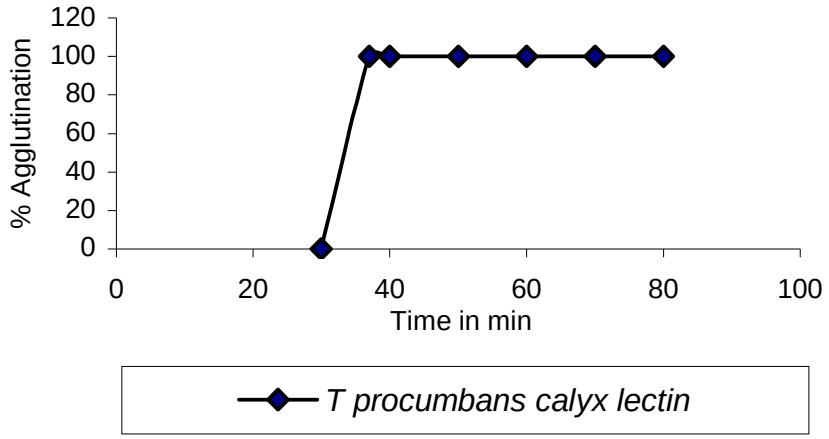

Fig - 5: Thermal inactivation on agglutination by *T. procumbens* calyx lectin (TPL-C)

TPL – C. The km for β –galactosidase for β – oNPG was 0.40 mM for TPL – C. In the presence of trypsin the enzyme activity was decreased but no loss of agglutination was found.

DISCUSSION

A lectin from the calyx of *T. procumbens L.* was purified to homogeneity by ammonium sulphate precipitation and affinity chromatography on cross-linked guar gum. The affinity chromatography purified lectin was found to be homogenous as it exhibited a single band on SDS - PAGE. The lectin gave a single band expressing itself to be a monomeric lectin as found in the *Vigna mungo* (black gram) as reported by Suseelan *et al.,* (1997). Molecular weight of TPL - C was found to be 23kD when compared with the standard proteins on SDS - PAGE. Similar results were reported by Nagata and Burger (1972) for *wheat germ agglutinin* with a molecular weight of 23.15 kD.

Hemagglutination units (HAU) for erythrocytes of all the three blood groups 'A', 'B', 'O', were calculated, as shown in Table - 1, HAU obtained with 'O' group erythrocytes were used throughout the study as TPL - C manifested maximum activity with 'O' group erythrocytes. TPL -C was able to agglutinate papain treated erythrocytes of human 'A', 'B', 'O'. *Ptilota filicina L*, a galactose specific lectin also agglutinated papain treated erythrocytes of human 'A', 'B', 'O', (Sampioa *et al.,* 1998). TPL - C was unable to agglutinate untreated erythrocytes.

The purified lectin was inhibited by galactose and galactose derivatives indicating the

lectin to be galactose specific. Suseelan *et al.,* (1997) reported *Vigna – mungo* (black gram) lectin to be galactose specific. The optimum pH for TPL – C was found to be 7.0, indicating the lectin to be stable at neutral pH. TPL – C lost its agglutination activity below pH 4 and above pH 12. Similar results were reported for E*rythrina veluntina and Forma aurantica*, where the lectin exhibited optimum pH of 7.5 for agglutination as reported by Moraes *et al.,* (1996).

TPL - C could not loose its agglutination activity when heated at 80°C for 1 hour, indicating the lectin to be thermostable. Similarly lectins from *Vigna mungo* (Suseelan *et al.,*1997) and *Erythrina veluntina and Forma aurantica* (Mores *et al*.,1996) were also stable at 70°C.

EDTA treated TPL – C was inhibited by Hg^{++}. Similarly *sea mussel Crenomytilis gryans* showed agglutination inhibition in the presence of Mg^{++} and Ca^{++} ions (Belogortsea *et al.,* 1998).

TPL – C exhibited α and β galactosidase activity with α - pNPG and β – oNPG. In the presence of galactose, the lectin did not loose the enzyme activity hence it can be stated that the lectin posses an enzyme activity as well as agglutination activity. Similar property was obtained by Suseelan *et al.,* (1997) with V*igna mungo lectin*. The α galactosidase activity was predominant than β galactosidase in TPL - C suggesting to have a bifunctional property. Lectin from *mung beans*, (Hankins and Shannon (1978), (Dey 1984), *Vicia faba seed lectin* (Dey et al., 1982) showed A galactosidase activities. *Black gram lectin* (Singh and Rao, 1991) showed B galactosidase activities, whereas Sharma and Salahuddin (1993) reported *black gram lectin* without any galactosidase activity.

In the presence of trypsin the enzyme activity was decreased and it agglutinated the erythrocytes indicating that the lectin contained an agglutinin site and an enzyme active site, which were situated away from each other on the same molecule, and both the sites were not affected by the trypsin action. Thus TPL – C was found to exhibit both enzymatic and agglutination activity as reported by Hankins and Shannon (1978) for *mung bean lectin*.

TPL - C was able to agglutinate human erythrocytes of 'A', 'B', and 'O' indicating the lectin to be "non - specific lectin". Lectins like *wheat germ agglutinin* and *black gram lectin* were also shown to be nonspecific as studied by Nagata and Burger (1972) and Suseelan and Mitra (2001). TPL - C was able to agglutinate papain treated erythrocytes of animals such as Rabbit, Hen, Cock, Goat, Dog, Buffalo, Bullock, Cow, Guinea Pig and Sheep. Agglutination with rabbit blood cells was predominant than the red blood cells of other animals as shown in

Table - 2. Similar studies were reported for agglutination of different animals like Mouse, Rat, Cat, Chicken, Sheep, Pig, Horse, Guinea Pig, Ox and Fishes etc. with the lectin from *Ricin* and *Abrin* as studied by Gold and Balding (1975) and *Phaseolus vulgaris* by Pusztai and Watt (1974). Thus the calyx lectin from *T. procumbens L* appears to be unique with respect to pH and temperature stability and its bifunctional nature.

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