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# Enhancement of sialyltransferase-catalyzed transfer of sialic acid onto glycoprotein oligosaccharides using silkworm hemolymph and its 30K protein

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**Abstract**

Silkworm hemolymph (SH) has been reported to inhibit apoptosis in both insect and human cells, and increase the high-sialylation structure of recombinant glycoprotein in insect cells. This indicates that SH might increase glycosyltransferase activity. Therefore, this study examined the effect of SH on the activity of sialyltransferase, which catalyzes the sialylation of the glycoprotein. When 10 μg/mL of SH was added to the reaction mixture, almost complete sialylation was observed even under the reaction conditions where sialyltransferase-catalyzed sialylation rarely occurs. The effect of deproteinized SH (dSH) and the 30K protein, which is a major plasma protein in SH, was examined to determine which component in SH enhances sialylation. The 30K protein promoted sialylation, while the dSH did not. This suggests that SH and its 30K protein can be used as an additive to a medium for efficient glycosylation when mammalian cells are being cultured for the production of valuable biopharmaceuticals, many of which are glycoproteins.


*Keywords:* Sialic acid; Sialyltransferase; Silkworm hemolymph; 30K protein; Glycoprotein

## 1. Introduction

Many recombinant proteins for a therapeutic and diagnostic use are produced in mammalian cells, which can perform post-translational modifications including glycosylation. Protein glycosylation plays a key role in the folding, activity, immunogenicity, and protease sensitivity *in vivo* [1]. Deglycosylation significantly decreases the *in vivo* activity of erythropoietin (EPO), which is an important recombinant pharmaceutical [2]. In general, the incomplete sialylation of glycans, which are normally sialylated leads to more rapid clearance *in vivo* through the asialoglycoprotein receptors in the liver [3]. Moreover, in the case of EPO, the addition of novel sites for sialic acid-containing glycans can increase its biological activity and prolong the duration of action [4]. Inside cells, sialyltransferases catalyzes the transfer of *N*-acetylneuraminic acid (NANA), which is the most common sialic acid, from cytidine 5-monophospho-*N*-acetylneuraminic acid (CMP-NANA), a sugar-nucleotide donor, to an oligosaccharide chain on the glycoproteins (Fig. 1).

It was reported that silkworm hemolymph (SH), which is a component in insect serum, inhibited apoptosis in both insect [5] and human cells [6,7]. The apoptosis-inhibiting component of SH was isolated and characterized [8]. A database search based on the N-terminal amino acid sequence of this component revealed that this protein shares a 95% homology with one of the '30K proteins', which are a group of structurally related proteins with molecular weights of approximately 30,000 Da [9]. SH was also reported to increase the productivity of recombinant proteins [10]. SH increased the production of EPO in CHO cells by suppressing the sodium butyrate-induced cell apoptosis in serum-free medium [11]. SH was shown to increase the high-sialylation structure of recombinant secreted human placental alkaline phosphatase (SEAP) in an insect cell system [12]. Therefore, it was suggested that SH might contain components that increase the glycosyltransferase expression level and/or activity. This study investigated the effect of SH and its 30K protein on the activity of α2,3-sialyltransferase, which catalyzes the sialylation of glycoprotein, using an *in vitro* asialofetuin sialylation system.

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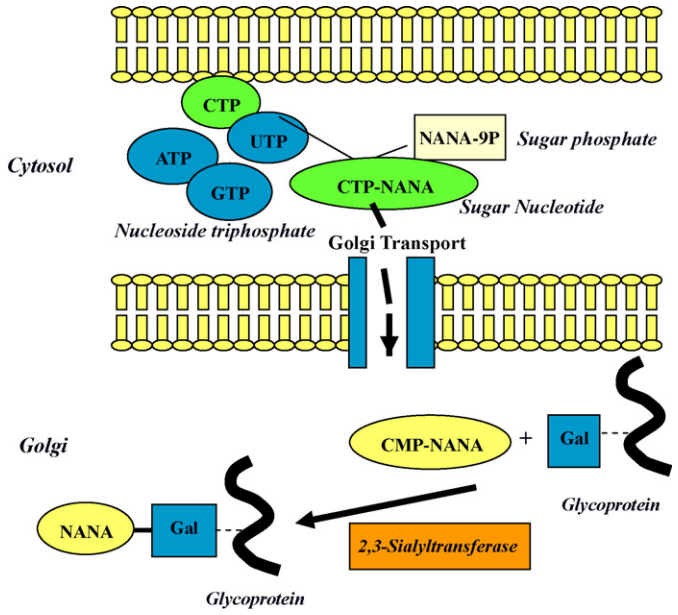



Fig. 1. Schematic diagram of sialyltransferase-catalyzed sialylation. *N*-acetylneuraminic acid (NANA, sialic acid) are transferred from cytidine 5-monophospho-*N*-acetylneuraminic acid (CMP-NANA) to the galactosylated (asialo) glycoprotein.

## 2. Experimental

### 2.1. Materials

The asialofetuin, fetuin from fetal calf serum, and CMP-NANA were purchased from Sigma, and the recombinant rat α2,3-sialyltransferase was obtained from Calbiochem.

### 2.2. Collection of SH

The SH was obtained from the fifth-instar larvae of *Bombyx mori* by clipping the side of an abdominal leg. It was collected into a sterilized 15 mL tube placed on ice. On average, 0.5 mL of hemolymph was obtained from each larva. To prevent the SH from being oxidized, 1 mL of mineral oil (Sigma) was added, and the SH was heat-treated at 60 °C for 30 min. This heat treatment inactivates the tyrosinase, which catalyzes the oxidation reaction. Then the SH was chilled and centrifuged at 12,000 rpm for 30 min after removing the mineral oil from the tube [7]. The supernatant was filtered through a 0.2 μm membrane and added directly to the enzyme reaction mixture or fractionated for the purification of 30K protein.

### 2.3. Purification of 30K protein

The SH collected was loaded on a Superdex 200 HR (1 cm × 3 cm × 30 cm, Amersham-Pharmacia Biotech) and eluted with a 50 mM sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl at a flow rate of 0.5 mL/min. The eluant was monitored at a wavelength of 280 nm, and 0.5 mL fractions containing the 30K protein were collected. The fractions from the Superdex 200 HR column were desalted into the starting buffer (120 mM Tris and 80 mM Bis–Tris, pH 9.0) using a HiTrap desalting column (1.6 cm × 3 cm × 2.5 cm, Amersham-Pharmacia Biotech). The desalted fractions were injected onto a Mono Q HR (0.5 cm × 3 cm × 5 cm, Amersham-Pharmacia Biotech), which had been pre-equilibrated with the same starting buffer, and eluted with a linear gradient of 20 column volumes of the elution buffer (120 mM Tris and 80 mM Bis–Tris, pH 5.0) at a flow rate of 0.5 mL/min. The eluant was monitored at a wavelength of 280 nm, and 1-mL fractions containing 30K protein were collected. The purified fractions were concentrated using lyophilizer and analyzed by MALDI-TOF-mass spectrometry and N-terminal amino acid sequencing to confirm the purification of 30K protein [8].

### 2.4. Sialylation of asialofetuin

Four micrograms of asialofetuin in 0.1 mL of reaction buffer (50 mM MOPS, 0.5 mg/mL BSA, 0.2% Triton CF-54 detergent, pH 7.4) was incubated with 10 mM CMP-NANA, 100 mU/mL α2,3-sialyltransferase, and various concentrations of SH (1–100 μg/mL) or the 30K protein (1–100 μg/mL) at 37 °C for 2 days [13].

### 2.5. Western blot analysis

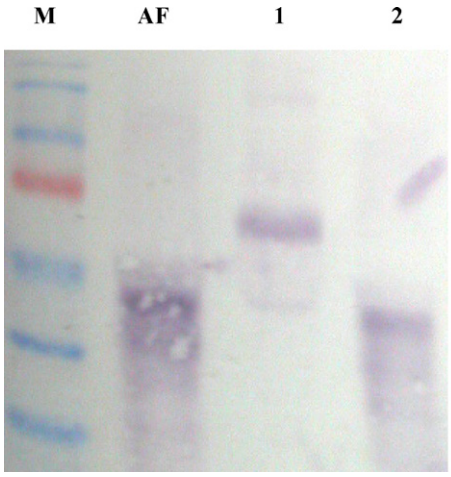
After the sialylation of asialofetuin, the proteins were denatured by boiling, and then loaded on a 12% polyacrylamide gel. The proteins separated by SDS-PAGE were transferred electrophoretically onto a PVDF membrane, and detected by Western blot analysis using the rabbit polyclonal antibody to bovine fetuin and alkaline phosphatase-conjugated goat anti-rabbit IgG [11].

### 2.6. Lectin assay

The terminal sialylation of the fetuin glycans was assayed using a DIG glycans differentiation kit (Roche). After the sialylation of asialofetuin, the protein was transferred onto a PVDF membrane after SDS-PAGE. The membranes were incubated in 20 mL blocking solution at 4 °C overnight, and further incubated with 50 μL MAA (Maackia amurensis agglutinin) lectin at room temperature for 1 h. Lectin binding was detected by incubating the membranes with anti-digoxigenin-alkaline phosphatase for 1 h. Nitriblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 5 mM $MgCl_2$ plus 100 mM Tris–buffer were used as the substrate. The membranes were washed five times with a TBS buffer (pH 7.5) for 5–10 min each.

### 2.7. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF mass spectrometry was carried out using a Voyager-DE STR (Applied Biosystems) instrument in positive ion mode. After the sialylation of asialofetuin, 10 μL of the matrix solution was mixed with 1 μL of the sample, and a 0.7 μL aliquot was dried in air. For the intact proteins, sinapinic acid



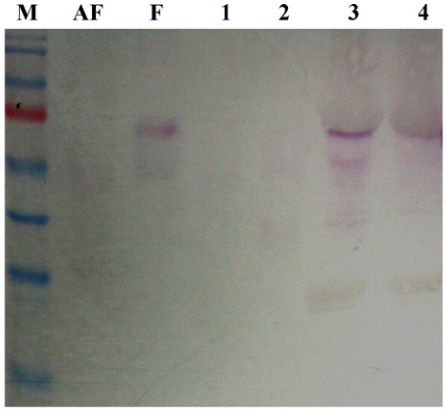

| Lane | |
|---|---|
| M | Marker |
| AF | Asialofetuin |
| 1 | AF + CMP-NANA + ST + SH |
| 2 | AF + CMP-NANA + ST |

Fig. 2. Western blot examining the effect of SH on the sialylation of asialofetuin. The reaction mixtures were separated by 12% SDS-PAGE after *in vitro* sialylation of asialofetuin using sialyltransferase for 2 days. The protein was transferred to a PVDF membrane and detected by the monoclonal anti-fetuin antibody. AF: asialofetuin, CMP-NANA: cytidine 5-monophospho-*N*-acetylneuraminic acid, ST: sialyltransferase, SH: silkworm hemolymph (10 μg/mL).

was used as the matrix, approximately 100 shots were combined, and the total laser irradiance was 5–6 μJ.

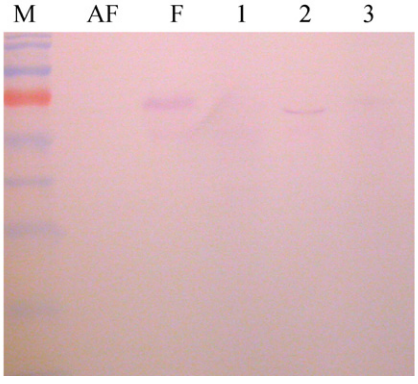
## 3. Results and discussion

Oligosaccharides of glycoprotein fetuin contain terminal glycan moieties with sialic acid (NANA) linked to *N*-acetylglucosamine [14]. In this study, an *in vitro* enzymatic system, i.e. the sialyltransferase-catalyzed sialylation of bovine serum asialofetuin, was used to determine the effect of SH on the sialylation of these moieties. In this reaction, NANA is transferred from CMP-NANA to the asialofetuin. The effect of SH on the sialylation of asialofetuin was examined by the Western blot analysis of the reaction products. Fig. 2 shows that the molecular




(A)

| Lane | |
|---|---|
| M | Marker |
| AF | Asialofetuin |
| F | Fetuin |
| 1 | AF + CMP-NANA + ST |
| 2 | AF + CMP-NANA + ST + dSH |
| 3 | AF + CMP-NANA + ST + SH |
| 4 | AF + CMP-NANA + ST + 30K |




(B)

| Lane | |
|---|---|
| M | Marker |
| AF | Asialofetuin |
| F | Fetuin |
| 1 | AF + CMP-NANA + SH |
| 2 | AF + CMP-NANA + ST + SH |
| 3 | AF + ST + SH |

Fig. 3. MMA lectin assay for analyzing the effect of SH and 30K protein on the sialylation of asialofetuin. The reaction mixtures were separated by 12% SDS-PAGE after *in vitro* sialylation of asialofetuin using sialyltransferase for 2 days. The protein was transferred to a PVDF membrane and detected by MAA lectin specific to sialic acid. AF: asialofetuin, F: fetuin, CMP-NANA: cytidine 5-monophospho-*N*-acetylneuraminic acid, ST: sialyltransferase, SH: silkworm hemolymph (10 μg/mL), dSH: deproteinized SH (5 μg/mL), 30K: 30K protein (1 μg/mL) purified from SH.

weight of the reaction product increased when SH was added to the reaction mixture (lane 1). However, under the same conditions used in the enzyme reaction, sialyltransferase did not transfer sialic acid from CMP-NANA to the terminal residue of asialofetuin in the absence of SH (lane 2). In this reaction, 4 μg of asialofetuin was incubated with 10 mM CMP-NANA and 100 mU/mL sialyltransferase at 37 °C for 2 days. This reaction condition, in which sialylation barely occurs in the absence of SH, was chosen in order to clearly show the effect of SH on sialylation. Sialylation occurs in the absence of SH if a larger amount of enzyme is used. The upper shift in the protein band using SH represents the sialylation of the oligosaccharides chain on asialofetuin. This suggests that SH enhances the sialylation of asialofetuin. The asialofetuin band (lane AF) appeared rather broad due to the heterogeneity of the oligosaccharides [15].

MAA (Maackia amurensis agglutinin) lectin, which recognizes sialic acid α(2–3)-linked to galactose and binds to the terminal sialic acid moieties, was used to determine if the upper shift of fetuin protein band was the result of terminal sialylation by sialyltransferase. As shown in Fig. 3A, sialylated fetuin in the reaction mixture supplemented with SH was clearly detected by the lectin assay (lane 3). The size of the sialylated fetuin produced in this reaction mixture was identical to that of the standard fetuin shown in lane F. The 30K protein or deproteinized SH were tested in order to investigate which component in SH enhanced the sialylation. The 30K protein, a major plasma protein in SH, was prepared by isolating and purifying SH by column chromatography. The deproteinized SH (dSH) was obtained by boiling SH. dSH did not promote sialylation (lane 2), while the 30K protein increased the level of sialyltransferase-catalyzed sialic acid transfer (lane 4). Sialylated fetuin, which is similar in size to standard fetuin, was produced in this reaction mixture containing the 30K protein.

SH was further examined to determine if might catalyze the sialylation itself. As shown in Fig. 3B, in the absence of sialyltransferase (lane 1) or CMP-NANA (lane 3), SH did not produce any sialylated fetuin from asialofetuin. Sialylation occurred only when both sialyltransferase and CMP-NANA were in the reaction mixture (lane 2). This indicates that SH does not contain the components functioning as sialyltransferase or CMP-NANA, and only enhances the enzymatic sialylation. In a previous study, SH and its 30K protein were investigated in the aspect of apoptosis inhibition [6–8]. However, their effect on sialylation or glycosylation is also important.

The effect of the 30K protein on the sialylation of glycoprotein was analyzed by MALDI-TOF mass spectrometry. Fig. 4 shows the results of the reaction mixtures with and without the 30K protein. After the enzymatic sialylation reaction without the 30K protein, a large peak was observed in the mass range of 40747–41547, which was assigned to the molecular mass of the unsialylated forms of fetuin (Fig. 4A). In the case of the enzymatic sialylation reaction with the 30K protein, another major peak was observed in the mass range of 42174–43445, which was assigned to the molecular mass of the sialylated forms of fetuin (Fig. 4B). The mass spectrometry data agrees with the results obtained from Western blot and MAA lectin analyses. Considering that the molecular mass of sialic acid is

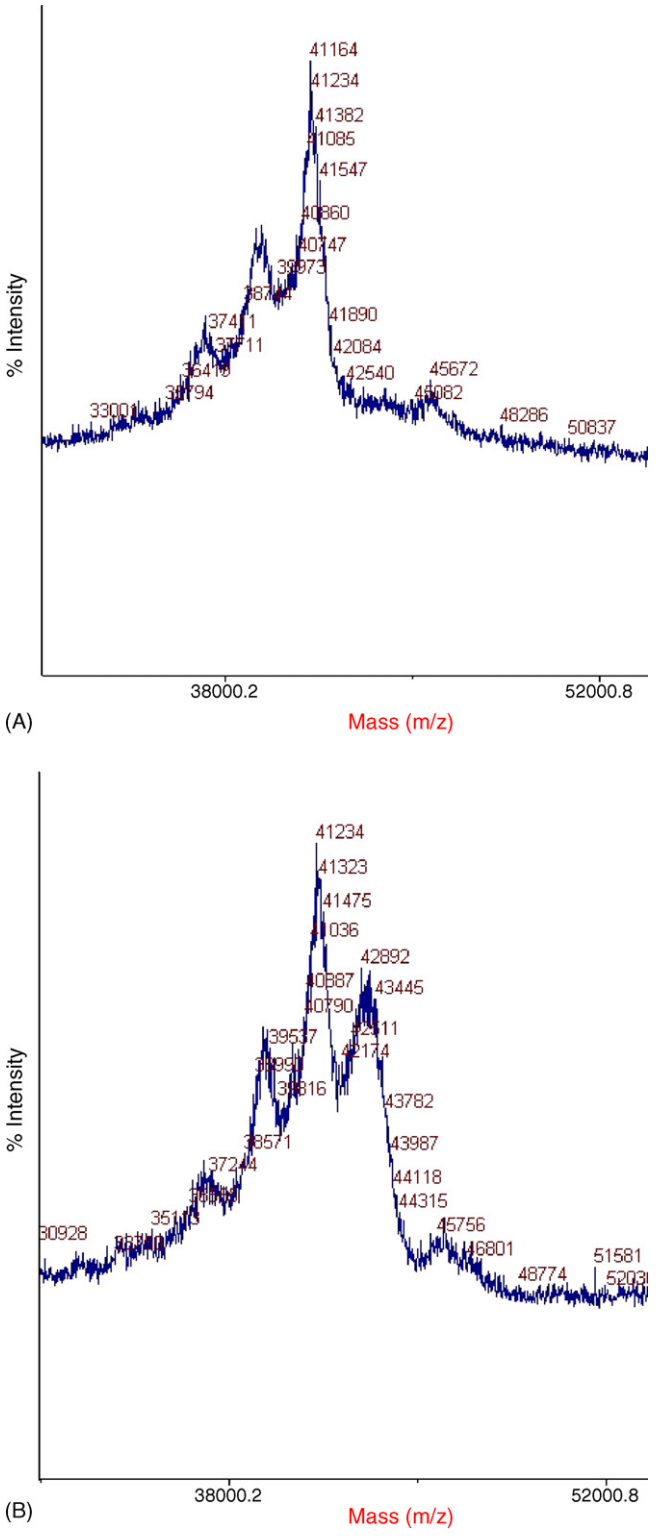



Fig. 4. MALDI-TOF mass spectrometry examining the effect of the 30K protein on the sialylation of asialofetuin. (A) Reaction without the 30K protein, (B) reaction with 30K protein (1 μg/mL). The masses of the components in the reaction mixture were analyzed by MALDI-TOF after the *in vitro* sialylation of asialofetuin using sialyltransferase for 2 days.

309.27, approximately five sites of the sialic acid residue were expected to be sialylated as a result of the enzyme reaction with the assistance of the 30K protein. More quantitative analysis of sialylation should be further performed to examine the sialylation sites more accurately.

SH or 30K protein could be effectively used for the production of biopharmaceutical glycoproteins such as EPO and follicle-stimulating hormone (FSH) in CHO cells. The α2,3-sialyltransferase used in this study is the enzyme that transfers sialic acid to the terminal residue of asialo protein in CHO cells. Co-expression of 30K protein with a target pharmaceutical protein would produce highly sialylated glycoproteins in CHO cells.

## 4. Conclusion

This study examined the effect of SH and its 30K protein on the activity of sialyltransferase, which catalyzes the sialylation of glycoprotein, through the *in vitro* sialylation of asialofetuin. The enhancement of sialyation by SH and the 30K protein was analyzed by Western blot, a lectin assay, and MALDI-TOF mass spectrometry. When either SH or 30K protein was added to the reaction mixture, sialyltransferase effectively catalyzed the sialylation process even under the reaction conditions where sialylation barely occurs. Incomplete sialylation of the glycans that are normally sialylated leads to the more rapid clearance *in vivo* through the asialoglycoprotein receptors in the liver. A higher sialic acid content at the end of the sugar moiety on the glycoprotein is favorable for its biological activity and *in vivo* stability. In this sense, SH and the 30K protein can be used as an additive to achieve efficient glycosylation when mammalian cells are cultured for the production of valuable biopharmaceuticals, many of which are glycoproteins.

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