

Targeted Functionalization of Spruce Galactoglucomannans with Aid of Galactose Oxidase (FunMan)

Annual Report 2009

Academy Decision Number 117765
Funding period: 1.1.2007-31.12.2009

Partners in the consortium:

Professor Maija Tenkanen¹
Professor (acting) Stefan Willför²

Participating researchers: —

Dr. Kirsti Parikka¹, Ann-Sofie Leppänen², Dr. Patrik Eklund³

Researchers / advisors involved in the project:

Doc. Liisa Virkki¹, Doc. Päivi Tuomainen¹ —
Prof. Rainer Sjöholm³, Em. Prof. Bjarne Holmbom², Doc. Anna Sundberg²,
Prof. Markku Auer²

PhD students working closely related to the project:

Dr. Kirsi Mikkonen (Ph.D. 11.12.2009)¹, Leena Pitkänen¹

ABSTRACT

The galactose units of spruce galactoglucomannan (GGM) were oxidized to the corresponding aldehydes with galactose oxidase (GO). A multienzyme system utilizing GO, horseradish peroxidase (HRP) and catalase was used in the reactions. The degree of oxidation of the product was 65% at the best. Other polysaccharides, guar galactomannan (GM, guar gum), larch arabinogalactan (AG), corn arabinoxylan (AX) and tamarind xyloglucan (XG) were oxidized as well, of which the highest degree of oxidation based on total carbohydrates was obtained with guar gum (28%). The investigation of the physicochemical properties of the products showed that the molecular weight of GGM increased in the oxidation, and that the highly viscous GM and XG formed gels. No significant changes were observed in the rheology of AG and AX due to their low degrees of oxidation.

The further modifications of the oxidized GGM, allylation and further oxidation to carboxylic acid, were first optimized with the aldehyde derivatives of methyl- α -D-galactopyranoside or raffinose. The reactions were performed directly in the same reaction media. Indium mediated allylation of oxidized GGM was successful using allyl bromide, a 100% conversion was obtained after 24 h reaction. Preliminary experiments were performed for the chemical oxidation with NaClO₂, GC-MS analysis indicating complete conversion. The molecular weight of the allylation product had slightly decreased due to the low pH, elevated temperature and long reaction time. Allylation restored the solubility of GGM in water, which had decreased due to the oxidation.

Effect of addition of GGM, oxidized GGM and allylated GGM to chemical pulp fibres was studied. Addition of oxidized GGM to bleached chemical pulp gave higher density and strength to the paper than addition of the native GGM.

¹ Department of Applied Chemistry and Microbiology, University of Helsinki

² Laboratory of Wood and Paper Chemistry, Process Chemistry Centre, Åbo Akademi

³ Laboratory of Organic Chemistry, Åbo Akademi

RESULTS

1. University of Helsinki

Oxidation of GGM

A multienzyme system utilizing galactose oxidase (GO), horseradish peroxidase (HRP) and catalase was used in the oxidations. GO is a copper metalloenzyme which catalyzes the oxidation of primary alcohols to corresponding aldehydes with high selectivity for galactose, and uses molecular oxygen in the reaction. HRP activates GO, and catalase removes H_2O_2 formed in the reaction by converting it to H_2O and O_2 . GGM is a substrate of GO as it contains terminal galactose units attached to the glucomannan backbone (ratio of monosaccharide units Gal:Glc:Man = 1:2:8).

During the first and second year of the project, the oxidation conditions of GO-catalyzed reaction were optimized for the production of aldehyde using methyl- α -D-galactopyranoside as a substrate, and the optimized conditions were then applied to the oxidation of spruce galactoglucomannan resulting in a product (ox-GGM) with 65% degree of oxidation (of Gal units) at the best (1% w/v solution in water at RT, 48 h reaction, Figure 1 A). During the third year, the reaction conditions, such as substrate concentration and reaction temperature, were further investigated to improve the conversion.

The reactivity of different GGM batches was studied and found to vary considerably depending on the purification or isolation technique. The sample having the highest purity, obtained by a laboratory-scale method involving purification by dialysis, had the highest degree of oxidation (65%), and the samples of lower purity, that had been precipitated from TMP mill process water without further purification, were converted to the aldehydes with a significantly lower degree of oxidation (10-35%).

Oxidation of other polysaccharides

Other polysaccharides with varying structure and galactose content were also oxidized with GO. Guar galactomannan (GM, guar gum) has a high galactose content (40%) and also had the highest degree of oxidation of the polysaccharides studied based on total carbohydrates (16%, Figure 1 B). Larch arabinogalactan (AG) and corn arabinoxylan (AX) were oxidized with a low degree of oxidation. Tamarind xyloglucan (XG) had the highest degree of oxidation of all the polysaccharides studied, based on the reacted galactosyls, 85% (Figure 1 A).

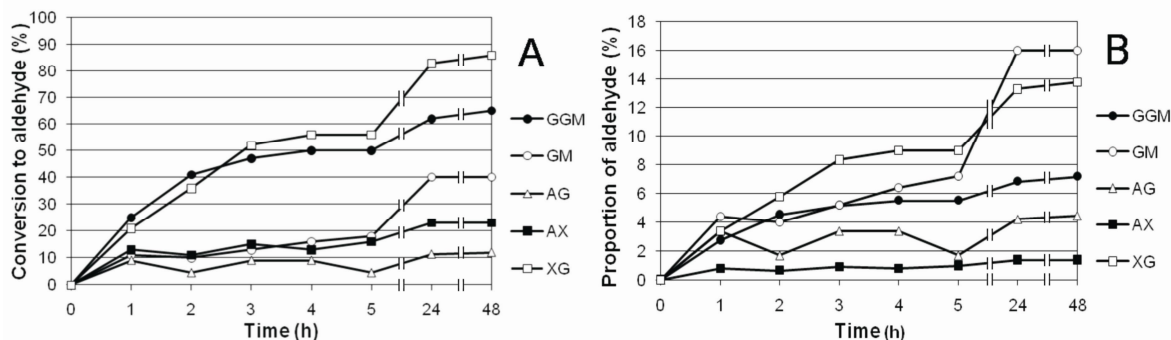


Figure 1. The degree of oxidation of polysaccharides during 48 h oxidation at RT, **A** based on the amount of oxidized terminal galactose; **B** based on the proportion of oxidized galactosyls to the total carbohydrates.

During the third year of the project, the effect of the substrate concentration on the degree of oxidation was studied. Decreasing the concentration of guar gum, which has a high M_w (ca. 2600 kDa) and forms a highly viscous solution with water, was found to increase the degree of oxidation (from 40% of 1% w/v solution up to 70% of a 0.1% w/v solution). To investigate the effect of M_w and viscosity, enzymatically hydrolyzed guar gum, having M_w of ca. 50 kDa, was prepared and oxidized. In a 1% w/v solution, the degree of oxidation was high, 78%, and the viscosity was low, 0.6 dl/g. This indicated that both viscosity and M_w of the starting material had an effect on the product formation and properties.

Oxygen consumption in the reaction

Oxygen is consumed in the GO-catalyzed reaction. The measurement of oxygen consumption in the reaction for the following of the oxidation and for testing new substrates for GO was investigated. The measurements were performed with a submersible oxygen meter at room temperature, and the reactions conducted without catalase to prevent extra oxygen being present. According to these measurements, GGM, XG and GM were the best substrates, their reactions showing fast disappearance of oxygen. The reactions of AG and AX did not consume oxygen significantly, predicting their low degrees of oxidation. The use of an excess amount of the enzymes was investigated with AX, but did not accelerate the reaction. The oxygen consumption was verified to be a suitable easy method for screening of potential new substrates for GO.

Physicochemical properties of oxidized GGM and the other polysaccharides

The particle size distribution of GGM was investigated with dynamic light scattering (DLS) and was found to increase in the oxidation. High performance size exclusion chromatography (HPSEC) analysis was in agreement with DLS, the M_w of ox-GGM had increased from 20 kDa to 35 kDa. No changes were observed in AG and AX apparently due to their low degrees of oxidation.

The rheology of the highly viscous GM and XG was investigated by different viscosity measurements. The dynamic viscosities of the oxidized products of GM and XG (1% w/v solutions) were higher than those of the starting materials at all shear rates. Oscillation measurements suggested that the oxidized GM and XG were gels. Especially the rheological properties of XG changed extensively in the oxidation.

Further oxidation

Preliminary experiments of the further chemical oxidation to obtain carboxylic acids from the aldehydes were conducted with raffinose and NaClO_2 . The aim was to continue the reaction in the same aqueous reaction medium without need for isolating the aldehydes. The GC-MS method involving NaBD_4 reduction of the aldehydes was suitable analysis method for the further oxidation as well, giving information on the unreacted starting material, and the carboxylic acid derivatives, which were stable under the required hydrolysis and sample preparation. The galacturonic acid derivative of raffinose was obtained with 100% conversion and the first experiments with GGM also indicated a complete conversion.

2. Åbo Akademi

Modification of oxidized methyl- α -D-galactopyranoside

The indium mediated allylation reaction of oxidized methyl- α -D-galactopyranoside (ox-Gal) was investigated. Since this reaction can be done in aqueous media, ox-Gal can be modified directly without the need of drying after the enzymatic oxidation. The reaction proceeded well at room temperature both in a mixture of water:THF (1:1) and in plain water. Three different allylhalides

(allyl bromide, crotyl chloride, and cinnamyl chloride) were tested. The corresponding homoallylic alcohols were obtained in good yields. The products were isolated and characterized by NMR.

Modification of oxidized GGM

Drying of GGM often makes re-dissolution difficult, so the possibility to perform reactions without the need of drying between reaction steps is an aspect worth considering when planning new routes for GGM modification. Drying and re-dissolution steps are avoided as the metal mediated allylation reaction can be performed with water as solvent. The aldehyde groups in ox-GGM were modified by the indium mediated allylation reaction. The allyl halides tested were allyl bromide, crotyl chloride, and cinnamyl chloride. Using the reaction conditions developed with ox-Gal, and a solvent system consisting of water and THF (1:1) at room temperature, only very little, if any, product was formed. By removing the organic co-solvent, i.e. performing the reaction in plain water, the yield increased significantly. The reaction between ox-GGM and allyl bromide in water proceeded well at 55 °C. According to the GC-MS analysis of the deuterated sample, the degree of oxidation had decreased during the first three hours to 20 %, and after 24 h all the aldehyde units had reacted. The incorporation of the allyl group was verified by ¹H-NMR.

Even if both crotyl chloride and cinnamyl chloride react with ox-Gal in water at room temperature producing the corresponding homoallyl alcohols in good yields, no reaction was observed with ox-GGM at the same reaction conditions. Warming the reaction mixture did not improve the results. By addition of HCl in the reaction, less than 10 % of the aldehyde units had reacted after 24 h. The difference in reactivity between the monomer and the polymer might be explained by the immiscibility of the allylic halides with water and the difference in the concentration of the aldehyde in the reaction solution.

Physicochemical properties of modified GGM

The average molecular weight of unoxidized, oxidized, and derivatized GGM was determined by HPSEC-MALLS. During the allylation reaction done at 55 °C the pH of the solution decreases to ~3. Even if GGM has been shown to be fairly stable against acid hydrolysis at pH 3, the elevated temperature and long reaction time is expected to cause some hydrolytic degradation. As expected, the M_w was slightly decreased during the allylation reaction.

The solubility of GGM, ox-GGM, and allyl-GGM in water, DMSO, and acetone was determined. The solubility of GGM in water and DMSO decreased after the oxidation step. The incorporation of allyl groups restored the solubility in water. The allylated GGM dissolved better than ox-GGM in DMSO, but a clear decrease in solubility compared to unmodified GGM was seen. No difference in solubility in acetone was observed.

Evaluation of the effects on paper properties

The interaction of modified GGM with cellulose fibres was investigated. GGM, ox-GGM, and allylated GGM (allyl-GGM) were deacetylated and adsorbed to bleached chemical pulp. The effect of the different GGMs on paper properties, such as strength and density, were measured and compared. It seemed that the addition of GGM, either modified or not, increased the strength of the paper sheet. The addition of ox-GGM to bleached chemical pulp gave the highest density and strength. The functional groups on the allyl-GGM were probably too small to give a significant difference to unmodified GGM.

Evaluation of the analytical methods

The analysis of modified GGM is challenging since the normally used depolymerisation and derivatization steps, that are required for gas chromatographic (GC) analysis, destroy both the aldehyde and the homoallylic alcohols. Liquid chromatography (LC) does not require any derivatization step prior to analysis and identification of the separated compounds could be done

with mass spectrometry (MS). An alternative to the commonly used reversed-phase columns is a porous graphitic column (PGC). On-line coupled to an MS detector, the PGC-LC-ESI-MS is a sensitive and fast method for identification of carbohydrates. The suitability of the PGC column for the analysis of mannoooligomers was tested, and the preliminary results showed that it is possible to analyse oligosaccharides. Thus PGC-LC-ESI-MS combined with a depolymerization step that does not destroy the formed homoallyl alcohol (e.g. enzymatic hydrolysis), might be applicable for the analysis of derivitized GGM samples and will be tested in the future work.

PROGRESS

The research and collaboration between the partners has proceeded as planned and project meetings have been organized regularly. The research on this topic and collaboration between the groups will continue for the next three years in the frame of Ann-Sofie Leppänen's PhD thesis study "Chemical modification of water-soluble spruce O-acetyl-galactoglucomannan" funded by the national Graduate School of Biomass Refining and in Kirsti Parikka's post doctoral research project "Production of aldehydes and functionalized polysaccharides by galactose oxidase and chemical modifications" funded by the Academy of Finland. The know-how obtained in the FunMan-project will also be utilized in future national and international projects.

DISSEMINATION

Participation and poster presentations in EUROCARB 15 conference in Vienna, Austria (Parikka) and in ITALIC 5 conference in Varenna, Italy (Leppänen), and oral presentations in the Japanese-European Workshop on Cellulose and Functional Polysaccharides, Hamburg, Germany (Willför) and in the Cost 928 3rd Annual Workshop, Krakow, Poland (Tenkanen). Two peer-reviewed articles have been published and one manuscript is in preparation.

Abstracts

- Parikka K., Leppänen A.-S., Reunanen M., Willför S., and Tenkanen M., Targeted functionalization of polysaccharides with galactose oxidase, EUROCARB 15, 19-24 July 2009, Vienna, Austria, Book of abstracts p. 191 (poster presentation).
- Leppänen A.-S., Parikka K., Tenkanen M., Eklund P., Sjöholm R. and Willför S., Chemical modification of oxidized O-acetyl-galactoglucomannan, Italic 5 - Science and Technology of Biomasses, 1-4 September 2009, Varenna, Italy, Proceedings pp. 197-200 (poster presentation).
- Willför, S., Galactoglucomannans – Functional and bioactive polysaccharides from softwoods. Japanese-European Workshop on Cellulose and Functional Polysaccharides, 16-18 September 2009, Johann Heinrich von Thünen-Institut, Hamburg, Germany, Proceedings pp. 34-35 (invited lecture).
- Parikka K., Leppänen A.-S., Pitkänen L., Willför S. and Tenkanen M., Targeted oxidation of polysaccharides by galactose oxidase, Cost 928 3rd Annual Workshop, 23-25. September, Krakow, Poland. Book of abstracts (oral presentation).

Articles

- Parikka K. and Tenkanen M. Oxidation of methyl α -D-galactopyranoside by galactose oxidase: products formed and optimization of reaction conditions for production of aldehyde, Carb. Res. 344 (2009) 14-20.
- Parikka K., Leppänen A.-S., Pitkänen L., Reunanen M., Willför S. and Tenkanen M., Oxidation of polysaccharides by galactose oxidase, J. Agric. Food Chem. 58 (2010) 262-271.

Manuscripts

- Leppänen A.-S., Parikka K., Tenkanen M., Eklund P., Sjöholm R. and Willför S., Metal mediated allylation of enzymatically oxidized methyl- α -D-galactopyranoside.