CHEMICAL REVIEWS

REVIEW

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Precision Polysaccharide Synthesis Catalyzed by Enzymes

Jun-ichi Kadokawa*

Graduate School of Science and Engineering, Kagoshima University, 1-21-40 Korimoto, Kagoshima 890-0065, Japan

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1. INTRODUCTION

Polysaccharides are one of three major classes of natural polymers found in the plant, animal, and microbial kingdom, which are vital materials for important in vivo functions, e.g., providing an energy source, acting as a structural material, and conferring

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specific biological properties.[1,2] They are structurally composed of monosaccharide residues linked through glycosidic linkages (Figure 1), which have very complicated structures owing to not only a variety of the monosaccharide structures but also the differences in stereo- and regio-types of the glycosidic linkages.[2] In contrast, the other two major natural polymers, i.e., nucleic acids and proteins, have relatively simple structures because these natural polymers are constructed by a type of specific linkage between 20 kinds of amino acids and several kinds of nucleotides, respectively (Figure 1).[3] A great variety of the polysaccharide structures contributes to serve a whole range of their functions in the host organism, and a subtle change in the structure of the monosaccharide unit or a type of glycosidic linkage has a profound effect on the properties and functions of the polysaccharides.[4−6]

Chemical synthesis of the polysaccharide was first performed by an attempt of cellulose synthesis in 1941,[7] and then attempts have been made to produce cellulose by traditional polymerization of glucose derivatives. For example, hydroxy groups of 2-, 3-, and 6-positions of glucose were protected and polycondensation of this glucose monomer with phosphorus pentoxide was performed to give the glucose polymer.[8] This product was claimed to be composed of β-(1→4)-glycosidic linkages; however, definite evidence of the structure was not sufficiently obtained. Then, although many efforts on polymerization had been devoted to chemical synthesis of polysaccharides with well-defined structures in stereo- and regioselectivities, only a few approaches successfully led to production of such polysaccharides. One approach is ring-opening polymerization of anhydrosugar monomers catalyzed by Lewis acid.[9] After many attempts for this polymerization using various anhydrosugar monomers,[10,11] cellulose with degree of polymerization (DP) of 19.3 was synthesized by the cationic ring-opening polymerization of 3,6-di-*O*-benzyl-α-D-glucose 1,2,4-orthopivalate and subsequent removal of the protective groups.[12] On the other hand, acid-catalyzed ring-opening polyaddition of sugar oxazoline monomers derived from *N*-acetyl-D-glucosamine (GlcNAc) derivatives gave aminopolysaccharides with relatively controlled structures.[13−19] For example, a 3,6-*O*-benzylated sugar oxazoline was polymerized with acid catalyst via ring-opening addition to give a dibenzyl chitin with molecular weights up to 4 900 (DP = ca. 13).[13] Then, removal of benzyl groups was carried out to give chitin; however, complete deprotection did not take place.[14] Because sugar monomers with protective groups have to be employed for the chemical synthesis of polysaccharides, subsequent deprotection procedure is necessarily conducted to give free polysaccharides. Furthermore, perfect stereocontrol of glycosidic linkages has not often been achieved.

On the basis of the above backgrounds and viewpoints, almost in the past two decades, an enzymatic approach as a superior method to the traditional chemical one has been employed for the polysaccharide synthesis with highly stereo- and regioselectivities,[20−35] which has attracted much attention and is still developing to provide a variety of new polysaccharides. This review focuses on precision polysaccharide synthesis catalyzed enzymes and describes in vitro synthetic approaches by isolated enzymes. Therefore, synthesis of polysaccharides from uridine diphosphate (UDP)−sugar substrates catalyzed by synthetic enzymes is not included.[36,37]

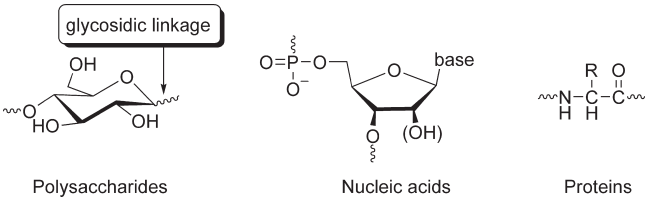
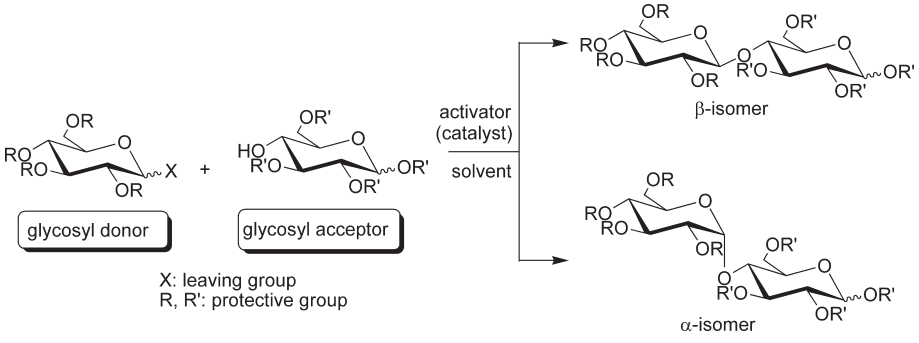


Figure 1. Typical unit structures of polysaccharide, nucleic acid, and protein.

2. CONCEPT IN POLYSACCHARIDE SYNTHESIS

Polysaccharides are produced by the repeated glycosylations of a glycosyl donor with a glycosyl acceptor to form a glycosidic linkage.[38−41] Scheme 1 shows a typical reaction of glycosylation for the possible formation of two isomers of a glucose dimer. For design of the substrates, an anomeric carbon (C1) of the glycosyl donor is activated by introducing a leaving group (X), and a hydroxy group in the glycosyl acceptor, which participates in the reaction, is employed as a free form, whereas other hydroxy groups in both the glycosyl donor and acceptor are protected. There are two important selectivities that should be controlled to form the desired glycosidic linkages. Because two possible geometric isomers related to the geometry of the anomeric carbon of a monosaccharide, namely, α-isomer and β-isomer, are conceived, the control of formation in such two glycoside linkages, i.e., stereoselectivity, is one of the two important selectivities in the glycosylation. The other prerequisite selectivity is regioselectivity. Monosaccharides have multiple hydroxy groups that participate in the formation of glycosidic linkages. Because the saccharide chains can be formed by connecting a hydroxy group at the anomeric position in a monosaccharide unit and one of other hydroxy groups of the adjacent monosaccharide unit, there are many possibilities for the regioselective formation of the glycosidic linkages. For example, glucose has four hydroxy groups (excluding an anomeric hydroxy group) that can participate in the glycosylation, i.e., 2-, 3-, 4-, and 6-hydroxy groups. In the glycosylation using

Scheme 1. Typical Reaction Manner of Glycosylation



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the two glucose substrates, therefore, α- and β-isomers arise with respect to stereoselectivity of the anomeric carbon, and four isomers are conceivable with respect to regioselectivity owing to the four hydroxy groups in the acceptor. Thus, the reaction involves a possibility for the production of eight isomers of the glucose dimer (Figure 2). The large numbers of isomers for the oligosaccharides composed of glucose residues are theoretically calculated.[39] Among the multiple fashions of the glycosidic linkages, only one kind of linkage must be formed to construct polysaccharides with well-defined structures on the basis of the two important selectivities in the glycosylation, i.e., stereo- and regioselectivities.

For stereo- and regioselective construction of the glycosidic linkage, an appropriate leaving group, protective groups, an activator (a catalyst), and a solvent should be selected. Over the past century, the reaction control in the glycosylation has been one of the main research areas in the carbohydrate chemistry. For example, the classical Koenigs−Knorr reaction[42] utilizes a combination of peracetylated glycosyl halides as a glycosyl donor and heavy metal salts such as silver oxide and mercury(II) cyanide as an activator in organic solvents.[43,44] Although many chemical glycosylations using various glycosyl donors, representatively such as glycosyl acylates,[45] orthoesters,[46] imidates,[47,48] fluorides,[49] and thioglycosides,[50] have been developed, the perfection of the glycosylating process still remains as a challenging problem.[41] As appeared in two representative natural polysaccharides, i.e., cellulose and starch, the importance of fashions of the glycosidic linkages in polysaccharides is significant for their functions.[2] These two natural polysaccharides are composed of the same structural unit, i.e., the glucose unit, but linked through the different β-(1→4)- and α-(1→4)-glycosidic linkages, respectively; starch contains amylose with a linear structure and amylopectin having a branched structure with α-(1→6)-linked branching points (Figure 3). Owing to the difference in such stereochemistry of glycosidic linkages of cellulose and starch, their roles in nature are completely different; the former is the structural material, and the latter acts as the energy source.[51−53] In the synthesis of polysaccharides, therefore, perfect control of stereo- and regiochemistries in the glycosidic linkages is strictly demanded. Furthermore, all the aforementioned chemical glycosylation methods require the protection−deprotection procedures of the hydroxy groups. During the multiple reaction steps for the synthesis of the saccharide chains via the chemical glycosylations, therefore, undesired side-reactions often take place.

To develop a superior method for the synthesis of polysaccharides, the in vitro synthesis by enzymatic catalysis has been significantly investigated.[20−35] Enzymes have several remarkable catalytic properties compared with other types of catalysts in

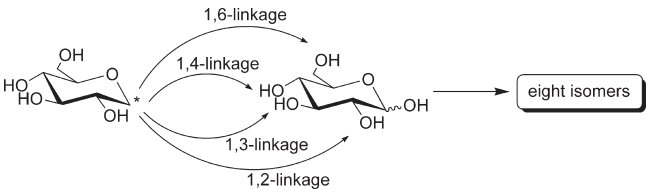

Figure 2. Four hydroxy groups in a glucose can participate in glycosylation toward C1 of the other glucose.

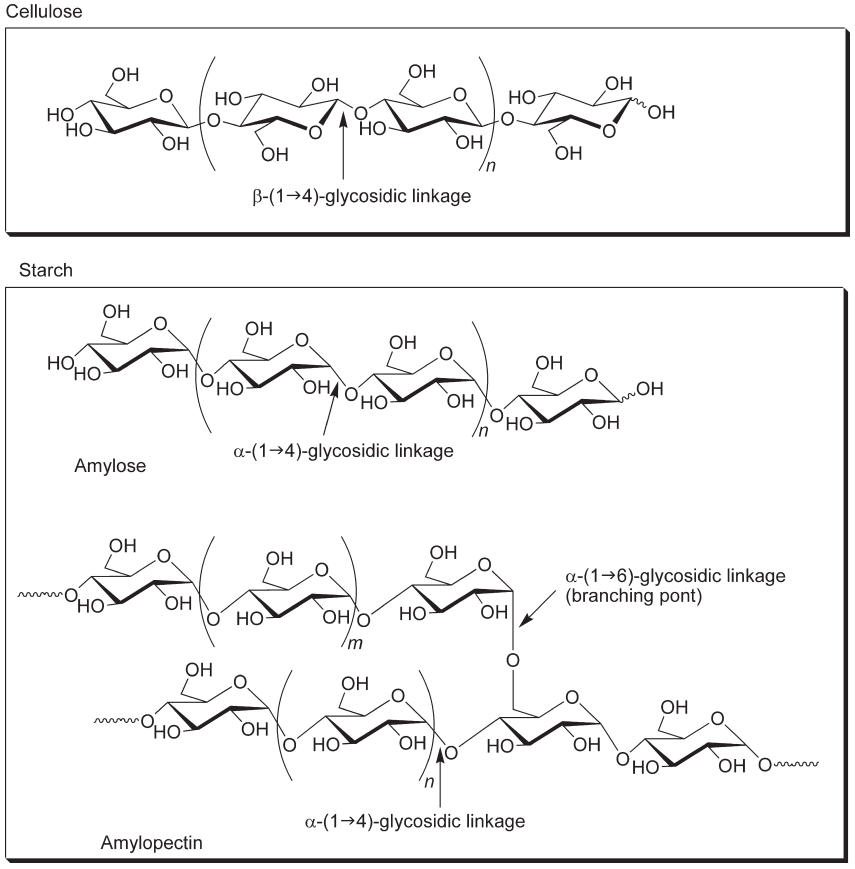

Figure 3. Structures of cellulose and starch.

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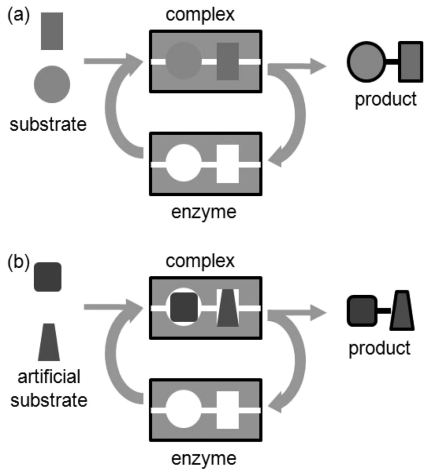


Figure 4. "Key and lock" theory showing the enzyme−substrate relationships for (a) an in vivo biosynthetic pathway and (b) an in vitro nonbiosynthetic pathway.

terms of the stereo- and regioselectivities.[54] In addition, enzymatic reaction is one of the most promising basic technologies with a simple operation under mild conditions, eliminating undesirable side-reactions.

3. CHARACTERISTIC FEATURES OF ENZYMATIC REACTIONS FOR POLYSACCHARIDE SYNTHESIS

All the biosubstances including polysaccharides are produced in vivo by enzymatic catalysis via biosynthetic pathways. Because enzymatic catalysis has attracted much attention and grew in importance in many research fields such as biochemistry, organic chemistry, and polymer chemistry,[55−57] fundamental research studies on enzymes and enzymatic reactions are still one of main topics in these research fields. In six main groups of enzymes, two enzymes, i.e., hydrolase[58,59] and transferase,[27,31,60−62] have been practically applied as catalysts for the in vitro enzymatic synthesis of polysaccharides. In general, enzymatic catalysis has the following advantageous characteristics: (i) progress of reactions under mild conditions, (ii) high selectivities not only of stereo- and regioregulations, but also of enantio- and chemoregulations, leading to structurally controlled products, and (iii) large turnover numbers. These general characteristics of enzymatic catalysis induce the following expectations for the precision synthesis of polysaccharides: (i) perfect control of stereo- and regioselectivities in glycosidic linkages, (ii) production of new non-natural polysaccharides, and (iii) achievement of a green process without use of harmful catalysts such as heavy metals and formation of byproduct.[33]

Furthermore, the following two aspects should be emphasized further as fundamental and important characteristics in enzymatic reactions. The first is a "key and lock" theory proposed by Fischer in 1894, which points out the relation of enzyme to native substrate.[63] The theory implies that a specific substrate and an enzyme correspond strictly in a 1:1 fashion like a key and lock relationship in biosynthetic pathways, as shown in Figure 4. In the enzyme−substrate complex, the substrate is located in the enzyme with geometrical adaptation, and the substrate is activated to induce the reaction, leading to a product with perfect structural control (cycle a). However, the key and lock relationship observed for in vitro enzymatic reactions is not absolutely strict in many cases. Enzymes often have loose specificity for

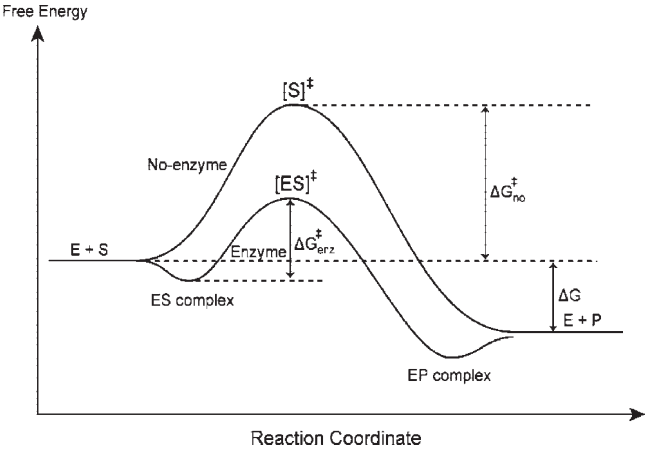
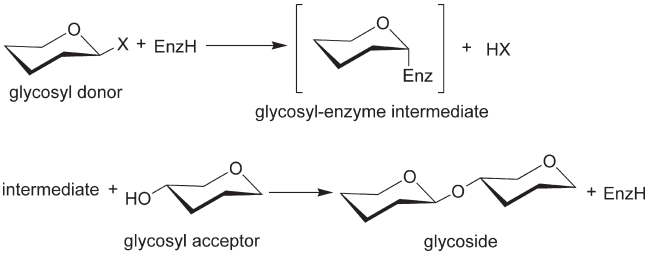


Figure 5. Energy diagram for a chemical reaction: An enzyme-catalyzed reaction proceeds much faster than a no-enzyme reaction, by lowering the activation energy while stabilizing the transition state of the reaction. Reprinted with permission from ref 34. Copyright 2009 American Chemical Society.

Scheme 2. General Reaction Route for Enzymatic Glycosylation



recognition of the substrate structure and can interact with not only a natural substrate but also a non-natural one. In the case of in vitro enzymatic synthesis of polysaccharides via nonbiosynthetic pathways, the non-natural (artificial) substrate can be employed for the enzymatic catalysis. The substrate is recognized by an enzyme to form enzyme−artificial substrate complex, resulting in progress of the desired reaction (cycle b).

The second characteristic is an energy diagram of the enzymatic reaction. Pauling demonstrated in 1946 the reason why enzymatic reactions proceed under mild conditions.[64,65] The formation of the enzyme−substrate complex stabilizes the transition state and lowers the activation energy compared with the no-enzyme case (Figure 5). Owing to such stabilization of the transition state, the rate acceleration reaches normally 10^6−10^{12}-fold, and in a specific case even 10^{20} fold.[66]

Similar to the reaction of the general glycosylation in Scheme 1, enzymatic formation of a glycosidic linkage between the C-1 atom of a monosaccharide and one of the hydroxy groups of the other monosaccharide can be realized by the reaction of an activated glycosyl donor and a glycosyl acceptor (Scheme 2).[22] First, the glycosyl donor is recognized by an enzyme to form a glycosyl−enzyme intermediate (or transition state). Then, the intermediate is attacked by the hydroxy group of the glycosyl acceptor, giving a glycoside. On the basis of the above characteristics of the enzymatic reactions, it has generally been well accepted that the enzymatic glycosylation is a very powerful tool for the stereo-

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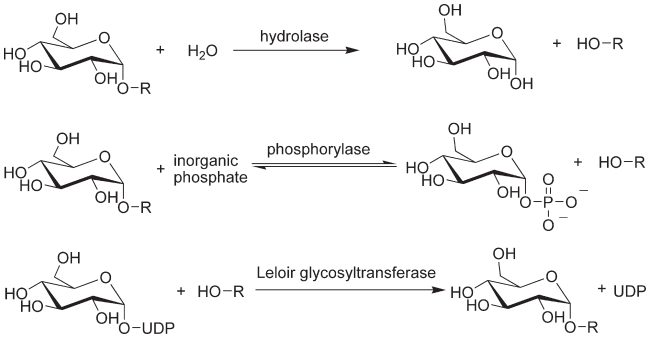
Scheme 3. Typical Enzymes Involved in the Synthesis of Polysaccharides (The Reaction Catalyzed by Sucrase-Type Enzyme Is Not Shown, Which Appears in the Text of Section 6.1.)



and regioselective construction of glycosidic linkages under mild conditions,[28] where a glycosyl donor and a glycosyl acceptor can be employed in their unprotected forms, leading to the direct formation of unprotected saccharide chains in aqueous media. Thus, repetition of the enzymatic glycosylations forms polysaccharides with a well-defined structure. For the enzymatic glycosylation using the artificial substrate,[30,67,68] its structure should be designed according to a concept of a "transition state analogue substrate" (TSAS);[25,33−35] the structure is close to that of the transition state in the in vivo enzymatic reaction. Because enzyme stabilizes the transition state via complexation with the substrate,[69] the artificial substrate, which is appropriately designed, forms readily an enzyme−artificial substrate complex and the reaction is induced to give the product with liberating enzyme again as shown in the cycle b in Figure 4.

As aforementioned, enzymes involved in the synthesis of polysaccharides are categorized into two main classes; hydrolytic enzymes (hydrolases)[58,59] and glycosyltransferases (Scheme 3).[27,31,60−62] The latter is precisely subclassified further into synthetic enzymes (Leloir glycosyltransferases),[62] phosphorolytic enzymes (phosphorylases),[27] and others.[31] Hydrolases have been frequently employed in the hydrolysis of polysaccharides, and they are industrially important in the utilization of polysaccharides such as starch.[70,71] The hydrolase-catalyzed reactions using natural substrates suitably proceed in the way to hydrolysis under the general conditions due to the presence of an abundance of water molecules in the reaction mixture. However, when an enzyme−substrate complex is formed, hydrolases catalyzing hydrolysis in vivo are able to catalyze a glycosylation in vitro to produce the saccharide chains. This view is based on a hypothesis that structure of a transition state is very close in both in vivo and in vitro reactions if the in vitro reaction would be induced.[25,33−35] Leloir glycosyltransferases are biologically important because they perform the role of synthesizing saccharide chains in vivo.[62,72−77] The reactions of the enzymes are irreversible in the synthetic direction due to the requirement for cleavage of the high-energy bond of the glycosyl nucleotide of a substrate in the reaction. However, Leloir glycosyltransferases are generally transmembrane-type proteins, present in nature in very small amounts, and unstable for isolation and purification with difficulty in handling. Thus, the enzymes are expensive and hardly available. Phosphorylases catalyze phosphorolytic cleavage of a glycosidic linkage in the saccharide chains in the presence of inorganic phosphate to give a monosaccharide 1-phosphate (glycose 1-phosphate) and the saccharide chain with one smaller DP.[27] Because the bond energy of the produced phosphate is comparable with that of the glycosidic linkage, the phosphorylase-catalyzed reactions have reversible nature. Therefore, phosphorylases can be employed in the practical synthesis of saccharide chains via glycosylation. In such phosphorylase-catalyzed glycosylations, the glycose 1-phsophates are used as a glycosyl donor and the glycose unit is transferred from the substrate to a nonreducing end of a glycosyl acceptor to form the stereo- and regiocontrolled glycosidic linkage accompanied with production of inorganic phosphate. The other enzyme in glycosyltransferases, which are often employed in the synthesis of polysaccharides, is sucrase-type enzyme.[31,78] This class of enzymes is the glycosyltransferase that is highly specialized in transfer either of a glucose or of a fructose moiety in a substrate of sucrose. Thus, sucrase-type enzymes form either glucose-based polysaccharides (glucans) or fructose-based polysaccharides (fructans) of different types with respect to glycosidic linkages and side chains.

The present review describes precision polysaccharide synthesis catalyzed by three types of enzymes, i.e., hydrolases, phosphorylases, and sucrase-type enzymes. Besides the polysaccharide synthesis, some significant studies on the enzymatic synthesis of oligosaccharides are also included. The synthetic methods disclosed in this review are defined as an "in vitro chemical synthesis of polysaccharides by enzyme-catalyzed polymerization (enzymatic polymerization)".[20−35,79−81] Therefore, it can be considered that these methods strongly contribute to developing the research field of "green polymer chemistry".[23−25,34,35] Because this review mainly focuses on the polysaccharide or oligosaccharide synthesis by successive enzymatic glycosylations of the small substrates, such as mono- and disaccharides, enzymatic cyclization and branching of the saccharide chains to produce cyclic and branched polysaccharides are not included.[82,83]

4. POLYSACCHARIDE SYNTHESIS CATALYZED BY HYDROLASES

4.1. Concept in Hydrolase-Catalyzed Synthesis of Polysaccharides

For the synthesis of polysaccharides by the polymerization manner catalyzed by hydrolases, the substrates (the monomers) have been designed according to the aforementioned TSAS concept.[25,33−35] On the basis of this concept, two types of monomers, that is, glycosyl fluorides and sugar oxazolines, have been designed to be efficiently recognized by hydrolases.[33] The anomeric carbon of the monomers is activated by introducing fluoride or an oxazoline group (1,2-oxazoline derived from 2-acetoamido-2-deoxy sugar), which have structures close to a transition state of enzymatic reactions and, thus, efficiently form the enzyme−substrate complexes. In the two types of hydrolases, i.e., endotype and exotype,[84,85] the former has been found to be an efficient catalyst for the enzymatic synthesis of the polysaccharides. Glycosyl fluorides have been investigated as a substrate for hydrolases (glycosidases), mainly in the fields of biochemistry and enzymology,[86−89] and they have also been found to act as a glycosyl donor for enzymatic glycosylation catalyzed by endotype hydrolases (endoglycosidases).[90] Endoglycosidases cleave a glycosidic linkage of the inner unit of polysaccharides, and their shapes at catalytic domain look like cleft.[84] The merit of using glycosyl fluorides as the glycosyl donor originates from the unique properties of a fluorine atom.[22,24,28,29,33,34] First, the size of a

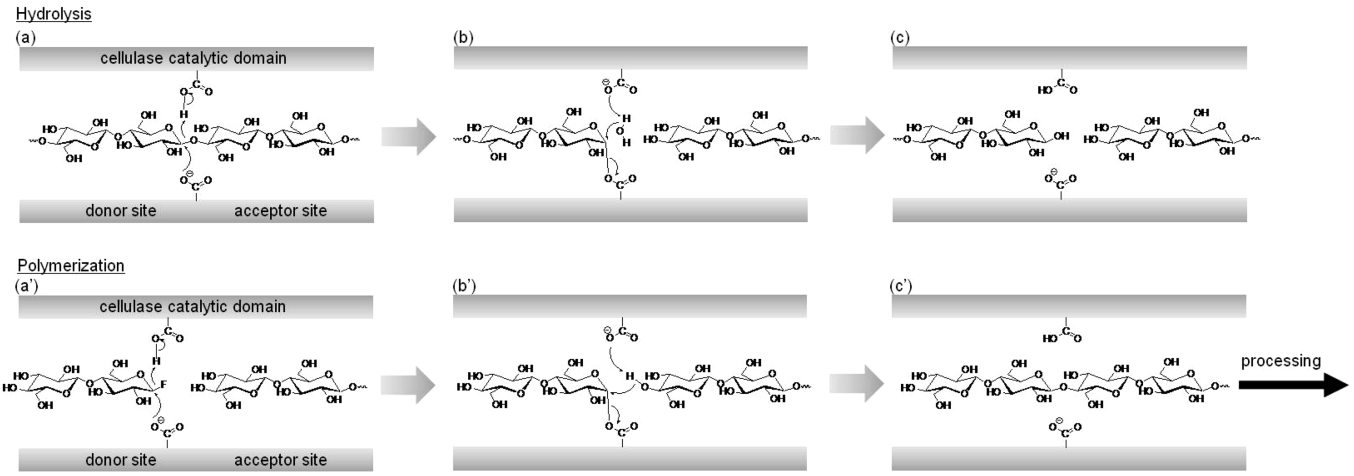
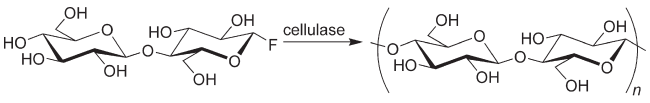


Figure 6. Postulated mechanism of cellulase catalysis for hydrolysis of cellulose and polycondensation of β-cellobiosyl fluoride.

Scheme 4. Cellulase-Catalyzed Polycondensation of β-Cellobiosyl Fluoride to Synthetic Cellulose



fluorine atom is comparable to that of a hydroxy group, so that it can be accepted by an active site of an enzyme. Second, of glycosyl halides, only glycosyl fluoride is stable as an unprotected form, which is necessary for most enzymatic reactions carried out in aqueous media. Third, because a fluorine atom behaves as a good leaving group, a C−F linkage in the glycosyl fluoride can be cleaved readily. On the other hand, sugar oxazolines having no protecting groups were also found to be an efficient glycosyl donor for the enzymatic glycosylation.[91−95] By enzymatic polymerizations using the glycosyl fluorides, cellulose, amylose, xylan, and related polysaccharides have been synthesized,[29,33−35] and enzymatic polymerizations using sugar oxazolines produced chitin, hyaluronan, chondroitin, and related aminopolysaccharides.[33−35,67,68,96−98] The former proceeds via the polycondensation through leaving hydrogen fluoride, whereas the ring-opening polyaddition is conceived in the polymerization manner of the latter case. The synthesis of nonnatural polysaccharides composed of two different monosaccharide units was also achieved by enzymatic polymerization catalyzed by hydrolases.[33,34,98]

4.2. Polysaccharide Synthesis by Hydrolase-Catalyzed Polycondensation

4.2.1. Synthesis of Cellulose and Its Derivatives by Hydrolase-Catalyzed Polycondensation of Glycosyl Fluorides. Cellulose is a representative natural polysaccharide and the most abundant organic substance on the earth, which consists of a chain of β-(1→4)-linked glucose residues (Figure 3).[51] Since the dawn of polymer science in 1920s,[99] it has been a symbolic molecule in polymers or macromolecules, and so far, the various fundamental and practical studies on cellulose have been carried out, which concern its structure, chemical and physical properties, biosynthesis, and morphology. The synthesis of cellulose by enzymatic polymerization was achieved in 1991 by polycondensation of β-cellobiosyl fluoride catalyzed by cellulase from *Trichoderma viride* (EC 3.2.1.4) (Scheme 4).[100−103] Cellulase is an enzyme that catalyzes the in vivo hydrolysis of β-(1→4)-glycosidic linkage between two anhydrous glucose units in cellulose. As a typical example, cellulase catalyzed the repeating glycosylations of β-cellobiosyl fluoride in a mixed solvent of acetonitrile/buffer (pH 5.0) (5:1) to produce synthetic cellulose having perfectly stereo- and regiocontrolled β-(1→4)-glycosidic linkages with DP of ∼11 based on the disaccharide (Scheme 4). The polymerization proceeded via polycondensation with liberating hydrogen fluoride. On the basis of the viewpoint of the glycosylation reaction, β-cellobiosyl fluoride behaves as both the glycosyl donor and acceptor for cellulase in this polycondensation. Mixing organic solvents with buffer was necessary as a medium to prevent the hydrolysis of produced cellulose by cellulase catalysis. The other effect of the organic cosolvent is to suppress nucleophilic attack of a water molecule to the glycosyl−enzyme intermediate, which leads to that of a hydroxy group of another saccharide chain to form a glycosidic linkage. Acetonitrile was most appropriate as the cosolvent, whereas *N*,*N*-dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) deactivated cellulase catalysis.

Figure 6 shows the proposed mechanism of cellulase catalysis in the hydrolysis of cellulose and in the polymerization of β-cellobiosyl fluoride.[24,29,33,34] Some cellulases (such as those belonging to glycosyl hydrolase family 5; GH5 (http://www.cazy.org/)) have two carboxylic acid residues for catalysis located in a cleft-like catalytic domain.[84,85,104,105] In the retention-type hydrolysis (inversion-type hydrolysis by cellulase catalysis has also been known[106]), one residue pulls the glycosidic oxygen by a protonation, and the other pushes the anomeric carbon in a general acid−base mode to assist the cleavage of the glycosidic linkage (stage a). Then, a highly reactive intermediate (or transition state) of a glycosyl−carboxylate structure of α-configuration is formed. A water molecule attacks the anomeric center of the intermediate from the β-side to complete the hydrolysis (from stage b to c). Therefore, this cellulase is a retaining enzyme with double-displacement mechanism, which involves two inversions of anomeric stereochemistry. In the polymerization, β-cellobiosyl fluoride is readily recognized by cellulase and activated at the donor site via a general acid−base mode to cleave the C−F bond and to from a highly reactive glycosyl−carboxylate intermediate having α-configuration (from stage a′ to b′), whose structure is similar to that of stage b in the hydrolysis. The C-4

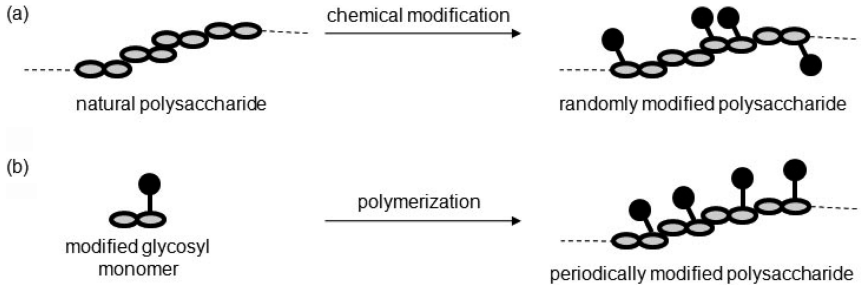
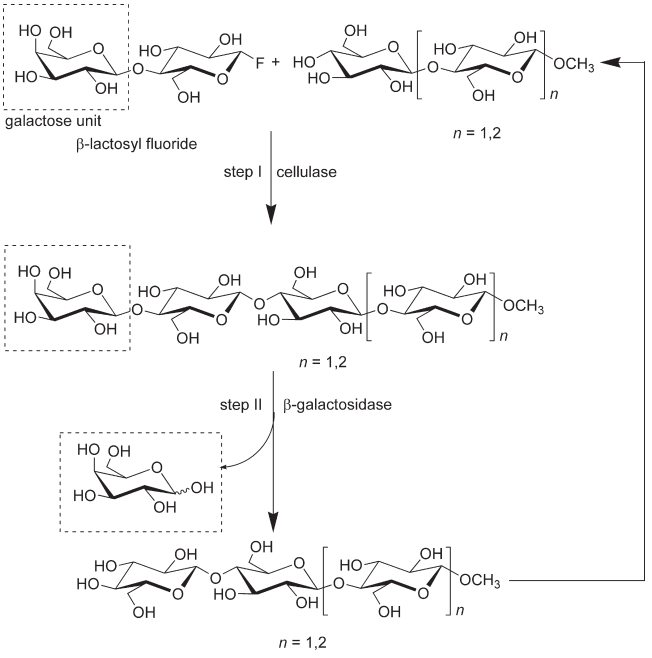


Figure 7. Synthesis of substituted polysaccharides by chemical modification of naturally occurring polysaccharides (a) and polymerization of modified glycosyl monomer (b).

Scheme 5. Stepwise Synthesis of Cellooligosaccharide Derivatives by Combined Use of Cellulase and β-Galactosidase



hydroxy group of another monomer or the growing chain end located at the acceptor site attacks the anomeric carbon from the β-side to form a new β-(1→4)-glycosidic linkage (stage c′). Thus, β-cellobiosyl fluoride can be considered as a TSAS monomer on the basis of the transition state (or intermediate) structures involved from stage a to b and stage a′ to b′ in both reactions. On the basis of the viewpoint of polymer chemistry, this polymerization proceeds via an "activated monomer mechanism" in contrast to an "active chain-end mechanism".

By means of the stepwise enzymatic glycosylations by cellulase catalysis combined with the catalysis of another enzyme, β-galactosidase (EC 3.2.1.23), cellooligosaccharides were prepared (Scheme 5).[107−112] A first step is β-lactosylation of methyl cellobioside (n = 1) catalyzed by cellulase using β-lactosyl fluoride as a glycosyl donor to produce a new oligosaccharide having a galactose unit at the nonreducing end (step I). A second step is a regioselective cleavage of the terminal galactose unit by β-galactosidase catalysis to afford the corresponding cellotriose (n = 1) (step II). The resulting cellotriose derivative was further subjected to the condensation with β-lactosyl fluoride, followed by the enzymatic degalactosylation, leading to the formation of methyl β-cellotetraose (n = 2). The reactions proceeded with perfect control of stereo- and regioselectivities without use of protective groups. This enzymatic β-lactosylation was also used for the synthesis of a bifunctional cellotetramer as an efficient substrate for evaluation of cellulase acitivity.[113]

Besides control of stereo- and regioselectivities in the construction of polysaccharide structures, another merit of enzymatic polymerization can be seen in the formation of modified polysaccharides with well-defined structures.[22,28] The conventional synthetic strategy for functionalized polysaccharides has been based on the modification of hydroxy groups of naturally occurring polysaccharides by chemical reactions (Figure 7a).[114,115] For example, various derivatizations of cellulose have been studied for the development of cellulose-based practical materials. However, chemical structures of the resulting derivatives by these modification methods are not precisely controlled in terms of the regioselectivities of different hydroxy groups at 2-, 3-, and 6-positions and of the degree of substitution because such modifications give a mixture of randomly substituted polysaccharides. An alternative method for the construction of a modified polysaccharide with well-defined structure is to polymerize a modified saccharide monomer (Figure 7b). By means of this method, a novel functional polysaccharide having a modified unit periodically in the main chain is produced.

For example, synthesis of an alternatingly 6-*O*-methylated cellulose by cellullase-catalyzed polymerization of 6-*O*-methyl- or 6′-*O*-methyl-β-cellobiosyl fluoride was examined (Scheme 6).[116,117] The polymerization of 6-*O*-methyl-β-cellobiosyl fluoride took place smoothly in a mixed solvent of acetonitrile/acetate buffer (pH 5.0) to give an alternatingly C-6 methylated cellulose derivative. The gel permeation chromatographic (GPC) measurement after acetylation of the product indicated that the DP was 7 (based on the disaccharide). The resulting cellulose derivative has a very unique structure in the main chain, where alternating glucoses have the methyl group at C-6 position. Formation of such a structure cannot be realized by the conventional modification method of cellulose. On the other hand, only the cellooligosaccharides such as tetrasaccharide were formed by cellulase-catalyzed reaction of 6′-*O*-methyl-β-cellobiosyl fluoride.

The cellulase-catalyzed polycondensation of the other methylated β-cellobiosyl fluoride derivative, 2′-*O*-methyl-β-cellobiosyl fluoride, also took place in a mixed solvent of acetonitrile/acetate buffer (pH 5.0) to produce an alternating 2′-*O*-methylated cellulose derivative (Scheme 7).[118] The matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spec-

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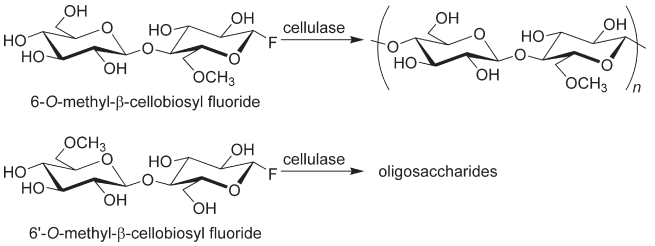
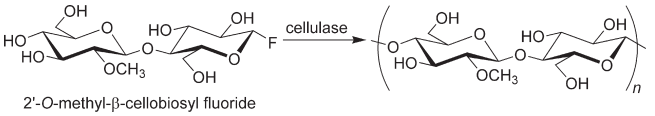
Scheme 6. Cellulase-Catalyzed Polycondensation of 6-*O*-Methyl- and 6′-*O*-Methyl-β-cellobiosyl Fluorides



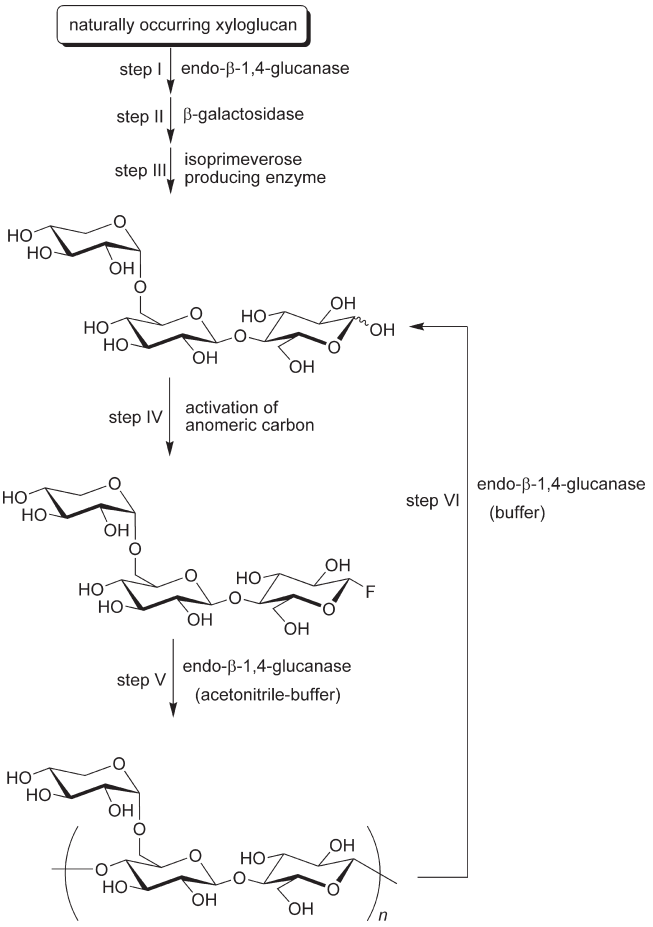
Scheme 7. Cellulase-Catalyzed Polycondensation of 2′-*O*-Methyl-β-cellobiosyl Fluoride



trum of the acetylated product of the water-insoluble fraction showed the peaks due to the cellulose derivatives with DPs up to 13 (based on the disaccharide).

The enzymatic synthesis of polysaccharide using the glycosyl fluoride was extensively studied to give an artificial xyloglucan oligomer, which has a β-(1→4)-glucan (cellulose) main chain with α-(1→6)-xylopyranosyl residue linked to the alternating glucose residues in the main chain (Scheme 8).[119,120] A monomer, α-(1→6)-xylopyranosyl-β-cellobiosyl fluoride, was prepared from a naturally occurring xyloglucan according to the following procedures; the natural xyloglucan has a structure that consists of β-(1→4)-glucan chain with single xylopyranosyl residue linked via α-(1→6)-glycosidic linkage to the main chain, and some xyloglucan extracted from *Tamarindus* seeds contains a galactopyranosyl residue linked via β-(1→2)-glycosidic linkage to the xylopyranosyl residue. First, the naturally occurring xyloglucan was treated with *endo*-1,4-β-glucanase to cleave the glycosidic linkage between the xylosylated glucose unit and the unsubstituted glucose unit regioselectively (step I); *endo*-1,4-β-glucanase is an endotype enzyme that catalyzes the random hydrolysis of the β-(1→4)-glucan chain. A mixture of the resulting oligosaccharides was then treated with β-galactosidase to remove the galactopyranosyl residue (step II), and the subsequent treatment with isoprimeverose-producing enzyme yields smaller oligosaccharides (step III). After purification by GPC, α-(1→6)-xylopyranosyl-β-cellobiose was obtained. The introduction of fluorine atom to the anomeric center of this trisaccharide was achieved by the general chemical procedure to give α-(1→6)-xylopyranosyl-β-cellobiosyl fluoride (step IV). The enzymatic polymerization of this monomer was performed in the presence of *endo*-1,4-β-glucanase in a mixed solvent of acetonitrile/buffer to produce artificial xyloglucan oligomers, which have an alternating xylosylated structure (step V). It is impossible to synthesize such a structure by the chemical modification of the natural polysaccharide. The MALDI-TOF mass measurement of the products indicated that the oligosaccharides with DPs up to 10 (based on the trisaccharide) were obtained. Interestingly, the resulting oligosaccharides can be degraded to the starting trisaccharide catalyzed by *endo*-1,4-β-glucanase in buffer solvent (step VI), which is able to convert again into the artificial xyloglucan

Scheme 8. Chemoenzymatic Synthesis of Xyloglucan Oligosaccharides



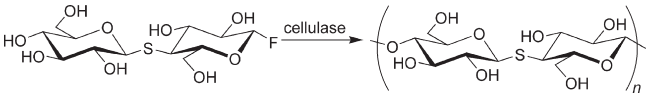
oligomer by the activation of the anomeric carbon by fluorine atom, followed by the enzymatic polymerization.

Xyloglucan endotransglycosylase (EC 2.4.1.207) was also employed for the enzymatic polymerization, giving xyloglucans. Although xyloglucan endotransglycosylase is a transglycosidase, which catalyzes the rearrangement of xyloglucans by endolytic cleavage and relegation, this enzyme is structurally and mechanically related to microbial glycosyl hydrolases and grouped into glycosyl hydrolase family 16 (GH16). Homo- and heteropolycondensations of α-xyloglucooligosaccharyl fluoride catalyzed by the E85A mutated xyloglucan endotransglycosylase from *Populus tremula* and *Populus tremuloides* (E = Glu, A = Ala) took place to give xyloglucans composed of β-(1→4)-glucan with a regular side-chain substitution pattern.[121]

An analogous substrate of β-cellobiosyl fluoride, the *S*-linked β-cellobiosyl fluoride, was also polymerized by cellulase from *Trichoderma viride* to produce oligosaccharides composed of alternating *O* and *S* linkages (hemithiocellodextrins) (Scheme 9).[122] Tetra-, hexa-, octa-, and decasaccharides were isolated from the products in 4.5, 7.5, 5.7, and 5.0% yields, respectively.

It is always a problem that enzymatic polymerization by hydrolase accompanies the hydrolysis (a reverse reaction of the glycosylation) of the products, which are the native substrates for hydrolytic reaction by the enzyme. To suppress this unsuitable reverse reaction, the cellulase-catalyzed polycondensation

Scheme 9. Cellulase-Catalyzed Polycondensation of S-linked β-Cellobiosyl Fluoride



of β-cellobiosyl fluoride was conducted under the preferable conditions for the polymerization by using a mixed solvent of acetonitrile/buffer.[100] Because the suppression of the hydrolysis was incomplete as long as the wild-type cellulase was used, mutant cellulase was designed to make that the polymerization take place more efficiently. There have been two approaches for the mutation of cellulase, i.e., removing cellulose-binding-domain (CBD), which is necessary to capture and take crystalline cellulose chain into the catalytic domain, and excluding the carboxylic acid group of a proton donor or a nucleophile in the catalytic domain.

The former approach of the mutation is performed using a CBD-deleted mutant cellulase. Cellulase (endoglucanase II, EG II) consists of three domains: CBD, a linker domain, and a catalytic domain. In the hydrolysis of cellulose, CBD first binds the crystalline part of cellulose and then the catalytic domain catalyzes the hydrolysis of cellulose chains. Because the molecular packing of cellulose chains is loosened to accelerate a consecutive cleavage by the catalytic domain upon binding of the CBD to crystalline cellulose,[123] it can be considered that the use of CBD-deleted mutant cellulase suppresses the hydrolysis reaction. Two types of protein enzymes were prepared from yeast; one had all domains (EG II), and the other had only the catalytic and linker domains (EG II core). Both enzymes showed high polymerization activity on β-cellobiosyl fluoride, giving cellulose. With the progress of the reaction time, the produced cellulose gradually hydrolyzed by EG II, whereas it was hardly hydrolyzed by EG II core.[124] These results indicated that the CBD played an important role for the hydrolysis of cellulose but not for the polymerization of β-cellobiosyl fluoride. In the following study, a new mutant enzyme, EG II core2 having sequential two active sites of EG II, was prepared. This mutant enzyme showed higher polymerization and hydrolysis activities than EG II core. This was mainly because of the suitably stabilized conformation with the sequential arrangement.[125]

As to the latter approach, it was reported that the E358A mutant of *exo*-β-glucosidase from *Agrobacterium* sp. recognized α-glucosyl and α-galactosyl fluorides and catalyzed the formation of the β-(1→4)- and β-(1→3)-glycosidic linkages.[126] In the mutated enzyme, termed "glycosynthase",[127] the acidic catalytic nucleophile (E) is replaced by the smaller non-nucleophilic residue (A), resulting in hydrolytically inactive nature. However, the enzyme can catalyze transglycosylation of the glycosyl fluoride donor that has the opposite anomeric configuration (α-form) to that of the normal substrate (β-form) of the parental wild-type enzyme, in which the substrate for the glycosynthase mimics the glycosyl-enzyme intermediate, which is formed during the catalysis of the wild-type enzyme. By means of this approach using mutated cellulases, which were prepared by the E197A and E197S (S = Ser) mutations of recombinant retaining cellulase endoglucanase I (Cel17B) from *Humanicola insolens*, synthesis of cellulose and cellooligosaccharides using α-cellobiosyl fluoride was performed.[128,129] The Cel7B mutant was able to recognize branched oligosaccharides as a glycosyl acceptor to catalyze the

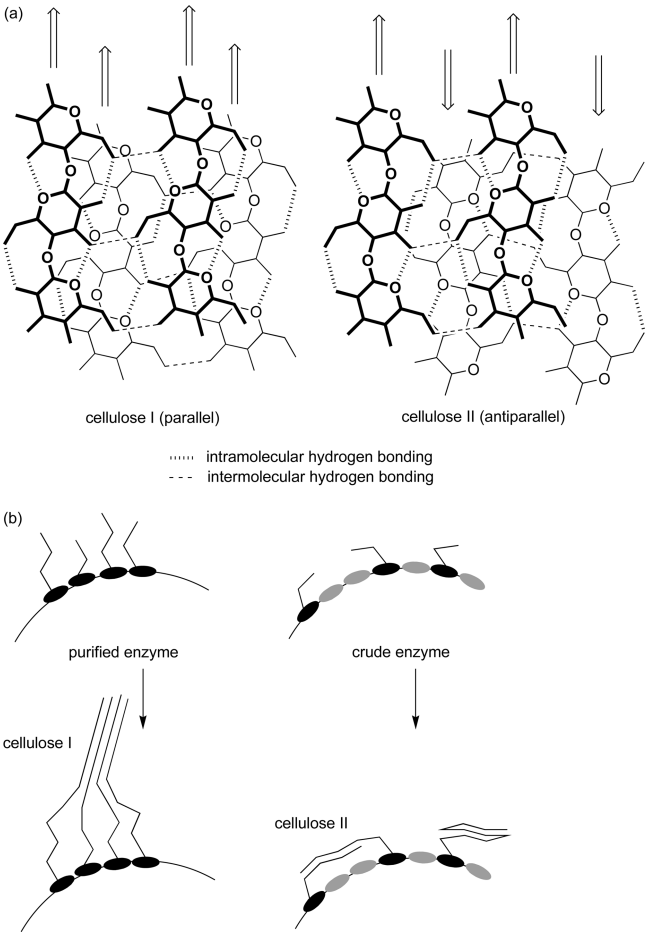
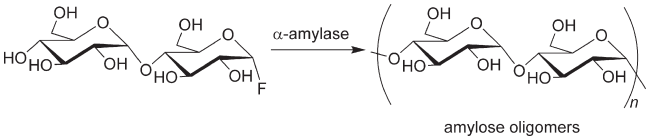


Figure 8. Two allomorphs of cellulose I and cellulose II crystalline structures (a) and postulated models for the formation of cellulose I by purified enzyme and cellulose II by crude enzyme mixture (b).

β-(1→4)-glycosidic linkage formation.[130−133] Synthesis of cellooligosaccharides catalyzed by a rice BGlu1 β-glucosidase (EC 3.2.1.21) mutant was also performed.[134] The enzyme is an exotype hydrolase, which is active on *p*-nitrophenyl-β-glycosides. The E414G (G = Gly) mutant of the enzyme catalyzed the β-(1→4)-glycoside linkage formation using α-glucosyl fluoride as a glycosyl donor and *p*-nitrophenylcellobioside as a glycosyl acceptor to produce cellooligosaccharides.

4.2.2. Higher-Ordered Self Assembly of Synthetic Celluloses. Cellulose typically forms two allomorphs as the higher-ordered crystalline structures (Figure 8a).[135,136] One is thermodynamically metastable cellulose I, which is of parallel molecular chain packing structure, and the other is thermodynamically stable cellulose II, which is of antiparallel molecular chain packing structure. Naturally occurring cellulose forms cellulose I in spite of thermodynamically less favored allomorph. Once cellulose I is converted into cellulose II by such method as mercerization, it does not return back to cellulose I. The enzymatic polymerization of β-cellobiosyl fluoride catalyzed by a crude cellulase mixture led to form a synthetic cellulose II spherulite, which was observed by transition and scanning electron microscopies (TEM and SEM, respectively).[137,138] On the other hand, the enzymatic polymerization of β-cellobiosyl fluoride catalyzed by a partially purified cellulase provided a microfibril of synthetic cellulose I, which was directly verified by TEM measurement.[139] The difference

Scheme 10. α-Amylase-Catalyzed Polycondensation of α-Maltosyl Fluoride

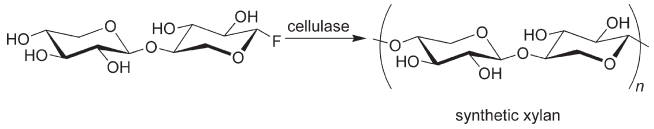


in the behaviors using the crude and purified cellulases in the enzymatic polymerization of β-cellobiosyl fluoride was explained as follows (Figure 8b). Cellulases formed a micellar molecular assembly in a mixed solvent of acetonitrile/buffer. A crude enzyme is believed to generally contain active sites in ≪1% for the total proteins. Because active sites of cellulase are separately located on the surface of the micelle, the elongating cellulose chains by cellulase catalysis form cellulose II allomorph by the thermodynamically controlled formation of inter- and intramolecular hydrogen bonding. On the other hand, active sites of the purified cellulase are densely distributed on the surface of the micelle. Therefore, cellulose chains elongate from such condensed active sites with the same direction to form metastable cellulose I allomorph by the kinetically controlled process. This study was the first example of the formation of cellulose I via the nonbiosynthetic pathways. The control in the higher-ordered molecular assembly during the polymerization process was proposed as a new concept termed "choroselective polymerization".[140]

The observation in a self-assembling process of synthetic cellulose during the cellulase-catalyzed polymerization of β-cellobiosyl fluoride was investigated in real time and in situ by a combined small-angle scattering technique including small-angle neutron scattering (SANS), small-angle X-ray scattering (SAXS), ultra-SANS, and ultra-SAXS methods together with wide-angle X-ray scattering and field-emission SEM.[141−143] The studies revealed the following: (1) Even in the aqueous reaction medium free from monomers, cellulases aggregate themselves into associations with a characteristic length of >200 nm. (2) Cellulose molecules, which are created at each active site of the enzymes, associate themselves around the enzyme associations into cellulose aggregates having surface dimensions D_s, increasing from 2 to 2.3 with reaction time. (3) The fractal structure, which is formed at the end of the reaction, extends over a surprisingly wide length scale ranging from 30 nm to 30 μm (3 orders of magnitude).

4.2.3. Synthesis of Amylose by Hydrolase-Catalyzed Polycondensation of Glycosyl Fluoride. Amylose is a polysaccharide composed of glucose residues linked through α-(1→4)-glycosidic linkages (Figure 3).[52,53] Starch is composed of both amylose with a linear structure and amylopectin having a branched structure with α-(1→6)-linked branching points. The enzymatic polymerization of α-maltosyl fluoride catalyzed by an endotype enzyme, α-amylase (EC 3.2.1.1), which catalyzes random hydrolysis of α-(1→4)-glucan chain, proceeded in a mixed solvent of methanol/phosphate buffer (pH 7.0) to give amylose oligomers up to heptasaccharide (Scheme 10).[144] The production not only of even-numbered, but also of odd-numbered, maltooligosaccharides was confirmed by high-performance liquid chromatographic (HPLC) analysis. This result suggested that enzymatic hydrolysis or transglycosylation of the products took place in parallel with the polymerization.

Scheme 11. Cellulase-Catalyzed Polycondensation of β-Xylobiosyl Fluoride



4.2.4. Synthesis of Xylans by Hydrolase-Catalyzed Polycondensation of Glycosyl Fluorides. Naturally occurring xylan is composed of a β-(1→4)-linked xylose main chain, which has some saccharide side chains. β-Xylobiosyl fluoride was designed as a monomer for the synthesis of xylan by the enzymatic polymerization, on the basis of the TSAS concept for the catalysis of cellulase, because its commercial-grade enzyme has been known to show xylanase activity. The enzymatic polymerization of β-xylobiosyl fluoride catalyzed by cellulase from *Trichoderma viride* proceeded in a mixed solvent of acetonitrile/buffer (pH 5.0) to give a synthetic xylan (Scheme 11).[145]

The mutation of the catalytic nucleophilic Glu (E) residue to the Gly (G) residue was conducted using xylanase from *Cellulomonas fumi* (CFXcd).[146] When *p*-nitrophenyl and benzylthio β-xylobiosides were used as a glycosyl acceptor, the mutant xylanase catalyzed the formation of β-(1→4)-glycosidic linkages using β-xylobiosyl fluoride as a glycosyl donor, giving xylan oligomers with DPs (based on the disaccharide) of 4−12. Mutated xylanases from different origins were also prepared according to the same way as that for CFXcd and used for the enzymatic polymerization of α-xylobiosyl fluoride, giving the synthetic xylan.[147,148] The synthetic xylan was also produced by the enzymatic polymerization of α-xylopyranosyl fluoride by the combined use of two mutated enzymes, i.e., XynB2-E335G and XT6B2-E265G; the former was prepared from GH52 β-xylosidase from *Geobacillus stearothermopilus*, and the latter was prepared from xylanase (EC 3.2.1.37) from *Geobacillus stearothermophilus* XT6.[148] The DP of the product ranged from 6 to >100 xylose units. The following two-step enzymatic reactions were considered to form the synthetic xylan from α-xylopyranosyl fluoride. First, α-xylobiosyl fluoride was produced by XynB2-E335G-catalyzed dimerization of α-xylopyranosyl fluoride, and then it was polymerized by XT6B2-E265G catalysis.

4.2.5. Synthesis of (1→3)-β- and (1→3,1→4)-β-Glucans by Hydrolase-Catalyzed Polycondensation of Glycosyl Fluorides. Polysaccharides composed of glucose residues linked through β-(1→3)-glycosidic linkages, i.e., (1→3)- β-glucans, such as curdlan, laminarin, and schizophyllan, are found in nature. They have increasingly attracted much attention because of their high immune properties and anticancer activities.[149] When the enzymatic polymerization of β-laminaribiosyl fluoride catalyzed by (1→3)-β-D-glucan endohydrolase (EC 3.2.1.39), which catalyzes the hydrolysis of β-(1→3)-glycosidic linkages, was carried out, the yields of the products were low.[150] The mutated (1→3)-β-D-glucan endohydrolase E231G was prepared, and the polymerization of α-laminaribiosyl fluoride by this mutated enzyme was performed in a mixed solvent of acetonitrile/acetate buffer (pH 5.0) to produce (1→3)-β-glucan with DPs of 28−44 (Scheme 12).[142] The catalysis of this mutated enzyme was extended to the polymerization of 3-thio-α-laminaribiosyl fluoride, giving rise to the corresponding polysaccharide with DPs of 6−18.[150]

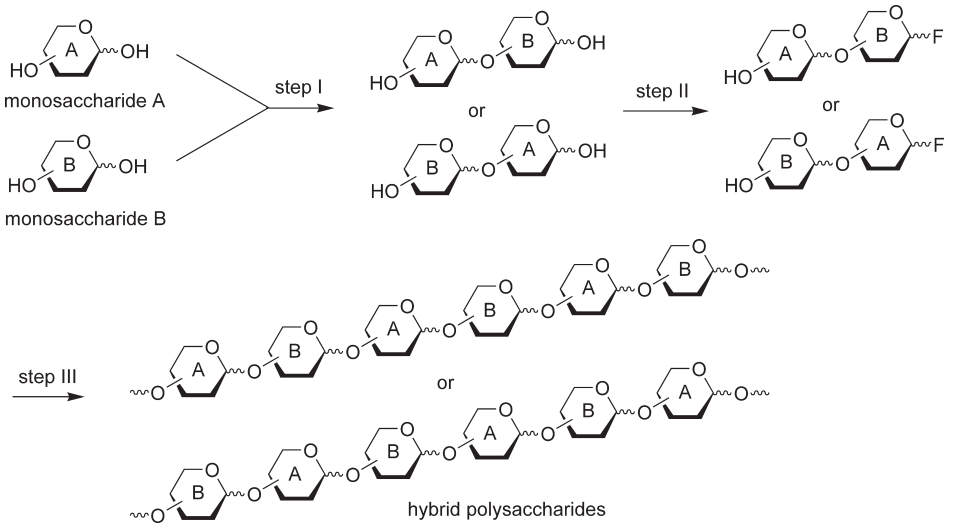
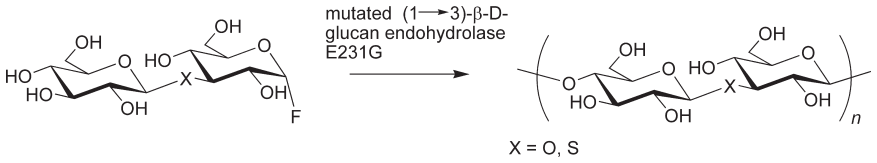


Figure 9. Procedure for synthesis of hybrid polysaccharides composed of the alternating monosaccharide units A and B by enzymatic polycondensation.

Scheme 12. Mutated (1→3)-β-D-Glucan Endohydrolase-Catalyzed Polycondensation of α-Laminaribiosyl Fluoride and 3-Thio-α-laminaribiosyl Fluoride



(1→3,1→4)-β-Glucan is one of the major components in the cell wall of the higher plants besides cellulose. The glycosylation of cellooligosaccharide acceptors with β-laminaribiosyl fluoride catalyzed by (1→3,1→4)-β-D-glucanase (EC 3.2.1.73) from *Bacillus licheniformis*, which catalyzes the hydrolysis of β-(1→4)-glycosidic linkages on 3-*O*-substituted glycopyranosyl residues, was carried out to form β-(1→4)-glycosidic linkages.[151,152] The mutated E134A enzyme of (1→3,1→4)-β-D-glucanase from *Bacillus licheniformis* catalyzed the polymerization of α-laminaribiosyl fluoride to give a polysaccharide with an alternatingly aligned β-(1→4)- and β-(1→3)-glucose units (DPs = 6−12).[153]

4.2.6. Synthesis of Non-natural Hybrid Polysaccharides by Hydrolase-Catalyzed Polycondensation of Glycosyl Fluorides. Hydrolase-catalyzed polycondensation of glycosyl fluorides has been extended to synthesis of non-natural hybrid polysaccharides.[22,33,34] Hybrid polysaccharides have been defined as non-natural polymers composed of different kinds of repeating monosaccharide units in a polymer chain, such as the alternating monosaccharide units A and B in Figure 9, which should completely be distinguished from polymer blends of natural polysaccharides.[68,98] It is considered that such polysaccharides are difficult to be synthesized by the conventional chemical methods. Hybrid polysaccharides have the potential to display novel functions derived from the specific structures of intramolecular hybridization. As shown in Figure 9, there are two possible disaccharide structures composed of monosaccharides A and B, according to the following two sequences, A−B and B−A. The skeleton of the two disaccharides can be constructed by a chemical coupling of the two monosaccharides A and B (step I). The resulting disaccharides are converted into the corresponding glycosyl fluoride monomers by activation at the anomeric carbons on the basis of the TSAS concept (step II). In step III, the enzymatic polycondensation of the monomers are performed to give the designed hybrid polysaccharides.

Cellulose−xylan hybrid polysaccharide was synthesized by the hydrolase-catalyzed polymerization of glycosyl fluoride monomers.[154] On the basis of Figure 9, two kinds of candidate monomers were designed from the viewpoint of the repeating structure of a cellulose−xylan hybrid polysaccharide, that is, Glc-β-(1→4)-Xyl-β-fluoride and Xyl-β-(1→4)-Glc-β-fluoride (Glc = glucose, Xyl = xylose) (Figure 10). Consequently, both the monomers were polymerized by catalysis of xylanase from *Trichoderma viride* to produce the cellulose−xylan hybrid polysaccharide.

Synthesis of cellulose−mannan hybrid polysaccharide was performed by the enzymatic polymerization of Man-β-(1→4)-Glc-β-fluoride (Man = mannose) catalyzed by cellulase from *Trichoderma reesei* (Scheme 13).[118] The MALDI-TOF mass spectrum indicated that the water-insoluble fraction in the products was composed of oligosaccharides up to hexadecasaccharide.

For the synthesis of cellulose−chitin hybrid polysaccharides, it was possible to design[155] two kinds of monomers, i.e., GlcNAc-β-(1→4)-Glc-β-fluoride and Glc-β-(1→4)-GlcNAc oxazoline (GlcNAc = *N*-acetyl-D-glucosamine), on the basis of the TSAS concept, the same as the aforementioned case of cellulose−xylan hybrid (Figure 9). The polymerization of the latter monomer is described in the following section (section 4.3.3). The former monomer was polymerized by cellulase catalysis to give the cellulose−chitin hybrid polysaccharide with the M_n of 2800 (DPs of ca. 8 based on the disaccharide) (Scheme 14).

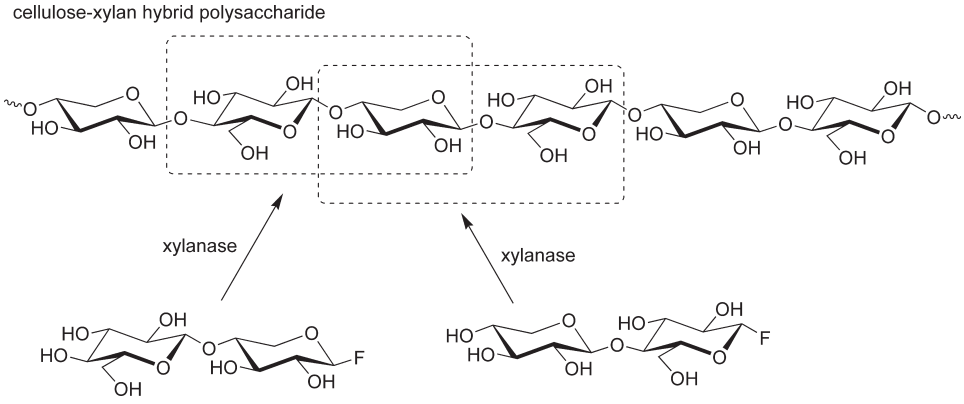
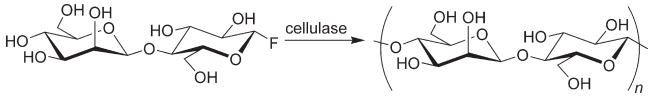
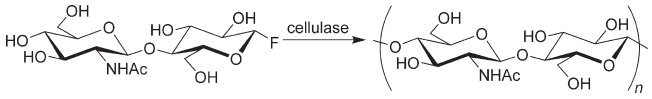


Figure 10. Potential two monomers for synthesis of cellulose−xylan hybrid polysaccharide by xylanase-catalyzed polycondensation.

Scheme 13. Cellulase-Catalyzed Polycondensation of Man-β-(1→4)-Glc-β-fluoride to Cellulose−Mannan Hybrid Polysaccharide



Scheme 14. Cellulase-Catalyzed Polycondensation of GlcNAc-β-(1→4)-Glc-β-fluoride to Cellulose−Chitin Hybrid Polysaccharide



4.2.7. Hydrolase-Catalyzed Polycondensation Using Other Substrates Besides Glycosyl Fluorides. A novel approach for the enzymatic polycondensation using cellobiose as a starting substrate without activation of the anomeric carbon was reported (Scheme 15).[156] This was achieved using a cellulase/surfactant ($2C_{18}\Delta^9$GE) complex as the catalyst in a nonaqueous LiCl/*N*,*N*-dimethylacetamide (DMAc) system. Cellobiose was polymerized by the catalysis of the complex to give cellulose with high molecular weight (DP reaching to 100 based on the monosaccharide).

A new glycosyl donor was found to be recognized by hydrolase, *endo*-1,4-β-glucanase, to give oligosaccharides.[157,158] The substrate was activated by a 4,6-dimethoxy-1,3,5-triazin-2-yl (DMT) group at the anomeric carbon, which can be directly prepared from a free sugar such as lactose (Scheme 16). This glycosylation process is the first example of chemoenzymatic glycosylation, where both the glycosyl donor synthesis and the successive glycosylation can be achieved in water without any protection and deprotection steps.

4.3. Polysaccharide Synthesis by Hydrolase-Catalyzed Ring-Opening Polyaddition

4.3.1. Synthesis of Chitin and Its Derivatives by Chitinase-Catalyzed Ring-Opening Polyaddition of Sugar Oxazolines. Chitin is the second most abundant organic substance on the earth after cellulose,[159,160] which is found as a skeletal component of invertebrates. Chitin is composed of GlcNAc residues linked through β-(1→4)-glycosidic linkages (Figure 11), which is synthesized in vivo by the chitin synthase catalysis (EC 2.4.1.16) with UDP-GlcNAc as a substrate.[161] Chitosan is an *N*-deacetylated derivative of chitin (Figure 11). They have been frequently utilized as a functional resource in medicines, pharmaceuticals, food supplements, and cosmetics because of their excellent characteristics such as biocompatibility, biodegradability, and low immunogenicity.[162−165]

In vitro synthesis of chitin was first reported by utilizing chitinase (EC 3.2.1.14) from *Bacillus sp.* (glycoside hydrolase family 18; GH18) as catalyst (Scheme 17).[166,167] On the basis of the TSAS concept, the *N*,*N*′-diacetylchitobiose (GlcNAc-β-(1→4)-GlcNAc) oxazoline monomer was designed. The enzymatic polymerization of this monomer proceeded via ring-opening polyaddition even under weak alkaline conditions (pH 9.0−11.0), giving a synthetic chitin with perfectly controlled stereo- and regioselectivities. The DPs were evaluated as 10−20 depending on the reaction conditions.

Chitinase, which catalyzes the ring-opening polyaddition, is classified into GH18, having a cleft-like catalytic domain.[168] As to the mechanism of the chitinase catalysis (Figure 12), two conserved carboxylic acid residues are involved and considered to act as an acid−base catalyst. In the hydrolysis of chitin, the glycosidic oxygen is protonated by one of the carboxylic acid residues immediately after the recognition of chitin at the catalytic domain (stage a). The carbonyl oxygen of C-2 acetamido group in a chitin unit at the donor site attacks the neighboring anomeric carbon from α-side to form an oxazolinium stabilized by the other carboxylic acid residue, and the C1−O linkage is cleaved completely (stage b), which is thus called a "substrate-assisted mechanism".[169] A nucleophilic attack by a water molecule from β-side induces the ring-opening of the oxazolinium to accomplish the hydrolysis reaction, giving rise to the hydrolysate having β-configuration (stage c). Therefore, it can be considered that the oxazolinium acts as a transition state (or intermediate) in the hydrolysis.

In the polymerization, *N*,*N*′-diacetylchitobiose oxazoline is developed as a TSAS monomer for chitinase because it already has an oxazoline moiety. First, the monomer is recognized at the donor site and simultaneously protonated by the carboxylic acid residue to form the corresponding oxazolinium ion (stage b′). Then, the C-4 hydroxy group of the GlcNAc unit located at the acceptor site attacks the anomeric carbon of the oxazolinium group from β-side to induce the ring-opening, giving a new β-(1→4)-glycosidic linkage (stage c′). Repetition of the glycosylations,

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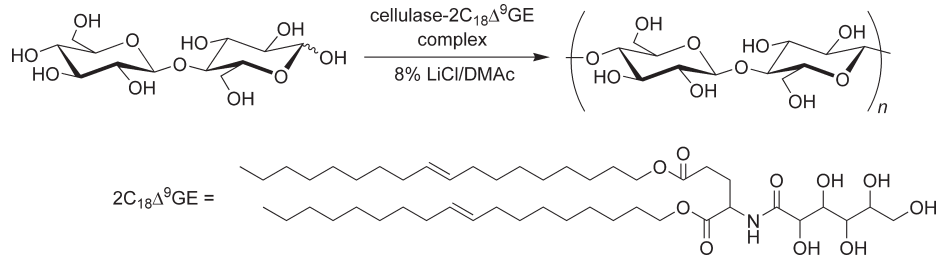
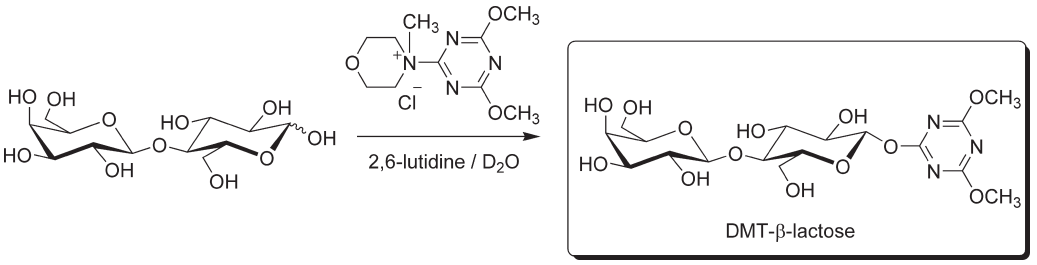
Scheme 15. Polycondensation of Cellobiose by Using Cellulase/Surfactant Complex in LiCl/DMAc



Scheme 16. One-Pot Synthesis of DMT−β-Lactose from Lactose in Aqueous Solution



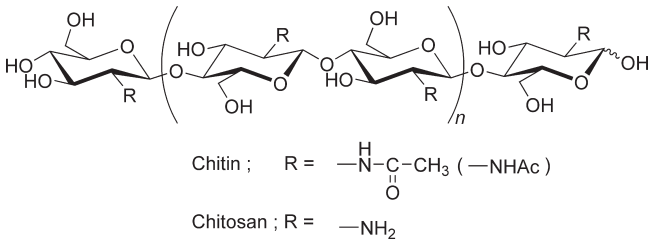
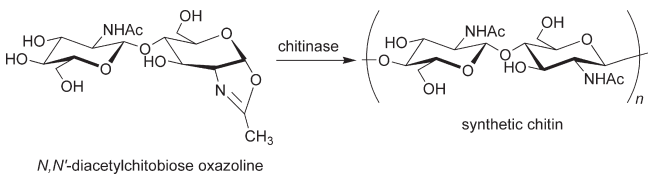


Figure 11. Structures of chitin and chitosan.

Scheme 17. Chitinase-Catalyzed Ring-Opening Polyaddition of *N,N′*-Diacetylchitobiose Oxazoline to Synthetic Chitin



where the monomer acts as both glycosyl donor and acceptor, takes place through the ring-opening polyaddition to produce a synthetic chitin. The key point for the chitinase-catalyzed ring-opening polyaddition is the similarity in the structure of the transition state involved at stages b and b′ in both the reactions.[33,34] The pH value for the optimal condition of the hydrolysis reaction by chitinase was reported to be ca. 8.0.[170] However, the chitinase-catalyzed ring-opening polyaddition took place even under the weak alkaline conditions at around pH 10−11 while simultaneously suppressing the hydrolysis of the produced chitin.

The chitinase-catalyzed glycosylation using the sugar oxazoline substrate was applied to the stepwise elongation of GlcNAc unit by utilizing two enzymes, chitinase and β-galactosidase (Scheme 18).[28,112,171−174] Because chitinase recognizes the 4-OH group of GlcNAc as a glycosyl acceptor, *N*-acetyllactosamine (Gal-β-(1→4)-GlcNAc) oxazoline (Gal = galactose), which has the axial 4-OH group at the nonreducing end, only acts as a glycosyl donor but does not act as a glycosyl acceptor for the chitinase catalysis. Thus, a first step for the chain elongation involved the chitinase-catalyzed glycosylation of the GlcNAc acceptor ($m = 1$) with *N*-acetyllactosamine oxazoline, giving rise to a new oligosaccharide having a galactose unit at the nonreducing end (step I). A second step was the removal of the galactose unit from the oligosaccharide by β-galactosidase-catalyzed hydrolysis, affording a new glycosyl acceptor with the GlcNAc unit at the nonreducing end ($m = 1$) (step II). Repetition of these procedures using the two enzymes enabled the synthesis of chitooligosaccharides with desired chain lengths.

Interestingly, *N*-acetyllactosamine oxazoline was found to be polymerized by chitinase A1 from *Bacillus circulans* WL-12 catalysis under basic conditions, giving rise to a novel oligosaccharide having the β-(1→4)-β-(1→6)-linked repeating unit in the main chain (Scheme 19).[175] The DP of the resulting oligosaccharides was up to 5 based on the disaccharide. This was the first example of enzymatic glycosylation forming β-(1→6)-glycosidic linkage by chitinase catalysis.

Various *N,N′*-diacetylchitobiose oxazoline derivatives were synthesized and subjected to the chitinase-catalyzed polymerization.[176] The chitinase-catalyzed ring-opening polyaddition using 3-*O*-methyl- and 3′-*O*-methylchitobiose oxazolines produced only chitooligosaccharide derivatives in rather low yields (Scheme 20a, b).[177] Polymerizability of the chitobiose oxazolines modified at C-6 position depended on the bulkiness of the substituents. Thus, 6-O- and 6′-O-carboxymethyled chitobiose oxazolines were hardly polymerized by chitinase catalysis, and only tetrasaccharide was produced from the latter substrate.[178,179] Despite the lower polymerizability of these four

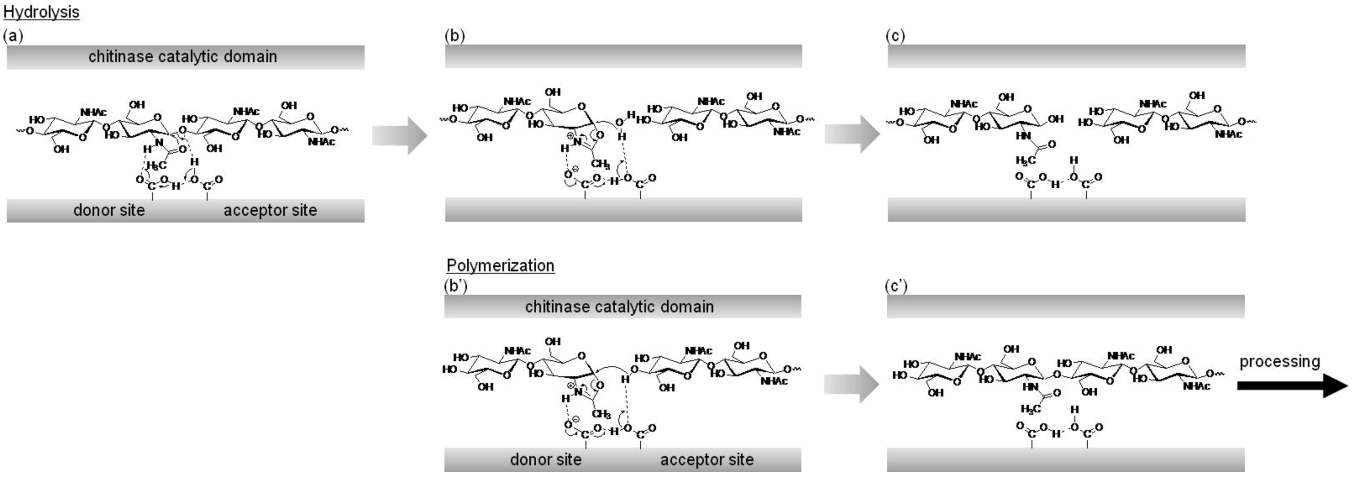
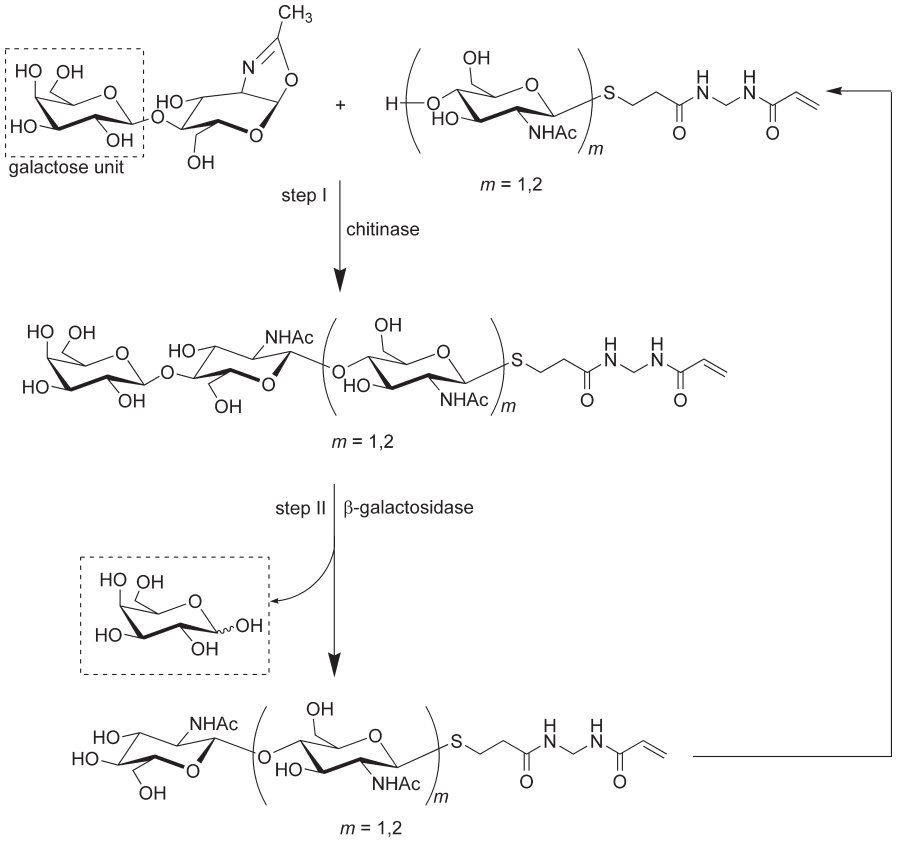


Figure 12. Postulated mechanism of chitinase catalysis for hydrolysis of chitin and ring-opening polyaddition of *N*,*N*′-diacetylchitobiose oxazoline.

Scheme 18. Stepwise Synthesis of Chitooligosaccharide Derivatives by Combined Use of Chitinase and β-Galactosidase



substrates, their conversion was accelerated in the presence of chitinase in the reaction mixture, indicating that these sugar oxazoline derivatives were recognized at the donor site of chitinase and hydrolyzed. Indeed, 6′-*O*-carboxymethylated chitobiose oxazoline acted as a good glycosyl donor for the chitinase-catalyzed glycosylation of the appropriate glycosyl acceptors to produce the corresponding tri- and tetrasaccharides in good yields.[178] On the other hand, the chitobiose oxazoline derivatives substituted with fluorine atom at C-6, C-6′, or both showed good polymerizability (Scheme 20c−e).[180,181] Under the weak alkaline conditions, the chitinase-catalyzed ring-opening polyaddition of these three substrates took place to produce the corresponding fluorinated chitins, which had average molecular weights of 1400−1700 (DPs of ca. 4 based on the disaccharide).

Chitobiose oxazoline derivatives modified at C-2′ position also showed good polymerizability.[176] For example, *N*-acetylchitobiose oxazoline, which has a free amino group at 2′-position, was polymerized by chitinase to give a water-soluble aminopolysaccharide composed of β-(1→4)-linked *N*-acetyl-D-glucosamine

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and D-glucosamine units alternatingly; this was named a chitin−chitosan hybrid polysaccharide (Scheme 21a).[182] Chitosan is generally prepared by *N*-deacetylation of chitin, and 50% *N*-deacetylated chitin exhibits good solubility in water (water-soluble chitin).[183,184] Therefore, the good solubility of the chitin−chitosan hybrid polysaccharide in water was anticipated reasonably. Chitinase-catalyzed copolymerization of this *N*-acetylchitobiose oxazoline with the aforementioned *N,N′*-diacetylchitobiose oxazoline was carried out to give chitin derivatives with the deacetylated extents ranging from 0 to 50%.[185] Because the polymerizability of these two monomers by GH18 chitinase catalysis was comparable, the extent of the deacetylation in the produced polysaccharide was controlled by the feed molar ratio of the two monomers. Because the above results indicated that chemical structure at C-2′ of chitobiose oxazolines did not strongly affect chitinase recognition, a chitobiose oxazoline derivative having an *N*-sulfonate group was designed.[186] The chitinase-catalyzed polymerization of this monomer proceeded to give the corresponding anionic aminopolysaccharide (Scheme 21b).

To suppress the chitinase-catalyzed hydrolysis of the product (or monomer) during the enzymatic polymerization, mutation of chitinase was performed. A DXE (D = Asp, X = any amino acid, E = Glu) had been found to be a general sequence at the catalytic domain of chitinase,[187,188] and, for example, wild-type chitinase A1 from *Bacillus circulans* WL-12 has D202 and E204 residues. In the case of this enzyme, D202 serves as a stabilizer for the oxazolinium intermediate and E204 protonates an oxygen atom of the glycosidic linkage to be cleaved. On the basis of the above, a mutant chitinase E204Q (Q = Gln) was prepared, which would have less protonation ability toward the oxygen of the glycosidic linkage by replacement of COOH in Glu with $CONH_2$ in Gln. This mutant enzyme exhibited less hydrolysis activity of the produced oligosaccharides by the enzymatic glycosylation.[189]

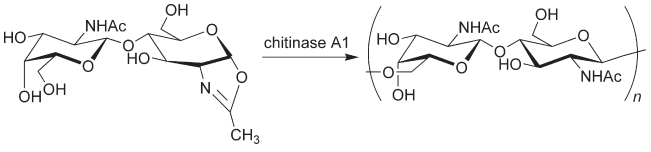
4.3.2. Higher-Ordered Self-Assembly of Synthetic Chitins. The process of the higher-ordered self-assembly of the synthetic chitin during the chitinase-catalyzed ring-opening polyaddition of *N,N′*-diacetylchitobiose oxazoline monomer was directly observed by phase-contrast and polarization microscopies in combination with SEM and TEM.[190] During the first 30 min, a small number of rectangular platelike solids were observed, which had 25 nm width, 10 nm height, and 50 nm−1 μm lengths, respectively. The electron microdiffraction measurement of the plates showed the pattern due to the thermodynamically stable crystalline structure, i.e., α-chitin. This suggested that the plate was a single crystalline of α-chitin, in which chitin chains formed an antiparallel structure via intra- and intermolecular hydrogen bonding. Then, the crystal plate grew and stacked on each other as time elapsed and shaped into ribbons, followed by the formation of bundlelike assemblies, leading to the synthetic chitin spherulites.

4.3.3. Synthesis of Non-natural Hybrid Polysaccharides by Chitinase-Catalyzed Ring-Opening Polyaddition of Sugar Oxazolines. As aforementioned, GlcNAc-β-(1→4)-Glc-β-fluoride was polymerized by cellulase catalysis to give the cellulose−chitin hybrid polysaccharide.[155] For the synthesis of the same hybrid polysaccharide, the other monomer, i.e., Glc-β-(1→4)-GlcNAc oxazoline, was also designed and synthesized on the basis of the TSAS concept.[155] This monomer was smoothly polymerized via ring-opening polyaddition catalyzed by chitinase to give the chitin−cellulose hybrid polysaccharide (Scheme 22a). Despite a high crystalline structure of both cellulose and chitin, the resulting hybrid polysaccharide showed no crystalline structure, which was confirmed by the X-ray diffraction measurement. It was also found that the presence of a larger amount of *endo*-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) induced the polymerization of Glc-β-(1→4)-GlcNAc oxazoline to produce the cellulose−chitin hybrid polysaccharide in 79% yield.[191]

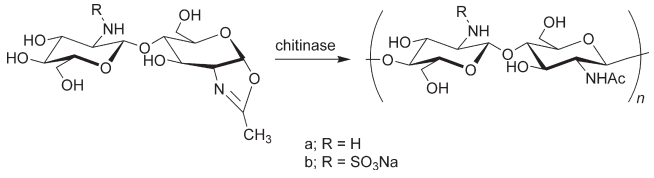
A chitin−xylan hybrid polysaccharide was synthesized by chitinase-catalyzed ring-opening polyaddition of Xyl-β-(1→4)-GlcNAc oxazoline monomer (Scheme 22b).[192] The resulting polysaccharide showed good solubility in water.

4.3.4. Synthesis of Glycosaminoglycans by Hyaluronidase-Catalyzed Ring-Opening Polyaddition of Sugar Oxazolines. Glycosaminoglycans are heteropolysaccharides, which are normally linked to various proteins to form proteoglycans. Together with collagens, fibronectins, and others, proteoglycans fill the interstitial spaces between living cells, so-called extracellular matrices,[193] and act as a compression buffer against the stress

Scheme 19. Chitinase A1-Catalyzed Ring-Opening Polyaddition of *N*-Acetyllactosamine Oxazoline



Scheme 21. Chitinase-Catalyzed Ring-Opening Polyaddition to *N*-Acetyl- and *N*-Acetyl-*N′*-sulfonylchitobiose Oxazolines



Scheme 20. Chitinase-Catalyzed Ring-Opening Polyaddition of Various *N,N′*-Diacetylchitobiose Oxazoline Derivatives

a; $R_1 = OCH_3$, $R_2 = R_3 = R_4 = OH$
b; $R_1 = R_2 = R_4 = OH$, $R_3 = OCH_3$
c; $R_1 = R_3 = R_4 = OH$, $R_2 = F$
d; $R_1 = R_2 = R_3 = OH$, $R_4 = F$
e; $R_1 = R_3 = OH$, $R_2 = R_4 = F$

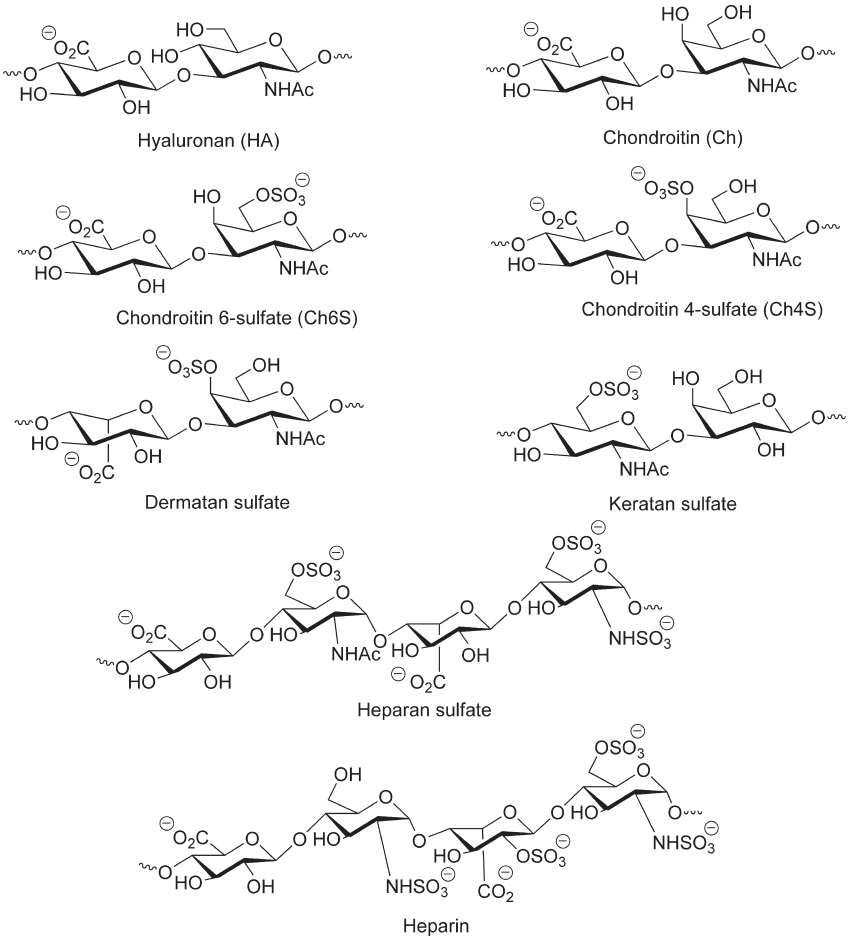
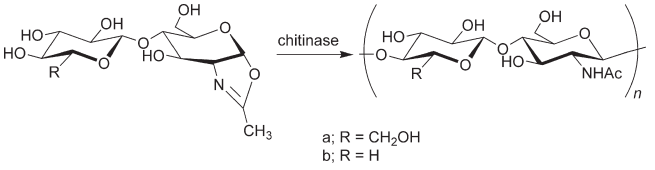


Figure 13. Structures of typical glycosaminoglycans.

Scheme 22. Chitinase-Catalyzed Ring-Opening Polyaddition of Glc-β-(1→4)-GlcNAc and Xyl-β-(1→4)-GlcNAc Oxazolines to Chitin−Cellulose and Chitin−Xylan Hybrid Polysaccharides



placed in the extracellular matrices.[194] Glycosaminoglycans include hyaluronic acid (hyaluronan, HA), chondroitin (Ch), chondroitin sulfate (ChS), dermatan sulfate, keratan sulfate, heparan sulfate, and heparin (Figure 13).[195] All glycosaminoglycans are composed of hexosamine units such as GlcNAc and *N*-acetyl-D-galactosamine (GalNAc) with uronic acid units and their sulfated derivatives. Because some glycosyl hydrolases involve an oxazolinium intermediate during the hydrolysis of the glycosidic linkages composed of GlcNAc units, on the basis of the repeating unit structures of glycosaminoglycans, the appropriate disaccharide oxazolines have been designed as a monomer for the synthesis of glycosaminoglycans by the enzymatic ring-opening polyaddition.[67,68] Hyaluronidase is one of the *endo*-β-*N*-acetylglucosaminidases responsible for metabolism of HA, Ch, and ChS.[195] The enzyme catalyzes the hydrolysis of glycosaminoglycans via a substrate-assisted mechanism similar as a chitinase involving an oxazolinium intermediate.[195−197]

Hyaluronan is a linear polysaccharide having a repeating unit of β-(1→3)-GlcNAc-β-(1→4)-GlcA (GlcA = glucuronic acid). GH56 hyaluronidase (EC 3.2.1.35) is an *endo*-type glycosyl hydrolase, which hydrolyzes β-(1→4)-glycosidic linkage between GlcNAc and GlcA. On the basis of the TSAS concept, as the design of the monomer for the synthesis of HA by the enzymatic polymerization, there are two possibilities for the monomer structures, an oxazoline-type and a fluoride-type. Because of the aforementioned characteristic of the hyarulonidase catalysis, the oxazoline-type monomer, i.e., GlcA-β-(1→3)-GlcNAc oxazoline, was synthesized and employed as a monomer. The oxazoline monomer was readily recognized and catalyzed by hyaluronidase, giving synthetic HA in a high yield (Scheme 23).[198] Stereo- and regioselectivities of the product were perfectly controlled as HA structure.

Ch exists predominantly as a carbohydrate part of proteoglycans or in the higher organisms as a precursor of ChS, mainly found in cartilage, cornea, and brain matrices. ChSs in nature are classified according to the site of sulfation.[199] The structural difference between HA and Ch chains is the difference in stereochemistry of C-4 in hexosamine unit. Therefore, on the basis of the TSAS concept, GlcA-β-(1→3)-GalNAc oxazoline monomer was designed for the synthesis of Ch via the hyaluronidase-catalyzed ring-opening polyaddition. Polymerization of this monomer was induced by hyaluronidase to give the nonsulfated Ch (Scheme 24).[200]

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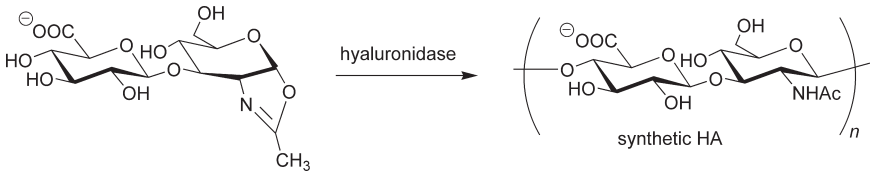
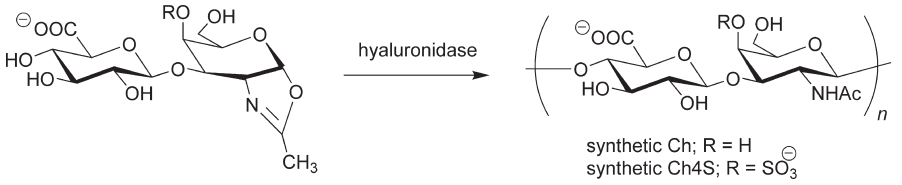
Scheme 23. Hyaluronidase-Catalyzed Ring-Opening Polyaddition of GlcA-β-(1→3)-GlcNAc Oxazoline to Synthetic Hyaluronan



Scheme 24. Hyaluronidase-Catalyzed Ring-Opening Polyaddition of GlcA-β-(1→3)-GalNAc Oxazoline Derivatives to Synthetic Chondroitin and Chondroitin 4-Sulfate



The preparation of ChS having a well-defined sulfation pattern is important for the fundamental study on the relation of the polysaccharide structure to the bioactivity. Therefore, three GlcA-β-(1→3)-GalNAc oxazoline monomers sulfated at C-4, C-6, and C-4,6 of GalNAc were synthesized, and their hyaluronidase-catalyzed polymerization was examined.[201] Consequently, the oxazoline monomer having a sulfate group at C-4 was successfully polymerized by hyaluronidase to give the corresponding ChS (Ch4S) in a high yield (Scheme 24). The other two monomers were not polymerized by hyaluronidase, and only the hydrolyzed disaccharide products were obtained. The above results suggest that hyaluronidase distinguished a sulfate group at the different position on the glycosaminoglycan chains.

For the extension of the hyaluronidase-catalyzed HA synthesis, 2-ethyl, 2-*n*-propyl, 2-isopropyl, 2-vinyl, 2-isopropenyl, and 2-phenyl oxazoline HA monomers were polymerized by the hyaluronidase catalysis, giving the corresponding HA derivatives.[202] Polymerizability of these oxazoline monomers by hyaluronidase catalysis depended on the structure of the 2-substituent in the oxazoline group, and some of the HA monomers showed a relatively close reactivity toward hyaluronidase. Therefore, copolymerization of 2-methyl, 2-vinyl, 2-ethyl, and 2-*n*-propyl oxazoline HA monomers proceeded in a stereo- and regioselective manner to yield the corresponding non-natural HA derivatives having the different *N*-acyl groups at the C-2 position.[203] By varying the comonomer feed ratios, compositions of the *N*-acyl groups were easily controlled.

The hyaluronidase-catalyzed polymerization of 2-ethyl and 2-vinyl oxazoline Ch monomers was also achieved to give the corresponding Ch derivatives.[200] On the other hand, the Ch monomers bearing 2-*n*-propyl, 2-isopropyl, and 2-phenyl groups were difficult to be polymerized, probably because of the steric hindrance of the substituents.

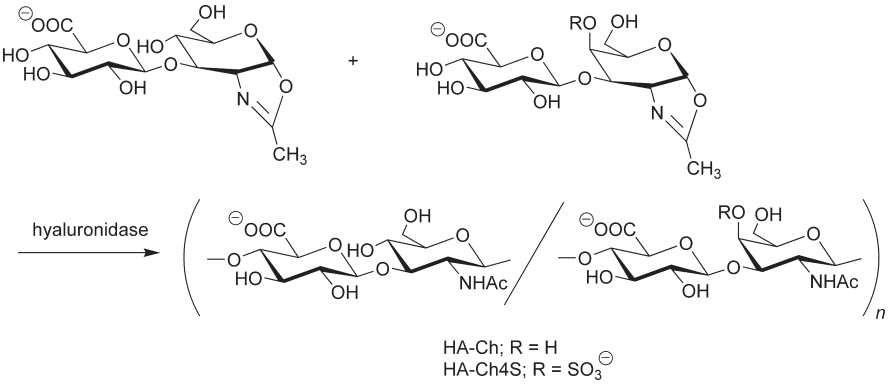
4.3.5. Synthesis of Glycosaminoglycan Hybrid Polysaccharides by Hyaluronidase-Catalyzed Ring-Opening Polyaddition of Sugar Oxazolines. Hyaluronidase-catalyzed copolymerization of HA oxazoline monomer with Ch oxazoline monomer was performed to give HA−Ch hybrid polysaccharide (Scheme 25).[204] The two monomers showed a similar copolymerizability, and the copolymerization proceeded homogeneously. Similarly, the synthesis of HA−Ch4S hybrid polysaccharide was also achieved by the copolymerization of the corresponding two oxazoline monomers by hyaluronidase catalysis (Scheme 25).[204] Copolymer compositions of the obtained hybrids were controllable by varying the comonomer feed ratios.

During the course of the studies on the hyaluronidase-catalyzed polymerization, a supercatalytic nature of hyaluronidase for the glycosaminoglycan synthesis has been found.[33,97] As described above, hyaluronidase catalyzes the polymerizations of a wide variety of sugar oxazoline monomers to yield not only natural glycosaminoglycans such as HA and Ch but also non-natural glycosaminoglycans, including the derivatives of HA and Ch as well as HA−Ch hybrid polysaccharides.

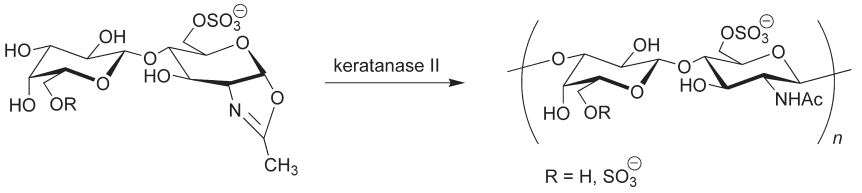
4.3.6. Synthesis of Other Poly- and Oligosaccharides by Enzymatic Ring-Opening Polyaddition of Sugar Oxazolines. Besides the chitinase-catalyzed glycosylation using the sugar oxazoline derivatives such as GlcNAc and *N,N′*-diacetylchitobiose oxazolines,[91,93] the enzymatic glycosylation using the sugar oxazolines has been extended to the catalysis of *endo*-β-*N*-acetylglucosaminidase from *Mucor hiemalis* (Endo-M) and Endo-A.[94] Endo-M- or Endo-A-catalyzed glycosylation of *p*-nitrophenyl *N*-acetyl-β-D-glucosaminoside as a glycosyl acceptor with Man-β-(1→4)-GlcNAc oxazoline as a glycosyl donor took place to give the core trisaccharide derivative (Man-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc derivative) of *N*-linked glycoproteins. Interestingly, the produced trisaccharide was not hydrolyzed by Endo-M and Endo-A. Chitinase A1 from *Bacillus circulans* WL-12 was also found to catalyze the similar glycosylation to give the *N*-linked trisaccharide core structure (Man-β-(1→4)-GlcNAc-β-(1→4)-GlcNAc) by employing Man-β-(1→4)-GlcNAc oxazoline as a glycosyl donor.[95] When the reaction was performed in the presence of 20 v/v% acetone, the trisaccharide was produced in 32% yield.

Keratan sulfate is one of the glycosaminoglycans, which has a structure of *N*-acetyllactosamine (Gal-β-(1→4)-GlcNAc) polymer linked through β-(1→3)-glycosidic linkages. C-6 of GlcNAc unit and a portion of C-6 of Gal unit are sulfated, and the main chain is covalently linked to core proteins thorough oligosaccharide linker. Keratan sulfate is hydrolyzed in vivo by keratanase and keratanase II catalyses, which catalyze the hydrolysis of Gal-β-(1→4)-GlcNAc and GlcNAc-β-(1→3)-Gal glycosidic linkages, respectively. Although there have

Scheme 25. Hyaluronidase-Catalyzed Ring-Opening Copolyaddition of GlcA-β-(1→3)-GlcNAc Oxazoline with GlcA-β-(1→3)-GalNAc Oxazoline Derivatives to HA−Ch and HA−Ch4S Hybrid Polysaccharides



Scheme 26. Keratanase II-Catalyzed Ring-Opening Polyaddition of Gal-β-(1→4)-GlcNAc Oxazoline Derivatives to Keratin Sulfate Oligomers



been two types monomers on the basis of the TSAS concept, the oxazoline-type monomers were designed for keratanase II by taking consideration of the enzymatic catalysis. Two sulfated oxazoline monomers, i.e., C-6 sulfated and C-6,6′ disulfated Gal-β-(1→4)-GlcNAc oxazolines, were recognized by keratanase II and polymerized to give keratin sulfate oligomers (Scheme 26).[205]

5. POLYSACCHARIDE SYNTHESIS CATALYZED BY PHOSPHORYLASES

5.1. Concept in Phosphorylase-Catalyzed Synthesis of Polysaccharides

Various phosphorylases have been known and all of them catalyze an *exo*-wise phosphorolysis of the glycosidic linkage at the nonreducing end.[27,31] Phosphorylases are generally classified by the anomeric forms of the glycosidic linkages in the substrates that are phosphorolyzed or by the anomeric forms of the glycose 1-phosphates that are produced. The other way employed to classify phosphorylases is describing them in terms of the anomeric retention or inversion in the reaction. The stereo- and regiospecificities of phosphorylases are very strict, and they catalyze the phosphorolysis of the specific type of glycosidic linkages (Figure 14). The characteristics are important in the exploitation of phosphorylases for the synthesis of oligo- or polysaccharides with well-defined structure via the reverse reactions. Several phosphorylases can be employed for the synthesis of polysaccharides or even oligosaccharides with relatively high DPs, but other phosphorylases recognize only disaccharide substrates and catalyze the reversible phosphorolysis to produce the corresponding glycose 1-phosphates and monosaccharides. For example, sucrose phosphorylase (EC 2.4.1.7), which is one of the most extensively studied phosphorylases, catalyzes the reversible phosphorolysis of sucrose into α-glucose 1-phosphate (α-Glc-1-P) and fructose in the presence of inorganic phosphate (Scheme 27).[206,207] Therefore, this enzyme has been used with α-Glc-1-P and fructose for the synthesis of sucrose. This type of enzyme, however, cannot be employed for the synthesis of poly- or oligosaccharides with DPs > 2. Only a few phosphorylases, i.e., α-glucan and cellodextrin phosphorylases (EC 2.4.1.1, and 2.4.1.49, respectively), have been used in various investigations for the practical synthesis of poly- or oligosaccharides with relatively higher DPs, as well as the related poly- and oligosaccharide-based materials. Although it has been reported that some phosphorylases, such as β-1,3-oligoglucan and kojibiose phosphorylases (EC 2.4.1.30 and EC 2.4.1.230), recognize glucans with the higher DPs and catalyze their phosphorolysis, there have not been many studies on the synthesis of poly- or oligosaccharides catalyzed by these enzymes.

5.2. Amylose Synthesis Catalyzed by α-Glucan Phosphorylase

Of the phosphorylases, α-glucan phosphorylase (glycogen phosphorylase, starch phosphorylase, or simply phosphorylase) is the most extensively studied and is found in animals, plants, and microorganisms.[208,209] The role of α-glucan phosphorylase is considered to be in utilization of storage polysaccharides in the glycolytic pathway.[208] This enzyme catalyzes the reversible phosphorolysis of α-(1→4)-glucans at the nonreducing end, such as glycogen and starch, in the presence of inorganic phosphate, giving rise to α-Glc-1-P.[208] By means of the reversibility of the reaction, α-(1→4)-glycosidic linkage can be constructed by the α-glucan phosphorylase-catalyzed glycosylation using α-Glc-1-P as a glycosyl donor (Scheme 28).[210] As a glycosyl acceptor in the glycosylation reaction, maltooligosaccharides with DPs higher than the

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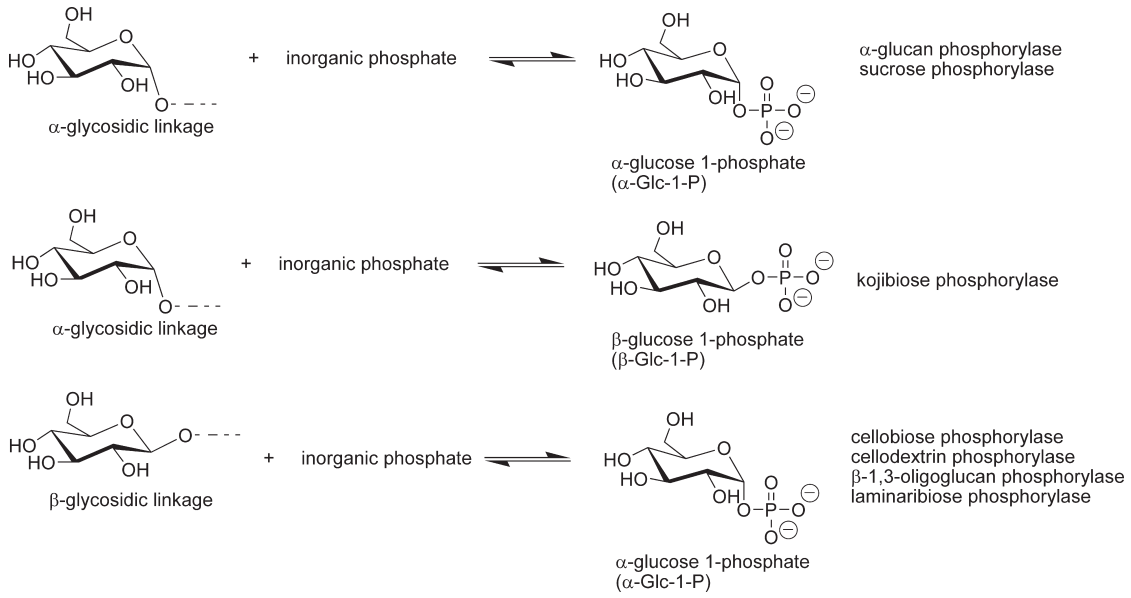
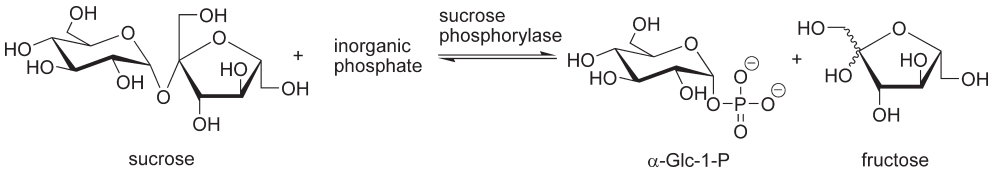


Figure 14. Catalytic patterns of phosphorylases described in this review.

Scheme 27. Catalysis of Sucrose Phosphorylase



smallest one, which is recognized by α-glucan phosphorylase, are used. The glycosyl donor is often called a "primer". The smallest glycosyl acceptor for the α-glucan phosphorylase-catalyzed glycosylation is typically maltotetraose (Glc_4), whereas that for the phosphorolysis is maltopentaose (Glc_5). In the glycosylation, a glucose unit is transferred from α-Glc-1-P to a nonreducing end of the primer to form α-(1→4)-glycosidic linkage. Then, successive reactions in the same manner occur as a propagation of polymerization to produce the α-(1→4)-glucan chain, i.e., amylose. Although amylose has been expected to be used in various industries as a functional material, a pure amylose is currently not available since the complete separation of natural amylose from amylopectin in starch is difficult. So far, α-glucan phosphorylases from various sources such as potato,[211,212] rabbit muscle,[213] and *T. aquaticus*[214] have been successfully employed in the synthesis of amylose in vitro. Because the α-glucan phosphorylase-catalyzed polymerization proceeds analogously to a living polymerization, the molecular weight of the produced amylose has a narrow distribution ($M_w/M_n < 1.2$) and can be controlled by the α-Glc-1-P/primer feed molar ratios.[211,215]

It has been reported that α-glucan phosphorylase from rabbit muscle is more suitable for the synthesis of amylose with low molecular weight in DPs of 10−20 than that from potato.[213] The amylose synthesis at elevated temperature (70 °C) was performed using the thermostable α-glucan phosphorylase from *T. aquaticus*, which resulted in the synthesis of amylose with very narrow distribution ($M_w/M_n < 1.01$).[214] Interestingly, the thermostable α-glucan phosphorylase has distinct substrate specificity, where maltotriose (Glc_3) is the smallest primer for the amylose synthesis and Glc_4 is the smallest substrate for the phosphorolysis.[216−218]

As aforementioned, α-glucan phosphorylase is useful for the practical production of amylose; however, the substrate, α-Glc-1-P, is relatively expensive. One possible solution to this problem has been reported by introduction of another enzyme that produces α-Glc-1-P. For example, the combined use of sucrose phosphorylase with α-glucan phosphorylase was reported for the in situ production of α-Glc-1-P from sucrose as the substrate for the amylose synthesis (Figure 15).[219] By using thermostable sucrose phosphorylase from *Streptococcus mutans* and triple mutant thermostable α-glucan phosphorylase (F39L/N135S/T706I, F = Phe, L = Leu, N = Asn, S = Ser, T = Thr, and I = Ile), amylose can be obtained from inexpensive sucrose in a very high yield.[220] In this case, the molecular weight of the produced amylose is strictly controlled by the sucrose/primer molar ratio.

For the purpose to produce α-Glc-1-P in situ, use of cellobiose phosphorylase (EC 2.4.1.20) combined with α-glucan phosphorylase was also examined (Figure 15).[221−223] Cellobiose phosphorylase catalyzes the phosphorolysis of cellobiose in the presence of inorganic phosphate to produce α-Glc-1-P and glucose (Scheme 29).[224] When partially purified cellobiose phosphorylase was incubated with cellobiose and α-glucan phosphorylase in the presence of inorganic phosphate, amyloses with various molecular weights (4.2×10^4−7.3×10^5, DPs = 2.5×10^2−4.3×10^3) were produced, but yield was not high. However, the yield was increased by adding mutarotase (EC 5.1.3.3) and glucose oxidase (EC 1.1.3.4) into the initial reaction mixture, which removed glucose accumulated in the reaction mixture.

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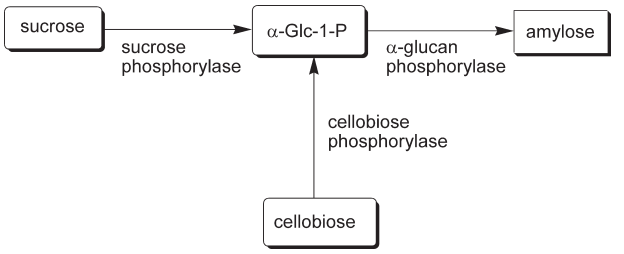
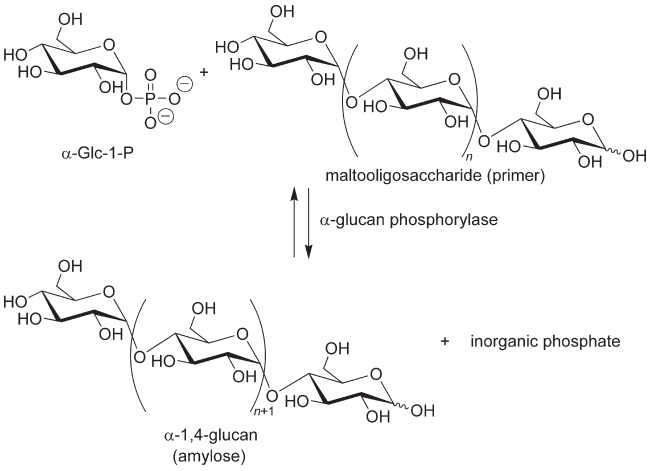


Figure 15. Production of amylose by combined use of sucrose or cellobiose phosphorylase and α-glucan phosphorylase.

Scheme 28. α-Glucan Phosphorylase-Catalyzed Glycosylation and Phosphorolysis

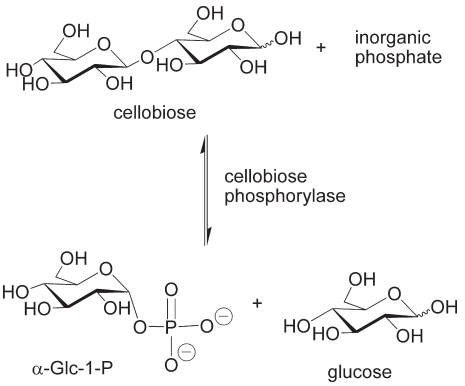


Deinococcus geothermalis glycogen branching enzyme (EC 2.4.1.18) is known to catalyze the redistribution of short α-glucans via inter- and intramolecular chain transfer from α-(1→4) positions to α-(1→6) positions. The combined use of α-glucan phosphorylase and glycogen branching enzyme gave highly branched amylose from α-Glc-1-P.[225]

Apparent production of an enzymatically synthesized amylose in DMSO was performed by means of the calcium alginate hydrogel beads/DMSO system as the reaction field of the α-glucan phosphorylase-catalyzed polymerization.[226] When the calcium alginate hydrogen beads including α-Glc-1-P, maltoheptaose (Glc_7), and α-glucan phosphorylase were suspended in DMSO and the system was slowly stirred at 40 °C for 12 h, the reaction proceeded to produce amylose, which eluted to the DMSO solution. The time-course experiment in this system revealed that the α-glucan phosphorylase-catalyzed polymerization took place for 15 min on the inside of the calcium alginate beads and the produced amylose gradually eluted to the surrounding DMSO solution.

One of the significant characteristics in the α-glucan phosphorylase-catalyzed polymerization is to initiate the reaction from a nonreducing end of the primer.[227] Therefore, the modified primers can be employed for the α-glucan phosphorylase-catalyzed polymerization to introduce the functional groups at the chain end of amylose or maltooligosaccharides. For example, the α-glucan phosphorylase-catalyzed polymerization using 2-chloro-4-nitrophenyl β-maltoheptaoside (Glc_7-CNP) was performed to produce CNP−maltooligosaccharides with longer chain lengths in the range of DPs = 8−11.[228] These maltooligosaccharide substrates were indispensable tools in the study on the binding sites and the actions of α-amylases having longer binding area than that of human α-amylase.

Scheme 29. Catalysis of Cellobiose Phophorylase



Glycogen is known as a high-molecular-weight and water-soluble polysaccharide, composed of linear chains containing an average of 10−14 (1→4)-linked α-glucose residues, which are interlinked by α-(1→6)-glycosidic linkages to form highly branched structure.[229,230] Besides glycogen being a substrate for the in vivo phosphorolysis by glycogen phosphorylase, it was used as a primer for the α-glucan phosphorylase-catalyzed polymerization. When the α-glucan phosphorylase-catalyzed chain elongation of glycogen using α-Glc-1-P was carried out, the reaction mixture turned into a hydrogel form, which was probably caused by formation of junction zones based on the double helix structure of the elongated amylose chains among the glycogen molecules (Figure 16).[231]

Because α-glucan phosphorylase has shown loose specificity for recognition of the structure of the glycosyl donor, the α-glucan phosphorylase-catalyzed glycosylation using different glycose 1-phosphates has been carried out. The α-glucan phosphorylase-catalyzed chain elongation of glycogen with 3- or 4-deoxy-α-D-glucose 1-phosphates was attempted.[232] However, only averages of up to 1.5 units were transferred. 1,2-Dideoxy-D-glucose (D-glucal) was also applied as a glycosyl donor with a glucal/primer ratio of 15:1 for occurrence of the α-glucan phosphorylase-catalyzed chain elongation of 2-deoxy-α-glucose unit to the primer in the presence of inorganic phosphate (Scheme 30).[233] In this system, 2-deoxy-α-D-glucose 1-phosphate (dGlc-1-P) was enzymatically produced in the following two reactions (Scheme 30a).[234] First, 2-deoxy-D-glucose unit is transferred to the α-glucan primer that is catalyzed by inorganic phosphate. Second, 2-deoxy-D-glucose is released by phosphorolysis to yield dGlc-1-P, and in the overall reaction the primer remains unchanged. After the α-glucan phosphorylase-catalyzed reaction in the presence of D-glucal, Glc_4, and only 0.05 equiv of inorganic phosphate for 6 h, 2-deoxy-α-D-glucosylated penta-, hexa-, and heptasaccharides were separated by size-exclusion chromatography in 17, 12, and 8% yields, respectively (Scheme 30b). Additionally, a fraction of higher molecular weight with an average DP of 12 was obtained in 33% yield.

Other glycose 1-phosphates, such as α-D-xylose, α-D-mannose, α-D-glucosamine, and *N*-formyl-α-D-glucosamine 1-phosphates, have also been recognized by α-glucan phosphorylase as a glycosyl donor, and each one monosaccharide residue has transferred to a nonreducing end of the primer (Figure 17).[235−238]

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However, further transfer of the monosaccharide residues from the glycose 1-phosphates did not take place because of no recognition of the produced oligosaccharides as the glycosyl acceptor by α-glucan phosphorylase, owing to which there was a different monosaccharide residue from glucose at the nonreducing end.

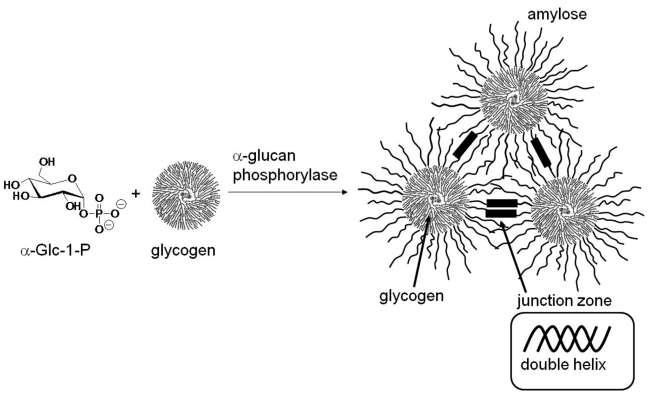
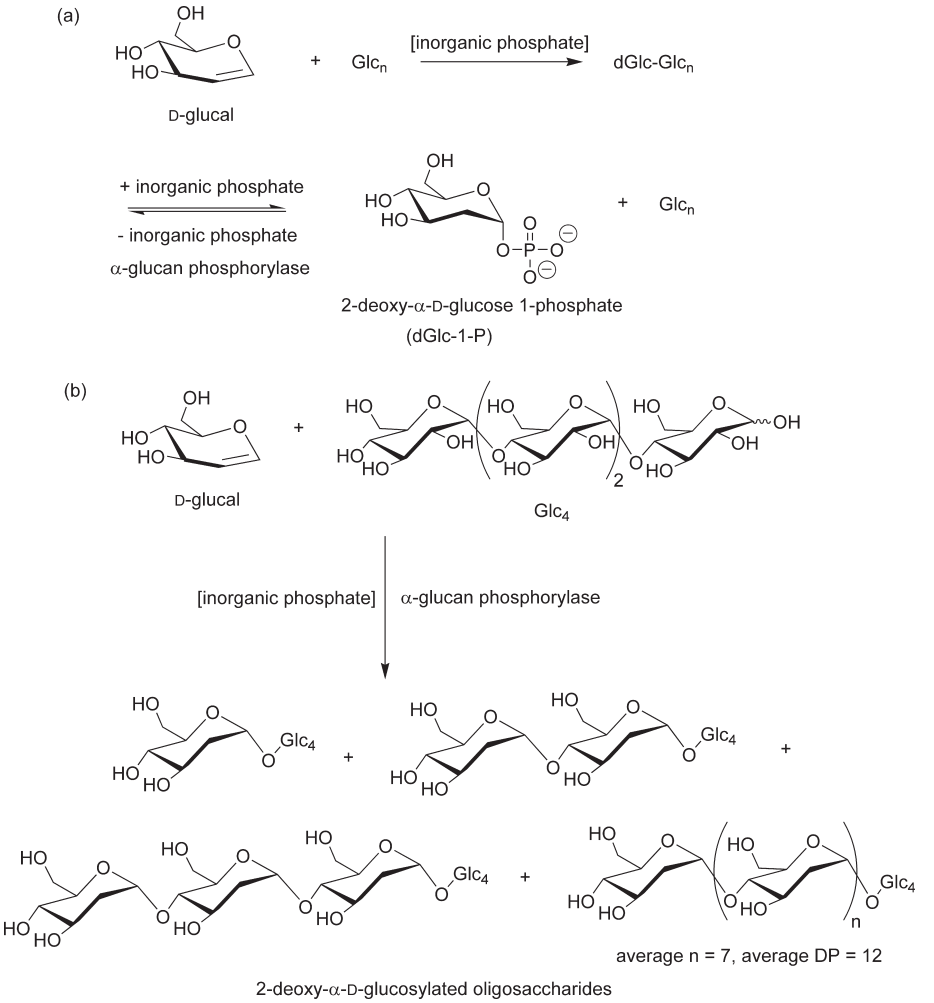


Figure 16. Schematic reaction for α-glucan phosphorylase-catalyzed chain elongation of glycogen to form hydrogels.

5.3. Chemoenzymatic Synthesis of Amylose-Grafted Polysaccharides and Polypeptide by Utilizing α-Glucan Phosphorylase Catalysis

Branched heteropolysaccharides are often found in nature. For example, arabinoxylan, arabic gum, and guar gum play important roles in moisture maintenance and protection against bacteria.[2] These materials are composed of two or more different kinds of saccharide components, which contribute to their prominent functions. Therefore, the preparation of artificial heteropolysaccharides with a branched or grafted structure is expected to give rise to novel high-performance biomaterials. On the basis of these viewpoints, amylose-grafting heteropolysaccharides have been synthesized by the combined method of the α-glucan phosphorylase-catalyzed polymerization with the chemical reaction according to Figure 18.[239,240] This chemoenzymatic method was achieved by the introduction of maltooligosaccharide primers to the abundant polysaccharide chain and the subsequent α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P. For the introduction of the primers to the polysaccharide main chain, a reductive amination was employed because it has been reported as the reaction that efficiently occurred between a reducing end of the oligosaccharide chain and the amino group in an aminopolysaccharide.[241]

Scheme 30. Two-Step Synthesis of dGlc-1-P in the Presence of Inorganic Phosphate and Phosphorylase (a) and α-Glucan Phosphorylase-Catalyzed Synthesis of 2-Deoxy-α-D-glucosylated Oligosaccharides (b)



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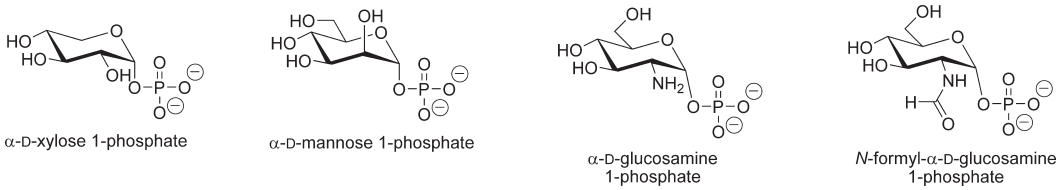


Figure 17. Glycose 1-phosphates that are recognized by α-glucan phosphorylase as a glycosyl donor.

By this chemoenzymatic method, for example, amylose-grafted chitin and chitosan were synthesized (Scheme 31). First, the Glc_7 primer was introduced to chitosan by the reductive amination using $NaBH_3CN$ in a mixed solvent of aqueous acetic acid/methanol to give a maltooligosaccharide-grafted chitosan. This material was converted into a maltooligosaccharide-grafted chitin by *N*-acetylation using acetic anhydride. The α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the maltooligosaccharide primers on these chitin and chitosan derivatives was then performed to obtain the amylose-grafted chitin and chitosan.[242,243]

The same method was also applied to cellulose main chain (Scheme 32). It was necessary to introduce amino groups into cellulose main chain for the subsequent reductive amination with maltooligosaccharide. Thus, the reductive amination of an amine-functionalized cellulose with Glc_7 was performed to give a maltooligosaccharide-grafted cellulose. Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the maltooligosaccharide primers on the cellulose main chain was carried out to produce the amylose-grafted cellulose.[244] When the reaction mixture of the enzymatic polymerization was left standing at room temperature for several days, the hydrogel was obtained.

The chemoenzymatic method was extended to an anionic polysaccharide of alginic acid[245] to give the amylose-grafted alginate.[246] For this approach, an amine-functionalized maltooligosaccharide was chemically introduced into sodium alginate by condensation with carboxylates of the alginate to produce a maltooligosaccharide-grafted alginate. Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the maltooligosaccharide primers on the alginate main chain was conducted to produce the amylose-grafted alginate. Beads were produced from the product by treatment with calcium chloride aqueous solution, which exhibited the enzymatic disintegratable property by β-amylase.

Naturally occurring saccharide−polypeptide conjugates such as glycoproteins and proteoglycans play important roles in living systems.[193,194,247] In relation to these substances, an amylose-grafted poly(L-glutamic acid) was synthesized by the chemoenzymatic method.[248] First, maltopentaosylamine was condensed with the pendant carboxyl groups of poly(L-glutamic acid) using a condensing agent to give a maltopentaose-grafted poly(L-glutamic acid). Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the maltooligosaccharide primers on the poly(L-glutamic acid) main chain was performed to produce the amylose-grafted poly(L-glutamic acid).

5.4. Chemoenzymatic Synthesis of Amylose-Grafted Synthetic Polymers and Amylosic Block Copolymers by Utilizing α-Glucan Phosphorylase Catalysis

The aforementioned chemoenzymatic approach has been the efficient method not only for the synthesis of the amylose-grafted natural polymers but also for the synthesis of the amylose-grafted synthetic polymers. There are generally two typical synthetic

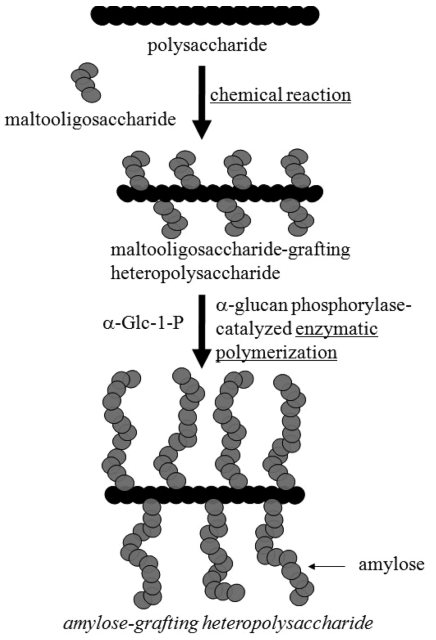
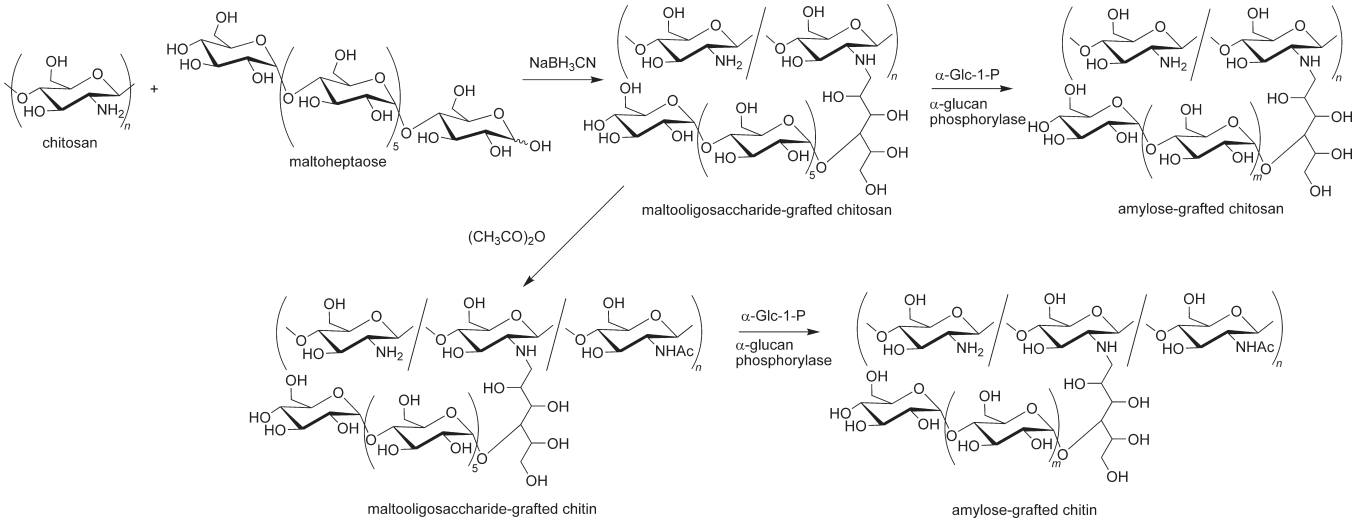


Figure 18. Schematic reaction for chemoenzymatic synthesis of amylose-grafting heteropolysaccharides by means of α-glucan phosphorylase-catalyzed enzymatic polymerization.

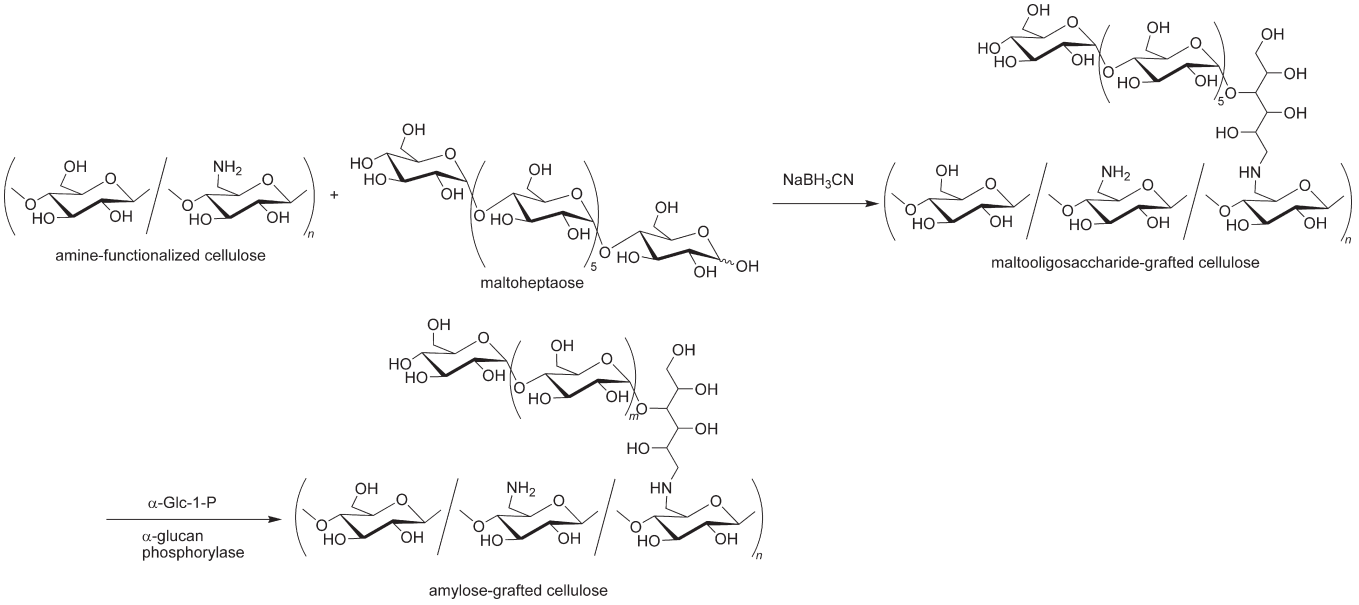
ways, i.e., a polymer reaction method and a macromonomer method (Figure 19), for the preparation of the amylose-grafted synthetic polymers by utilizing α-glucan phosphorylase-catalyzed polymerization; the former is the same manner as that for the amylose-grafted natural polymers described in section 5.3.[240,249]

An amylose-grafted polystyrene was prepared by two different approaches from a styrene-type macromonomer having a maltooligosaccharide chain (Scheme 33), which was prepared by the reaction of a Glc_5 lactone with 4-vinylbenzylamine.[250,251] In route I, the α-glucan phosphorylase-catalyzed chain elongation of α-Glc-1-P from the macromonomer was first performed to give a styrene-type macromonomer having an amylose chain. The radical polymerization of the product gave the desired amylose-grafted polystyrene. In route II, on the other hand, the radical polymerization of the macromonomer having a maltooligosaccharide chain was first carried out, followed by the enzymatic chain elongation by α-glucan phosphorylase catalysis, giving rise to the amylose-grafted polystyrene. Every repeating unit in the produced polystyrene derivative by route I has the amylose chains, whereas the amylose chains are probably present partially in the repeating units of the polystyrene derivative obtained by route II because of the probable occurrence of the enzymatic chain elongation from a part of the maltooligosaccharide primers on the polystyrene main chain due to steric hindrance in the latter case. The α-glucan

Scheme 31. Chemoenzymatic Synthesis of Amylose-Grafted Chitin and Chitosan by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



Scheme 32. Chemoenzymatic Synthesis of Amylose-Grafted Cellulose by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



phosphorylase-catalyzed polymerization was also performed using the maltooligosaccharide-grafted polystyrene prepared by 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)-mediated living radical polymerization.[252]

Amylose-grafted polyacetylenes were synthesized by both the macromonomer and polymer reaction methods (Scheme 34). In the macromonomer method (Scheme 34a),[253] an acetylene-type macromonomer having a maltooligosaccharide chain was polymerized by Rh-catalyst to give a polyacetylene with pendant maltooligosaccharide graft chains. Moreover, the Rh-catalyzed copolymerization of the macromonomer with another acetylene monomer was also carried out. However, the DPs of the products obtained by both the above polymerization and copolymerization were not high (ca. 10), probably due to steric hindrance of the bulky oligosaccharide graft chains in the macromonomer and the produced polyacetylene derivatives. The α-glucan phosphorylase-catalyzed enzymatic polymerization of α-Glc-1-P from the maltooligosaccharide graft chains on these polyacetylene derivatives was performed to yield two types of the amylose-grafted polyacetylenes (homopolyacetylene and copolyacetyne). The complex formation with iodine has been a well-known characteristic property of amylose.[254] Accordingly, the colorless solutions of the amylose-grafted polyacetylenes in DMSO turned into violet by adding a standard iodine-iodide solution to the polymer solutions, as the same color change in the complex formation of amylose with iodine. The values of the λ_{max} in the UV−vis spectra of the iodine complexes with the amylose graft chains on the homopolyacetylene and copolyacetylene main chains, and a sole amylose, were 577, 586.5, and 586.5 nm,

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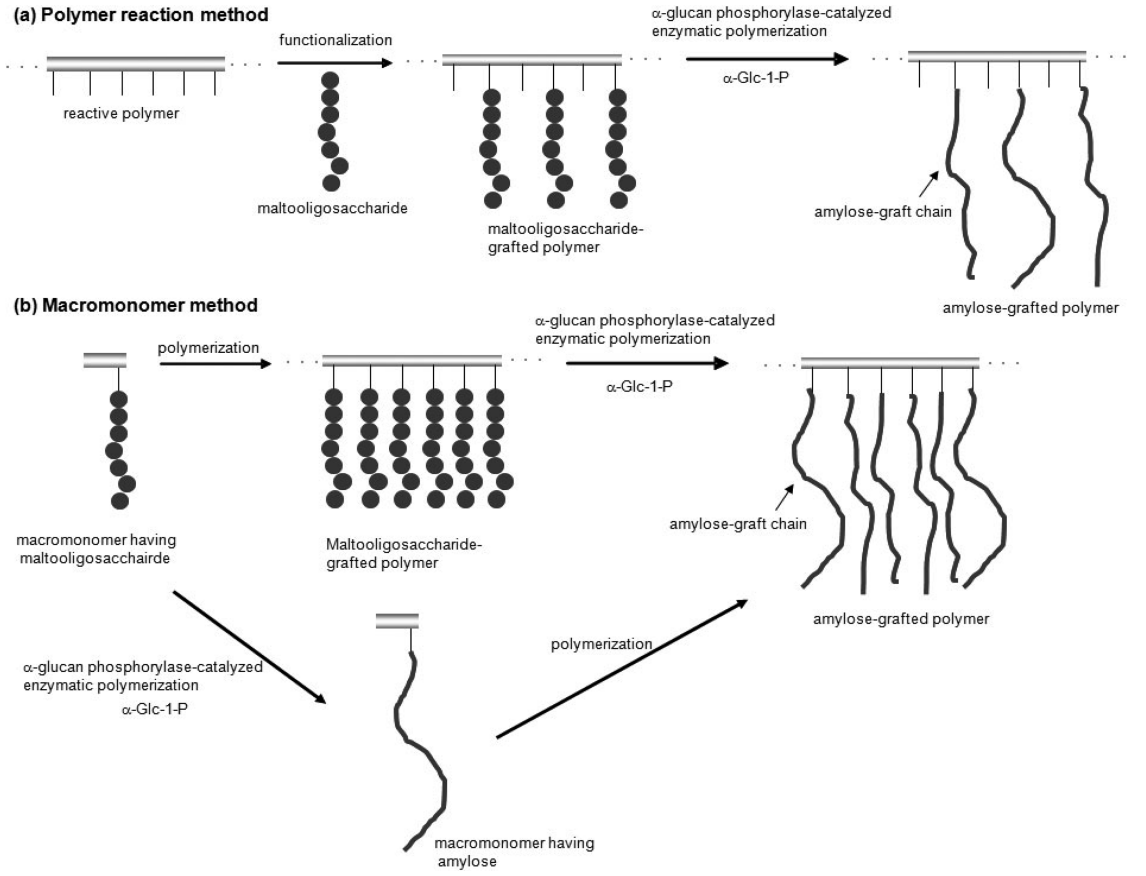
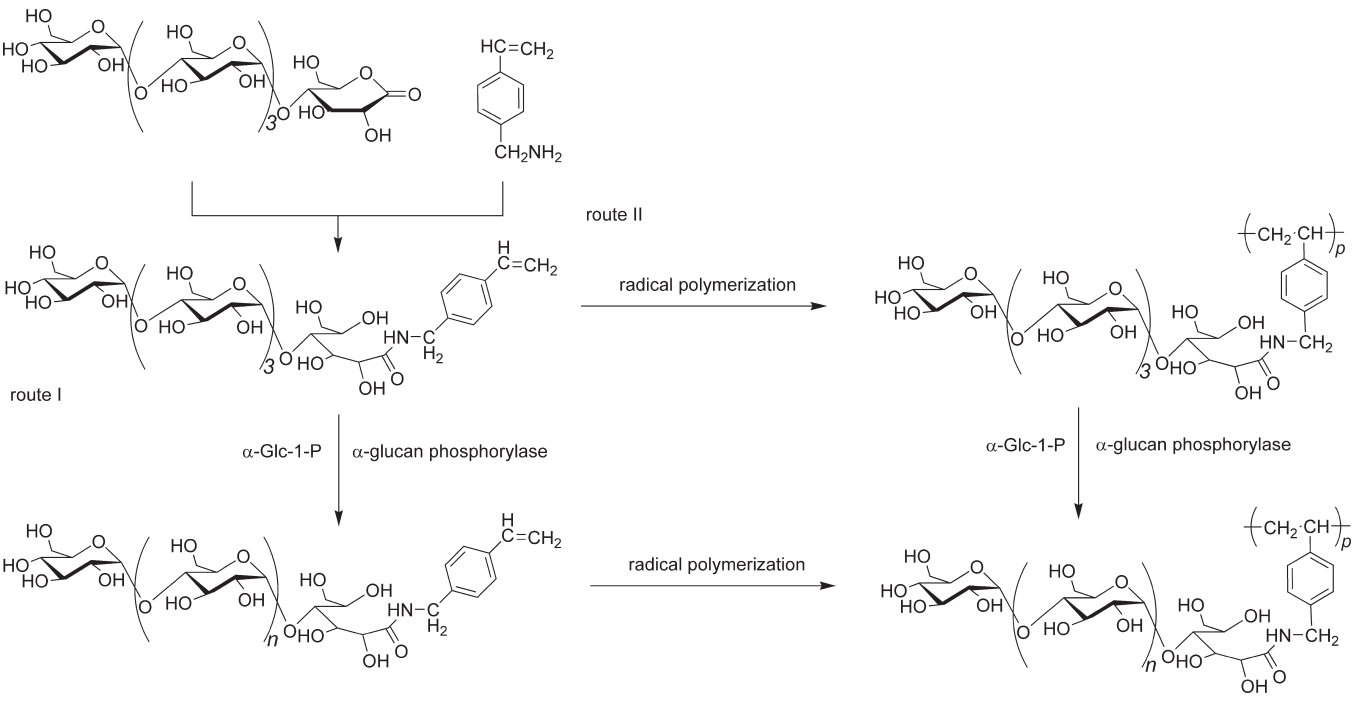


Figure 19. Two approaches for chemoenzymatic synthesis of amylose-grafted polymers: polymer reaction method (a) and macromonomer method (b).

Scheme 33. Chemoenzymatic Synthesis of Amylose-Grafted Polystyrene by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



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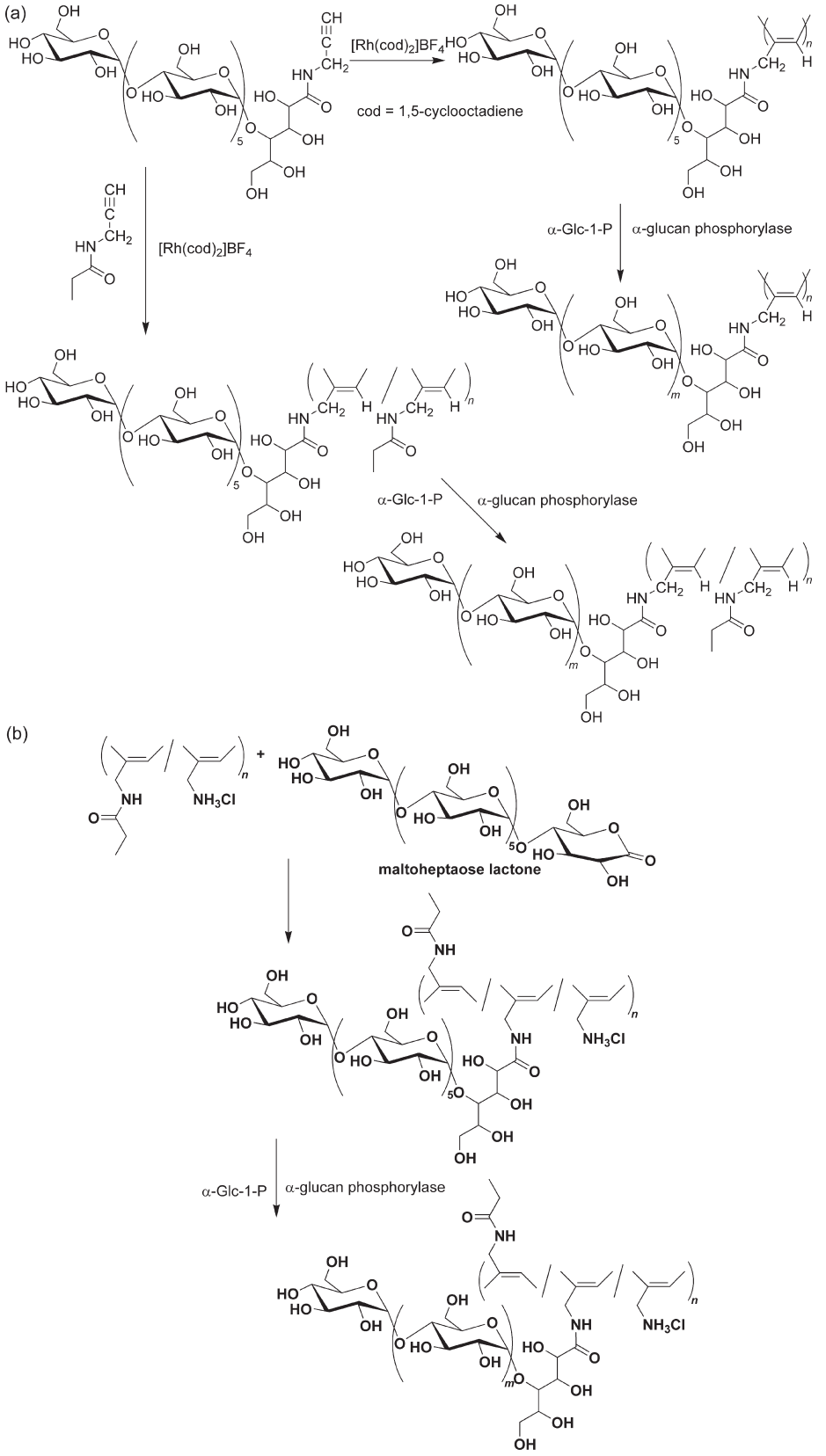
Scheme 34. Chemoenzymatic Synthesis of Amylose-Grafted Polyacetylenes by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization by Macromonomer Method (a) and by Polymer Reaction Method (b)



respectively; the sole amylose was prepared by the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using Glc_7 as a primer under the same conditions as those for the amylose-grafted polyacetylenes. These data indicated that the average DP

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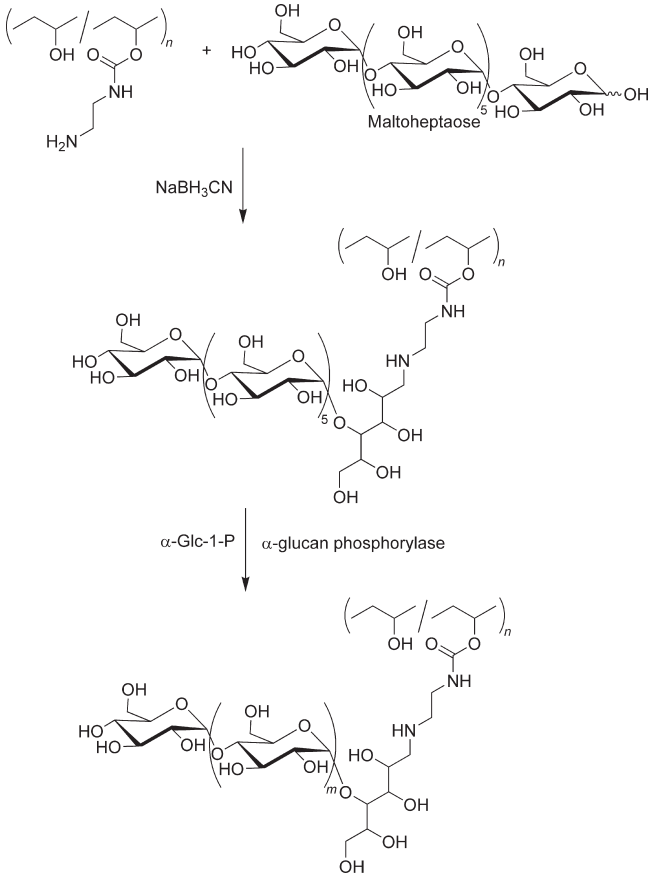
Scheme 35. Chemoenzymatic Synthesis of Amylose-Grafted Poly(vinyl alcohol) by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



of the amylose graft chains on the copolyacetylene was probably comparable to that of the sole amylose, whereas the average DP of the amylose graft chains on the homopolyacetylene might be lower in comparison with the sole amylose. On the basis of the above results, the following difference in the manners during the enzymatic polymerization using the maltooligosaccharide-grafted homopolyacetylene and copolyacetylene was assumed. The enzymatic polymerization was hardly initiated from all the potential sites of the maltooligosaccharide primers on the homopolyacetylene main chain due to steric hindrance, resulting in the lower average DP. On the other hand, the less-hindered orientation of the maltooligosaccharide primers on the copolyacetylene main chain was probably more suitable for initiation from most of the potential sites for the enzymatic chain elongation.

To obtain the amylose-grafted polyacetylene with higher DP of the main chain, the approach according to the polymer reaction method was conducted (Scheme 34b).[255] First, the reaction of a Glc_7 lactone with an amine-functionalized polyacetylene with the high DPs (ca. 72−112) was performed, giving rise to a maltooligosaccharide-grafted polyacetylene. Then, the α-glucan phosphorylase-catalyzed polymerization from the maltooligosaccharide primers on the polyacetylene derivative was carried out to produce the desired amylose-grafted polyacetylene with the high DPs.

An amylose-grafted poly(vinyl alcohol) (PVA) was also synthesized by the chemoenzymatic method (Scheme 35).[256] First, Glc_7 was introduced to an amine-functionalized PVA by the reductive amination using $NaBH_3CN$ to produce a maltooligosaccharide-grafted PVA. Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the maltooligosaccharide graft chains on the PVA main chain was conducted to obtain the amylose-grafted PVA. A film of the product was soaked in an iodine−iodide ethanol solution to form an iodine-doped film. The UV−vis analysis of the doped film indicated that iodine was retained in this film even after it was left standing for 24 h. This was probably because the iodide ions were contained in the cavities of the amylose graft chains in this film.

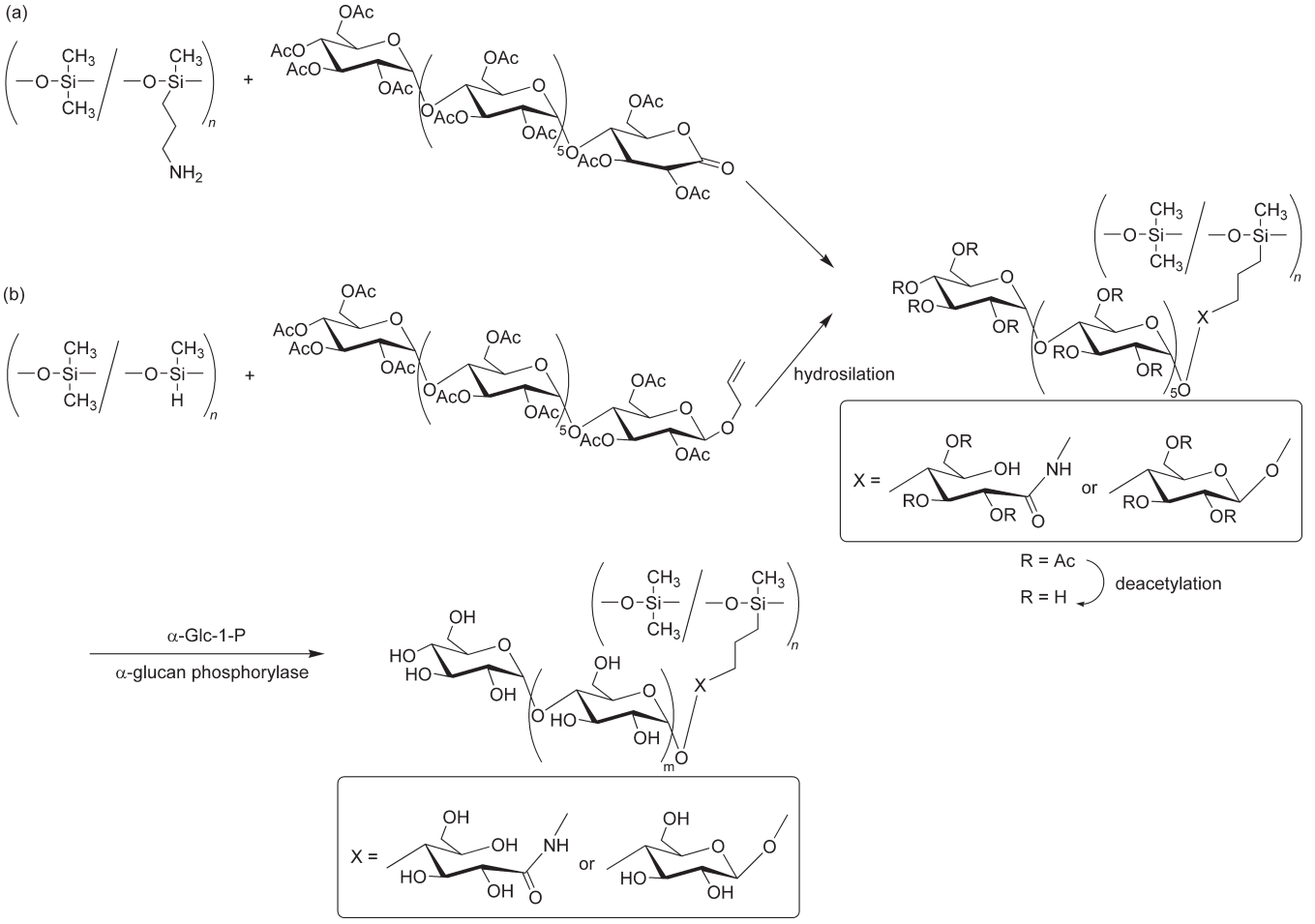
The chemoenzymatic approach by utilizing the α-glucan phosphorylase-catalyzed polymerization was applied to the preparation of amylose-grafted inorganic polymeric materials.[257] Inorganic polymers have various interesting properties, e.g., high oxygen permeability, low toxicity, and biocompatibility, which are advantages as practical biomaterials.[258] Therefore, saccharide−inorganic polymeric hybrids would be expected to have a significant potential for biological applications. Thus, amylose-grafted polydimethylsiloxanes (PDMSs) were synthesized as follows.[259,260] First, maltooligosaccharide-grafted PDMSs were prepared by the reaction of a Glc_7 lactone derivative with an amine-functionalized PDMS (Scheme 36a) or the hydrosilylation of an allylated Glc_7 with a PDMS derivative having Si−H linkage (Scheme 36b), followed by deacetylation. Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using the maltooligosaccharide-grafted PDMSs was carried out to give the amylose-grafted PDMSs.

The chiral recognition ability of amylose derivatives is one of the significant functions for their practical use of the amylose-conjugated materials.[261] On the basis of this viewpoint, silica-gel bounded by amylose through the α-glucan phosphorylase-catalyzed polymerization was prepared, and the chiral recognition ability of its phenylcarbamate derivative was investigated.[262] Amylose chains having reactive groups at the reducing end were first prepared by the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using the appropriate maltooligosaccharide derivatives as a primer. Then, the products were chemically bounded to silica gel, followed by derivatization by 3,5-dimethylphenyl isocyanate to give the silica gels bounded by the phenylcarbamoyl amylose, which were used as the chiral stationary phase in HPLC. The chiral recognition abilities of these materials were investigated using racemic *trans*-stilbene oxides by HPLC. The enantiomers eluted at different retention times, indicating that racemic *trans*-stilbene oxides were completely separated.

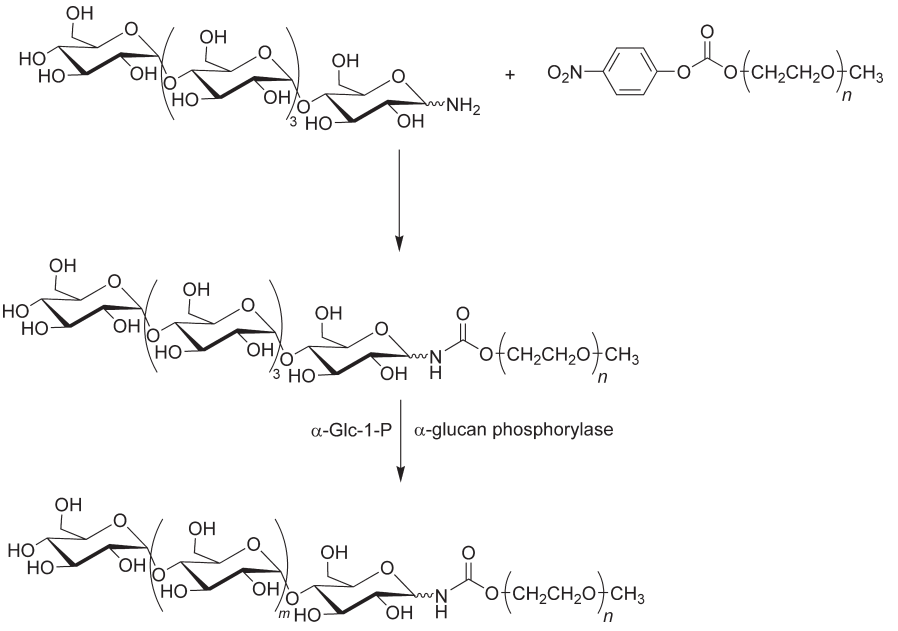
Amylosic diblock copolymers were also synthesized by utilizing the α-glucan phosphorylase-catalyzed polymerization using the polymeric primers having a maltooligosaccharide moiety at the chain end. For example, amylose-*block*-polystyrenes were synthesized by the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using the polystyrene having a maltooligosaccharide moiety at the chain end as a primer, which was prepared by the reaction of a Glc_7 lactone with an amine-terminated polystyrene.[263−265] The kinetics of the enzymatic polymerization showed an interesting dependence on the molecular weight of polystyrene due to the micellar structure of the primer in water.

The synthesis of an amphiphilic methoxy poly(ethylene oxide) (MPEO)-*block*-amylose and its complexation with methyl orange (MO) were investigated (Scheme 37).[266,267] First, an MPEO-primer was prepared by the condensation of maltopentanosylamine with MPEO-*p*-nitrophenylcarbonate. Then, the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from the primer was carried out to give the MPEO-*block*-amylose. A sole

Scheme 36. Chemoenzymatic Synthesis of Amylose-Grafted Polydimethylsiloxanes Using Glc_7 Lactone Derivative (a) and Allylated Glc_7 (b) by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



Scheme 37. Synthesis of Amphiphilic Methoxy Poly(ethylene oxide)-*block*-amylose by Means of α-Glucan Phosphorylase-Catalyzed Enzymatic Polymerization



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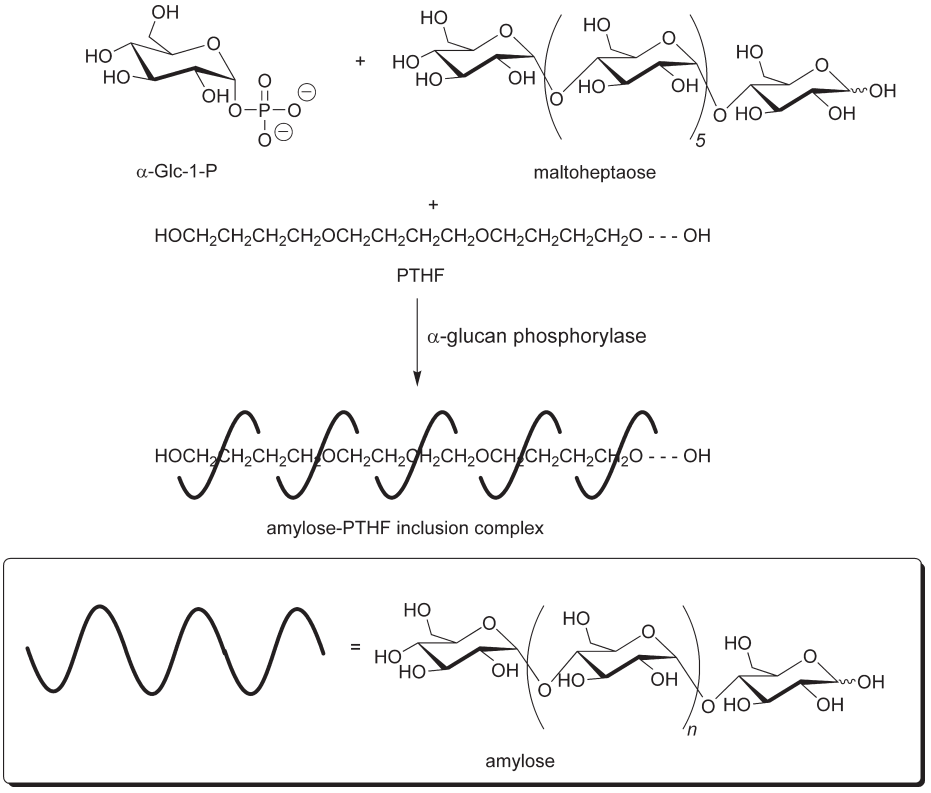
Scheme 38. Formation of Amylose-PTHF Inclusion Complex in α-Glucan Phosphorylase-Catalyzed Polymerization in the Presence of PTHF



amylose is insoluble in chloroform, but the product was slightly soluble in chloroform. It was confirmed that the complexation of the MPEO-*block*-amylose with MO was significantly enhanced in the amylose domain of the associate in chloroform.

Maltopentaose-*block*-alkyl chain surfactants ($C8Glc_5$, $C12Glc_5$, $C16Glc_5$) were synthesized, where an alkyl group (C8, C12, C16) is linked to the reducing end of Glc_5.[268,269] The primers were prepared by the reaction of Glc_5 with octyl-, dodecyl-, or hexadecylamine. The primer surfactants formed micelles in water, which were dissociated upon the α-glucan phosphorylase-catalyzed polymerization. The enzymatic polymerization of α-Glc-1-P using the primer surfactants was performed in the presence of phosphorylase b and adenosine 5′-monophosphate sodium salt (AMP) in a Bis-tris buffer at 40 °C; this enzyme is activated by AMP. By using the property of the micelle formation of the primer surfactants, the micelle-to-vesicle transition of the mixed lipid/primer systems was caused by the enzymatic polymerization and could be controlled. Consequently, $C12Glc_5$ micelles were viewed as enzyme-responsive molecular assembly systems. An enzyme-responsive artificial chaperone system using the amphiphilic primer ($C12Glc_5$) as a surfactant and phosphorylase b was designed to enable protein refolding. Effective refolding of carbonic anhydrase B after both heat denaturation (70 °C for 10 min) and guanidine hydrochloride (6M) denaturation was observed by controlled association between the protein molecules and the $C12Glc_5$ primer micelle through the enzymatic polymerization.

Branched amylosic polymers of star and comb shapes were also obtained by the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using difunctional primers composed of oligo(methylene)- and oligo(oxyethylene)-blocks and multifunctional primers composed of poly(vinylamine) chains.[210]

5.5. Formation of Inclusion Complexes of Amylose with Synthetic Polymers in the α-Glucan Phosphorylase-Catalyzed Polymerization System

Amylose has been known as a host molecule, which forms inclusion complexes with various monomeric organic guest molecules owing to its helical conformation.[270] However, little had been reported regarding the formation of inclusion complexes between amylose and polymeric molecules. The principal difficulty for incorporating polymeric materials into an amylose cavity is that the driving force for the binding is only due to weak hydrophobic interactions between host and guest molecules. Amylose, therefore, does not have sufficient ability to include the long chain of the polymeric guest into its cavity. A method for the formation of such inclusion complexes between amylose and polymeric guest molecules has been developed by means of the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P.[34,35,112,173,249,271−275] A first example of the method was achieved using poly(tetrahydrofuran) (PTHF) as a guest polymer (Scheme 38).[276] When the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P using Glc_7 as a primer was performed in the presence of the dispersed PTHF in a buffer solvent, the polymerization proceeded with forming the amylose−PTHF inclusion complex, which was obtained as a precipitate in the reaction mixture. Mixing amylose and PTHF in the buffer solution did not afford the formation of the inclusion complex, suggesting that the inclusion complex formed during the enzymatic polymerization. When the effect of the end groups (OH, OCH_3, OCH_2CH_3, OCH_2Ph) of the guest PTHFs was investigated,[277] the results indicated that the formation of the inclusion complex was strongly affected by the bulkiness of the

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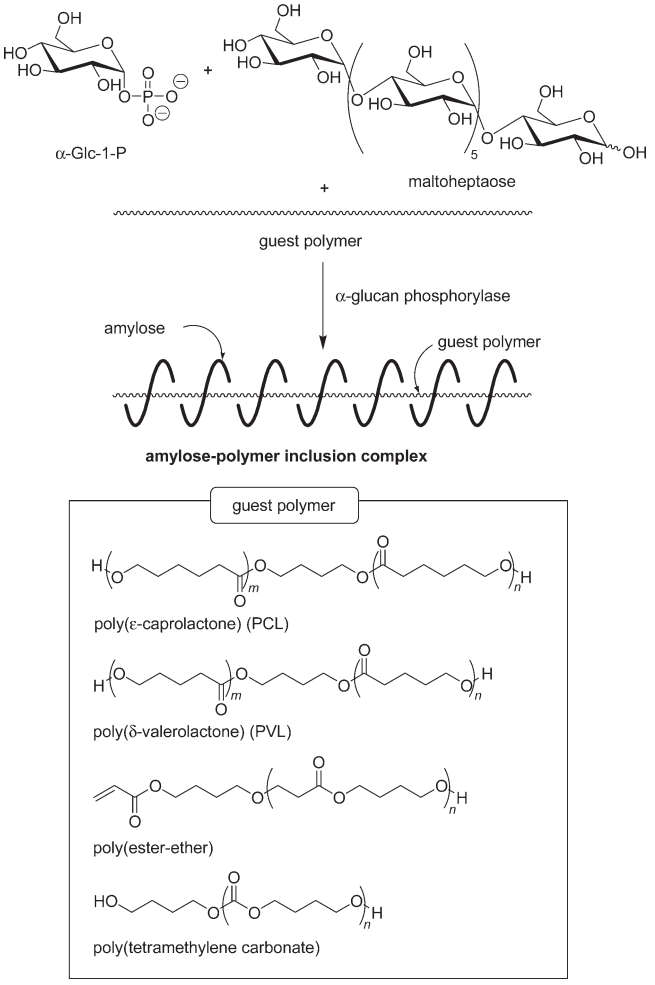
Scheme 39. Formation of Amylose-Polymer Inclusion Complexes in α-Glucan Phosphorylase-Catalyzed Polymerization in the Presence of Hydrophobic Guest Polymers



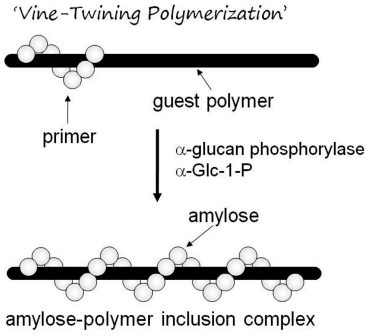


Figure 20. Image of "vine-twining polymerization".

end groups of PTHFs. Furthermore, it has been confirmed that the hydrophobicity of the guest polymers is a very important factor in whether the inclusion complex is formed by this method. Thus, the inclusion complex was produced in the α-glucan phosphorylase-catalyzed polymerization in the presence of poly(oxetane) ($-CH_2CH_2CH_2O-$, POXT) of another hydrophobic polyether, whereas no inclusion complex was formed from PEO of a hydrophilic polyether.[277] In addition, the formation of the amylose−polymer inclusion complexes was found in the α-glucan phosphorylase-catalyzed polymerization in the presence of various hydrophobic polymers such as polyesters of poly(δ-valerolactone) (PVL) and poly(ε-caprolactone) (PCL), poly(ester−ether), and poly(tetramethylene carbonate) (Scheme 39).[278−280] The representation of this method as shown in Figure 20 is similar to the way that plant vines grow twining around a rod. Accordingly, this method to produce amylose−polymer inclusion complexes has been named "*vine-twining polymerization*".[271−275]

By means of the vine-twining polymerization technique, selective inclusion toward two resemblant polymers by amylose has been achieved (Figure 21). For example, amylose selectively included one side of the polyethers, that is, PTHF from a mixture of PTHF/POXT.[281] The selective inclusion by amylose was also found when the vine-twining polymerization was performed in the presence of a mixture of two resemblant polyesters, PVL/PCL, in which PVL was selectively included by amylose.[282] Furthermore, it was found that amylose selectively included a specific range of molecular weights in any PTHFs when the vine-twining polymerization was conducted in the presence of PTHFs with different average molecular weights.[283]

The inclusion complexes composed of amylose and strongly hydrophobic polyesters were formed in a parallel enzymatic polymerization system (Scheme 40).[284] This was achieved by conducting the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from Glc_7, giving rise to the host amylose, and the lipase-catalyzed enzymatic polycondensation of dicarboxylic acids and diols, leading to the guest polyesters,[285,286] simultaneously. When the numbers of methylene units in a dicarboxylic acid and a diol as the monomers for the guest polyester were 8, the corresponding amylose−polyester inclusion complex was obtained. On the other hand, use of the monomers having methylene units of 10 and 12 hardly gave the inclusion complexes.

The preparation of hydrogels through the formation of an inclusion complex of amylose in the vine-twining polymerization was reported.[287] This was achieved by the α-glucan phosphorylase-catalyzed polymerization of α-Glc-1-P from Glc_7 in the presence of a water-soluble copolymer having the hydrophobic PVL graft chains (poly(acrylic acid sodium salt-*graft*-VL)) (Figure 22). The enzymatic reaction mixture turned into a gel during the polymerization process. During the enzymatic polymerization, the produced amylose included the PVL graft chains in the intermolecular guest copolymers. Therefore, the formed inclusion complexes acted as the cross-linking points for the formation of the hydrogel as shown in Figure 22. Furthermore, the enzymatic disruption and reproduction of the hydrogels were achieved by the combination of the β-amylase-catalyzed hydrolysis of the amylose component and the formation of amylose by the α-glucan phosphorylase-catalyzed polymerization.

5.6. Polysaccharide Synthesis Catalyzed by Other Phosphorylases

Cellodextrin phosphorylase is an enzyme that catalyzes the reversible phosphorolysis of cellooligosaccharides larger than cellotriose to produce α-Glc-1-P.[288] This enzyme has been found in cells of *Clostridia*.[289] Cellooligosaccharides have been synthesized by the cellodextrin phosphorylase-catalyzed chain elongation using various cellobiose acceptors and the α-Glc-1-P donor (Scheme 41).[290] When cellobiose was used as a glycosyl acceptor, various cellooligosaccharides ranging from water-soluble

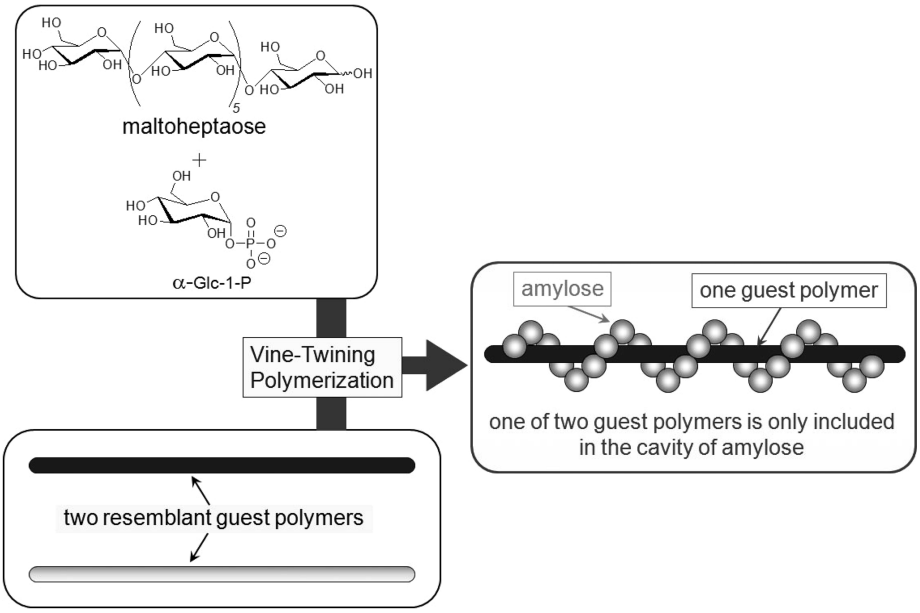
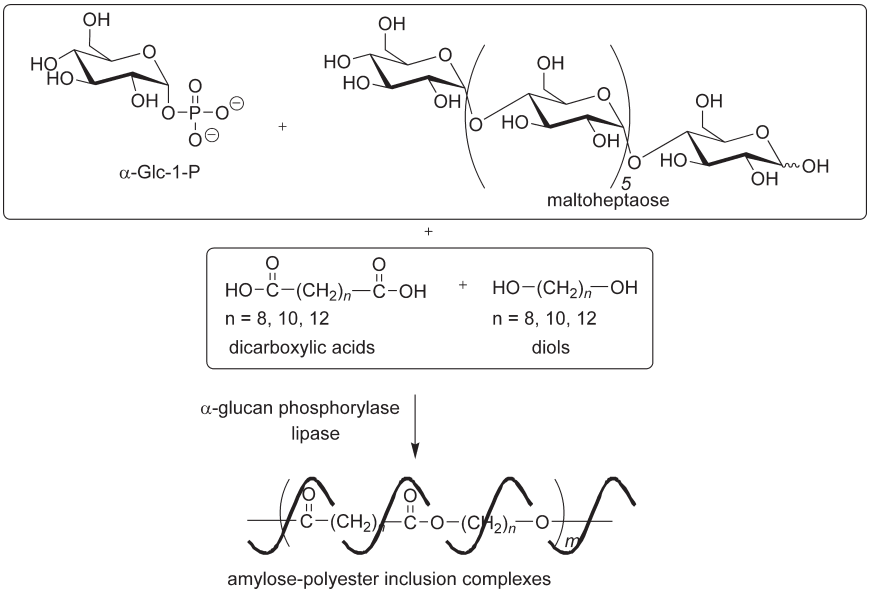


Figure 21. Amylose selectively includes one of two resemblant polymers in vine-twining polymerization.

Scheme 40. Formation of Inclusion Complexes Composed of Amylose and Strongly Hydrophobic Polyesters in Parallel Enzymatic Polymerization System



products to crystalline precipitates were obtained, depending on the concentration of the acceptor. The NMR analysis of the crystalline precipitate indicated an average DP of ~8. The precipitates showed the diffraction diagrams of low-molecular-weight cellulose II. The cellodextrin phosphorylase-catalyzed synthesis of the cellooligosaccharides substituted at their reducing end was also achieved using various cellobiose derivatives and analogues as a glycosyl acceptor (Scheme 41). When the cellodextrin phosphorylase-catalyzed reaction was performed using α-Glc-1-P as a glycosyl donor and glucose as a glycosyl acceptor, cellooligosaccharides with an average DP of 9 were produced and the products formed highly crystalline cellulose II.[291] Although glucose had been believed to not act as the glycosyl acceptor for the cellodextrin phosphorylase catalysis, a significant amount of insoluble cellulose was precipitated without accumulation of soluble cellooligosaccharides in this enzymatic reaction system using the glucose acceptor. This result was explained in terms of the large difference in the acceptor reactivity between glucose and cellooligosaccharides as the general acceptor of the cellodextrin phosphorylase catalysis.

Cellodextrin phosphorylase was found to recognize α-Xyl-1-P as a glycosyl donor and xylose-containing disaccharides as a glycosyl acceptor.[292] Therefore, the enzymatic synthesis of a library of β-(1→4)-hetero-D-glucose and D-xylose-based oligosaccharides was attempted by the cellodextrin phosphorylase-catalyzed glycosylation using α-Glc-1-P or α-Xyl-1-P as a donor

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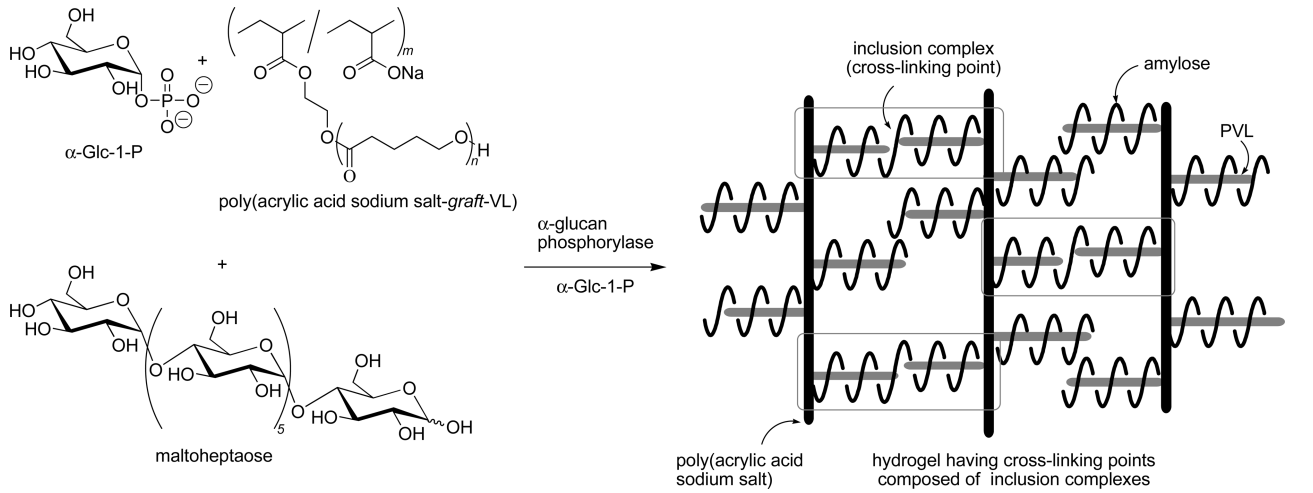
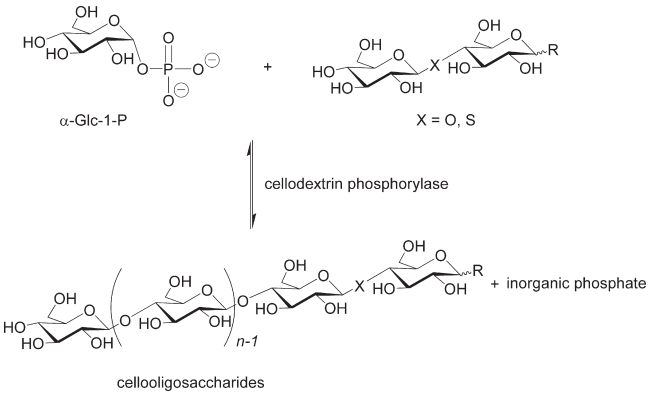


Figure 22. Preparation of hydrogel by vine-twining polymerization in the presence of poly(acrylic acid sodium salt-*graft*-VL).

Scheme 41. Cellodextrin Phosphorylase-Catalyzed Synthesis of Cellooligosaccharides Using Cellobiose Derivatives and Analogues As a Glycosyl Acceptor



and cellobiose, xylobiose, Glc-β-(1→4)-Xyl, or Xyl-β-(1→4)-Glc as an acceptor. Consequently, the enzymatic glycosylation by the cellodextrin phosphorylase catalysis successfully produced all six heterotrisaccharides and 10 of the 14 possible heterotetrasaccharides. On the other hand, it was not found possible to synthesize the four tetrasaccharides with a Xyl→Glc sequence at their nonreducing ends by this enzymatic reaction.

β-(1→3,1→4)-Oligosaccharides and thiooligosaccharides were synthesized by the cellodextrin phosphorylase-catalyzed chain elongation of α-Glc-1-P using β-(1→3)-linked oligosaccharide acceptors.[152,293] Furthermore, cellobiosylated dimer and trimer, and cellobiose-coated polyamidoamine (PAMAM) dendrimers, were synthesized as a glycosyl acceptor for the cellodextrin phosphorylase-catalyzed reaction. Then, the cellodextrin phosphorylase-catalyzed chain elongation of α-Glc-1-P was conducted using these acceptors to give the corresponding materials containing cellooligosaccharides at chain ends (Scheme 42).[294]

Kojibiose phosphorylase was discovered in the cell-free extract of a thermophilic bacterium accompanied with trehalose phosphorylase (EC 2.4.1.64).[295,296] This enzyme catalyzes the reversible phosphorolysis of kojibiose into β-D-glucose 1-phosphate (β-Glc-1-P) and glucose. The reverse reaction catalyzed by this enzyme was utilized for the synthesis of kojioligosaccharides (oligosaccharides composed of the α-(1→2)-glycosidic linkage). When the mixtures of various proportions of glucose and β-Glc-1-P were allowed to react in the presence of kojibiose phosphorylase, kojioligosaccharides having only the α-(1→2)-glycosidic linkage were produced (Scheme 43).[297] The average DPs of the products increased with decreasing proportions of glucose.

β-1,3-Oligoglucan phosphorylase and laminaribiose phosphorylase (EC 2.4.1.31) were found in *Euglena gracilis* cells, and both the enzymes phosphorolyze a series of laminarioligosaccharides to produce α-Glc-1-P.[298,299] Mixtures of laminarioligosaccharides with the varying DPs were synthesized from glucose and α-Glc-1-P by the combined actions of these two enzymes.[300]

6. POLYSACCHARIDE SYNTHESIS CATALYZED BY SUCRASE-TYPE ENZYMES

6.1. Concept in Sucrase-type Enzyme-Catalyzed Synthesis of Polysaccharides

Non-Leloir-type glycosyltransferases that use sucrose as a substrate are able to catalyze the synthesis of poly- and oligosaccharides in high yields under kinetic control, even in dilute aqueous solution of sucrose.[31] Most enzymes of this class highly specialize in transfer of either the glucose or the fructose moiety of sucrose, resulting in glucose-based polysaccharides (glucans) or fructose-based polysaccharides (fructans) of different types with respect to glycosidic linkages and side chains. The simplified reaction schemes are represented as follows:

Glucosyltransferases:

$$n\text{Suc} \rightarrow \text{glucan} + n\text{Fru}$$

Fructosyltransferases:

$$n\text{Suc} \rightarrow \text{fructan} + n\text{Glc}$$

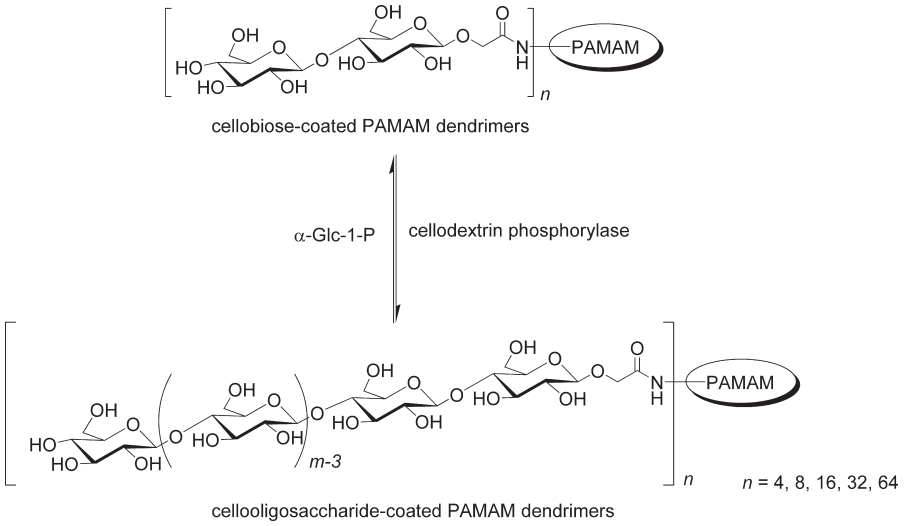
where Suc = sucrose, Fru = fructose, and Glc = glucose.

The enzymes of this group are often called sucrase-type enzymes, i.e., glucosyltransferases are glucansucrases and fructosyltransferases are fructansucrases.

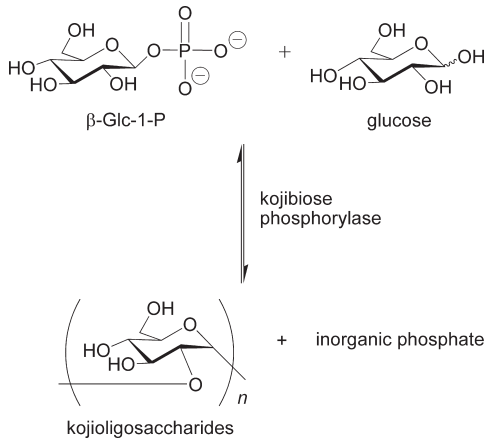
6.2. Polysaccharide Synthesis Catalyzed by Glucansucrases

Glucansucrases are typically extracellular enzymes, which are produced mainly by lactic acid bacteria.[301] They are composed of two functional domains, which are a core region containing a

Scheme 42. Cellodextrin Phosphorylase-Catalyzed Synthesis of Cellooligosaccharide-Coated Polyamidoamine (PAMAM) Dendrimers



Scheme 43. Kojibiose Phosphorylase-Catalyzed Synthesis of Kojioligosaccharides Using Glucose As a Glycosyl Acceptor



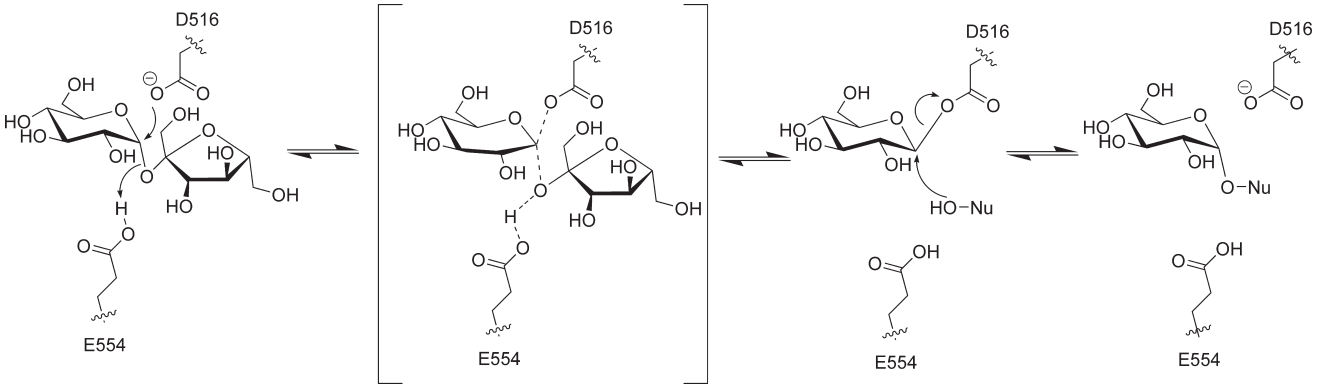
highly conserved catalytic domain, involved in sucrose binding and glucosyl transfer, and a C-terminal region composed of a series of tandem repeats, involved in glucan binding.[301] The mechanism of glucansucrases proceeds via a covalent glucosyl−enzyme intermediate.[302,303] The glucosyl transfer involves two amino acids of D516 (D = Asp) and E554 (E = Glu), which function as a nucleophile for covalent binding of the glycosyl residue and a proton donor, respectively, as shown in Scheme 44. Typically, dextran (α-(1→6)-glycosidic linkage), mutan (α-(1→3)-), alternan (alternating α-(1→3)- and α-(1→6)-), reuteran (α-(1→4)- and α-(1→6)-), and amylose (α-(1→4)-) are produced by dextran-, mutan-, alternan-, reuteran-, and amylosucrases, respectively (Figure 23).[78,304] Furthermore, the modifications with respect to the glycosidic linkage pattern in polysaccharide synthesis have been demonstrated.[305,306]

Amylosucrase (EC 2.4.1.4) is the most extensively studied glucansucrase. For example, the catalytic properties of the highly purified amylosucrase from *Neisseria polysaccharea* were characterized.[307] Consequently, it was revealed that, in the presence of sucrose alone, several reactions are catalyzed by the enzyme in addition to the amylose synthesis, which are sucrose hydrolysis, maltose and maltotriose synthesis by successive transfers of the glucose moiety of sucrose onto the released glucose, and finally turanose and trehalulose synthesis obtained by glucose transfer onto fructose. When glycogen was used as an acceptor for the amylosucrase catalysis, the sucrose hydrolysis decreased strongly with increasing the concentration of glycogen, as did oligosaccharide synthesis, by glucose transfer onto glucose and fructose.[308] The glucosyl units consumed were then preferentially used for the elongation of glycogen chains. Moreover, when various polysaccharides were tested as acceptors for the amylosucrase catalysis, the chain elongation proceeded only on the polysaccharides with α-(1→4)- or α-(1→4)- and α-(1→6)-linkages.[309]

Recombinant amylosucrase was used to synthesize amylose from sucrose without use of an acceptor.[310] The products had the DP of 35−58. By changing only the initial sucrose concentration, it was possible to obtain amyloses with different morphology and structure. The recombinant amylosucrase was also used for the chain-elongation reaction in the presence of glycogen as an acceptor.[311] The morphology and structure of the resulting insoluble products were shown to strongly depend on the initial sucrose/glycogen ratio. For the lower ratio, all glucose molecules produced from sucrose were transferred onto glycogen, giving rise to a slight elongation of the external chains and their organization into small crystallites at the surface of the glycogen particles. With a high initial sucrose/glycogen ratio, the external glycogen chains were extended by the enzyme, leading to dendritic nanoparticles with a diameter 4−5 times that of the initial particle.

Three different glucosyltransferases, GTF-S1, GTF-S3, and GTF-S4, which were isolated from three strains of *Streptococcus sobrinus*-K1-R, -6715-13-201, and -6715-13-27, respectively, were used to synthesize, from sucrose, water-soluble α-glucans of quite different structure.[312] The GTF-S1-type enzymes synthesized highly branched (up to 34%) glucans having single α-(1→3)-linked glucose residues attached to ∼1 in 2 of the

Scheme 44. Proposed Mechanism of Glucansucrase Catalysis



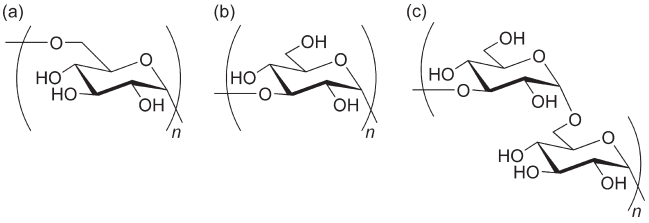


Figure 23. Typical structures of dextran (a), mutan (b), and alternan (c).

α-(1→3)-linked glucose units of the main chain. The GTF-S3 enzymes produced low-molecular-weight linear α-(1→3)-linked glucans. The GTF-S4 enzymes formed glucans with branching ranging from 8 to 11%.

6.3. Polysaccharide Synthesis Catalyzed by Fructansucrases

Fructansucrases transfer the fructose units of sucrose onto polysaccharides or appropriate acceptors with release of glucose. Hydrolysis is generally a minor reaction, depending on conditions such as concentration of sucrose. Fructans, thus produced, are either levan composed of β-(2→6)-linked fructose residues by levansucrase catalysis or inulin composed of β-(2→1)-linked fructose residues by inulosucrase catalysis (Figure 24).[78] Some fructansucrases show high specificity for the polysaccharide formation such as the inulosucrase from *Leuconostoc citreum*.[313] As a possible enzymatic pathway, the following mechanism for *Bacilus subtilis* levansucrase catalysis was proposed.[314] Amino acid residues D86 (D = Asp) and E342 (E = Glu) form the pair of essential catalytic side chains and the fructosyl unit of sucrose is bound tightly and with high specificity in the active site to form the complex with the enzyme. Meanwhile, D247 forms strong hydrogen bonding interactions with the C-3′ and C-4′ hydroxy groups of the fructosyl unit. An amino acid residue E342 as general acid/base catalyst presumably protonates the glycosidic oxygen of the substrate and forms an oxonium ion. The retaining mechanism further proceeds through the formation of a covalent glycosyl−enzyme intermediate and a nucleophilic attack of the acceptor.

Sucrose analogues are non-natural substrates with a similar glycosidic linkage to sucrose, which have been used for the synthesis of new poly- and oligosaccharides by catalyses of the sucrase-type enzymes. For this purpose, a whole range of sucrose analogues, such as those which were composed of galactose, mannose, xylose, fucose, and rhamnose in place of

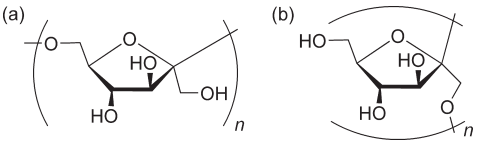


Figure 24. Typical structures of levan (a) and inulin (b).

glucose, have been prepared.[314−317] A wide range of fructansucrases recognize most of them, giving rise to novel poly- and oligosaccharides (Scheme 45).[318,319]

7. CONCLUDING REMARKS

In this review, precision polysaccharide synthesis by enzymatic catalysis has been overviewed. The significant studies on the enzymatic synthesis of oligosaccharides were also included. Because the enzymatic glycosylations proceed with highly controlled stereo- and regioselectivities, the poly- and oligosaccharides with well-defined structure have efficiently been synthesized and even the synthesis of structurally complicated polysaccharides, which are very difficult to be produced via conventional chemical routes, was achieved via the enzymatic reaction. Beyond the key-and-lock theory for the enzymatic reaction, the enzymatic synthesis of the polysaccharides has been extended to structural variation of non-natural substrates, providing the various types of non-natural polysaccharides. Because of the production of the structurally defined saccharide chains by the enzymatic catalysis, furthermore, the strict control of their higher-ordered assemblies has been achieved. Additionally, the motivation for the studies on the enzymatic synthesis of the polysaccharides has strongly been based on the viewpoints that the greener and sustainable processes should be developed in the fields not only of fundamental research but also of practical application on the polymer and material chemistries. Thus, besides the aforementioned precision process such as control of stereo- and regiochemistries, the enzymatic polysaccharide synthesis has the advantage over the conventional chemical process in some additional points, for example: (i) the reactions proceed under the mild conditions in aqueous media; (ii) renewable resources from natural and related sources can be employed as the substrates of the reactions; (iii) the products have biodegradability in most cases; and (iv) enzymes are renewable and nontoxic.

The polysaccharides and the related compounds have been attracting much attention because of their potential for the

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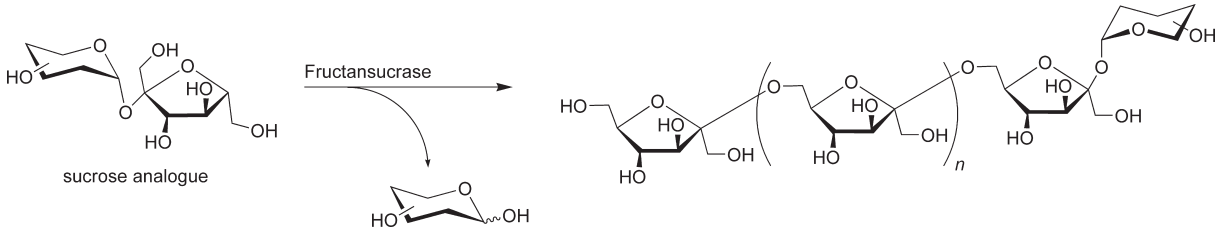
Scheme 45. Fructansucrase-Catalyzed Synthesis of Poly- And Oligofructosides from Sucrose Analogues



application as the new functional materials in many research fields such as medicines, pharmaceutics, foods, and cosmetics. Therefore, the precision synthesis of polysaccharides by enzymatic catalysis will be increasingly important in the future.

AUTHOR INFORMATION

Corresponding Author

*Fax: +81-99-285-3253. E-mail: kadokawa@eng.kagoshima-u.ac.jp.

BIOGRAPHY

Jun-ichi Kadokawa was born in Matsuyama, Japan, in 1964. He studied applied chemistry and materials chemistry at Tohoku University, where he received his M.S. degree in 1989 and his Ph. D. in 1992 under the supervision of Prof. Shiro Kobayashi. He then joined Yamagata University as a Research Associate. From 1996 to 1997, he worked as a visiting scientist at the Max-Planck-Institute for Polymer Research in Germany. In 1999, he became an Associate Professor at Yamagata University and moved to Tohoku University in 2002. He was appointed as a Professor of Kagoshima University in 2004. His research interests focus on new enzymatic glycosylations, new polysaccharide-based materials, and control of the higher-ordered structures of polysaccharides. He received the Award for Encouragement of Research in Polymer Science (1998) and the Cellulose Society of Japan Award (2010).

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