Isolation and Characterization of Multiple-Lectins from Serum of the Desert Locust Schistocerca gregaria (Orthoptera: Acrididae)

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Isolation and characterization of multiple-lectins from serum of the desert locust Schistocerca gregaria (Orthoptera: Acrididae)

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Abstract

Three lectins, designated as Sg_1, Sg_2 and Sg_3, were identified in the serum of the desert locust *Schistocerca gregaria*. With the use of $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on DEAE-cellulose and affinity chromatography on CNBr–activated Sepharose 4B, three pools, each containing one of the putative lectins, were obtained. IEF, native PAGE and SDS/PAGE revealed that the three pools contained Sg_1, Sg_2 and Sg_3, respectively, with pI = 6.39, 8.11 and 6.8; native PAGE Rm = 0.44, 0.32 and 0.32; SDS/PAGE subunits approximate molecular weights = 21.5, 44.5, and 43.9 kDa, respectively. Under reducing conditions, SDS/PAGE has separated each of Sg_2 and Sg_3 into two bands that indicate the presence of covalent interactions between their subunits, which are not present in Sg_1. The three lectins are more highly specific for rabbit RBCs than for those of other vertebrates. The HA of lectins are Ca^{2+}–dependent, heat-labile, and are inhibited strongly by α-linked-D-galactosides, followed by L-(+)-rhamnose.

Keywords

multiple lectins, isolation, serum, hemagglutination, sugar inhibition, α-linked-D-galactosides, rhamnose, *S. gregaria*

Abbreviations

HA, hemagglutinating activity
IEF, isoelectric focusing
PAGE, polyacrylamide gel electrophoresis
pl, isoelectric point
RM, relative mobility
SDS, Sodium dodecyl sulfate

Introduction

Lectins can be defined as proteins that recognize specific carbohydrate structures and thereby agglutinate cells by binding to cell-surface sugars, glycoproteins, and other glycoconjugates (Lis & Sharon 1998). They are usually structurally complex molecules with one or more carbohydrate-recognition domains and therefore with possible multiple binding sites (Gillespie *et al.* 1997, Dodd & Drickamer 2001). Among these lectins, those that require Ca^{2+} for their activity are called C–type lectins (Drickamer 1988). Animal lectins have been found in various invertebrates as well as vertebrates (Barondes 1984), either in soluble or in membrane-bound form. These lectins can play a variety of physiological roles: in particular, they are crucial in the innate immune system where they bind, as referred to above, to the carbohydrates present on the surface of potential pathogens (Rudd *et al.* 2001).

The most extensively studied functions of hemolymph lectins have been their roles in insect immune defense systems (Ratcliffe & Rowley 1980, Vasta *et al.* 2007). Furthermore, induced or endogenous hemolymph lectins and/or hemocyte-associated lectins have been shown to be involved in phagocytosis (Kawasaki *et al.* 1993, Wheeler *et al.* 1993, Wilson *et al.* 1999), encapsulation (Komano & Natori 1985), nodule formation (Kyriakides *et al.* 1993), activation of the prophenoloxidase system (Chen *et al.* 1995) and hemolymph coagulation (Minnick *et al.* 1986).

Relatively few insect lectins have been purified and characterized. The majority of insect lectins characterized to date have been detected in the hemolymph and in most cases they are thought to be synthesized in the fat body (Kubo *et al.* 1984, Amanai *et al.* 1991) and/or the hemocytes (Amiranti 1976, Stiles *et al.* 1988, Amanai *et al.* 1991, Boucias & Pendland 1993). Lectins have been isolated and characterized from some insects, *e.g.*, dipterans (Komano *et al.* 1980, Stynen *et al.* 1985, Ingram & Molyneux 1990, McKenzie & Preston 1992, Haq *et al.* 1996, Chen & Billingsley 1999, Volf *et al.* 2002), lepidopterans (Pendland & Boucias 1986, Castro *et al.* 1987, Qu *et al.* 1987, Amanai *et al.* 1990, Gül & Ayvali 2002, Ourth *et al.* 2005, Chai *et al.* 2008), orthopterans (Stebbins & Hapner 1985, Drif & Brehelin 1994, Ayaad 2004), dictyopterans (Kubo & Natori 1987, Chen *et al.* 1993), phasmids (Richards *et al.* 1988), and hemipterans (Gomes *et al.* 1991). Their structure, function, and carbohydrate-binding properties still need further investigation to clarify their role in the innate immune system of insects.

The present work reports on isolation and on the biological, physicochemical and molecular characterization of multiple lectins from serum of the 5th-instar desert locust *S. gregaria*.

Materials and Methods

Insects, sample preparation and biological and physicochemical tests.—Insect rearing, preparation of hemolymph sera, preparation of erythrocyte suspension, assay of hemagglultinating activity (HA), effect of divalent cations, inhibition assays of sugars and glycoconjugates, and stability tests — were carried out for the whole-hemolymph sera lectins (Ayaad *et al.* 2009 this issue). Recounted briefly: solutions of the isolated lectins were used instead of the prepared sera. Rabbit erythrocytes, 2% suspension in TBS/Ca^{2+}-Mg^{2+} (pH 7.0) were used for any of the HA assays. The effect on HA of the bivalent cations (20 mM) Ca^{2+}, Mg^{2+}, Zn^{2+}, and Mn^{2+}, and EDTA (5-10 mM) was tested at pH 7.0. The inhibitory effect of carbohydrates on HA was tested in the presence of free and conjugated sugars in TBS/Ca^{2+}-Mg^{2+} at pH 7.0. Heating for 25 min at 60 and at 100°C, and storage at both 4 and at -20°C over a period of 1 week to 3 months, were tested for their effect on HA (Ayaad *et al.* 2009 this issue).

Determination of total protein concentration.—The total protein concentration was determined according to the method of Bradford (1976), using Coomassie Brilliant Blue (COBB). Bovine serum albumin (BSA) fraction V (Sigma-Aldrich), dissolved in 0.15 M NaCl, was used as a protein standard.

Isolation of lectins [$(NH_4)_2SO_4$ – salting out of serum proteins].— Insect serum (25 ml) was diluted with an equal volume of 0.02 M TBS pH 7.0 containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (TBS/Ca^{2+}-Mg^{2+}). Proteins were precipitated by addition of $(NH_4)_2SO_4$ (Sigma-Aldrich) at a concentration of 70%, with gentle stirring at 4°C . The resulting precipitate was collected by centrifugation and dialyzed in Visking® dialysis tubing (mol. wt cut-off [MWCO]: 12000-14000 Da) (Serva Electrophoresis GmbH, Germany) against distilled water for 24 h at 4°C with three changes. The dialyzed solution was then centrifuged at 8000 rpm for 5 min at 4°C to remove insoluble materials.

Ion-exchange chromatography of lectins.—Twenty grams of DEAE-cellulose (Amersham Pharmacia Biotech) were washed in 0.5 M NaOH for 10 min, then rinsed in distilled water, followed by 0.8 M HCl, and finally 0.5 M NaOH, to remove contaminants. The washed matrix was thoroughly rinsed free of NaOH with distilled water and mixed with a sufficient quantity of starting buffer (0.01 M TBS containing 0.01 M $CaCl_2$ and 0.01 M $MgCl_2$, pH 7.0) to produce a thin suspension. The treated matrix was packed into a column (20 cm × 1.0 cm i.d.) and thoroughly washed with the starting buffer. A sample from the last step of $(NH_4)_2SO_4$ fractionation (approximately 20 ml of 2.85 mg protein/ml) was applied to the DEAE-cellulose column. The column was then washed with the starting buffer until the baseline of absorption at 280 nm was achieved, and then elution carried out with a linear gradient of 0-0.25 M NaCl in 200 ml of the starting buffer. The flow rate was adjusted to 10 ml/h. Fractions (tubes) constituting the separated peaks were tested separately for HA against rabbit RBCs; all fractions displaying HA were then pooled for use in affinity chromatography.

Affinity chromatography of lectins.—Lectins of the same peak were isolated from the other proteins contained therein, by use of CNBr-activated Sepharose 4B (Sigma-Aldrich) affinity chromatography as described by Komano *et al.* (1980) with slight modifications. CNBr-activated Sepharose 4B matrix was packed into a column (10X1 cm i.d.) and extensively washed with about 500 ml of TBS/Ca^{2+}-Mg^{2+}, pH 7.0 containing 0.02% NaN_3 (Sigma-Aldrich). Each separate pool of fractions, having HA from the DEAE-cellulose column, was separately applied (slowly) to the CNBr-Sepharose 4B column at 4 °C. This latter column was washed successively with TBS, to which 0.5 M NaCl was added, until no further protein was detected in the eluate by monitoring the absorbance at 280 nm. Lectins (the bound material) were then eluted with 0.3 M raffinose solution in TBS/ Ca^{2+}-Mg^{2+} (Ayaad 2004, Ayaad *et al.* 2009). The collected fractions of each peak were pooled and each pool separately dialyzed extensively against TBS/ Ca^{2+}-Mg^{2+}, then against de-ionized distilled water to remove raffinose; then HA of each pool was assayed against rabbit RBCs. Each pool of fractions of the resulting peaks was lyophilized and stored at -20°C until use.

IEF of lectins.—The isoelectric points of lectins, contained in pools collected from affinity chromatography columns, were estimated on a pH 3.6 to 9.3 gradient polyacrylamide gel, according to the method of O'Farrell (1975), in slab gels (140 × 120 × 0.75 mm). Electrophoresis ran at 350 V for 17-18 h, yielding approximately 6000 V/h at 22°C. The pI values of the isolated lectins were determined by running a mixture of standard proteins of known isoelectric points on the same gel. We obtained a number of mixtures of proteins with different pI values (IEF MIX-Sigma, USA, pH 3.6-9.3) containing amyloglucosidase (pI 3.6), trypsin inhibitor (pI 4.6), β-lactoglobulin A (pI 5.1), carbonic anhydrase II (bovine, pI 5.9), carbonic anhydrase I (human, pI 6.6), myoglobin (pI 6.8, 7.2), lectin from *Lens culinaris* (pI 8.2, 8.6, 8.8), and trypsinogen (pI 9.3); identifications of the different pI values were made using an image densitometer G 700 (Bio-Rad, USA).

Native PAGE of lectins.—The identity of the destined lectins contained in the pools collected from affinity chromatography columns (raffinose-eluents) was confirmed by native PAGE. Electrophoresis was carried out on 10% polyacrylamide gel under nondenaturating conditions, according to the method of Schägger and von Jagow (1991), using a Tris-glycine running buffer without SDS.

After isolation of protein bands by the nondenaturating PAGE, the characteristic bands were detected on the gel by staining and matching procedures, then were sliced away from the remainder of the gel with a sharp razor blade. Each of these specific gel areas was cut into pieces, approximately 1mm² for each, and the pieces of each band soaked in 2 ml of distilled water in a separate tube overnight at room temperature. Each tube was centrifuged at 6000 rpm for 10 min; the eluate (supernatant) was transferred into a new tube. The eluted proteins were concentrated by speed vacuum for 30 min and then stored at -20°C until used.

SDS/PAGE of lectins (under nonreducing and reducing conditions).— SDS/PAGE of isolated lectins was carried out by the discontinuous buffer system of Laemmli (Laemmili 1970). Some samples were denatured with 2% SDS containing 5% β-mercaptoethanol by boiling for 3 min. Treated samples were centrifuged at 10000 g for 5 min before being loaded onto the gels. Electrophoresis was carried out at a constant voltage of 200 V for 90 min. The gels were calibrated with standard molecular weight proteins [New England Biolabs Ltd. (low range: 116, 91, 46.4, 34.3, 28.7, and 21 kDa) and/or Titan Biotech Ltd. (high and low range: 200, 116, 97, 66, 45, and 21 kDa)] and the quantifications of the different molecular weights were made using an image densitometer G 700 (Bio-Rad).

Results and Discussion

The isolated lectins

Isolation of lectins.—The methods and procedures used for isolation of the 5th-instar *S. gregaria* lectins are presented in Figure 1.

After $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography, the HA was found to be present in fractions of peak I (18 ml), peak II (24 ml), and peak III (18 ml), but not in peaks IV and V (Fig. 2). Therefore, fraction pools of each of peaks I, II, and III were indicated to contain lectins. These pools were separately, and slowly applied to the prepared CNBr-activated Sepharose 4B columns for affinity chromatography.

On washing each column successively with TBS to which 0.5 M NaCl was added until no further protein was detected, it was shown that most proteins were not adsorbed to the column, appeared in the through-flow fractions, and had no HA (Fig. 3a, b, c). When

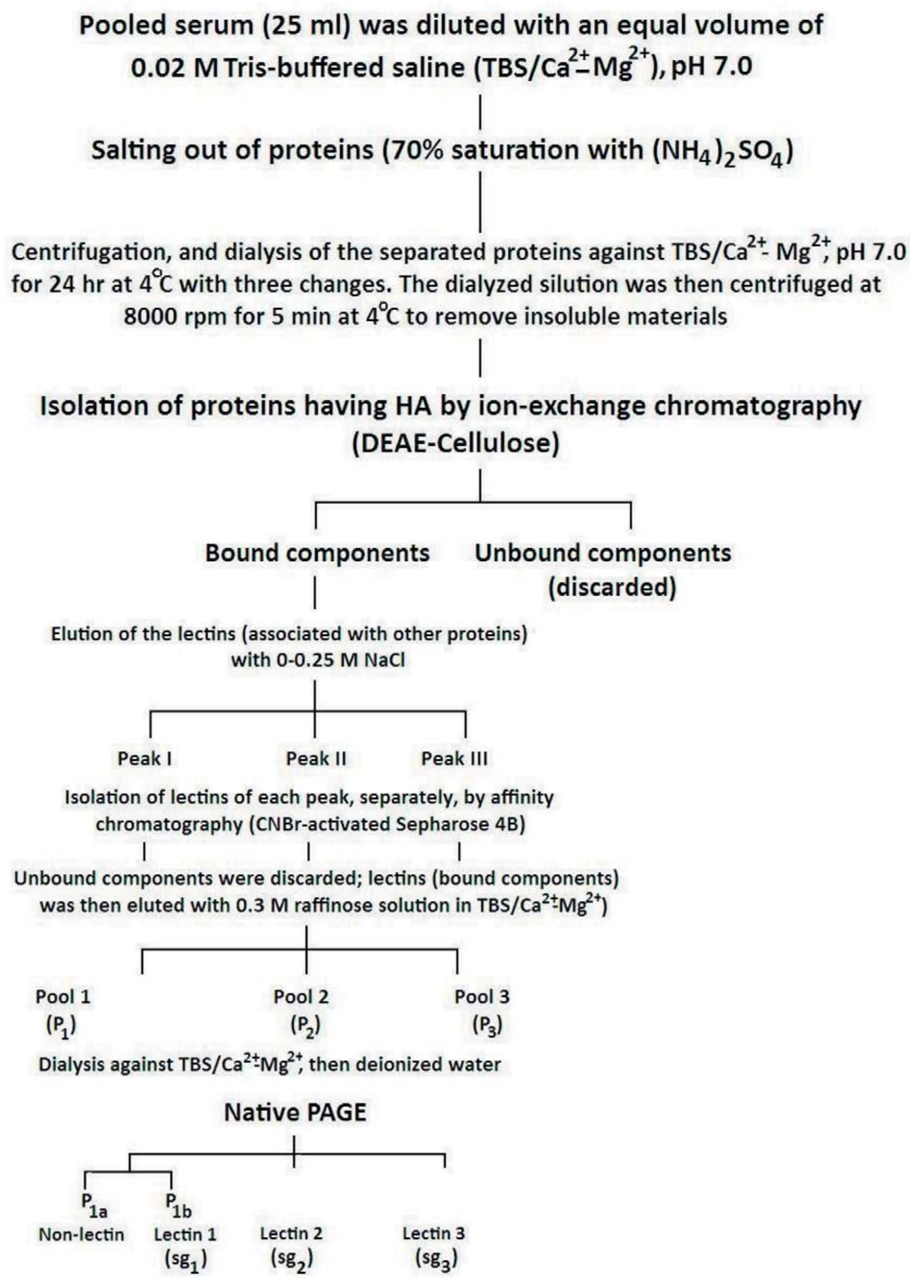

Fig. 1. Summary of methods and procedures used to isolate serum lectins of 5th instar *S. gregaria*.

Table 1. Purification schedule of the 5th instar *S. gregaria* serum lectins.

Purification step	Volume (ml)	Protein (mg/ml)	Hemagglutinating activity (HA)	Specific activity (HA/mg protein)	Yield (%)
A- Crude lectins and salting out					
Whole serum (crude lectins)	25	2.28	512	224.5	100
70% saturated $(NH_4)_2SO_4$	150	0.62	256	412.9	50
B- DEAE cellulose chromatography					
Peak I	18	1.99	8	4.02	1.562
Peak II	24	1.73	16	9.24	3.125
Peak III	18	2.04	16	7.84	3.125
C- Affinity chromatography					
Pool 1	7.5	0.85	128	150	25
Pool 2	7.5	0.66	256	387.8	50
Pool 3	6	0.55	256	465.4	50

Table 2. Hemagglutinating activity (HA)[a] of the 5th instar *S. gregaria* serum lectins contained in the collected pools from affinity chromatography, against a group of vertebrate RBCs.

	HA [b]		
	P_1	P_2	P_3
Rabbit	128	256-512	256
Sheep	4	32	16
Human:			
A^+	4	4	0
B^-	0	16	0
O^-	4	4	0
Rat	0	4	0
Guinea pig	0	0	0
Horse	0	0	0

[a] Measured in a sample pool of 20 insects for each RBC type.
[b] Standard assay condition: using TBS/Ca-Mg. HA expressed as $\log_2$ titer.

the bound material (lectins) was then eluted with 0.3 M raffinose solution in TBS/ Ca^{2+}–Mg^{2+}, small protein peaks containing HA were realized (Fig. 3a, b, c). Fractions constituting each peak were considered as different pools [pool 1 (P_1), pool 2 (P_2), and pool 3 (P_3)], each containing different lectins. After dialysis to remove raffinose, the three resulting pools exhibited prominent HA; therefore, they seemed to contain different lectins. The specific activity of lectins recovered from serum by use of these methods and procedures showed a 1.5 to 4-fold increase in the specific activity (Table 1), indicating prominent reproducibility.

HA of the isolated lectin pools.—The determined HA patterns of lectins, of the 5th instar *S. gregaria*, contained in the three collected pools from affinity chromatography, are presented against a range of vertebrate RBCs, (Table 2).The isolated lectin pools have the highest HA against rabbit RBCs, when compared to the other RBC types, in the order $P_2 > P_3 > P_1$. For the other RBC types, sometimes there was a very low level of HA, or even none. These results demonstrate a high specificity of the 5th instar *S. gregaria* isolated serum lectins to RBCs of rabbit, when compared to those of the other vertebrates (Table 2).

Homogeneity and molecular characteristics of lectins

Homogeneity and molecular characteristics of the isolated lectins contained in the pools P_1, P_2 and P_3 were monitored and confirmed by use of IEF, and both the native PAGE and SDS/PAGE (under nonreducing and reducing conditions).

Isoelectric focusing (IEF).—The obtained results (Fig. 4) reveal some characteristics of these lectins. P_2 and P_3 both focused as single bands at pH 8.11 and pH 6.84, respectively. However, P_1 showed a major band (~84%) focused at pH 6.39 and a minor band (16%) focused at pH 6.53 and realized in association to the major one. Therefore, each of the P_2 and P_3 seems to be formed of a single and separate lectin. The nature of the two-band components of P_1 were confirmed by native PAGE, accompanied with HA determination to each separated band (see below).

The pI values of P_2 protein appear to lie in the slightly alkaline

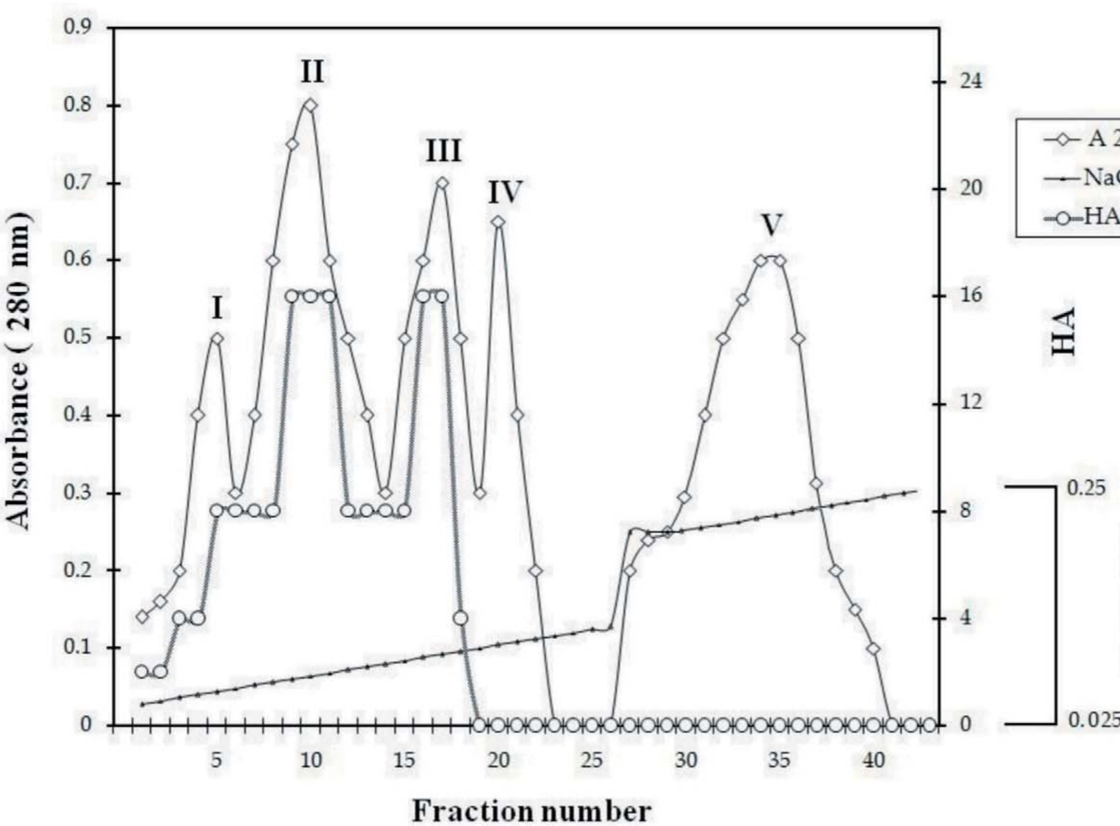

Fig. 2. Ion-exchange chromatography on DEAE-cellulose, of the 5th instar *S. gregaria* serum proteins, resulting from salting out by 70% $(NH_4)_2SO_4$. Elution with a linear gradient of 0 – 0.25 M NaCl. Fractions of peaks I, II, III have HA against rabbit RBCs; but those of peaks IV, V have none.

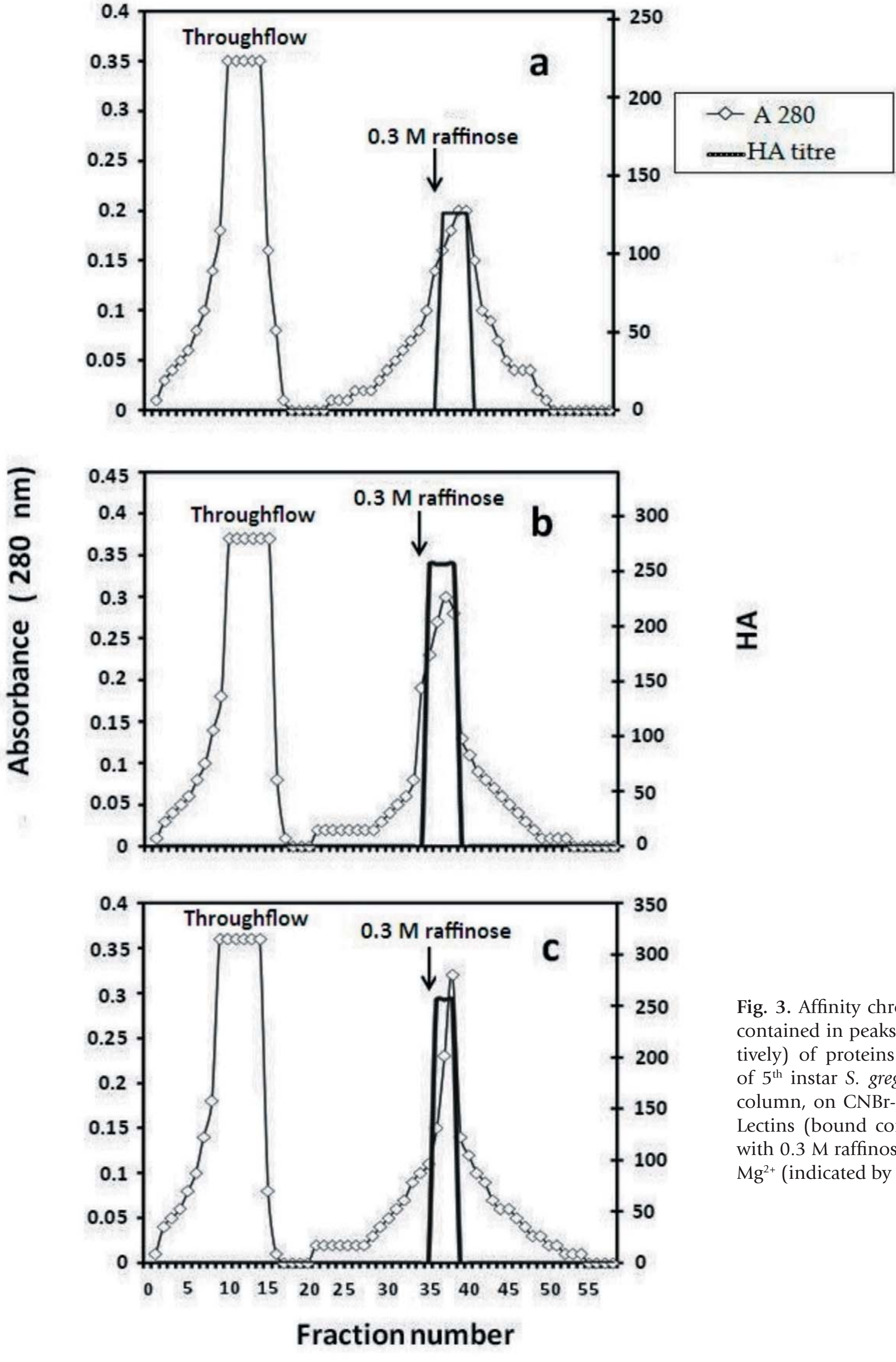

Fig. 3. Affinity chromatography of lectins, contained in peaks I, II, III (a, b, c, respectively) of proteins separated from serum of 5th instar *S. gregaria* by DEAE-cellulose column, on CNBr-activated Sepharose 4B. Lectins (bound components) were eluted with 0.3 M raffinose solution in TBS/ Ca^{2+}-Mg^{2+} (indicated by arrows).

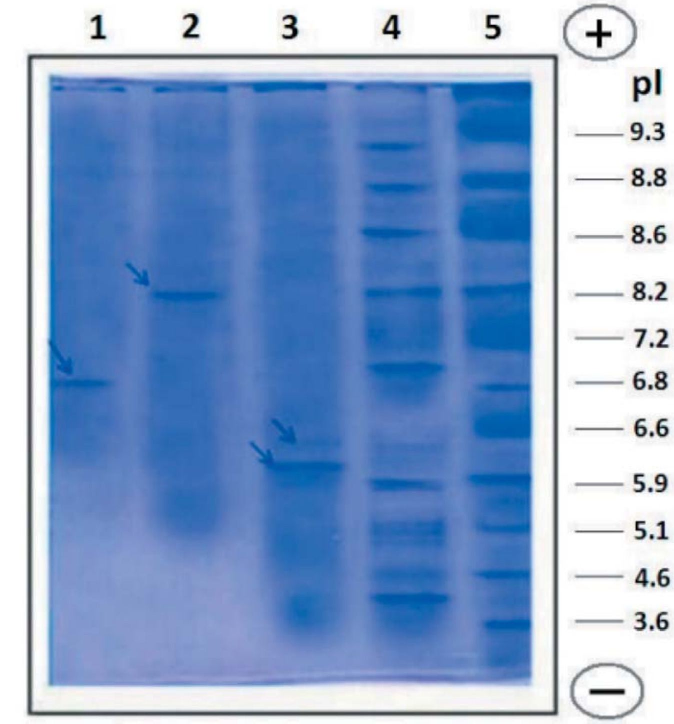

Fig. 4. IEF/PAGE of the 5th instar *S. gregaria* lectins contained in the three pools P_1, P_2, and P_3 (lanes 3, 2, and 1, respectively) collected from Sepharose 4B affinity chromatography columns, and also of the whole-serum proteins (lane 4). IEF standards (lane 5) (Sigma) with different pH ranges are shown to the right. The gel was stained with COBB R-250.

range, whereas those of both P_1 and P_3 appear to lie in the slightly acidic range. In other insects, the pI values range from 4.7 to 6.4, as in the orthopterans *M. sangunipes* (Stebbins & Hapner 1985), and *L. migratoria* (Drif & Brehelin 1994), the dictyopteran *B. discoidalis* (Chen *et al.* 1993), the lepidopterans *M. sexta* (Minnick *et al.* 1986), and *H. cecropia* (Castro *et al.* 1987), the coleopteran *A. dichotoma* (Umetsu *et al.*, 1984), and the dipteran *G. fuscipes* (Ingram & Molyneux 1990).

Native PAGE.—The identity of the lectins contained in the three pools P_1, P_2 and P_3, collected from affinity chromatography columns (raffinose-eluents) (Fig. 3 a-c) was confirmed by native PAGE. The electophoretograph obtained (Fig. 5) shows that P_2 and P_3 appear as single bands. This confirms the nature of both P_2 and P_3 that appeared on IEF gel. Therefore, each of these two bands was considered as a discrete lectin and they are designated as Sg_2 and Sg_3, respectively. Both bands have Rm values of almost 0.32, (Fig. 5) that were revealed in the high molecular weight range.

On the other hand, P_1 was separated into two bands with different relative mobility values of 0.38 (P_{1a}) and 0.44 (P_{1b}), respectively. The first band P_{1a} (Rm = 0.38) did not display any HA, whereas the second band P_{1b} (Rm = 0.44) enclosed HA, and was considered as a third lectin in the serum of *S. gregaria*; it was designated as Sg_1. The nonagglutinating band may be a nonlectin glycoprotein that binds to, and is eluted from, the ion exchange and the affinity columns, in association with the isolated lectin Sg_1. Co-isolated proteins, during the isolation of multiple lectins, that are eluted from both ion exchange and affinity chromatography, present a case observed before in other insects such as *E. tiaratum* (Richards *et al.* 1988) and *B. discoidalis* (Wilson *et al.* 1999).

Appearance of native PAGE patterns of Sg_1 (P_{1b}), Sg_2 and Sg_3 as a single band with high molecular weight range, indicates that each band may be formed of aggregates (oligomers) of subunits. This assumed oligomeric native form of lectins of the 5th instar *S. gregaria* serum is reported before in other insects, *e.g.*, the orthopterans *M. sangunipes* (Stebbins & Hapner 1985), *L. migratoria* (Drif & Brehelin 1994), and adult *S. gregaria* (Ayaad 2004), and in the dipteran *G. fuscipes* (Ingram & Molyneux 1990).

SDS/PAGE.—When the lectins Sg_1 (P_{1b}), Sg_2 and Sg_3 were sliced away from the native PAGE, eluted, then electrophoresed by SDS/PAGE, under both nonreducing (Fig. 6), and reducing (Fig. 7) conditions, using 10% separating gel and 5% stacking gel, additional characteristics were revealed. Under the nonreducing conditions, each of the Sg_1, Sg_2, and Sg_3 was separated by SDS/PAGE as a single band with approximate molecular weights of 21.5, 44.5, and 43.9 kDa respectively (Fig. 6). This may indicate that subunits of each lectin are of approximately the same molecular weight and are held together by noncovalent interactions. Noncovalent linking of protein subunits was reported in lectins of other insects, *e.g.*, the dipteran *S. peregrina* (Komano *et al.* 1980), lepidopterans *H. cecropia* and *S. exigua* (Castro *et al.* 1987, Boucias & Pendland 1993), and the phasmid *E. tiaratum* (Richards *et al.* 1988).

Under the reducing conditions, SDS/PAGE separation (Fig. 7) showed a single band only from Sg_1 (21.7 kDa), whereas each of Sg_2 and Sg_3 were separated into two bands. Those resulting from Sg_2 have approximate molecular weight of 32.6 and 31.7 kDa, and those of Sg_3 are of 32.9 and 31.5 kDa. The separation of two different bands with two different molecular weights from each of the Sg_2 and Sg_3 (Fig. 8) indicates that each of these latter is formed of two different types of subunits linked with disulphide bonds. This character was reported before for some insect lectins, for example, the orthopterans *T. commodus* (Hapner & Jermyn 1981), and *M. sanguinipes* (Stebbins & Hapner 1985), BDL1, and BDL2 of the dictyopteran *B. discoidalis* (Chen *et al.* 1993), and allo A-I and -II of the coleopteran *A. dichotoma* (Umetsu *et al.* 1984).

The low molecular-weight range of the single-banded Sg_1 separated by the nonreducing and reducing SDS/PAGE (21.5 to 21.7 kDa) is near to those of other insects, for instance, 30-kDa lectin of *P. americana* (Kubo & Natori, 1987), 20-kDa lectin of *S. peregrina* (Fujita *et al.* 1998).

Physicochemical properties of the isolated lectins

Stability.— The obtained data revealed that HA was completely abolished after 25 min exposure to 100°C, but reduced to 75 % only upon exposure to 60°C for the same time. On the other hand, it was observed that HA was stable when exposed to 25°C (room temperature) for the same period of time. These observations indicate that lectins of the 5th instar *S. gregaria* are heat-labile in nature. Heat instability is characteristic for lectins of some other insects, *e.g.*, the orthopterans *T. commodus*, (Hapner & Jermyn 1981), and *M. sanguinipes* (Stebbins & Hapner 1985), the phasmid *E. tiaratum* (Richards *et al.* 1988), and the dipteran *G. fuscipes* (Ingram & Molyneux 1990). However, in contrast to these cases, in the coleopteran *L. decemlineata* (Minnick *et al.* 1986), and the orthopteran *L. migratoria* (Drif & Brehelin 1994) the lectins were reported to be heat resistant when subjected to elevated temperatures of 70-100°C.

For the storage temperature and period, the HA of lectins of 5th instar *S. gregaria* were stable when stored at -20°C, with an extremely slow insignificant decline observed on prolonged storage (3 mo).

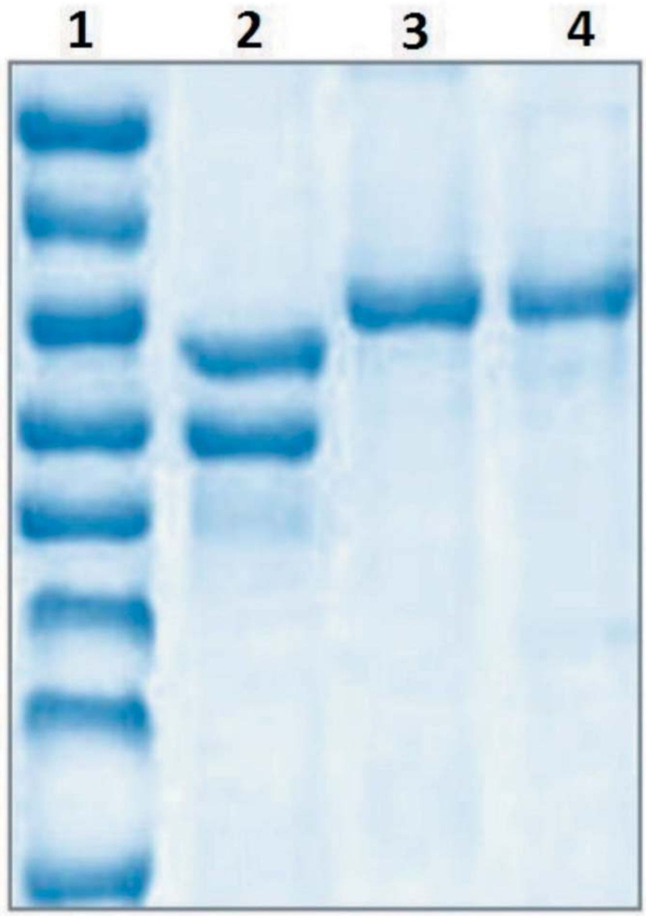

Fig. 5. Native PAGE of the 5th instar *S. gregaria* serum proteins (lane 1), and the lectins contained in the pools P_1, P_2, and P_3 (lanes 2, 3, and 4 respectively) collected from Sepharose 4B affinity chromatography columns. The gel was stained with COBB R-250.

Since the storage medium of the isolated lectins under investigation was without any additive-sugars, it seems that these lectins do not need the presence of sugars for their stability. This situation is analogous to that reported in *S. peregrina* (Komano *et al.* 1980). However, in contrast to this case, isolated lectins from other insects require addition of sugar to the storage medium to maintain their activity. For example, in *T. commodus* (Hapner & Jermyn 1981), the lectin required the addition of galactose. Also the lectins of *M. sanguinipes* (Stebbins & Hapner 1985), once isolated, were so unstable that disaggregation of the multimer occurred readily in the absence of D-galactose; and in *E. tiaratum* instability was observed when the lectin was not stored in lactose (Richards *et al.* 1988).

Divalent cation requirement for HA.— The present data show that the isolated lectins Sg_1, Sg_2, and Sg_3 require Ca^{2+} to express their HA. Some cations such as Mg^{2+} or Zn^{2+} can only partly replace Ca^{2+}; however other divalent cations, such as Mn^{2+}, have no effect (Table 3). As expected, addition of EDTA caused complete inhibition to HA. Also removal of Ca^{2+} by dialyzing the lectin solutions against either TBS alone or TBS with 10 mM EDTA, similarly resulted in a complete loss of HA of these lectins. EDTA presumably exerted its effect through chelation of divalent cations that structurally associated with the lectins. The HA of C-type lectins of *T. commodus*, *M. sanguinipes*, *S. exigua*, *E. tiaratum* and adult *S. gregaria* were also lost in the presence of EDTA (Pendland & Boucias 1986, Stebbins & Hapner 1985, Ayaad 2004, Richards *et al.* 1988, Hapner & Jermyn 1981). In most insect lectins a complete loss of HA was observed in the presence of EDTA (Ratcliffe *et al.* 1985). Therefore, for the lectins isolated from serum of the 5th instar *S. gregaria*, the observed requirement of Ca^{2+} to enhance and maintain HA of the three lectins Sg_1, Sg_2, and Sg_3, prominently indicates that these lectins are Ca^{2+} - dependent. This property is a general characteristic of a group of lectins denoted as C-type lectins. This type of lectin was originally named to reflect the special importance of Ca^{2+} in the mechanism of carbohydrate binding (Zelensky & Gready 2005). Each of the known C-type lectins has carbohydrate-recognition domain structures containing a conserved Ca^{2+}- binding site; and may also contain a second site (Drickamer 1999).

Sugar specificity and inhibition to HA.—The obtained profile of sugar inhibition to HA of lectins isolated from serum of 5th instar *S. gregaria* against rabbit RBCs shows certain characteristics. The inhibition of sugars and of glycoconjugates to HA of Sg_1, Sg_2 and Sg_3 showed great resemblance to each other (Table 4). The HA of these lectins was preferentially inhibited (IC_{50} = 6.25 mM) by raffinose, α-m-nitrophenyl-D-galactose, α-*p*-nitrophenyl-D-galactose, and α-methyl-D-galactose, and the monosaccharide L-(+)-rhamnose. None of these lectins were inhibited by D-(+)-mannose, trehalose and N-acetylglucosamine (200 mM), or laminarin (> 1 %). Low sensitivities (IC_{50} = 50-100 mM) were observed in case of the free sugars D-galactose, D-glucose, sucrose, lactose, and also by N-acetyl-D-galactosamine. These results allow speculation that the carbohydrate-binding site of the carbohydrate recognition domain of these lectins prefers (within the range of the tested sugars) α-linked-D-galactosides over

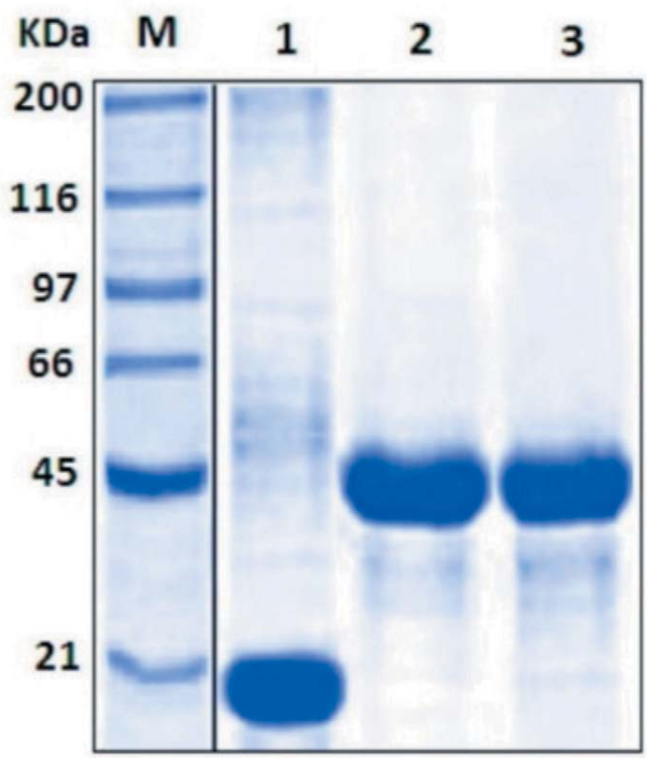

Fig. 6. SDS-PAGE analysis (under nonreducing conditions) of lectins Sg_1, Sg_2, and Sg_3 (lanes 1, 2 and 3 respectively) isolated from serum of the 5th instar *S. gregaria*. The gel was stained with COBB R-250. Molecular weights (kDa) of protein standards (lane M) are shown on the left.

Table 3. Effects of divalent cations and EDTA (cation chelator) on hemagglutinating activity (HA) of the lectins Sg_1, Sg_2, and Sg_3 isolated from serum of 5th instar *S. gregaria*.

	HA		
	Sg_1	Sg_2	Sg_3
Ca^{2+} - free medium	0	0	4
20 mM Ca^{2+} [a]	128	256-512	256
20 mM Mg^{2+}	64-128	128	128
20 mM Zn^{2+}	128	128	128
20 mM Mn^{2+}	4	4	4
10 mM EDTA	0	0	0
5 mM EDTA	4	4	4

[a] Standard assay condition; *i.e.*, reference value of HA.

both the β-form and also the free D-galactose.

The affinity of the 5th instar isolated *S. gregaria* serum lectins toward α-linked galactosides is a feature that has also been reported in other acridids, such as *L. migratoria* (Drif & Brehelin 1994) and adult *S. gregaria* (Ayaad 2004). On the other hand, affinity of lectins to β-linked-D-galactosides, such as lactose and lactulose, was recorded in the coleopteran *A. dichotoma* (Umetsu *et al.* 1984), its lectins being inhibited by this group of carbohydrates. The latter lectin specificity is also known in vertebrates, where the best ligands are β-galactosides (Barondes 1984). Some lectins purified from other insect species, especially from orthopterans (Stebbins & Hapner 1985, Lackie 1981, Jurenka *et al.* 1982, Hapner 1983, Drif & Brehelin 1989) show an affinity for a broad spectrum of carbohydrates. Another orthopteran *T. commodus* (Hapner & Jermyn 1981), and the dipteran *P. duboscqi* (Volf *et al.* 2002) possess lectins with amino sugar-binding affinity. In numerous other insect species, mainly lepidopterans, hemolymph lectins show affinity for galactose and lactose (Pendland & Boucias 1986) or to glucosides (Minnick *et al.* 1986, Qu *et al.* 1987). Lectins from another group

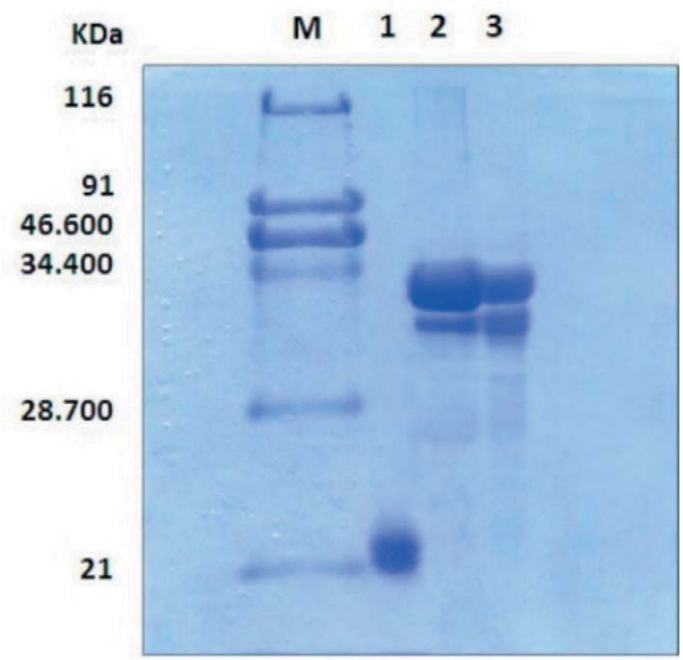

Fig. 7. SDS-PAGE analysis (under reducing conditions) of lectins Sg_1, Sg_2, and Sg_3 (lanes 1, 2, and 3, respectively) isolated from serum of the 5th instar *S. gregaria*. The gel was stained with COBB R-250. Molecular weights (kDa) of protein standards (lane M) are shown on the left.

Table 4. Inhibition by sugars and glycoconjugates to hemagglutinating activity (HA), of lectins Sg_1, Sg_2, and Sg_3, isolated from serum of the 5th instar *S. gregaria*, against rabbit RBCs.

	Minimum concentration[a] (mM/or %) required for 50 % inhibition (IC_{50}) to HA		
	Sg_1	Sg_2	Sg_3
Monosaccharide			
D-(+)-galactose	100	100	100
D-(+)-glucose	>100	50	>100
L-(+)-rhamnose	25	≥ 6	25
Oligosaccharides			
Lactose	100	100	100
Raffinose	12	6	6
Sucrose	100	100	>100
N-acetylated sugars			
N-acetyl-D-galactosamine	100	50	>50
Others			
α-methyl-D-galactose	12	12	25
α- m-nitrophenyl-D-galactose	6	6	6
α- *p*-nitrophenyl-D-galactose	6	< 6	< 6

[a] Data presented are from experiments repeated three times. Values > 200 mM [(D-(+)-mannose, trehalose, and N-acetyl-D-glucosamine] or >1 % (zymosan and laminarin) indicate that no inhibition of agglutination was observed. All inhibitions were of two wells unless otherwise indicated.

of insects have binding affinity to mannose, *e.g.*, the dictyopteran *B. discoidalis* (Chen *et al.* 1993), and the lepidopteran *H. virescens* (Ourth *et al.* 2005), and to mannan, *e.g.*, the dipteran *A. stephensi* (Chen & Billingsley 1999).

In this context, C-type lectins are known to have a common carbohydrate recognition domain (Weis *et al.* 1991, Drickamer 1992), or two domains (Yu & Kanost 2000), that may increase their binding affinity to carbohydrates. This domain contains a conserved Ca^{2+}-binding site, and may contain a second one (Drickamer 1999). The binding mechanism involves interaction with carbohydrate and the conserved Ca^{2+}, which may form hydrogen bonding with acid and amide side groups (Weis & Drickamer 1996).

The ligand specificity of the carbohydrate recognition domain is largely dependent on the position of hydroxyl groups on the free or conjugated sugar, particularly the 3-OH and 4-OH (Ng *et al.* 1996, Kolatkar & Weis 1996). In addition to these interactions further specificity may be achieved by hydrogen bonds and electrostatic interactions with the surface of the protein (Ng & Weis 1997, Kolatkar *et al.* 1998). In the present work, agglutination of rabbit RBCs by C-type lectins Sg_1, Sg_2, and Sg_3 of the serum of the 5th instar *S. gregaria* was inhibited most efficiently by the α-linked-D-galactosides followed by L-(+)-rhamnose. However, the free monosaccharide D-(+)-galactose and the β-linked-D-galactosides (as in lactose) were of very low efficiency. In the latter cases, the orientation of 3-OH and 4-OH are the same for the same sugar, D-(+)-galactose; and the same was observed for 2-OH and 4-OH in D-(+)-galactose and L-(+)-rhamnose. Therefore, not only the orientation of 2-OH, 3-OH and 4-OH, but also the configuration (α or β) and the nature of the substituents at C_1 or the functional group at C_6, seem to affect the binding specificity and affinity of the 5th instar *S. gregaria* lectins to carbohydrates.

According to these parameters, carbohydrate-binding specificity is crucial in recognition of these cell-surface carbohydrates, including those of the invading pathogens (McGreal *et al.* 2004). Therefore, animal C-type lectins are among the important proteins in pathogen recognition and cellular interaction (Rudd *et al.* 2001, Weis *et al.* 1998) by binding to the carbohydrate component of the surface molecular patterns of these targets (Janeway 1989).

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