



Enzymatic cross-linking of carboxymethylpullulan grafted with ferulic acid



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ABSTRACT

Carboxymethylpullulan (CMP) has been modified in a two-step grafting reaction of ferulic acid (FA). Acid adipic dihydrazide (ADH) was first reacted with FA activated with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC). Then the product of this first reaction was reacted with CMP (activated with EDC). Grafted polysaccharides structure was confirmed by FTIR and ¹H NMR spectroscopy. Analyses by size-exclusion chromatography (SEC) coupling on-line with a multi-angle light scattering detector (MALS), a viscometer and a differential refractive index detector (DRI) (SEC/MALS/DRI/Visco) showed that associations between FA moieties occurred due to hydrophobic interactions. The grafting rates of FA were determined by the Folin-Ciocalteu method and were found between 1.0% and 11.2% (mol/mol anhydroglucose unit). The CMP-FA were then enzymatically cross-linked with laccase from *Pleurotus ostreatus*. The crosslinking reactions were followed by rheological measurements, demonstrating the influence of laccase concentration on kinetics. Elastic modulus and swelling rates of hydrogels depends on FA content only for low values.

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1. Introduction

Polysaccharides are natural polymers with interesting viscosifying and/or gelling properties for wide domains of application, including food or cosmetic industries. To broaden their applications, polysaccharides can be chemically modified to bring them new properties such as amphiphilic properties (Hassani, Hendra, & Bouchemal, 2012), thermosensitive properties (Dulong, Mocanu, Picton, & Le Cerf, 2012; Prabaharan and Mano, 2006) or photosensitive properties (Tsai, Lin, & Lin, 2006; Vázquez et al., 2009). Pullulan, a neutral water-soluble polysaccharide produced by *Aureobasidium pullulans*, has been chemically modified to adjust its biological activity and chemical properties (Shingel, 2004; Singh, Kaur, & Kennedy, 2015). Its linear flexible chains are formed from α -1,4-linked glucose units, which are included in α -1,6-linked maltotriose units. Carboxymethylation of pullulan (Duval-Terrié, Hugué, & Muller, 2003) brings carboxylate groups which are further used to graft various molecules or oligomers. In our laboratory, carboxymethylpullulan (CMP) has been widely studied

and used to obtain many grafted pullulan with various physico-chemical properties. For example, amphiphilic pullulan derivatives have been obtained by grafting of alkyl chains (Bataille, Hugué, Muller, Mocanu, & Carpov, 1997; Duval, Le Cerf, Picton, & Muller, 2001), perfluoroalkyl chains (Glinel, Hugué, & Muller, 1999) or polyether chains (Dulong et al., 2012; Mocanu, Mihai, Dulong, Picton, & Lecerf, 2011; Mocanu, Mihai, Dulong, Picton, & Le Cerf, 2012). These amphiphilic carboxymethylpullulans can be used to synthesize hydrogels for drug delivery systems. The cross-linking reaction is generally achieved by a chemical reaction. Classically, glutaraldehyde (Wu and Imai, 2011) or epichlorhydrin (Mocanu, Mihai, Le Cerf, Picton, & Muller, 2004) are used to obtain hydrogels but these cross-linking agents are toxic; hydrogels can also be obtained with a water soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, EDC) (Dulong, Le Cerf, Picton, & Muller, 2006), leading to by-products (urea). Sodium trimetaphosphate is also a good candidate to cross-link polysaccharides but the conditions of the cross-linking reaction are very hard (pH 13) (Lack et al., 2004).

A biomimetic approach is to use enzymes for cross-linking reactions of polysaccharides. Water extractable arabinoxylans (WEAX, residues of the main non-starch polysaccharides of cereal grains) are known to form viscous solutions or soft gels by enzymatic cross-linking reaction with laccase, an enzymatic free radical

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generating agent. WEAX naturally contains ferulic acid (FA) (3-methoxy-4-hydroxycinnamic acid) substituents (Ishii, 1997; Smith and Hartley, 1983) which undergoes oxidation by reaction with laccase, leading to dimerization of two ferulic acid substituents, permitting the cross-linking of WEAX chains (Adelakun et al., 2012; Carvajalmillan, Guigliarelli, Belle, Rouau, & Micard, 2005; Carvajalmillan, Guilbert, Morel, & Micard, 2005; Carvajal-Millan et al., 2005; Carvajal-Millan, Guilbert, Doublie, & Micard, 2006; Figueroa-Espinoza and Rouau, 1998; Vansteenkiste, Babot, Rouau, & Micard, 2004). Ferulic acid, the most abundant hydroxycinnamic acid in the plant world (Mathew and Abraham, 2004), possesses antioxidant, antibacterial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Choi and Park, 2015; Lemos et al., 2014; Itagaki et al., 2009; Takahashi, Kashimura, Koiso, Kuda, & Kimura, 2013). Laccase belongs to the blue-copper family of oxidases (Thurston, 1994). Laccase is produced by fungi and is a biocatalyst already industrially used, for example, to decontaminate industrial wastewaters or in the textile, dye or printing industries (Riva, 2006).

Some other polysaccharides have been chemically grafted with FA moieties to obtain polysaccharide derivatives with antioxidant properties. Cellulose (Trombino et al., 2008; Trombino et al., 2009) or dextran (Cassano, Trombino, Muzzalupo, Tavano, & Picci, 2009) have been grafted with FA by acylation in organic solvent using dicyclohexylcarbodiimide as condensation agents. FA was grafted onto hyaluronic acid backbone through an ester bond with bisimidazole in formamide (Valacchi et al., 2015), and onto chitosan in acidic water using EDC as coupling agent (Woranuch and Yoksan, 2013). But these authors did not use laccase to cross-link the FA grafted polysaccharides and to our knowledge, laccase has never been used to enzymatically cross-link other polysaccharides than arabinoxylans. Indeed, the hydrogels obtained by enzymatic cross-linking of WEAX are soft with low values of elastic modulus ($G' < 50$ Pa), mainly due to the very low content of FA substituents in WEAX, to our knowledge, 2.3 mg per g of AX is the maximum value found in literature (Carvajalmillan, Guilbert et al., 2005; Carvajal-Millan et al., 2005).

The aim of this work is to obtain natural hydrogels issued from polysaccharide chemically grafted with ferulic acid, with a controlled grafted degree, by a green cross-linking reaction. For this purpose, we have chosen to graft ferulic acid onto CMP, a model of anionic polysaccharide, to further obtain hydrogels with suitable and controlled mechanical properties, including good elastic properties, by enzymatic cross-linking reaction with laccase. This study is expected to be generalized to other anionic polysaccharides such as hyaluronic acid, alginate, etc.

These CMP hydrogels could find applications in food or cosmetic applications as texturizing or stabilizing agents.

In this work, we covalently graft FA onto CMP with different molar ratios, using a water soluble carbodiimide, via a two steps reaction. The CMP-FA derivatives have been characterized by different techniques (FTIR, NMR, size-exclusion chromatography (SEC) coupling on-line with a multi-angle light scattering system (MALS), a viscometer and a differential refractive index detector (DRI)) and cross-linked with laccase from *Pleurotus ostreatus* to obtain hydrogels. The cross-linking reaction has been followed by rheology to evaluate the gel point and to determine the viscoelastic properties of the hydrogels. The swelling properties of the hydrogels have been studied in citrate/phosphate buffer (0.1 mol L⁻¹, pH 5.5).

2. Materials and methods

2.1. Materials

Pullulan was purchased from Hayashibara Biochemical Laboratory (Japan). Ferulic acid (FA), acid adipic dihydrazide (ADH),

1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC), laccase (EC 1.10.3.2) from *Pleurotus ostreatus* were purchased from Sigma-Aldrich (France); sodium hydroxide (NaOH), hydrochloric acid (HCl), citric acid, ethanol (96%), acetone, sodium hydrogenophosphate (Na₂HPO₄), Folin-Ciocalteu reactive and anhydrous sodium carbonate were purchased from VWR (France) and sodium chloroacetate from Acros Organics (France). Water was purified with the milli-Q water reagent system (Millipore, USA). All compounds were used without further purification.

Citrate/phosphate buffer (0.1 mol L⁻¹, pH 5.5) was prepared by adding 44.6 mL of 0.5 mol L⁻¹ Na₂HPO₄ and 17.7 mL of 0.5 mol L⁻¹ citric acid into a 200 mL gauged flask, adjusted with milli-Q water (ionic strength 0.1 mol L⁻¹).

2.2. Synthesis of carboxymethylpullulan (CMP)

CMP was synthesized beforehand by the reaction of pullulan with sodium chloroacetate in the presence of NaOH by a method previously described (Duval-Terrié et al., 2003). CMP was synthesized with a substitution degree in carboxylate groups of 0.80 ($DS_{COONa} = 0.80$, the number of carboxylate groups by anhydroglucose unit (AGU) was determined by conductimetric titration).

2.3. Synthesis of CMP-FA

For example, ferulic acid (5.1 mmol) was dissolved in ethanol (50 mL) and ADH (5.1 mmol) was dissolved in a mixture of ethanol and acidified (50 μ L of HCl 1 mol L⁻¹) milli-Q water (80/20 V/V). The coupling reaction of ADH on FA was activated by EDC (0.52 mM in ethanol) and the reaction was let under stirring at ambient temperature during 24 h. At the end of the reaction and after evaporation of the solvent, a volume of milli-Q water was added to the solid derivative FA-ADH to remove unreacted ADH and EDC urea. After filtration and washing with milli-Q water, the FA-ADH derivative was dried and then dissolved in 40 mL of a mixture of ethanol and milli-Q water (50/50 V/V) and slowly added to a solution of CMP (1 g) in milli-Q water (100 mL) adjusted to pH 4.75 (with HCl 1 mol L⁻¹) beforehand. The coupling reaction of FA-ADH with carboxylate groups of CMP was activated by EDC (at a molar ratio of EDC over AGU of CMP equal to 1) and the reaction was conducted at ambient temperature during 24 h. During the reaction, the volume ratio ethanol/milli-Q water was 20/80.

At the end of the reaction, the pH was checked and adjusted to 7 if necessary. Then, the CMP-FA derivative was precipitated in acetone, washed three times with acetone, dissolved in milli-Q water and dialysed against NaOH (0.1 mol L⁻¹) during 2 h to eliminate unreacted FA and by products (EDC urea), then against milli-Q water until low conductivity of dialysis water (equivalent to milli-Q water one). The CMP-FA derivatives were then lyophilised.

2.4. FTIR spectroscopy

The infrared spectroscopy was performed on a spectrometer Nicolet IS50 FT-IR (Thermo Scientific, USA). The samples were analysed by transmission from 500 to 4000 cm⁻¹ (128 scan resolution 4) using the OMNIC software.

2.5. NMR spectroscopy

¹H NMR spectra of the CMP-FA derivatives were performed with a Bruker Advance AC-P 300 MHz spectrometer (USA). Chemical shifts are reported in ppm relative to the deuterated solvent resonances. Dimethyl sulfoxide-d₆ (DMSO-d₆, 99.9 atom % D) and deuterium oxide (D₂O, 99.9 atom % D) were used as NMR solvents and purchased from Sigma-Aldrich (France). The spectra were

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