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Enzymatic and chemical oxidation of polygalactomannans from the seeds of a few species of leguminous plants and characterization of the oxidized products

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ABSTRACT

Plant polysaccharides are used in a growing number of applications, in their native or in chemically and/or biochemically modified forms. In the present work, we compare TEMPO-mediated oxidation with laccase of polygalactomannans (PGM) from different species of plant leguminous to chemical oxidation with NaClO/NaBr/TEMPO. We have investigated the gums from: locust bean (*Ceratonia siliqua*), tara (*Caesalpinia spinosa*), guar (*Cyamopsis tetragonolobus*), sesbania (*Sesbania bispinosa*) and fenugreek (*Trigonella foenum-graecum*).

Upon laccase/TEMPO oxidation, PGM viscosity and concentration of reducing groups increased up to five-fold and structured, elastic, stable gels were formed, which could be degraded by hydrolysis with β-mannanase.

Conversely, chemical oxidation with NaClO/NaBr/TEMPO caused a rapid, intermediate transition of the gum solutions to compact gels, that immediately reverted to liquid, with a lower viscosity than at the start and an increased concentration of reducing groups, similar to the reaction with laccase.

We interpret the above as due to, in the case of laccase, oxidation of primary hydroxyl groups to aldehydes, able to form stable hemiacetalic bonds with free hydroxyl groups. While upon chemical oxidation, primary OH's are only transiently oxidized to aldehydes, followed by rapid oxidation of all carbonyl groups to carboxylates.

In either cases, TEMPO appeared to cause a limited splitting of glycosidic bonds of PGM.

Native and oxidized PGM were further characterized by 1D and 2D NMR spectroscopy and by rheology.

1. Introduction

Plant polysaccharides often represent a valuable and sustainable alternative to traditional synthetic polymers produced from monomers of fossil, non-renewable origin and are increasingly applied in a growing number of industrial fields, either in their natural or chemically/biochemically modified forms. Indeed, the production/modification processes of polysaccharides largely conform to the Twelve Principles of Green Chemistry (Anastas and Warner, 1998). These biodegradable compounds are mostly used as: emulsion stabilizers; thickeners; rheology modifiers; agents for coating, binding, suspending, conditioning, leveling; in drilling fluids; for water-retention, etc., in a wide variety of food, feed, cosmetics and industrial fields (e.g., oil operations, detergency, textile printing, building materials, paint and coatings, adhesives, inks, paper making, etc.). If appropriately modified and adapted to film forming, they can also be used in the production of packaging material for food and non-food contact, eventually in combination with cellulosic material (Kenawy El et al., 2007; Das et al., 2011).

The most studied and exploited polysaccharides are: cellulose, starch, galactomannans, chitosan, alginates, pectins, but also exudative gums (Verbeken et al., 2003). Cellulose is the main biomaterial used for paper making, packaging, textiles, etc., and several chemical derivatives of cellulose are widely used on an industrial scale. Chitosan, obtained by de-acetylation of shellfish chitin, finds application as a wetting agent in cosmetic, and is being studied as a "functional" packaging material when bound to antimicrobial compounds (Itzincab-Meja et al., 2013). Biomaterials for

Abbreviations: ABTS, 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid); FG, fenugreek gum; GG, guar gum; LG, locust gum; TG, tara gum; SG, sesbania gum; GO, galactose oxidase; Lcc, laccase; LMS, laccase mediator system; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy radical; BCA, disodium 2,2-bicinchoninate.

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"functional" packaging and coating actually represent a promising and rapidly growing field (Salwiczek et al., 2014).

Second only to starch and cellulose, galactomannans from leguminous plants are commonly employed to produce a considerable range of derivatives with numerous applications in food, feed and industrial fields. Galactomannans are high molecular weight polysaccharides found in the seeds of some *Leguminosae* (belonging to the family *Fabaceae*) where they serve as reserve source for carbon and energy upon germination (Prajapati et al., 2013).

Commercial galactomannans are mostly obtained from the seed endosperms of a shrub (*Cyamopsis tetragonolobus*), commonly referred to as guar. Guar gum (GG) is obtained by thermo mechanical, solvent-free milling/sieving of seeds with no generation of by-products or environmental pollution. The polymeric structure of guar galactomannan is composed of a backbone of mannose units linked by β-1,4 glycosidic bonds and side units of galactose linked to mannose by α-1,6 glycosidic bonds, with an average ratio of mannose to galactose of 1.4–1.6 (Daas et al., 2000, 2002; Crescenzi et al., 2004), but also claimed to be as high as 1.8–2.0 (McCleary et al., 1985). Contrary to starch, this peculiar structure makes it rather soluble in cold water, flexible in application and chemically/biochemically quite reactive (Cheng et al., 2002a).

Galactomannans from other leguminous plants have different ratios of monomers, thus different properties, more specifically: 1:4.5–5.0 (cassia), 1:3.5–4.0 (locust bean), 1:2.5–3.0 (tara), 1:1.3–1.5 (sesbania), 1:1 (fenugreek) (see Daniel et al., 1994; Daas et al., 2002). These, as well as other polysaccharides from tamarind or spruce, are investigated by several R&D groups, but are still relatively less exploited for industrial uses (Pollard et al., 2010, 2011; Parikka et al., 2012; for comprehensive reviews, see Srivastava and Kapoor, 2005; Prajapati et al., 2013).

A chemical reaction commonly applied to guar polygalactomannan is hydroxyalkylation, performed with either ethylene or propylene oxide, or both, in order to modify its solubility and viscoelastic properties (Lapasin and Pricl, 1995; Nussinovitch, 1997; Cheng et al., 2002b). Other chemical modifications carried out in industry are: carboxymethylation, hydrophobization, depolymerization, cationization, crosslinking, eventually combined with hydoxyalkyklation, as required by the end-use (Risica et al., 2005).

Enzymatic reactions can also be applied to PGM under mild conditions, with no generation of side products, e.g.,: depolymerization with β-mannanase, debranching with α-glycosidase, oxidation with oxidases (e.g., laccase, peroxidase, galactose oxidase), but also "elimination" of unwanted insoluble proteins with a protease (Baldaro et al., 2012). Enzymatic hydrolysis of guar gum is applied in the production of a nutraceutical (PHGG) commercialized as Benefibra® by Novartis and to the study of its biostability (Cheroni et al., 2012). Enzymatic oxidation of guar galactomannans has been described using either a native galactose oxidase (GO), followed by reductive amination (Hall and Yalpani, 1980) or by halogen oxidation (Frollini et al., 1995), or with a highly engineered GO by Delagrave et al. (2001, 2002), Parikka and Tenkamen (2009), Parikka et al. (2010, 2012), Leppanen et al. (2010) and Mikkonen et al. (2014). On the other hand, oxidation of polysaccharides with the more versatile enzyme laccase, under mild reaction conditions, can generate reactive groups (e.g., carbonyls, carboxyls) on cellulose (Viikari et al., 1999a), starch (Viikari et al., 1999b), pullulan (Jetten et al., 2000), guar galactomannan (Lavazza et al., 2011). These latest Authors have described the use of a fungal laccase (benzene-diol: oxygen oxidoreductases, E.C. 1.10.3.2), in combination with TEMPO as mediator, to selectively oxidize primary hydroxyl groups of guar galactomannan. The laccase enzyme family comprises an extended group of blue multi-copper oxidases known to oxidize a wide range of substrates, mainly phenols and substituted amines, by catalyzing the four electron reduction of molecular oxygen to water, which is well described in recent reviews (Claus, 2004; Riva, 2006; Witayakran and Ragauskas, 2009; Rodgers et al., 2010). Laccases cannot directly oxidize aliphatic alcohols, but this limitation can be overcome by using as electron acceptor an intermediate mediator, such as the stable radical TEMPO (Bragd et al., 2004). This molecule is firstly oxidized to an oxonium ion, which in turn selectively oxidizes primary hydroxyl groups to the corresponding aldehydes and (in the case of mono-, oligo- and poly-saccharides) also to carboxylates (Viikari et al., 1999a; Marzorati et al., 2005; Ding et al., 2008). The combination of enzyme and mediator is referred to as a "laccase-mediator system" or LMS (Eggert et al., 1997; Fabbrini et al., 2002; Galante and Formantici, 2003; Kulys and Vidziunait, 2005; Morozova et al., 2007). It was previously reported that incubation of laccase from *Trametes versicolor* plus TEMPO in an unbuffered, aqueous solution of 1% (w/w) guar galactomannan causes a substantial viscosity increase and the viscous gum solution is converted to an elastic gel, as confirmed by its rheological profile (Almdal et al., 1993; Lavazza et al., 2011). Presumably, the enzymatic oxidation brings about the formation of a structured, cross-linked gel *via* the establishment of intra- and/or inter-chains hemiacetalic bonds between newly formed carbonyls and free hydroxyl groups, as also suggested in the literature (Donnelly, 1999).

In the present work, this LMS was applied to five different PGM from: locust bean, tara, guar, sesbania and fenugreek (all with different Gal:Man ratios, molecular weights, reducing ends concentrations and viscosity profiles) and, upon enzymatic oxidation, similar transitions to elastic gels were observed. The reaction time course was further characterized and the end products of enzymatic oxidation were compared to the "classic" NaClO/NaBr/TEMPO chemical oxidation of polysaccharides described by several Authors (De Nooy et al., 1995; Sierakowski et al., 2000; Kato et al., 2003; Matsushiro et al., 2006; De Souza et al., 2011). In either cases a partial TEMPO-induced oxidative depolymerization and/or debranching of the polysaccharides was considered likely to occur, as also reported by others (Kato et al., 2003; Shibata and Isogai, 2003; De Souza et al., 2011).

Native and oxidized PGM were characterized by 1D and 2D NMR spectroscopy and their rheological profiles were studied.

These results of PGM's enzymatic oxidation should be preliminary to the development of new "functional" polymeric entities or biomaterials to be further investigated.

2. Materials and methods

2.1. Materials

Laccase from *T. versicolor* in powder form, from Sigma–Aldrich, was dissolved with mild stirring in MilliQ water. TEMPO, BCA and all other chemicals were from Sigma–Aldrich or Fluka. Beta-mannanase was from Megazyme (E-BMANN) with a declared activity of 400 U/ml.

Non purified gum powders from locust bean (LG), tara (TG), guar (GG), sesbania (SG) and fenugreek (FG) with indicative Brookfield viscosity of 1% (w/v) aqueous solutions at 20 rpm and 25 °C of: 2100–2700, 4500, 5000, 3000 and 1500–2500 mPa s, respectively, were from commercial sources and sampled from Lamberti S.p.A. industrial stocks. Actual PGM content of unpurified gums varied between 76 and 80% (w/w).

2.2. Preparation, purification and viscosity measurements of PGM solutions

2.2.1. Preliminary purification

PGM's were purified by dispersion in H_2O/ethanol (7:3), followed by vacuum filtration, further dispersion in acetone and final