Sialyltransferases Obtained from Marine Bacteria

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Abstract: Sialyltransferases transfer N-acetylneuraminic acid (Neu5Ac) from cytidine monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) to the acceptor substrate. Up to the present, many sialyltransferases have been cloned from mammalian and bacterial sources. All the sialyltransferases have been classified into five families in the CAZy (carbohydrate-active enzymes) database (families 29, 38, 42, 52 and 80), and all of the marine bacterial sialyltransferases are classified into the family 80. During the course of our study, we have isolated several marine bacteria producing sialyltransferases. Many of them were identified as the bacteria which were classified into genera Photobacterium and Vibrio. Furthermore, we have also demonstrated that these marine bacterial sialyltransferases have unique acceptor substrate specificity, compared with those of mammalian sialyltransferases. N-Acetylneuraminic acid is usually linked to the terminal position of glycan moiety of glycoconjugates, including glycoprotein and glycolipid. Enzymatic sialylation using sialyltransferase is a single-step process with high positional and anomeric selectivity and high reaction yield under mild reaction conditions. Therefore, sialyltransferase is believed to be one of the most important enzymes in the field of glycotechnology and to be a powerful tool for the study of glycobiology.

Key words: marine bacteria, Photobacterium, sialyltransferase, Vibrio

Sialic acids (Sias) are important components of carbohydrate chains and are linked to terminal positions of the carbohydrate moiety of glycoconjugates, including glycoproteins and glycolipids. A lot of studies have focused on clarifying the structure-function relationship of Sias and have revealed that N-acetylneuraminic acid (Neu5Ac) is the major Sia component of glycoconjugates, and that the sialylated glycoconjugates play significant roles in many biological processes.7

In the sialylated glycoconjugates, mainly four linkage patterns, including Neu5Acα2-6Gal, Neu5Acα2-3Gal, Neu5Acα2-6GalNAc and Neu5Acα2-8Neu5Ac are found.12 These structures are formed by specific sialyltransferases in the cell. Therefore, the sialyltransferases are considered key enzymes in the biosynthesis and in vitro enzymatic synthesis of sialylated-glycoconjugates and/or glycans. Here, all known sialyltransferases use cytidine 5′-monophosphate N-acetylneuraminic acid (CMP-Neu5Ac) as the common donor substrate.21

The sialyltransferases have been cloned from various sources, including mammalian organs, viruses and bacteria.12,24 Generally, the enzymes from a bacteria are more stable and productive in Escherichia coli protein expression systems than the mammalian-derived enzymes. These advantages highlight the capacity of bacterial enzymes as efficient tools for the in vitro enzymatic synthesis of sialosides.

Genes encoding sialyltransferases have been cloned from various types of bacteria, including Neisseria gonorrhoeae,7 Neisseria meningitidis,6 Neisseria meningitidis,7 Campylobacter jejuni,7 E. coli,6 Photobacterium damselae,9 Photobacterium phosphoreum,10 Photobacterium leiognathi,11 Photobacterium sp.,12 Vibrio sp.,13 Pasteurella multocida,14 Haemophilus influenzae15 and Streptococcus agalactiae.16 All the sialyltransferases, including mammalian, viral and bacterial origin, have been classified into five families in the CAZy database. However, all bacterial sialyltransferases are classified into four families in the CAZy database: (1) glycosyltransferase (GT) family 38 (polysialyltransferase from E. coli and N. meningitidis); (2) GT family 42 (lipooligosaccharide α₂,3-sialyltransferase and α₂,3-α₂,6-sialyltransferase from C. jejuni and H. influenzae); (3) GT family 52 (α₂,3-sialyltransferase from H. influenzae, N. gonorrhoeae and N. meningitidis) and (4) GT family 80 (α₂,6-sialyltransferase and α₂,3-α₂,6-sialyltransferase from P. damselae and P. multocida).

An abundant supply of sialylated oligosaccharides is essential for investigation of the biological function of sialylation. Enzymatic glycosylation using glycosyltransferases is a single-step process with high positional and anomeric selectivity and high reaction yield. For example, with sialylation, the transfer of Neu5Ac by sialyltransferases to the appropriate substrate from CMP-Neu5Ac as the donor substrate can be readily achieved in the final step under mild reaction conditions.19 Therefore, sialyltransferases are thought to be a powerful tool for the study of glycotechnology, especially in producing various sialylated glycoconjugates. In this report, we review the character of marine bacterial sialyltransferases we have isolated.

Screening of bacterial strains producing sialyltransferases.

The screening method of sialyltransferase was as follows. From the many kinds of samples, including seawa-
ter, sea sand and fishes, many bacteria were isolated. Normally, Marine broth 2216 was used as the culture medium. One colony of isolated bacteria on a Marine broth 2216 agar plate were inoculated into 5 mL of Marine broth 2216 medium in a 30 mL test tube and cultivated at 30°C for 12 h on a rotary shaker each. After cultivation, those cells were recovered by centrifugation and suspended in extraction buffer. The suspended cells were sonicated until absorbance at 600 nm became 30% of that of the cell suspension. The sonicated solutions were used as crude enzyme solution. Sialyltransferase activity was routinely assayed by measuring \^[4,5,6,7,8,9\textsuperscript{14}C]\text{Neu5Ac} transferred from CMP-[^4,5,6,7,8,9\textsuperscript{14}C]\text{Neu5Ac} as a donor substrate to lactose as an acceptor substrate. During the course of the study, we have isolated many bacteria that produce sialyltransferase. New species of bacteria were found among them. It was evident likely that many of the bacteria that produced sialyltransferases were classified in genus \textit{Photobacterium} or closely related genus \textit{Vibrio}. For instance, \textit{P. phosphoreum} JT-ISH467 showed \(\alpha\text{2,3-sialyltransferase} \) activity, \(\alpha\text{2,6-sialyltransferase} \) activity. The relationship between type of sialyltransferase and its origin is summarized in Table 1.

### Table 1. Relationship between enzyme type and bacteria.

<table>
<thead>
<tr>
<th>GT family</th>
<th>Origin</th>
<th>Enzyme type</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>\textit{Escherichia coli} K1 \textit{Neisseria meningitidis}</td>
<td>(\alpha\text{2,8/2,9, \alpha\text{2,8-sialyltransferase} ) (\alpha\text{2,8-sialyltransferase} )</td>
</tr>
<tr>
<td>42</td>
<td>\textit{Campylobacter jejuni} \textit{Haemophilus influenzae}</td>
<td>(\alpha\text{2,3/2,8, \alpha\text{2,3-sialyltransferase} ) (\alpha\text{2,3/2,8-sialyltransferase} )</td>
</tr>
<tr>
<td>52</td>
<td>\textit{Neisseria gonorrhoeae} \textit{Neisseria meningitidis}</td>
<td>(\alpha\text{2,3/2,6, \alpha\text{2,3-sialyltransferase} ) (\alpha\text{2,3-sialyltransferase} )</td>
</tr>
<tr>
<td>80</td>
<td>\textit{Photobacterium damselae} \textit{Photobacterium leigonathi} \textit{Photobacterium phosphoreum} \textit{Pasteurella multocida} \textit{Vibrio sp.}</td>
<td>(\alpha\text{2,6-sialyltransferase} ) (\alpha\text{2,6-sialyltransferase} ) (\alpha\text{2,3-sialyltransferase} ) (\alpha\text{2,3-sialyltransferase} ) (\alpha\text{2,3-sialyltransferase} )</td>
</tr>
</tbody>
</table>

Fig. 1. Multiple alignments among the marine bacterial sialyltransferases.

The amino acid sequences from \textit{P. damselae} \(\beta\text{-galactoside} \(\alpha\text{2,6-sialyltransferase} \) (Pd-0160, accession no. BAA25316), \textit{Photobacterium} sp. JT-ISH224 \(\beta\text{-galactoside} \(\alpha\text{2,6-sialyltransferase} \) (ISH224 \(\alpha\text{2,6, accession no. AB293985), \textit{P. leigonathi} JT-SHIZ145 (SHIZ145, accession no. AB306315), \textit{P. phosphoreum} JT-ISH467 \(\alpha\text{-galactoside} \(\alpha\text{2,3-sialyltransferase} \) (ISH467, accession no. BAF63530), \textit{Photobacterium} sp. JT-ISH224 \(\alpha\text{-galactoside} \(\alpha\text{2,3-sialyltransferase} \) (ISH224, accession no. AB293984) and \textit{Vibrio} sp. JT-FAJ16 \(\alpha\text{-galactoside} \(\alpha\text{2,3-sialyltransferase} \) (FAJ16, accession no. AB308042) were aligned using Genetyx ver. 7.0. The conserved amino acids are boxed in black.
sialyltransferase, the \( K_m \) value for the lactose is about 33-fold higher than that of \( N \)-acetyllactosaminide.\(^{29}\) On the other hand, in the case of \( \alpha_2,6 \)-sialyltransferase of \( P. \) damselae \( JT0160 \), lactose has almost the same \( K_m \) value as \( N \)-acetyllactosaminide.\(^{20} \) From these results, it is demonstrated that \( \alpha_2,6 \)-sialyltransferase of \( P. \) damselae \( JT0160 \) does not recognize the 2-acetamido group in the \( N \)-acetylgalactosaminyl residue.

In addition, the structure of \( \alpha_2,6 \)-sialyltransferase seemed to be quite different from those of the mammalian enzymes. Many mammalian sialyltransferases were cloned and comparison of their amino acid sequences has revealed conserved regions, which were named as the sialyl-motifs in the catalytic domains.\(^{20} \) However, the analysis of the \( \alpha_2,6 \)-sialyltransferase from \( P. \) damselae \( JT0160 \) did not detect any regions homologous to the cloned mammalian sialyltransferases.\(^{20} \) The deduced primary sequences of the cloned mammalian sialyltransferases were predicted to carry a short amino-terminal cytoplasmic domain, a signal-membrane anchor domain, a stem region, and a large catalytic domain on the carboxy-terminal side of the mature proteins.\(^{20} \) On the other hand, when the carboxy-terminus of the \( \alpha_2,6 \)-sialyltransferase from \( P. \) damselae \( JT0160 \) was truncated, the protein became soluble and still retained the sialyltransferase activity.\(^{20} \) From these results, it was predicted that the catalytic domain of the enzyme was in the amino-terminal side of the protein, and the \( \alpha \)-helix structures, which might be formed in the carboxy-terminal regions of the enzyme, seemed to be involved in membrane binding. Then, we constructed a number of expression vectors, which contained a series of truncated sequences of the sialyltransferase, and proteins were produced in \( E. \) coli to identify portions of the protein essential for the activity. The shortest, active truncated enzyme, which lacked 107 amino acids residues from the amino-terminus and 176 amino acids residues from carboxy-terminus of the full-length enzyme was found in the soluble fraction of the lysate of \( E. \) coli.\(^{20} \) The properties of the truncated enzyme were examined further, and the kinetic parameters of this protein for donor and acceptor substrate were similar to those of the native enzyme.

\( \alpha_2,6 \)-sialyltransferase produced by \( P. \) leiognathi \( JT-\) SHIZ-145.

Many of sialyltransferases obtained from mammals and bacteria have their optimum pH under acidic conditions, at around pH 6.0. However, the maximal activity of monofunctional \( \alpha_2,6 \)-sialyltransferase produced by \( P. \) leiognathi is achieved at pH 8.\(^{11} \) CMP-Neu5Ac and the sialylglycoconjugates (the sialyltransferase reaction products) are both more stable under basic conditions than under acidic conditions. The high functionality of \( P. \) leiognathi-derived \( \alpha_2,6 \)-sialyltransferase under basic conditions provides a unique advantage of this enzyme for the efficient production of sialosides.

An increase in the concentration of sodium chloride also increases the activity of \( P. \) leiognathi \( \alpha_2,6 \)-sialyltransferase. Approximately 500 mM NaCl in the reaction mixture is required for maximum activity of this enzyme. The conditions in the periplasma are thought to
be similar to those of the environment in which the bacterium grows. The optimal conditions for the α,2,6-sialyltransferase from *P. leioognathi* are very similar to those of average seawater (pH 8.0, 500 mM NaCl). The enzyme of *P. leioognathi* JT-SHIZ-145, a Gram-negative marine bacterium, has a candidate signal peptide in the NH2-terminal region of its deduced sequence. Thus, this enzyme is thought to be translocated across the cytoplasmic membrane to the periplasm like α,2,6-sialyltransferase produced by *P. damselae* JT0160.10

α/β-galactoside α,2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467.

The α/β-galactoside α,2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 has optimal enzyme activity at pH 5.5 and 25°C.10 Acceptor substrate specificity of the α,2,6-sialyltransferase for several mono- and disaccharides was shown in Table 2. The anomer selectivity of this enzyme was quite low. So, this enzyme was able to transfer Neu5Ac to both methyl-α-galactopyranoside and methyl-β-galactopyranoside at almost the same level. Similar specificity has been reported for α,2,3-sialyltransferase from *N. meningitidis* immunotype L1. Among the mono-saccharides and disaccharides, the most preferred acceptor substrate of this sialyltransferase is methyl α-galactoside 2,3-disaccharides and disaccharides, the most preferred acceptor specificity has been reported for 2,6-sialyltransferase from marine bacterial sialyltransferases.

Table 2. Acceptor substrate specificity of α,2,3-sialyltransferase from *P. phosphoreum*.

<table>
<thead>
<tr>
<th>Acceptor substrate</th>
<th>Neu5Ac transferred (nmol/min)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl-α-D-galactopyranoside</td>
<td>10.4</td>
<td>452</td>
</tr>
<tr>
<td>Methyl-β-D-galactopyranoside</td>
<td>5.8</td>
<td>251</td>
</tr>
<tr>
<td>Methyl-α-L-glucopyranoside</td>
<td>1.1</td>
<td>48</td>
</tr>
<tr>
<td>Methyl-β-D-glucopyranoside</td>
<td>0.8</td>
<td>33</td>
</tr>
<tr>
<td>Methyl-α-D-mannopyranoside</td>
<td>1.4</td>
<td>60</td>
</tr>
<tr>
<td>Methyl-β-D-mannopyranoside</td>
<td>1.5</td>
<td>63</td>
</tr>
<tr>
<td>N-Acetylglactosamine</td>
<td>1.0</td>
<td>43</td>
</tr>
<tr>
<td>Gal-β-1,4-Glc (lactose)</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td>Gal-β-1,4-GlcNAc (N-acetyllactosamine)</td>
<td>2.4</td>
<td>103</td>
</tr>
<tr>
<td>Gal-β-1,3-GlcNAc-β-OMe</td>
<td>3.0</td>
<td>129</td>
</tr>
<tr>
<td>Gal-β-1,3-Gal-β-OMe</td>
<td>5.2</td>
<td>316</td>
</tr>
<tr>
<td>Gal-α-1,3-Gal-α-OMe</td>
<td>7.2</td>
<td>438</td>
</tr>
</tbody>
</table>

*a*Lactose is 100% value.

Syntheses of sialylgalactopyranosides using α/β-galactoside α,2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 and β-galactoside α,2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224.

The enzymatic syntheses were done using methyl-β-D-galactopyranoside as an acceptor substrate with α/β-galactoside α,2,3-sialyltransferase from *P. phosphoreum* JT-ISH-467 and β-galactoside α,2,6-sialyltransferase from *Photobacterium* sp. JT-ISH-224, independently. The reaction mixture was composed of acceptor substrate (51.5 μmol, 10 mg), CMP-Neu5Ac (83.5 μmol, 55 mg) and 0.5 U of the each purified recombinant sialyltransferase in 0.5 mL of 100 mM Bis-Tris buffer (pH 6.0) containing 0.03% Triton X-100. Each reaction mixtures were incubated at 30°C for 3 h. The products were purified as follows: after the reaction, each reaction mixture was diluted with 10 mL of distilled water and put on separate column (1.5 × 14 cm) of AG 1-X2 (phosphate form, 200–400 mesh, Bio-Rad). The column was washed with distilled water, and the enzymatic reaction product was eluted with 5 mM of sodium phosphate buffer (pH 6.8). The fractions containing glycosidic Neu5Ac were pooled and evaporated to dry residues. Each dried residue was dissolved in 2 mL of distilled water and then put on column (2.6 × 100 cm) of Sephadex G-15 superfine. The product was eluted with distilled water. The fractions found to contain glycosidic Neu5Ac were pooled and evaporated to dry residues. The purified products were analyzed by 1H-NMR spectroscopy in D2O at 298K. The products turned out to be Neu5Ac β-2,3-Gal-β-OMe and Neu5Acc2,6-Gal-β-OMe, respectively. Neu5Acc2,3-Gal-β-OMe was obtained in a total yield of 54.4% (13.6 mg) based on the acceptor substrate Gal-β-OMe. On the other hands, Neu5Acc2,6-Gal-β-OMe was obtained in a total yield of 66.8% (16.7 mg) based on the acceptor substrate Gal-β-OMe (Fig. 3). The structural reporter group signals of Neu5Acc2,3-Gal-β-OMe were as follows: δ4.32 (d, 1H, Gal H-1), δ3.51 (s, 3H, OMe), δ3.48 (dd, 1H, Gal H-2), δ2.71 (dd, 1H, Neu5Ac H-3 eq), δ1.97 (s, 3H, Ac), δ1.73 (dd, 1H, Neu5Ac H-3 ax). The structural reporter group signals of Neu5Acc2,6-Gal-β-OMe were as follows: δ4.24 (d, 1H, Gal H-1), δ3.51 (s, 3H, OMe), δ3.42 (dd, 1H, Gal H-2), δ2.67 (dd, 1H, Neu5 Ac H-3 eq), δ1.97 (s, 3H, Ac), δ1.63 (dd, 1H, Neu5Ac H-3 ax).

Conclusion.

Sialylated glycoconjugates play a significant role in many biological processes. In particular, the importance of complex-type oligosaccharides for protein function has been demonstrated.22 However, practical scale production of sialylated glycoconjugates or glycans has still some problem. During the course of our study, we isolated many bacteria producing sialyltransferases and clarified...
the fundamental characteristics of these sialyltransferases. These marine bacterial sialyltransferases showed unique donor and acceptor substrate specificity. In general, the acceptor substrate specificity of mammalian sialyltransferases is generally very strict. In contrast, the acceptor specificity of bacterial sialyltransferases is broad, and this property is considered to be useful when various sialylated glycans are to be prepared. Therefore, these properties would make marine bacterial sialyltransferases powerful tools for the industrial production of glycans and the modification of glycoconjugates such as glycoproteins and glycolipids.

REFERENCES

各種海洋性細菌が生産する
シアル酸転移酵素の性質とその可能性

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シアル酸は、複合糖質糖鎖の非還元末端に存在するこ
多が変異であり、シアル酸を含む複合糖質が生体内で
様々な役割を有していることが多くの研究により示され
ている。そのため、シアル酸含有糖鎖の糖鎖修飾は、重
要な研究テーマの一つとなっている。シアル酸転移酵素
を用いるシリアル化は、複合糖質の機能を損なうことな
く、温度な条件下で効率よくシアル酸を付加させるこ
が可能であることから、極めて有効な手法と考えられる。

近年ではタンパク質の糖鎖修飾は、真核生物のみにみ
られる特徴と考えられていたが、細菌においても糖鎖修
飾された糖タンパク質が存在することが明らかにされ、
多くの糖転移酵素が種々の細菌から得られている。我々
は、これまでにシアル酸転移酵素活性を示す細菌を20菌
株以上単離している。これらの細菌を同定した結果、
Photobacterium 属およびVibrio 属にシアル酸転移酵素を
生産する種が多いことが明らかになった。具体的には、α
2,3-シアル酸転移酵素生産菌株として、P. phosphoreum JT-
ISH-467 株、Vibrio sp. JT-FAJ-16 株等がある。α2,6-シア
ル酸転移酵素の生産菌株としては、P. damselae JT-0160
株、P. leiognathi JT-SHIZ-145 株等が挙げられる。また、
種の同定には至っていないが、α2,3-およびα2,6-シアル酸
転移酵素の両酵素を生産する菌株として、Photobacterium
sp. JT-ISH-224 株を単離している。これら海洋性細菌由来
のシアル酸転移酵素の生産レベルはいずれも高く、いず
れの酵素も大量供給が可能である。

※※※※※※※※※※

[質問] 京大・生命科学 山本
1) Sialyltransferaseは特に海洋性細菌において、他の
細菌よりも活性が高いのか。

2) 基質特異性は、ほ乳類の酵素などと同じであるか。
[答]
1) シアル酸転移酵素を生産する他の細菌との酵素の
性質についてはわかりかねますが、これまでに我々の
グループでクローニングしている海洋性細菌由来のシアル
酸転移酵素遺伝子は、大腸内での大量に発現すること
を確認しております。また、それらの比活性も他の細菌
由来の酵素と比較して、高いことを確認しております。

2) 乳動物由来の酵素と比較した場合、海洋性細菌
由来シアル酸転移酵素の糖受容体および糖供与体基質に
対する特異性は、非常に広いことを確認いたしております。

[質問] 京大・生命科学 萊田
1) 細菌のどの部に酵素が局在しているのか、可溶性
タンパク質か。
2) 細菌表面のシアル酸が付加した産物は何か、コロ
ミン酸のようなホモポリマーの可能性はあるか。
[答]
1) 直接の証拠は得ておりません。しかしながら、海
洋性細菌から精製した酵素は、シグナル配列と考えられ
るアミノ酸配列を欠損しておりましたことから、細胞質
で合成された後に、ベリプラズマ空間に移行しているの
ではないかと考えています。

2) 細菌の表面に存在するシアル酸を含む化合物につ
いては、現在検討を行っているところです。結果が得ら
れ次第、別途報告でできればと考えています。

[質問] 農研機構・食衛研 徳安
糖転移酵素による反応の収率としては、66%程度とい
うのは低いのではないのか。
[答]
今回お示しした結果は、酵素反応生成物の収率より精
製度を優先するために、ご指摘のとおり収率が低くなっ
たと考えています。