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Purification in high yield and characterisation of a new galactose-specific lectin from the seeds of *Trichosanthes cucumerina*

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Abstract

Ten Cucurbitaceae species have been investigated for the presence of seed lectins of which only two species, *Trichosanthes cucumerina* and *T. palmata*, have displayed agglutination activity which was inhibited by galactose. The lectin from *T. cucumerina* seeds has been purified in high yield (~350 mg lectin/100 g deshelled seeds) by affinity chromatography on cross-linked guar gum. The purified *T. cucumerina* seed lectin (TCSL) moved as a single symmetrical peak on gel filtration on Sephadex G-150 with an apparent molecular weight of 62 (±5) kDa and gave a single band on PAGE under non-denaturing conditions. In SDS-PAGE, TCSL gave a single band at 69 kDa in the absence of 2-mercaptoethanol, whereas in the presence of 2-mercaptoethanol two bands corresponding to 41 and 22 kDa were observed, suggesting that the lectin is made up of two non-identical subunits that are linked by one or more disulphide bridges. TCSL is a glycoprotein with about 3.0% covalently bound neutral sugar. The lectin cross-reacted with rabbit antiserum raised against the *Trichosanthes anguina* (snake gourd) seed lectin (SGSL), yielding a single precipitin line and SGSL cross-reacted with the anti-TCSL antiserum raised in rabbits, indicating that the two lectins are antigenically very similar. This was further confirmed by Western blot analysis where the two subunits of TCSL were found to react with both anti-TCSL and anti-SGSL antisera and vice versa. On the other hand, TCSL did not cross-react with the antiserum raised against *Momordica charantia* lectin and vice versa, suggesting that these two cucurbit seed lectins are antigenically dissimilar. Haemagglutination-inhibition data show that TCSL is specific for the β-anomer of galactose with MeβGal and lactose being the best mono- and disaccharide inhibitors, respectively.

Keywords: *Trichosanthes cucumerina*; *T. anguina*; Cucurbitaceae; Agglutinin; Lectin; Galactose-specific; Affinity chromatography; Saccharide specificity; Carbohydrate-binding protein

1. Introduction

The ability of certain lectins to distinguish between normal and malignant cells (Rapin & Burger, 1974) and to recognise specifically different types of human blood groups (Sharon & Lis, 1989) has led to intense efforts being focused on the purification and characterisation of lectins from a variety of species (Goldstein & Poretz, 1986). Now lectins are established to be ubiquitous, their presence being demonstrated in a variety of plant and animal species from bacteria to human beings. Because of their ability to specifically recognise unique carbohydrate structures, lectins are being used in the purification and characterisation of glycoconjugates and in the study of cell-surface architecture. Additionally, lectins are also found to be useful in the fractionation of cells for their use in bone marrow transplantation (Lis & Sharon, 1986).

Abbreviations: ; BSA, bovine serum albumin; MCL, *Momordica charantia* (bitter gourd) seed lectin; SGSL, *Trichosanthes anguina* (snake gourd) seed lectin; TCSL, *Tricosanthes cucumerina* seed lectin; PBS, 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.1% sodium azide; PBS–βME, 20 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NACl, 10 mM β-mercaptoethanol and 0.1% sodium azide; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; MeβGal, methyl-β-D-galactopyranoside; MeαGal, methyl-α-D-galactopyranoside; GalNAc, *N*-acetyl-D-galactosamine; MeαGlc, methyl-α-D-glucopyranoside; MeβGlc, methyl-β-D-glucopyranoside; MeαMan, methyl-α-D-mannopyranoside; $GlcNH_2$, D-glucosamine; LacNAc, *N*-acetyllactosamine; PNPβGal, *p*-nitrophenyl-β-D-galactopyranoside; MeUmbβGal, 4-methylumbelliferyl-β-D-galactopyranoside; Thiodigalactoside, D-galactopyranosyl-β-D-thiogalactopyranoside; Galβ13GalNAc, 3-*O*-*N*-acetylgalactopyranosyl-β-D-galactopyranoside; Galβ13GalβOMe, methyl 3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside.

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Because of their presence in large quantities in the seeds of Leguminosae, the legume seed lectins have been most widely studied, leading not only to their use in a variety of applications, but also to a detailed understanding of the similarities between them in terms of primary, secondary and tertiary structure as well as at the level of 3-dimensional structure resulting in the unraveling of the evolutionary relationships between them (Lis & Sharon, 1984; Etzler, 1985; Strosberg, Buffard, Lauwereys, & Foriers, 1986; Rougé, Cambillau, & Bourne, 1991; Rini, 1995). Similar information is lacking on the lectins from other plant families. From Cucurbitaceae only three seed lectins, namely, those from *Momordica charantia* (bitter gourd), *Trichosatithes anguina* (snake gourd) and *Trichosanthes kirilowii*, have been purified and characterised in some detail (Mazumder, Gaur, & Surolia, 1981; Khan, Mazumder, Pain, Gaur, & Surolia, 1981; Das, Khan, & Surolia, 1981; Falasca, Abbondanza, Barbieri, Bolognesi, Rossi, & Stirpe, 1989; Komath, Nadimpalli, & Swamy, 1996; Komath, Nadimpalli, & Swamy, 1998). With the objective of identifying new lectin activities from the seeds of Cucurbitaceae species and characterising the evolutionary relationships between them, we have been screening several species from this family for lectin activities and reported on the affinity purification in high yield and macromolecular characterisation of a galactose-specific lectin from the seeds of *T. anguina* and showed that histidine residues are essential for its sugar-binding and cell-agglutinating activities (Komath et al., 1996, 1998). In the present study, we report on the affinity purification and characterisation of a galactose-specific lectin from the seeds of *Trichosanthes cucumerina* with respect to its macromolecular and carbohydrate-binding properties. Additionally, the seed extracts of several other cucurbit species have been tested for lectin activity, of which only the seed extracts of *Trichosanthes palmata* showed agglutination activity which was inhibited by galactose and its derivatives.

2. Results and discussion

Interest in the seed lectins of the Cucurbitaceae arises due to the fact that the fruits of various species in this family are used in the diet in different parts of the world, particularly, in the tropics. Additionally, the Cucurbitaceae seed lectins, unlike those from legume seeds, have not been investigated in much detail and thus there is a need to purify lectins from this family and to characterise them in a systematic manner. This is likely to lead to an understanding of the properties of these molecules in detail as well as to the unraveling of any evolutionary relationships that they might share. In view of this, we have taken up the screening of various Cucurbitaccae species for lectin activities in their seed extracts and the purification of the activities identified. In an earlier paper we reported the screening of three Cucurbitaceae species i.e. *Trichosanthes anguina*, *Luffa acutangula* and *Lagenaria vulgaris* for lectin activities and large-scale purification of a galactose-specific lectin from the seeds of *T. anguina* (snake gourd) (Komath et al., 1996). Further, using chemical modification studies we have shown that histidine residues are essential for the saccharide-binding and cell-agglutinating activities of the *T. anguina* lectin (Komath et al., 1998). In the present study, several other Cucurbitaceae species, namely, *Trichosanthes cucumerina*, *Trichosanthes palmata*, *Benincasa cerifera*, *Coccinia indica*, *Citrullus colocynthis*, *Citrullus vulgaris*, *Cucumis sativus*, *Cucumis trigonus*, *Luffa amara* and *Luffa echinata* were examined for lectin activities in their seed extracts. Of these, the extracts of only *T. cucumerina* and *T. palmata* exhibited agglutination activity which could be inhibited, among monosaccharides, by galactose and some of its derivatives. In this study, the galactose-specific lectin present in the seeds of *T. cucumerina* has been purified by affinity chromatography on cross-linked guar gum and characterised in considerable detail with respect to its macromolecular and saccharide-binding properties. The results obtained are discussed here.

2.1. Purification and macromolecular properties of the *T. cucumerina* seed lectin (TCSL)

The purification of the *T. cucumerina* lectin involved ammonium sulphate fractionation followed by affinity chromatography (Table 1). The lectin eluted as a single peak from the affinity column when 0.2 M lactose in PBS was used as the eluant (Fig. 1). The ammonium sulphate precipitation step resulted in a twofold increase in the specific activity of the lectin with about 77% recovery of the activity, whereas the final affinity purification step gave the lectin in about 60% yield with a 25-fold purification as compared to the crude extract. Routinely, about 350–400 mg of the purified lectin were obtained from 100 g of deshelled seeds.

The affinity purified *T. cucumerina* lectin was found to be pure by PAGE, where it moved as a single band (Fig. 2A). In SDS-PAGE, the lectin yielded a single band of M_r 69,000 in the absence of β-mercaptoethanol, whereas in the presence of β-mercaptoethanol it gave two bands corresponding to M_rs of 41,000 and 22,000, respectively, indicating that the two subunits are covalently linked by one or more disulphide bridges (Fig. 2B). This suggests that the subunit structure of the *T. cucumerina* lectin is similar to that of the seed lectins from *T. anguina* (snake gourd) and *T. kirilowii*. In gel permeation chromatography experiments

Table 1
Purification of *T. cucumerina* seed lectin (from 40 g of deshelled seed)

Purification step	Total activity (units $\times 10^{-4}$)	Protein content (mg)	Specific activity (units/mg)	Recovery (%)
Aqueous extraction	24.08	5875	41	100
Ammonium sulfate precipitate	18.45	2250	82	77
Affinity chromatography	14.34	140	1024	60

the affinity-purified TCSL eluted as a single symmetrical peak, both in the absence and in the presence of 0.2 M lactose. By comparing with the elution profiles of standard proteins its M_r was estimated to be 62,000 ($\pm$5000) (Fig. 3). This is also comparable to the M_r of the seed lectins from snake gourd and *T. kirilowii* (Falasca et al., 1989; Komath et al., 1996). Carbohydrate estimation indicated that TCSL contains about 3.0% neutral sugar which again is similar to that observed with the lectins from *T. anguina* and *T. kirilowii* (Falasca et al., 1989; Komath et al., 1996).

2.2. Ouchterlony double-immunodiffusion experiments

Polyclonal antiserum was raised against the affinity purified *T. cucumerina* agglutinin in rabbits. In Ouchterlony double-immunodiffusion experiments, the lectin reacted with the antiserum giving a single precipitin line, indicating that the antiserum specifically recognises the *T. cucumerina* lectin (Fig. 4A). Preimmune serum did not give any precipitin line, clearly demonstrating that the precipitin line observed with the antiserum is due to the specific interaction of the antibody with TCSL and not because of the interaction of the lectin with the serum glycoproteins through its carbohydrate-binding site.

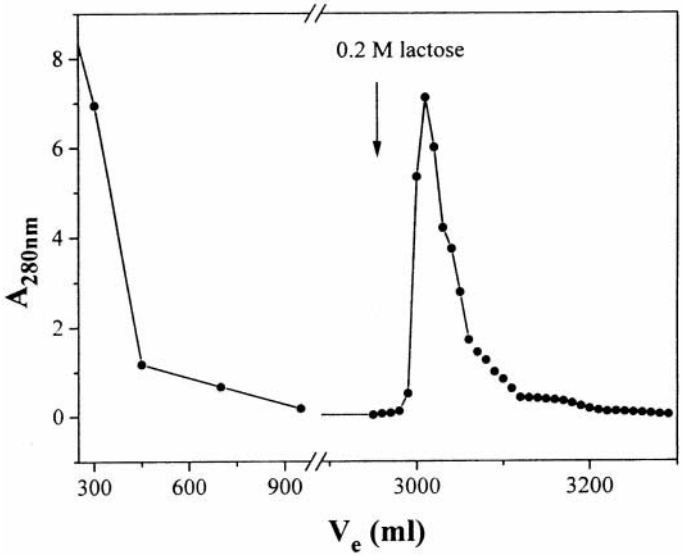

Fig. 1. Affinity chromatography of *Trichosanthes cucumerina* lectin on cross-linked guar gum. The soluble protein obtained in the 20–80% saturation range in the ammonium sulphate precipitation step was dialysed and loaded onto the guar gum affinity column. After extensive washing with PBS-βME, the bound lectin was eluted with 0.2 M lactose in PBS (indicated by the arrow in the figure).

In order to find out if there are any similarities between TCSL and SGSL, immunodiffusion experiments were carried out where the cross-reactivity between anti-SGSL antibody and TCSL and between anti-TCSL antiserum and SGSL were investigated. These experiments showed that anti-SGSL antiserum recognises TCSL and vice versa, demonstrating that the two lectins are antigenically very similar (Fig. 4A). This suggests that these two lectins are likely to be closely related in primary sequence as well as in the 3-dimensional structure. On the other hand, the anti-TCSL antiserum did not recognise another Cucurbitaceae seed lectin, namely the *Momordica charantia* lectin (Fig. 4A) and anti-MCL antiserum failed to cross react with the *T. cucumerina* lectin (data not shown). In an earlier study we showed that the snake gourd seed lectin also did not cross-react with the anti-MCL antiserum (Falasca et al., 1989). These observations suggest that both SGSL and TCSL are structurally different from the *M. charantia* lectin. Additionally, it may also be noted that while both SGSL and TCSL are dimeric in nature with a molecular mass of about 62 kDa, MCL is a tetramer with a molecular mass of 116 kDa.

2.3. Western blot analysis

The ability of the two separated subunits of TCSL and SGSL to interact with the anti-TCSL and anti-SGSL antisera was investigated by the Western blot technique. Fig. 4B shows the results of these experiments, where it can be clearly seen that the two subunits of TCSL were recognised by both the anti-TCSL and anti-SGSL antibodies. Similarly, the two subunits of SGSL were also recognised by both antibodies, further confirming the immunological cross-reactivity observed in the immunodiffusion experiments with native SGSL and TCSL.

2.4. Carbohydrate-binding properties

The carbohydrate-binding specificity of the *T. cucumerina* agglutinin was probed by the haemagglutination-inhibition method. Though this method is only

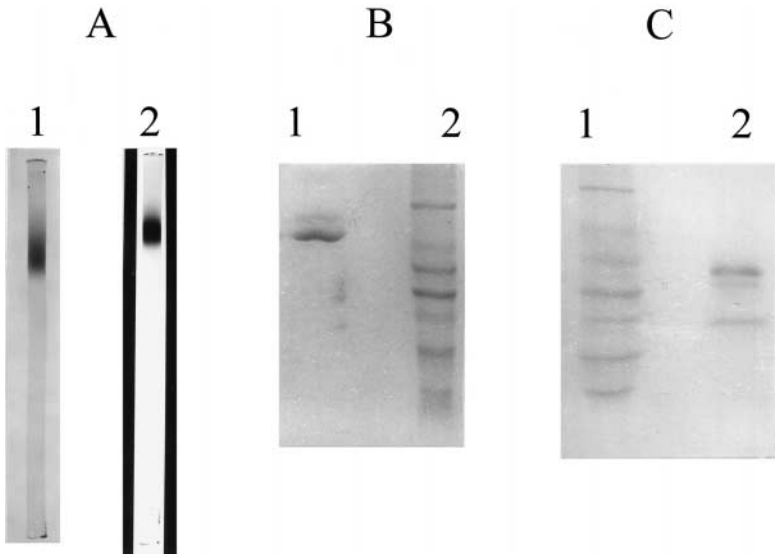

Fig. 2. Electrophoresis of the *T. cucumerina* lectin. (A) PAGE under non-denaturing conditions. This was done in tube gels containing 7.5% acrylamide at pH 8.3 (lane 1) and at pH 4.5 (lane 2). (B) SDS-PAGE without β-mercaptoethanol; lane 1: TCSL, lane 2: M_r markers. (C) SDS-PAGE with β-mercaptoethanol; lane 1: molecular-weight markers, lane 2: TCSL. The standards used are phosphorylase B (molecular-weight 97,000), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), α-lactalbumin (M_r 14,200) and aprotinin (M_r 6,500). For SDS-PAGE, gels contained 5% acrylamide in the stacking gel and 12.5% acrylamide in the resolving gel.

semi-quantitative, it can provide information regarding relative abilities of various saccharides to inhibit the lectin activity and therefore is useful in screening a large number of sugars before more accurate methods such as titration microcalorimetry or spectroscopic methods can be used on selected structures.

2.4.1. Binding of monosaccharides

Results of haemagglutination-inhibition experiments with *T. cucumerina* agglutinin are given in Table 2. The data presented here clearly shows that among the monosaccharides and their glycosides, *p*-nitrophenyl-β-D-galactopyranoside is the best inhibitor. Galactose,

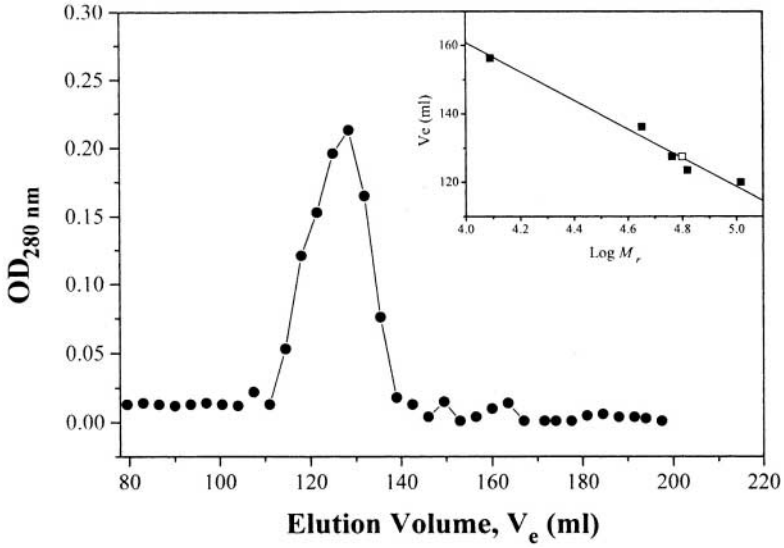

Fig. 3. Gel filtration of *T. cucumerina* seed lectin. One ml of a 3.0 mg/ml sample of the *T. cucumerina* lectin in 20 mM phosphate buffered saline containing 0.15 M NaCl was loaded onto a column of Sephadex G-150 superfine (104 × 1.8 cm), which was preequilibrated with the same buffer. Three ml fractions were collected and the elution pattern was monitored by $A_{280\ mn}$. Inset, plot of log M_r vs the elution volume, V_e. The standards used were: (1) peanut agglutinin (M_r 105,000), (2) bovine serum albumin (M_r 66,000), (3) winged bean agglutinin (M_r 58,000), (4) ovalbumin (M_r 45,000) and (5) bovine cytochrome *c* (M_r 12,300).

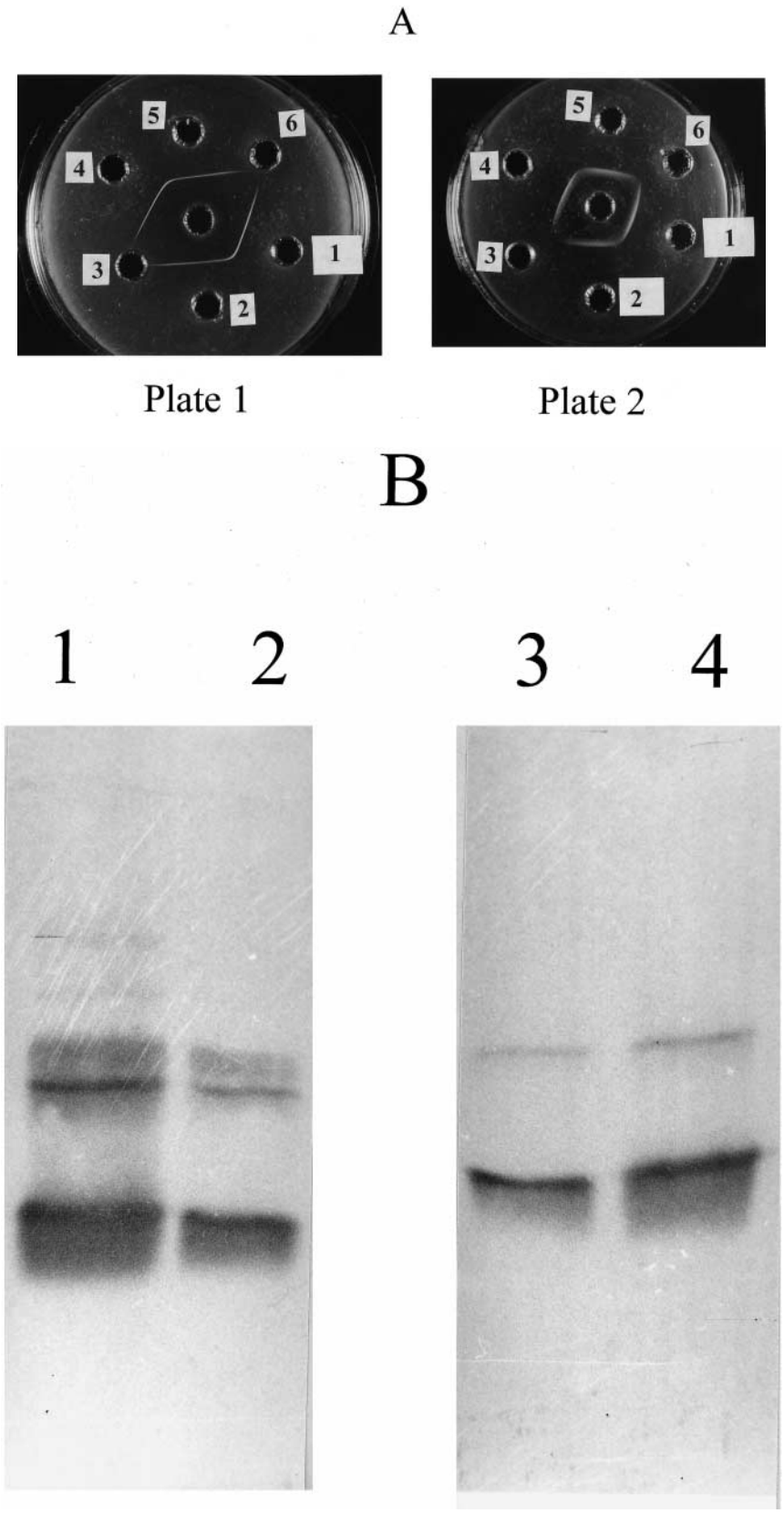

Fig. 4. Immunodiffusion and Western blot of *T. cucumerina* lectin. (A) Immunodiffusion. Plate 1. The anti-TCSL antiserum was placed in the central well. Samples in the outer wells were: 1 and 4, TCSL; 2 and 5, SGSL; 3 and 6, MCL. Plate 2. The anti-SGSL antiserum was placed in the central well. Samples in the outer wells were: 1 and 4, SGSL; 2 and 5, TCSL; 3 and 6, MCL. (B) Western blot: lanes 1 and 4, TCSL; lanes 2 and 3, SGSL. Lanes 1 and 2 were probed with anti-TCSL antiserum and lanes 3 and 4 were probed with anti-SGSL antiserum.

Table 2
Inhibition, by various saccharides, of the agglutination activity of *T. cucumerina* seed lectin

Sugar	Concentration required for 50% inhibition (mM)	Relative inhibitory potency (galactose = 1.0)
p-Nitrophenyl-β-D-galatopyranoside	0.3	1.33
4-Methylumbelliferyl-β-D-galactopyranoside	0.4	1.00
Galactose	0.4	1.00
Methyl-β-galactoside	0.4	1.00
Methyl-α-galactoside	0.8	0.50
N-Acetylgalactosamine	0.8	0.50
D-Galactosamine·HCl	1.5	0.26
p-Nitrophenyl-α-D-galactopyranoside	2.5	0.16
2-Deoxy-D-galactose	3.0	0.13
D-Fucose	3.0	0.13
L-Arabinose	6.0	0.07
Lactose	0.2	2.00
Melibiose	1.5	0.26
Thiodigalactoside	1.5	0.26
Raffinose	1.5	0.26

All sugars are D-sugars unless otherwise specified.The following sugars did not show any inhibition at concentrations upto 100 mM: glucose, mannose, MeαGlc, MeβGlc, MeαMan, $GlcNH_2$, cellobiose, maltose. Other non-inhibitory sugars are (maximum concentration tested indicated in parentheses): stachyose (25 mM), LacNAc (2.5 mM), Galβ13GalNAc (1.0 mM), Galβ13GalβOMe (0.15 mM).

MeβGal and MeUmbβGal display comparable inhibitory capacities and are marginally weaker as compared to PNPβGal. MeαGal is only half as potent as MeβGal, indicating that the lectin exhibits a preference for the β-anomer of galactose over the α-anomer. Binding of GalNAc is weaker than that of galactose, which shows that the acetamido group at the C-2 position is not as effective as the hydroxy group in forming favourable interactions with the lectin combining site. Binding of 2-deoxygalactose is almost 8-fold weaker than galactose, which clearly shows that the equatorial hydroxy group at the C-2 of the sugar is an important locus for the interaction of the sugar with the lectin. Similarly, D-fucose binds to the lectin with an affinity that is 8-fold weaker than galactose, clearly identifying that the hydroxy group on the C-6 of galactose as another position of strong favourable interaction with the lectin combining site. Removal of the hydroxymethyl group entirely as in L-arabinose results in a further 2-fold decrease in the affinity, suggesting that the C-6 methyl moiety of D-fucose also has some favourable interaction with the lectin-binding site. Other monosaccharides tested, such as glucose, mannose, MeαGlc, MeβGlc, MeαMan, $GleNH_2$ did not inhibit the activity of *T. cucumerina* agglutinin upto 100 mM concentration (Table 2). The haemagglutination-inhibition data therefore shows that an equatorial OH at C-2, an axial OH at C-4 and the C-6 hydroxyl group in the galactose configuration are important for the interaction of the saccharides with the *T. cucumerina* lectin.

2.5. Binding of di- and oligo-saccharides

The disaccharide, lactose exhibits an affinity that is 2-fold higher than galactose, suggesting some additional interactions of the glucopyranosyl unit with the lectin combining site. Other disaccharides tested, melibiose and thiodigalactoside are almost four times weaker than galactose, indicating that some unfavourable interactions are introduced by the addition of the second monosaccharide unit. The trisaccharide, raffinose is also about 4-fold weaker than galactose in its ability to inhibit the activity of the *T. cucumerina* lectin, which indicates that there must be some unfavourable steric factors which restrict the interaction of the non-reducing terminal galactose unit with the lectin. The tetrasaccharide, stachyose does not show any inhibition upto 25 mM concentration, which is also likely to be due to steric hindrance caused by the rest of the molecule, resulting in the inability of the non-reducing terminal galactose unit with the lectin combining site.

The haemagglutination-inhibition experiments thus show that the *T. cucumerina* lectin resembles the *T. anguina* lectin and *M. charantia* lectin, both of which are also purified from the seeds of Cucurbitaceae species, in their preferential recognition of the β-anomer of galactose and stronger binding of the disaccharide, lactose over other saccharides (Mazumder et al., 1981; Komath et al., 1996).

In summary, this is the first report on the purification of a new galactose-specific lectin in high yield by affinity chromatography from the seeds of *T. cucumerina*. It has been shown that the lectin exhibits a

preference for the β-anomer of galactose and binds the disaccharide, lactose most strongly among a battery of mono-, di-, and oligo-saccharides tested. The *T. cucumerina* lectin strongly resembles the *T. anguina* lectin in its subunit structure and saccharide specificity. The two lectins also share immunological cross-reactivity, suggesting that they are closely related. Although these two lectins exhibit similar saccharide specificity as the *M. charantia* lectin, they seem to be structurally and immunologically different from it. Further characterisation of these two lectins, particularly with respect to their primary structure, is necessary to understand the evolutionary relationship between them at the molecular level.

3. Experimental

3.1. Materials

All the seeds were procured from United Chemicals and Allied Products (Calcutta, India). Guar gum, bovine serum albumin (BSA), ovalbumin, cytochrome *c*, trypsin, acrylamide, 2-mercaptoethanol, sodium dodecyl sulphate (SDS), bisacrylamide, molecular weight markers used in electrophoresis, Freund's complete and incomplete adjuvants and all the sugars except for those indicated below were purchased from Sigma (St. Louis, MO). Alkaline phosphatase conjugated to goat anti-rabbit IgG and its substrate, 5-chloro 4-bromo 3-indolyl phosphate/nitro blue tetrazolium, were obtained from Bangalore Genie (Bangalore, India). Peanut agglutinin was purified by affinity chromatography on cross-linked guar gum (Appukuttan, Surolia, & Bachhawat, 1977). Winged bean basic lectin purified by affinity chromatography (Khan, Sastry, & Surolia, 1986), thiodigalactoside, Galβ13GalNAc, Galβ13GalβOMe, *N*-acetyllactosamine (LacNAc), raffinose and stachyose were generous gifts from Prof. A. Surolia of the Indian Institute of Science (Bangalore, India). Sephadex G-150 was from Pharmacia (Uppsala, Sweden). Glycine and Tris base were obtained from SRL (Mumbai, India). Ammonium persulphate, glycerol and bromophenol blue were procured from Ranbaxy (Mumbai, India). All other reagents used were obtained from local suppliers and were of the highest purity available.

3.2. Purification of Momordica charantia (bitter gourd) lectin (MCL) and snake gourd (Trichosanthes anguina) seed lectin (SGSL)

MCL and SGSL were purified by affinity chromatography on cross-linked guar gum (Appukuttan et al., 1977) as described by Mazumder et al. (1981) and Komath et al. (1998), respectively.

3.3. Purification of Trichosanthes cucumerina seed lectin

About 100 g of deshelled *T. cucumerina* seeds were homogenised in a kitchen blender and defatted with 3×300 ml of distilled acetone. The defatted seed-meal was dried, mixed with 1 l of 20 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 10 mM β-mercaptoethanol (PBS-βME), and stirred at 4°C for 12 h. This suspension was then filtered and the filtrate centrifuged at 6000 rpm for 30 min at 4°C in a Remi C23 refrigerated centrifuge. The supernatant was subjected to ammonium sulphate precipitation and the fraction obtained in the 20–80% saturation range was thoroughly dialysed against PBS-βME. This was centrifuged again at 6000 rpm for 30 min and the supernatant obtained was loaded onto a column of cross-linked guar gum (3.5 cm × 25 cm), preequilibrated at 4°C with PBS-βME. The column was washed extensively with PBS-βME, until the absorbance of the column effluent at 280 nm fell below 0.04. The bound lectin was then eluted with 0.2 M lactose in PBS. Ten ml fractions were collected and the elution was monitored by $A_{280\ nm}$. Fractions with OD > 0.1 were pooled and dialysed thoroughly against PBS.

3.4. Haemagglutination and haemagglutination-inhibition

Haemagglutination assays were carried out using normal as well as trypsin-treated rabbit or human A, B, O erythrocytes in 96-well ELISA plates. Seed extract or the purified protein solution (100 μl) was placed in the first well and serially diluted. Then 100 μl of 4% erythrocyte suspension was added and after incubating the plate for 1 h at 4°C the haemagglutination titer was scored visually. Haemagglutination-inhibition assays with the purified TCSL were done using the following procedure. In the first well of the microtiter plate 50 μl of the sugar solution (400 or 100 mM) in PBS was added. Then 50 μl of the protein solution (25 μg/ml, which is 8 times the concentration required for 50% agglutination) was added to each well. After incubating this at 4°C for 1 h 100 μl of a 4% erythrocyte suspension was added, the plate was incubated for another hour, and the titer was scored visually.

3.5. Gel electrophoresis

SDS-PAGE was carried out according to Laemmli (1970) on 12.5% slab gels containing a 5% stacking gel. PAGE under non-denaturing conditions was performed in 7.5% tube gels using the Tris–glycine buffer system at basic pH (8.3) as described in (Laemmli, 1970). PAGE at acidic pH (4.5) was carried out according to Davis (1964). The gels were stained with Coomassie Brilliant Blue R-250.

3.6. Gel filtration

Gel filtration was performed on a column of Sephadex G-150 fine (104 cm × 1.7 cm). About 3.0 mg of protein in ~1.0–1.5 ml of PBS containing 2 mM sodium azide was loaded onto the column preequilibrated with the same buffer. Elution was also performed with the same buffer. The column was calibrated with peanut agglutinin (M_r 105,000), BSA (M_r 66,000), winged bean agglutinin (M_r 58,000), ovalbumin (M_r 45,000) and cytochrome *c* (M_r 12,400) as standards. Three ml fractions were collected and the elution was monitored by $A_{280\ nm}$.

3.7. Raising antibodies to purified SGSL, TCSL and MCL

Antibodies against purified snake gourd seed lectin, bitter gourd seed lectin and *T. cucumerina* seed lectin were raised in New Zealand white rabbits. Purified lectin (0.5 mg) in 0.5 ml of 0.9% saline was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the rabbits. At intervals of 4, 6 and 8 weeks, a booster dose of 0.5 mg of the lectin in Freund's incomplete adjuvant was given. Blood was collected from the ear vein one week after the second and subsequent injections, allowed to clot and the serum was collected and stored at −20°C. The specificity of the antiserum was detected by the Ouchterlony double-immunodiffusion technique (Ouchterlony, 1948). The immunodiffusion experiments were performed in petri dishes (7.5 cm diameter) containing 1% agar in PBS.

3.8. Western blot analysis

The immunoreactivity of the two subunits of TCSL towards the anti-TCSL antiserum and anti-SGSL antiserum was assessed by Western blot analysis, performed according to the protocol given in the Promega Technical Manual (1987). The purified protein samples of TCSL and SGSL were heat-denatured and the disulphide bonds were cleaved by treatment with β-mercaptoethanol and the two subunits were separated by SDS-PAGE on 12.5% slab gels as described above. The protein bands were electrophoretically transferred to a nitrocellulose membrane according to the procedure of Towbin, Staehelin, & Gordon (1979). The membrane was blocked to prevent non-specific binding and then probed with either anti-SGSL antiserum or anti-TCSL antiserum. The protein–antibody complex was then detected by the use of a secondary antibody conjugated with alkaline phosphatase and visualised by incubating with the substrate, 5-bromo 4-chloro 3-indolyl phosphate/nitro blue tetrazolium.

3.9. Estimation of protein and carbohydrate

Protein was estimated by the method of Lowry, Rosebrough, Farr, & Randall (1951) using bovine serum albumin as standard or by monitoring absorbance at 280 nm. Neutral sugar was estimated by the phenol-sulphuric acid assay (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956) using galactose as the standard.

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