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Authors: Ayaad, Tahany H., Dorrah, Moataza A., Mohamed, Amr A., and Bassal, Taha T.M.

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Tahany H. Ayaad, Moataza A. Dorrah, Amr A. Mohamed, and Taha T.M. Bassal*

Entomology Department, Faculty of Science, Cairo University, Giza, Egypt.
*Author for correspondence. Email: tahabasal@gmail.com

Abstract

Hemagglutinating activity (HA) of the serum of the 5th-instar desert locust *Schistocerca gregaria* was detected against a range of vertebrate red blood cells (RBCs). Serum strongly agglutinates RBCs of rabbit. In contrast, no agglutination was observed for RBCs from guinea pig or horse and a very low level was observed for human, sheep, and rat RBCs. HA is Ca^{2+}-dependent, heat-labile, and was strongly inhibited by α-linked-D-galactosides and rhamnose. Developmentally, a relatively low level of HA (≤ 32) is present in the extracted fluids from centrifuged homogenate of 1 to 12 day-old eggs. Also, a limited level of HA was observed in the 2nd and 3rd instars. However, prominent and cyclical patterns were observed in the 4th and 5th instars. In each of these cycles, HA starts low (64), maximizes (128, 512, in the two instars respectively) at about midstadium, then declines again prior to ecdysis to the next stage. On the other hand, adult stage HA starts at a low value (512), then maximizes and is sustained at a fairly constant value (1024), without any difference in the sexes throughout the period of measurements (up to day 73 of the life cycle). HA is independent of sex and season but conspicuously and reproducibly varies with stadium, stage and age.

Key words

Introduction

Insects, as most invertebrates, have an innate immune system, which keeps up effective defense mechanisms against infectious microbes and foreign intruders (Hultmark 1993, Hoffmann 1995, Hoffmann *et al.* 1996, Vilmos & Kurucz 1998). The major defense mechanisms in the hemocoel comprise two different components. The first is a cellular one, involving phagocytosis, nodule formation, and encapsulation (Lackie 1988, Ratcliffe 1993, Gillespie *et al.* 1997, Kurata 2006). The second is a humoral one, characterized by soluble factors circulating in the plasma that participate in the immune defense (Gillespie *et al.* 1997, Ashida & Brey 1998, Bulet *et al.* 1999, Vasta *et al.* 2007).

Lectins are of special consideration, and 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). 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). Some insect hemolymph lectins have been implicated in developmental events, including regeneration

(Kubo *et al.* 1990), moulting (Amanai *et al.* 1991, Chai *et al.* 2008), metamorphosis and embryogenesis (Takahashi *et al.* 1986). However, the most extensively studied aspects of hemolymph lectins has been their role in insect immune defense (Acton & Weinheimer 1974, Ratcliffe & Rowely 1980, Vasta *et al.* 2007).

The presence of lectins in different developmental stages of insects are reported in hemimetabolous insects, *e.g.*, nymphs of the orthopteran *Locusta migratoria* (Drif & Brehelin 1994), adults of the dictyopteran *Periplaneta americana* (Kubo & Natori 1987), and adults of the phasmid *Extatosoma tiaratum* (Richards *et al.* 1988). They are reported as well in stages of holometabolous insects, such as eggs of the hymenopteran *Oecophylla smaragdina* (Hassan & Absar 1995), larvae of the dipteran *Sarcophaga peregrina* (Komano *et al.* 1980), adults of the lepidopteran *Spodoptera exigua* (Pendland & Boucias 1986), pupae of the lepidopterans *Antheraea pernyi* (Qu *et al.* 1987) and *Heliothis virescens* (Ourth *et al.* 2005), and in the adult dipteran female *Phlebotomus duboscqi* (Volf *et al.* 2002).

The present work reports on hemagglutinating activity (HA) of serum from the 5th instar of the desert locust against different types of red blood cells (RBCs). We examined the physicochemical characteristics of serum lectins and their binding affinities and specificities toward carbohydrates and glycoconjugates, a crucial element in the recognition of foreignness. To identify patterns of change and progress, HA was estimated in the different developmental stages and at different ages within each instar and/or stage.

Materials and Methods

Insect rearing.—Desert locusts, *S. gregaria*, were from a well-established laboratory colony at the Entomology Department, Faculty of Science, Cairo University, Egypt. The insects were reared at $30\pm2°C$ and 16:8 h (L: D) photoperiod. Cages were supplied with oviposition pots containing sterile moistened sand. The different instars and adult stage were fed upon clover (*Trifolium alexandrinum*, Papillionaceae) from October to June and then upon *Sesbania seban* (Papillionaceae). Detailed descriptions of the rearing techniques are given by Hinks and Erlandson (1994).

Preparation of egg extract and sera.—Groups of 25 locust eggs were homogenized in TBS/Ca^{2+}-Mg^{2+}, pH 7.0, and then centrifuged at 3000 rpm and 4°C for 20 min; the supernatant was used for the assay of HA in eggs. Sera were collected and pooled from 30 individuals of the same age. For bleeding and collecting hemolymph, individuals were first anaesthetized by placing them at -0°C for approximately 20 min: they were then bled. Hind legs were severed with fine scissors and the exuded hemolymph collected in sterile centrifuge tubes, containing a few crystals of phenylthiourea (East-

man-Kodak, U.S.A) to inhibit phenoloxidase activity. These tubes were kept for 10 min, and then centrifuged at 10,000 rpm and 4°C for 10 min to remove the hemocytes. The supernatant representing the serum was pooled and used for HA assays against rabbit RBCs. The samples were tested immediately after collection to avoid counterfeit negative results.

Preparation of erythrocyte suspension.—Rabbit, sheep, horse, and guinea-pig blood were collected aseptically, each in an equal volume of either Alserver's solution (Alserver & Ainslie 1941) or in heparin (Nile Co. for Pharmaceuticals and Chemical Industries, Cairo, Egypt). Human blood (types A+, B−, and O−) in citrate/dextrose was obtained from Kasr El aini Hospital, Cairo, Egypt. All blood samples were stored at 4°C, and used within 1 wk of collection. RBCs were separated by centrifugation at 1500 rpm and 4°C for 10 min (and the buffy coat was carefully removed), washed three times in phosphate-buffered saline pH 7.2 (PBS) (0.15M NaCl, 0.075M Na_2HPO_4/KH_2PO_4; 0.02% sodium azide), and centrifuged for 10 min at 1500 rpm and 4°C. The obtained RBCs were used for preparation of 2% suspension in Tris-buffered saline (TBS): 25 mM Tris-HCL, 137 mM NaCl and 3 mM KCl, containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (TBS/Ca^{2+}-Mg^{2+}), pH 7.0.

Assay of HA.—HA of the serum was determined against the range of vertebrate RBCs mentioned above and performed at room temperature (RT) in 96-well U-bottomed microtiter plates (Cooke Laboratories, Alexandria, Virginia, USA). Serum, 25 µL, was serially diluted with TBS/Ca^{2+}-Mg^{2+} (see below), and 25 µL of the erythrocyte suspension added to each well. End points of agglutination were recorded 2 h later (after incubation at RT), initially by eye, and then by examining aliquots of each well under a microscope. The HA titre was defined as the reciprocal of the highest dilution showing visual agglutination of the tested RBCs. Controls containing TBS/Ca^{2+}-Mg^{2+} buffer, pH 7.0, instead of the serum, were used.

Effect of divalent cations.—To examine divalent cation requirements for HA, the agglutination test was performed as described by Richards *et al.* (1988). Aliquots of 100 µl of diluted serum were dialyzed for 24 h at 4°C against 200 mL TBS, pH 7.0, either without or with 20 mM Ca^{2+}, 20 mM Mg^{2+}, 20 mM Zn^{2+}, 20 mM Mn^{2+}, or 10 mM or 5 mM EDTA at pH 7.0, for 24 h with two changes of buffer. HA assays were then performed using rabbit RBCs washed in the corresponding dialyzing buffer.

Inhibition assay.—The binding preference to sugars by lectins in serum was investigated by competitively binding and measuring HA in the presence of rabbit RBCs. The following sugars and glycoproteins were tested: the monosaccharides D-(+)-galactose, D-(+)-glucose [Sigma], D-(+)-mannose [MP Biomedicals, Inc. Ohio, U.S.A], and L-(+)-rhamnose [Ptrap Chemical Industries PVT.LTD. India]; the oligosaccharides sucrose, lactose, trehalose, and D-(+)-raffinose [Sigma]; and the sugar derivatives N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, α-*p*-nitrophenyl-D-galactose [Fluka, Switzerland], α-methyl-D-galactose, and α-m-nitrophenyl-D-galactose [Acros organics, New Jersey, USA]. Other glycoconjugates employed were zymosan and laminarin (Sigma). Stock solutions of sugars and glycoconjugates were prepared in TBS/Ca^{2+}-Mg^{2+}. For each inhibition test, serial two-fold dilutions of 25 µL of the inhibitors in TBS/Ca^{2+}-Mg^{2+} in microtiter plates were used. Subsequently, 25 µL of serum were added to each well, mixed by shaking, and incubated for 1 h at RT. Then 50 µL of 2% rabbit RBCs suspension were added. The minimum concentration of these sugars and glycoconjugates

required to cause 50% inhibition (IC_{50}) of HA was indentified.

Effect of temperature and storage time.—Separate sera were heated, in a water bath, for 25 min at 60 and 100°C. Tubes were slackly covered with small caps to reduce evaporation. HA was subsequently assayed against rabbit RBCs. Control experiments were carried out for the same period of time, at RT (25°C). To study the effect of storage time on HA, aliquots of sera were stored at both 4°C and at -20°C over a period of time (1 wk to 3 mo), and the HA was assayed at intervals.

Results and Discussion

HA of whole serum.—The determined HA patterns of the whole serum of nymphal *S. gregaria* against certain vertebrate RBCs are presented (Table 1). The whole serum contains relatively strong HA against rabbit RBCs. Detectable, but much less activity (≤ 32) was observed against human (A+, B−, O−), rat, and sheep RBCs. On the other hand, no HA was observed with RBCs of either Guinea pig or horse. The obtained results demonstrate a high specificity of nymphal *S. gregaria* lectins, contained in the whole serum, for agglutination of RBCs of rabbit, as compared to lectins of the other tested vertebrate RBCs.

Such specificity is already reported for acridids: *S. gregaria* adults (Lackie 1981, Jurenka *et al.* 1982, Ratcliffe & Rowley 1983, Ayaad 2004), *L. migratoria*, (Ratcliffe & Rowley 1983; Drif & Berhelin 1989, 1994), *Melanoplus sanguinipes, M. differentialis* (Stebbins & Hapner 1985), and others (Hapner 1983). The observed high specificity of the acridid lectins toward rabbit RBCs is also detected in other insects, *e.g.*, the dictyopteran *P. americana* (Lackie 1981), the lepidopterans *Hyalophora cecropia, A. pernyi* (Castro *et. al.* 1987) and *Helicoverpa armigera* (Chai *et al.* 2008), the phasmid *E. tiaratum* (Richards *et al.* 1988), and the dipteran *Ph. duboscqi* (Volf *et al.* 2002). On the other hand, other insects have a wide variety of vertebrate RBC specificity (major specificity), *e.g.*, bovine (Haq *et al.* 1996), chicken (Gül and Ayvali, 2002), human type A (Umetsu *et al.* 1984), Human type AB (Ingram and Molynneux, 1990), pigeon (Hapner & Jermyn 1981), and sheep (Komano *et al.* 1980, Kubo & Natori 1987). From these reports, it can be observed that sometimes one type of RBC is strongly agglutinated by hemolymph lectins of a given insect species, but not by the lectins of another one. These variations seem to be due to differences in the nature of the carbohydrate-binding sites of hemolymph lectins: these sites define lectin specificity to these carbohydrates, and so also to differences in conjugated carbohydrates of the surface plasma membrane of the different RBCs.

The present study also indicated a requirement of nymphal *S. gregaria* whole-serum lectins for the presence of divalent cations in order to display their HA. The obtained data (Table 1) show that addition of Ca^{2+} to the serum solution has enabled its lectins to express their HA. Also, Mg^{2+} and Zn^{2+} can partly replace Ca^{2+}, but Mn^{2+} cannot. Removal of Ca^{2+} by dialyzing the lectin solutions against either TBS alone or TBS with 10 mM EDTA, nearly obliterated the HA of the whole serum. The same result was obtained through chelation of Ca^{2+} from the serum by addition of 10 or even 5 mM EDTA to its solution (Table 1). Therefore, the hemolymph serum seems to contain Ca^{2+}-dependent lectins; these latter are classified as the other Ca^{2+}-dependent lectins (Drickamer 1999) *i.e.*, as C-type lectins.

The requirement of HA of *S. gregaria* serum lectins for Ca^{2+} is a general characteristic of lectins of some insects such as *Teleogryllus commodus* (Hapner & Jermyn 1981), *M. sanguinipes* (Stebbins & Hapner 1985), *S. exigua* (Pendland & Boucias 1986), *P. americana*

Table1. Hemagglutinating activity (HA)[a,b] of 5th instar *S. gregaria* whole serum against a range of vertebrate RBCs; effects of divalent cations and EDTA (cation chelator); and inhibition[c] of rabbit RBCs agglutination by certain sugars and glycoconjugates.

Erythrocyte type	HA	Additives	HA	Inhibitors	Minimum concentration (mM/or %) required for 50% inhibition (IC_{50}) to HA
Rabbit	512	20 mM Ca^{2+}	512	**Monosaccharide**	
Sheep	32	20 mM Mg^{2+}	128	D-(+)-galactose	100
Human:		20 mM Zn^{2+}	64-128	D-(+)-mannose	100
A$^+$	16	20 mM Mn^{2+}	16	L-(+)-rhamnose	25
B$^-$	32	10 mM EDTA	4		
O$^-$	32	5 mM EDTA	16	**Oligosaccharides**	
Rat	16-32			Lactose	100
Guinea pig	0			Raffinose	6
Horse	0			Sucrose	50
		Control[d]	4-16	**N-acetylated sugars**	
		(*i.e.*, Ca^{2+}– free medium)		N-acetyl-D-galactosamine	100
				Others	
				α-methyl-D-galactose	12
				α- m-nitrophenyl-D-galactose	6
				α- p-nitrophenyl-D-galactose	6

[a] Standard assay condition (*i.e.*, reference value of HA) using TBS/Ca^{2+}-Mg^{2+}; HA expressed as log$_2$ titer.

[b] Each test was carried out with a sample pool of 20 insects for each erythrocyte type.

[c] Data presented are from one representative experiment repeated three times. Values > 200 mM sugars (D-(+)-glucose, 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.

[d] HA was detected when each erythrocyte type was suspended in TBS only.

(Kubo & Natori 1987), *E. tiaratum* (Richards *et al.* 1988), *Blaberus discoidalis* (Chen *et al.* 1993), *L. migratoria* (Drif & Brehelin 1994), *Drosophila melanogaster* (Haq *et al.* 1996), adult *S. gregaria* (Ayaad 2004), and *H. armigera* (Chai *et al.* 2008).

The profile of inhibition by certain sugars and other glycoconjugates to HA of nymphal *S. gregaria* whole-serum lectins is presented in Table 1. The presented data show that HA was strongly inhibited by the α-linked D-galactosides raffinose, α-m-nitrophenyl-D-galactose, α-*p*-nitrophenyl-D-galactose, and α-methyl-D-galactose; and also strongly inhibited by the monosaccharide L-(+)-rhamnose. The tested sugars sucrose, D-(+)-galactose, D-(+)-mannose, lactose, and also N-acetyl-D-galactosamine were considered of very low preference. The following sugars and glycoconjugates D-(+)-glucose, trehalose, N-acetyl-D-glucosamine, or laminarin, and zymosan were considered to be noninhibitors. Therefore, within the limits of the tested range of sugars and glycoconjugates, we may speculate that lectins of serum of nymphal *S. gregaria* have a preferential binding affinity for the α-linked-D-galactosides, particularly those containing aryl groups, followed by L-(+)-rhamnose. This inhibition presumably means that these lectins have preferred binding affinity to these sugars and glycoconjugates, over that to the employed rabbit RBCs.

Affinity of the nymphal *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 in particular, was recorded in the Coleopteran *Allomyrina dichotoma* (Umetsu *et al.* 1984), where its lectins were inhibited by this group of carbohydrates, such as lactose and lactulose. Some lectins of other insects, especially orthopterans (Lackie 1981, Jurenka *et al.* 1982, Hapner 1983, Stebbins & Hapner 1985, Drif & Brehelin 1989), show an affinity for a broad spectrum of carbohydrates. Other

orthopterans such *T. commodus* (Hapner & Jermyn 1981), and the dipteran *Ph. 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).

The effects of elevated temperature, as well as storage at low temperature and storage time, were tested on HA of whole serum. The obtained data reveal that HA was completely abolished after 25 min exposure to 100°C; but reduced only to 75% upon exposure to 60°C for the same interval. On the other hand, it was observed that HA was stable when exposed to 25°C (RT) for the same period of time.

These observations indicate that serum lectins of nymphal *S. gregaria* are heat-labile in nature, and so the 25°C-condition may be considered as a suitable experimental condition. Heat instability is characteristic for lectins of some other insects, *e.g.*, the orthopterans *T. commodus*, (Hapner & Jermyn 1981), *M. sanguinipes* (Stebbins & Hapner 1985), the phasmid *E. tiaratum* (Richards *et al.* 1988), and the dipteran *Glossina fuscipes* (Ingram & Molyneux 1990). However, in the coleopteran *Leptinotarsa decemlineata* (Coleoptera) (Minnick *et al.* 1986), the hemipteran *Halys dentata* (Pathak 1991), 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 serum lectins of the nymphal *S. gregaria* were stable when the serum was stored at -20°C, with an extremely slow, insignificant, decline observed on prolonged storage (3 mo).

Developmental changes in HA.—The pattern of changes, and progress of HA in the different developmental stages (from egg up to the adult), and at different ages within each instar and/or stage of *S.*
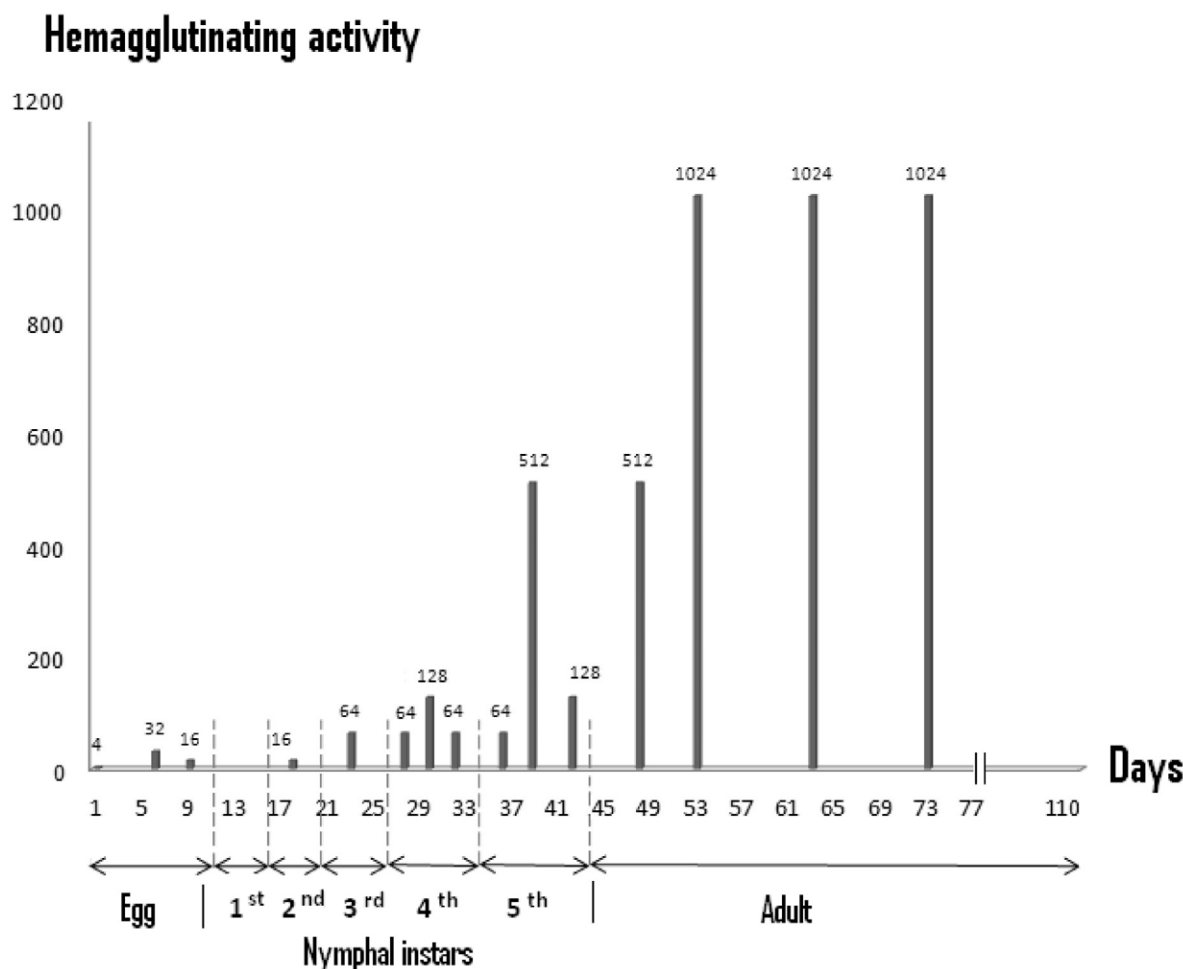
Hemagglutinating activity

Fig. 1. Developmental changes in hemagglutinating activity of the whole serum of different developmental stages (and of extracted fluids from egg homogenate after centrifugation) of *S. gregaria*, against rabbit RBCs. Duration of the whole life cycle was about 110 d at 30 ± 2°C. Each column depicts the mean of values recorded in three separate experiments.

gregaria are shown (Fig. 1). The presented results show a low level of HA (≤32) in the fluids extracted from homogenate of 1 to 12-day old eggs. Also, limited levels of HA were observed in the 2nd and 3rd instars. However, prominent and cyclical patterns were observed in the 4th and 5th instars. In each of these cycles, HA starts low, maximizes (128 and 512 in the two instars respectively) at about midstadium, then declines again to a low value prior to ecdysis to the next developmental form. On the other hand, HA of the adult stage starts at a low value (512), then maximizes and sustains at a fairly constant value (1024), without observable difference in both sexes throughout the period of measurements for the adult (30 days). So age, instar, and the stage of *S. gregaria* appear to affect remarkably the levels of HA.

The cyclical HA change in *S. gregaria is* similar to that reported for another hemimetabolan, *Halys dentata* (Pathak 1991). In *H. dentata*, the HA was found to decrease during the apolysis-ecdysis phase in each instar, and to increase in the prevailing period. Further, HA increased with each developmental stage and was the highest in adults.

In another hemimetabolan, the acridid *M. sanguinipes* (Jurenka *et al.* 1982), HA was reported to exhibit no dramatic change from the 4th to the 5th instar, nor to the adult stage, nor between different ages of adults. Furthermore, in yet another acridid, *L. migratoria*,

Drif and Brehelin (1989) measured the hemagglutinin titer daily, between the last day of the 4th instar and the 1st day of adult development they observed that HA started with a medium titer (512) at the end of 4th instar (before molting). This activity was increased (up to 4096) in the course of the 5th instar on the 6th day, then fell again after molting (1024) on the 1st day of the adult stage.

In holometabolous insects, other patterns of change were shown. Suzuki and Natori (1983) reported a rhythmic pattern in the activity of hemagglutinins during larval-pupal and pupal-adult development in *Bombyx mori*. They reported also that HA was relatively high in the early fifth instar, during the spinning period, and at the emergence of the adult, and decreased during prevailing periods of that larval instar and pupa. They suggested that these changes were necessary for development. They additionally suggested that agglutinins may be absorbed to the surface of decomposed larval tissues and therefore, may be functional in the immune surveillance of old tissue. A related pattern was reported in the beetle *A. dichotoma* (Umetsu *et al.* 1984), where slight HA was detected in the egg and in the first two instars. The activity was observed to increase in the third instar, reaching a maximum at its maturity, then to decrease to about half its value in the pupa; this was followed by another decrease in the adult stage to about one half of the pupal value. In *H. cecropia* (Yeaton 1981), HA increases throughout early instars up

to the fourth, where it is sustained at this level in both the pupal and adult stages. Komano *et al.* (1980, 1981) reported the presence of agglutinins in the larvae of *S. peregrina* and a decrease in HA on pupation; they proposed that these lectins may be responsible for immunosurveillance, playing a role in scavenging of both invading foreign bodies (on injury) and of decomposed self-tissue fragments on pupation.

In *S. gregaria*, it being a hemimetabolous insect, the cyclical change of serum HA, where the apolysis-ecdysis phase is short and restricted to the formation of new cuticle, cannot be explained on the basis proposed by Komano *et al.* (1980, 1981), Suzuki and Natori (1983), and Umetsu *et al.* (1984). This is because in holometabolous insects complete reconstruction occurs during metamorphosis and lectins may be absorbed by degenerating larval tissue. However, growth and developmental events, other than apolysis-ecdysis, are continuous at different rates throughout the immature stages in hemimetabolans; therefore, the cyclical changes in HA may correspondingly be correlated to these events.

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