KSÜ Tarım ve Doğa Derg 27 (5), 1192-1198, 2024
KSU J. Agric Nat 27 (5), 1192-1199, 2024
DOI:10.18016/ksutarimdoga.vi.1430234

ß-Galactoside α 2-6 Sialyltransferase Gene Expression in *Bombyx mori* Tissues

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ABSTRACT

Bombyx mori is an important insect due to its genome, homology to humans, and ease of growth. Sialylation has been reported in some insects, but sialic acid biosynthesis cannot be observed in these insects. Sialic acids are negatively charged nine-carbon sugars located terminally on glycoconjugates. Sialylation, which occurs after translation and is regulated by enzymes, has been studied in prokaryotes, deuterostomes, and protostomes. One of the enzymes that is important for sialylation is sialyltransferases. This enzyme plays a role in linking sialic acid to glycoconjugates. In this study, we investigated sialyltransferase (ß-Galactoside α-2,6-Sialyltransferase I) gene expression in tissues of *B. mori*. *B. mori* larvae, which were fed with fresh mulberry leaves since hatching, were divided into two groups; the control group, which continued to be fed with fresh mulberry leaves in the 5th instar (last instar) and the treatment group, which was fed with mulberry leaves treated with a sialic acid solution. Midgut, fat body, hemocyte, ovary, and testicular tissues were dissected, and gene expressions were examined with Real-Time PCR. The expression level is observed in every tissue, but an increase is seen in only the fat body. The fat body is a vital tissue for insects and plays a fundamental role in immunity, endocrine, and detoxification processes. The reason for the highest gene expression in the fat body can be attributed to the similarities in the functions of the fat body and sialic acid and their roles in insects.

Animal Physiology

Research Article

Article History
Received : 01.02.2024
Accepted : 29.03.2024

Keywords
Sialic acid
Sialyltransferase
Gene expression
RT-PCR
Bombyx mori

Bombyx mori Dokularında ß-Galaktozit α 2-6 Siyaliltransferaz Geninin İfadesi

ÖZET

Bombyx mori genomunun bilinmesi, insanlarla homoloji göstermesi ve kolay yetiştirilmesi açısından en önemli böcek türlerinden biridir. Bazı böceklerde sialilasyona ilişkin raporlar mevcuttur, hatta sialile edilmiş moleküllerin çeşitli yapıları bildirilmiştir ancak böceklerde sialik asit biyosentezi gözlenmemektedir. Sialik asitler, terminal olarak glikoproteinler ve glikolipitler üzerinde bulunan negatif yüklü dokuz karbonlu şekerlerdir. Sialilasyon, enzimler tarafından düzenlenen bir translasyon sonrası modifikasyondur. Prokaryotlar, dueterostomlar ve protostomlarda çalışılmıştır. Sialilasyon için önemli olan enzimlerden biri de sialiltransferazlardır. Bu enzim, sialik asidin glikokonjugatlara bağlanmasında rol oynar. Bu çalışmada ipek böceği *Bombyx mori* dokularında sialiltransferaz (ß-Galaktozit α 2-6 Sialiltransferaz) geninin ifade düzeylerinin belirlenmesidir. Yumurtadan çıkışından itibaren taze dut yaprakları ile beslenen *B. mori* larvaları, 5. instar (son instar)'da taze dut yaprakları ile beslemeye devam edilen (kontrol grubu) ve sentetik sialik asit solüsyonu uygulanmış dut yaprakları ile beslenilen uygulama grubu olarak ayrıldı. 5. instarın orta bağırsak, yağ doku, hemosit, ovayum ve testis dokuları alınarak Real-Time PZR ile gen ifade düzeyleri incelendi. Ekspresyon düzeyi her doku için ayrı ayrı belirlendi, ancak artış sadece yağ dokuda tespit edildi. Yağ doku böcekler için oldukça önemlidir, bağışıklık, endokrin ve detoksifikasyon süreçlerinde temel rol oynar. Yağ dokuda sialiltransferaz gen ekspresyonunun en fazla

Hayvan Fizyolojisi

Araştırma Makalesi

Makale Tarihçesi
Geliş Tarihi : 01.02.2024
Kabul Tarihi : 31.03.2024

Anahtar Kelimeler
Sialik asit
Sialiltransferaz
Gen ifadesi
RT-PZR
Bombyx mori

olmasının sebebinin yağ doku ve sialik asidin fonksiyonlarındaki benzerliklere ve böceklerdeki rollerine bağlanabilir.

Atıf Şekli: Ersoy, C., İzzetoğlu, S., & Turgay-İzzetoğlu, G., (2024). *Bombyx mori* dokularında ß-Galaktozit α 2-6 Siyaliltransferaz geninin ifadesi. *KSÜ Tarım ve Doğa Derg 27* (5), 1192-1198. https://doi.org/ 10.18016/ksutarimdoga.vi.1430234

To Cite : Ersoy, C., İzzetoğlu, S., & Turgay-İzzetoğlu, G., (2024). ß-Galactoside α 2-6 Sialyltransferase gene expression in *Bombyx mori* tissues. *KSU J. Agric Nat 27* (5), 1192-1198. https://doi.org/ 10.18016/ksutarimdoga.vi. 1430234

INTRODUCTION

Glycoconjugates have significant roles in various biological processes, such as cell interaction, development, differentiation, and viral infection (Varki, 2017; Miyazaki et al., 2019). N-glycosylation is important post-translational modification. Mammalian cell lines produce glycoproteins with glycan patterns that terminate in sialic acid residues. In insect cell lines, the final N-glycosylation pattern is mostly mannose or paucimannosidic structures (Hsu et al., 1997; Altmann et al., 1999; Lawrence et al., 2001). Some studies suggested that insects can perform sialylation, but others indicated that essential genes may not be present or active (Lawrence et al., 2001). The study of insect systems has revealed that N- and O-glycans produced by all insect systems are similar or identical to those produced by all eukaryotes. The role of sialic acids in glycobiology is crucial. They are found as terminal residues on cell-surface glycoconjugates and are involved in immunological reactions, many cell-cell interactions, and the clearance of circulating glycoproteins (Marchal et al., 2001). Sialylation is the formation of sialyl glycoconjugate by binding of sialic acids to the terminal ends of glycans of glycoconjugates by sialyltransferases. In some insects, sialyl glycoconjugates were found and sialyltransferases, NeuAc phosphate synthase, CMP- sialic acid synthase (CMAS) was determined (Koles et al., 2007; Kuijura et al., 2015; Ghosh, 2018).

The recent availability of complete genomic information has the potential to make *B. mori* an effective model system in elucidating the biochemical components of insect glycosylation pathways. The silkworm, *B. mori, is* one of the most important insects used in silk production for centuries and in protein production for the cosmetics, and medicine industries (Resh & Carde, 2003). It is the model organism for research and the first Lepidopteran whose genome was available (Shimomura et al., 2009). Having a short generation time, known genome, rich genetic resources, and homolog genes to humans, silkworms have widely been used in studies (Meng et al., 2017). The present study was aimed at sialyltransferase (ß-Galactoside α-2,6-Sialyltransferase I) gene expression in tissues of 5. instar *B. mori* larvae.

MATERIAL and METHOD

Silkworm breeding and sialic acid application

The domestic silkworm *Bombyx mori* hybrid eggs, which constitute the study material, were obtained from Koza Birlik (Bursa, TÜRKİYE). The breeding of insects was actualized in the Silkworm Culture Laboratory of Ege University Biology Department. The leaves of mulberry trees were used three times a day for larvae nutrition. In our study, in parallel with the control group fed with normal mulberry leaves, a treatment group fed with mulberry leaves with 0.1M concentration of sialic acid (Sigma A0812) solution, (sialic acid solution was sprayed on the leaves and allowed to dry) prepared freshly with ultrapure water every day from the 0th day of the 5th instar was formed. Dissection was performed on the 1st, 4th, and 7th days of both control and treatment group 5th instar silkworms, which continued this feeding procedure for 7 days. *Bombyx mori* sialyltransferase (BmST) expression profile in *B. mori* was determined using Real-Time PCR. Specific primers for BmST (Kajuira et al., 2015) and cDNAs from *B. mori* 5th instar larvae were used.

Total RNA isolation:

The midgut, fat body, ovary, testis, and hemocyte, which were dissected from 10 silkworms belonging to the control (feeding directly on mulberry leaves) and treatment (feeding mulberry leaves with sialic acid) groups, were placed in Eppendorf containing triazole (Invitrogen 15596026) separately. A homogeneous and pink image is obtained by adding triazole to the tissues and vortexing them. Centrifugation is performed for RNA isolation and RNAse-free water is added to the remaining precipitate. After isolation, RNA concentrations were measured by spectrophotometric methods.

cDNA synthesis:

Transcriptor First Strand cDNA Synthesis Kit (Roche) was used for cDNA synthesis. For each sample, 11 µl of RNA+RNAse free water mixture was added and 2 µl of random hexamer primer was added. RNAs were denatured for 10 min at 65°C by placing the tubes in a thermal cycler.

Real- Time PCR analysis:

The primers used for B. mori ST6Gal I gene expression were benefited from the work of Kajiura et al. (2015). The expressions of BmST were determined by amplifying Bmrp49 as a control and BmST using following gene-specific primer sets;

Bmrp49 gene:

(forward: 5′-CAGGCGGTTCAAGGGTCAATAC-3′, reverse: 5′-TGCTGGGCTCTTTCCACGA-3′),

BmST:

(forward: 5′- GAGTCGCCGGTGTCATTACT-3′, reverse: 5′-CCTCGTTGAAAGGTGTCGAT-3′).

Master mix content created in real time PCR experiments; Cyber Green 2X Rox Dye Master mix (Qiagen), forward and reverse primers designed for genes, cDNAs as templates and nuclease-free water. After the master mixes were prepared, the samples were analyzed in the Real Time PCR device [The real time PCR device basically consists of a thermocycler, excitation light source, a fluorescence detection system and software (Tutar et al., 2015)] and run in the optimized program. The ΔΔCT method was used to provide the quantitative results obtained (Yıldırım et al., 2018).

Statistical analysis:

Statistical analysis was performed by comparing the control and treatment groups from the results obtained from the PCR analysis. For this analysis, the t-test method was applied to independent groups in the "IBM SPSS Statistics 26" statistical program.

RESULTS and DISCUSSION

In this study, potential sialyltransferase gene expression in *B. mori* tissues was examined directly. RNA amount measurements of the midgut, hemocyte, fat body, testis, and ovary tissues from which RNA was isolated were determined using nanodrop. Considering the A260/A280 absorbance ratios, control groups and treatment groups were found in ideal purity. The amounts resulting from RNA isolation were diluted with RNAase-free water to be 100 nanograms in a total volume of 20 µl. When examining the real-time PCR results, the expression levels of silkworms on the first, fourth, and seventh days of the fifth instar in the midgut, fat body, ovary, testis, and hemocyte tissues are different in all of them. The expression level was observed after the sialic acid application with the normal-fed control group. Expression levels are noted to be different in each organ and day. Sialyltransferase gene expression levels were determined by giving substrate to *B. mori*. Similar to this study, different expression levels were detected in each tissue and day. Statistically insignificant differences were found on some days in the midgut, hemocyte, ovary, and testis (Fig. 1-4). Although there were differences in expression levels, gene expression activity was observed in all tissues of the groups given sialic acid.

Sialylated glycans have been identified to be responsible for neural regulation in *Drosophila*, and sialioglycoconjugates have been found to play a role in the viral transmission of dengue virus on mosquito tissues (Koles et al., 2004; Cime-Castillo et al., 2015).

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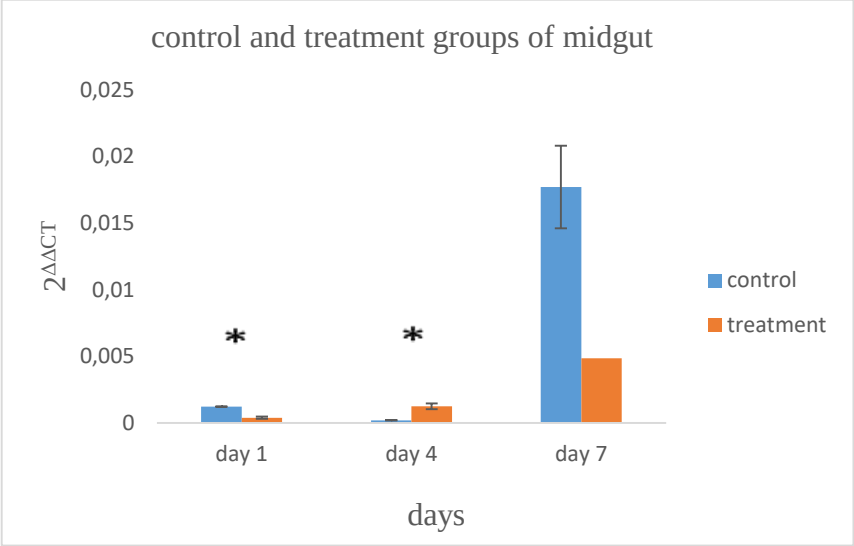

Figure 1. Comparison of sialyltransferase gene expression levels of control and treatment groups in midgut with independent samples T-test (*; p<0.05).

Şekil 1. Kontrol ve uygulama gruplarının orta bağırsaklarındaki sialiltransferaz gen ifade düzeylerinin bağımsız örnekler T- testi ile karşılaştırılması (; p<0.05).*

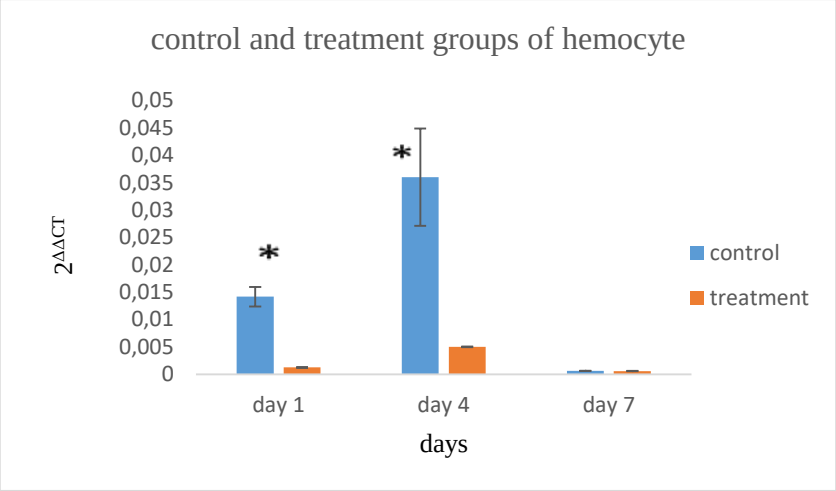

Figure 2. Comparison of sialyltransferase gene expression levels of control and treatment groups in hemocyte with independent samples T-test (*; p<0.05).

Şekil 2. Kontrol ve uygulama gruplarının hemositlerindeki sialiltransferaz gen ifade düzeylerinin bağımsız örnekler T- testi ile karşılaştırılması (; p<0.05).*

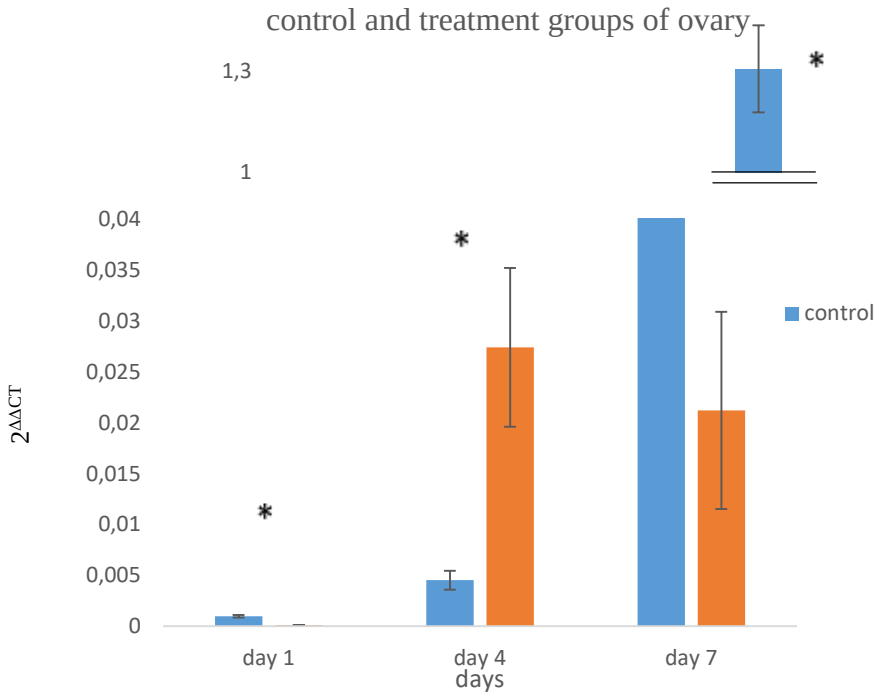

Figure 3. Comparison of sialyltransferase gene expression levels of control and treatment groups in the ovary with independent samples T-test (*; p<0.05).

Şekil 3. Kontrol ve uygulama gruplarının ovaryumlarındaki sialiltransferaz gen ifade düzeylerinin bağımsız örnekler T- testi ile karşılaştırılması (; p<0.05).*

The gene expressions determined in *B. mori* differ in each tissue in the treatment and control groups. Therefore, it cannot be interpreted that gene expression increases or decreases when sialic acid is given. A definite increase in fat body only after treatment and the highest expression levels are in fat body among the tissues treated with sialic acid (Fig 5).

The sialylation of glycoproteins and glycolipids plays a significant role in numerous biological functions. Sialylglycoconjugates are involved in cell-cell communication, immune responses, and the removal of circulating glycoproteins (Marchal et al., 2001). A family of sialyltransferases plays a role in transferring sialic acid (NeuAc) from active sugar donor CMP-NeuAc to terminal nonreducing positions of a variety of oligosaccharide chains found on glycoconjugates (Weinstein et al., 1987; Li & Chen, 2012; Petit et al.,

2015; Teppa et al., 2016). Sialylation and sialyltransferases are found in some insects like *B. mori*, *Drosophila melanogaster*, *Aedes aegypti*, *Galleria mellonella*, *Philaenus spumarius*, *Bactrocera dorsalis* (Ghosh, 2018).

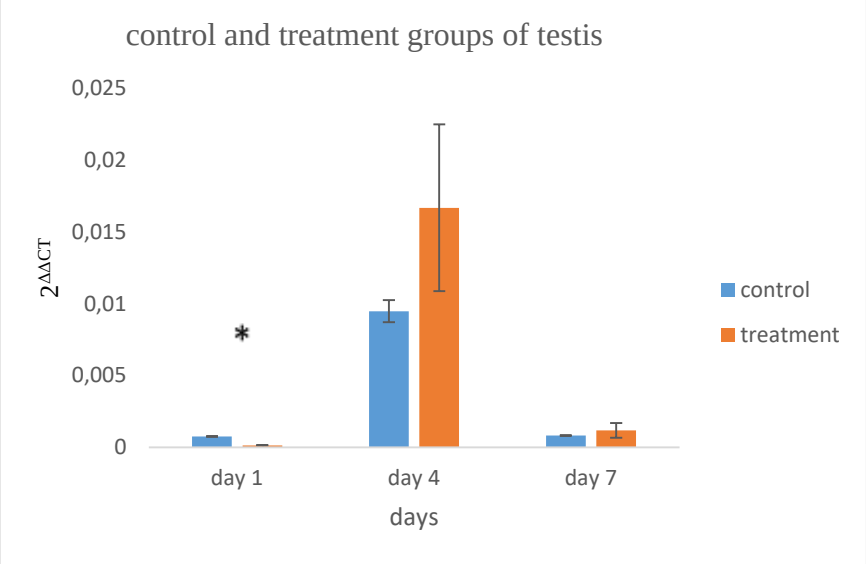

Figure 4. Comparison of sialyltransferase gene expression levels of control and treatment groups in testis with independent samples T-test (*; p<0.05).

Şekil 4. Kontrol ve uygulama gruplarının testislerindeki sialiltransferaz gen ifade düzeylerinin bağımsız örnekler T- testi ile karşılaştırılması (; p<0.05).*

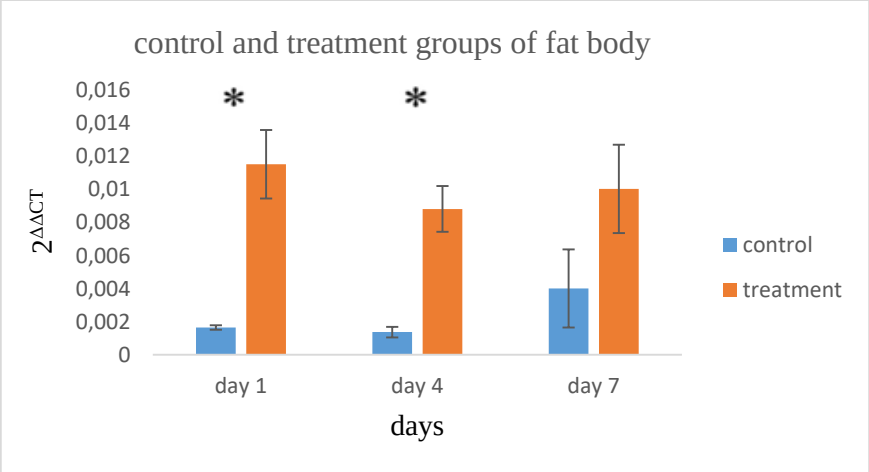

Figure 5. Comparison of sialyltransferase gene expression levels of control and treatment groups in fat body with independent samples T-test (*; p<0.05).

Şekil 5. Kontrol ve uygulama gruplarının yağ dokularındaki sialiltransferaz gen ifade düzeylerinin bağımsız örnekler T- testi ile karşılaştırılması (; p<0.05).*

In insects, sialic acids play a significant role in phylogenetics and evolution. *Drosophila* genome database searches have revealed the existence of several genes encoding putative orthologs of vertebrate enzymes of the sialic acid biosynthetic pathway, including Neu5Ac phosphate synthase, CMP-Neu5Ac synthase, CMP-Neu5Ac/CMP antiporter and sialyltransferase (Koles et al., 2004). The first sialyltransferase that is identified in insects is *D. melanogaster* sialyltransferase (DmST). It was found that sialylated N-glycans are present in *Drosophila* embryos and the nervous system (Koles et al., 2004; Koles et al., 2007). In *A. aegypti*, on the other hand, sialyltransferases play a role in mediating contagious dengue infection (Malykh et al., 1999). Kajiura et al. (2015) identified a *B. mori* sialyltransferase involved in the vertebrate ST6Gal I-like located in the Golgi. Unlike human α2,6-sialyltransferase, BmST required post-translational modification, specifically N-glycosylation, for its full activity. On the other hand, Kajiura et al. (2015) identified the BmST gene in their study and performed RT-PCR analysis on 5th-instar tissues as a method.

The fat body of insects has a mesodermal origin, growing in the embryonic phase through the increase in the number and differentiation of present cells. The fat body is a distinct organ that only insects have. It spreads throughout the body of insects (Law & Wells, 1989; Arrese & Soulages, 2010). The fat body is commonly described as a resemblance to vertebrate liver (Chapman, 2013; Zhang & Xi, 2014). It plays numerous roles in insects. For example, synthesis, absorption, and storage of nutrients from hemolymph (Roma et al., 2010; Turgay-İzzetoğlu & Gülmez, 2019), immunity, endocrine and detoxification processes, show high biosynthetic activity (Resh & Cardé, 2009; Arrese & Soulages, 2010; Martins et al., 2012; Chapman, 2013; Assis et al., 2014), reproduction (Roma et al., 2010). Most hemolymphatic proteins are synthesized in the fat body, which also stores lipids, carbohydrates, and proteins (Feitosa et al., 2006; Alves et al., 2010; Park et al., 2013; Zhang & Xi, 2014; Nation, 2016). In addition to its numerous metabolic functions, the fat body in insects serves as a receptive tissue for the regulatory actions of all major insect hormones (Roma et al., 2010).

According to this information, the higher expression of sialyltransferase in *B. mori* than in other tissues indicates that the enzyme has an important place in fat body functions.

Sialic acids typically play roles in modulating cellular adhesion processes, by either facilitating binding interactions or concealing recognition sites. These functions are integral to processes such as cell proliferation, ageing, phagocytosis, apoptosis, transportation, and receptor-mediated functions (Schauer, 2001). The function of sialic acid in organisms is to control major biochemical events. Sialic acids have a pivotal role in biological masking, effectively shielding sites of recognition, such as the penultimate sugar moiety in receptors and antigens. This masking effect can be attributed to their substantial size and negative charge, which grants cells a "self-like" property. In parallel, sialic acids function as ligands for various entities, including hormones, lectins, antibodies, and inorganic cations, thereby contributing to processes like adhesion, inflammation, immune responses, and embryogenesis in the nervous system (Buschiazzo & Alzari, 2008; Schauer, 2009; Ghosh, 2018).

Sialylation contributes to the fat body in insects. This showed that fat body and sialic acid have similar features and they work together in the same functions. Sialic acid was found on the prothoracic gland cells of *G. mellonella* larvae. After sialylation, this monosaccharide had a protective role on the juvenile cells against degeneration (Schauer, 2001).

DmST plays a critical role in the biological functions. Indeed, mutation of DmST resulted in a deficiency of the sialylation of N-glycans and was associated with decreased longevity, abnormal locomotor activity and temperature-sensitive paralysis because of the loss of voltage-gated sodium channels (Repnikova et al., 2010; Kajiura et al., 2015).

It was observed that the fat body is one of the most important tissues, especially for the larvae stage. Sialylateglucoconjugates play important roles in mammals and insects. Therefore, it was thought that the sialyltransferase expression profile is most in fat body tissue.

CONCLUSION

This study demonstrated that *B. mori* larvae tissue has shown sialyltransferase gene expression. We considered not only sialyltransferase but the other enzymes, that are responsible for the sialylation pathway, were also activated. The provided substrate led to sialyltransferase gene expression activity in all tissues, thereby demonstrating the active state of the sialylation pathway. The increase was determined only in the fat body. This can be attributed to the similarities in the functions of fat body and sialic acid and their roles in insects. It was determined that sialyltransferases are important for insect development. Because of the sialyltransferase activity, sialic acid has affected the tissues of the insect.

ACKNOWLEDGEMENT

This work was supported by the Ege University Scientific Research Fund (FYL-2021-23087).

Author's Contribution

The contribution of the authors is equal.

Conflict of Interest

The authors declare that there is no conflict of interest.

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