"An aerogel obtained from chemo-enzymatically oxidized fenugreek galactomannans as a versatile delivery system"

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Abstract

We describe a new aerogel obtained from laccase-oxidized galactomannans of the leguminous plant fenugreek (*Trigonella foenum-graecum)* and suggest its potential practical use.

Laccase/TEMPO oxidation of fenugreek in aqueous solution caused a viscosity increase of over fifteen-fold. A structured, elastic, stable hydrogel was generated, due to formation of carbonyl groups from primary OH of galactose side units and subsequent establishment of hemiacetalic bonds with available free hydroxyl groups.

Upon lyophilization of this hydrogel, a water-insoluble aerogel was obtained (EOLFG), capable of uptaking aqueous or organic solvents over 20 times its own weight. The material was characterized by scanning electron microscopy, FT-IR, elemental analysis and ^{13}C CP-MAS NMR spectroscopy and its mechanical properties were investigated.

To test the EOLFG as a delivery system, the anti-microbial enzyme lysozyme was used as model active principle. Lysozyme was added before or after formation of the aerogel, ~~was~~ entrapped or absorbed in the gel, retained and released in active form, as proven by its hydrolytic glycosidase activity on lyophilized *Micrococcus lysodeikticus* cells wall peptidoglycans.

This new biomaterial, composed of a chemo-enzymatically modified plant polysaccharide, might represent a versatile, biocompatible “delivery system” of active principles in food and non-food products.

Keywords: *galactomannans, fenugreek, laccase oxidation, aerogel, lysozyme, delivery system.*

Abbreviations*: ABTS, 2,2’-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid);FG, fenugreek gum; GaO, galactose oxidase; GM, galactomannans; Lcc, laccase; LMS, laccase-mediator system; PBS, phosphate buffer saline; TEMPO, 2,2,6,6-Tetramethyl-1-piperidinyloxy radical.*

1. *Introduction*

Development of new biomaterials and of "functionalized polymers", namely polysaccharides from renewable sources, by means of mild, enzymatic reactions, is of great interest and of great practical potential for applications in biomedical and industrial fields, including packaging (Mitrus, Vojtowicz & Moscicki, 2009).

Galactomannans (GM) are the most widely used polysaccharides, next to cellulose and starch. GM are high molecular weight polysaccharides found in the seed endosperms of some *Leguminosae* (belonging to the family *Fabaceae*), where they serve as reserve source for carbon and energy upon germination (Prajapati et al., 2013).

They have a branched polymeric structure composed of a backbone of mannose units linked by β-1,4 glycosidic bonds and side units of galactose bound to mannose by a α-1,6 glycosidic bond. The average ratio of galactose to mannose (Gal: Man) is variable, depending on the plant source, and ranges from 1 : 4.5 in cassia (*Cassia tora*) to 1 : 1 in fenugreek (*Trigonella foenum-graecum*) (McCleary, Clark, Dea & Rees, 1985; Daniel, Whistler, Voragen & Pilnik, 1994; Daas, Schols & de Jongh, 2000; Daas, Grolle, van Vliet, Schols & de Jongh, 2002; Crescenzi et al., 2004; Sittikijyothin, Torres & Gonçalves, 2005; Merlini, Boccia, Mendichi & Galante, 2015; Liyanage, Abidia, Auldb & Moussa, 2015; Wei et al., 2015). This peculiar structure makes them rather soluble in water at different temperatures, flexible in application and chemically/biochemically quite reactive (Cheng, Prud'homme, Chick & Rau, 2002).

GM are commonly employed to generate a considerable range of derivatives with wide applications as rheology modifiers, thickening and suspending agents in food, feed and industry (Mathur, 2011). They are also used as excipients and co-formulants in the biomedical field, more specifically in pharmaceutical formulations of tablets and in orally controlled drug delivery systems (ODDS), but also as binders, disintegrants, suspending, thickening, gelling, stabilizing and protective agents, to add cohesiveness to drug powder, as they are susceptible to microbial degradation in the large intestine (Meghwa & Goswami, 2012). Not least, GM are increasingly consumed as dietary fibers with atoxic bioactivities, to lower calories intake and for weight reduction, to control blood glucose, cholesterol and insulin levels, to reduce the risks of heart diseases and colon cancer, as texture modifiers and stabilizers in "specialty" foods (Murthy, Moorthy, Prabhu & Puri, 2010).

Enzymatic reactions can be applied to GM under mild conditions, with no generation of side products, e.g.: depolymerization with β-mannanase, debranching with α-glycosidase, oxidation with oxidases (i.e., laccase, peroxidase, galactose oxidase), but also for the "elimination" of unwanted insoluble proteins with proteases (Baldaro et al., 2012). Enzymatic oxidation of guar GM has been described using either a wild type galactose oxidase (GaO), followed by reductive amination (Hall & Yalpani, 1980) or by halogen oxidation (Frollini, Reed, Milas & Rinaudo, 1995), or with a highly engineered GaO by Parikka and co-workers (Delagrave et al., 2001, 2002; Parikka & Tenkanen, 2009; Parikka et al., 2010; Parikka et al., 2012; Leppanen et al., 2010; Mikkonen et al., 2014; Ghafar et al., 2015; Parikka, Master & Tenkanen, 2015). More generally, oxidation of polysaccharides with the enzyme laccase can generate reactive groups (e.g., carbonyls, carboxyls) on cellulose (Viikari, Buchert & Kruus, 1999a), on starch (Viikari et al., 1999b), on pullulan (Jetten, van den Dool, van Hartingsveldt & Besemer, 2000), and on guar galactomannan (Lavazza et al., 2011).

We have previously reported (Lavazza et al., 2011) that a fungal laccase from *T. versicolor* (benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2, see Riva, 2006; Witayakran & Ragauskas, 2009; Rodgers et al., 2010), in combination with TEMPO as mediator and for laccase regeneration (Bragd, van Bekkum & Besemer, 2004), oxidizes primary hydroxyl groups of GM in an unbuffered aqueous solution of guar galactomannan, causing a substantial viscosity increase of the polysaccharide solution, which is converted to an elastic gel, as confirmed by its modified rheological profile. In a follow-up publication (Merlini et al., 2015), we reported that the laccase/TEMPO oxidation system yields elastic gels also in the case of four other leguminous gum solutions (i.e., locust bean, tara, sesbania and fenugreek) and have also shown that the higher the galactose content of the GM, the higher the viscosity increase. Indeed, fenugreek gum (FG) oxidation gave the most dramatic results under the experimental conditions used, with a surge in viscosity of over fifteen-fold, to generate a very compact, elastic hydrogel, that, even after partial depolymerization with a β-mannanase, was able to preserve some of its "gel-like" structure.

FG is a leguminous plant grown in northern Africa, the Mediterranean basin, western Asia, northern India, and more recently also in Canada (www.agriculture.gov.sk.ca). The storage polysaccharide found in its seed endosperm is a galactomannan, similar to locust bean, guar and tara gum, but more extensively branched (Wei et al., 2015). The Gal : Man ratio in fenugreek GM was estimated to be close to unity by HPLC analysis (Brummer, Cui & Wang, 2003) and confirmed by NMR (Merlini et al., 2015).

In the present paper, we report that lyophilization of chemo-enzymatically oxidized fenugreek hydrogel leads to formation of a stable aerogel (referred to as EOLFG, for Enzymatically Oxidized, Lyophilized Fenugreek Gum), with a high water uptake capability. This new biomaterial was characterized by scanning electron microscopy, FT-IR, elemental analysis and ^{13}C CP-MAS NMR spectroscopy and its mechanical properties were investigated.

As a model delivery system (DS), EOLFG was evaluated in combination with lysozyme (LSZ, EC 3.2.1.17), a natural antimicrobial enzyme present in several mammalian secretion fluids, which is industrially obtained from hens egg white (HEW) (Brasca et al., 2013). This enzyme has the ability to hydrolyze the β-1,4 glycosidic bond between *N*-acetyl-muramic acid and *N*-acetyl-glucosamine in the cell wall peptidoglycans of gram-positive bacteria (Silvetti et al., 2010) and to inhibit the growth of *Clostridium tyrobutyricum* vegetative cells in cheese (Ávila, Gómez-Torres, Hernández & Garde, 2014). It is also effective in controlling *Listeria monocytogenes* growth on raw minced tuna and salmon roe (Takahashi et al., 2011). Lysozyme is non-toxic to humans, is allowed as a food additive (E1105) in ripened cheeses and milk products (EU No. 1129/2011) and is also active on processed meat (Tiwari et al., 2009).

Incorporation of lysozyme in EOLFG and its release was studied by two different approaches: a) addition of the enzyme to the fenugreek solution, before laccase, TEMPO-mediated, oxidation and formation of the aerogel; b) absorption and retention in the gel from an aqueous solution of lysozyme. In both cases, release of the muramidase was tested by diffusion in Petri dishes of agar layered with lyophilized *Micrococcus lysodeikticus* cells and halo formation due to cell wall peptidoglycans hydrolysis.

We propose that this new biomaterial might have promising potential in several applications and mostly as a versatile DS of various active principles. Indeed, we are investigating its properties as a DS of: anti-microbial peptides (e.g., nisin) for food and food packaging; of anti fouling enzymes (e.g., proteases, lipases) for surface coating; of pesticides (e.g., an anti *Botrytis* fungicide) for crop protection); of non steroidal anti-inflammatory drugs (e.g., ibuprofen) and antibiotics (e.g., amoxicillin) for the biomedical field; of industrial biocides (e.g., 1,2-benzoisothiazol-3(2H)-one) for in-can preservation). The results of these studies will be reported in a forthcoming publication.

2. *Experimental*

2.1. Material

Laccase from *Trametes versicolor*, in powder form from Sigma-Aldrich with a measured activity of 4,300U/g on ABTS as substrate, was dissolved with mild stirring in MilliQ water. TEMPO and all other chemicals were from Sigma-Aldrich or Fluka. β-mannanase was from Megazyme (E-BMANN) with a declared activity of 400 U/ml.

Non purified gum powder from fenugreek (FG) with Brookfield viscosity at 1% (w/v) in aqueous solution at 20 rpm and 25°C of 1500-2500 mPa*s, was from a commercial source of Canadian origin and kindly supplied by Lamberti S.p.A. Actual GM content of unpurified gum varied between 76 and 80% (w/w), while the remaining components were represented by aleuronic proteins, seed coat residues, low mol wt sugars, ashes. Lysozyme hydrochloride was supplied by Sacco (Cadorago (CO), Italy).

2.2. Laccase assay.

Laccase activity was determined using as substrate 2.48 mM ABTS in 100 mM sodium acetate at pH 5 (Niku-Paavola, Karhunen, Salola&Raunio, 1988).

One laccase unit is defined as the amount of enzyme that catalyzes the oxidation of one μmole of ABTS in one min at 25 °C and pH 5.

2.3. Purification and viscosity measurements of fenugreek solutions.

Fenugreek gum was purified by dispersion (at 10% w/w) in a 3:7 solution of H_2O/ethanol, under stirring at room temperature for 30 min, followed by vacuum filtration. The recovered FG was dispersed (at 10% w/w) in acetone, under stirring as before and was finally recovered by vacuum filtration. Before use, it was oven-dried at 60°C overnight. Yield of this procedure was 85-90% (w/w); the residual 10-15%, composed of proteins and other minor components, was discarded.

“Purified” FG was dissolved in MilliQ water at room temperature at 1200 rpm with an IKA overhead stirrer. The solution was kept standing overnight at room temperature without stirring before any further experiment. Compared to “non purified” FG, viscosity increased by about 10-15% at equal polysaccharide concentration in water. FG solutions for all experiments were prepared the same way.

Viscosity measurements were performed in a volume of 300-400 ml in a beaker at room temperature using a Brookfield DV-I Prime, at 20 rpm, mounted with the appropriate spindle.

2.4. Preparation of the aerogels.

Laccase TEMPO-mediated oxidation of FG was carried out following the procedure previously described by Merlini et al. (2015). Purified FG was dissolved under constant mechanical stirring in 100 ml of MilliQ water (1.075% w/w) at room temperature for 30 min in order to develop a final viscosity around 1800 mPa*s and the solution was kept still overnight at room temperature. The mediator TEMPO was added to a final 0.64 mM concentration (10 mg), followed by 60 U/g $_{(GM)}$ of laccase (eq. to about 15 mg). The reaction was continued for 3 h at 35°C, with constant mechanical stirring at 500 rpm, after which Brookfield viscosity was measured and the mixture was left standing at room temperature. Viscosity was measured again after 6 and 24 h from the start of the reaction.

To obtain the aerogel, chemo-enzymatically oxidized FG was distributed either into 24 well plates, to obtain 16-20 mm x 12-16 mm cylindrically shaped samples, or in Petri dishes (90×12 mm),to give a flat aerogel wafer of about 80x5 mm and frozen for 12 h at -80°C, followed by freeze-drying at -55 °C for 48 h (this material is referred to as EOLFG).

The lysozyme-loaded aerogel was prepared following a similar approach: lysozyme was firstly dissolved under constant mechanical stirring in MilliQ water for 30 min to give an aqueous solution of 2000 ppm protein, in which the purified FG was later dissolved and the oxidation was carried out as described before. This material is referred to as EOLFG-LYS1.

Pristine, non-oxidized FG was lyophilized under the same conditions and used as control

2.5 Characterization of the aerogels.

Elemental analysis was performed with a Costech ECS 4010 analyzer, while the solid-phase FTIR spectra of the powdered samples, with infrared grade KBr, were obtained using a Varian 640-IR spectrometer.

The ^{13}C cross-polarization magic-angle-spinning (CP-MAS) spectra were recorded with an FT-NMR Avance TM 500 (Bruker BioSpinS.r.l) with a superconducting ultra-shield magnet of 11.7 Tesla operating at 125.76 MHz ^{13}C frequency. The following conditions were applied: repetition time 4 s, ^{1}H 90 pulse length 4.0 μs, contact time 1.2 ms, and spin rate 8 kHz. The material was placed in a zirconium rotor, 4 mm diameter and 18 mm length. The chemical shifts were recorded relative to a glycine standard, previously acquired (C=O signal: 176.03 ppm, relative to a tetramethylsilane reference).

Scanning electron microscopy (SEM) was performed using a variable-pressure instrument (SEM Cambridge Stereoscan 360) at 100/120 Pa with a VPSE detector. The operating voltage was 20 kV withan electron beam current intensity of 150 pA. The focal distance was 8 mm. Samples were analyzed with no preliminary treatment.

2.6. Mechanical features.

Cylindrically shaped samples of both pristine FG and EOLFG aerogels were evaluated by a compressive test according to the procedure described by Deszczynski, Kasapis & Mitchell (2003). Two consecutive cycles of compression were performed with a dynamometer (mod. Z005, Zwick Roell, Ulm, Germany) fitted with a 100 N load cell and connected to two plates (30 mm diameter), placed at a distance of 22 mm apart. Each compression cycle accounted for a maximum deformation of the sample of 2 mm, at a crosshead speed of 2 $mm*s^{-1}$. Both stress–strain and force–time profiles were recorded and the following parameters were elaborated by software (TestXpert V10.11 Master): compressive modulus (i.e., E-mod, expressed in kPa, as the slope of the initial rising part of the first stress–strain curve), determined according to a secant method; maximum compressive force (i.e, F_{max}, expressed in N, as the peak force of the first compression cycle); cohesiveness (i.e., the ratio of the area of the second cycle to the area of the first cycle); springiness (i.e, the area of compression of the second cycle divided by the area of compression of the first cycle); resilience (i.e, the ratio of the area of decompression to the area of the compression of the first cycle) (Ghafar et al., 2015). All tests were carried out at 23 ± 0.5 °C and 40 ± 2.5 % relative humidity (RH). At least ten replicates were tested for each sample,either of pristine, lyophilized FG or of EOLFG. Statistical difference between mean values was determined by Student's *t*-test, with a significance level (p) < 0.05, using Statgraphic Plus 4.0 software.

2.7. Solvent uptake measurements

Solvent uptake was determined by immersing EOLFG weighted samples either in MilliQ water or in phosphate buffer saline (PBS), pH 7.4, or in DMSO at room temperature. At pre-determined time intervals (i.e., 2, 5, 10, 20, 60, 120 min), the samples were removed with a spatula and gently blotted on filter paper, leaving only interstitial solvent molecules trapped in the polymer network, and weighted again.

The percentage of solvent uptake (SU) was calculated using equation (1):

$$SU = 100 \times \frac{w_s - w_d}{w_d} \qquad (1)$$

Where $w_s$is the weight of the hydrogel at different uptake times and $w_d$is the weight of the dry sample.

2.8. Lysozyme adsorption in the aerogel.

Plugs were carved with a sterilized cork borer (Ø 8 mm) froman EOLFG wafer, lyophilized in a Petri dish. Average weight of the plugs was 7 ± 2 mg. Lysozyme was incorporated in the gel by immersing the plugs for 1 h at room temperature in an Eppendorf tube containing 1 ml of 20, 200 or 2000 ppm of enzyme dissolved in sterile water. The plugs were then rinsed three times in 1 ml of sterile distilled water and blotted on UV-sterilized filter paper. The average weight of the "loaded" hydrogel plugs so obtainedwas 103 ± 29 mg, with a mean weight increase of almost 15 fold, from which the "theoretical" amount of lysozyme absorbed was calculated. This material is referred to as EOLFG-LYS2a, b, or c, if obtained from a 20, 200, 2000 ppm lysozyme aqueous solution, respectively.

EOLFG-LYS2c was re-lyophilized for ease of handling, before characterization of the material, as described in § 2.5 and § 3.2.

2.9. Lysozyme assay

Activity of lysozyme was determinedby a modified biochemical assay described by Silvetti et al. (2010), as follows. Fifteen ml of a sterilized medium composed of 1% agar dissolved in 0.1 M citrate buffer (pH 6.2) were poured in a Petri dish (Ø 9 cm) and left to solidify at room temperature. An aliquot of 5 ml of agar containing 0.2% of lyophilized cells of *Micrococcus lysodeikticus* (ATCC No. 4698 from Sigma, St. Louis, MO, USA) was layered above. Equidistant wells (Ø 8 mm) were carved with a sterilized cork borer and the liquid mixture or the plug specified in the figure legends was added. Inoculated plates were incubated at 35°C for 24 h.

Release of lysozyme from the EOLFG-LYS1 or EOLFG-LYS2 was assessed by evaluating the lysis halo around each well in comparison to EOLFG control and to diffusion of free lysozyme.

Lysozyme entrapped in EOLFG-LYS1 was also estimated after complete disruption of the gel, as follows: 250 mg were aseptically transferred to a sterile stomacher bag containing 10 ml of 0.1 M citrate buffer (pH 6.2) and homogenized in a laboratory homogenizer (BagMixer®, Interscience, France) at high speed for 15 min, the homogenized mixture was diluted with 0.1 M citrate buffer and 0.1 ml were added to the wells.

As control a 2000 ppm standard solution of lysozyme was prepared in 0.1 M citrate buffer (pH 6.2), dilutions were freshly made before use in the same buffer and 0.1 ml were added to control wells.

All manual operations were performed under a vertical laminar flow hood and all experiments were run in triplicate.

3. Results and Discussion

3.1. Preparation of EOLFG.

The protocol for the preparation of EOLFG is outlined in Fig.1 and is described in details in Materials & Methods.

The process was carried out in water at 35 °C, with no production of by-products or waste, and involves two main steps. In the first step, primary hydroxyl groups of FG galactose residues are oxidized to carbonyls by the laccase/TEMPO catalytic system (Lavazza et al., 2011; Merlini et al., 2015). In the reaction, TEMPO is firstly oxidized to an oxoamonium ion, which in turn selectively oxidizes primary OH's to the corresponding aldehydes (see also: Ding et al., 2008; Marzorati, Danieli, Haltrich & Riva, 2005; Viikari et al., 1999a). This combination of enzyme and mediator is referred to as a "laccase-mediator system" or LMS (Fabbrini, Galli & Gentili, 2002; Galante & Formantici, 2003; Kulys & Vidziunait 2005; Morozova, Shumakovich, Shleev & Yaropolov, 2007).

In the second step, the newly formed carbonyl groups react with neighboring hydroxyl groups to form intra- and/or inter-molecular hemiacetalic bonds, as previously suggested by Donnelly (1999). The generation of this chemically cross-linked network caused a progressive increase in viscosity from 1,790 mPa*s (at about 1% w/w of FG in water) to 7,500 mPa*s after 3 h, under constant stirring, as reported in Table S.1 in "Supplementary Material". The mixture was then left standing at room temperature, with no stirring, and viscosity was measured after 6 h and 24 h, when it reached 30,000 mPa*s. Accordingly, a progressive thickening of the mixture was observed, that eventually displayed the typical behavior of a structured, elastic hydrogel, which could hold its structure up to 1 h following depolymerization with a β-mannanase down to 10,000 mPa*s, but was completely de-structured after 24 hours of enzymatic hydrolysis (Merlini et al., 2015).

Fig. S.1 in "Supplementary Material" offers a visual image of the different appearance of the FG solution, before and after enzymatic oxidation and hydrogel formation.

When enzymatic oxidation was performed in the presence of 2000 ppm lysozyme (see § 2.4 for details), viscosity increase was somewhat lower at each step of the process, as reported in Tab. S.1. The solution reached a viscosity of 3000 mPa*s after 3 h and of 5,800 mPa*s after 24 h, equivalent to half and one fifth the value without lysozyme, which however did not compromise its ability to form a gel and to be freeze-dried. This different behavior is likely due to interference of the entrapped protein with the "structuring" of the gel, causing a more limited cross-linking of the polymer.

Finally, hydrogels, without or with lysozyme, were freeze-dried to yield the corresponding aerogels, referred to as EOLFG and EOLFG-LYS1, respectively. The latter appeared to be more fragile and brittle than the former, and was, only to a limited extent, evaluated as a possible delivery system of lysozyme in agar gel.

3.2 Characterization of EOLFG.

Molding of the oxidized FG hydrogel during lyophilization can determine the final shape of the aerogel.Fig. 2(a) shows cylindrically shaped EOLFG samples obtained by freeze-drying the hydrogel poured into a 24 well-plate used as mold. The material is light (the weight of each specimen was in the range 25-30 mg), with a spongy texture, somewhat resembling polystyrene packing “peanuts”. The density of EOLFG, calculated from the weight of the samples and their volumewas about 10-14mg*cm^{-3}, close to the values reported for guar gum oxidized with GaO in the presence of peroxidase and catalase (Mikkonen et al., 2014).

SEM images of EOLFG are shown in Fig. 2(b) – (d), where the internal morphology of the aerogel appears to be formed by thin polymer layers tightly stacked on top of one another. The thickness of each sheet is estimated to be below 1μm.

In Fig. 3, the kinetic curves of solvent uptake by EOLFG samples, immersed either in MilliQ water, in PBS (pH 7.4) or in DMSO, are reported. PBS was tested in order to mimic “physiological” conditions and to evaluate the effect of salts on water uptake of this non-charged polymer. DMSO was taken into consideration as a representative aprotic organic solvent, with a high dipole moment. After immersion in solvent, no significant changes in the overall sizeof the EOLFG cylindrical samples was noticed. Nevertheless, they were able to rapidly uptake and retain MilliQ water or PBS, forming a stable hydrogel. After 2h, weight increase was about 20-fold the initial average value, but for longer contact times, water uptake increased to almost 40-fold the initial weight. In DMSO, weight increase was 15-fold after 2 h and just over 18-fold after 24 or 48 h. When the same experimental conditions were applied to pristine, non-oxidized, lyophilized FG, the material completely dissolved in water.

EOLFG and re-lyophilized EOLFG-LYS2c (i.e., a lysozyme-loaded hydrogel from a 2000 ppm enzyme aqueous solution for 2 h at 25 °C, followed by blotting on filter paper and a second cycle of freeze drying) were further characterized by different techniques: elemental analysis, FT-IR spectroscopy, ^{13}C CP-MAS NMR spectroscopy, and compared to pristine FG.

Data of elemental analysis are reported in Tab. 1. The background nitrogen content (0.75%) in the pristine FG can be attributed to little remaining impurities in the material used in our experiments. The slight increase in nitrogen content found in EOLFG is very likely be due to laccase protein nitrogen and residual TEMPO entrapped into the structured gel. The amount of laccase and TEMPO in the final product can be roughly estimated around 10 mg/g$_{(GM)}$ and 15 mg/g$_{(GM)}$, respectively. TEMPO is considered as non-toxic and it is used in a wide number of biomedical applications (Yoshitomi, Hirayama & Nagasaki, 2011; Narain, 2011; Yoshitomi, Yamaguchi, Kikuchi &

Nagasaki, 2012). Therefore, neither residual components of the oxidation reaction should be considered as drawbacks in future practical applications of this new biomaterial.

As expected, a higher content of nitrogen (2.20%) was found in EOLFG-LYS2c, thus confirming the presence of “entrapped” lysozyme in the EOLFG.

Fig.4 shows the FTIR spectra of: (a) pristine FG, (b) EOLFG and (c) EOLFG-LYS2c. Spectra of FG and EOLFG appear to be quite similar. The characteristic band in the wave number range between 3000 $cm^{-1}$and 3700 cm^{-1} and between 2700 cm^{-1} and 3000 cm^{-1} can be ascribed to O-H and C-H stretching vibration, respectively. The peaks in the range of 1020 cm^{-1} and 1400 cm^{-1} are consistent with C-H and O-H bending vibrations. However, the EOLFG spectrum does not show any peak in the range that can be ascribed to carboxylic groups. It is worth noticing that previous^{1}H NMR experiments carried out in deuterated aqueous solution of chemo-enzymatically oxidized FG revealed also the presence of aldehyde proton signals in the 9.2-9.5 ppm range (Merlini et al., 2015). It is likely that the freeze-drying process favors the conversion of the residual aldehydes to hemiacetalic bonds, thus enhancing the cross-linking of GM polymer chains, as a prerequisite to formation of an aerogel.

The presence of lysozyme in EOLFG-LYS2c is confirmed by the appearance of the amidic NH_2 vibration peak at 1541 cm^{-1} (Abidi, Cabrales, &Haigler, 2014), while the peak associated to lysozyme amidic C=O stretching, which should appear at 1655 cm^{-1}, is not clearly visible, because it overlaps with the bending signal of residual water tightly bound to the polymer in all three samples (Olsson & Salmén, 2004).

The ^{13}C CPMAS NMR spectra of FG and EOLFG, reported in Fig. S.2, are quite similar to each other. The peaks are rather broad, offering proof that the material is essentially amorphous. The signal of the anomeric carbons is clearly visible at 100 ppm, while all remaining carbons of the mannose and galactose units give a broad and intense signal in the range 55-85 ppm. In both FG and EOLFG spectra it is possible to observe a small signal in the range 170-180 ppm, which is ascribed to impurities existing in the pristine FG. The presence of lysozyme in EOLFG-LYS2c is confirmed by a clearly detectable signal associated with amidic carbons at 175 ppm.

3.3 Mechanical properties

Large deformation tests were applied to quantify the effect of enzymatic oxidation and internal cross-linking on the mechanical properties of the EOLFG aerogel. The compressive force–time curves obtained from native, lyophilized FG and EOLFG gave two clearly distinct profiles, as summarized in Table 2 and shown in Fig. S.3.

E-mod and F_{max}values of EOLFG were approximately 35 times stiffer and 28 times harder than lyophilized FG, respectively, thus confirming the lighter and more breakable structure of the latter.

A similar trend was observed with the other mechanical parameters. Cohesiveness represents the ability of the sample to stand a second deformation in relation with the sample behavior during the first deformation cycle. Springiness indicates the capability of the sample to spring back after the first compression. Resilience is a measure of the ability of the sample to recover its original shape. Cohesiveness, springiness, and resilience were lower with EOLFG, consistent with the inverse relationship between these parameters and the compressive modulus and maximum force, in accordance with what described by Ghafar et al. (2015) on chemo-enzymatically oxidized guar gum. Overall, these results would further support that enzymatic oxidation of fenugreek gum, followed by lyophilization, causes formation of a stable network of FG polymer, less prone to be deformed and more able to absorb an applied stress.

3.4 Uptake and release of active lysozyme from EOLFG

The potential of the EOLFG aerogel described above to function as a delivery system (DS) was investigated with lysozyme as an active compound.

As mentioned before, in a first approach lysozyme was entrapped in the gel before chemo- oxidation and lyophilization (EOLFG-LYS1). Alternatively, the enzyme was incorporated in carved plugs of EOLFG by immersion, under sterile conditions, in an aqueous solution of 20, 200 or 2000 ppm of lysozyme (EOLFG-LYS2a, b, c, respectively). This concentration range was chosen considering that a dosage of 100 - 500 ppm is generally used in the food industry (Brasca et al., 2013; Carrillo, Garcia-Ruiz, Recio& Moreno-Arribas 2014). A rough estimation of the enzyme content in the final material, before rinsing and blotting, can be calculated from the mean weight increase of the plugs (i.e., 15-fold).

Release of lysozyme was qualitatively evaluated by a standard biochemical test of its hydrolytic activity in agar plates layered with lyophilized cells of *Micrococcus lysodeikticus*.

Fig. 5 shows the diffusion pattern of lysozyme from a plug of EOLFG-LYS1 aerogel, compared to free lysozyme and to lysozyme freed from a homogenized EOLFG-LYS1. The figure can only offer a qualitative evidence of enzyme release, because a quantitative estimation based on halo diameters would not be accurate, considering that the aerogel in the central well has to first absorb water before releasing the entrapped muramidase, prior to diffusion of the latter and display of its enzymatic activity. On the other hand, lysozyme in the lateral wells, either as free protein or freed upon homogenization of the gel, can promptly diffuse.

Fig. 6 shows the release and diffusion of lysozyme from EOLFG-LYS2c. The central well (a) was fitted with a plug of a three-times rinsed EOLFG-LYS2c hydrogel.Based on its weight increase, the amount of incorporated lysozyme could be extimated close to 200 μg. The central wellshould be compared to the peripheral control wells: b) a plug of control EOLFG and (c) 200 µg of free lysozyme. The wells marked as (d), (e) and (f) each contained 100 µl of undiluted rinse water of the experimental plug in (a). It proves that the third rinse developed almost no halo, which supports the conclusion that the large halo formed around the central well was due to release of lysozyme loaded and incorporatedinto the gel, not just washed off from its surface. Similar experiments were performed with 20 and 200 ppm enzyme solutions, which meant fitting in the central well a plug loaded with 2 and 20 µg muramidase, respectively. In either case, the central halo was quite evident, but essentially no halo formation was noticed in the second and third rinses (see Fig. S.4 and Fig. S.5 in "Supplementary Material"). The control EOLFG never showed any halo formation, proving that this biochemical test is very specific for lysozyme activity.

4. Conclusions

We applied the laccase/TEMPO oxidation reaction to fenugreek gum in aqueous solution, followed by lyophilization, and have obtained a water-insoluble aerogel (EOLFG), with high water and DMSO uptake capacity. The believe that the underlying mechanism involves generation of carbonyl groups by the chemo-enzymatic reaction, that are able to form hemiacetalic bonds with adjacent free OH's, thus causing internal cross-linking of the polymer and its "structuring" to yield a compact, highly elastic gel, as a prerequisite to aerogel formation by lyophilization. This is, to our knowledge, the first description of an aerogel from chemo-enzymatically oxidized FG, whose general features appear to resemble the material obtained by others with guar gum oxidized by GaO (Mikkonen et al., 2914; Ghafar et al, 2015).

However, in view of future developments and possible industrial transfer, we have chosen to focus on a commercial polysaccharide, as well as available enzymes (i.e., laccase and lysozyme), rather than on pure substrates and reagents supplied for the sole purpose of research.

As proof of concept that this biomaterial can function as a delivery system, lysozyme was loaded and entrapped in EOLFG, and was released in active form in agar gel, as proven by the hydrolysis of peptidoglycans from the cell wall of *M. lisodeikticus*. Other ongoing studies are yielding similar results with peptides and larger enzymes.

The potential of this new chemo-enzymatically modified material from renewable source as a versatile delivery system of other active principles (e.g., anti-inflammatory drugs, antibiotics, fungicides, biocides, etc.) is under investigation and will be reported in a following publication..

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