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Antioxidant properties and bioactivity of Carboxymethylpullulan grafted with ferulic acid and of their hydrogels obtained by enzymatic reaction

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ABSTRACT

Antioxidant and cytocompatible chemically modified polysaccharides and their hydrogels were obtained by a biomimetic approach. For this purpose, carboxymethylpullulan grafted with ferulic acid (CMP-FA) was firstly synthesized with different substitution degrees (DS_{FA}). Their hydrogels were secondly obtained by enzymatic cross-linking with laccase. Hydrogel swelling has been found dependent on both DS_{FA} and media ionic strength. The CMP-FA antioxidant properties were evaluated by the DPPH method and ABTS assays. The DPPH radical scavenging effect was high for CMP-FA solutions (80% after 30 min) and lower for the corresponding hydrogels (70% after 7 h). The antibacterial properties of ferulic acid and CMP-FA derivatives were tested against *Staphylococcus aureus* but the minimal inhibitory concentration of CMP-FA was not reached in the range of concentrations studied. Finally the CMP-FA derivatives showed no cytotoxicity towards mouse fibroblast cells.

1. Introduction

Polysaccharides are interesting raw materials because they are natural renewable polymers with good biocompatibility and biodegradability. Their applications in food industry (Puoci et al., 2008) or biomedical (Basu, Kunduru, Abtew, & Domb, 2015) are already numerous and they can still be expanded by chemical modification with molecules of interest. For example, pullulan, a water soluble exopolysaccharide issued from *Aureobasidium pullulans,* owns excellent film forming properties (Oğuzhan & Yangılar, 2013). The oxygen-impermeable and good mechanical properties of the pullulan films make them suitable as coating or packaging material of dried foods (Xiao, Lu, Tong, & Liu, 2015). Moreover, pullulan has food, pharmaceutical and cosmetic grades and is listed on the USP-NF (United States Pharmacopoeia—National Formulary) and JP (Japanese Pharmacopoeia)[1].

For applications in food, cosmetic or pharmaceutical industries, antioxidant and antibacterial properties with no-cytotoxicity are highly sought after. Many plants present good antioxidant and antibacterial properties and more particularly those containing flavonoid and phenolic derivatives (Hafsa et al., 2016). Natural polysaccharides issued from fruits with antioxidant activity also exist (Dou et al., 2015; Hua, Zhang, Huang, Yi, & Yan, 2014; Zhu et al., 2017). Polysaccharides issued from plants such as arabinoxylans (AX) (Katapodis et al., 2003) or from microalgae such as sulphated polysaccharides (Fimbres-Olivarria et al., 2017) also present antioxidant properties. In the case of AX, the antioxidant activity is due to the presence of ferulic acid (FA, 4-hydroxy-3-methoxycinnamic acid), known for its antioxidant activity by radical scavenging effect (Ishii, 1997). But the AX antioxidant properties are weak because the amount of FA naturally present in AX is low (1.4–2.3 μg/mg AX). FA is the most abundant phenolic acid in the plant world, including cereals, fruits, vegetables, herbs or spices. FA possesses antioxidant, antibacterial, anti-inflammatory, anti-thrombosis, and anti-cancer activities (Ou & Kwok, 2004) and is rapidly absorbed from the stomach or the small intestine and excreted through the urine (Bourne & Rice-Evans, 1998). So, its grafting onto a polysaccharide could enable the development of new materials for a wide range of applications including food, cosmetic, pharmaceutical and biomedical applications. Moreover, FA is capable of dimerizing by the action of laccase (an oxidase), leading to the possible synthesis of hydrogels by the formation of cross-links (Micard & Thibault, 1999).

Aljawish et al. grafted FA and ethyl ferulate (EF) on chitosan via an oxidation reaction with *Myceliophtora thermophyla* laccase to evaluate their antioxidant and antibacterial activities (Aljawish et al., 2012). They obtained FA-chitosan derivatives with better antioxidant properties than EF-chitosan derivatives. Hydrogels of dextran grafted with FA have also been obtained by Cassano et al. for the stabilization and transdermal delivery of vitamin E (Cassano, Trombino, Muzzalupo, Tavano, & Picci, 2009). Hyaluronic acid have been grafted with FA to obtain hyaluronate derivatives with wound healing properties (Cappelli et al., 2014). They studied the cytotoxicity on keratinocyte cells and the

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[1] (Http://www.intl.hayashibara.co.jp/products.php?jpml=products_pharmaceuticals_pullulan, n.d.)

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derivatives demonstrated interesting wound healing properties in both *in vitro* and *in vivo* preclinical models (Valacchi et al., 2015).

In a previous work (Dulong, Hadrich, Picton, & Le Cerf, 2016) we synthesized new carboxymethylpullulan (CMP) grafted with FA to subsequently obtain hydrogels by a biomimetic approach using the action of laccase from *Pleurotus ostreatus*. We obtained CMP-FA derivatives with various substitution degrees of FA (DS_{FA} up to 11%). The most grafted derivatives showed an aggregative character in aqueous solutions due to the presence of hydrophobic FA moieties. We studied the enzymatic cross-linking with laccase by rheological measurements and we showed that the kinetics of the enzymatic cross-linking reaction was dependent on the DS_{FA}. The set of rheological properties (storage and loss moduli) as well as the preliminary swelling study of the hydrogels (in the crosslinking medium) makes it possible to envisage food applications as thickening or gelling systems. This approach should lead to the conception of systems without synthetic antioxidant molecules such as BHT (butylhydroxytoluene), suspected to be endocrine disruptors (Chen, Zhao, Lin, Su, & Wang, 2014). The grafting of FA onto a polysaccharide can also avoid the release and diffusion of the antioxidant molecule and limits its possible toxicity. In the present article, we focused on the swelling properties of the hydrogels in different media (ionic strength, pH) and on the antioxidant properties of CMP-FA derivatives and their hydrogels. The antioxidant properties (by the DPPH method and the ABTS assays) and the antibacterial properties against *Staphylococcus aureus* of the CMP-FA derivatives were also evaluated. Finally, tests of cellular viability of fibroblast cells in the presence of CMP-FA derivatives were performed.

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), 2,2-diphenyl-1-picrylhydrazyl (DPPH), phosphate buffered saline (PBS), 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 (Na_2HPO_4), sodium chloride, potassium dihydrogeno phosphate (KH_2PO_4), Folin-Ciocalteu reactive and anhydrous sodium carbonate from VWR (France); Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate ($K_2S_2O_8$) and sodium chloroacetate from Acros Organics (France); and finally 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) from Alfa Aesar (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 $mol.L^{-1}$ Na_2HPO_4 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 $mol.L^{-1}$). Simulating Gastric Fluid pH 1.2 (SGF) consisted in a solution of HCl pH 1.2 in NaCl 0.35 $mol.L^{-1}$. Simulating Intestinal Fluid pH 6.8 (SIF) was prepared by adding 250 mL of KH_2PO_4 0.2 $mol.L^{-1}$ in 118 mL of NaOH 0.2 $mol.L^{-1}$. Phosphate buffer saline 0.15 $mol.L^{-1}$ pH 7.4 was prepared as indicated by the provider, by dissolving one tablet in 200 mL of milli-Q water to obtain 0.01 $mol.L^{-1}$ phosphate buffer, 0.0027 $mol.L^{-1}$ potassium chloride and 0.137 $mol.L^{-1}$ sodium chloride at pH 7.4.

2.2. Grafting of CMP with FA (CMP-FA derivatives)

The CMP-FA derivatives were synthesized as described in our previous article (Dulong et al., 2016). The reactions were performed with a CMP having a degree of substitution of carboxylate groups, DS_{COONa} = 0.8 and in a water/ethanol mixture in the presence of EDC as activator.

The substitution degree of FA (corresponding to the molar ratio of FA onto anhydroglucose unit of CMP) was determined by the Folin-Ciocalteu method described in Dulong et al. (2016).

The FA content was calculated according to Eq. (1).

$$FA_{content} = \frac{M_{FA} DS_{FA}}{M_{CMP-FA}} \times \mathbf{10^3} \quad (1)$$

With $M_{FA} = 194.2\, g.mol^{-1}$ and $M_{CMP\text{-}FA} = 162 + 80DS_{COONa} + 310DS_{FA}$

The weight average molar mass of CMP precursor was equal to 201,800 $g.mol^{-1}$ with a polydispersity of 1.6. The CMP-FA derivatives were obtained without degradation of the polymer chains and showed a tendency to aggregation when DS_{FA} increases (Dulong et al., 2016).

2.3. Hydrogels of CMP-FA

The hydrogels were obtained by enzymatic reaction with laccase (Dulong et al., 2016). Hydrogels were prepared as follows: 80 mg of CMP-FA samples were dissolved in 4 mL of citrate/phosphate buffer (0.1 $mol.L^{-1}$ pH 5.5) then laccase was added (at an activity of 2 nkat, 20 µL of 1 $g.L^{-1}$ solution). The cross-linking reaction was let at 25 °C during 24 h. The hydrogels obtained were washed with milli-Q water until conductivity of water was low. After washing, hydrogels were either frozen then freeze-dried during 3 days or air-dried at 40 °C during 24 h.

2.4. Swelling of hydrogels

For swelling degree measurements, a known amount (between 10 and 20 mg) of dried hydrogel (freeze-dried or air-dried) was immersed in a medium. The swollen hydrogel was then weighted (after elimination of the excess water) and the swelling degree (SD) was calculated according to Eq. (2) where m is the weight of the swollen hydrogels and m_o, the weight of the dried hydrogels.

$$SD = \frac{m - m_o}{m_o} \quad (2)$$

SD is the average of 3 different swelling measurements.

2.4.1. Swelling in different media

The freeze-dried or air-dried hydrogels were immersed in water, PBS, NaCl 0.15 $mol.L^{-1}$, SIF or SGF and weighted after different times of immersion to evaluate the SD of the hydrogels in these media.

2.5. Antioxidant properties

2.5.1. DPPH scavenging effect

The antioxidant properties of the CMP-FA derivatives and their freeze-dried hydrogels were evaluated by the adapted DPPH method (Brand-Williams, Cuvelier, & Berset, 1995). Briefly, 5 mg of CMP-FA derivatives or CMP-FA dried hydrogels were dissolved in 1 mL of NaCl (0.15 $mol.L^{-1}$) then 1 mL of DPPH solution at 100 µ$mol.L^{-1}$ (prepared by dissolving 4 mg of DPPH in 100 mL of ethanol) was added to the sample solution and let in the dark during 30 min (or 2 h and 7 h for the hydrogels). For a better comparison, FA was also tested by this method and solutions were prepared in ethanol (at different concentrations: 0.0005–0.11 $g.L^{-1}$) because FA is insoluble in water. The absorbance of the solutions was measured at 517 nm and the radical scavenging effect was measured according to Eq. (3).

$$Scavengingeffect\,(\%) = \frac{A_0 - A_1}{A_0} \times \mathbf{100} \quad (3)$$

where A_0 corresponds to the absorbance of a standard solution of DPPH prepared in the same medium (1 mL NaCl 0.15M + 1 mL solution DPPH in ethanol for CMP-FA derivatives or 1 mL ethanol + 1 mL solution