

The Determination of N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) Types of Sialic Acids in Hematopoietic Organ of The Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) ^[1]

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Summary

In insects, for a long time, it was generally accepted that members of insect do not have sialic acids which are typical terminal glycans of cells surface glycolipids and glycoproteins, and thus especially important in cell interactions and signaling events. Here, it was reported the occurrence of sialic acid known the most common in all organisms (Neu5Ac and Neu5Gc) in hematopoietic organ of larvae of the silkworm, *Bombyx mori*. Histochemical and cytochemical analysis of hematopoietic organ sections with the FITC- and Gold-conjugated lectins (*Limulus polyphemus*, LPA) showed the presence of sialic acids in hematopoietic organ. The results provide further evidence for the existence of sialic acids in insects.

Keywords: N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), Sialic acid, Lectin, *Bombyx mori*

İpekböceği, *Bombyx mori* L. (Lepidoptera: Bombycidae)'nin Hematopoietik Organında N-asetilneuraminik asit (Neu5Ac) ve N-glikolilneuraminik asit (Neu5Gc) Tip Sialik Asitlerinin Belirlenmesi

Özet

Hücre yüzey glikolipit ve glikoproteinlerin en uç kısımlarında yer alan ve özellikle hücre ilişkilerinde ve sinyal iletimi olaylarında önemli rolleri olan sialik asitler, uzun zamandır böceklerde olmadığı kabul edilirdi. Bu çalışmada, ipekböceği *Bombyx mori* larvalarının hematopoietik organında çoğu organizmada en çok bilinen Neu5Ac ve Neu5Gc tip sialik asitlerin bulunduğu tespit edilmiştir. FITC- ve altın bağlanmış lektinler (*Limulus polyphemus*, LPA) kullanılarak hematopoietik organ kesitlerinde histokimyasal ve sitokimyasal yöntemlerle sialik asitlerin varlığı gösterildi. Bu sonuçlar böceklerde sialik asit bulunduğuna dair ek bir kanıt oldu.

Anahtar sözcükler: N-asetilneuraminik asit (Neu5Ac), N-glikolilneuraminik asit (Neu5Gc), Sialik asit, Lektin, *Bombyx mori*

INTRODUCTION

Sialic acids are biosynthesized as a 9-carbon carboxylated monosaccharide and frequently found at the nonreducing terminal position of various glycoconjugates. Sialic acids, primarily N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), are existed by almost all organisms ¹⁻⁴. However, small amounts of sialic acid have been reported in a few insect species ⁵⁻¹⁴. Sialic acids are involved in numerous biological properties of glycoconjugates, some of which are modulated by sialic acid modifications ^{2,15}. Sialic acid residues of glucoconjugates

are cell type-specific and developmentally regulated ¹². The specific sialylation pattern of glycoconjugates is due to regulated expression of specific sialyltransferases ¹⁶. Negatively charged sialic acids contribute to many properties of the cell surface and play an important role in a number of biological activities such as in signal transduction events in cell-cell and cell-matrix interactions. Biological roles of sialic acids are gradually being clarified as important regulators in many cellular and molecular interactions. Sialylated glycoconjugates exhibit remarkably



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diverse structures, and their expression has been shown to change during embryonic differentiation, many kinds of cellular interaction ageing and pathologic transformations^{9,17}.

Hematopoietic organ (HPO) in insects produces and contains premature and pre-differentiated hemocytes (insect blood cells) in order to survive in habitats that are highly infected with microorganisms¹⁸. The cellular and humoral defense system is based on these hemocytes in HPO or hemocoel by phagocytosis, multicellular encapsulation and nodule formation¹⁸⁻²³. HPOs are generally located near the dorsal vessel in the thorax and the abdominal segments. In Lepidoptera species; HPOs of *Bombyx mori*²³⁻²⁵, *Manduca sexta*²⁶⁻²⁷ are attached to imaginal wing discs. However, İzzetoğlu and Karaçalı²⁸ found a novel site for HPO that closely associated with dorsal vessel in larvae of *Bombyx mori*. HPO is composed of several lobes located very close to the dorsal vessel in the thorax and in the abdominal segments of the larvae. Anterior lobes were primarily composed of big secretory cells that probably responsible for the synthesis of defense molecules for humoral and cellular immune response. The posterior lobes of HPO include secretory cells and loose-associated different hemocyte clusters²⁸. The immune system is able to distinguish between self and non-self^{22,29} by changing in sialylation patterns of their cell surface⁹. Since maturation of all hemocytes occur in HPO, these results indicate sialic acids as ideal points of investigation in the HPO.

In this present work, it was reported the occurrence of sialic acids in HPO of *Bombyx mori* larvae by immunohistochemical staining (fluorescent microscopy) and post-embedding immunogold labeling (Electron microscopy) of larval HPO sections with sialic acid-specific lectin, which are known to be a valuable methods.

MATERIAL and METHODS

Insects and Hematopoietic Organ

The larvae of silkworm, *Bombyx mori* were cultured on the leaves of the mulberry at 24±1°C, 70% relative humidity, and under a photoperiod of 12:12 (light and dark)^{28,30}. In this study, we used HPOs of the fifth or last instar larvae of silkworm. Dissected HPOs were immediately transferred to the mold containing the embedding medium for immunohistochemical investigations by fluorescent microscopy, or fixed in 4% paraformaldehyde in 0,1M PBS for postembedding immunogold labeling by electron microscopy.

Lectins

Lectins, due to their high specificity toward recognizing carbohydrates of defined structures, have been used in cytochemical and histochemical studies to identify modifications in glycosylation³¹. The lectin from *Limulus polyphemus*, LPA that selectively binds to Neu5Ac and

Neu5Gc³², were used for immunostaining of sialic acids. FITC-conjugated and Colloidal gold-conjugated lectins (LPA) were purchased from EY laboratories (San Mateo, CA).

Lectin Immunohistochemistry and Postembedding Immunogold Labelling

Lectin staining of HPO sections (5 µm) in frozen microtome (Leica CM1850) were observed by fluorescent microscopy. Briefly, FITC-conjugated lectin was diluted in Tris buffered saline 0.05M TBS (0.05M Tris base, 0.15M NaCl, 0.01M CaCl₂, pH 8.0) to give final concentrations of 20 µl/ml LPA. Samples on glass slides were washed with TBS. They were rinsed once blocking buffer (TBS containing 0.5% bovine serum albumin (BSA), 0.1% Tween 20, 0.1% NaAzid, 0.1% Gelatin) for at least 30 min, followed by incubation with diluted FITC-LPA solution in TBS for 1 h at room temperature in humidity chamber. After samples were washing there times in TBS for 5 min each, slides were coverslipped with the glycine. Then, samples were immediately viewed with the appropriate fluorescence blue filters using a differential interference fluorescence microscope (Leica DM 4000B with DP71 video camera system).

In order to determine sialic acids in HPO cells by colloidal gold-conjugated LPA, HPOs were fixed in freshly prepared 4% paraformaldehyde in PBS (pH 7.3) and dehydrated in ascending concentrations of ethanol. The resulting samples were embedded in Lowicryl HM20 (-35°C, low temperature embedding medium - Polyscience Inc.). In semithin sections, HPO structures were identified, and ultra-thin sections were cut with a diamond knife on a Reichert OMU3 ultramicrotome. All immunostaining was performed on sections mounted on nickel grids. Gold labeled lectin was diluted in 0.05M TBS to give final concentrations of 20 µl/ml LPA. Ultrathin sections, which placed on formvar-coated nickel grids were rinsed with 0.1 M Glycine for blocking of aldehyde groups. After the sections were washing there times in TBS for 5 min each, incubated with diluted Gold labeled-LPA for 1 h at room temperature in humidity chamber. Lectin-labelled samples were double contrasted with saturated uranyl acetate and Reynold's lead and examined in Jeol 100C electron microscope (TEM).

RESULTS

In this study, it is reported that the occurrence of the sialic acids (Neu5Ac and Neu5Gc) were determined in HPO of *Bombyx mori* larvae using optical methods; Neu5Ac and Neu5Gc specific FITC-lectin (LPA, *Limulus polyphemus*) in the fluorescent microscopy and gold conjugated-lectin (LPA) in electron microscopy. LPA recognizes both Neu5Ac and Neu5Gc³². The distribution of fluorescent labeled lectin as indicated to sialic acids, interacted with all lobes of the total HPO (Fig. 1a,b). Electron microscopic immunogold labeling was detected the presence of sialic acids (Neu5Ac

and Neu5Gc) in cells of HPO. The gold particles are distinct and readily visualized. They are easily quantifiable and do not diffuse on the sections. There is little binding of gold to the nucleus heterochromatin area of secretory cells of HPO (Fig. 2a). Many gold particles were especially located over the secretion granules in secretory cells (Fig. 2b).

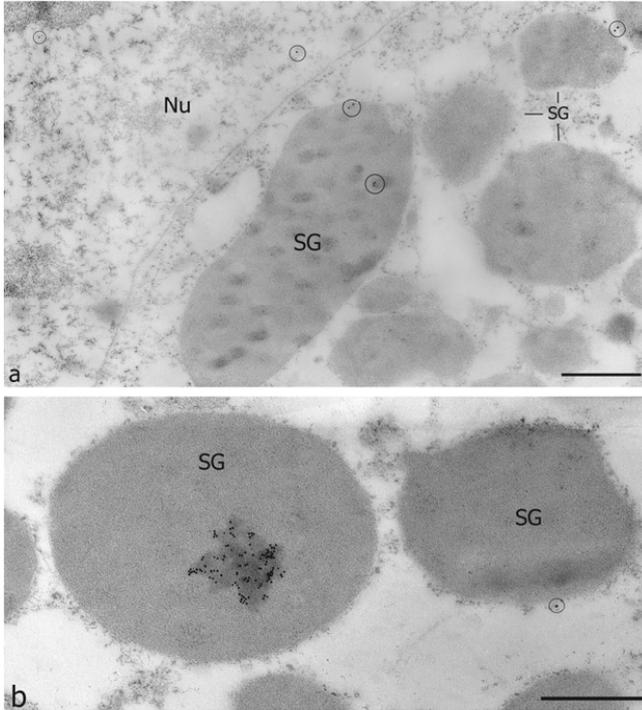


Fig 1. Ultra-thin sections from Lowicryl HM20-embedded HPO showing colloidal gold labeling LPA binding to cells of HPO. (a) Little binding of gold (in circles) is seen over the nucleus heterochromatin area (Nu) and cytoplasmic secretory cells of HPO. (b) Many gold particles are especially located on the secretion granules (SG). Section stained with uranyl acetate and Reynold's lead, Bar: 0.5 μ m

Şekil 1. HPO hücrelerine bağlanan kolloidal altın işaretlenmiş LPA'nın Lowicryl HM20'ye gömülmüş HPO'da ince yapısında gösterilmesi. (a) nükleus heterokromatin bölgesine (Nu) ve HPO'nun sitoplazma salgı granüllerine (SG) bağlanan altınların (daire içinde) varlığının gösterilmesi. Uranil asetat ve Reynold's'ın kurşun sitrat boyalı kesit, Bar: 0.5 μ m

DISCUSSION

There have been many reports concerning the analysis of specific sialic acids in related with their function in animals. Recently, however, some evidence for the presence of sialic acids in insects has started to appear. Roth et al.⁵ were the first to detect sialic acid in insect, in embryonic nervous system of *Drosophila melanogaster*. Later, some other reports of sialic acid in insects were published. Presence of Neu7,9Ac₂5Gc type of sialic acid in prothoracic glands of *Galleria mellonella* has been determined⁶. The occurrence of Neu5Ac was found in Malpighian tubules of *Philaenus spumarius* by GLC-MS and cytochemical analysis⁸. Seven types of sialic acids-Neu5Ac, Neu5Gc, Neu4,5Ac₂, Neu5,9Ac₂, Neu2en5Ac, Neu9Ac5Gc, and Neu5,8,9Ac₃ were found in developing testis of *G. mellonella* by GC and

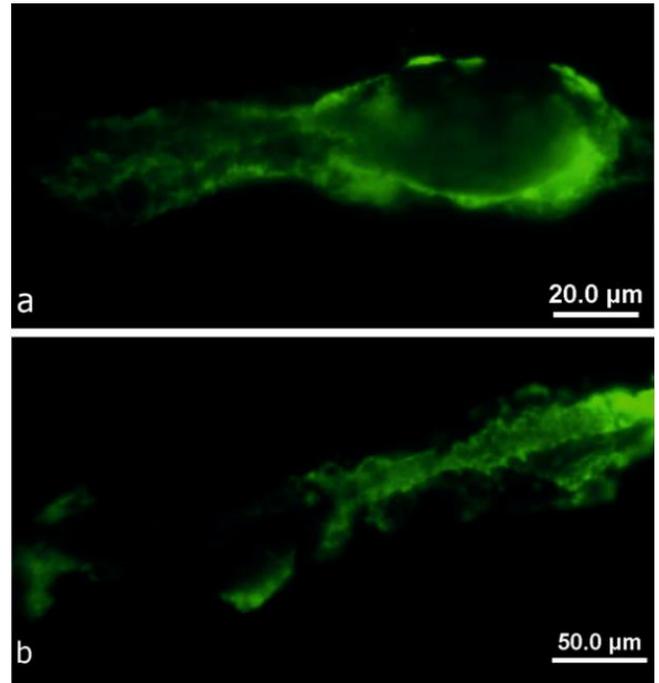


Fig 2. Immunohistochemical detection of sialic acid in total HPO using FITC-conjugated lectin, LPA (green), which selectively binds to Neu5Ac and Neu5Gc (a,b) Determination of sialic acid is indicated by the presence of green fluorescence

Şekil 2. Total HPO'ya Neu5Ac ve Neu5Gc'ye seçici olarak bağlanan FITC-LPA lektinin (yeşil) immunohistokimyasal olarak gösterilmesi (a,b) yeşil ışımının olması ile sialik asidin belirlenmesi

GC-MS analysis⁷. The expression level and distribution of Neu5Ac and its α 2,6 linkage in the third and final larval, mature prepupa, and pupa stages of *Galleria mellonella* were found by SNA (*Sambucus nigra agglutinin*) lectin immunofluorescence and lectin blot analysis³³. Moreover, Neu5Ac, Neu5Gc, Neu5,7Ac₂, Neu5,8Ac₂, Neu5,9Ac₂ types of sialic acids were determined in prothoracic gland cells of *Galleria mellonella* larvae by LC-ESI-MS/MS system (Karacali et al., 2011 in preparation).

In *Drosophila* larvae, the first lobes of the hematopoietic organ contain numerous active secretory cells that synthesize extracellular matrix and defense molecules³⁴. The cells with similar secretory characteristics were observed in HPO of *Bombyx mori* larvae. At present, nothing is known about the glycoconjugates containing the Neu5Ac or Neu5Gc sugars detected in HPO of *Bombyx mori*. We therefore can only speculate that secretory cells in the anterior lobes of the HPO participate to synthesis of defense molecules possessing sialylated glycoprotein or glycolipid for humoral immune system.

In conclusion, the findings obtained by optic methods provide an evidence that sialic acid is present in the HPO of *Bombyx mori*. It has been commonly accepted that plants are not capable of synthesizing sialic acids. No evidence has previously been reported to suggest that they contain sialic acids^{8,35-36}. Silkworms were traditionally reared on mulberry leaves. The food of the silkworm larvae did not

have sialic acid^{37,38}. Thus, the uptake of sialic acid by the silkworm larvae with its food can be excluded. These results support to the study of Kato et al.³⁹. They found that strong evidence emerged for existence of sialic acid in silkworm larvae. According to the known functions of sialic acids in other systems, these molecules must have important roles during hemocyte differentiation, maturation and releasing from HPO to hemocoel. The results provide further evidence for the existence of sialic acids in insect tissues. Hence, Neu5Ac and Neu5Gc in silkworm larvae is very likely of endogenous origin. Further studies on the function of sialic acids in secretory granules of HPO secretory cells of *B. mori* are necessary to confirm. This may provide interesting new insights into the origin of sialic acid and function in insects.

ABBREVIATIONS: Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid; Neu7,9Ac₂5Gc, 7,9-Di-O-acetyl-5-N-glycolyl-neuraminic acid; Neu4,5Ac₂, 5-N-acetyl-4-O-acetyl-neuraminic acid; Neu5,9Ac₂, 5-N-acetyl-9-O-acetylneuraminic acid; Neu2en5Ac, 5-N-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; Neu9Ac5Gc, 9-O-acetyl-5-N-glycolyl-neuraminic acid; Neu5,8,9Ac₃, 5-N-acetyl-8,9-di-O-acetylneuraminic acid; Neu5,7Ac₂, 5-N-acetyl-7-O-acetylneuraminic acid; Neu5,8Ac₂, 5-N-acetyl-8-O-acetylneuraminic acid.

REFERENCES

- Varki A: Diversity in the sialic acids. *Glycobiology*, 2 (1): 25-40, 1992.
- Klein A, Diaz S, Ferreira I, Lamblin G, Roussel P, Manzi AE: New sialic acids from biological sources identified by a comprehensive and sensitive approach: Liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) of SIA quinoxalinones. *Glycobiology*, 7 (3): 421-432, 1997.
- Morimoto N, Nakano M, Kinoshita M, Kawabata A, Morita M, Oda Y, Kuroda R, Kakehi K: Specific distribution of sialic acids in animal tissues as examined by LC-ESI-MS after derivatization with 1,2-diamino-4,5-methylenedioxybenzene. *Anal Chem*, 73 (22): 5422-5428, 2001.
- Lamari FN, Karamanos NK: Separation methods for sialic acids and critical evaluation of their biologic relevance. *J Chromatogr B Analyt Technol Biomed Life Sci*, 781 (1-2): 3-19, 2002.
- Roth J, Kempf A, Reuter G, Schauer R, Gehring WJ: Occurrence of sialic acids in *Drosophila melanogaster*. *Sci*, 256 (5057): 673-675, 1992.
- Karaçalı S, Kırmızıgül S, Deveci R, Deveci O, Onat T, Gürcü B: Presence of sialic acid in prothoracic glands of *Galleria mellonella* (Lepidoptera). *Tissue Cell*, 29 (3): 315-321, 1997.
- Karaçalı S, Kırmızıgül S, Deveci R: Sialic acids in developing testis of *Galleria mellonella* (Lepidoptera). *Invertebr Repr Dev*, 35 (3): 225-229, 1999.
- Malykh YN, Krisch B, Gerardy-Schahn R, Lapina EB, Shaw L, Schauer R: The presence of N-acetylneuraminic acid in Malpighian tubules of larvae of the cicada *Philaenus spumarius*. *Glycoconj J*, 16 (11): 731-739, 1999.
- Karaçalı S, Deveci R, Pehlivan S, Özcan A: Adhesion of hemocytes to desialylated prothoracic glands of *Galleria mellonella* (Lepidoptera) in larval stage. *Invertebr Repr Dev*, 37 (2): 167-170, 2000.
- Martensen I, Schauer R, Shaw L: Cloning and expression of a membrane-bound CMP-N-acetylneuraminic acid hydroxylase from the starfish *Asterias rubens*. *Eur J Biochem*, 268, 5157-5166, 2001.
- Schauer R: The occurrence and significance of sialic acids in insects. *Trends Glycosci. Glycotechnol*, 13, 507-517, 2001.
- Kim K, Lawrence SM, Park J, Pitts L, Vann WF, Betenbaugh MJ, Palter KB: Expression of a functional *Drosophila melanogaster* N-acetylneuraminic acid (Neu5Ac) phosphate synthase gene: evidence for endogenous sialic acid biosynthetic ability in insects. *Glycobiology*, 12 (2): 73-83, 2002.
- Gollub M, Shaw L: Isolation and characterization of cytidine-5'-monophosphate-N-acetylneuraminic acid hydroxylase from the starfish *Asterias rubens*. *Comp Biochem Physiol B Biochem Mol Biol*, 134 (1): 89-101, 2003.
- Schauer R, Srinivasan GV, Coddeville B, Zanetta JP, Guéardel Y: Low incidence of N-glycolylneuraminic acid in birds and reptiles and its absence in the platypus. *Carbohydr Res*, 344 (12): 1494-1500, 2009.
- Varki A: Biological roles of oligosaccharides: All of theories are correct. *Glycobiology*, 3 (2): 97-130, 1993.
- Schauer R, Kelm S, Reuter G, Roggentin P, Shaw L: Biochemistry and role of sialic acids. In, Rosenberg A (Ed): *Biology of Sialic Acids*. pp. 7-67, Plenum Press, New York, 1995.
- Kelm S, Schauer R: Sialic acids in molecular and cellular interactions. *Int Rev Cytol*, 175, 137-240, 1997.
- Lackie AM: Immune mechanisms in insects. *Parasitol Today*, 4 (4): 98-105, 1988.
- Ratcliffe NA, Rowley AF, Fitzgerald SW, Rhodes CP: Invertebrate immunity: Basic concepts and recent advances. *Int Rev Cytol*, 97, 183-221, 1985.
- Trenczek T, Kanost MR: Insect haemocytes-multifunctional cells in defense mechanisms. Sbornik Jihoceske Univerzity Zemedelske Fakulty V Ceskych Budejovicich, Fytotechnica Cislo: 2/XIV: 18, 1997.
- Jarosz C: Active resistance of entomophagous rhabditid Hetero-rhabditis bacteriophora to insect immunity. *Parasitology*, 117, 201-208, 1998.
- Vilkinkas A, Götz P: Parasitic fungi and their interactions with the insect immune system. *Adv Parasitol*, 43, 267-313, 1999.
- Ling E, Shirai K, Kanekatsu R, Kiguchi K: Hemocyte differentiation in the hematopoietic organs of the silkworm, *Bombyx mori*: Prohemocytes have the function of phagocytosis. *Cell Tissue Res*, 320 (3): 535-543, 2005.
- Han SS, Lee MH, Kim WK, Wago H, Yoe SM: Hemocytic differentiation in hemopoietic organ of *Bombyx mori* larva. *Zool Sci*, 15, 371-379, 1998.
- Nakahara Y, Kanamori Y, Kiuchi M, Kamimura M: *In vitro* studies of hematopoiesis in the silkworm: Cell proliferation and hemocyte discharge from the hematopoietic organ. *J Insect Physiol*, 49 (10): 907-916, 2003.
- Nardi JB, Pilas B, Ujhelyi E, Garsha K, Kanost MR: Hematopoietic organs of *Manduca sexta* and hemocyte lineages. *Dev Genes Evol*, 213 (10): 477-491, 2003.
- Nardi JB: Embryonic origins of the two main classes of hemocytes-granular cells and plasmatocytes in *Manduca sexta*. *Dev Genes Evol*, 214 (1): 19-28, 2004.
- İzzetoğlu S, Karaçalı S: A novel site for hematopoietic organ in *Bombyx mori* L. (Lepidoptera: Bombycidae). *Kafkas Univ Vet Fak Derg*, 16 (Suppl-B): S243-S247, 2010.
- Yokoo S, Götz P, Tojo S: Phagocytic activities of haemocytes separated by two simple methods from larvae of two lepidopteran species, *Agrotis segetum* and *Galleria mellonella*. *Appl Entomol Zool*, 30 (2): 343-350, 1995.
- İzzetoğlu GT, Özkorkmaz F, Zeka Ö, Öber A: İpekböceği (*Bombyx mori*)'nde juvenil ve ekdizon hormonları uygulaması sonucu olası değişimler. *Kafkas Univ Vet Fak Derg*, 15 (4): 525-530, 2009.
- Sierra C, Guevara J, Lascurain R, Perez A, Agundis C, Zenteno E, Vazquez L: Sialylation is modulated through maturation in hemocytes from *Macrobracon rosenbergii*. *Comp Biochem Physiol C Toxicol Pharmacol*, 130 (2): 179-189, 2001.
- Angata T, Varki A: Chemical diversity in the sialic acids and related α -keto acids: An evolutionary perspective. *Chem Rev*, 102 (2): 439-470, 2002.
- Saribek B, Erden S, Karaçalı S: Determination of alpha-2,6 sialic acid in developmental stages of *Galleria mellonella* (Lepidoptera). *Invert Rep Dev*, 53 (1): 45-52, 2009.
- Lanot R, Zachary D, Holder F, Meister M: Postembryonic hematopoiesis in *Drosophila*. *Dev Biol*, 230 (2): 243-257, 2001.
- Shah MM, Fujiyama K, Flynn CR, Joshi L: Sialylated endogenous glycoconjugates in plant cells. *Nat Biotechnol*, 21, 1470-1471, 2003.
- Zeleny R, Kolarich D, Strasser R, Altmann F: Sialic acid concentrations in plants are in the range of inadvertent contamination. *Planta*, 5, 1-6, 2006.
- Schauer R, Kamerling JP: Chemistry, biochemistry and biology of sialic acids. In, Montreuil J, Vliegthart JFG, Schachter H (Eds): *Glycoproteins II*. pp. 243-402, Elsevier, Amsterdam, 1997.
- Ratanapo S, Ngamjunyaporn W, Chulavatnatol M: Sialic acid binding lectins from leaf of mulberry (*Morus alba*). *Plant Sci*, 139, 141-148, 1998.
- Kato Y, Nakamura T, Takeuchi T: Hemagglutination activity of hemolymph of *Bombyx mori* treated with a juvenile hormone analogue. *J Seric Sci*, 63, 221-228, 1994.