www.bti.org.in
ISSN 0974-1453
Research Article

IMMUNOAGGREGATION TECHNIQUE FOR IDENTIFICATION OF GENETIC HARDINESS IN SELECTED SILKWORM *Bombyx mori* (L.) GERMPLASM STOCKS

S. VishnuPriya[*] and P. Somasundaram[1]

[1] Scientist D, Central Sericultural Germplasm Resource Centre, CSB, Biotechnology Laboratory, Hosur, TamilNadu.

[*]Corresponding Author: vishnupriyabiotechnology@gmail.com

ABSTRACT

Lepidopteran insect silkworm *Bombyx mori,* provide important model system for innate immunity of insects. Ten silkworm samples were collected and investigated by bacterial endotoxin Lipopolysaccharide. In this study, we characterized the tolerant and susceptible silkworm races with bacterial endotoxin antigen (LPS) and to quantify the immune-diversity among the ten races based on their serum antibodies. The level of serum antibodies were detected by coagulation and haemolymph density studies and the immuno titre value was identified by qualitative and quantitative analysis. The titer of agglutination in the micro technique varied depending upon the concentration of antigen in haemolymph and midgut samples of ten races. The effect of different immune response factors (IRF) from immunized selected silkworms (i.e., treated with LPS) haemolymph and midgut were assayed. Correlations between the races based and immune functions viz., coagulation and haemocyte density shown a highly significant (P<0.001), Pure Mysore, Nistari, Kollegal Jawan, OS-616, A13 and moderate correlation (P<0.01) in MY1 (SL), HosaMysore and no significant correlation (P<0.05) in PMX, Tamil Nadu white. From qualitative and quantitative analysis, the race Pure Mysore, Nistari, A13 showed maximum number of aggregation with titre value (1:120) and minimum number of titre value (1:20) were observed in PMX and Tamil Nadu white in LPS treated both haemolymph and midgut samples. Thus the maximum titre of Pure Mysore, Nistari and A12 races are considered being hardy races, because of the presence of lectins. Hence, these lectins are also used as a marker for identification of disease tolerant and susceptible races.

Keywords: Endotoxin-Lipopolysaccharide, Silkworm Bomyx *mori*, Agglutination, Immunity, Genetic Hardiness.

INTRODUCTION

The term agglutination is the phenomenon that occurs when a cellular/ particulate antigen such as a saline suspension of bacteria is mixed with the homologous antiserum, under suitable conditions of temperature, salinity, pH. The clumping of cells in flocculent or granular masses, and subsequent settling to the bottom of the mixture, indicates agglutinating antibody (Steinhaus, 1963). Coagulation is one of the first responses to injury in insects as in other animals, and the clot prevents infection as well as stops bleeding and contributes to wound healing (Gregoire, 1974., Bohn, 1986., Theopold *et al*., 2004). Lectins/agglutinin is a glycoprotein usually without catalytic activity that has the ability to bind to specific carbohydrates and exist in almost all living organisms (Marques and Barracco, 2000). They can bind cells and an agglutination reaction occurs. Interaction between lectins and carbohydrates is involved in various biological activities, for instance the cellular and tissue transport of carbohydrates (Goldstein *et al*., 1980; Ravindranath and Copper, 1984), glycoproteins (Vasta,1992), cell adhesion (Kasai and Hirabayashi,1996; Ni and Tizard,1996; Vasta *et al*., 1999), opsonization (Jomori and Natori, 1992., Cerenius *et al*.,1994), and nodule formation (Koizumi *et al*., 1999). Especially, C-type lectins, calcium dependent lectins are reported to be involved in immune recognition in invertebrates (Weis *et al*.,1998., Vasta *et al*.,1999). Constitutively expressed lectins that have an LPS-binding property have been characterized from the silkworm *Bombyx mori* (Koizumi *et al*., 1999) and the American cockroach *Periplaneta Americana* (Jomori and Natori,1992; Natori *et al*.,1999). It was found that the biological functions of these LPS binding proteins are involved in bacterial clearance activity and an opsonic effect, respectively. Therefore, we designed a novel approach to identify components of humoral Immunity by inducing coagulation in the lepidopteron *Bombyx mori* using a bacterial endotoxin (LPS) antigen and the level of genetic hardiness was analysed by their heamagglutination activities.

MATERIALS AND METHODS

Test material

The ten MV silkworm races maintained at Central Sericultural Germplasm Resources Centre, Hosur were selected for the study. The silkworm races were reared as per the standard rearing procedure (Krishnaswamy, 1978). The ten selected multivoltine (MV) silkworm races (Table 1) were screened for bacterial toxicity viz., LPS (Lipopolysaccharide) administration.

LPS Administration and sample collection

The 5^{th} instars 4^{th} day larvae were treated with an LPS (*E.coli* 0111:B1) by intravenous injection of 20µl of saline containing 100µg of LPS in 0.75% NaCl solution. In control larve only saline was administrated)" (Takahiro and Yusuke, 1999), 0.75% NaCl solution. In control larvae saline only was administrated (Takahiro and Yusuke, 1999; Ishii *et al*.,

2008., Genome Pharmaceticals Institute Co., Ltd, 2000). Haemolymph and midgut were collected separately from each larva. Sample was collected by cutting an abdominal leg of the larvae in a pre-cooled microfuge tube containing approximately 0.05% of phenylthiourea (PTU) to prevent melanization (Gupta, 1979). For haemolymph, phosphate buffer saline (PBS) pH 7.4) was added and the midgut was grounded and dissolved in extraction buffer (pH 7.2) and then the samples were transferred to microfuge tube. The haemolymph were centrifuged at 5000 rpm for 5 minutes at 4°C and midgut samples were centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected in a separate pre-cooled microfuge tube and stored at-20°C.

Table 1. List of selected Multivoltine silkworm races

S.No.	Accession No.	Race Name
1	BMI-0001	Pure Mysore
2	BMI-0017	Nistari
3	BMI-0009	Kollegal Jawan
4	BMI-0014	OS-616
5	BMI-0056	MY1(SL)
6	BMI-0036	PMX
7	BMI-0034	AP12
8	BMI-0006	HosaMysore
9	BMI-0035	A13
10	BMI-0004	TamilNadu White

Measurement of Immune function traits

To assess haemocyte density, haemolymph were added in the ratio of 2:1 to EDTA- PBS anticoagulant and added glycerol in an eppendorf tube . The contents of the tube were gently mixed and pipetted onto a haemocytometer with improved Neubauer ruling. The numbers of haemocytes in five non adjacent squares were counted on each side of the haemocytometer at X400 magnification. The counts for each chamber were summed and averaged to give an estimate of the haemocyte density of each individual. The hemocyte density was expressed as cells per 0.2µL (Cotter and Wilson, 2002; Cotter *et al*., 2004).

Coagulation assay

Calibration curve: A 2% solution of Amaranth Red was made up in Insect Ringer solution (IRS), and a second solution was made up in Insect Ringer supplemented with LPS (0.5 mg/ml). 5 µl of Amaranth solution was diluted in 25, 50, 100, 200, 300 and 400 µl in Insect Ringer or LPS (0.5 mg/ml in Ringer), respectively. A 5 µl aliquot of these dilutions was added to 195µl LPS/Ringer and the optical density at 520nm (OD520)

was measured photometrically in a microplate reader. The OD was plotted against dilution volume in order to construct standard curves for LPS/Ringer dilutions. The coagulation activity was measured by $\log_e$ OD units (Cotter and Wilson, 2002; Haine *et al*., 2007). All the statistical analysis was performed by PAST - PAlaeontological STatistics, ver. 1.34 (Hammer *et al*., 2001).

Measuring clotting in vivo

Experiments were assigned to two treatments: "Ringer" and "LPS". Ten MV silkworm races were anaesthetized for 30 seconds with CO2 then injected with 5 µl Amaranth in Ringer, or Amaranth in Ringer with LPS, respectively. After a set period of time (5, 15, 30, 45 and 60 minutes after injection), individuals were briefly CO_2 anaesthetized and a 5 µl sample of haemolymph was taken from an anterior leg. Three consecutive 5 µl samples were taken from each individual. Each sample was added to 195 µl of Ringer/LPS solution in a 96-well plate and the OD_{520} measured. The mean OD_{520} for the three measures for each individual was converted to dilution of the dye in insect haemolymph, based on the standard curves in order to obtain a relative measure of in vivo coagulation between treatments (Haine *et al*., 2007).

Antigen–Antibody Agglutinations /aggregations

Ten silkworm races are tested against LPS antigens by slide agglutination and by micro agglutination/aggregation.

Qualitative assay: Slide Agglutination Tests

The control aliquots of two adjacent suspensions were added with the drops of saline on a slide. Then a drop of antiserum (LPS treated) to one suspension was added in one suspension and examined for agglutination (clumping) of the suspension (with antiserum) and clearing of the saline. Slide agglutination was read macroscopically after 3 minutes of mixing at room temperature (Myrick and Ellner,1982., NSM, 2010).

Quantitative assay on Biochip/therasaki plates

Appropriate positive and negative serum controls were included with all tests, as well as controls for diluents and antigen. Dilutions of antisera ranged from 1:20 to 1:120 by making appropriate two fold dilutions in 0.5ml of 0.85% saline containing 0.2% of Safranin O and then adding 0.5ml of the appropriate antigen to each series of Biochip or Therasaki plates. The microtiter plates were sealed with plastic covers and incubated at 37˚C. The plates were then placed in a refrigerator at 4˚C for 2 hr before being read (Gaultney *et al*., 1971; Amanai *et al*., 1990).

Reading the pattern of agglutination in microtiter plates

Nonagglutinated antigen appeared like a sedimented form in the micro technique. Agglutinated antigen was either completely dispersed and did not form sediment or, if it settled, formed poorly defined sediment with a cremated edge that was easily differentiated from the button of nonagglutinated antigen. The titer of the agglutination reaction in microtiter plates was determined by observing the highest

dilution of antiserum that permitted sedimentation of a round button of nonagglutinated antigen. The end points were thus designated by a negative reaction rather than by a positive one because the end point of nonagglutinated antigen was easier to define as a round button than was the dispersed or poorly sedimented agglutinated antigen. The difference between agglutinated and nonagglutinated antigen is easier to read. Addition of Safranin 0 to diluents enhances visibility of nonagglutinated antigen by making the button bright red. The buttons are easily observed at lower dilutions, but as the end point of titration is approached the button becomes small, and experience is required to determine the precise titer in microtiter plates (Myrick and Ellner,1982).

RESULTS AND DISCUSSIONS

The present study clearly identified the agglutination properties of the silkworm races. Further it was documented that lectins bound to LPS antigen produce agglutinating antibodies in LPS treated races. Hemagglutinins or lectins have been found in various invertebrates as well as vertebrates but their functions seem to differ in different species. In silkworm, the lectins have ability to agglutinate certain species of bacteria. Microbial surfaces bear many of the sugar residues capable of interacting with lectins. Lepidopteron lectins are thus found to be associated in several biological and biomedical applications. The recent studies examining the effect of successful immune defense against pathogens reported a decrease in survivorship of silkworm (Tsakas and Marmaras, 2010) and support the present findings.

Coagulation assay

The results of coagulation in response to an immune elicitor viz., LPS were measured as the retention of a dye (Amaranth Red) injected into the ten silkworm races at the same time as an immune challenge (LPS) or IRS. From the absorbance (OD 520nm) values less coagulation was observed in Pure Mysore, Nistari and A13 whereas remaining races showed higher values (Fig.1).

Trait correlations

Table 2 shows the correlations between the selected races and their immune function viz., coagulation and haemocyte density. The selected races are positively correlated with their immune functions. Significant and moderate correlation was found in the races viz., Pure Mysore, Nistari, Kollegal Jawan,OS-616, MY1(SL), AP12, Hosa Mysore, A13, whereas PMX and TamilNadu while shown non-significant values as evidenced from the table (Table No. 2).

Qualitative assay: Slide Agglutination Tests

A sensitive antigen suspension was used with a simple slide agglutination method which makes possible a serological diagnosis or exclusion of endotoxin level of ten races. The reaction showed aggregations with different time interval of all the LPS – treated races. The races which are not treated by LPS are termed as control races. It was observed that there were no aggregation reactions in the control races.

Among the races, PMX and TamilNadu White had taken more minutes to agglutinate whereas others are moderate. The quantitative analysis of immune titre was shown in Fig. 2, Pure Mysore race shows more agglutination/aggregations when compared to PMX. The intensity and rapidity of the slide agglutination reaction provide a rough measure of the titre of an immunity level.

Table 2. ANOVAs for the effect of immune challenge (LPS) correlation between the races and immune functional traits.

Races Name → / Immune functions ↓	**Pure Mysore**	**Nistari**	**Kollegal Jawan**	**OS-616**	**MY1 (SL)**	**PMX**	**AP12**	**Hosa Mysore**	**A13**	**Tamil Nadu White**
Coagulation assay	0.85**	0.83**	0.66*	0.73*	0.70*	0.51 ^{NS}	0.70*	0.65*	0.84**	0.48 ^{NS}
Haemocyte density	0.85**	0.80**	0.80**	0.86**	0.68*	0.55 ^{NS}	0.63*	0.75*	0.85**	0.44 ^{NS}

Table 3. Cellular reactions associated with immunity under LPS stress.

S. No.	Race No.	Race Name	Agglutination titre
1	BMI-0001	Pure Mysore	1:120
2	BMI-0017	Nistari	1:120
3	BMI-0009	Kollegal Jawan	1:60
4	BMI-0014	OS-616	1:120
5	BMI-0056	MY1(SL)	1:120
6	BMI-0036	PMX	1:20
7	BMI-0034	AP12	1:40
8	BMI-0006	Hosa Mysore	1:60
9	BMI-0035	A13	1:60
10	BMI-0004	TamilNadu White	1:20

Quantitative Assay: Microagglutination technique

For aggregation/agglutination assay, a series of inducible immune proteins in fifth instars larvae of selected silkworm races was analysed. The tendency to aggregate was found higher in healthy silkworm samples.The titer of agglutination in the microtechnique varied depending upon the concentration of antigen in haemolymph and midgut samples of ten races. Endotoxin antigen was diluted 1: 20, the optimal concentration as determined by block titration. The titer obtained with the optimal concentration was consistently greater, being one to four dilutions higher than the titers obtained with a more concentrated amount of antigen. LPS stimulates the micro aggregation reactions in a dose dependant manner viz., 1:20, 1:40, 1:60, 1:80, 1:100, 1:120. Among the races the Pure Mysore, Nistari, OS-616 and MY1 (SL) showed maximum titre value (1:120)

and exhibited high level of tolerance against LPS whereas PMX, TamilNadu White showed minimum titre value (1:20) and considered as susceptible to diseases and others are moderate which has titre of 1:40 and 1:60.The quantitative analysis of immune titre are shown in Table 3 & Fig.3.A quick and distinct agglutination indicates a titre of 1: 120 indicating high level of tolerance to diseases. A slow and indistinct result was obtained when the titre of the serum (LPS-treated sample) was about 1: 20.

Lectins act as a biospecific recognizer because lectins also bind mono-oligosaccharides and specifically precipitate polysaccharides and glycoproteins; the precipitation is inhibited by sugars, as in the case of the agglutination reaction. A lectin was purified in *Bombyx mori* larval hemolymph and the hemagglutinating activity (HA activity) of the hemolymph was present in 4^{th} and 5^{th} instars (Amanai *et al*.,1990., Amanai *et al*., 1991). Alternatively, the hemolymph lectin may differ from the lectins in tissues in its physiological function and site of synthesis. Higher agglutination properties of the individual races may link with hardiness of the silkworm. The future work must be examined by purification and characterization of the lectins from individualraces.

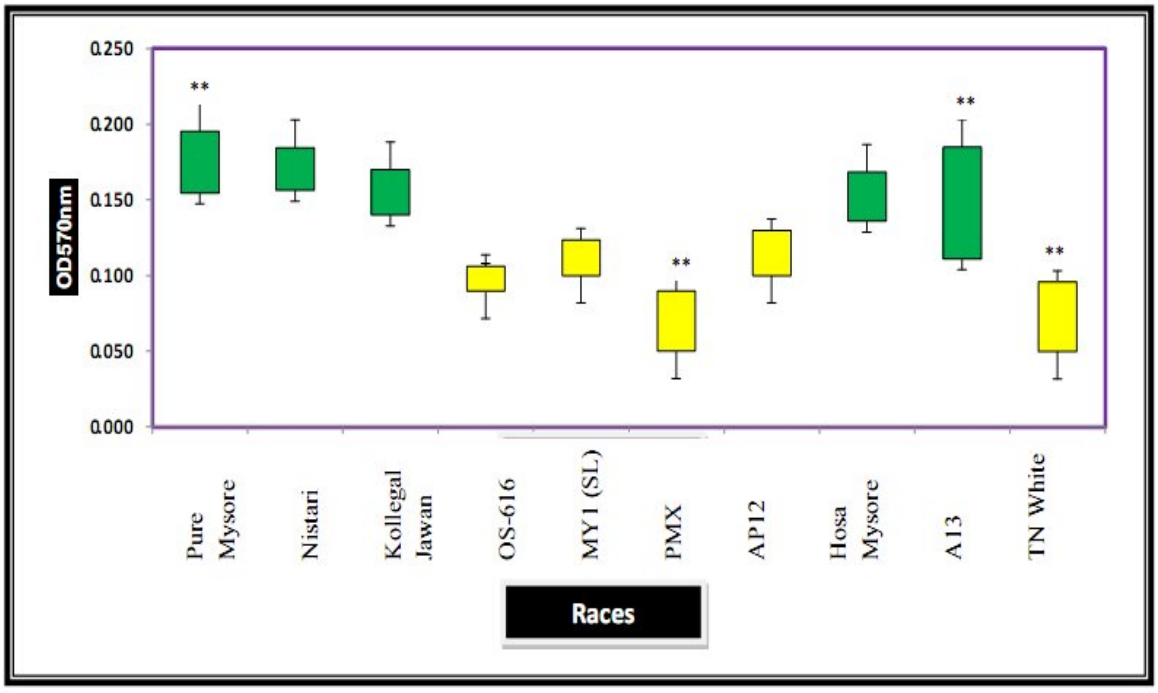

Fig.1 Coagulation activity of ten MV silkworm races under biotic stress (LPS). Green colour indicates more coagulation activity, Yellow colour indicates less coagulation activity).These experiments were performed in triplicate (mean ±SD), and the bar represented the mean ±SD of the triplicate experiments results.

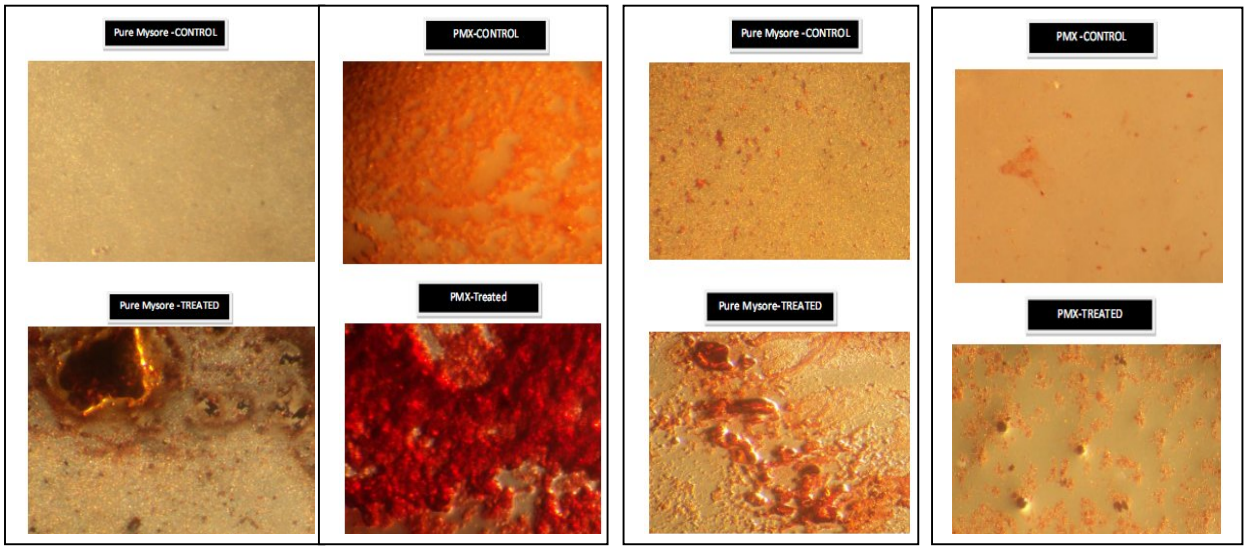

Fig. 2 Aggregation reactions with LPS (A-B;Haemolymph samples)(C-D;Midgut samples).

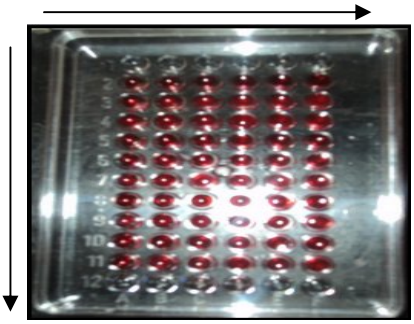

Fig.3 Determination of immune titre value using BIOCHIP-Quantitative analysis of immunity in haemolymph of ten MV silkworm races.Right arrows indicates the dilutions. Down arrow indicates the races no.

CONCLUSION

The present findings will be of great use to identify the silkworm biodiversity under a pathogenic environment. The immuno parameters may be used as markers to transfer the concerned genetic character to any breed that needs to be genetically enhanced for hardiness against any biotic environmental conditions.

ACKNOWLEDGEMENTS

The authors are thankful to the Director, Central Sericultural Germplasm Resources Centre (CSGRC), Hosur, for providing lab facilities and also acknowledge with thanks the scientists for their help. We further acknowledge the guidance of

Dr. Balakrishnan, Reader, Department of Immunology, Madurai Kamaraj University, for immunological aspects.

REFERENCES

Amanai, K., Sakurai, S., and Ohtaki T. (1990). Purification and characterization of hemagglutinin in the haemolymph of the silkworm, *Bombyx mori*. Comp Biochem Physiol. 978: 471- 476.

Bohn, H. (1986). Hemolymph clotting in insects. Brehelin M. editor. Immunity in invertebrates. Berlin: Springer, 188–207.

Cerenius, L., Liang, Z., Duvic, B., Keyser, P., Hellman, U., and Palva, ET., Iwanaga, S. and Söderhäll K. (1994). Structure and biological activity of a 1,3-β-D-glucan-binding protein in crustacean blood. J Biol Chem. 269: 29462-29467.

Cotter, S. ., C and Wilson, K. (2002). Heritability of immune function in the caterpillar Spodoptera *littoralis*. Heredity. 88: 229–234.

Cotter, S.C., Kruuk, L.E.B., and Wilson, K. (2004). Costs of/ resistance: genetic correlations and potential trade-offs in an insect immune system. J Evol Biol.17: 421-429.

Genome Pharmaceticals Institute Co., Ltd. Bacteria and fungi infection models. 2000, Japan patent application no.2000-177565 (2001-583180).

Goldstein, IJ., Hughes, RC., Monsigny, M., Osawa, T., and Sharon, N. (1980). What should be called a lectin? Nature. 285: 66.

Gregoire, C. (1974). Hemolymph coagulation. Rockstein M,editor. Physiology of insecta, New York: Academic Press. 5:309–360.

Gupta, A. P. (ed.) (1979) Haemocyte types their structure species interrelationship and taxonomic significance; in Insect haemocytes (Cambridge: Cambridge University Press). 85-127.

Haine, ER., Roiff, J., and Siva-Jothy, M.T. (2007). Functional consequences of blood clotting in insects, Developmental and comparative Immunoology, 31:456-464.

Ishii. K., Hamamoto, H., Kamimura, M., and Sekimizu, K. (2008). Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. J Biol Chem. 283: 2185-2191.

Gaultney, J.B., Wende, R.D., and Robert, P. (1971). Williams Microagglutination Procedures for Febrile Agglutination Tests. Appl Environ Microbiol. 22 (4): 635.

Jomori, T and Natori, S. (1992). Function of the lipopolysaccharide- binding protein of *Periplaneta americana* as an opsonin. FEBS Lett., 296: 283-286.

Kasai, K., and Hirabayashi, J. (1996). Galectins: a family of animal lectins that decipher glycocodes. J Biochem. 119: 1-8.

Amanai K., Sakai M., Sakurai S., Mori T., Nikaido, O., and Ohtaki, T. (1991). Occurrence of Lectin in the Silkgland of the Silkworm, *Bombyx mori* (lectin /silkgland / haemolymph).Develop Growth & Differ., 33 (4): 421-427.

Koizumi, N., Imamura, M., Kadotani, T., Yaoi, K., Iwahana, H., and Sato, R. (1999). The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with

two different tandem carbohydrate-recognition domains. FEBS Lett. 443: 139-143.

Krishnaswamy, S. (1978). New technology of silkworm rearing, Bulletin No. 2, Central Sericulture and Training Institute: Mysore, India. 1-23.

Marques, M.R.F., and Barracco, MA. (2000). Lectin, as non-self- recognition factors, in crustaceans. Aquaculture. 191: 23-44.

Myrick, B.A., and Ellner, PD. (1982). Evaluation of the latex slide agglutination test for identification of *Staphylococcus aureus*. J Clin Microbiol.15: 275-277.

Natori, S., Shiraishi, H., Hori, S., and Kobayashi, A. (1999). The role of Sarcophaga defense molecules in immunity and metamorphosis. Dev Comp Immunol. 23:317-328.

Ni, Y., and Tizard, I. (1996). Lectin-carbohydrate interaction in the immune system. Vet Immunol Immunopathol. 55:205-223.

NSM , Agglutination Test (2010). Standards Unit, Department for Evaluations, Standards and Training. 2: 9 -11.

Hammer, Ø., Harper, D.A.T ., and Ryan, P.D. (2001). PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4(1).

Ravindranath, M. H., and Copper, EL. (1984). Crab lectins: receptor specificity and biomedical applications. Prog Clin Biol Res., 157: 83-96.

Steinhaus, EA. (1963). Background for the diagnosis of insect diseases. In insect pathology, an advanced treatise, Vol. 2 Ed. EA. Steinhaus 549-589. Academic Press, New York.

Takahiro, S., and Yusuke, K. (1999). Induction of carboxylesterase isozymes in *Bombyx mori* by *E.Coli* infection. Insect Biochem Mol Biol, 29:731-736.

Theopold, U., Schmidt, O., Soderhall, K and Dushay, MS. (2004). Coagulation in arthropods: defence, wound closure and healing. Trends Immunol., 25:289–294.

Tsakas, S., and Marmaras, V.J. (2010). Insect immunity and its signalling: an overview. Inform Sys J. 7:228-238.

Vasta, GR., Quesenberry, M., Ahmed, H., and O Leary, N. (1999). C- type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. Dev Comp Immunol. 23: 401-420.

Vasta, G.R. (1992). Invertebrate lectins: distribution, synthesis, molecular biology, and function. Allen HJ, Kisalius EC, editor. Glyco conjugates. Marcel Dekker, New York, 593-634.

Weis, WI., Taylor, ME., and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. Immunol Rev., 163: 19-34.