

Contribution

Synthesis of Bioactive Glycoconjugates Using the Transglycosylation Activity of Microbial Endoglycosidase

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1. Introduction

Glycoconjugates such as glycoproteins and glycolipids have structures in which proteins or lipids are conjugated with oligosaccharide moieties, and are essential components of biological organisms, including various organs of animals, tissues of plants, and cell walls and membranes of microorganisms. However, the exact roles of these glycoconjugates were not clearly understood until recently. This is mainly because it has been considered that these oligosaccharide moieties might play a role only in maintaining the structure of protein or lipid. However, since it was found that the antigen specificity of human ABO type blood is expressed in the oligosaccharide moiety of glycoconjugate located on the membrane of the erythrocyte, it has gradually been recognized that the oligosaccharide moieties indeed play important roles in various biological phenomena such as cellular recognition, embryogenesis and differentiation of biological organisms and oncogenesis. Significant progress has been particularly made in this field in the last decade, resulting in the development of a new terminology for oligosaccharide moieties (sugar chain) as “the third chain” corresponding to nucleic acids and proteins as the “first” and “second” chains, respectively. Thus, research in this field to understand roles and functions of glycoconjugates has recently been termed “glycobiology” or “glycotechnology” being ranked with “gene engineering” or “protein engineering”. In addition, glycotechnology has now been recognized as a very important target area in the post-genomic research.

One of the essential requirements in “glycotechnology” is to develop a system by which a specific, desired function can be added to a certain substance, by addition of an oligosaccharide chain or modification of the attached oligosaccharide chain. However, despite the fact that in current genetic engineering and protein engineering, genes and proteins can be easily “cut and pasted” or “replaced” with the desired mutated ones, the technical skills in

glycotechnology, whose goals are aimed at addition or modification of sugar chains, have not yet reached the levels of those of “gene engineering” or “protein engineering”. Recently, attention has been paid to various glycosyltransferase enzymes as tools to artificially add oligosaccharide moieties onto the protein or lipid, and genetic cloning of these enzymes has been actively conducted. However, these enzymes can add only one saccharide residue onto the receptor in the presence of sugar nucleotide as the saccharide donor; this system appears not to be suitable for the synthesis of complex polysaccharides such as sugar chain.

By using the transglycosylation activity of microbial endoglycosidase, we recently succeeded in adding oligosaccharide moieties onto other compounds, which had been thought impossible by conventional methods. In this report, we introduce microbial endoglycosidases as a tool for making “impossible” addition of sugar chain a reality and describe the synthesis of bioactive glycoconjugates using the transglycosylation activity of microbial endoglycosidases.

2. Endo- β -N-acetylglucosaminidase

Unlike the exoglycosidases that hydrolyze oligosaccharides and sugar chain in glycoconjugates at the non-reducing end of saccharides moiety and release monosaccharides, endoglycosidases exert their actions by recognizing an internal structures of substrates and release oligosaccharides.

Because of this property that endoglycosidases can release oligosaccharide without causing damage to either the oligosaccharide or protein/lipid moiety, endoglycosidases have been shown to be a useful tool for structural or functional analyses of various sugar chains (Fig. 1).

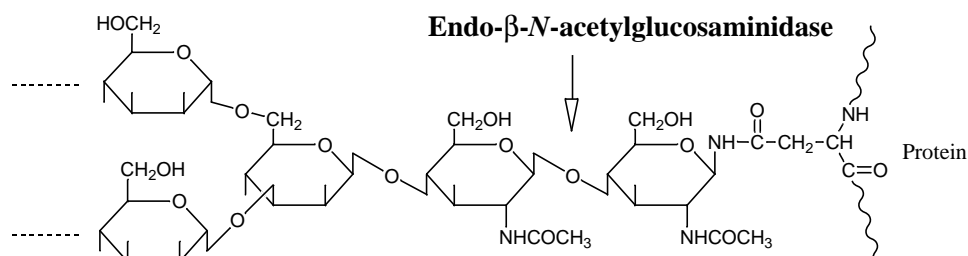


Fig. 1 Enzymatic action of Endo-β-N-acetylglucosaminidase.

Endoglycosidase was found by Muramatsu in 1971, who demonstrated that there existed an enzyme that removed the oligosaccharide moiety from murine immunoglobulin in a cultured medium of *Diplococcus pneumoniae*.¹⁾ This enzyme named endo-β-N-acetylglucosaminidase (Endo-β-GlcNAc-ase) acts on the N-glycoside linked oligosaccharide moiety, which binds to an asparagine residue of the protein, and cleaves the diacetylchitobiose linkage (N-acetylglucosaminyl β-1,4 N-acetylglucosaminide; GlcNAc-GlcNAc) between the protein and oligosaccharide moiety, thus releasing oligosaccharides. Unlike the peptide N-glycanase that releases oligosaccharide moieties from protein at a site linked to the asparagine residue, endoglycosidase is unique in its action that leaves one N-acetylglucosamine residue bound to the protein (Fig 1). Although endoglycosidase has been widely expressed not only in microorganisms but also in animal and plant tissues, the most well studied endoglycosidase is the one derived from a microbial origin.

Asparagine-linked oligosaccharide moieties are generally divided into 3 groups; high-mannose type, hybrid type and complex type. Microbial Endo-β-GlcNAc-ase acts particularly on oligosaccharide moieties of the first two types, the high-mannose type that is composed of mannose oligomers, and hybrid type that has a combined structure of oligosaccharide moieties of high-mannose and complex types. Endoglycosidase H (Endo-H) derived from *Streptomyces plicatus*²⁾ is the endoglycosidase that has been most commonly used for the analyses of sugar chains of glycoproteins in glycotecnology or cellular biology. While this Endo-H responds very well to the high-mannose type oligosaccharide moieties, it has no effect at all on oligosaccharide moieties of the complex type, which are widely expressed in almost all animal glycoproteins. Different to the high-mannose type oligosaccharide moieties, oligosaccharide moieties of the complex type consist of various saccharides, including sialic acid (NeuAc), Galactose (Gal), N-acetylglucosamine (GlcNAc) and mannose (Man).

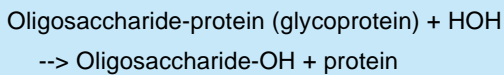
In 1982, Elder and Alexander identified a new type of Endo-β-GlcNAc-ase (Endo-F) that acted on the complex type oligosaccharide moieties in culture media of *Flavobacterium meningosepticum*.³⁾ Endo-F is comprised of 3 isozymes with different substrate specificities. Endo-F1, a primary component of Endo-F, is an isozyme that acts on only the high-mannose type oligosaccharide moieties,⁴⁾ while the other two, Endo-F2 and Endo-F3, act on the high-mannose and complex types oligosaccharide moieties.⁵⁾ Similarly, we also identified another Endo-β-GlcNAc-ase that acted on the complex type oligosaccharide moieties in culture media of *Mucor hiemalis* isolated from soil, and termed this Endo-β-GlcNAc-ase as Endo-M after its source.⁶⁾ Endo-M has a wide spectrum of substrate specificity and acts on all types of oligosaccharide moieties with high-mannose, hybrid and complex types. Moreover, Endo-M has a unique specificity different from the conventional microbial endoglycosidases, that it has an action on sialo-complex type oligosaccharide moieties with sialic acid at their non-reducing ends.^{7,8)}

3. Transglycosylation Activity of Endoglycosidase

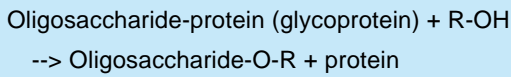
Many glycosidases possess hydrolytic activity that hydrolyzes glycosidic linkages as well as transglycosylation activity that transfers the released saccharide moieties onto compounds containing hydroxyl groups. The transglycosylation reaction has been considered to be a specific reaction in the hydrolysis of saccharides. In other words, hydrolysis of glycosidase is a reaction in which a saccharide moiety released from the substrate is transferred to water. On the other hand, transglycosylation activity is a reaction in which the released saccharide is transferred to a compound with a hydroxyl group instead of to water.⁹⁾ Transglycosylation activity of exoglycosidase has been widely utilized for enzymatic synthesis of various

oligosaccharides, whereas little is known regarding transglycosylation activity of endoglycosidase. However, if endoglycosidase also possesses transglycosylation activity, it can be considered that endoglycosidase can release saccharide moieties from glycoproteins or glycolipids and transfer them onto compounds with hydroxyl groups. Thus, it is quite possible to add saccharide moieties to various compounds, and this, in turn, means that we can take advantage of transglycosylation activity of endoglycosidase as a means of glycosylation.

Hydrolysis of Endoglycosidase:



Transglycosylation reaction of Endoglycosidase:



Transglycosylation activity of endoglycosidase was first found in Endo-F by Trimble *et al* in 1986.¹⁰⁾ They found that, when Endo-F acted on the high-mannose type oligosaccharide linked to an asparagine residue (GP-V), which was obtained from hen egg white and had a structure of Man₅GlcNAc₂Asn, it released the oligosaccharide, and the released oligosaccharide was further transferred to the glycerol that was added in the Endo-F enzyme preparation as a stabilizer. Later, Takegawa *et al*

also demonstrated that Endo-β-GlcNAc-ase of *Arthrobacter protophormiae* (Endo-A) had transglycosylation activity.¹¹⁾ Endo-A is an endoglycosidase that acts only on the high-mannose type oligosaccharide chain. They found that, when glycopeptide was hydrolyzed by Endo-A in the presence of GlcNAc or glucose, the released oligosaccharide moiety after hydrolysis was further transferred and added onto the monosaccharide, resulting in increased hydrolysis. Thus, it was found that this Endo-A also had transglycosylation activity.

We also found that Endo-M has transglycosylation activity.¹²⁾ When Endo-M acted on glycopeptide from human serum transferrin that possessed complex type bi-antennary oligosaccharide, saccharide moieties were released. In addition, the released saccharide moieties were further transferred and added to the GlcNAc residue of the acceptor, in the presence of GlcNAc or appropriate acceptor including GlcNAc residue (Fig. 2).

4. Chemo-Enzymatic Synthesis of Bioactive Glycopeptide

Endo-M can transfer the oligosaccharide moiety from glycopeptide to not only GlcNAc but also 4-L-asparatylglycosylamine, which has the structure of GlcNAc linked to the asparagine residue (GlcNAc-Asn), and its derivatives. That is, when GlcNAc is bound to the asparagine residue of a peptide or protein, the oligosaccharide moiety will be transferred and added to the peptide or protein by Endo-M, resulting in the synthesis of glycopeptide or glycoprotein. The addition of oligosaccharide moiety can prevent degradation of the protein by degradative enzymes, stabilize it and induce additional functions on the protein.

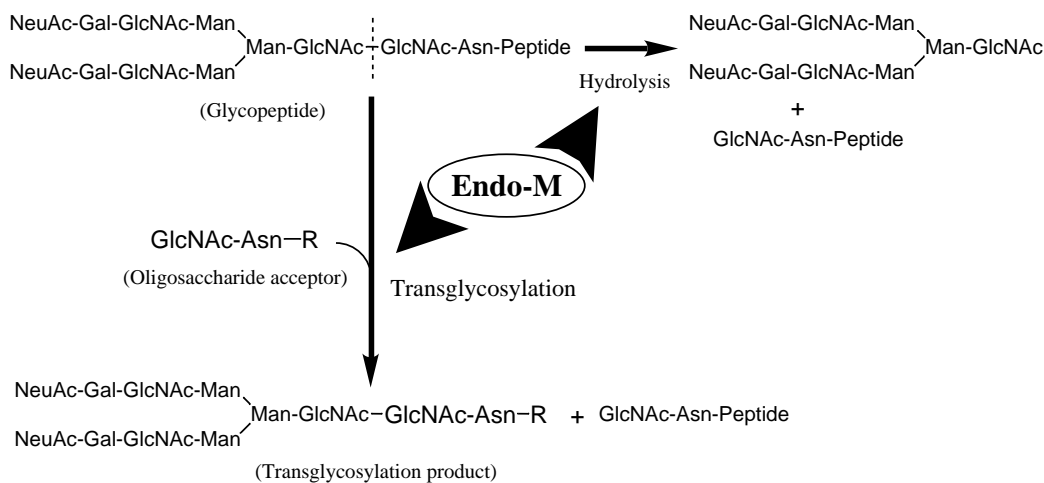
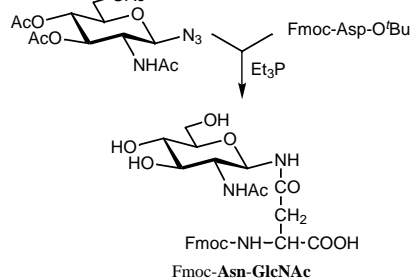
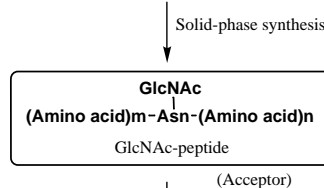


Fig. 2 Enzymatic action of Endo-β-N-acetylglucosaminidase from *Mucor hiemalis* (Endo-M).

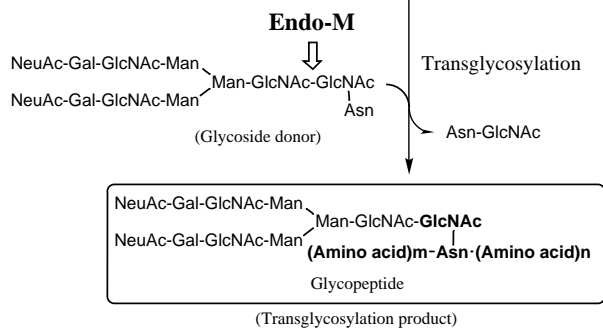
1. Glycosylasparagine synthesis



2. *N*-Acetylglucosaminyl peptide synthesis



3. Transglycosylation reaction of Endo-M



Fmoc-Asp-O^tBu: Fmoc-aspartic acid α -*t*-butyl ester, Et₃P: triethylphosphine

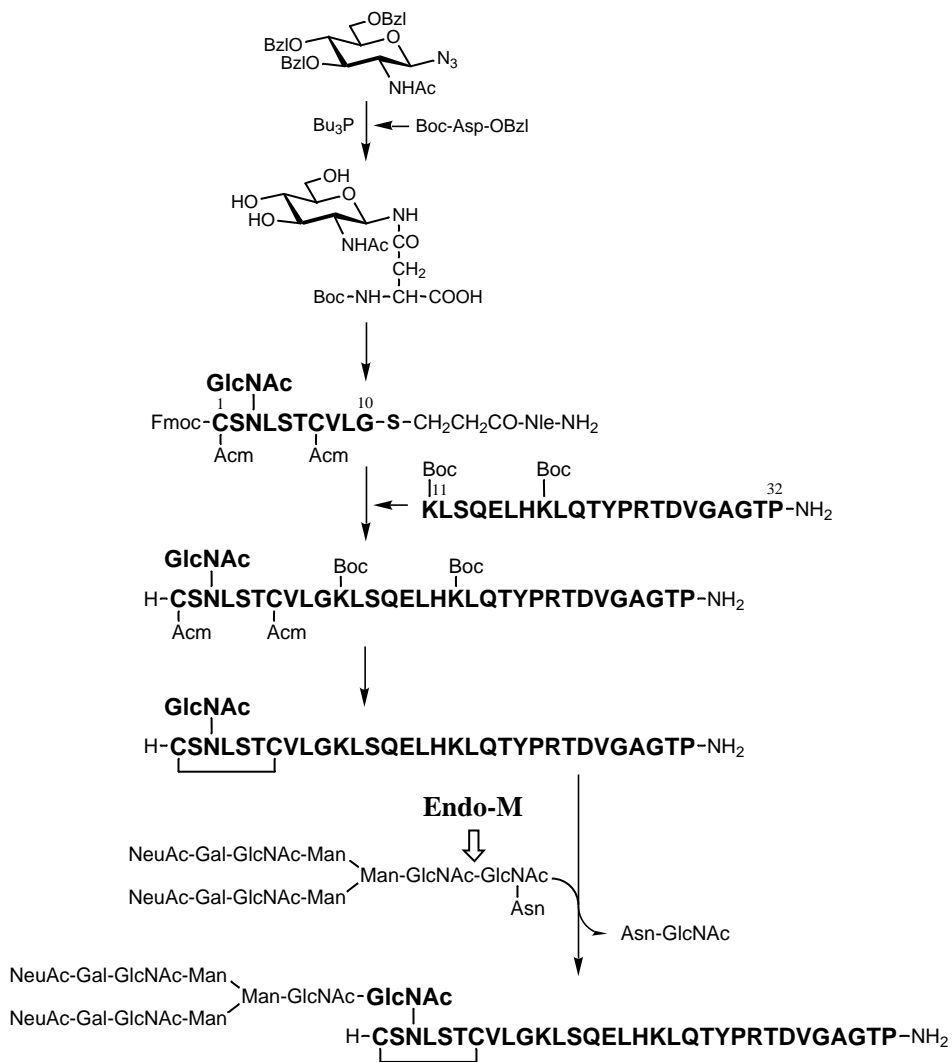
Fig. 3. Strategy of chemo-enzymatic synthesis of glycopeptide.

Accordingly, we attempted to add an oligosaccharide moiety to a bioactive peptide using Endo-M. The chemo-enzymatic method is shown in Fig 3.¹³ The first step of the strategy is a chemical synthesis of glycosylasparagine, a material for the synthesis of *N*-acetylglucosaminylpeptide, which is a peptide attached to GlcNAc at the asparagine residue. That is, Fmoc (9-fluorenylmethyloxycarbonyl) - Asn-GlcNAc is synthesized using an azide form of GlcNAc and Fmoc-aspartate butyl ester as starting materials.¹⁴ In the second step, solid phase synthesis of peptide is carried out by the Fmoc method using the Fmoc-Asn-GlcNAc as a material that can be substituted for Fmoc-Asn, and *N*-acetylglucosaminylpeptide will be synthesized. In the third step, glycopeptide is synthesized by transferring the oligosaccharide moiety from its donor to *N*-acetylglucosaminylpeptide by the transglycosylation activity of Endo-M. We have successfully synthesized many bioactive glycopeptides by this chemo-enzymatic method.

Peptide-T is a peptide composed of 8 amino acids (ASTTTNYT), which is considered to be an agent for the treatment of AIDS since it has been shown to inhibit binding of HIV to cell receptors.¹⁵ In order to stabilize peptide-T and prevent its degradation by degradative

enzymes, we attempted to add an oligosaccharide to peptide-T by the transglycosylation activity of Endo-M according to the strategy described above. First, we synthesized *N*-acetylglucosaminyl peptide-T, which was made by adding GlcNAc to the asparagine residue of peptide-T. Then, using human serum transferrin glycopeptide with sialo complex type oligosaccharide moiety as a donor and *N*-acetylglucosaminyl peptide-T as a receptor of the oligosaccharide moiety, respectively, peptide-T bound to the sialo complex oligosaccharide moiety was enzymatically synthesized, and isolated by HPLC.¹⁶

In order to prevent degradation by degradative enzymes such as peptidases, peptide-T was conventionally modified by linking it to glucose or lactose at the serine or threonine residue of peptide-T.¹⁷ When the peptide-T linked to the sialo-complex type of oligosaccharide moiety was examined for its stability by assessing its protease-induced degradation, it was demonstrated that the sialo-complex type glycosylated peptide T was significantly more stable than native peptide-T and *N*-acetylglucosaminyl peptide-T.¹⁶



Bzl: benzyl, Boc-Asp-OBzl: Boc(*tert*-butyloxycarbonyl)-aspartic acid benzyl ester, Bu₃P: tri-*n*-butylphosphine, Acm: acetamidomethyl

Fig. 4. Procedure for synthesizing calcitonin glycopeptide.

Calcitonin is a bioactive peptide, which plays a role in inhibition of calcium release from bone as a calcium regulating hormone, and has been shown to be effective for the treatment of osteoporosis. Consisting of 32 amino acids, calcitonin has an asparagine residue at position 3 from the N-terminus. We introduced a GlcNAc at this asparagine residue to synthesize a new peptide, to which a sialo-complex oligosaccharide moiety was then added by Endo-M transglycosylation activity, and obtained a glycopeptide of calcitonin.¹⁸⁾ A summary of the procedure for the chemo-enzymatic synthesis is shown in Fig.4. First, the following two compounds were separately synthesized by solid phase synthesis: the first compound was Fmoc derivative of an N-terminal 1-10 fragment, including the asparagine residue at position 3, that is

Fmoc peptide (CSNLSTCVLG) attached by a thioester segment, and the second one was Boc derivative of an amide form of the remaining C-terminal 11-32 fragment in calcitonin (KLSQELHKLQTYPRTDVGAGTP). These two compounds were applied to thioester condensation to yield *N*-acetylglucosaminyl calcitonin. Using Endo-M transglycosylation activity, the sialo-complex type oligosaccharide moiety of human serum transferrin glycopeptide was added to the obtained *N*-acetylglucosaminyl calcitonin. When in vitro bioactivity of the synthesized glycopeptide calcitonin was assessed by inhibition of formation of actin ring in osteoclasts, the bioactivity of the glycopeptide was well remained although it was slightly reduced compared to that of native calcitonin.¹⁹⁾

5. Chemo-enzymatic Synthesis of Bioactive Glycopeptide with Glutamine-linked Oligosaccharide

It is well known that in nature an *N*-glycoside-linked oligosaccharide of a glycoprotein or glycopeptide is bound to the asparagine residue of a consensus tri-peptide sequence, -Asn-X-Thr/Ser-. The chemo-enzymatic synthetic method using transglycosylation activity of Endo-M, however, enables us to attach oligosaccharides to any peptides with any sequences, so long as the peptide has an asparagine residue. This property has been considered to be the best advantage for this chemo-enzymatic synthetic method. However, there exist peptides without the asparagine residues.

Substance P is a bioactive neurotransmitter peptide that causes certain physiological actions such as reducing blood pressure. This peptide consists of 11 amino acids (RPKPQQFFGLM) but has no asparagine residue in its sequence. In nature, this peptide will never be able to get bound to a *N*-glycoside linked oligosaccharide, and this means that it is impossible to biosynthesize a glycopeptide of substance P even by genetic engineering. On the other hand, Endo-M can also add oligosaccharide to GlcNAc-Gln as well as GlcNAc-Asn. Accordingly, we attempted to create a new, non-naturally occurring, glycopeptide of substance P with a glutamine-linked oligosaccharide, based

on the fact that substance P has a couple of glutamine residues as its components. Thus, we first synthesized a peptide linked to GlcNAc at the glutamine residue, to which we added a sialo-complex type bi-antennary oligosaccharide of the glycopeptide obtained from hen egg yolk, using transglycosylation activity of Endo-M. That is, we successfully chemo-enzymatically synthesized two different glycopeptides of substance P with oligosaccharides at the two-glutamine residues of position 5 and 6 from N-terminus.²⁰ (Fig 5) We then examined the bioactivity of these glycopeptides as assessed by measurement of the contractile response of longitudinal muscles of guinea pig ileum, and found that the glycosylated substance P at Gln-5 possessed a bioactivity almost equivalent to that of native substance P, while the bioactivity of the glycosylated substance P at Gln-6 was markedly reduced. Since it has been shown that the region of substance P essential for its bioactivity is located in the C-terminus, the data suggested that introduction of the oligosaccharide at the glutamine residue of position 6, closer to the C-terminus, might have caused structural hindrance, which in turn might have reduced its bioactivity. Moreover, substance P is generally degraded by angiotensin-converting enzyme (ACE), one of the dipeptidylcarboxypeptidases. However, neither glycosylated form of substance P was degraded by ACE at all.²⁰

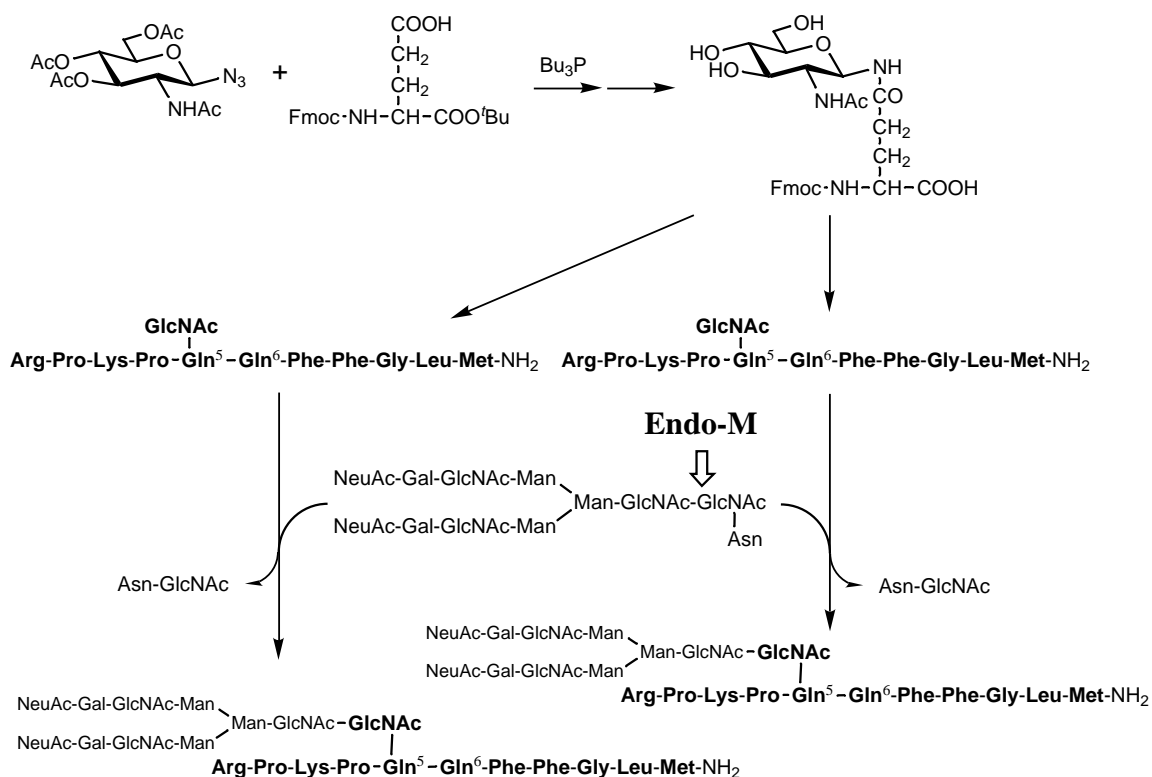


Fig. 5. Procedure for synthesizing glycosylated Substance P containing glutamine-linked oligosaccharide.

We also synthesized α -mating factor (WHWLQLKPGQPMY) of yeast that was glycosylated at the glutamine residues. This peptide is a hormone-like substance secreted by yeast (*Saccharomyces cerevisiae*). This α -mating factor is degraded by yeast-secreted proteases and inactivated. Accordingly, we synthesized two glycosylated α -mating factors linked to the sialo complex type bi-antennary oligosaccharide by Endo-M transglycosylation activity, and examined their bioactivity as assessed by a growth arrest assay using a protease-deficiency mutant strain of the yeast α -mating type. It was found that the glycosylated α -mating factor bound to the sialo oligosaccharide at the glutamine residue of position 5 from the N-terminus markedly reduced its bioactivity compared to that of the native one, while the glycosylated α -mating factor bound to the asialo oligosaccharide maintained its bioactivity, similar to that of native α -mating factor. The latter has a structure in which sialic acid located at the terminus of the oligosaccharide was removed by sialidase. Thus, it was suggested that sialic acid might inhibit binding between α -mating factor and yeast. On the other hand, it was demonstrated that *N*-acetylglucosaminyl α -mating factor, which was linked to GlcNAc at the glutamine residue, rather increased its bioactivity.²¹⁾

6. Synthesis of Infection Inhibitor against Influenza Viruses

It is well known that human influenza is an infectious disease mediated via viruses, which repeatedly infects many people every year. It is also well known that the viruses that cause influenza can recognize the sialic acid located at the termini of oligosaccharides of glycoprotein or glycolipid in the plasma membrane of host cells, and infect them. Amongst them, A type virus recognizes the α -2,6-linked sialic acid and binds to its receptor, hemagglutinin (erythrocyte coagulation factor). We focused on the fact that a complex type bi-antennary oligosaccharide of the glycopeptide extracted from hen egg yolk has α -2,6-linked sialic acid at the non-reducing end, and investigated the possibility that we could suppress viral infection on host cells by binding the oligosaccharides of the glycopeptide to viruses. In addition, since it has been shown that saccharide polymers generally exhibit a better effect per mole unit than corresponding monomers, the so-called "multivalent effect",²²⁾ it was postulated that sialosaccharide polymers might be effective compounds as inhibitors of viral infection. Accordingly, we chose chitosan as a base oligosaccharide polymer, and first, synthesized

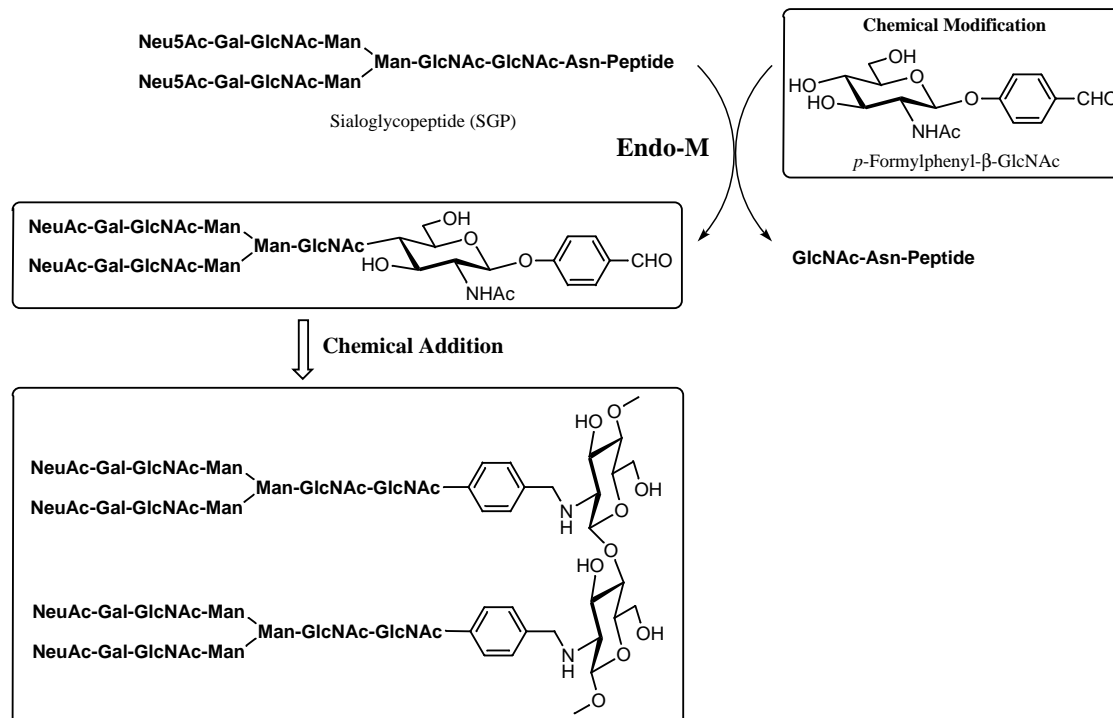


Fig. 6. Chemo-enzymatic synthesis of multivalent sialo-oligosaccharides conjugated with chitosan.

p-Formylphenyl β -GlcNAc. We then transferred sialo-oligosaccharide moieties of the hen egg yolk glycopeptide to this *p*-Formylphenyl β -GlcNAc as a receptor of the oligosaccharide moiety by the transglycosylation activity of Endo-M. Next, this oligosaccharide conjugates were added to chitosan as a multipolymer by reduced amination, resulting in the synthesis of multivalent sialo-oligosaccharide conjugated polymer with chitosan (Fig 6). When the binding inhibition activity of influenza virus of this synthetic high mass molecule compound was examined, the multivalent sialo-oligosaccharide conjugated polymer with chitosan was 300-times more potent in its inhibitory effects than Fetuin, a glycoprotein that possesses sialo-oligosaccharide moieties with a similar structure. Since both chitosan and hen egg yolk-derived glycopeptide are harmless to the human body, and materials for their synthesis are abundant in our resources, this multivalent sialo-oligosaccharide conjugated polymer with chitosan has been thought to be very promising as an agent for the treatment of influenza virus infection with industrial mass production in the future.

7. Preparation of Monoclonal Antibody against the Oligosaccharides of Glycoprotein

Since monoclonal antibodies against oligosaccharide chains of glycolipids can be easily prepared using the glycolipids itself as an antigen, a relatively large number of antibodies have been developed. However, it is often difficult to develop monoclonal antibodies against oligosaccharides of glycoprotein because of potent antigenicity of their protein molecules. It is well known that oligosaccharides on the surface membranes of cancer cells can be oncogenetically mutated. Therefore, many monoclonal antibodies against oncogenetically mutated oligosaccharides of glycolipid have been developed and used for diagnosis of cancer. Furthermore, it has been shown that oligosaccharide moieties of glycoproteins can also be oncogenetically mutated. For instance, it has been reported that oligosaccharides of α -fetoprotein, a glycoprotein detected in the serum of patients with hepatoma, were oncogenetically mutated.²³⁾ Therefore, it has gradually been recognized that oligosaccharides of glycoproteins may play an essential role as a cancer marker, and it is urgently required to efficiently develop antibodies against oligosaccharide moieties of glycoproteins. Accordingly, we developed a chimeric glycolipid by using transglycosylation activity of Endo-M, and added an oligosaccharide moiety of glycoprotein to a synthetic lipid with weak antigenicity. Thus, we have developed an efficient method to prepare monoclonal antibodies against oligosaccharide moieties of glycoproteins. That is, complex type bi-antennary oligosaccharides from hen egg yolk-derived glycopeptide were used as a donor of oligosaccharides, and using transglycosylation activity of Endo-M, the complex type bi-antennary oligosaccharides were transferred and added to *p*-Formylphenyl β -GlcNAc. Next, L- α -phosphatidyl ethanolamine dimyristoyl residues were added to this *p*-Formylphenyl β -GlcNAc with the complex type bi-antennary oligosaccharides by reductive amination

reaction. Then, a monoclonal antibody was successfully developed by immunizing the obtained product as an antigen in BALB/c mice. The developed monoclonal antibody can specifically recognize the complex type bi-antennary oligosaccharide that contains α -2,6-linked sialic acid at the non-reducing end of the oligosaccharide of the glycopeptide. Therefore, this monoclonal antibody did not respond to asialo-oligosaccharides or high-mannose type oligosaccharides at all. Moreover, it had no response against saccharide moieties of glycolipids.

8. Summary

Endoglycosidases have been widely used as tools for structural or functional analyses of oligosaccharides. However, it has never been attempted to use these enzymes as tools for the synthesis of new compounds by using the specific activity of these endoglycosidases. As reported here, various applications of endoglycosidases can be explored, and will be possibly developed for mass production of synthetic compounds in the near future. Endo-M is almost the only enzyme that can add any oligosaccharide moieties to proteins or peptides, and is currently commercially available from Tokyo Chemical Industry. Thus, it is highly expected that the use of Endo-M will significantly expand in the future. Moreover, Endo-M has been considered to be a tool for "remodeling" of naturally occurring saccharides into saccharides specifically modified for our own purposes. For instances, human-derived glycoprotein produced by recombinant yeast has high level of high-mannose type oligosaccharide moieties instead of its native complex type oligosaccharide moieties. This oligosaccharide moiety of the recombinant glycoprotein can be modified to its original type of oligosaccharide moiety, a human compatible complex type oligosaccharide moiety.²⁴⁾ Thus, massive supply of the enzyme is essential as the use of endoglycosidase expands. It is highly expected that exploration of better endoglycosidases that possess more defined functions and to identify the genes of corresponding endoglycosidases by cloning will occur.

Acknowledgement

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 1976-1991 Research Associate, Kyoto University, Faculty of Agriculture, Department of Food Science and Technology.
 1980 Jun -1981 Sep Visiting Scientist, Tulane University, New Orleans, LA, USA.
 1991-1997 Associate Professor, Kyoto University, Faculty of Agriculture, Department of Food Science and Technology.
 1997-1999 Associate Professor, Kyoto University, Graduate School of Agriculture, Division of Applied Life Sciences.
 1999-present Professor, Kyoto University, Graduate School of Biostudies, Division of Integrated Life Science.

Awards:

1987 Outstanding Scientist Award in the field of Agricultural Chemistry
 2004 Arima Kei Memorial Award, Bio-Industry Association

Expertise and Speciality:

Applied Microbiology, Glycobiology, Applied Enzymology

(Received September, 2006)

TCI's Related Compound

Endo-M (= *endo*- β -*N*-Acetylglucosaminidase)

100 munits* [A1651]

Recombinant: from *Mucor hiemalis* expressed in *Candida boidinii* [Purity: single band by SDS-PAGE (85KDa)]

* One unit will catalyze the release of 1 μ mol of Fmoc-Asn(GlcNAc) from Fmoc-Asn[(NeuAc-Gal-GlcNAc-Man)₂-Man-GlcNAc₂]-OH per min. at pH 6.0 at 37 °C.

This Endo-M was merchandised as the fruition of NEDO project under licenses from patent-holding companies of Takara Bio Inc. and Kirin Brewery Co., LTD.