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An isolectin complex from *Trichosanthes anguina* seeds

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Abstract

Sepharose 4B affinity chromatography of *Trichosanthes anguina* seed extract and subsequent elution with galactose resulted in the isolation of an apparently single lectin with molecular weight of $45{,}000 \pm 700$. However, major amount of the hemagglutinating activity was recovered as unadsorbed protein fraction. High affinity matrix Lactamyl Seralose could retain most of the galactose specific lectin activity from fraction 'A' which was eluted with lactose. It is evident from PAGE and SDS-PAGE analysis of the purified protein that *T. anguina* seeds contains a mixture of isolectins ranging in molecular weight from 30,000 to $50{,}000 \pm 1300$. Periodic Acid Schiff's staining of the gels revealed this lectin complex to be a combination of glycosylated and non-glycosylated lectins. Two Isolectins SLc and IEL from within this complex have been isolated by affinity and ion exchange chromatography respectively. Apparent homology of these two lectins is indicated by their identical molecular weight (45 kDa), sub unit composition, non glycoprotein nature and immunological identity. However, these two lectins show minor differences in their biological and physicochemical properties. The peptide maps of the two lectins obtained after digestion with Trypsin and Pronase E also indicate minor changes in the primary structure.

Keywords: *Trichosanthes anguina*; Cucurbitaceae; Galactose specific isolectins; Lactamyl Seralose

1. Introduction

Lectins are carbohydrate binding proteins, predominantly isolated from seeds of leguminous plants (Etzler, 1985), though they are also present in other vegetative tissues. In the family Cucurbitaceae, seed lectins have been purified from *Momordica charantia* (Barbieri et al., 1980), phloem lectins from *Cucurbita maxima* (Sabnis & Hart, 1978). Purification of isolectins from root tubers and a seed lectin from *Trichosanthes kirilowii*, a Chinese Cucurbit, have been reported (Yeung, Wong, Ng & Li, 1986; Yeung, Wong, Ng, Wong & Li, 1986; Falasca et al., 1989; Dong, Ng, Ricky, Yeung & Xu, 1993). A number of legume and other seeds contain isolectins, which exhibit differences in structural and biological properties (Sharon & Lis, 1989). These isolectins differ in blood group specificity (Lis, Latter, Adar & Sharon, 1988), carbohydrate specificity (Rutherford, Dick, Cavine, Dombrink-Kutzman & Sladki, 1986), charge (Hoedmacker, Richardson, Diaz, Pater & Kyne, 1994) and differences in amino acid sequence of the isolectins (Richardson, Rouge, Sousa-Cavada & Yarwood, 1984). Ghosh, Dasgupta and Sircar (1981) have reported the purification of two isolectins from *Trichosanthes anguina* seeds, while Komath, Nadimpalli and Swamy (1996) have reported the isolation of a single seed lectin from *T. anguina*.

Present work describes the isolation and physicochemical characterization carried out to detect similarities and differences in terms of glycosylation, primary structure and biological properties of two isolectins (SLc) and (IEL) from a complex of isolectins (LSL) from *T. anguina* seeds.

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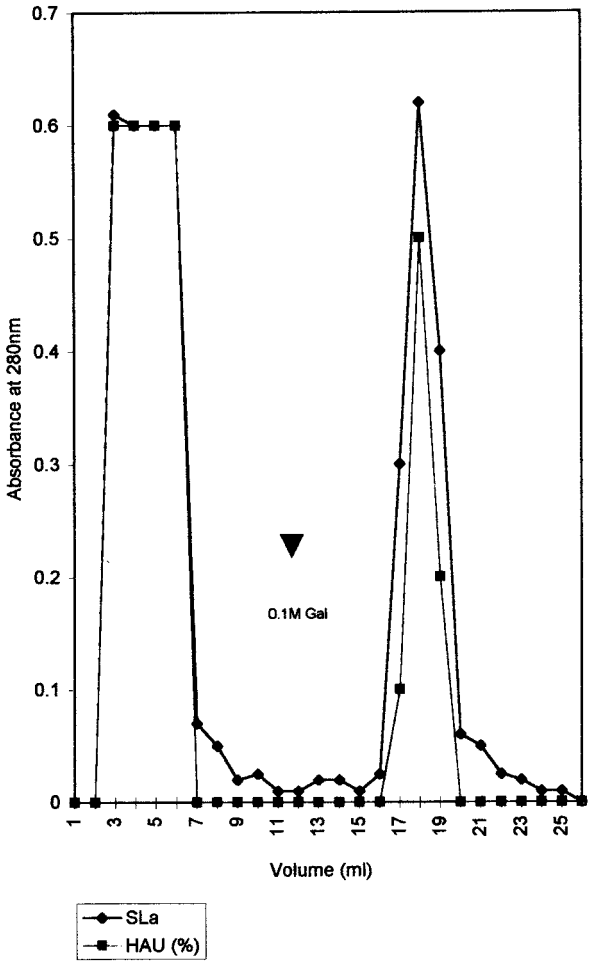

Fig. 1. Affinity chromatography of *T. anguina* fraction 'A' on Sepharose 4b column: 3 ml fractions were collected and monitored for protein at 280 nm. Hemagglutination Activity Units are represented as ×100% activity, with the highest value set at 100%.

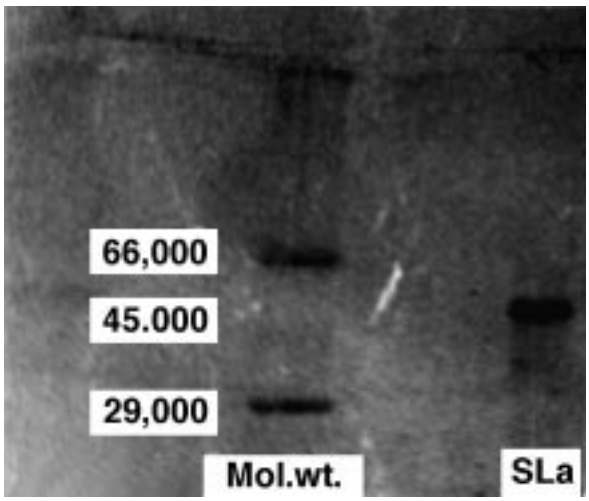

Fig. 2. SDS — Alkaline polyacrylamide gel electrophoresis at pH 8.9: SLa protein (10 μg) was run on a 10% gel, protein bands were stained with Coomassie blue.

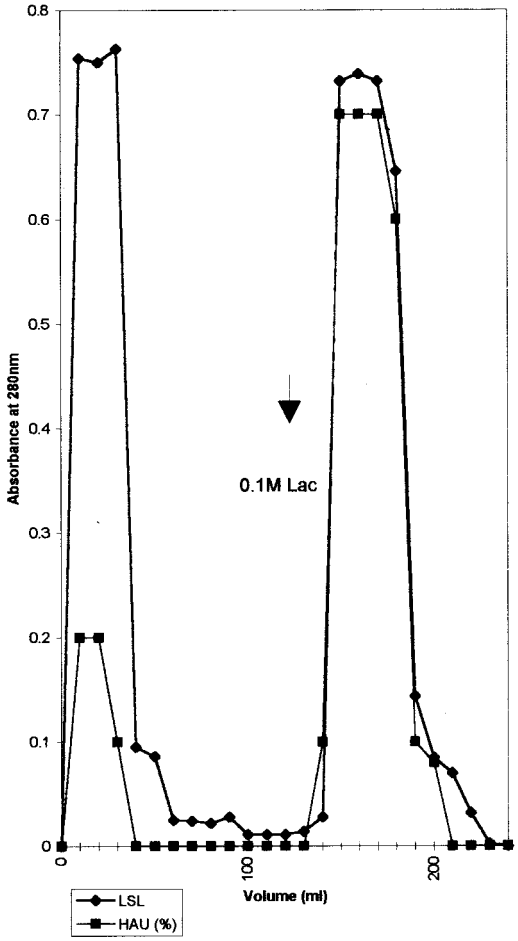

Fig. 3. Affinity chromatography of *T. anguina*, fraction 'A' on Lactamyl Seralose column: 3 ml fractions were collected and analyzed for protein at A 280. Hemagglutination Activity Units are represented as ×100% activity, with the highest value set at 100%.

2. Results and discussion

When fraction 'A' was loaded on Sepharose 4B column it was observed that only a minor portion of the lectin activity was retained while substantial lectin activity exhibiting galactose specificity as examined by sugar inhibition of hemagglutination, remained unadsorbed and washed off with equilibrating buffer. This was indicative of the presence of other galactose specific lectins which were unable to bind or interact with the galactose residues of Sepharose (Hegde, Maiti & Podder, 1991). The adsorbed protein fraction was eluted with 0.2 M galactose in buffer 1 (Fig. 1) and the presence of an apparently single lectin (SLa) was detected by PAGE pH 8.9 and by SDS-PAGE having a M_r of 45 K (Fig. 2). On the other hand, chromatography on high affinity matrix Lactamyl Seralose, resulted in the retention of most of the galactose specific activity from Frac. 'A'. Elution profile of the adsorbed protein fraction with 0.1 m lactose in buffer

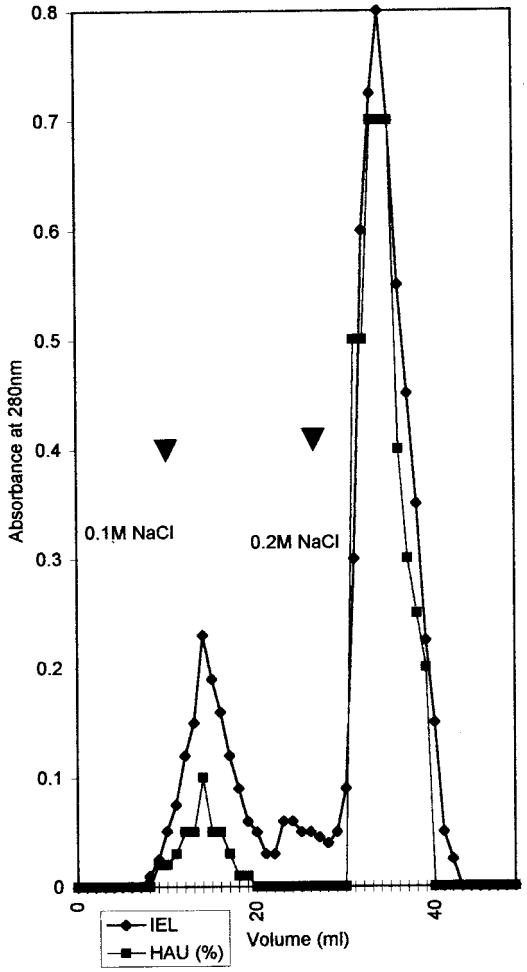

Fig. 4. Ion exchange chromatography of *T. anguina* fraction 'A'on DEAE cellulose: After column was washed free of unbound protein, elution was performed with a discontinuous gradient of NaCl in PB and 3 ml fractions were collected. Hemagglutination Activity Units are represented as ×100% activity, with the highest value set at 100%.

1 (Fig. 3) shows a skewed peak indicating the presence of more than one protein (LSL). As *T. kirilowii* lectins have been purified on DEAE Cellulose matrix, the same was done for *T. anguina* seed extract, but it was observed that DEAE Cellulose matrix resulted in poor retention of galactose specific lectin activity, only a very minor portion was adsorbed. Elution by a discontinuous salt gradient resulted in the purification of a single lectin (IEL), (Fig. 4), and was similar to SLa having a M_r of 45 K.

Electrophoretic analysis of LSL by PAGE pH 8.9 showed it to be composed of two broad, closely associated, diffuse bands. Individual bands were eluted and were shown to possess hemagglutinating activity. The major bands were not artifacts generated by intra molecular associations during electrophoresis because the bands 'run true' after their isolation by elution and re-electrophoresis. In non reducing SDS-PAGE the LSL

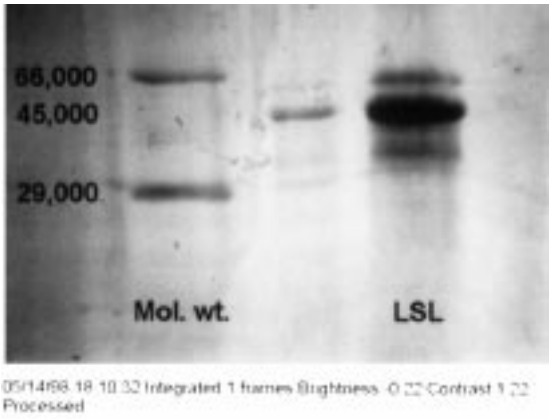

Fig. 5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) LSL from *T. anguina* was run on a 12% resolving gel and 5% stacking gel in the absence of β-mercaptoethanol, protein bands were stained with Coomassie blue, (L-R), Lane 1 — Molecular weight markers (1) Bovine Serum Albumin (66,000); (2) Egg Albumin (45,000); (3) Carbonic Anhydrous (29,000), Lane 2 — LSL (5 μg); Lane 3 — LSL (20 μg).

complex shows presence of five individual closely spaced bands ranging in M_r between 30 and 50 K (Fig. 5). In the presence of mercaptoethanol the LSL bands split into seven components; one subunit with 25 K and the remaining six bands are seen in the M_r range of 30–37 K.

PAS staining of gels with the lectin bands revealed that only two of the five LSL bands were PAS positive

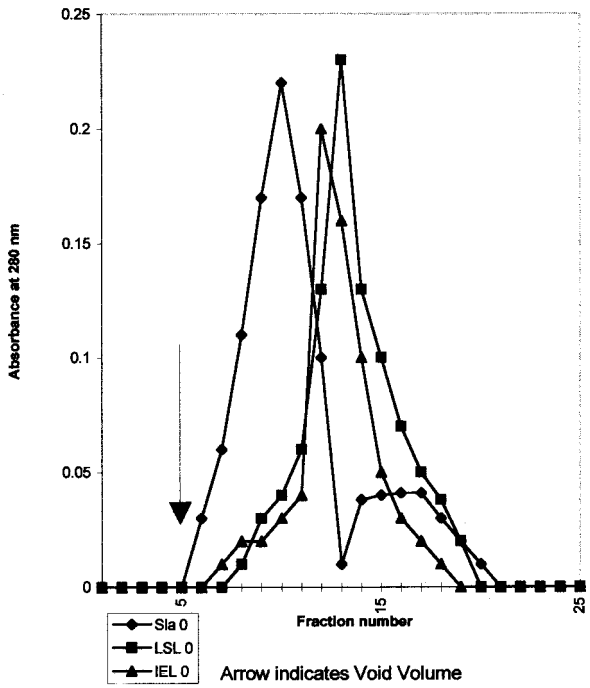

Fig. 6. Gel filtration profiles of *T. anguina* isolectins (SLa, LSL and IEL): on Sephadex G-100 column, elution with 0.145 M NaCl; 3 ml fractions were collected and analyzed for protein at 280 nm and hemagglutinating activity.

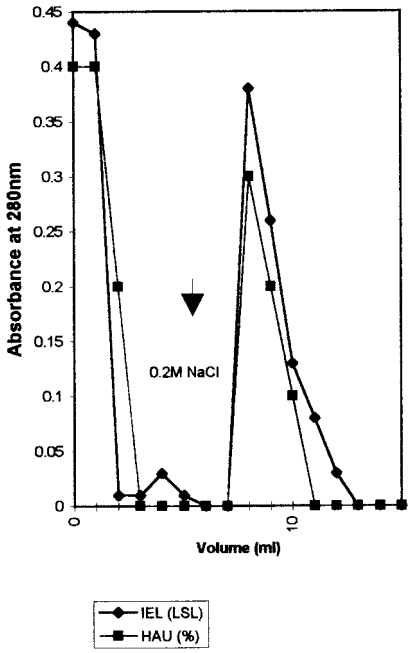

Fig. 7. Purification of IEL isolectin from LSL: re chromatography of LSL on DEAE Cellulose column, elution with 0.2 M NaCl in 0.025 M PB pH 7.5; 1 ml fractions were collected and analyzed for protein at 280 nm and hemagglutinating activity. Hemagglutination Activity Units are represented as ×100% activity, with the highest value set at 100%.

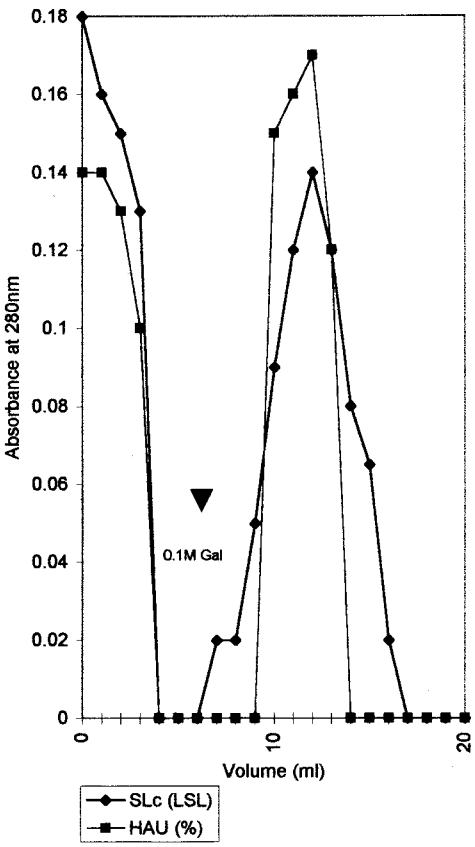

Fig. 8. Purification of SLc isolectin from LSL: re chromatography of LSL of *T. anguina* on Sepharose 4B column, elution with 0.1 M galactose in buffer 1; 1 ml fractions were collected and analyzed for protein at 280 nm. Hemagglutination Activity Units are represented as ×100% activity, with the highest value set at 100%.

and SLa and IEL remained PAS negative. This result correlated with the carbohydrate content analysis by Phenol Sulfuric assay, whereby LSL contained 3% neutral sugar and SLa and IEL lacked carbohydrate.

In contrast to the above results Ghosh et al. (1981) reported the presence of two isolectins with M_r 90 K and 60 K respectively. Komath et al. (1996) report the purification of a single seed lectin with M_r 62 K on cross linked guar gum, with approx. 3% carbohydrate content in this single lectin.

Gel filtration studies of the three isolated lectins, LSL, SLa and IEL was done on Sephadex G-100, and revealed interesting observations. Multiple components of LSL did not segregate, probably due to the narrow differences in their molecular weights. IEL also eluted as a single symmetrical peak. Surprisingly the apparently single lectin SLa separated into two distinct peaks. The first eluting larger peak, initially thought to be inactive was found to be active only at higher pH (8–11), this lectin (SLb) is currently being studied. The latter eluting smaller peak was active at neutral pH (SLc) (Fig. 6) and has been used in the present work.

Re chromatography of LSL on DEAE cellulose resulted in the retention of a single lectin identical to IEL (Fig. 7). Similarly LSL re chromatography on Sepharose 4B showed retention of a lectin identical to SLc (Fig. 8). During re chromatography both matrices had unretained galactose specific lectin activity in their respective flow through fractions. Table 1 gives the molecular weights of the isolectins by SDS-PAGE. Table 2 shows the blood group specificity of the isolectins towards human blood groups. Inhibitory concentrations of galactose containing sugars required to

Table 1
Molecular weight determination and subunit composition of *T. anguina* lectins

T. anguina lectins	Non-reducing SDS-PAGE (kDa)	No. of protein bands	Reducing SDS-PAGE	No. of subunits
LSL	30–50	5	25–45	7
SLc	45 ± 0.7	1	25 and 37	2
IEL	45 ± 0.7	1	25 and 27	2

Table 2
Blood group specificity of *T. anguina* isolectins

T. anguina lectins	A^{+*}	B^{+}	AB^{+}	O^{+}
LSL	425	3404	851	851
SLc	–	4000	2000	8000
IEL	–	1333	666	1333

* Hemagglutinating Activity Units.

inhibit hemagglutination are shown in Table 3. IEL and SLc had molecular weight of approx. 45 kDa in SDS PAGE without β-mercaptoethanol, two subunits with M_r of 25 and 37,000 were obtained in the presence of β-mercaptoethanol. Two of the multiple bands of LSL on SDS-PAGE were PAS positive and the lectins IEL and SLc were found to be PAS negative. Total carbohydrate content of LSL complex was 3%, IEL and SLc lacked carbohydrate. The pH and temperature optima required for activity of the isolectins are shown in Figs. 9 and 10, respectively. The anti LSL serum formed single precipitin arc with the isolectins in double immunodiffusion tests. IEL and SLc obtained by re chromatography of LSL was subjected to partial proteolysis with Pronase E and showed differences in their respective peptide maps on 15% SDS-PAGE gels, SLc showed the presence of an extra peptide fragment that was absent in IEL (Fig. 11). Trypsin digestion of IEL and SLc also revealed variable maps with IEL having only two peptide fragments and SLc having 5–6 peptide fragments. Chymotrypsin treatment of IEL and SLc also gave different peptide maps (data not shown).

N-terminal amino acid sequence analysis of SLc and IEL will be carried out to study the relatedness of these two isolectins as the tryptic digests of *Lathyrus ochrus* isolectins were identical, though small but distinct differences were seen with chymotrypsin and V8 digests. These variations were due to the amino acid differences near the C-termini (Richardson et al., 1984).

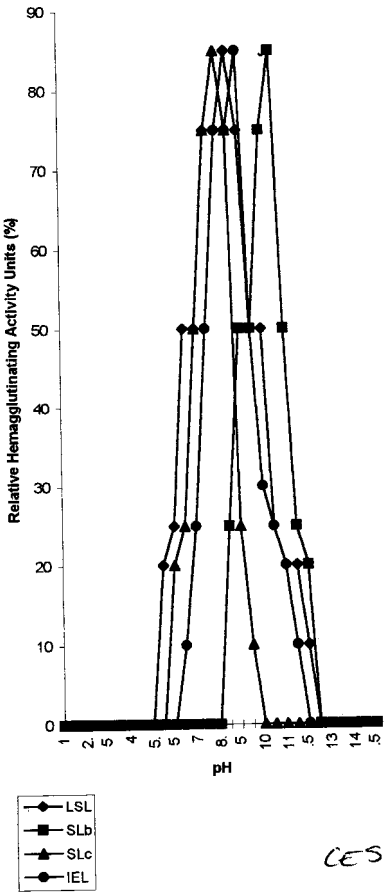

Fig. 9. pH optima of *T. anguina* isolectins. Protein was incubated at various pH for 30 min and analyzed for hemagglutinating activity.

The above data shows *T. anguina* isolectins to be composed of glycosylated and non-glycosylated lectins; attempts to isolate the glycosylated isolectins from the LSL complex by lectin affinity chromatography are being currently carried out. The apparent homology of the isolectins IEL and SLc is evident from the similarity in molecular weights, subunit composition, sugar specificity, non glycoprotein nature and immunological identity, but support for the fact that they are two dis-

Table 3
Carbohydrate specificity of *T. anguina* lectins

Sugars	Minimum inhibitory concentration (mM)		
	LSL	SLc	IEL
D-galactose	12.5	25.0	25.0
N-acetyl-D-galactosamine	50.0	50.0	50.0
D-galactosamine HCl	100.0	50.0	50.0
1-*O*-methyl-α-D-galactopyranoside	12.5	50.0	50.0
p-nitrophenyl-β-D-galactopyranoside	6.25	50.0	50.0
Melibiose	50.0	50.0	50.0
Lactose	25.0	25.0	25.0

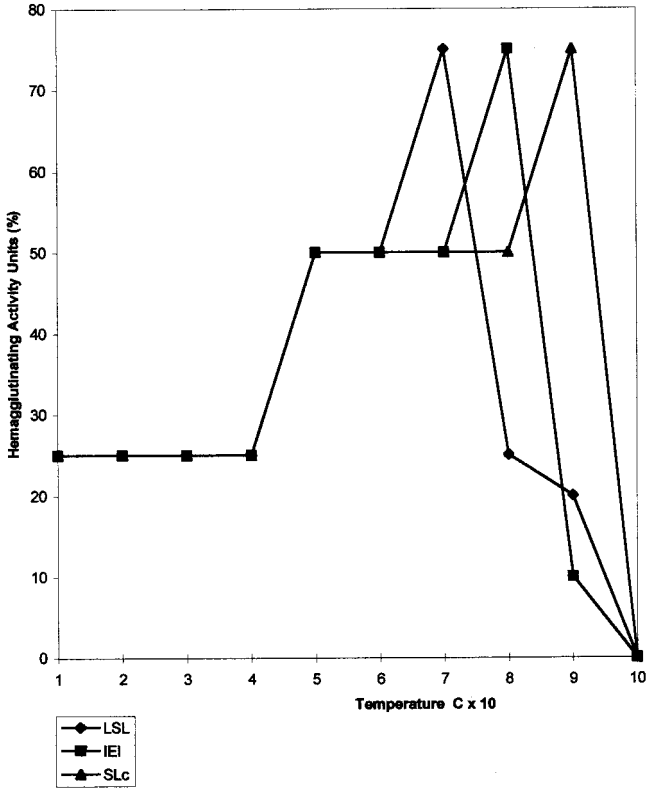

Fig. 10. Temperature optima of *T. anguina* isolectins. Protein was incubated at various temperatures for 15–30 min and assayed for hemagglutinating activity.

tinct lectins is derived from biological and physicochemical studies. Moreover the peptide fragment variation reflects micro heterogeneity in the primary structure of these two isolectins.

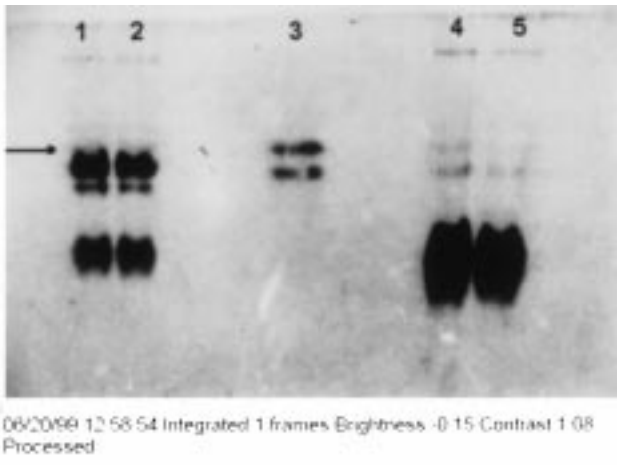

Fig. 11. Peptide mapping of IEL and SLc after digestion with Pronase E: In a 15% SDS-PAGE gel, gel was silver stained. Lanes 1 and 2 — SLc (2.5 μg), Lane 3 — Pronase E (2.5 μg), Lanes 4 and 5 — IEL (2.5 μg), arrow at lane 2 indicates the extra peptide fragment.

3. Experimental

3.1. Materials

Sephadex G-100 was purchased from Pharmacia, DEAE Cellulose, Pronase E, Trypsin and Chymotrypsin and SDS molecular weight markers were purchased from Sigma USA; Sepharose 4B, Acrylamide, Bisacrylamide, Epichlorohydrin and Glutaraldehyde were from SRL Mumbai; Amino Hexyl Seralose 4B was obtained from Dr G. S. Murthy, Indian Institute of Science, Bangalore. Seeds of *T. anguina* were purchased from local markets. Human erythrocytes were obtained from the Health Center, University of Pune and Jankalyan Blood Bank, Pune, India. Rabbit antiserum to *T. anguina* lectin was raised at Serum Institute, Pune, India.

3.2. Fraction 'A' preparation

The saline homogenate of *T. anguina* seeds (100 g) was delipidated by repeated treatment with *n*-butanol and acetone precipitated at -4°. The acetone dried powder was extracted with saline (0.145 M NaCl, 100 ml/10 g) twice, and centrifuged at 10,000 rpm. The supernatant was subjected to ammonium sulphate fractionation. The protein fraction that pptd. between 30 and 80% saturation was isolated and dissolved in a minimum amount of distilled water, extensively dialyzed against distilled water and finally against 0.145 M saline (Sawhney & Bhide, 1990). The clear supernatant or (fraction 'A') obtained after centrifugation was stored at -20°, until further use.

3.3. Purification of galactose specific lectins from T. anguina seeds

Affinity chromatography and ion exchange chromatography was employed to purify and separate the galactose specific lectins from fraction 'A'.

Sepharose 4B column (30×1.5 cm) was pre equilibrated with Phosphate buffer saline pH 7.5, (0.025, 0.145 M NaCl) (Buffer 1). Fraction 'A' containing $\approx$100 mg of protein was loaded, unbound proteins were washed with buffer 1. Elution of adsorbed proteins was carried out with 0.2 M galactose in buffer 1. Peak fractions were pooled, extensively dialyzed and lyophilized for storage at -20°, until further use.

3.4. Preparation of affinity matrix

Lactose was covalently linked to AH-Seralose 4B (Ito, Yamasaki, Seno & Matsumoto, 1986), matrix was packed into (30×1.5 cm) column. The column was pre-equilibrated with buffer, $\approx$100 mg of fraction 'A' was loaded on the affinity column. The unretained pro-

teins were washed with buffer 1 and checked for lectin activity. The retained protein was eluted with 0.1 M lactose in buffer 1. The peak fractions were pooled, dialyzed against distilled water and lyophilized.

3.5. Ion exchange chromatography

DEAE Cellulose matrix was activated as per the manufacturer's instructions, and packed into a (40 × 2 cm) column. Fraction 'A' was dialyzed against Phosphate buffer, (0.025 M, pH 7.5) (PB).

Protein, ≈80 mg, was loaded on the DEAE Cellulose column pre-equilibrated with PB. Elution was carried out with a discontinuous gradient of 0.1–0.2 M NaCl in PB and monitored for protein.

3.6. Gel filtration

Affinity purified lectins LSL, SLa and the lectin purified on DEAE Cellulose, IEL was dissolved in ≈2 ml of 0.145 M saline and individually gel filtered on a Sephadex G-100 column (50 × 2.5 cm) previously equilibrated with 0.145 M saline. The peak protein fractions were checked for hemagglutinating activity, pooled and dialyzed against distilled water. These gel filtered proteins were lyophilized and used for all further characterization.

Protein concentrations were determined by absorption measurements at 280 nm using Bovine Serum Albumin (BSA) as standard with $A^{1\%}/cm = 6.45\ cm^{-1}$ (Sawhney & Bhide, 1990), alternatively by the Lowry, Rosebrough, Farr and Randal method (1951) with BSA as standard.

3.7. Gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) at pH 8.9 (Perez, 1984) was done. SDS-PAGE under reducing and non reducing conditions were performed with Laemmli's system (1970). The protein bands were visualized by staining the gels with Coomassie Brilliant Blue R-250, alternatively by silver staining (Merril, Goldman, Sedman & Ebert, 1981). Glycoproteins were detected by the Periodic Acid Schiff's (PAS) staining of gels (Fairbanks, Steck & Wallach, 1971). Protein from individual bands on the gels was eluted into buffer 1 and assayed for lectin activity and sugar specificity. Hemagglutination assay was performed as in Sawhney and Bhide (1992).

3.8. Blood group specificity and sugar inhibition assays

The blood group specificity of the lectins towards human erythrocytes A^+, B^+, O^+, and AB^+ and the inhibitory effect of various sugars was measured by the two fold dilution method in a microtitre plate (Sawhney & Bhide, 1992).

3.9. Physicochemical studies

Influence of pH and temperature for lectin activity was done as in Sawhney and Bhide (1992).

3.10. Immunological studies

LSL (5 mg) was emulsified with 0.5 ml of Freund's complete adjuvant and injected subcutaneously into a rabbit. A booster schedule of 3 doses with 10-day intervals between each dose was followed. Serum from the blood of immunized rabbit was separated by 50% ammonium sulphate saturation to precipitate the IgG. The specificity of the lectin antiserum was tested by Ouchterlony's (1948) double immunodiffusion test.

3.11. Peptide mapping

Partial proteolysis of purified lectins was done with Pronase E, Trypsin and Chymotrypsin as in Cleveland, Fischer, Kirshner and Laemmli (1977). Digested proteins were run on a 15% SDS-PAGE and the peptide maps were developed by silver staining of the gels.

3.12. Neutral sugar content

Estimation of the carbohydrate content of the lectin samples was done by the Phenol Sulphuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), with D-glucose as standard.

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