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# Thermally stable hydrogels from enzymatically oxidized polysaccharides

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ABSTRACT

Polysaccharides guar galactomannan (guar gum), locust bean galactomannan (locust bean gum) and tamarind galactoxyloglucan were selectively oxidized by galactose oxidase. The degrees of oxidation of the products were 18–28% for guar galactomannan, 10–16% for locust bean galactomannan and 12–14% for tamarind galactoxyloglucan, calculated from the ratio of oxidized galactose units and total carbohydrates. The rheological properties of the unoxidized and oxidized polysaccharide solutions were investigated by determining their viscosities, storage and loss moduli, and temperature dependence of moduli from 20 °C to 90 °C. All the studied oxidized polysaccharides formed hydrogels throughout the entire temperature range. Concentration (0.2–1% w/v) and degree of oxidation had an effect on the gel formation. The oxidized galactomannans formed stable gels already in low concentrations, such as 0.2–0.4% w/v, while oxidized galactoxyloglucan required a concentration of 0.8% w/v to be stable up to 90 °C. The oxidized polysaccharide hydrogels are highly potential materials for food and medical applications requiring thermal stability.



## 1. Introduction

Hydrogels are polymeric materials, which adsorb large amounts of water, and have applications in food, medical, and various other industries. Various biopolymers, such as polysaccharides, are interesting materials for hydrogel formation due to their renewability, non-toxicity, and biodegradability (Coviello, Matricardi, Marianecci, & Alhaique, 2007). Tamarind seed (*Tamarindus indica*) galactoxyloglucan (XG) is one of the polysaccharides whose potential as a hydrogel has been studied. XG is a commonly used food additive in Asia, especially in Japan (Nishinari, Yamatoya, & Shirakawa, 2000). XG forms a gel in the presence of sugar or alcohol (Nishinari et al., 2000) or in aqueous solutions with concentrations above 1% w/v (Miyazaki et al., 1998). However, the formation of strong XG hydrogels requires an even higher concentration, such as 3% w/v (Nisbet et al., 2006). A technique commonly used to improve the gelation has been the enzymatic removal of the galactose side groups of XG by β-galactosidase. The removal of at least 30% of the galactose residues facilitates gelation at lower concentrations, such as 1.5%–2.5% w/v (Brun-Graeppi et al., 2010). The β-galactosidase-treated XGs have been studied especially as drug releasing agents, for example, in oral (e.g. paracetamol, Miyazaki et al., 2003), rectal (e.g. indomethacin, Miyazaki et al., 1998), and ocular (e.g. pilocarpine, Miyazaki et al., 2001) delivery. By the partial removal of the galactosyl residues, the molar mass and viscosity of XG decrease, the polymer adopts a more compact structure, and forms a gel when heated to 40 °C (Brun-Graeppi et al., 2010).

Guar and locust bean galactomannans are polysaccharides, which are used as thickeners and stabilizers in various food products due to their water-adsorbing ability, and in pharmaceutical and cosmetic applications, as well as in the textile, paper, mining, and oil industries (Wielinga, 2000). Guar galactomannan (guar gum, GM) is obtained from the seeds of guar bean (*Cyamopsis tetragonoloba*) and locust bean galactomannan (carob gum, LGM) from carob tree (St. John's bread, *Ceratonia siliqua*) seeds, which are known as locust beans. The rheological behaviour of GM and LGM is different. Native GM does not form gels, but weak gels can be obtained from LGM by freeze–thaw cycles, where the solution is quickly frozen to −78 °C and then slowly heated to 25 °C (Wielinga, 2000). Mixing LGM and GM with other substances, such as whey protein isolate (Rocha, Tiexeira, Hilliou, Sampaio, & Goncalves, 2009; Fitzsimons, Mulvihill, & Morris, 2008) and carob protein isolate (Zárate-Ramirez, Bengoechea, Cordobés, & Guerrero, 2010) has been studied aiming to improve the gel strength of the protein solutions. The rheological properties of chemically modified GM have been extensively studied. Recent examples of the chemical modifications include, for example, the cross-linking of GM with

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polyethylene glycol diglycidyl ether, thus forming a thixotropic hydrogel (Barbucci, Pasqui, Favaloro, & Panariello, 2008). Another hydrogel has been prepared by cross-linking *N*,*N*-methylenebisacrylamide with acid hydrolyzed and native GM, which were first oxidized by nitrogen oxides, $NO_x$ (Chauhan, Chauhan, & Ahn, 2009). Glutaraldehyde has also been used as the cross-linker of GM (Coviello, Alhaique, Dorigo, Matricardi, & Grassi, 2007; Gliko-Kabir, Yagen, Penasi, & Rubinstein, 1998; Sandolo, Matricardi, Alhaique, & Coviello, 2007; Sandolo, Matricardi, Alhaique, & Coviello, 2009). The glutaraldehyde-linked GM hydrogels have been investigated as drug carrier matrices (Coviello et al., 2007; Gliko-Kabir et al., 1998).

In the present work XG, GM, and LGM were modified with a simple enzymatic method. The enzyme utilized, galactose oxidase (GO, EC 1.1.3.9), is a copper metalloenzyme, which oxidizes primary alcohols to aldehydes with high regioselectivity for the hydroxyl group at C-6 of galactose (Whittaker, 2003). GO is secreted by the fungus *Fusarium* spp. To improve the enzyme production, the corresponding gene has been isolated and expressed, for example, in *Pichia pastoris* (Hartmans et al., 2004) and *Escherichia coli* (Sun, Petrounia, Yagasaki, Bandara, & Arnold, 2001) of which the *E. coli* system has recently been evaluated the most efficient for large-scale production (Spadiut, Olsson, & Brumer, 2010). Various analytical techniques are based on GO, for example, the lactose concentration of dairy products is often determined using GO biosensors (Adanyi, Szabo, & Varadi, 1999). We have recently investigated the GO-catalyzed selective oxidation of different galactose-containing polysaccharides and optimized the reaction conditions for aldehyde production (Parikka et al., 2010; Parikka & Tenkanen, 2009). The aim of this work was to utilize the GO-catalyzed oxidation to create hemiacetal cross-links between the carbonyl, formed in the oxidation, and hydroxyl functionalities of the polymer chains, and thus improve the gelation of the polysaccharides. The thermal stability of the modified polysaccharides and the effects of the concentration and degree of oxidation on the gel formation were studied.

## 2. Materials and methods

### 2.1. Enzymes and substrates

Galactose oxidase was produced by *P. pastoris* carrying the gene encoding GO from *Fusarium* spp. The GO preparation was a gift from Hercules (Barneweld, Netherlands), and was used without further purification. According to analysis with SDS-PAGE GO contained one major 65 kDa protein. The protein content was 2.0 mg/ml (the Bradford assay, Quick Start Bradford, Bio-Rad, United Kingdom). As the activity of the GO preparation was not known, the reported specific activity of a similar preparation (26 U/mg) was used to estimate the GO amounts (Schoevaart & Kieboom, 2002). Guar galactomannan (GM, 14K0168), $NaBD_4$, horseradish peroxidase (HRP, P8250, type II, 181 U/mg), and catalase (C30, from bovine liver, 22 000 U/mg) were purchased from Aldrich (St. Louis, MO, USA). Locust bean galactomannan (LGM, Lot 1075204) was purchased from Fluka (St. Louis, MO, USA). Tamarind seed galactoxyloglucan was a donation from Dainippon Sumitomo Pharma (Fukushima, Japan).

### 2.2. Oxidation of polysaccharides

Polysaccharide (GM, LGM, or XG) was stirred in Milli-Q water (concentration of the polysaccharide 2, 4, 5, 6, 8, or 10 mg/ml; 0.2%, 0.4%, 0.5%, 0.6%, 0.8%, and 1%, respectively) and the solution heated in a water bath to ensure dissolution. After reaching 80 °C the solution was cooled down to room temperature, and the enzymes were added (GO, HRP, catalase). HRP and catalase were added to enhance the action of GO. The enzyme dosages and reaction conditions were based on a previous study (Parikka et al., 2010). The amount of GO was related to the approximate amount of terminal galactose present in the polymer (0.052 U of GO/mg of galactose). The dosage of HRP 1 U/mg and catalase was 115 U/mg. For example, 21 U of GO, 400 U of HRP and 46 kU of catalase were used in the oxidation of 1 g of GM containing ca. 400 mg of galactose (ca. 40% of total sugars). After stirring the reaction at RT for 48 h the enzymes were inactivated by heating the solution in a boiling water bath for ca. 10 min.

### 2.3. Analysis of the degree of oxidation

The degree of oxidation of the products was determined by using a previously developed GC–MS method utilizing deuterium labelling of the oxidized product and comparison of the ratio 361 *m*/*z* and 362 *m*/*z* ions of silylated galactosyls, 361 corresponding to the unoxidized galactosyls and 362 the oxidized galactosyls (Parikka et al., 2010). For the analysis, samples of 5 mg of the oxidized polysaccharides were taken (e.g. 0.5 ml of the 1% solution), and $NaBD_4$ was added (3 mol eq related to the maximum molar amount of galactose in the sample). To the most viscous solutions (0.5–1%), 0.5–1 ml of $H_2O$ was added to ensure the proper mixing of the reagents. The solutions were stirred overnight. EtOH was added to obtain a ca. 1:3 $H_2O$:EtOH solution, which precipitated the polysaccharides. The samples were centrifuged for 5 min at 13,400 rpm. The supernatant was discarded, and the polymer placed in a pear-shaped flask and dried. Acidic methanolysis (Sundberg, Sundberg, Lillandt, & Holmbom, 1996) was performed: HCl/MeOH (2 M, 1 ml) was added, the flask sealed, and the solution kept at 100 °C for 3 h. The samples were allowed to cool, neutralized with pyridine (50 μl), and diluted with MeOH (4 ml). For the GC–MS analysis, 1 ml of the obtained solution was placed in a Kimax tube, evaporated, silylated with 200 μl of TMSCl/BSTFA/pyridine (1:100:100) at 60 °C for 30 min, and evaporated. Heptane (1 ml) was added, and the sample filtered. GC–MS analysis of the samples was performed with an HP 5890 gas chromatograph equipped with an HP-5 column and connected to an HP 5972 Mass Selective Detector mass spectrometer. The column temperature program was as follows: 150 °C; 2 °C/min; 220 °C (5 min).

### 2.4. Rheological experiments

Viscosity and oscillation measurements were performed with a TA Instruments (New Castle, DE, USA) AR 2000 controlled stress rheometer. The measuring geometry was a concentric cylinder with a conical end (DIN) rotor (radius, 14 mm; stator radius, 15 mm; immersed height, 42 mm; and gap, 5920 μm). Temperature was controlled with a Peltier element. For the determination of apparent viscosity, shear rates of 0.1–1000 $s^{-1}$ were used. For oscillatory measurements, the linear viscoelastic range was determined by strain sweep, and correspondingly, the oscillatory frequency sweeps were made in the linear regime at 20 °C. The oscillatory temperature ramps were made between 20 °C and 90 °C using a heating rate of 2 °C/min at 1 Hz oscillatory frequency.

## 3. Results

### 3.1. Oxidation of the polysaccharides

The polysaccharides studied differed from each other in terms of carbohydrate and galactose content and molecular weight (Table 1). The α-D-galactosyl residues of structurally very similar GM and LGM were directly attached to the C-6 of the mannosyl