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β-Lactoglobulin tablets as a suitable vehicle for protection and intestinal delivery of probiotic bacteria

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ABSTRACT

The use of succinylated β-lactoglobulin as a novel functional tablet excipient for the protection of probiotic bacteria against the adverse gastric conditions and their delivery in the intestine was studied. Tablets were produced by direct compression of a dry mixture of *Bifidobacterium longum* HA-135 and the tested excipient. The results showed that tablets made of native β-lg did not ensure cell survival while grafting carboxylic acid groups on the protein revealed to be an innovative method to create a gastroresistant matrix that could allow the survival of up to 10^8 CFU and 10^7 CFU after 1 h and 2 h gastric incubation, respectively. When compared to other polymers, succinylated β-lg promoted the best survival both upon compression and after simulated gastric passage. The proportion of succinylated β-lg in the formulation could be lowered to 60% without modifying the protective ability of the matrix. Additionally, the tablets proved to be stable over a period of 3 months when refrigerated. Succinylated β-lg tablets are an interesting vehicle for the protection of acid-sensitive bacteria during transit in the upper gastro-intestinal tract.

1. Introduction

Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host, as defined by the Food and Agriculture Organization and the World Health Organization (FAO/WHO, 2006). Since Metchnikoff (2004) first exposed his thoughts on the benefits of ingesting specific bacterial strains a hundred years ago, probiotics have made their way through the nutraceutical sector to become a multibillion dollars a year market (BCC Research, 2008). Global interest is linked to the numerous studies that have attributed, and continue to attribute, several health benefits to probiotic-containing products. These beneficial actions range between the alleviation of lactose intolerance, the reduction of symptoms caused by viral and antibiotic associated diarrhea, the modulation of the immune system, the prevention of inflammatory bowel disease and the reduction of risks associated with mutagenicity and carcinogenicity (Fooks et al., 1999; Saxelin et al., 2005; Vasiljevic and Shah, 2008).

However, to confer such health benefits to the host, probiotic microorganisms must be viable when they reach their site of action, meaning that they must survive the adverse conditions encountered in the upper gastro-intestinal tract (Mattila-Sandholm et al., 2002; Del Piano et al., 2006; Ding and Shah, 2007). One of the main issues in probiotic research is therefore to develop strategies to ensure survival during gastric transit and during storage of the product containing beneficial microorganisms. The design of tablets made of functional polymers to improve the stability and survival of probiotics is currently gaining attention, as they allow accurate dosage, ease of administration, good patient acceptance, stability upon storage and large-scale production (Klayraung et al., 2009). Different polymers have been studied to form the protective matrix, namely sodium alginate in combination with hydroxypropylcellulose (Chan and Zhang, 2002, 2005), carboxymethyl high amylose starch (Calinescu et al., 2005), carboxymethyl high amylose starch in combination with chitosan (Calinescu and Mateescu, 2008), hydroxypropylmethylcellulose acetate succinate (Stadler and Viernstein, 2003) and hydroxypropylmethylcellulose phthalate (Klayraung et al., 2009). In all cases, the tablets had the capacity to maintain their physical integrity in gastric fluid, minimize the penetration of solvent at acidic pH values and permitted some survival of the encapsulated bacteria.

Our research group recently demonstrated the effective use of succinylated food proteins, including β-lactoglobulin, as natural tablet excipients to protect acid-labile compounds from the gastric environment and to delay the release of active molecules (Caillard et al., 2009, 2011). β-Lactoglobulin is the main component of whey proteins. Thus, β-lactoglobulin constitutes a natural and largely available material. Moreover, β-lactoglobulin is a co-product from dairy industry that makes it a low cost biopolymer. Finally, because

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of its dairy origin, β-lactoglobulin seems well adapted to the protection of dairy bacteria such as probiotics.

The aim of the present work was therefore to extend the use of these novel excipients to the protection of probiotic microorganisms during the gastrointestinal transit. To achieve this, matrix-type tablets made of succinylated β-lactoglobulin and freeze-dried *Bifidobacterium longum* were characterized regarding their processability, their ability to maintain bacterial viability during gastric incubation and their stability in time. *B. longum* was chosen because of its sensibility to gastric pH (Boylston et al., 2004). Moreover, *B. longum* is a well recognized probiotic and is one of the 16 species eligible for probiotic claims in Canadian regulation (Health Canada, 2009).

2. Materials and methods

2.1. Materials

Freeze-dried *B. longum* HA-135, the model probiotic bacterium used in the present work, was kindly provided by Harmonium International (Mirabel, QC, Canada). BioPURE β-lactoglobulin (β-lg) isolate was donated by Davisco Foods International (Le Sueur, MN, USA). MRS agar, according to de Man, Rogosa and Sharpe, and cysteine hydrochloride used to prepare the culture media were purchased from EMD Chemicals (Gibbstown, NJ, USA) and Sigma–Aldrich (St. Louis, MO, USA) respectively. Pepsin from porcine gastric mucosa and other chemicals used for dissolution experiments, peptone water, sodium alginate and succinic anhydride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pectin DE35 was obtained from CP Kelco (Lille Skensved, Denmark) and hydroxypropylmethylcellulose acetate succinate (AQOAT) was given by Shin-Etsu Chemical Co. (Tokyo, Japan). Alginate (Alginic acid sodium salts) from brown algae was purchased from Sigma Aldrich (St. Louis, MO, USA). Gelatin gelcaps were bought from a local pharmacist.

2.2. Succinylation of β-lactoglobulin

β-Lactoglobulin was succinylated at levels of 50% and 100% using succinic anhydride according to the method of Caillard et al. (2009, 2011) for soy proteins with slight modifications. Briefly, succinic anhydride was added gradually to a 10% β-lg solution maintaining the pH value between 8.0 and 8.5 using 2 M NaOH. Once all the succinic anhydride was added and the pH stabilized, the solutions were dialyzed overnight at 4 °C using 1 kDa membranes. The solutions were finally freeze-dried and ground to a fine powder.

2.3. Tablet preparation

Tablets with a constant weight of 400 mg were prepared by direct compression of a homogeneous mixture of excipient and freeze-dried *B. longum* using a Carver press equipped with a 13 mm diameter flat-faced punch (Autopellet Laboratory press, Carver Incorporation, Wabash, IN, USA). All the punch pieces were disinfected with 70% ethanol before each tablet was produced. Unless otherwise specified, all tablets were formed at an applied pressure of 67 MPa.

2.4. Dissolution media

Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the US pharmacopoeia (The United States Pharmacopoeial Convention, 2004). SGF consisted of 2 g NaCl dissolved in 993 mL HPLC grade water and 7 mL 37% hydrochloric acid (final pH of 1.2). 3.2 g (924 units/mg of protein) pepsin were added to 1000 mL of SGF. SIF was prepared by dissolving 6.8 g monobasic potassium phosphate in 250 mL HPLC-grade water to which were added 190 mL 0.2 M sodium hydroxide and 400 mL HPLC grade water. The pH was adjusted to 7.5 ± 0.1 with 0.2 M NaOH and the final volume was brought to 1000 mL with HPLC grade water. Dissolution steps in SIF were conducted without the addition of pancreatin as it did not influence cell viability. Preliminary experiments showed that incubation of *B. longum* in SIF with ($10\,g\,L^{-1}$) or without pancreatin resulted in a difference of 0.1 log in the number of viable cells, a difference that proved to be statistically non-significant.

2.5. Bacterial mortality during tabletting

To determine the relationship existing between the applied pressure and bacterial mortality, 400 mg tablets composed of 10% freeze-dried *B. longum* and 90% native β-lg were formed at pressure values ranging from 30 to 300 MPa. After compression, the tablets were immediately dissolved in 100 mL simulated intestinal fluid. The resulting suspension was serially diluted in 0.1% sterile peptone water and 1 mL of each dilution was plated in duplicate in MRS agar supplemented with 0.05% cysteine hydrochloride (MRS-C). The Petri dishes were incubated for 48 h at 37 °C in a 2.5 L AnaeroJar (Oxoid Ltd, Hampshire, UK) using an AnaeroGen sachet (Oxoid Ltd, Hampshire, UK) to create an anaerobic environment. The colony forming units (CFU) were finally enumerated.

The initial number of CFU in 40 mg lyophilised *B. longum* was determined by following the same procedure without compressing the powder mixture.

2.6. Survival to gastric conditions

Tablets made of 360 mg native β-lg, 50% succinylated β-lg or 100% succinylated β-lg and 40 mg lyophilized *B. longum* were incubated in 400 mL simulated gastric fluid with pepsin at 37 °C for 30, 60 or 120 min. An incubator shaker (Lab Line Instruments Inc., Melrose Park, IL, USA) was used to promote a constant agitation of 160 rpm. Immediately after gastric incubation, the tablets were transferred to 25 mL simulated intestinal fluid and dissolved. To determine the number of viable bacteria remaining after gastric passage, culture and enumeration were conducted as described previously. If the tablet was completely dissolved during gastric incubation, no transfer to SIF was possible and the SGF was used as the mother suspension for the following steps.

As a control, 40 mg non-compressed freeze-dried *B. longum* was treated following the same procedure.

2.7. Comparison to other excipients

Other polymeric excipients were studied regarding their ability to prevent bacterial mortality during gastric incubation. The studied dosage forms were tablets composed of 360 mg of sodium alginate, pectin, or AQOAT and 40 mg bacterial lyophilizate, tablets combining 216 mg AQOAT mixed with 144 mg sodium alginate and 40 mg freeze-dried *B. longum* as well as gelatin gelcaps filled with 40 mg freeze-dried *B. longum* and 360 mg methylcellulose. 24 h after tablets preparation, tablets specific volumes were calculated as an approximation of tablets initial porosity. The weight of each tablet was determined and the height of tablets was measured using a Mitutuyo Indicator (Mitutuyo, Japan). Tablet specific volume (V_{sp}) was calculated as follows:

$$V_{sp} = \frac{\pi \times r^2}{w}$$

where w is the tablet weight, h is the tablet height and r is the tablet radius (6.5 mm). Each measurement was performed in triplicate.