Food Research International 88 (2016) 153–158

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Improving the performance of transglutaminase-crosslinked microparticles for enteric delivery

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ARTICLE INFO

Article history:
Received 15 September 2015
Received in revised form 21 February 2016
Accepted 26 February 2016
Available online 28 February 2016

Keywords:
Cross-linking
Complex coacervation
Enzyme
Microencapsulation
Gastrointestinal resistance

ABSTRACT

Various agents for cross-linking have been investigated for stabilizing and controlling the barrier properties of microparticles for enteric applications. Transglutaminase, in addition to being commercially available for human consumption, presents inferior cross-linking action compared to glutaraldehyde. In this study, the intensity of this enzymatic cross-linking was investigated in microparticles obtained by complex coacervation between gelatin and gum Arabic. The effectiveness of cross-linking in these microparticles was evaluated based on swelling, release of a model substance (parika oleoresin: colored and hydrophobic) and gastrointestinal assays. The cross-linked microparticles remained intact under gastric conditions, whereas the uncross-linked microparticles have been dissolved. However, all of the microparticles have been dissolved under intestinal conditions. The amount of oily core that was released decreased as the amount of transglutaminase increased. For the most efficient microparticles (50 U/g of protein), the performance was improved by increasing the pH of cross-linking from 4.0 to 6.0, resulting in a release of 17.1% rather than 32.3% of the core material. These results were considerably closer to the 10.3% of core material released by glutaraldehyde-cross-linked microparticles (1 mM/g of protein).

1. Introduction

Orally administered polymeric microparticles that encapsulate drugs must resist degradation in gastric juices while ensuring complete drug release in the intestinal tract (Song, Zhang, Yang, & Yan, 2009; Zhang, Pan, & Chung, 2011). In most cases, this ideal objective can be achieved through structural modifications of the polymeric wall, which can delay degradation of the microparticles until a specific time or location.

These polymeric carrier systems can be produced using various encapsulation techniques, including spray chilling, spray drying, ionic gelation, fluidized-bed coating and liposome entrapment (Desai & Park, 2005). Another such technique, complex coacervation, presents various advantages, including high encapsulation efficiency without the use of extreme production conditions; therefore, this technique can be used in the preparation of pharmaceuticals and foods. However, the nature of the ionic interactions between the polymers leads to low mechanical and thermal resistances of the resulting polymeric wall. Hence, it is necessary to strengthen the wall via cross-linking (Burgess & Ponsart, 1998; Sanchez & Renard, 2002) such that the rate of release of the active component can be precisely controlled.

For many years, aldehydes have been used as cross-linking agents (Thies, 2006). Aldehydes, such as glutaraldehyde (GLU), produce irreversible bonds and thus enhance the resistance of the wall to extreme pH and temperature conditions (Babin & Dickinson, 2001; Hernández-Muñoz, Villalobos, & Chiralt, 2004). These cross-linking agents covalently bind to the free unprotonated amino groups of a protein. During the reaction, aldehydes are also inserted into the chemical binding; however, they are toxic, which limits their use for human consumption (Gallieta, Di Giola, Guilbert, & Cuq, 1998).

Transglutaminase (TG) is a non-toxic enzyme that catalyzes intra- and intermolecular cross-linking (ε-(λ-glutamyl)-lysyl) between protein molecules (Motoki & Seguro, 1998). TG has also been studied as a cross-linking agent, particularly for materials that are produced for human consumption (Kuraishi, Yamazaki, & Susa, 2001; Mariniello, Di Pierro, Giosafatto, Sorrentino, & Porta, 2008; Tang, Chen, Li, & Yang, 2006). The conditions typically used for cross-linking with transglutaminase include a temperature of 20 °C, pH of 5.0 and long reaction times, such as 15 to 24 h.

In the present study, the intensity of transglutaminase cross-linking in microparticles produced by complex coacervation between gum Arabic and gelatin was investigated. The effects of adding different levels of TG (0, 10, 30 and 50 U/g of protein) on the mean size, morphology,

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http://dx.doi.org/10.1016/j.foodres.2016.02.020
0963-9969/

release of oily core material and resistance to gastrointestinal conditions were compared to those of the typical concentration of glutaraldehyde (1 mM/g of protein). Because the activity of enzymes such as TG is highly sensitive to pH, the pH of cross-linking was also evaluated, as well as its effects on the release of core material under simulated gastric conditions.

2. Materials and methods

2.1. Materials

Gum Arabic (IRX49345, Colloides Naturels, SP, Brazil) and type A gelatin (244 bloom, Gelita South America, SP, Brazil) were used as wall materials. Commercial soy oil (Liza®) containing 10:1 paprika oleoresin (Citromax, SP, Brazil) was used as the core material. Microbial transglutaminase (Ca^{2+} independent, 100 U/g of nominal enzymatic activity, Activa TG-S®, Ajinomoto, SP, Brazil) and glutaraldehyde (Sigma, St. Louis, USA) were used as cross-linking agents. Pepsin (3180 U/mg of protein) and pancreatin (3 × USP unit of enzyme activity) enzymes, which were used to simulate gastric conditions, were purchased from Sigma-Aldrich (St. Louis, USA). All solvents and other reagents were of analytical grade, and deionized water was used in all the experiments.

2.2. Production of microparticles

Microparticles were produced by emulsifying 2.5 g of the core material in 100 mL of gum Arabic solution (2.5 wt.%) for 1 min at 14,000 rpm at 50 °C using an Ultra-Turrax homogenizer (T-18 basic, IKA, Staufen, Germany) followed by incorporating the emulsion into 100 mL of gelatin solution (2.5 wt.%), also at 50 °C, and adding 400 mL of deionized water at 50 °C. The pH was reduced to 4.0 using 0.1 M HCl, and the system was gradually cooled to 10 °C. After 12 h in a refrigerator (5 °C), the particles settled and the excess liquid was drained. These moist microparticles were freeze-dried in an Edwards Pirani 501 Freeze Dryer (West Sussex, UK) and characterized.

To determine the efficiency of encapsulation, the freeze-dried microparticles (100 mg) were dissolved in a tube with 1 mL of pancreatin solution (0.30 mg/mL in $NaHCO_3$, 0.1 M, pH 7.0). Pancreatin, a protease, was used to hydrolyze the wall of particles and allow the release of the oily core. After incubating for 30 min at 37 °C, 20 mL of sunflower oil, a hydrophobic medium, was added to the tube to extract the hydrophobic core material released from the aqueous system. The system was continuously stirred at 25 rpm for 1 h at 37 ± 0.5 °C. The content of the tubes was filtered through glass wool to separate the particulate materials and centrifuged (10 min at 3500 rpm). An aliquot of the lighter oil phase was removed with a pipette. The amount of the core material present in the sunflower oil was quantified using a standard curve (460 nm) on a Beckman DU-70 spectrophotometer (Indianapolis, IN, USA); the curve was obtained at 5 concentration levels (0.020 to 0.150 mg/mL), with linearity ($R^2 = 0.998$) was determined using a least squares regression method in triplicate for each concentration level. The encapsulation efficiency (%) was calculated by comparing the amount of core material in the microparticles relative to the amount of core material used to produce the microparticles.

2.3. Cross-linking of microparticles

The wall of the original moist microparticles was strengthened by cross-linking with 3 different concentrations of TG (10, 30 and 50 units (U)/g of protein), using the same pH as that for the complex coacervation of the polymers (pH 4.0). These amounts represent, respectively, 0.1, 0.3 and 0.5 g of transglutaminase/g of protein. Cross-linking with the chemical agent glutaraldehyde (1 mM/g of protein or 0.39 mL of glutaraldehyde solution/g of protein) was performed under the same conditions as a control. The process, which was maintained under constant magnetic stirring, required 15 h at room temperature. The pH, time and temperature conditions were chosen based on previous work conducted by our group (Alvim & Grosso, 2010; Prata, Zanin, Ré, & Grosso, 2008) and other authors (Dong et al., 2008; Zhang, Zhang, Hu, Bao, & Huang, 2012). The cross-linked microparticles were washed with water at the same pH.

After evaluating the results obtained with TG, the higher concentration (50 U/g of protein) was used to cross-link the microparticles at pH 6.0. The cross-linking was performed at room temperature, with constant agitation over 15 h, and the same conditions were used to cross-link glutaraldehyde (1 mM/g of protein).

The strength of the cross-linking in the wall was determined by evaluating the release of the core material in an oily medium. The release of the core material from the freeze-dried microparticles was determined by adding 100 mg of these microparticles to 20 mL of sunflower oil in capped glass tubes covered with an aluminum sheet to avoid the effects of light. The tubes were agitated in a rotary shaker (AP22, Phoenix, SP, Brazil) moving at approximately 30 rpm. After 4, 8, 12 and 24 h, the tube contents were filtered using glass wool and centrifuged for 10 min at 3500 rpm.

The amount of core material released into the sunflower oil was quantified using the standard curve. All determinations were performed in triplicate.

Thus, the oil release assay was performed with dried particles (i.e., without hydrophilic hindrance) in another oily medium, sunflower oil (i.e., hydrophobic affinity), over a period of 24 h.

2.4. Analysis of microparticle swelling behavior

To analyze swelling, the morphologies and sizes of all microparticles were determined, with measurements taken both before freeze-drying and after rehydration with distilled water (1 h with constant agitation). The morphologies were observed using an optical microscope (Jenaval, Carl Zeiss, Göttingen, Germany) coupled to a digital camera and recorded using Image Pro Plus 4.0 software. The mean diameter was determined for at least 500 of the microparticles using the free Scion Image software (www.sciocorp.com).

2.5. Resistance of cross-linked microparticles to simulated gastrointestinal conditions

Rehydrated cross-linked microparticles (100 mg of dried particles plus 1 mL of deionized water, 15 min) were submitted to an in vitro gastrointestinal simulation with pepsin and pancreatin (Bermejo et al., 2002). These samples were incubated in 1 mL of simulated gastric medium (pepsin solution; 2.65 U/mg of protein) in 0.1 M HCl, adjusted to pH 1.2, for 2 h at 37 °C in a water bath with constant agitation. The solution was then neutralized with 1.5 M $NaHCO_3$ at pH 7.0, and 1 mL of intestinal medium (0.15 mg pancreatin/mL of 0.1 M $NaHCO_3$) was added. Incubation for 4 h at 37 °C with agitation in a water bath was followed by immersion in an ice bath to stop the action of the enzyme. The samples were observed using an optical microscope, as described above.

2.6. Release in simulated gastric medium

To verify the release of the core material under simulated gastric conditions, freeze-dried microparticles were incubated as described above in a solution simulating gastric conditions (pepsin, pH 1.2). After reaching the time of the simulated gastric assay, sunflower oil (20 mL) was added as a solvent to remove the oily core from the aqueous simulated gastric medium, and the tube was manually stirred for 3 min. The contents of each tube were filtered using glass wool and centrifuged at 3500 rpm for 10 min. The absorbance of the supernatant was measured at 460 nm using a spectrophotometer, and the quantity of core material released was estimated using the standard curve. To measure the amount of core material released under simulated gastric