

博士論文

**Characterization of Dextrin Derivatives  
by Chemical or Enzymatic Esterification**

(化学的または酵素学的エステル化法を用いた  
デキストリン誘導体の合成と物性評価)

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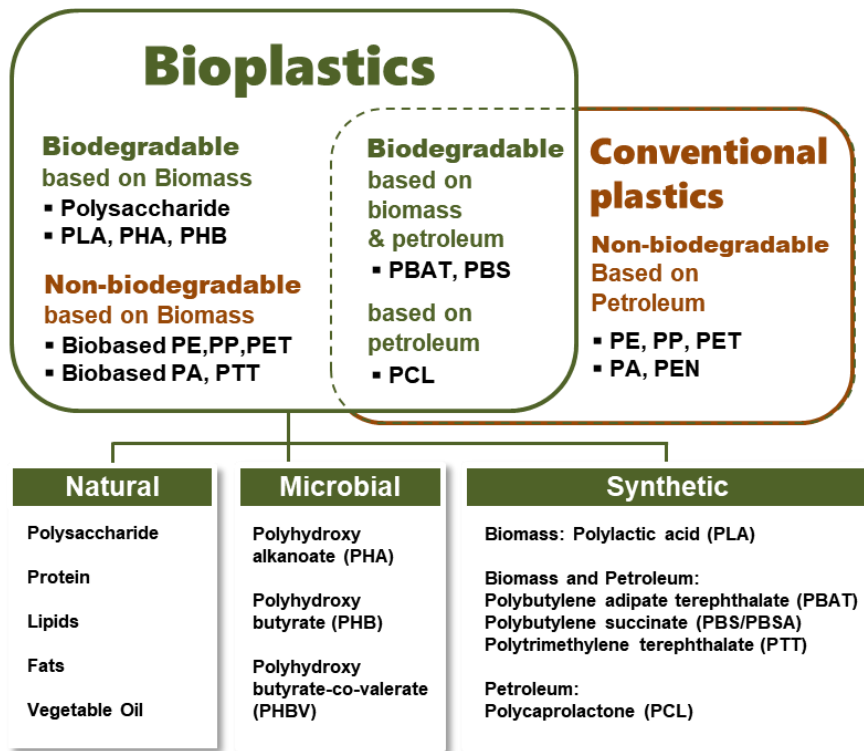
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# **Chapter 1**

## **General Introduction**

### **1.1. Introduction**

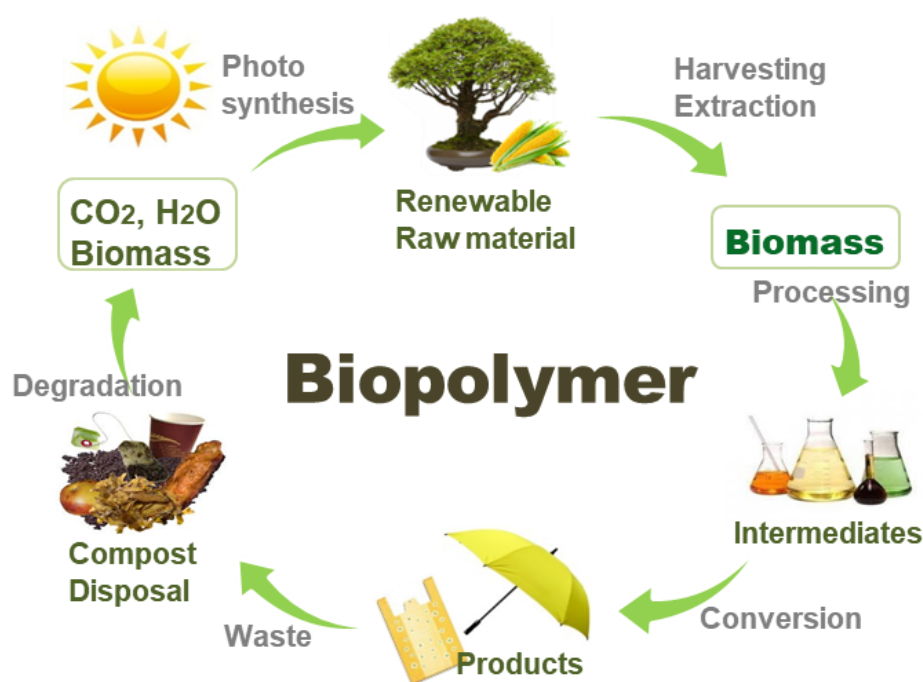
Since the rapid advances in industrial technology has developed polymeric chemical industry using petroleum resource, plastic materials have contributed to the abundant human life due to their advantages such as versatility, ease of manufacture and low cost [1, 2]. Plastics have been being used extensively in various application fields of textiles, packaging, engineering, medicine and electronics, therefore they are important and essential for human society [4]. However, prominent environmentalists have been constantly warning of the environmental threats of plastics, and in fact, the environmental pollution is a serious global issue [5]. Regarding the environmental issues, there are several problems of both the mass production and disposal of plastics. One of them is the exhaustion of petroleum resources. Commercial plastics materials rely mostly on petroleum resources as raw materials, and the expected depletion of petroleum resources is a major concern for the sustainable production of plastics [6]. In addition, mass consumption of petroleum resources is one of the causes of breaking down the carbon cycle, releasing anciently immobilized carbon as CO<sub>2</sub> into the atmosphere. At present, various effect to reduce the CO<sub>2</sub> emission caused by mass consumption of petroleum have been developed by recycling and energizing waste plastics [6]. But the fundamental solution is understandably to reduce dependence on petroleum resources. As an alternative solution, 'Bioplastic' has attracted plenty of worldwide attention to a green chemistry (Figure 1-1). Bioplastics are defined as whole biomass plastics derived from



**Figure 1-1.** Classification of bioplastics, and examples.

biomass resources as well as biodegradable plastics sourced from petroleum resources [7]. Therefore, bioplastic include biodegradable and non-degradable, and they can be classified into five types as shown in the Figure 1-1; <sup>1)</sup> Natural polymers obtained from biomass, <sup>2)</sup> polymers produced by microorganisms, <sup>3)</sup> polymers produced from monomers obtained through the fermentation of biomass, <sup>4)</sup> polymers produced by polymerizing biomass-based monomers, and <sup>5)</sup> petroleum-based monomers [8]. In recent bioplastics, the tendency of environment friendly turn from biodegradable plastics into biomass plastics [9]. This is because biodegradable plastics may cause persistent environmental problems for a long term as microplastics during the degradation completely. In addition, the biodegradable plastics have the limited utilization due to their low physical properties compared to petroleum-based plastics, and thus they have not achieved the eco-friendly [10]. On the other hand, biomass plastics are focused on CO<sub>2</sub> reduction, not degradability.

Since the carbon source of biomass plastics is CO<sub>2</sub> in the atmosphere fixed by photosynthesis of plants, "Carbon neutral" is established which does not affect the increase or decrease of the total amount of CO<sub>2</sub> even if disposing waste of plastics (Figure 1-2) [11]. Besides, the CO<sub>2</sub> emitted at the disposal can be recycled for the growth of biomass resource. Expanding the use of biomass plastics is extremely important for the creation of a sustainable recycling-oriented society [12]. The competitiveness of biomass plastics can be enhanced by the synthesis of modified biomass plastics through efficient process. This study introduces polysaccharides which are one kind of the biomass materials and presents results of modified properties of polysaccharide derivatives.



**Figure 1-2.** Carbon cycle of biomass plastics.



## **1.2. Dissertation overview**

Chapter 1 describes the general motivation of this research, which is environmental threat caused by the plastics industry based on petroleum resources. This chapter explains the necessity for bio-plastics as sustainable and alternative resources. The dissertation overview at the end of this chapter provides summarized information for each of the chapters, that have been divided for this research performed on modifying the polysaccharide as a one of the bioplastics.

Chapter 2 describes the background knowledge and strategies for modification of polysaccharides. This chapter introduce the origin, structure and properties of major natural polysaccharides as well as used polysaccharides in this research (i.e. dextrin, amylose, paramylon and dextran). And also, the description of esterification reaction catalyzed by chemical reagent or enzyme are included as modifying process of polysaccharides.

Chapter 3 describes the chemical esterification of dextrin in detail. The structural fully-substitution induced by chemical esterification of dextrin is indicated in this chapter, and the correlations between structure and properties are presented through structural, thermal, mechanical and hydrophobic analysis experimentally.

Chapter 4 describes the enzymatic esterification of dextrin in detail. Preferentially, the catalytic activity of four commercial lipase enzymes on dextrin were optimized in this chapter. After then, experimental results demonstrate that regioselectively mono-substituted dextrin esters were successfully synthesized under an optimal condition.

Chapter 5 describes catalytic activity of on other polysaccharides such as amylose, paramylon and daxtran. This chapter presents the possibility of whether the results

obtained from a specific polysaccharide can be applied to other polysaccharides as an extension experiment in Chapter 4.

Chapter 6 summarizes the results obtained from the experiments in this research for chemical and enzymatic esterification of polysaccharides. and also summarizes knowledge on biomass plastic materialization and material properties using two kinds of modification of polysaccharide. In addition, this chapter predicts the academic value of this research and suggests the future direction of the polymer industry.

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## **Chapter 2**

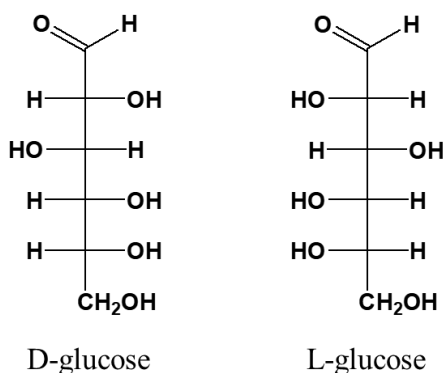
### **Research Background**

#### **2.1. Polysaccharides**

Polysaccharides are natural polymers commonly derived from renewable resources, and there are a wide range of naturally occurring polysaccharides possessing magnificent structural diversity and functional versatility [1]. Recently, polysaccharides and their derivatives have gained attention for various application due to fascinating benefits such as diverse, abundant, important for life and environment friendly [2-3]. Some of polysaccharides – in particular structural polysaccharide such as cellulose and chitin, and storage polysaccharides such as starch and glycogen – are actively used in commercial products today, although many others still remain underutilized [4, 5]. With the recent rapid advancements in chemical industry (related to purification into uniform molecules, synthetic pathway for well-designed molecular derivatives, efficient processing techniques, and demonstration of structure/properties relationships through adequate analysis), new opportunities for the use of polysaccharides and their derivatives are now being considered [6].

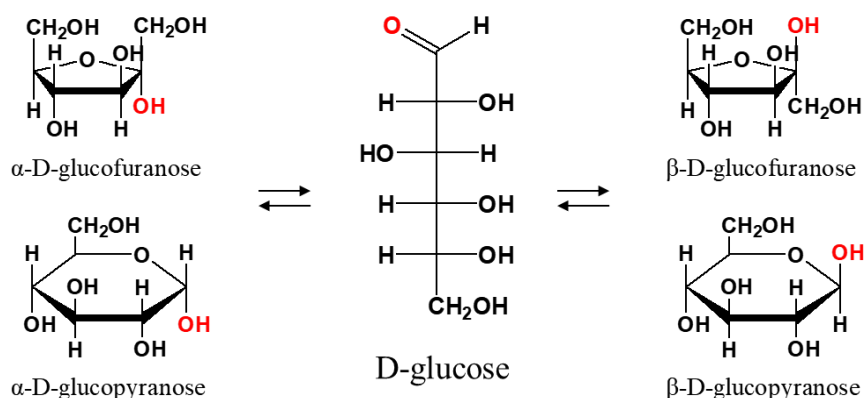
## 2.2. Structure and classification of polysaccharides

Polysaccharides are a large molecule with general formula  $-(CH_2O)_n-$  composed of repeating units either monosaccharides (such as glucose, fructose, galactose) or disaccharides (such as sucrose, lactose, maltose) joined together by glycosidic linkages [7]. The monomers, that might be polymerized to polysaccharides, are generally cyclic structure. An example of a polysaccharide is glucan, which is polymers of glucose containing 6 carbon atoms. Glucose molecular formula indicates two forms called enantiomers (i.e., D-glucose and L-glucose), because their molecular structures are mirror images of each other (Scheme 2-1). Regarding the production of glucose, only D-glucose occurs in nature and is of much abundance and importance compared to L-glucose [8].



**Scheme 2-1.** Structure of glucose enantiomers; D-glucose and L-glucose.

In solutions, glucose exist in equilibrium between open-chain form as described above (linear form) and closed-chain forms as mentioned below (cyclic forms). The cyclic forms of glucose exist several isomers, which were pyranose consisting of 6 membered cyclic and furanose with 5 membered cyclic. The point to be noted is pyranose occupy most of forms of more than 99%. Furanose exists in negligible



**Scheme 2-2.** Structural arrangements of D-glucose anomers.

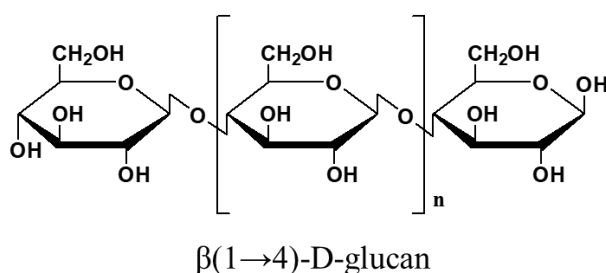
amounts and the linear form is limited to about 0.25% [9]. The cyclization of D-glucose from the form of carbonyl carbon asymmetric molecules due to the presence of an anomeric carbon, resulting in  $\alpha$ -D-glucopyranose and  $\beta$ -D-glucopyranose. These two isomers are referred to as the alpha ( $\alpha$ ) and beta ( $\beta$ ) anomers. Scheme 2-2 shows their configurations of constituent. In the  $\alpha$  anomer, the hydroxyl group at the anomeric carbon (carbon number 1) forms a trans-arrangement and is located in the opposite direction of the methyl group at the chiral center (carbon number 5). Contrastively, the  $\beta$  anomer presents that the hydroxyl group on the anomeric carbon is in the cis-arrangement, facing in the direction of the methyl group on the chiral center [9]. The physical and chemical properties of the anomers depend on the difference in the  $\alpha$  or  $\beta$  arrangement. The anomers have significantly different physical and chemical properties, which also have a significant effect on the final properties of the polysaccharide [10, 11].

Polysaccharide is a large polymer linked by glucosidic bonds, formed between the hydroxyl group of one carbohydrate unit and a hydroxyl group of another unit. For example, amylose and cellulose are linear polysaccharides consisting of glucose units linked between the first carbon of glucose and the fourth carbon of adjacent another.

It is common for this bond to be expressed in terms of the number of linked atoms and the stereoisomer of the unit, so that amylose and cellulose are represented by  $\alpha(1\rightarrow4)$  glucan and  $\beta(1\rightarrow4)$  glucan, respectively [11]. Polysaccharides are produced by various animals and plants, and there are various chemical structures depending on the kinds of constituent saccharides and the difference in binding mode. Polysaccharides are classified as homopolysaccharides (consisting of one type of monosaccharide) and heteropolysaccharides (consisting of more than one type of monosaccharide), and they range in structure from linear to branched [12]. This chapter introduces the origin, structure and properties of major natural polysaccharides as well as used polysaccharides in this research.

### 2.2.1. Cellulose

Cellulose is the main structural component of the cell wall of plants, and is the most abundant natural polysaccharide on Earth. It is mainly photosynthesized from carbon dioxide by plants, which is made up of hundred to many thousands of glucose units. According to a previous report on the cellulose content in the raw material, that of cotton fiber is 90%, that of wood is 40-50% and that of dried hemp is 57% approximately [13,14]. Cellulose is a homopolysaccharide in which D-glucopyranose is bound to  $\beta(1\rightarrow4)$  glycoside linkage, and it has three alcoholic hydroxyl groups per glucose residue ( $\beta(1\rightarrow4)$ -D-glucan; Scheme 2-3). It is a stiff linear polymer, and the molecule adopts extended rod-like conformation. In one chain, multiple hydroxyl groups in glucose form hydrogen bonds with oxygen atoms on the same or adjacent chains. This arrangement confers straightness between the chains together side-by-side and forming microfibrils and matrix [15]. Since cellulose forms a strong hydrogen bond between molecules, it is insoluble in ordinary organic solvents and water, and does not have thermoplasticity. Although its molecular weight varies depending on the raw material, in the case of cellulose industrially produced as a wood kraft pulp, the polymerization degree is about 800 to 1500 and the molecular weight has a value of about several hundred thousand. Various cellulose derivatives have been produced by esterifying or etherifying hydroxyl groups of cellulose in order to improve processability as a plastic material [16, 17]

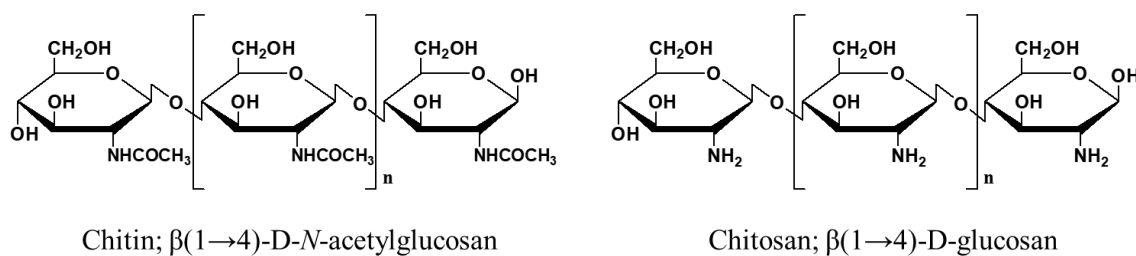


**Scheme 2-3.** Structural formula of cellulose.



### 2.2.2. Chitin and chitosan

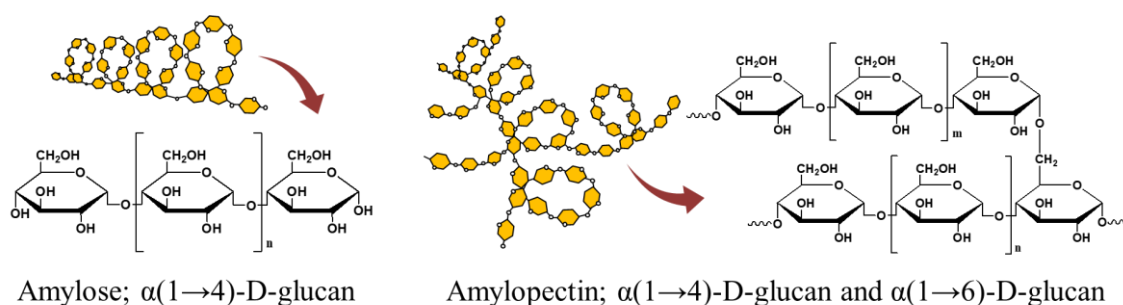
Chitin is the second abundant polysaccharide occurring in nature after cellulose. It is the structural supporting material of the exoskeleton of arthropods (e.g., crustaceans and insects), the cell walls of fungi and yeast or the scales of fish and lissamphibians [18]. Chitin is a linear homopolymer of  $\beta(1\rightarrow4)$  linked *N*-acetyl-D-glucosamine residue, which differs structurally from cellulose only in that each OH group on carbon number 2 is replaced by an C2-NHCOCH<sub>3</sub> (Scheme 2-4). This increases the strength of the chitin polymer matrix by increasing hydrogen bonding between adjacent polymers, for similar reasons as cellulose [19]. Although the structure of chitin and cellulose is similar in that both present the  $\beta(1\rightarrow4)$  linkages, their characteristics are not shared with each other. Chitin is highly hydrophobic and does not dissolve in most organic solvents as well as water [20]. Chitosan is the most important chitin derivative by partially or completely deacetylation, forming randomly distributed  $\beta(1\rightarrow4)$ -D-glucosan (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit). Chitosan derivatives are easily obtained under mild conditions in a controlled amount along the chitosan chain [21]. Chitin and chitosan are both crystalline polysaccharides and have a characteristic chemical structure having an amine as a repeating unit. Therefore, in addition to their properties such as biocompatibility and biodegradability, they exhibit specific properties such as cationic, antibacterial and physiological activities. Due to these properties, application studies on chitin, chitosan and their derivatives have been actively conducted, for example, in food fields using low-molecular substances and gelling materials, and in medical fields such as wound healing films [22, 23].



**Scheme 2-4.** Structural formula of chitin and chitosan.

### 2.2.3. Starch

Starch is polysaccharide as plant storage form of carbohydrate extracted from corn, wheat, rice and potatoes. Glucose by photosynthetic organisms is converted into insoluble granules such as oils, fats, and starch for storage. Therefore, starch is composed of two different glucose polymers, as the linear structure  $\alpha(1\rightarrow4)$ -D-glucan (amylose) and the branched structure  $\alpha(1\rightarrow4)$ ;  $\alpha(1\rightarrow6)$ -D-glucan (amylopectin) (Scheme 2-5). Depending on the plant, starch generally contains 20 to 25% amylose and 75 to 80% amylopectin by weight [24].



**Scheme 2-5.** Structural formula of amylose and amylopectin.

Amylose is a helix structure by formation of hydrogen bond between oxygen atoms bonded at carbons number 2 of one glucose unit and the carbon number 3 of neighboring glucose molecules. This extensive intermolecular hydrogen bonding results in amylose possessing a fairly lower solubility and hydrophobicity [25].

Amylopectin is highly branched structure consists of an amylose chain with  $\alpha(1\rightarrow6)$  linked branches. Usually, branches occur every 24 to 30 glucose units, resulting in higher solubility and, hence, quick degradation because it has many end points onto

which enzymes can attach [26]. Usually, amylose and amylopectin can be separated and modified by chemical, physical, or enzymatic treatment [27].

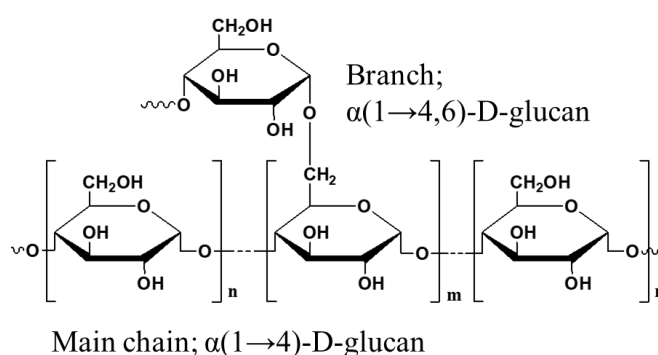
Starch is not only a major nutrient for humans as a carbohydrate, but also an important industrial material. Although starch is extracted from various plant species such as corn, potato, rice, wheat, and coconut, especially starch extracted from corn and potato is actively used for industrial purposes. Depending on the source of the plant, starches have different polymer structures and compositions therefore have different physicochemical properties in terms of gelatinization temperature, cooked paste and gel viscosity) [28].

#### **2.2.4. Glycogen**

Glycogen is a multibranched polysaccharide that is the energy storage form of glucose in human, animal, fungi and bacteria (Starch is not found in animal cells). When energy is required by living organism, glycogen is broken down to glucose is released into the bloodstream through the glycolytic or pentose phosphate pathway [29, 30]. Glycogen has a similar structure to amylopectin (a component of starch), but, consisting of shorter linear chain and more extensively branched chains compared to that extracted from starch. The linear form is linked by  $\alpha(1\rightarrow4)$  glycosidic bonds from one glucose to the next. And, branches are connected to a linear chain branched by  $\alpha(1\rightarrow6)$  glycosidic bonds, occurring every 8 to 12 glucose units [31]. Currently, applications using glycogen in the polysaccharide industry are not actively performed. This is more efficient to demonstrate the structure/properties relationship using starch instead, which is equally judged in the application field.

### 2.2.5. Dextrin

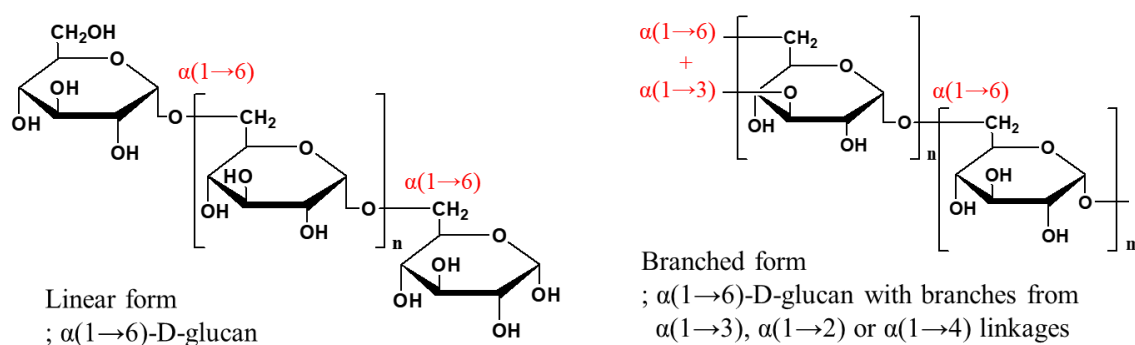
Dextrin is a polysaccharide produced by the partial hydrolysis of starch or glycogen. Its structure consists of an  $\alpha(1\rightarrow4)$  linked main chain with  $\alpha(1\rightarrow4,6)$  linked branches. Depending on the degree of hydrolysis, dextrin is classified as amylo-dextrin, erythro-dextrin, achro-dextrin or malto-dextrin, and each type exhibits different properties [32]. A general characteristic of dextrin is that it dissolves well in polar solvents, such as water with a low viscosity (a consequence of the extra degrees of freedom provided by rotation at the C6 position). This allows for its use in field of adhesives, paint, cosmetics and biomedical industries [33-36]. However, because neat dextrin does not exhibit thermoplasticity or hydrophobicity – a consequence of strong hydrogen bonding – its use in other applications is limited. The limited properties of dextrin can be modified by substitution reactions of the hydroxyl groups, which result in the insertion of functional molecular chains [37, 38]. The conditions of these substitution reactions, such as the components, positions and chain lengths of the substituents, have a significant effect on the final properties of the dextrin [39].



**Scheme 2-6.** Structure formula of dextrin.

## 2.2.6. Dextran

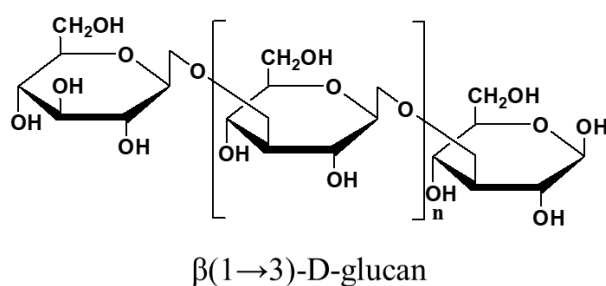
Dextran is a complex branched polysaccharide of glucose, composed of  $\alpha(1\rightarrow6)$  linked in major chains, usually containing more than 50% of total linkages (IUPAC defines dextran as branched poly- $\alpha$ -D-glucosides of microbial origin having glycosidic bonds predominantly  $\alpha(1\rightarrow6)$  linkages) [40, 41]. These major glucan also possess branched chains stemming from mainly  $\alpha(1\rightarrow3)$  linkages or  $\alpha(1\rightarrow2)$ ,  $\alpha(1\rightarrow4)$ , depending on the bacteria used. This distinct branches are distinguished from dextrin, that combines  $\alpha(1\rightarrow4)$  linked main chain and  $\alpha(1\rightarrow6)$  linked branches. [42]. Physicochemical properties of dextran vary depending on the specific chemical structure. Generally, dextrans with the highest content of  $\alpha(1\rightarrow6)$  linkages are the most soluble in water [43].



**Scheme 2-7.** Structural formula of two forms of dextran.

### 2.2.7. Curdlan and paramylon

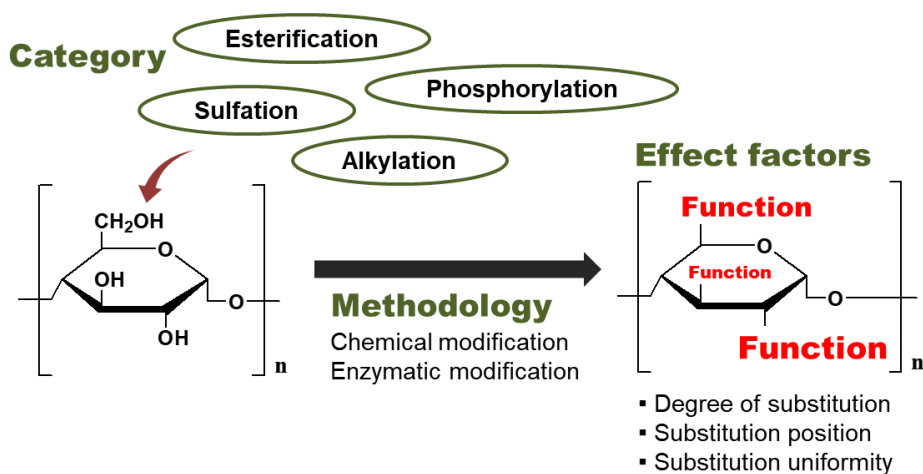
Curdlan is a microbial polysaccharide produced by *Alcaligenes faecalis*, a gram-negative bacterium, and is a homopolysaccharide in which D-glucose is linearly  $\beta(1\rightarrow3)$ -glycosidically linked of glucose. with the same structure, Paramylon is made from pyrenoids found in *Euglena*. (Scheme 2-8). Curdlan and paramylon are a crystalline polysaccharide and has been reported to have multiple crystal systems [44, 45]. Their use are mainly thickener and gelling agent to food field, medical field by application development. The main difference between curdlan and paramylon is molecular weight. In general, curdlan is known to have a higher molecular weight than paramylon, which has a sensitive effect on the morphological characteristics in the solvent [46].



**Scheme 2-8.** Structural formula of curdlan and paramylon.

## 2.3. Properties and modification of polysaccharides

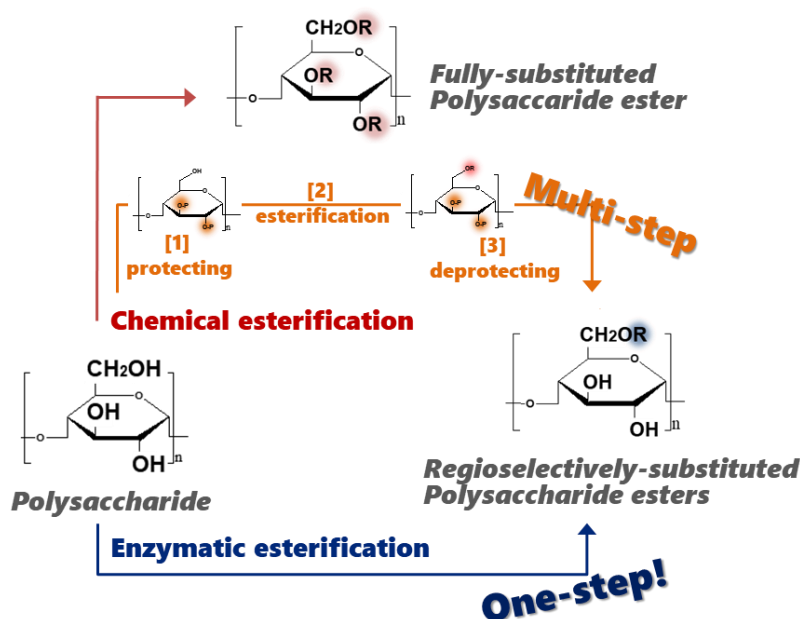
Polysaccharides have different complex structures and thus exhibit unique properties., depending on the constituent and conformation of repeating units ( $\alpha$  or  $\beta$  glucosidically linked structures based on about 40 different monosaccharides), polysaccharides have different complex structures and thus exhibit unique properties. Currently, various modifying methods are used to control their physicochemical properties such as hydrophilicity, thermoplasticity, biocompatibility, biodegradability, and polyfunctionality. An important and common example is substitution reaction, which is to replace the -OH, -NH or -COOH moieties at main chains to functional molecules [47]. In this context, the important factors in controlling the physicochemical properties of substitution reaction are the conditions of substituent on the polysaccharide, such as intrinsic properties of substituent (category) as well as degree of substitution (uniformity and position) (Figure 2-1). This functionalization is often proceeded with carboxyl, phosphate and/or sulfate ester groups (4), which facilitate structural modification and control the function of specific polysaccharides [48]. Among the substitution reactions, esterification reactions are particularly important in the modification of polysaccharides,



**Figure 2-1.** Substitution reaction of polysaccharides.

because most of unmodified polysaccharides generally tend to form strong hydrogen bonding networks by -OH, -NH or -COOH moieties on the main chain [49]. This extensive hydrogen bonding maintains the chains together and contributes to the high tensile strength due to influence of molecular arrangement. There have been various esterification methods for substitution of polysaccharides, and systematic studies on the relationship between structure and function have suggested the possibility of modifying the physicochemical properties of polysaccharides [50, 51]. This chapter describes the methodology for structural modification of polysaccharide through chemical and enzymatic esterification.

The esterification reaction is a kind of condensation reaction in which a usually carboxylic acid (-COOH) is reacted with an alcohol (-OH) under a catalyst to produce an ester group (-COO-). Formation of esters in polysaccharide is an efficient approach, that has been studied from the distant past, to obtain polysaccharide derivatives with modified properties. Almost polysaccharides are difficult to induce thermoplastic and



**Figure 2-2.** Esterification of polysaccharides ; difference of chemical and enzymatic esterification.



hydrophobicity because of strong hydrogen bonding. These characteristic makes the processability of the polysaccharide difficult and limits its commercial use. Esterification modification is mainly used to control thermoplastic, hydrophobicity and solubility in water (or the specific organic solvents). A lot of research has been reported on esterification of polysaccharides (such as cellulose [52], chitin [53], starch [54] and curdlan [55]) presenting that their functional properties can be controlled by introduction of substituents. More specifically, the relationship between structure and functionality related to substituent, i.e., chain length, chain shape, degree of substitution, and substitution position, is presented [56]. Esterification can be catalyzed by chemical reagents and enzymes as well. Differences between chemical and enzymatic esterification are described in the following sections.

### **2.3.1. Chemical esterification**

Chemical esterification reaction is one of the popular methods to obtain modified polysaccharide because the replacement of hydroxyl groups reduces the tendency to form hydrogen bonding networks. Chemical esterification mainly uses a strong acid as a catalyst and proceeds fast under extreme conditions at high temperature, resulting in a high degree of substitution due to the strong catalytic reactivity of chemical reagent. Chemical esterification is suitable for the production of polysaccharides with a high degree of substitution (DS), because both primary and secondary hydroxyl groups are substituted in a non-selective reaction [57]. In general, the esterification of polysaccharide can be carried out in homogenous or heterogeneous ways [58]. The homogenous way contains the soluble polymer in suitable solvent. On the other hands, the reaction using heterogeneous system occurs in slurry without dissolution of the polymer. The preferred method of polysaccharide modification is the heterogeneous way in industry.

### **2.3.2. Enzymatic esterification**

Enzymatic esterification is promising method for modifying functionality to the polysaccharides. This route is not only environmentally benign; it is also highly regioselective, allowing for the synthesis of polysaccharide esters with controlled structures and functionalities [59]. Enzymes are generally efficient catalysts for the hydrolysis of esters, but in the absence of water (i.e., in organic solvents) they catalyze the reverse reaction. Most enzymes are easily denatured and inactivated in organic solvents, and therefore many studies on enzymatic esterification have been reported for the synthesis of monosaccharides, oligosaccharides and polysaccharide derivatives [60]. These studies indicate a high DS for the enzymatic synthesis of carbohydrate derivatives, but the reactions exhibit a lack of regioselectivity, due to the specific conditions used to amplify the enzyme's catalytic activity (such as substrate pretreatment, microwave treatment and the addition of ionic liquids).

For a successful regioselective substitution reaction, it is important to choose an enzyme with excellent activity in organic solvent. Several reports have classified enzymes by their degree of solvent tolerance and have explored the industrial applications of solvent-tolerant enzymes [61]. Lipases have gained attention by many industries, because of their general ease of handling, broad substrate tolerance, high stability towards temperatures and solvents, and convenient commercial availability [62]. Furthermore, lipases are able to esterify the primary hydroxyl group (C6-OH) of glucose, which allows for the efficient synthesis of regioselectively-substituted polysaccharides without the need for protection and deprotection reactions [63]. Extensive research has been carried out on the modification of polysaccharides using lipases [64-66].

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## Chapter 3

### Chemical Esterification; Fully Substituted Dextrin Esters

#### Synthesis and Characteristic of Dextrin Derivatives

#### by Heterogeneous Esterification

### 3.1. Introduction

Chemical esterification is suitable to obtain fully-substituted polysaccharide derivatives because this is mainly performed by strong acid as a catalyst, resulting in a high degree of substitution. Dextrin is a polysaccharide produced by hydrolysis of starch or glycogen, which is consisting of  $\alpha(1\rightarrow4)$  and  $\alpha(1\rightarrow4,6)$  glucan resulting in branched structure [1]. Generally, unmodified dextrin does not exhibit thermoplastic and hydrophobic because of their strong molecular hydrogen bonding [2]. These functional properties can be controlled by altering their chemical structure using esterification reaction, because the esterification of these hydroxyl groups increases the thermoplastic, hydrophobicity and thermal stability by interference of the tendency to form hydrogen bonding networks [3]. Regarding research on modification of polysaccharide, there are many literatures on chemical esterification of polysaccharides such as cellulose [4, 5], starch [6], amylose [7], chitin [8] and curdlan [9, 10]. Despite the extensive research on polysaccharide fields, dextrin have not been explored relatively. Furthermore, the research using hyper-branched dextrin have not yet been reported. In this chapter, the main objective is to synthesize thermoplastic and hydrophobic dextrin derivatives by chemical esterification. A series of fully-substituted dextrin esters (DS = 3) with varying side-chain lengths (C2–12) were synthesized by heterogeneous esterification using



trifluoroacetic anhydride/carboxylic acid. After then, the effect of side-chain lengths on structure and properties were confirmed by structural, thermal, mechanical and hydrophobic analysis.

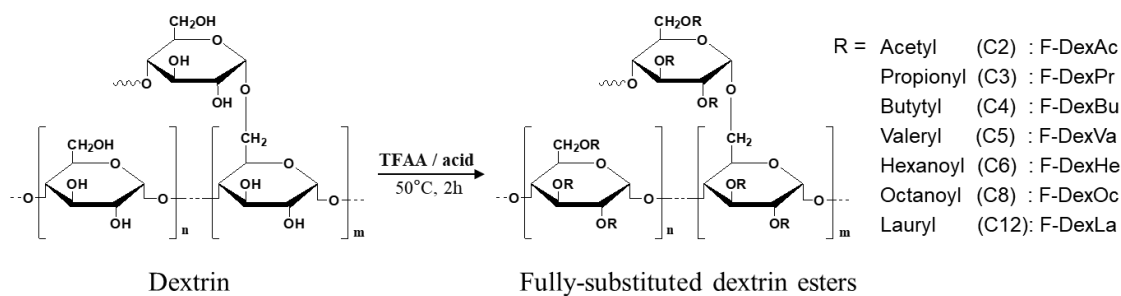
## 3.2. Experimental

### 3.2.1. Materials

Hyper-branched dextrin was prepared from waxy corn starch by partial amylase digestion, obtained from Hayashibara Co. Ltd. (Okayama, Japan). Trifluoroacetic anhydride (TFAA) and carboxylic acids (acetic, propionic, butyric, valeric, hexanoic, octanoic, and lauric acid) were purchased from Wako Pure Chemicals (Tokyo, Japan). All other chemicals were used without further purification.

### 3.2.2. Chemical synthesis of fully-substituted dextrin esters

Dextrin esters were synthesized according to the procedure described in previous other study [11]. Firstly, a heterogeneous mixture of TFAA (50 mL) and carboxylic acid (50 mL) had been stirred at 50 °C for 10 min. Freeze-dried dextrin (1 g) was immediately added into mixture and kept for 2 hours. After cooling to room temperature, the mixture was poured into 1L of methanol, and then precipitate was filtered. To remove the unreacted reagents and byproduct, reprecipitation was conducted in three time by using chloroform and methanol as a good and poor solvent. Then, the remaining solvent was completely dried *in vacuo* (Scheme 3-1).



**Scheme 3-1.** Synthesis of fully-substituted dextrin esters, and their abbreviations.

### 3.2.3. Preparation of films

Dextrin esters (0.5 g) were dissolved in chloroform (10 ml) except for dextrin (using water with same concentration). The solution were poured onto Teflon-plates. The solvent was evaporated at room temperature under atmospheric pressure for 1 week. Colorless and transparent casting films were obtained (Figure 3-1). Also, dipping films were prepared using a glass substrate.



**Figure 3-1.** Film pictures of dextrin and fully-dextrin esters.

### 3.2.4. Measurements

#### 3.2.4.1. Nuclear magnetic resonance (NMR) spectroscopy

Chemical structure were measured by  $^1\text{H}$ -NMR (JNM-A500, 500 MHz, JEOL Ltd. Japan) with *d*-chloroform as a solvent, where solution concentration was set to 10 mg/mL. Chemical shifts ( $\delta$  in ppm) were referenced to the resonance of tetramethylsilane (TMS;  $\delta = 0$ ) for *d*-chloroform. The degree of substitution (DS) was calculated based on the integration ratio of the acyl protons to the ring protons of the glucose unit.

$$\text{DS} = \frac{\text{Peak area } [\text{CH}_3]/3}{\text{Peak area } [\text{ring} - \text{H}]/7}$$

#### 3.2.4.2. Gel permeation chromatography (GPC)

Weight-average molecular weights ( $M_w$ ) and polydispersity indices ( $M_w/M_n$ ) were determined by GPC using a GPC system (CBM-20A, DGU-20A3, LC-6AD, SIL-20AHT, CTO-20A, RID-10A, Shimadzu Corp. Japan). Chloroform was used as the eluent at a flow rate of 0.8 mL/min at 40 °C. Polystyrene standards (Shodex<sup>®</sup> STANDARD SM-105, Showa Denko K.K.) were used to obtain the calibration curve.

#### 3.2.4.3. Fourier-transform infrared (FT-IR) spectroscopy

FT-IR spectra of dextrin and dextrin esters were acquired using Nicolet 6700 spectrometer (Thermo Scientific Corp. USA) using KBr disk technique. For FT-IR measurement, the samples were mixed with anhydrous KBr and then compressed into thin disk-shaped pellets. The spectra were obtained with a resolution of 2  $\text{cm}^{-1}$  and a wavenumber range of 400–4000  $\text{cm}^{-1}$ .

#### **3.2.4.4. Thermogravimetric analysis (TGA)**

Thermal decomposition behaviors were investigated using a Thermo plus TG8120 system (Rigaku Corp., Japan). 5mg of each sample was put in platinum pan and heated at heating rate of 10 °C/min from 40 °C to 600 °C under nitrogen gas.

#### **3.2.4.5. Differential scanning calorimetry (DSC)**

DSC thermograms were obtained by using DSC8500 (PerkinElmer Co. Ltd. Japan). The measurements were carried out using about 2 mg of sample packed in an aluminum pan under the nitrogen atmosphere. The first heating were conducted from 40 °C to 260 °C at a rate of 100 °C/min. After quenching to -10 °C (cooling rate of -100 °C/min, 5 min holding), the second heating were performed to 260 °C at a heating rate of 100 °C/min.

#### **3.2.4.6. Wide-angle X-ray diffraction (WAXD) measurements**

WAXD measurements were conducted using a RINT 2200 (Rigaku Corp., Japan) operating at 40 kV and 40 mA with Cu K $\alpha$  ( $\lambda = 0.15418$  nm) radiation in the symmetrical reflection mode, equipped with a graphite monochromator ( $2\theta_M = 26.58^\circ$  for Cu K $\alpha$ ) at the scattered X-ray position. Scanned  $2\theta$  range was set to 4–40° with a scan rate of 0.5°/min and a step size of 0.1°, where  $\theta$  is the Bragg angle. All measurements were carried out at room temperature under atmospheric pressure.

### **3.2.3.7. Dynamic mechanical analysis (DMA)**

Dynamic mechanical behaviors were investigated in shear mode by a DVA-200S (IT Measurement Control Co., Ltd., Japan). Temperature scans at a frequency of 1 Hz were carried out in the range -10 to 250 °C at a heating rate of 20 °C/min under nitrogen atmosphere. The specimens (5 mm × 5 mm × 0.5 mm) were prepared from casting film.

### **3.2.4.8. Tensile strength test**

Tensile strength measurements were conducted at room temperature using an EZ-test machine (Shimadzu, Japan). The crosshead speed was 10 mm/min, and the initial gauge length was 10 mm. Five specimens (30 mm × 5 mm × 0.2 mm) of casting films were used for each measurement, and the data were averaged for each sample.

### **3.2.4.9. Contact angle (CA) measurements**

The surface wettability of the coated glass substrates were measured by contact angle meter (Drop master 500, Kyowa Interface Science Co., Ltd., Japan). The static contact angle (SCA) was measured using 500 µL syringe and a needle, 0.5 mm in diameter and 38 mm in length and liquid (water) volume of 0.2 mL.

### 3.3. Results and discussion

Table 3-1 shows the characteristic of dextrin and dextrin esters. After esterification of dextrin, the  $M_w$  of dextrin esters gradually increased with the side-chain lengths, ranged from  $8.83 \times 10^5$  to  $1.27 \times 10^6$  g/mol. The obtained product yields were 83–95%.

**Table 3-1.** Characteristic of dextrin and dextrin esters

Name	$M_w$ $\times 10^{-5}$	$M_w/M_n$ ( <i>PDI</i> )	Yield (%)	DS	$T_{d,10\%}$ (°C) <sup>a</sup>	$T_g$ (°C) <sup>b</sup>	$T_g$ (°C) <sup>c</sup>	$\delta_m$ (MPa) <sup>e</sup>	$\varepsilon_b$ (%) <sup>f</sup>	$E'$ (MPa) <sup>g</sup>	CA (°) <sup>i</sup>
Dextrin	5.34	5.35	-	-	301	nd <sup>d</sup>	nd <sup>d</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	26.7±5.5
DexAc	8.83	4.07	84	3	349	162	171	n.d. <sup>h</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	71.8±1.6
DexPr	9.36	2.49	85	3	344	124	127	n.d. <sup>h</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	88.3±1.1
DexBu	9.73	2.63	83	3	348	78	94	n.d. <sup>h</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	94.5±0.6
DexVa	10.15	3.97	89	3	350	64	75	n.d. <sup>h</sup>	n.d. <sup>h</sup>	n.d. <sup>h</sup>	95.7±0.5
DexHe	11.88	2.15	93	3	355	53	63	4.5±0.6	34±9	4.5×10 <sup>-5</sup>	97.1±0.5
DexOc	11.38	3.28	95	3	352	50	67	3.5±0.1	70±17	2.1×10 <sup>-5</sup>	99.8±0.5
DexLa	12.77	2.18	89	3	340	49	62	2.2±0.1	78±12	1.0×10 <sup>-5</sup>	103.4±0.5

<sup>a</sup> Determined by TGA.

<sup>b</sup> Determined by DSC (second heating run).

<sup>c</sup> Determined by DMA (shear mode).

<sup>d</sup> Not detected.

<sup>e</sup> Tensile strength (maximal stress value) by UTM.

<sup>f</sup> Elongation at break by UTM.

<sup>g</sup> Young's modulus by UTM.

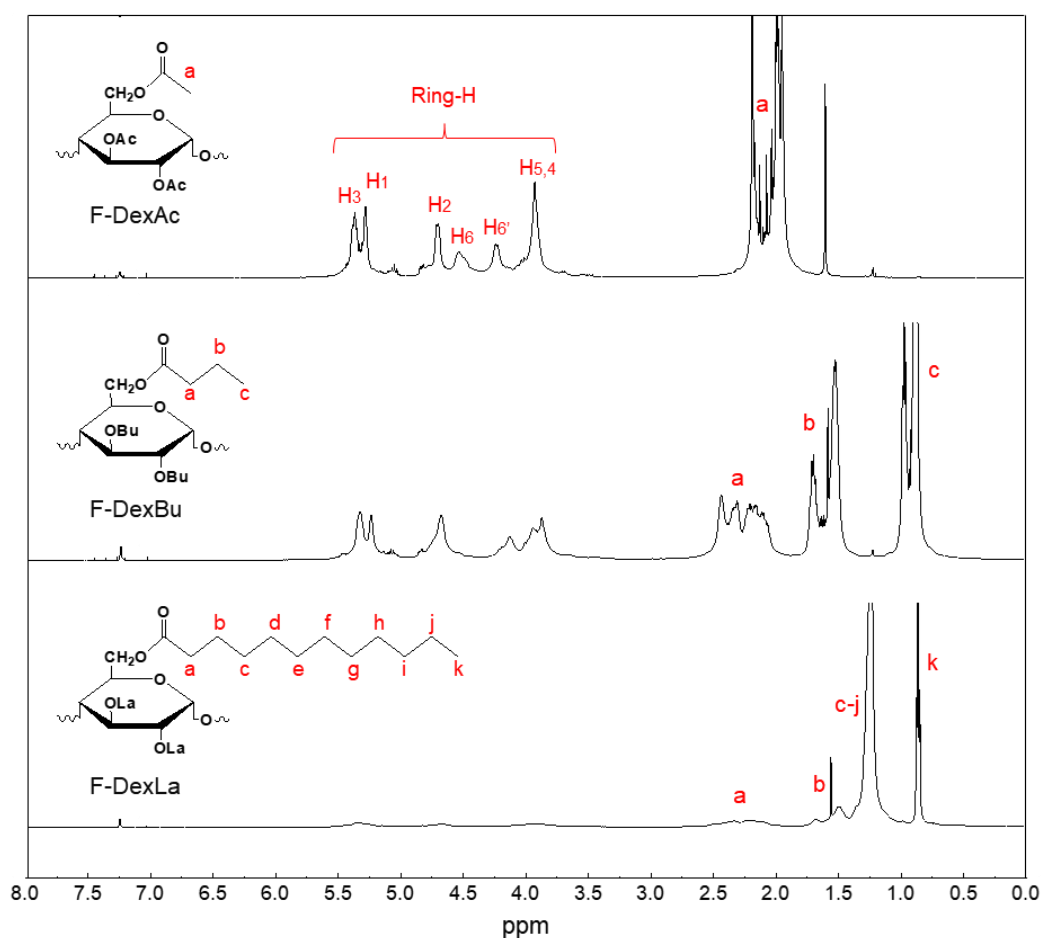
<sup>h</sup> Not conducted because of brittle property of specimen.

<sup>i</sup> Contact angle value.

### 3.3.1. Chemical compositions

By using a integration ratio of the acyl protons to the ring protons of the glucose unit in  $^1\text{H}$ -NMR spectrum, DS value was calculated. In fact, dextrin esters can't have a DS value of 3 even if a complete substitution reaction of hydroxyl groups takes place. This is because the dextrin is a branched structure composed to  $\alpha(1\rightarrow4)$  glucan and  $\alpha(1\rightarrow4,6)$  glucan containing three or two hydroxyl groups in glucose unit. However, the calculated DS of dextrin esters showed a value of 3 for all, which means that the degree of branching in dextrin is negligibly small as a macroscopic viewpoint.

Figure 3-2 shows the  $^1\text{H}$ -NMR spectra of fully-substituted dextrin esters (C2, C4 and C12) as representatives. The peaks derived from ring protons of the dextrin backbone

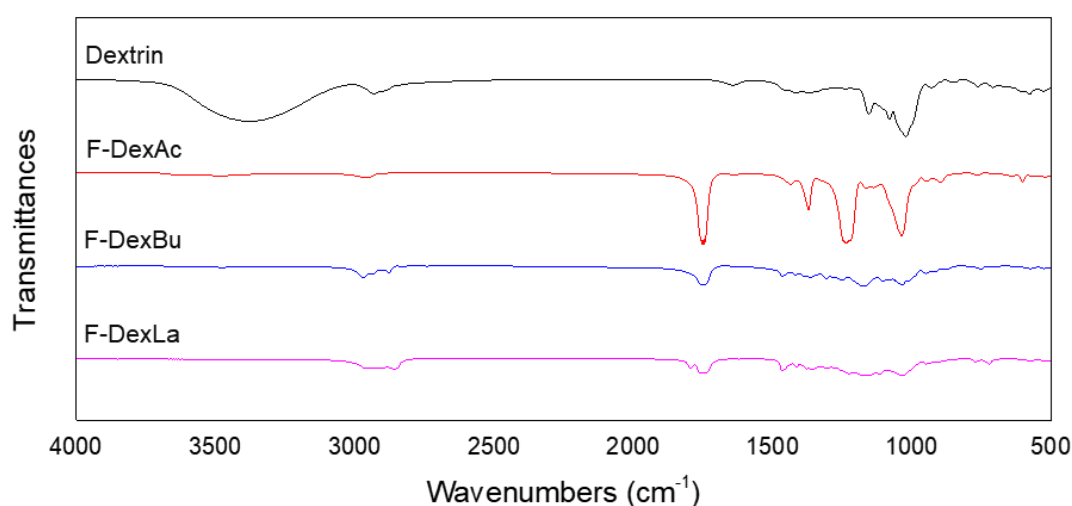


**Figure 3-2.**  $^1\text{H}$ -NMR spectra of fully-substituted dextrin esters.



were observed at 3.7-5.5 ppm in all spectra. The corresponding protons in the backbone ring were labeled on the DexAc spectrum, determined by 2D-NMR analysis (Not shown in this paper). With increasing the composition ratio of acyl chain, the intensity of the peaks related to the ring-proton gradually decreased. As a results of the esterification, all dextrin esters showed strong peaks assigned to methyl ( $-\text{CH}_3$ ) and methylene protons ( $-\text{CH}_2-$ ) of acyl group at 0.7-2.6 ppm. The length of the acyl chain affected the intensity of the peak and made it more complicated.

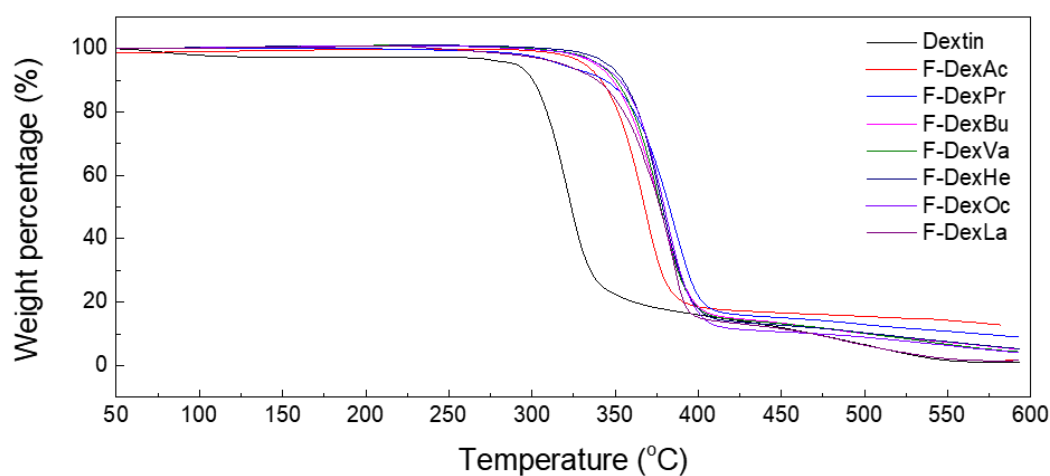
The FT-IR measurement showed similar results, presenting the hydroxyl group peak at  $3500\text{ cm}^{-1}$  disappeared, and the carbonyl group peaks at  $1750\text{ cm}^{-1}$  was newly observed by esterification (Figure 3-3). Moreover, the intensity of acyl group (C-H stretching;  $2910\text{ cm}^{-1}$ , C-H bending ;  $900\text{-}1500\text{ cm}^{-1}$ ) increased compared to native dextrin. From the NMR and FT-IR results, it was confirmed that the esterification reaction of dextrin completely proceed. In our previous experiments using a TFAA/acid homogeneous system, fully-substituted polysaccharides with similar results have already been reported [9, 12, 13].



**Figure 3-3.** FT-IR spectra of dextrin and fully-substituted dextrin esters.

### 3.3.2. Thermal degradation

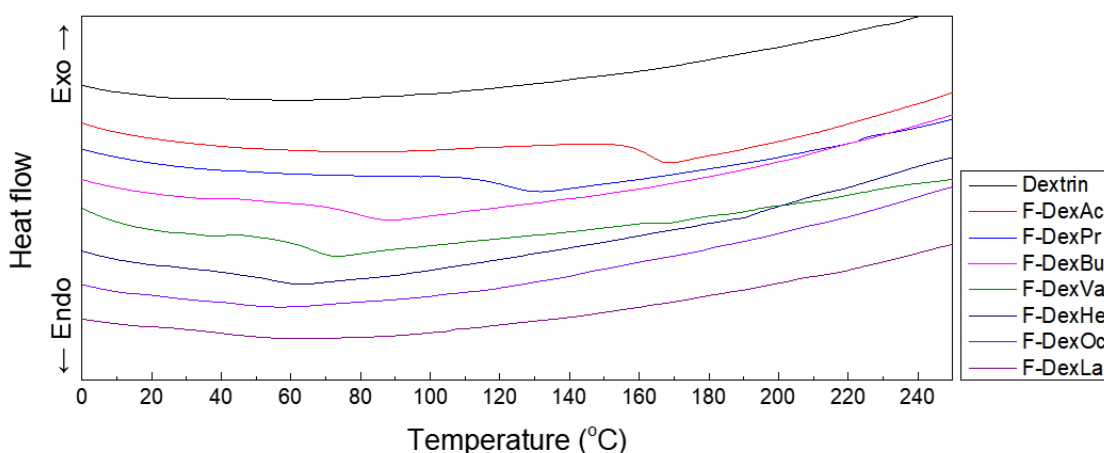
Figure 3-4 shows the thermogravimetric curves of dextrin and dextrin esters. As listed in Table 3-1, the decomposition temperature at 10 % weight loss ( $T_{d,10\%}$ ) of dextrin esters were 340-355 °C, which were higher than that of dextrin (301 °C). This results indicate that esterification reaction enhance thermal stability of dextrin in terms of  $T_{d,10\%}$ . In comparison of the thermal stability between the dextrin esters, the  $T_{d,10\%}$  were not significantly different and were independent of side-chain length. This suggests that the difference in thermal stability between dextrin and dextrin ester is influenced by the presence or absence of hydroxyl groups. Many research related to polysaccharide derivatives such as starch [6], curdlan [9], glucomannan [12] and xylan [14] have reported similar thermal behavior. It is generally known that levoglucosan (volatile) is produced in the pyrolysis process of polysaccharide regardless of their linkage position and anomericity [15, 16]. Commonly, the substitution of the hydroxyl groups by the esterification hinders formation of levoglucosan, resulting in improved thermal stability to polysaccharide esters.



**Figure 3-4.** TGA thermocurves of dextrin and fully-substituted dextrin esters.

### 3.3.3. Thermal properties

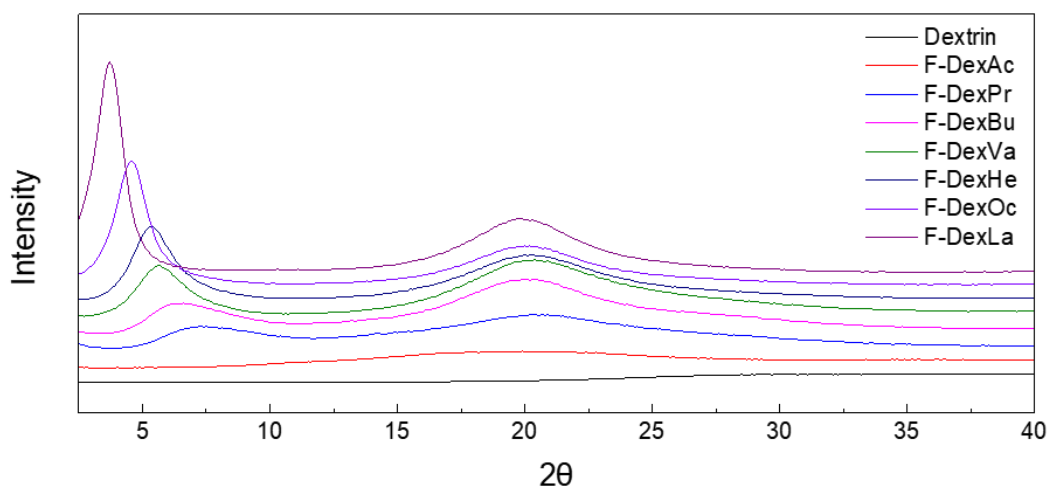
DSC second heating thermocurves of dextrin and dextrin esters are shown in Figure 3-5. The heating rate was set at 100 °C/min because no peak was observed at the usual heating rates of 10 or 20 °C/min, which means that the molecular motion of dextrin esters is quite restricted. The peaks of crystallization or melting were not observed because dextrin and dextrin esters were amorphous. The glass transition temperature ( $T_g$ ) was determined for all dextrin esters. As shown in Table 3-1, The  $T_g$ s of dextrin esters decreased with the increase of side chain lengths, ranged from 162.2 °C to 49.2 °C for DexAc (C2) and DexLa (C12), respectively. It is because the mobility of dextrin esters increase due to the increase of the free volume depending on acyl side-chain. At over DexHe (C6), there is no significant difference in  $T_g$ , showing around 50 °C. This might be because steric hindrance of neighboring bulky side chains restrict the rotation of glycosidic linkages in the dextrin backbone. Similar results were discussed in studies of amorphous polysaccharide esters such as pullulan [13] and xylan [14].



**Figure 3-5.** DSC second heating thermograms of dextrin and fully-substituted dextrin esters.

### 3.3.4. Structure analysis

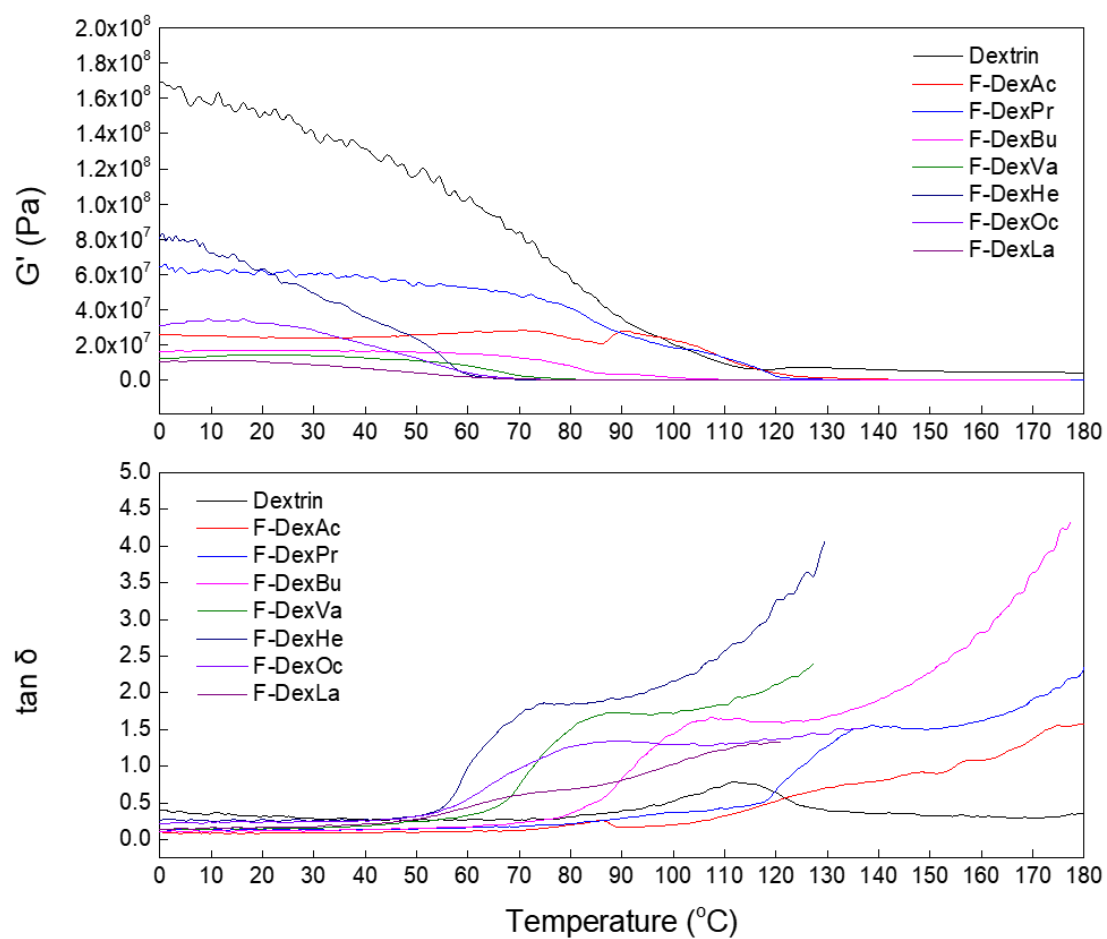
Figure 3-6 shows WAXD curves of casting films for dextrin and dextrin esters. All curves display a major reflection in the low angle region ( $4^\circ < 2\theta < 8^\circ$ ) and a broad and weak reflection at  $2\theta = 20^\circ$ , except dextrin. These reflection are a typical results of amorphous polysaccharide esters based on reports of other polysaccharides esters [13, 14]. The reflection at low angle region indicates the corresponding distance between the dextrin backbone chains. With increasing the side-chain length, the reflection of the backbone chains were shifted to the lower angle because the distance between the backbone-chains increases. The scattering at  $2\theta = 20^\circ$  was derived from other distances such as the possible distance between the acyl side-chains. The intensity of these peak increased as the length of side-chain increased.



**Figure 3-6.** WAXD curves of dextrin and fully-substituted dextrin esters.

### 3.3.5. Dynamic mechanical properties

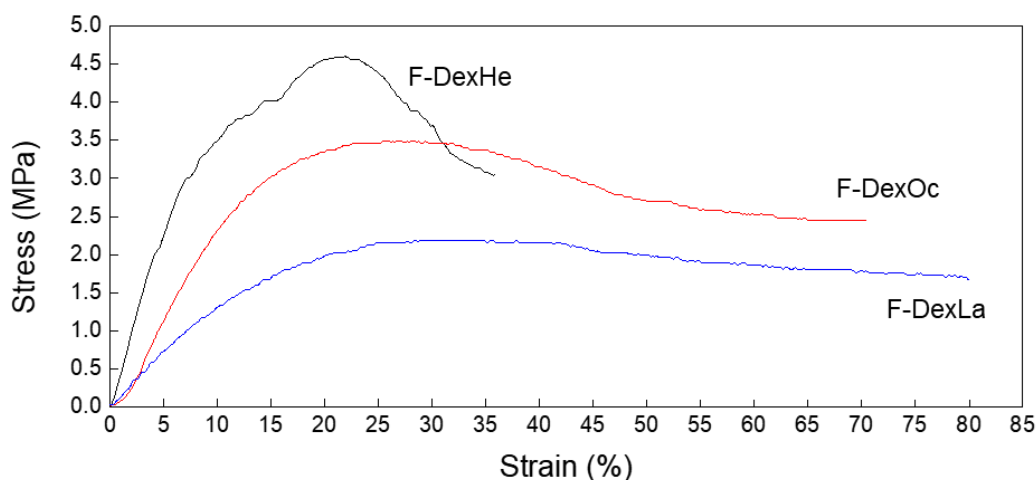
The dynamic properties provide significant information on molecular motion. Figure 3-7 shows the dynamic mechanical properties, storage modulus and tangent delta ( $\tan \delta$ ), of the dextrin and dextrin ester films as a function of temperature. All dextrin ester films showed marked drop in the storage modulus at room temperature and these values decreased with side-chain lengths, overall (Figure 6a). Dextrin ester films have single peak in the  $\tan \delta$ , assigned to the relaxation of acyl side-chains (Figure 6b). The decreasing temperature of relaxation peaks showed a corresponding tendency to the result of  $T_g$  revealed in DSC measurement. This results indicate that thermoplasticity of the dextrin is improved by addition of acyl side-chain. The same phenomenon has been observed for fatty acid esters of glucomannan [12] and pullulan [13]. Unlike dextrin esters, the single peak appearing in neat dextrin is not  $T_g$ . Because dextrin films were prepared from water solution, some amount of water molecules remain inside film. Water molecules might be evaporated at this temperature.



**Figure 3-7.** Storage modulus (top) and  $\tan \delta$  (bottom) curves of dextrin and fully-substituted dextrin esters.

### 3.3.6. Mechanical properties

To measure mechanical properties, the stress-strain behaviors of only dextrin ester films (C6-12) were investigated by tensile strength test at room temperature. Dextrin and dextrin esters with short side-chain (C2-5) were not measured because their casting films were too brittle to be fixed on a tensile testing machine. As shown in the Figure 3-8 and Table 3-1, the Young's modulus ( $E$ ) and tensile strength tended to decrease with increasing the side-chain lengths, whereas the elongation at break increased. Namely, dextrin ester films behave more soft and flexible with increasing the side chain lengths, which allowed the film exhibit better plasticization. The dependence of mechanical properties on dextrin ester films is considered to be due to the enhanced plasticizing effect by acyl side-chains [17]. As mentioned above in DSC results, this enhanced plasticizing effect has already affected  $T_g$ . A similar tendency of the mechanical properties has been observed in other polysaccharide esters with acyl side-chains [5, 6, 9, 13]. Compared to other polysaccharide esters, The reason that dextrin esters have a relatively low Young's modulus is predicted due to the low intermolecular interaction by branched structure of dextrin backbone.

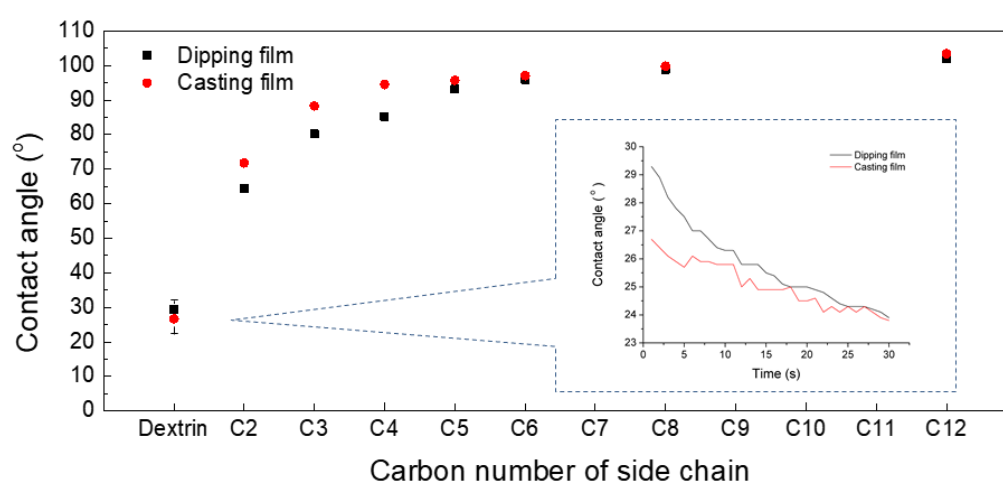


**Figure 3-8.** Strain-stress curves of the fully-substituted dextrin esters.

### 3.3.7. Surface hydrophobicity

The CA can be used to compare the hydrophobicity of the material by measuring the angle of liquid droplet between the liquid/vapor interface, where a liquid/vapor interface meets a solid surface. Figure 3-9 shows the CA values of dextrin and dextrin esters from dipping and casting films. The dextrin films showed the  $29.6^{\circ} \pm 10.2^{\circ}$  property and this value decreased over time because of their dissolution characteristic in water. As a result of esterification, the surface properties of the dextrin ester films showed considerable differences compared to unmodified dextrin. The CA of the dextrin ester films were checked as increasing side-chain lengths. The dextrin ester films showed increased hydrophobic properties up to  $102.1^{\circ} \pm 1^{\circ}$  due to the replacement of hydrophilic hydroxyl groups to hydrophobic acyl groups. The CA values of dextrin ester films sharply increased until DexVa (C5). At over the DexVa (C5), most of the dextrin ester films showed similar CA values. This indicates that the valeric acid (C5) is sufficient acyl donor for imparting hydrophobicity to the dextrin. It is well known that a component with lower surface energy in multicomponent polymeric systems is preferentially segregated at the air region in order to minimize the interfacial free energy [18]. The modified hydrophobic property of dextrin showed similar results to those of starch [19, 20], cellulose [21] and xylan [14].





**Figure 3-9.** Static contact angle graph of dextrin and fully-substituted dextrin esters

### 3.4. Conclusion

A series of dextrin fatty ester derivatives having a different acyl side-chain length (C2-12) were synthesized by heterogeneous system. The GPC,  $^1\text{H-NMR}$  and FT-IR analysis presented the fully-substituted dextrans (DS=3) with high molecular weights and high yields,  $8.8\text{-}12.7 \times 10^5$  g/mol and 83–95%, respectively. TGA revealed that the replacement of hydroxyl groups to acyl group by esterification improve thermal properties of dextrin, showing 41-54°C higher  $T_{d,10\%}$ . In DSC and XRD analysis, the transition temperature of melting and crystallization were not observed for all dextrin esters because they were amorphous polymers. The  $T_g$ s of dextrin esters decreased with increasing acyl side-chain lengths. This indicates that thermoplasticity of the dextrin is successfully improved by esterification. Colorless and transparent dextrin ester films were prepared to measure the mechanical properties for DMA and tensile strength test. With increasing acyl side-chain length, the mechanical behavior of dextrin ester films were gradually turned to more soft and flexible properties. The CA of dextrin ester films also increased with acyl side-chain, which means that hydrophobicity of dextrin can be controllable depending on the length of acyl chain. This study is expected to help resolve the limitations of commercialization of dextrin due to low thermoplastic and hydrophobicity.

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## **Chapter 4**

### **Enzymatic Modification; Regioselectively Substituted Dextrin Esters**

#### **Synthesis of Optimization of Lipase-catalyzed Dextrin Esters**

#### **for Regioselective Substitution**

### **4.1. Introduction**

Enzymatic esterification is a promising alternative method to chemical esterification for modifying functionality to the polysaccharide. Branched dextrin, with two types of glycosidic bonds, is a suitable substrate for enzymatic esterification, because it has good solubility in organic solvent (a consequence of the extra degrees of freedom provided by rotation at the C6 position) [1, 2]. Chemical esterification is suitable for the production of fully-substituted dextrin esters as above, Chapter 3, presenting that the thermoplasticity and hydrophobicity of the dextrin were significantly increased. However, this route has a limitation when regioselectively-substituted amphiphilic dextrin esters are desired [3]. Enzymatic esterification is efficient to produce regioselectively-substituted dextrin derivatives as a green chemistry due to regiospecificity of enzyme under environmentally benign condition [4, 5]. In addition, this route allows for the synthesis of polysaccharide esters with well-defined structures and functionalities. Actually, most of enzymes are generally efficient catalysts for the hydrolysis of esters, but in the absence of water (i.e., in organic solvents) they catalyze the reverse reaction. Among enzymes, lipases have gained attention by many industries, because of their general ease of handling, broad substrate tolerance, high stability towards temperatures and solvents, and convenient commercial availability [6].

In this chapter, Four lipase enzymes were investigated as catalysts in the synthesis of regioselectively monosubstituted dextrin esters from dextrin and vinyl acetate, and the parameters that affect the enzyme activity were identified. The regioselectively-substituted dextrin esters were then characterized in detail to determine their structure.

## 4.2. Experimental

### 4.2.1. Materials

Hyper-branched dextrin was prepared from waxy corn starch by partial amylase digestion ( $M_w = 1,070,000$  g/mol). The used enzymes were four commercial lipase enzymes, which are Lipozyme CALB, Novozyme 435, Lipozyme TL 100L and Lipozyme TL IM. All lipase enzymes were supplied by Novozymes (Bagsvaerd, Denmark) and their characteristic in aqueous system are shown in Table 4-1. Dextrin and liquid type enzymes were treated by freeze drying before reaction to remove the water. Vinyl acetate, propionate, hexanoate and laurate were purchased from Wako Pure Chemicals (Tokyo, Japan). All other chemicals were used without further purification.

**Table 4-1.** Types and characteristics of lipase enzymes

Product name	Source	Type	Activity
Lipozyme CALB	<i>Candida antarctica B</i>	Liquid lipase	$5 \times 10^3$ U/g
Novozyme 435	<i>Candida antarctica B</i>	Immobilized lipase	$1 \times 10^4$ U/g
Lipozyme TL 100L	<i>Thermomyces lanuginosa</i>	Liquid lipase	$1 \times 10^5$ U/g
Lipozyme TL IM	<i>Thermomyces lanuginosa</i>	Immobilized lipase	$2.5 \times 10^2$ U/g

\* Lipase unit is the amount of enzyme activity liberates  $1 \mu\text{mol}$  of each specific product represented by Novozymes. For the immobilized lipase, the enzyme unit (U/g) contains the mass of substrate resin for immobilization of enzyme.

### 4.2.2. Optimization of enzyme activity

Table 4-1 shows the activity of each enzyme, but the enzyme in the organic solvent system act quite differently unlike in the aqueous system. Therefore, the activity of four enzymes between dextrin and vinyl acetate were identified in organic solvent by varying influence factors to find optimal condition. The standard reaction were carried out at

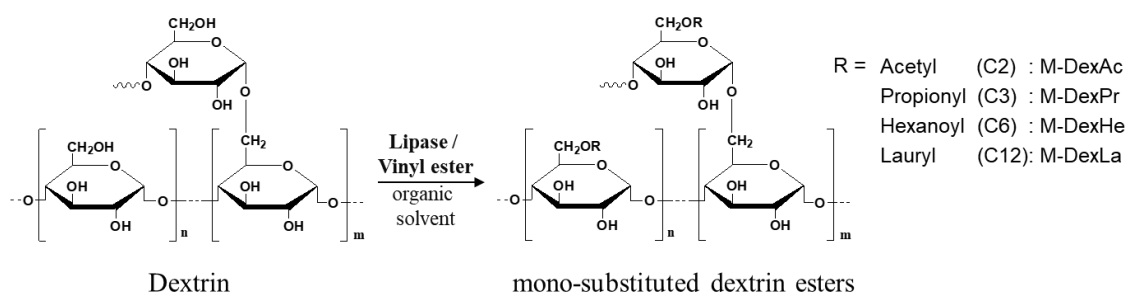
60 °C for 12 hours in super dehydrated dimethyl sulfoxide (DMSO, 10 ml) with freeze-dried dextrin (2.5 mmol) and vinyl acetate (7.5 mmol) and enzyme (liquid type; 10 ml or immobilized type; 0.1 g). And then, the influence parameters such as enzyme type, solvent system, reaction temperature, molar ratio of glucose unit to vinyl acetate, dextrin concentration, enzyme dosage and reaction time were investigated. The activity of enzyme was determined based on the DS value, calculated by the integration ratio of the acyl protons to the C1 proton of glucose in  $^1\text{H}$ -NMR spectrum.

$$\text{DS} = \frac{\text{Peak area } [\text{CH}_3]/3}{\text{Peak area } [\text{H1 of glucose}]}$$

#### 4.2.3. Enzymatic synthesis of mono-substituted dextrin esters

Regioselectively mono-substituted dextrin esters (*mono-DexEs*) were synthesized according to the following procedure using lipase enzymes as a biocatalyst (Scheme 4-1). The homogeneous mixtures were prepared in super dehydrated DMSO (10 ml) by mixing freeze-dried dextrin (2.5 mmol) and vinyl ester (7.5 mmol) and Lipozyme TL IM (0.1 g; 2.5 U/ml). The mixtures were kept at 60 °C with increasing reaction time up to 120 hours in air-bath shaker with 200 rpm. At this time, Lipozyme TL IM was replaced with new one every 2 days. After the reaction, the mixtures were cooled to room temperature, and enzyme was filtered by nylon mesh (50  $\mu\text{m}$  of pore size). The filtrate solution were precipitated with ethanol followed by centrifugation (8000 rpm, 5 min). The precipitate were washed with water and ethanol to remove the residual enzyme, solvent, unreacted reagents, and byproduct. Lastly, the obtained lipase-catalyzed dextrin esters were dried in vacuum at 100 °C for 24 h.





**Scheme 4-1.** Synthesis of regioselectively mono-substituted dextrin esters using lipase enzyme, and their abbreviations.

#### 4.2.4. Peracylation of mono-substituted dextrin esters

To compare the properties of substitution degree and position, fully-substituted dextrin esters (fully-DexEs) and random-substituted dextrin esters (*random*-DexEs) were synthesized by chemical esterification according to the procedure described in Chapter 3. Briefly, a heterogeneous mixture of trifluoroacetic anhydride (50 ml) and carboxylic acid (50 ml) had been stirred at 50 °C for 10 min. And freeze-dried dextrin (1 g) was immediately added into mixture and kept for 2 hours. After cooling to room temperature, the mixture was poured into methanol, and then precipitate was filtered. To remove the unreacted reagents and byproduct, reprecipitation was conducted in three time and was completely dried in vacuo.

#### 4.2.5. Confirmation of substitution position

The first method to identify the substitution position of mono-DexEs was directly performed by two-dimensional NMR analysis. Another method was to indirectly compare the structures of mono-DexEs and fully-DexEs, but the following procedure was additionally required because of the solubility differences in common solvents; The mono-DexEs obtained through enzymatic synthesis were converted to regioselectively

per-substituted dextrin esters (regio-DexEs) by following a stepwise chemical synthesis. The substitution position of mono-DexEs was indirectly confirmed by comparison to regio-DexEs and fully-DexEs.

## **4.2.6. Measurements**

### **4.2.6.1. Nuclear magnetic resonance (NMR) spectroscopy**

Chemical structure of were measured by  $^1\text{H}$ -NMR,  $^{13}\text{C}$ -NMR and two-dimensional NMR (JNM-A500, 500 MHz, JEOL Ltd. Japan) with dimethyl sulfoxide-*d*<sub>6</sub> as a solvent, where solution concentration was set to 20 mg/ml. Chemical shifts ( $\delta$  in ppm) were referenced to the resonance of tetramethylsilane (TMS;  $\delta = 0$ ) for dimethyl sulfoxide-*d*<sub>6</sub>.

### **4.2.6.2. Fourier-transform infrared (FT-IR) spectroscopy**

FT-IR spectra of dextrin and dextrin esters were acquired using Nicolet 6700 spectrometer (Thermo Scientific Corp. USA) using KBr disk technique. For FT-IR measurement, the samples were mixed with anhydrous KBr and then compressed into thin disk-shaped pellets. The spectra were obtained with a resolution of  $2\text{ cm}^{-1}$  and a wavenumber range of  $400\text{--}4000\text{ cm}^{-1}$ .

### **4.2.6.3. Thermogravimetric analysis (TGA)**

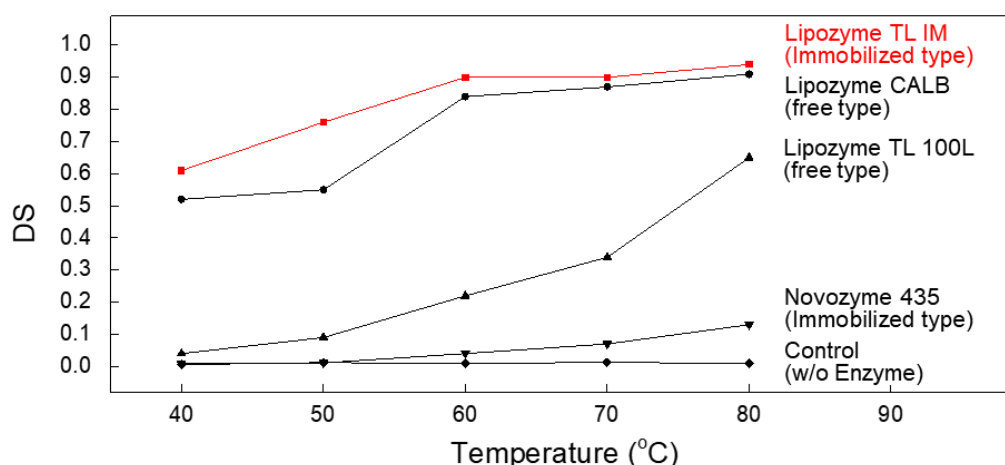
Thermal decomposition behaviors were investigated using a Thermo plus TG8120 system (Rigaku Corp., Japan). 5 mg of each sample was put in platinum pan and heated at heating rate of  $10\text{ }^\circ\text{C}/\text{min}$  from  $40\text{ }^\circ\text{C}$  to  $600\text{ }^\circ\text{C}$  under nitrogen gas.

## 4.3. Results and discussion

### 4.3.1. Optimization of enzyme activity

#### 4.3.1.1. Effect of reaction temperature on enzyme activity

An important property of enzymes is that they exhibit substrate specificity under the conditions required for activity. Initially, the effect of reaction temperature was studied, because enzyme activity is sensitive to temperature. The activity of four enzymes in the catalyzed reaction between dextrin and vinyl acetate was determined at various temperatures, ranging from 40 °C to 80 °C, after 12 hours. Figure 4-1 shows the DS values of the dextrin acetate (DexAc) for each type of enzyme. Overall, the catalytic activity of all enzymes increased with increasing temperature. However, two of the four enzymes, Lipozyme CALB and Lipozyme TL IM, displayed high catalytic activity. Lipozyme CALB is a non-immobilized enzyme extracted from *Thermomyces lanuginose*, and Lipozyme TL IM is an immobilized enzyme extracted from *Candida antarctica* B.

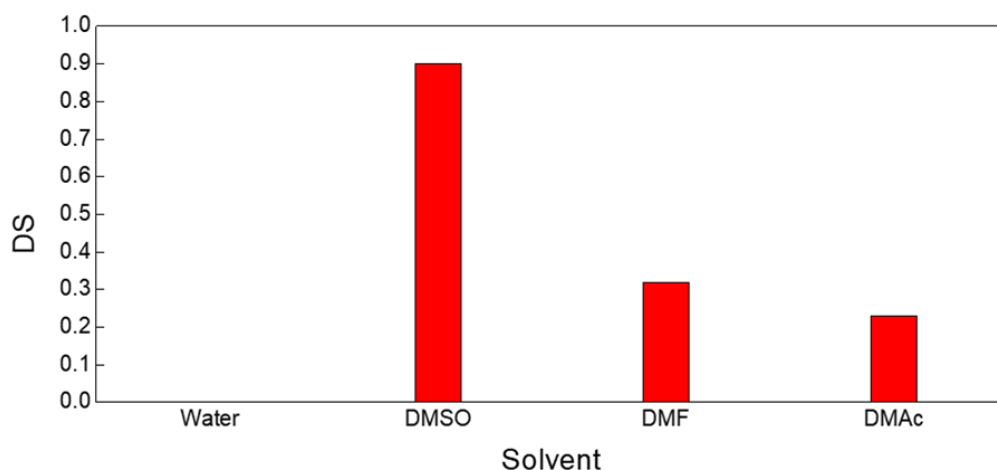


**Figure 4-1.** DS values of dextrin acetate for each type of enzyme versus reaction temperature.

Although both enzymes exhibited high catalytic activity, the reaction conditions were only optimized for Lipozyme TL IM in the following experiments. This is because the immobilized enzyme has superior thermal stability and reusability, which is commercially advantageous [11]. The DS values of DexAc produced by the Lipozyme TL IM-catalyzed reaction at 40 °C, 50 °C, 60 °C, 70 °C and 80 °C were 0.61, 0.76, 0.90, 0.90 and 0.94, respectively. The DS values gradually increase until 60 °C, and stay relatively constant, at around 0.90, beyond this temperature. This indicates that a reaction temperature of 60 °C is sufficient for imparting acetate groups to the dextrin regioselectively.

#### 4.3.1.2. Effect of solvent on DS value

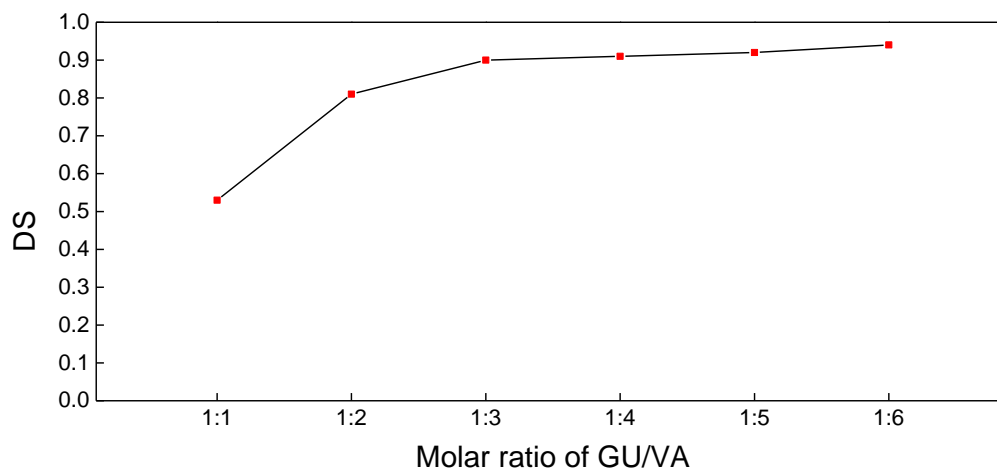
The selection of solvent is also an important factor in the optimization of enzymatic polysaccharide modification reactions. To obtain a high DS value, the polysaccharide should be fully dissolved and also have affinity for the enzyme. Dextrin characteristically dissolves in polar solvents. The catalytic activity of the enzyme was confirmed using typical polar solvents, such as water, dimethylsulfoxide (DMSO), *N,N*-dimethylformamide (DMF) and *N,N*-dimethylacetamide (DMAc) (Figure 4-2). In water, the substitution reaction was not detectable, because the enzyme-catalyzed hydrolysis reaction dominated. In DMSO, DMF and DMAc, the DS values were 0.90, 0.32 and 0.23, respectively. There is a positive correlation between the solvent polarities and the DS values. Therefore, the differences in the DS values might simply be due to the differences in polarity of the solvents. The most polar solvent (DMSO) gave the highest DS value (0.90). This solvent is commonly used in experiments involving polysaccharides [12, 13].



**Figure 4-2.** DS values of dextrin acetate obtained by modifying the solvent.

#### 4.3.1.3. Effect of glucose unit/vinyl acetate molar ratio on DS value

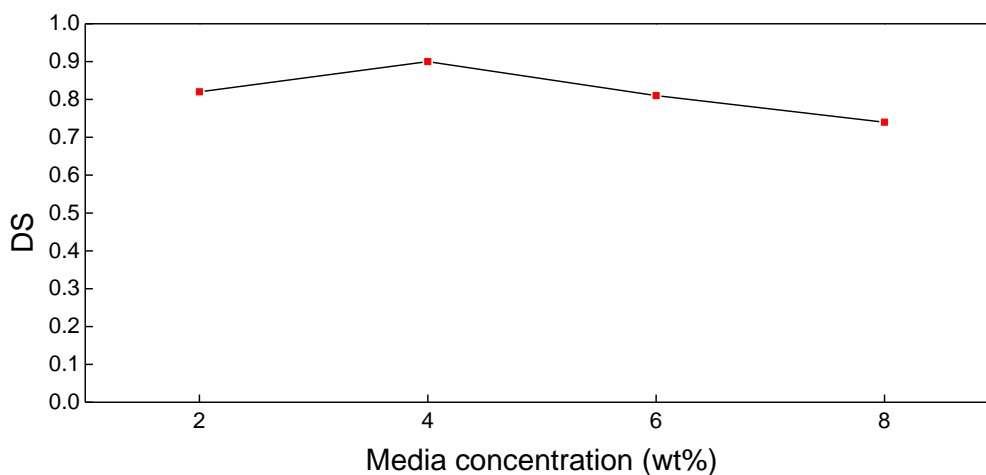
To confirm the effect of the acyl donor content on the DS value, the reaction was carried out using various molar ratios of the glucose unit to vinyl acetate (1:1, 1:2 1:3, 1:4, 1:5 and 1:6). The results are shown in Figure 4-3. As the molar ratio of vinyl acetate increases, the DS value also increases. This is because the reaction frequency between the hydroxyl group of the glucose and the ester group of vinyl acetate increases stochastically. However, there was no significant difference in the DS values at molar ratios higher than 1:3 (all conditions between 1:3 and 1:6 gave DS values between 0.90 and 0.94). These results indicate that a molar ratio of 1:3 is sufficient to obtain a high DS value. Previous studies on other polysaccharides have shown similar results.



**Figure 4-3.** DS values of dextrin acetate obtained by modifying the molar ratio of glucose unit/vinyl acetate (GU/VA).

#### 4.3.1.4. Effect of media concentration on DS value

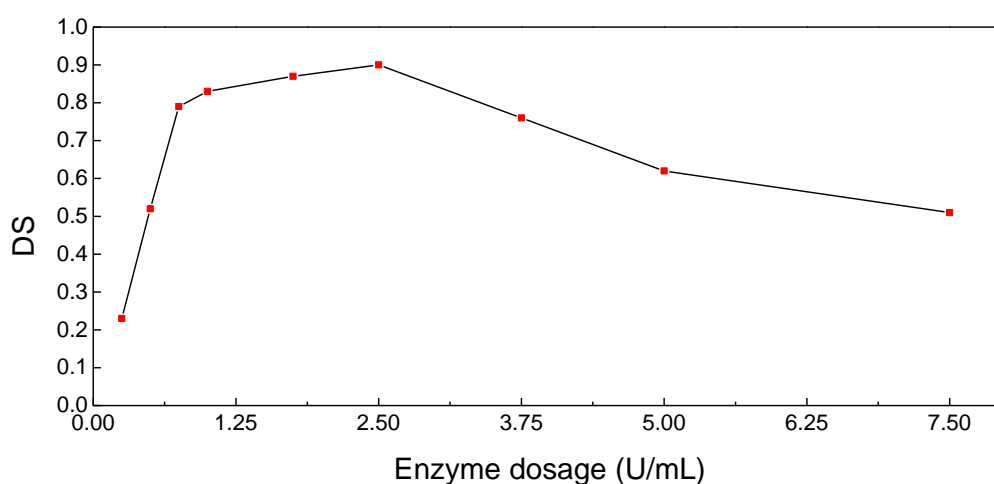
It is well known that branched polymers, such as dextrin, have unique viscosity properties [14]. Therefore, to confirm the effect of the dextrin media concentration, the catalytic activity of the enzyme was determined at various dextrin concentrations, ranging from 2 to 8 wt%. Dextrin showed good solubility in DMSO and low visible viscosity at all media concentrations. Figure 4-4 shows the DS values of DexAc produced by the lipase-catalyzed reaction versus media concentration. The DS values did not change significantly with media concentration, and the highest value obtained (0.90) was at 4 wt%. These results indicate that the most efficient media concentration for collision probability of dextrin, vinyl acetate and enzyme is 4 wt%.



**Figure 4-4.** DS values of dextrin acetate obtained by modifying the media concentration.

#### 4.3.1.5. Effect of enzyme dosage on DS value

The amount of used enzyme in an enzyme-catalyzed reaction is a factor that directly influences the degree of reaction. The DS values were determined while increasing the amount of enzyme from 0.25 U/mL to 7.5 U/mL (Figure 4-5). When a small amount of enzyme was used (between 0.25 U/mL and 1 U/mL), the DS value increased sharply with increasing enzyme dosage. Between 1 U/mL and 2.5 U/mL, the DS value gradually increased with increasing dosage. However, when the dosage exceeded 3.75 U/mL, the DS value began to decrease. The highest DS value (0.90) was observed at 2.5 U/mL, indicating that the enzyme is the most active at this dosage. The lower degree of reactivity with excess enzyme is thought to be due to the interruption of the movement of the enzyme to neighboring hydroxyl groups by other enzymes. It has been shown in other literature that the enzyme engages the hydroxyl groups of polysaccharide substrates in a sequential manner.

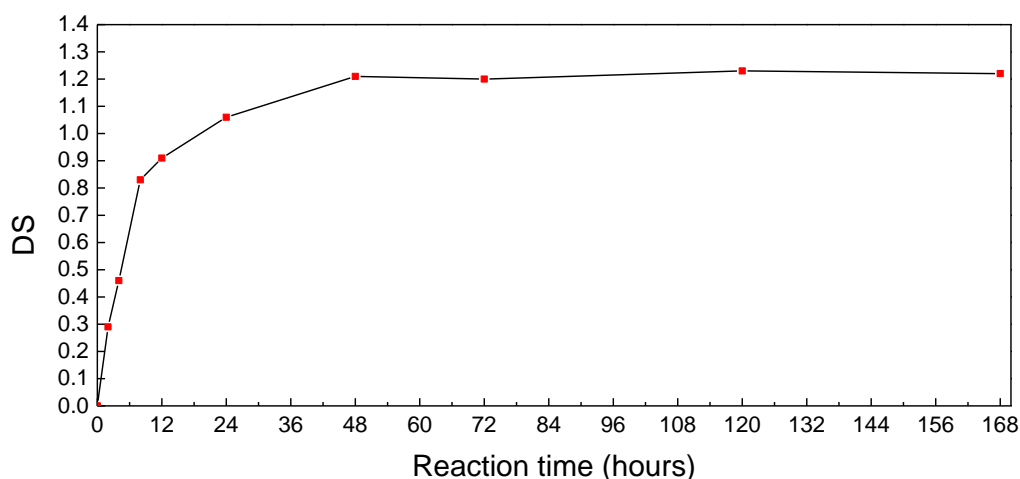


**Figure 4-5.** DS values of dextrin acetate obtained by modifying the enzyme dosage.



#### 4.3.1.6. Effect of reaction time on DS value

To obtain fully-modified DexAc (i.e., with a DS value of 1), the reaction time was increased from 2 hours to 120 hours, and the effect of reaction time on the DS values was determined. As shown in the Figure 4-6, the dextrin substitution reaction occurs rapidly within the first 24 hours, giving a DS value of 1.06 at the 24-hour time point. The DS values at 48, 72 and 120 hours were 1.21, 1.20 and 1.23, respectively. These values are similar to the DS value at 48 hours, indicating that only a slight substitution reaction takes place up to the equilibrium state of the reaction. These results suggest that substitution reactions at the C2 and C3 positions are expected only after the substitution reaction takes place preferentially at the C6 hydroxyl group (i.e., regioselectively). Therefore, the time required to obtain mono-substituted dextrin acetate (mono-DexAc) (DS = 1.06) is 24 hours.

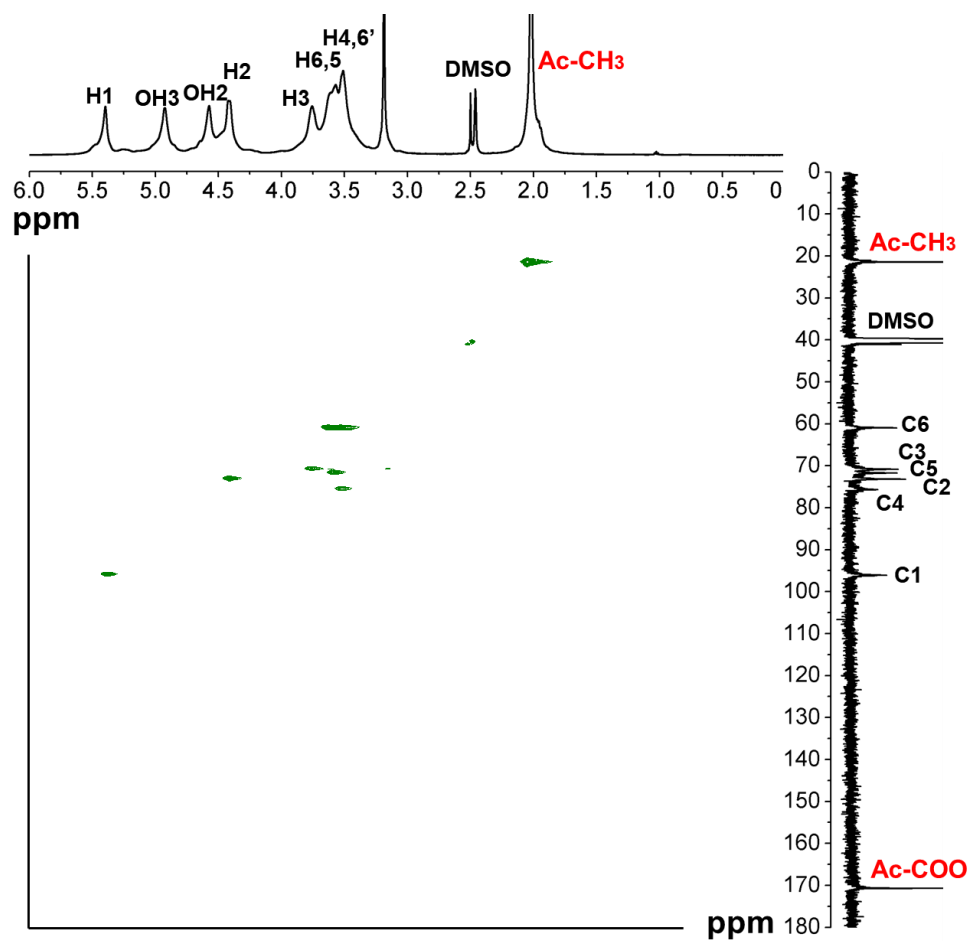


**Figure 4-6.** DS values of dextrin acetate versus reaction time under optimal conditions.

### **4.3.2. Characterization of mono-substituted dextrin acetate**

#### **4.3.2.1. Chemical compositions**

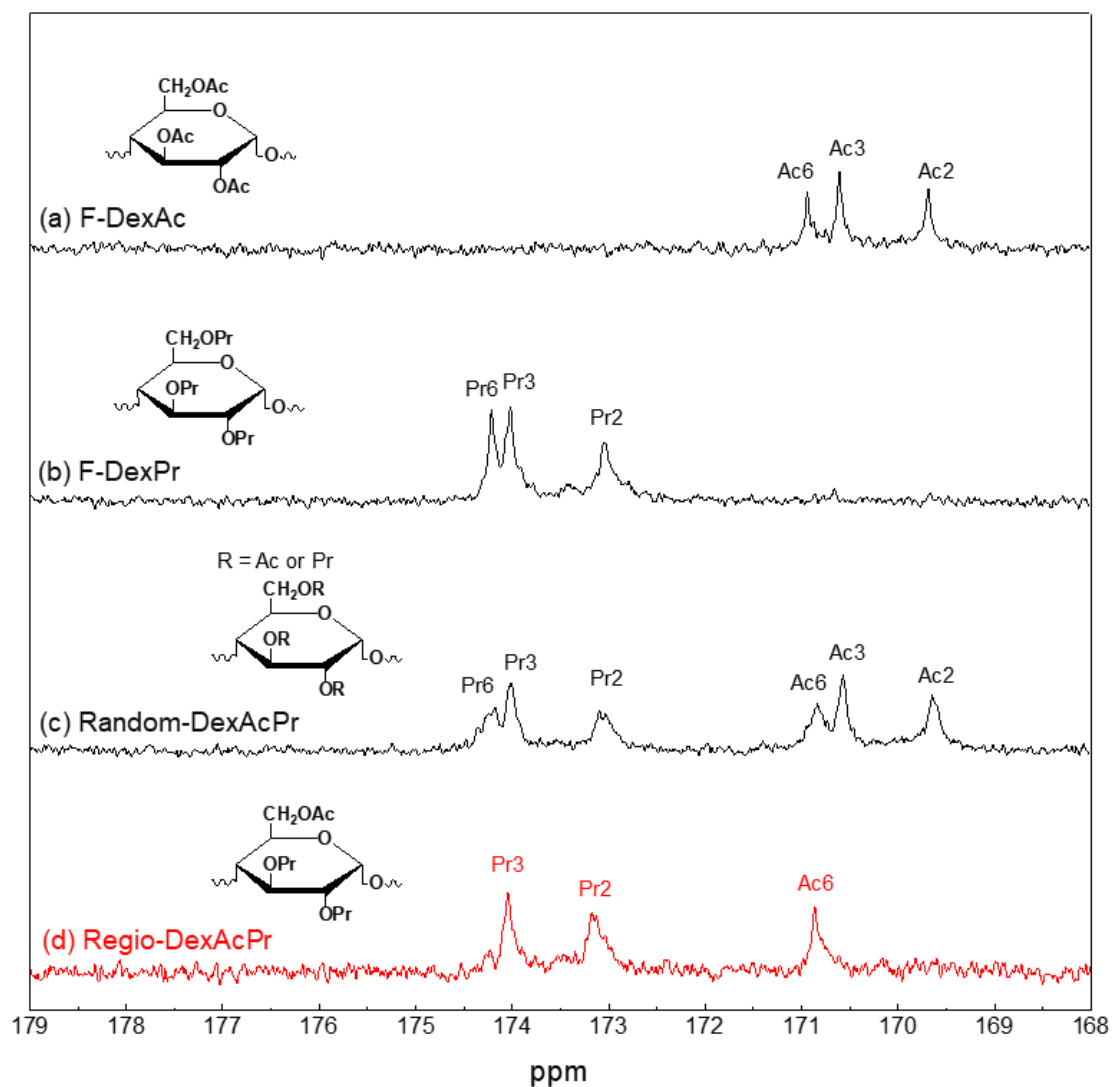
The structural analysis of mono-DexAc (with a DS value of 1.06) was carried out using  $^1\text{H}$ ,  $^{13}\text{C}$  and two-dimensional NMR. Figure 4-7 shows the HSQC spectrum of mono-DexAc with the respective protons marked on each of the peaks. The hydroxyl peaks in the dextrin  $^1\text{H}$ -NMR spectrum (X-axis), showed slight changes during the enzymatic reaction; the disappearance of the peak corresponding to the hydroxyl group at the C6 position demonstrates the regioselective substitution of the dextrin. Concurrently, a strong Ac-CH<sub>3</sub> peak is observed at 2.02 ppm, due to the addition of the acetate group. In the  $^{13}\text{C}$ -NMR spectrum (Y-axis), peaks corresponding to the Ac-CH<sub>3</sub> (21.5 ppm) and Ac-COO- (170.7 ppm) groups of the regioselectively substituted acetate appear together, and the peaks corresponding to the glucose carbons are observed in the region of 100–60 ppm. The results of the NMR study show that the regioselective reaction proceeds successfully.



**Figure 4-7.** HSQC spectrum of mono-substituted dextrin acetate (DS = 1.06).

#### 4.3.2.2. Confirmation of substitution position

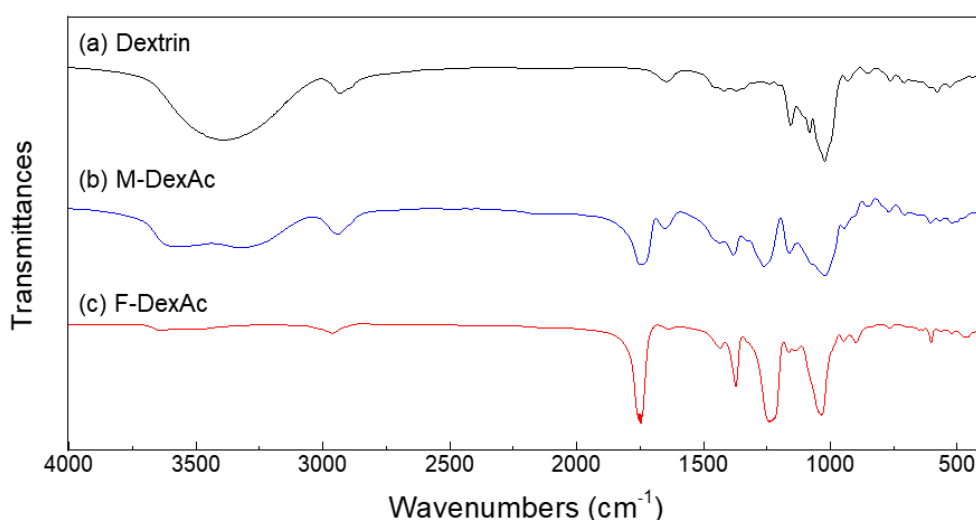
It was difficult to directly confirm the substitution positions of mono-DexAc and fully acetylated dextrin (fully-DexAc) using NMR analysis, because of the differences in solubility of the two substances in common solvents. To solve this problem, mono-DexAc was peracylated, replacing the remaining hydroxyl groups with propionate groups (regio-DexAcPr). The substitution positions of four dextrin esters, fully-DexAc, fully-DexPr, random-DexAcPr and regio-DexAcPr, all of which dissolved in chloroform, were identified by their carbonyl peaks in  $^{13}\text{C}$ -NMR (Figure 4-8). The carbonyl peaks of the fully-substituted dextrin esters appear in three regions of the spectrum, but those of fully-DexPr (Pr-COO-; 174.2, 174.0 and 173.0 ppm) were found to be higher than those of fully-DexAc (Ac-COO-; 170.9, 170.6 and 169.7 ppm). All carbonyl peaks corresponding to acetate and propionate were observed in the spectrum of random-DexAcPr, which was synthesized to determine the effect of the composition ratio. The spectrum of regio-DexAcPr includes a C6 acetate peak at 170.9 ppm, in addition to the C2 and C3 propionate peaks at 173.1 ppm and 174.0 ppm, respectively. These results prove that the lipase-catalyzed reaction proceeds regioselectively, in accordance with  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR data obtained for mono-DexAc.



**Figure 4-8.**  $^{13}\text{C}$ -NMR spectrum of peracylated dextrin esters. (a) fully-DexAc (DS = 3), (b) fully-DexPr (DS = 3), (c) random-DexAcPr (DS = 1.5/1.5) and (d) regio-DexAcPr (DS = 1.06/1.94).

#### 4.3.2.3. Functional group analysis

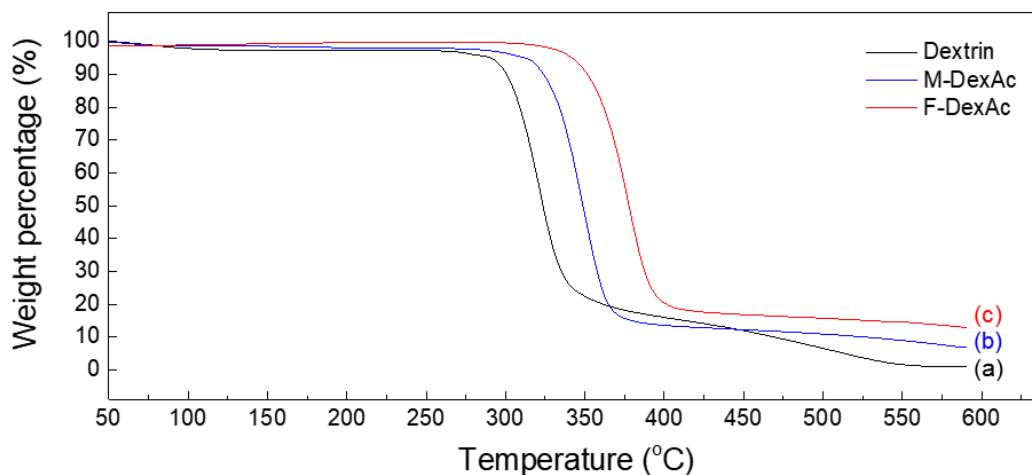
FT-IR analysis was performed to confirm the change in functional groups during the esterification reaction. The results are shown in Figure 6. Neat dextrin exhibited a strong and broad peak in the region of  $3800\text{--}3100\text{ cm}^{-1}$ . This is due to the  $\text{--OH}$  groups located at the C2, C3 and C6 positions. The peaks that appear at the regions of  $3000\text{ cm}^{-1}$  and  $1700\text{--}900\text{ cm}^{-1}$  correspond to C–H stretching and C–H bending, respectively (Figure 4-9(a)). In the case of mono-DexAc (obtained by enzymatic esterification), a strong peak appears at  $1750\text{ cm}^{-1}$ , which is not observed in the spectrum of neat dextrin. This peak is due to the C=O bond of acetate, which is present at the C6 position. In addition, the spectrum includes all of the peaks in the spectrum of neat dextrin (Figure 4-9(b)). Finally, Figure 4-9(c) shows the spectrum of fully-DexAc, which was obtained through chemical esterification. The substitution of all hydroxyl groups with acetate resulted in the disappearance of the  $\text{--OH}$  peak, while strong C=O and C–H peaks of the acetate were observed. These results are in accordance with the NMR results.



**Figure 4-8.** FT-IR spectra of dextrin and dextrin esters. (a) dextrin, (b) mono-DexAc (DS = 1.06) and (c) fully-DexAc (DS = 3).

#### 4.3.2.4. Thermal stability

The thermal stabilities of dextrin, mono-DexAc and fully-DexAc were analyzed by thermogravimetric analysis (TGA) and the results are shown in Figure 4-10. The thermal stability of mono-DexAc is between that of dextrin and fully-DexAc. The decomposition temperatures at 90% weight loss ( $T_{d,10\%}$ ) of dextrin, mono-DexAc and fully-DexAc were 301.2 °C, 323.7 °C and 349.8 °C, respectively. These results indicate that the esterification reaction enhanced the thermal stability of dextrin, as measured by  $T_{d,10\%}$ . Many previous studies on polysaccharide derivatives, including starch, curdlan, glucomannan and xylan, have shown that levoglucosan, a volatile organic compound, is produced during the pyrolysis of polysaccharides [15, 16]. The esterification of the hydroxyl groups in fully-DexAc inhibits the formation of levoglucosan, relative to mono-DexAc. mono-DexAc is less affected owing to its relatively low DS value.



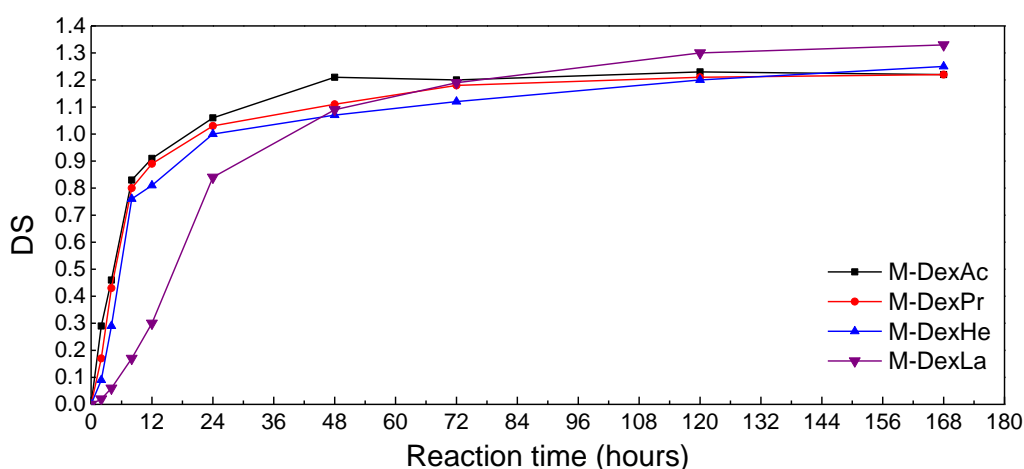
**Figure 4-10.** Thermogravimetric analysis curves of dextrin and dextrin esters. (a) dextrin, (b) mono-DexAc (DS = 1.06) and (c) fully-DexAc (DS = 3).

### 4.3.3. mono-substituted dextrin esters using the optimal conditions

The vinyl acyl donor plays an important role in the physicochemical properties of the final product, because it determines the length of the side-chain of the modified dextrin. The individual reactivity of each acyl donor (vinyl acetate, propionate, hexanoate and laurate) was confirmed for Lipozyme TL IM by increasing the reaction time. As shown in Table 4-2 and Figure 4-11, the lipase-catalyzed production of DexAc exhibited the fastest initial reaction rate. This is because the shorter acyl donor has less steric hindrance and a higher solvent affinity. In the case of the lipase-catalyzed production of laurate-substituted dextrin (DexLa), the reaction proceeded considerably slower than the others. This is because of the low solvent affinity the vinyl laurate (due to its high hydrophobicity). However, the DexLa produced from the lipase-catalyzed reaction

**Table 4-2.** DS values of dextrin esters for each reaction time under optimal conditions

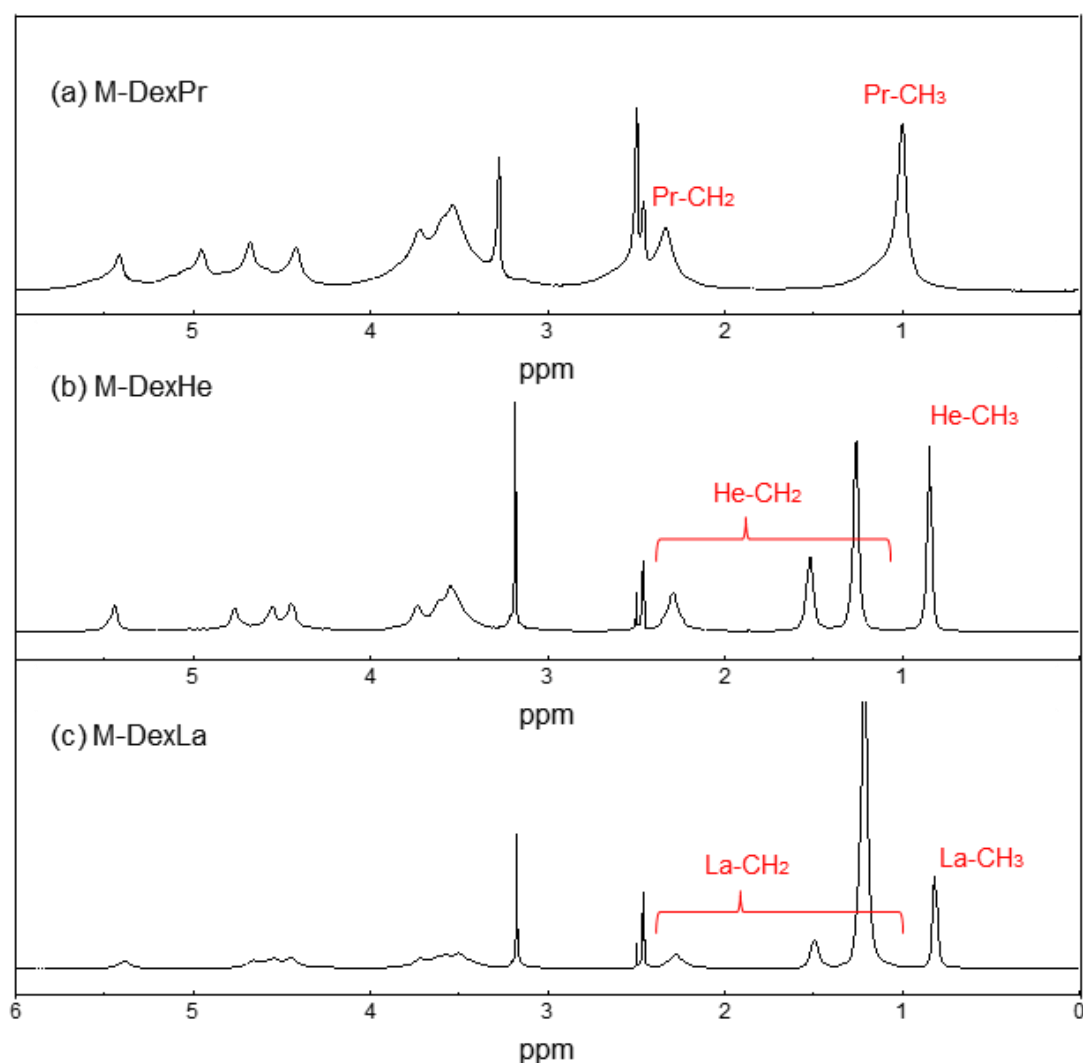
DexEs	2 h	4 h	8 h	12 h	24 h	48 h	72 h	120 h
M-DexAc	0.29	0.46	0.83	0.91	1.06	1.21	1.20	1.23
M-DexPr	0.17	0.43	0.80	0.89	1.03	1.11	1.18	1.21
M-DexHe	0.09	0.29	0.76	0.81	1.00	1.07	1.12	1.20
M-DexLa	0.02	0.06	0.17	0.30	0.84	1.09	1.19	1.30



**Figure 4-11.** DS values of dextrin esters versus reaction time under optimal conditions.



showed the highest DS value (1.30) at 120 hours. This result might be because of an increase in affinity between the vinyl laurate and the mono-DexLa (i.e., the hydrophobicity of the DexLa gradually increases with the addition of laurate to the dextrin chain, resulting in an increase in affinity with vinyl laurate). All final DS values of the dextrin esters (DexEs) are higher than 1. That indicates that the lipase-catalyzed substitution reaction occurs at the C2 and C3 positions, in addition to the C6 position.



**Figure 4-12.**  $^1\text{H}$ -NMR spectra of mono-substituted dextrin esters. (a) mono-DexPr (DS = 1.03), (b) mono-DexHe (DS = 1.00) and (c) mono-DexLa (DS = 1.09).

However, minimal substitution takes place at the C2 and C3 positions, because the hydroxyl groups at the C6 position are substituted first. The structure analysis of mono-substituted dextrin propionate (mono-DexPr), mono-substituted dextrin hexanoate (mono-DexHe) and mono-DexLa (with DS values of 1.03, 1.00 and 1.09, respectively) was carried out (Figure 4-12). The results obtained for the mono-DexEs were similar to those obtained for mono-DexAc, showing terminal methyl protons ( $-\text{CH}_3$ ) as well as methylene protons ( $-\text{CH}_2-$ ). These results demonstrate that the lipase enzyme exhibits regioselective modification of dextrin when using acyl donors other than vinyl acetate.

#### 4.4. Conclusion

The activities of lipase enzymes in the regioselective synthesis of dextrin acetate were investigated. The structure of the dextrin acetate produced by the lipase-catalyzed reaction was analyzed by NMR, which confirmed the regioselective substitution. An immobilized lipase enzyme (Lipozyme TL IM) showed the most efficient reactivity. The optimal conditions for obtaining a high DS value were determined to be: a reaction temperature of 60 °C, the use of DMSO as a solvent, a 1:3 molar ratio of glucose unit/acyl donor, an enzyme dosage of 2.5 U/mL and a dextrin concentration of 4 wt%. Based on these optimal conditions, dextrin esters with varying side-chain lengths (C2-12) were obtained using the lipase-catalyzed process with increased reaction time. The initial reaction rates and final DS values of the dextrin esters were affected by the chain length of the vinyl acyl donor. As the chain length increased, the initial rate slowed (because of increased steric hindrance), but the final DS value increased (because of increased hydrophobicity). Furthermore, the substitution reaction was confirmed to occur at the C6 position of dextrin preferentially. After all the C6 positions are substituted, the reaction occurs slightly at the C2 and C3 positions. The regioselectively mono-substituted dextrin esters showed thermal stabilities between those of unmodified dextrin and the fully-substituted dextrin esters. Overall, these results demonstrate the successful regioselective modification of dextrin using a lipase enzyme as a biocatalyst. This study contributes to the green chemistry available for the modification of polysaccharides.

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## Chapter 5

### Lipase-catalyzed polysaccharide esters

#### 5.1. Introduction

Enzymatic esterification is important not only for the enzyme activity in the organic solvent but also for the solution properties of substrate, such as its viscosity, concentration and polarity [1, 2]. In the Chapter 4, catalytic activity of commercial enzymes was confirmed using a specific polysaccharide, i.e., dextrin as a substrate, and it was confirmed that the immobilized lipase enzyme presents regioselectively mono-substituted dextrin esters at C6 position. Branched dextrin, with two types of glycosidic bonds, is a suitable substrate for enzymatic esterification, because it has good solubility in organic solvent (as a consequence of the extra degrees of freedom provided by rotation at the C6 position) [3]. The point to be noted is the results of lipase-catalyzed dextrin esters are not representative of other polysaccharides. Since the enzymes are influenced by the rheological properties of the substrate, it is difficult to predict the catalytic activity for the polysaccharide and, furthermore, their regioselectivity may differ due to the structural differences of polysaccharides [4, 5]. Namely, it is necessary to experimentally confirm the catalytic activity of specific enzyme to other polysaccharide respectively.

In this chapter, the catalytic activities of lipase enzymes on other polysaccharides were confirmed based on the enzymatically optimal condition to dextrin. The polysaccharides used in this work were amylose ( $\alpha(1\rightarrow4)$  glucan), paramylon ( $\alpha(1\rightarrow3)$ glucan) and dextran ( $\alpha(1\rightarrow6)$  glucan without branches). Amylose and paramylon exhibit high crystallinity due to the arrangement between their linear chains. Therefore,

their derivatives with delicate structural changes are expected to exhibit modified crystalline properties. Thus, catalytic activity of four kinds of lipase enzymes on amylose, paramylon and dextran was investigated, suggesting the research knowledge and possibilities for enzymatic modification.

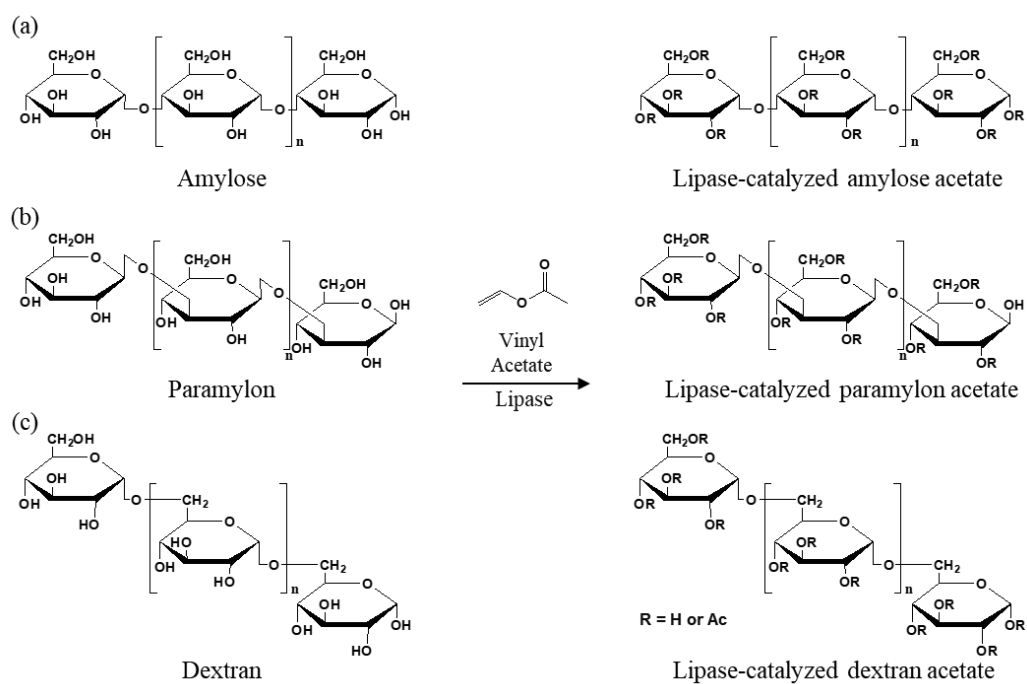
## **5.2. Experimental**

### **5.2.1. Materials**

Three kinds of polysaccharide, which are amylose ( $M_w = 6,000$  g/mol), paramylon ( $M_w = 200,000$  g/mol) and dextran ( $M_w = 70,000$  g/mol), were used as a substrate. The used commercial lipase enzymes were Lipozyme CALB, Novozyme 435, Lipozyme TL 100L and Lipozyme TL IM (Table 4-1). All polysaccharides and liquid type enzymes were treated by freeze drying before reaction to remove the water. Vinyl esters (acetate, propionate, hexanoate and laurate) were used as acyl donor without further purification.

### **5.2.2. Enzymatic synthesis of amylose, paramylon and dextran acetate**

Three kinds of polysaccharide acetate, lipase-catalyzed amylose acetate (L-AmyAc), lipase-catalyzed paramylon acetate (L-ParaAc) and lipase-catalyzed dextran acetate (L-DtraAc), were synthesized by enzymatic synthesis according to optimal condition described in Chapter 4, which is reaction at 60°C for 24 hours, using a 1:3 molar ratio of glucose unit/vinyl acetate and 2.5 U/mL enzyme dosage in super dehydrated DMSO (Scheme 5-1). With increasing reaction time up to 120 hours, lipase-catalyzed polysaccharide esters of various degree of substitution (DS) were obtained.



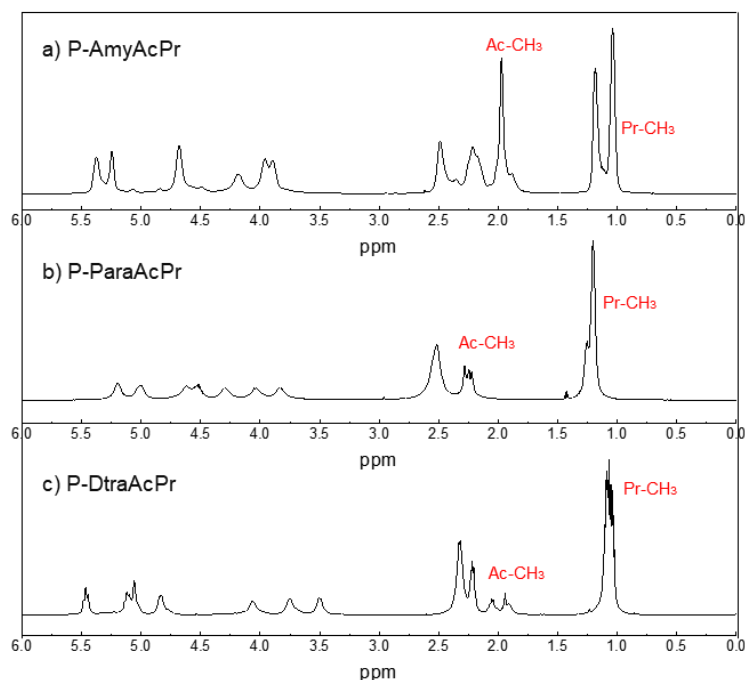
**Scheme 5-1.** Synthesis of lipase-catalyzed polysaccharide esters. (a) lipase-catalyzed amylose esters, (b) lipase-catalyzed paramylon esters and (c) lipase-catalyzed dextran esters



### 5.2.3. Determination of degree of substitution

It was difficult to confirm the catalytic activity by directly measuring the DS value from the  $^1\text{H}$ -NMR spectrum, because of the complexity in the spectrum of each polysaccharide esters. Therefore, an indirect approach (i.e. Peracylation) was used by synthesis of peracylated-amylose esters (peracylated-AmyAcPr), peracylated-paramylon esters (peracylated-ParaAcPr) and peracylated-dextran esters (peracylated-DtraAcPr), which is substitution of the remaining hydroxyl groups to propionyl group. Specifically, the DS value was calculated by the integration ratio of methyl protons of acetate ( $\text{Ac}-\text{CH}_3$ ) to those of propionate ( $\text{Pr}-\text{CH}_3$ ) after a stepwise peracylation by following chemical esterification described in Chapter 3. Figure 5-1 shows the examples of their  $^1\text{H}$ -NMR.

$$\text{DS} = \frac{\text{Peak area } [\text{Ac} - \text{CH}_3]}{\text{Peak area } [[\text{Ac} - \text{CH}_3] + [\text{Pr} - \text{CH}_3]]} \times 3$$



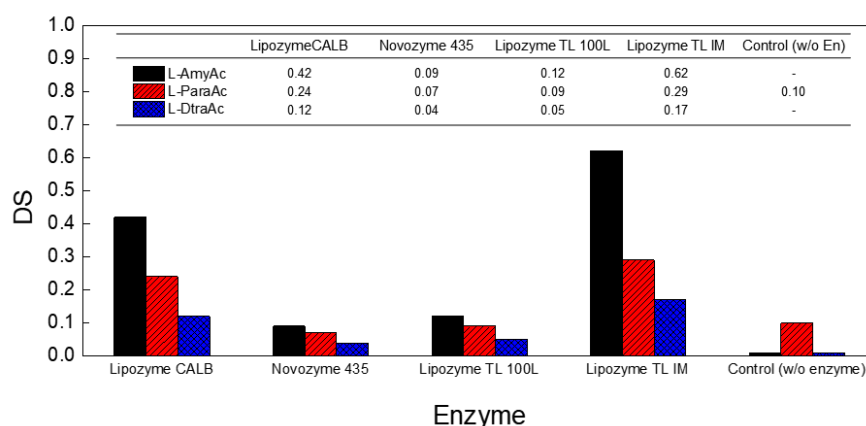
**Figure 5-1.**  $^1\text{H}$ -NMR spectra of peracylated-polysaccharides after enzymatic reaction.

a) peracylated-amylose ester (DS=0.95/2.05), b) peracylated-paramylon ester (DS=0.67/2.33) and c) peracylated-daxran ester (DS=0.35/0.65).

## 5.3. Results and discussion

### 5.3.1. Enzyme activity to amylose, paramylon and dextran

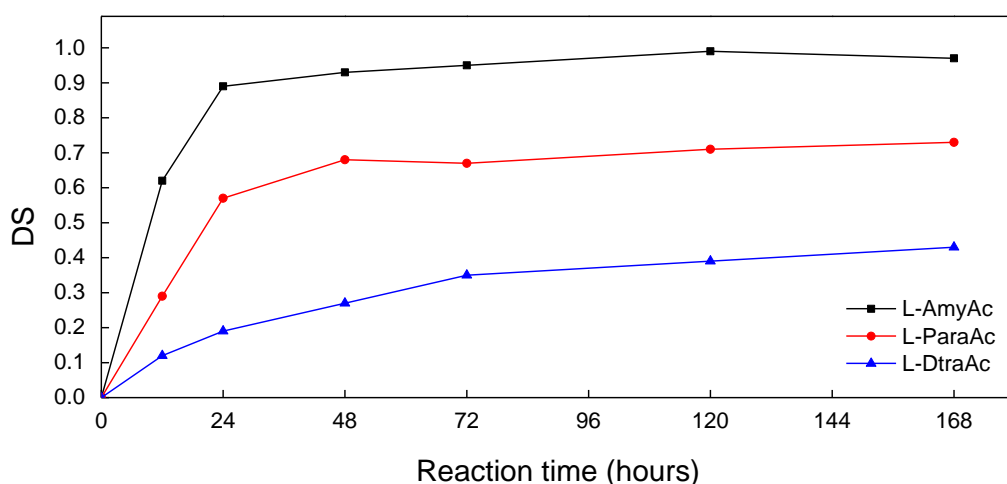
An important property of enzymes is that they exhibit substrate specificity under the conditions required for activity. It is well known that lipases are able to esterify the primary hydroxyl group (C6-OH) of polysaccharides [4, 5]. Therefore, the catalytic activity of lipases on polysaccharides possessing C6-OH, amylose and paramylon, were investigated, and at the same time dextran without C6-OH was also identified to compare the positional specificity. Initially, AmyAc, ParaAc and DtraAc catalyzed by four kinds of lipase enzymes were synthesized under the reaction at 60°C for 12 hours using a 1:3 molar ratio of glucose unit/vinyl acetate and 10 ml (liquid type) or 0.1 g (immobilized type) enzyme dosage in super dehydrated DMSO. Figure 5-2 shows the DS values of lipase-catalyzed polysaccharide acetate synthesized by each enzyme. As with the results of dextrin, the immobilized lipase enzyme, Lipozyme TL IM, showed the highest DS value in all polysaccharide acetate. This results suggest that lipase enzyme can act as a catalyst even with other polysaccharides such as amylose, paramylon and dextran. in the following step, Lipozyme TL IM was only used to confirm the DS value with increasing the reaction time.



**Figure 5-2.** DS values of polysaccharide acetate for each type of enzyme

### 5.3.2. Effect of reaction time on DS value using the optimal conditions

To obtain high DS values of lipase-catalyzed polysaccharide acetate, the reaction time was increased up to 168 hours, and their results are shown in Figure 5-3. The DS value of the polysaccharide acetate proceeded by Lipozyme TL IM increased with increasing reaction time. Amylose is a crystalline polysaccharide composed of linear  $\alpha(1 \rightarrow 4)$  glucan same to the main chain of dextrin. AmyAc by lipase-catalyzed process showed considerably high DS values, which are 0.62, 0.89, 0.93, 0.95, 0.99 and 0.97 for 12, 24, 48, 72, 120 and 168 hours, respectively. This is because the amylose has a same structure to the main chain of dextrin,  $\alpha(1 \rightarrow 4)$  glucan, then Lipozyme TL IM behaves similarly as a catalyst. However, due to its low molecular weight, it showed a low yield of 13.1-20.2 % (The yield of dextrin were 73.6-93.8 %). Lipase-catalyzed ParaAc exhibits a lower DS value than those of AmyAc, despite the presence of a primary hydroxyl group on the C6 carbon. Their DS values were 0.29, 0.57, 0.68, 0.67, 0.71 and 0.73. It is considered that the activity of the enzyme is disturbed by the high viscosity of the paramylon solution [6,



**Figure 5-3.** DS values on polysaccharide acetates for Lipozyme TL IM versus reaction temperature.

7]. The glucose linkages in dextran is connected between carbon number 1 and carbon number 6 and as a result there is no hydroxyl group at their C6 position. However, the DS values of DtraAc obtained by the enzymatic reaction is in the range of 0.12 to 0.43. This indicates that the lipase-catalyzed substitution reaction can occur not only at the C6 position but also at the C2, C3 and C4 positions, but does not show a large degree of reactivity as C6 position.

## **5.4. Conclusion**

To investigate the catalytic activity of lipase on polysaccharides, four kinds of lipase as a biocatalyst were used for reaction between three kinds of polysaccharides (amylose, paramylon and dextran) and vinyl acetate respectively. Immobilized lipase enzymes (Lipozyme TL IM) showed the most efficient reactivity, and their activity was in the order of amylose, paramylon, and dextrin. Lipase-catalyzed AmyAc showed a high catalytic activity with a DS value of 1. Lipase-catalyzed ParaAc exhibited lower catalytic activity due to their high viscosity characteristics. Finally, lipase-catalyzed dextran has lowest catalytic activity, indicating that the activity towards C2, C3, C4 positions is less than the C6 position. Overall, these results indicate that the lipase enzyme can be used as a biocatalyst in the modification of polysaccharides.

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## Chapter 6

### Summary and Conclusion

Dextrin is a polysaccharide produced by the partial hydrolysis of starch or glycogen. Its structure consists of an  $\alpha(1\rightarrow4)$  linked main chain with  $\alpha(1\rightarrow4,6)$  linked branches. A general characteristic of dextrin is that it dissolves well in polar solvents, such as water. This allows for its use in the adhesive, paint, cosmetic and biomedical industries. However, because neat dextrin does not exhibit thermoplasticity or hydrophobicity – a consequence of strong hydrogen bonding – its use in other applications is limited. The limited properties of dextrin can be modified by substitution reactions of the hydroxyl groups. The conditions of these substitution reactions, such as the components, positions and chain lengths of the substituents, have a significant effect on the final properties of the dextrin.

Esterification is one of the most popular modification reactions used to obtain thermoplastic and hydrophobic polysaccharides. This reaction can be catalyzed by chemical reagents or enzymes. Chemical esterification is suitable for the production of dextrin esters with a high degree of substitution (DS), because both primary and secondary hydroxyl groups are substituted in a non-selective reaction. Another route for the synthesis of dextrin esters is enzymatic esterification. This route is not only environmentally benign; it is also highly regioselective, allowing for the synthesis of polysaccharide esters with controlled structures and functionalities.

For chemical esterification for dextrin derivatives, a series of fully-acylated dextrin esters (DS = 3) with varying side-chain lengths (C2–12) were synthesized by heterogeneous esterification using trifluoroacetic anhydride/carboxylic acid. The

influence of side-chain lengths on structure and properties of dextrin esters were investigated by structural, thermal, mechanical and hydrophobic analysis. The thermal stability of dextrin was enhanced by esterification. Colorless and transparent dextrin ester films were prepared to measure the film properties. Dextrin ester films showed significantly increased thermoplastic and hydrophobicity.

In case of enzymatic esterification for dextrin derivatives, four lipase enzymes were investigated as catalysts in the synthesis of regioselectively mono-substituted dextrin esters from dextrin and vinyl acetate. An immobilized lipase enzyme (Lipozyme TL IM) exhibited the highest activity. This enzyme showed regioselective substitution of the dextrin at the primary hydroxyl group (C6 position) under optimal conditions (60°C for 24 hours, using a 1:3 molar ratio of glucose unit/vinyl acetate and 2.5 U/mL enzyme dosage in an organic solvent). To compare the reactivity of other vinyl esters, mono-substituted dextrin esters (degrees of substitution [DS]  $\approx$  1) with varying side-chain lengths (C2-12) were synthesized. With increasing side-chain length, the initial catalytic activity of the lipase enzyme decreased, resulting in lower DS values. However, the final DS values of the mono-substituted dextrin esters with longer side-chains were higher than those of the shorter chain analogues, because of an increase in affinity between the substrate and acyl donor.

As an extension of enzymatic esterification, the catalytic activity of lipase enzyme on amylose, paramylon and dextran was investigated, and the activity of an immobilized enzyme was in the order of amylose, paramylon, and dextrin. These results indicate that lipase enzyme reacts efficiently at C6 position and preferentially reacts to other positions such as C2, C3 and C4.

In this research, it is confirmed that the functional properties of dextrin can be controlled esterification reaction. In particular, the chemical esterification of dextrin is expected to help resolve the limitations of commercialization of dextrin caused by their low thermoplastic and hydrophobicity. Otherwise, the results of enzymatic esterification demonstrate successful regioselective modification of dextrin using a lipase enzyme as a biocatalyst. Furthermore, lipases enzyme has the potential for catalytic function to other polysaccharides, amylose, paramylon and dextran. This research will contribute to the environmental friendly chemistry in terms of polysaccharide modification.



## **Lists of Schemes and Tables**

**Scheme 2-1.** Structure of glucose enantiomers; D-glucose and L-glucose.

**Scheme 2-2.** Structural arrangements of D-glucose anomers.

**Scheme 2-3.** Structural formula of cellulose.

**Scheme 2-4.** Structural formula of chitin and chitosan.

**Scheme 2-5.** Structural formula of amylose and amylopectin.

**Scheme 2-6.** Structure formula of dextrin.

**Scheme 2-7.** Structural formula of two forms of dextran.

**Scheme 2-8.** Structural formula of curdlan and paramylon.

**Scheme 3-1.** Synthesis of fully-substituted dextrin esters, and their abbreviations

**Scheme 4-1.** Synthesis of regioselectively mono-substituted dextrin esters using lipase enzyme, and their abbreviations.

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## List of Publications

1. Hak Yong Lee, Satoshi Kimura, Tadahisa Iwata, Lipase-catalyzed Regioselective Synthesis of Dextrin Esters, *Biomacromolecules* (2018)
2. Hak Yong Lee, Takahiro Danjo, Tadahisa Iwata, Synthesis and Characteristic of Dextrin Derivatives by Heterogeneous Esterification, *Journal of Polymer Research* (2017) 24, 183-191.

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