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Starch granules as a vehicle for the oral administration of immobilized antigens

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ABSTRACT

Microparticles of diverse compositions are often used as carriers for interesting antigens. In this work, we propose the use of natural microparticulated starch as a vehicle for antigens. The proposed system is composed of raw starch microparticles and a starch-binding domain that when fused to another protein, allows for a stable protein immobilization onto the granule surface. To demonstrate the use of starch as an antigen carrier, a fusion combining fragment C of the tetanus toxin with the starch-binding domain was adsorbed to starch and administered orally to mice in two different doses and, importantly, without the use of any adjuvant. The results showed that the system allows the induction of specific antibodies; moreover mice given this immobilized protein presented a delay in the onset of tetanus symptoms compared to mice administered the non-immobilized protein. The study outlines the viability of this immobilization system as an antigen and protein carrier.

1. Introduction

Carbohydrate-binding modules (CBMs) are auxiliary domains that are usually found in carbohydrate processing enzymes, but in some instances they can be found as independent proteins. The primary role of a CBM is to promote enzyme attachment to the substrate and to increase the substrate concentration at the active site of a catalytic domain (Bolam et al., 1998). Amylases are among the enzymes with CBMs, specifically, they have a starch-binding domain (SBD) that plays a crucial role in raw starch hydrolysis. The lack of an SBD stops the enzyme adsorption to the insoluble substrate, which is a prerequisite for hydrolytic activity (Rodriguez Sanoja et al., 2000).

The adsorption capacity of the SBD has plenty potential for the field of biotechnology. In a previous study, fusions of a novel tandem arranged SBD (SBD_{tag}) and proteins from different microorganisms were constructed. In that work, the SBD_{tag} showed its capacity to immobilize several proteins. It was also used as a tag for protein recovery with only starch as a matrix, indicating that it could be an inexpensive method of recombinant protein purification compared to the currently available technologies (Guillen et al., 2013).

In this work, we propose to use this system as a vehicle for the oral administration of therapeutic proteins or antigens. Using SBD_{tag}, the system immobilizes proteins onto starch granules, with sizes in the microparticle range, instead of using chemical immobilization methods or synthetic micro- or nanoparticles, which are both expensive and polluting plus have a questionable biosafety.

Starch is an abundant polysaccharide in nature; it is economic and has already been approved for many alimentary and pharmaceutical uses; properties that make it an attractive candidate for an oral delivery system of proteins, peptides and other biomolecules; it has the advantages of oral administration and eliminates pain to the patient, while it does not require qualified personnel or sterile materials.

In the present study, the effectiveness of raw starch granules at carrying superficially loaded proteins was demonstrated using the green fluorescent protein (GFP). Furthermore, the fragment C of tetanus toxin fused to the SBD_{tag} and immobilized on starch microparticles ($\mu iTcSBD_{tag}$) or non-immobilized ($niTcSBD_{tag}$) was used as a model antigen in oral administration assays for the induction of specific antibodies.

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2. Materials and methods

2.1. Microbial strains and media

Escherichia coli strain BL-21(DE3) [F^- *ompT gal dcm lon* $hsdS_B(r_B^- m_B^-)$ λ(DE3 (*lacI lacUV5-T7gene1ind1 sam7 nin5*))] used for fusion protein production, was grown in Luria–Bertani medium (1% tryptone, 0.5% yeast extract and 1% NaCl) supplemented with ampicillin (100 μg/mL) for plasmid maintenance.

2.2. Protein production and purification

The fusion proteins of GFP or tetanus toxin fragment C (TetC) with the SBD_{tag} were constructed previously (Guillen et al., 2013). Briefly, GFP and the TetC gene were amplified by PCR from the templates pIRES-hrGFP-1 (Stratagene) and pBR327 + FragBC + COOH (donated by M.S. Munguía, ME), respectively. The amplified fragments were purified from a 1% agarose gel, ligated into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions, and transformed into *E. coli* DH5α by electroporation. The obtained pGEM-T-easy constructs were digested, and the released fragments were ligated to the pQE31-SBD_{tag} that was derived from vector pQE31 (QIAGEN). The constructs were named pQGFPSBD_{tag} and pQTcSBD_{tag}.

E. coli BL21(DE3) cells expressing fusion proteins of SBD_{tag} and GFP (GFPSBD_{tag}) or TetC (TcSBD_{tag}) were grown in LB medium supplemented with ampicillin (100 μg/mL) for 12 h at 29 °C ($O.D._{600} \cong 0.5–0.6$). Protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM, and incubation was continued at 29 °C for 6 h. Cells were harvested (8000 × *g* for 10 min at 4 °C) and washed in 20 mM Na_2HPO_4 at pH 7.4 before being lyzed. The obtained proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). GFPSBD_{tag} and TcSBD_{tag} were identified by Western Blotting with a primary anti-His antibody (Roche) and a secondary anti-mouse immunoglobulin G antibody that was labeled with alkaline phosphatase (Perkin-Elmer Life Sciences), according to the manufacturer's instructions.

TcSBD_{tag} purification was performed by affinity chromatography according to a previously reported protocol (Guillen et al., 2013), employing the SBD as the affinity tag.

2.3. Fluorescence assay with GFPSBD_{tag}

Fifty milligrams of corn starch (SIGMA) were washed three times with cold water and suspended in 300 μL of cold citrate–phosphate buffer at pH 5.0. A previously obtained lysate of *E. coli* cells expressing GFPSBD_{tag} was added to the starch suspension and incubated on ice for 30 min, protected from light with gentle stirring. After incubation, the starch was washed three times with the same buffer and analyzed by fluorescent microscopy using an Olympus BX-51 microscope and an Olympus DP70 camera. As a control, an *E. coli* lysate containing GFP without the SBD_{tag} was also incubated with starch granules and treated to the same conditions.

2.4. Antigen adsorption to starch

First, 100 mg of rice starch (SIGMA) was washed twice with 1 mL of cold water and once with 1 mL of 25 mM citrate–phosphate buffer at pH 7.0. Finally, the starch was suspended in 1 mL of the same buffer. The TcSBD_{tag} was incubated O.N. with the starch in a protein–starch ratio of 1:50. TcSBD_{tag} without starch, as well as the vehicle control, received the same treatment. Adsorption of TcSBD_{tag} to the starch was verified using SDS-PAGE and Western Blotting.

2.5. Fusion protein administration schedule

Female BALB/c mice, 6–8 weeks old, were orally administered with 0.2 mL of either 25 or 75 μg of μiTcSBD_{tag} or niTcSBD_{tag}. Some mice were only given starch microparticles as a vehicle control. In all cases, the treatments were administered *via* the gastric route with a feeding needle (Kent Scientific Corp.). In the administration protocol used in this study, the mice received three doses, with a week-long interval between each dose, and received a boost three weeks after the last administration. The use of an adjuvant was omitted to avoid a false positive response.

2.6. Sample preparation

The systemic antibody response was determined by measuring the IgG level in serum. Individual blood samples were taken before and after the gastric administration of the antigen. Blood samples were taken from the tail vein and centrifuged at 8000 rpm for 10 min. Serum was recovered and stored at −20 °C until its use (Wikingsson & Sjöholm, 2002).

2.7. Humoral response evaluation

Fusion protein TcSBD_{tag} was transferred to PVDF membranes for immunoblot assays. Individual membrane lanes containing the protein were cut, and each lane was incubated for 1.5 h at room temperature with serum from the immunized and control groups; the serum was in a 1:1000 dilution in PBS buffer (pH 7.2), Tween 0.05% (PBS-5T) and 3% dry milk. After incubation, the membrane strips were washed in the same buffer three times for 5 min each, and then they were incubated again for 1.5 h with goat anti-mouse IgG alkaline phosphatase conjugate (Invitrogen) diluted in the buffer to a ratio of 1:2500. The membranes were washed three more times, and substrate BCIP/NBT (Invitrogen) was added and incubated with them at room temperature until a signal appeared.

To compare the IgG response, ELISA assays were performed. Costar® 96-well EIA/RIA plates were coated with 100 μL of 10 μg/mL TetC in 0.05 M carbonate buffer at pH 9.6 and incubated over night at 4 °C. The wells were washed three times with PBS-5T; the plates were then blocked with 3% nonfat dried milk in PBS-5T for 30 min at room temperature, and the washes were repeated. The immune and control sera were diluted to a ratio of 1:500 in PBS-5T, and 100 μL of sample was added to each well. The plates containing samples were incubated for 2 h at 37 °C, followed by a wash step. As a secondary antibody, 100 μL of anti-mouse IgG alkaline peroxidase (Invitrogen) was used at a 1:1000 dilution in PBS-5T, and the plates were incubated for 2 h at 37 °C. The plates were washed again and incubated for 30 min at room temperature with the FAST OPD substrate (SIGMA), and the absorbance at 450 nm was determined on a Multiskan FC (Thermo Scientific). The data were analyzed with a two factor (time and treatment) ANOVA using GraphPad Prism 4 software.

For IgG anti-TetC quantification, the procedure described above for ELISA assays was followed and a standard curve was elaborated using serial dilutions from 1/50 to 1/3200 of pure mouse IgG antibody (Roche), with a stock concentration of 1 mg/mL. The optical density values obtained from the sera of treated mice were extrapolated along the standard curve to determine their antibody concentrations.

2.8. Challenging mice with tetanus toxin

Between 0.25 and 50 ng/mouse of commercially available tetanus toxin (SIGMA) was injected subcutaneously in a volume no greater than 30 μL to non-immunized mice to experimentally

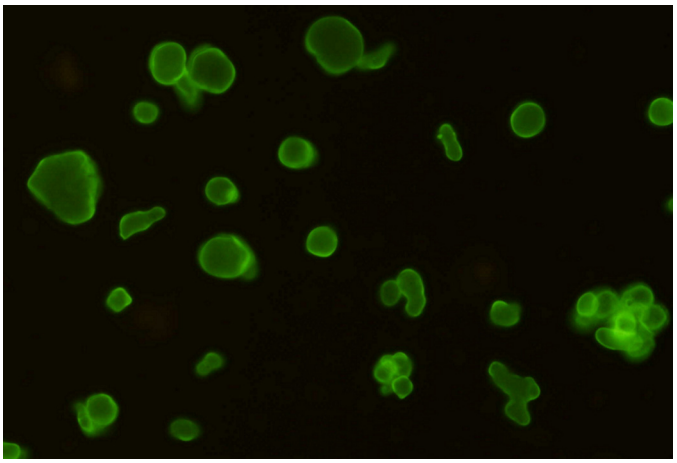

Fig. 1. An image at 40× magnification of starch granules fluorescing after being incubated with *E. coli* lysate containing the fusion protein GFPSBD$_{tag}$. The fusion protein is able to adsorb to the starch granule and retain its fluorescence capacity.

obtain the LD_{100}. The lethal dose was established as the tetanus toxin concentration at which 100% of mice were dead within 48 h.

Seven days after the last fusion protein administration, the treated animals were subcutaneously injected with the determined LD_{100} amount of tetanus toxin. The animals were observed closely for signs of paralysis or lameness, and mice that developed symptoms were euthanized. In all of the experiments, the animals were treated according to the accepted protocol by the institution's Animal Care and Use Committee.

3. Results

To assess the starch-binding faculty of the SBD$_{tag}$ in GFPSBD$_{tag}$, an *E. coli* lysate containing the fusion protein was incubated with starch granules and washed to avoid unspecific protein binding. When the starch granules were observed under a microscope, an image of fluorescing starch granules was obtained, denoting that the fusion protein had adsorbed to the starch granule (Fig. 1).

In the case of the fusion protein TcSBD$_{tag}$, adsorption to the starch was verified by Western Blotting. The protein was detected in the starch pellet fraction, whereas the supernatant did not reveal any visible signal, indicating that the protein was completely adsorbed onto the starch (results not shown).

Immunoblotting using the complete fusion protein TcSBD$_{tag}$ was performed on the sera collected on day 36 from the administered groups. Although the technique is not as sensitive as ELISA, it is a fast way to qualitatively verify the induction of specific antibodies. When TcSBD$_{tag}$ was administered orally, the presence of the specific antibody was favored in the group that received the higher dose of μiTcSBD$_{tag}$, followed by the group given 25 μg of μiTcSBD$_{tag}$. The humoral response in the group administered 25 μg of niTcSBD$_{tag}$ was not detectable by immunoblot assay, and sera from the control animals, who were administered only starch microparticles, did not show any cross reaction against the fusion protein (Fig. 2).

ELISA assays are more sensitive than immunoblotting and were performed to quantify and compare the humoral specific responses against the target protein TetC. The ELISA results denoted a good response against TetC at the end of the administration protocol, particularly for the group given 75 μg of μiTcSBD$_{tag}$. That group also mounted the best IgG specific response against TetC (Figs. 3 and 4).

It was observed that the mice immunized with 25 and 75 μg of μiTcSBD$_{tag}$ showed the fastest humoral response. Their responses began in just 15 days while the groups receiving the niTcSBD$_{tag}$ did not begin showing a response until day 36, without ever reaching

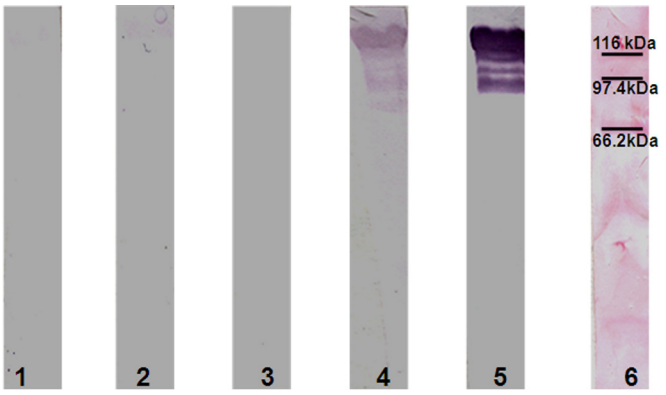

Fig. 2. An immunoblot with the sera of the different groups administered. 1: 25 μg of niTcSBD$_{tag}$; 2: 75 μg of niTcSBD$_{tag}$; 3: the control, given only starch microparticles; 4: 25 μg of μiTcSBD$_{tag}$; 5: 75 μg of μiTcSBD$_{tag}$; and 6: MWM.

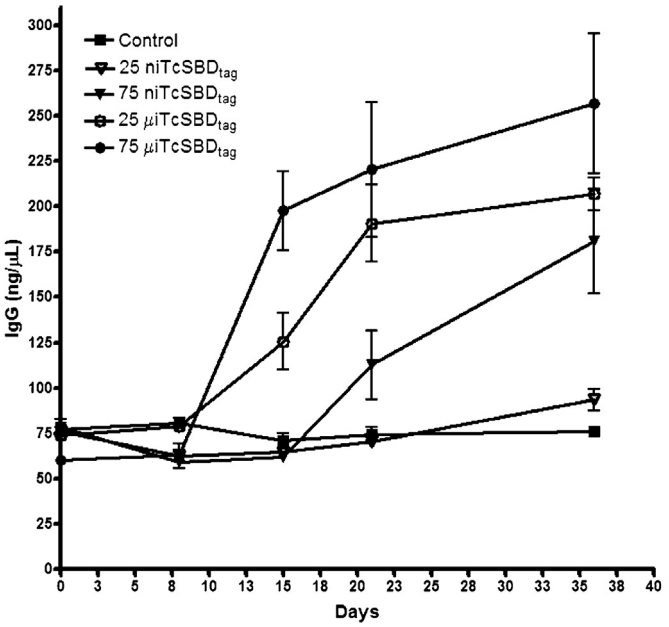

Fig. 3. Evaluation of the specific IgG against protein TetC in the mice immunized by single doses of μiTcSBD$_{tag}$ or niTcSBD$_{tag}$ with seven-day intervals between each administration.

the IgG levels obtained with the same amount of protein adsorbed to the starch granules. All of the mentioned differences were statistically significant, with $p < 0.05$.

To test the biological activity of the observed responses, the orally immunized mice were challenged subcutaneously with a lethal dose of tetanus toxin that was experimentally established (12.5 ng/mouse). A delay in symptom development was observed when the groups were treated with μiTcSBD$_{tag}$ when compared to the control group, which received only starch. The control group showed clear signs of tetanus within 36 h after a subcutaneous injection of tetanus toxin, while the mice given 75 μg of niTcSBD$_{tag}$ or 25 μg μiTcSBD$_{tag}$ showed a 24 h delay in symptom appearance. The group that received 75 μg of μiTcSBD$_{tag}$ had a larger delay of up to 36 h (Fig. 5).

4. Discussion

After proving that GFP could be immobilized to a starch granule through the SBD and fluoresce, we explored other proteins as fusion partners, aiming to use the starch microparticles as vehicles for

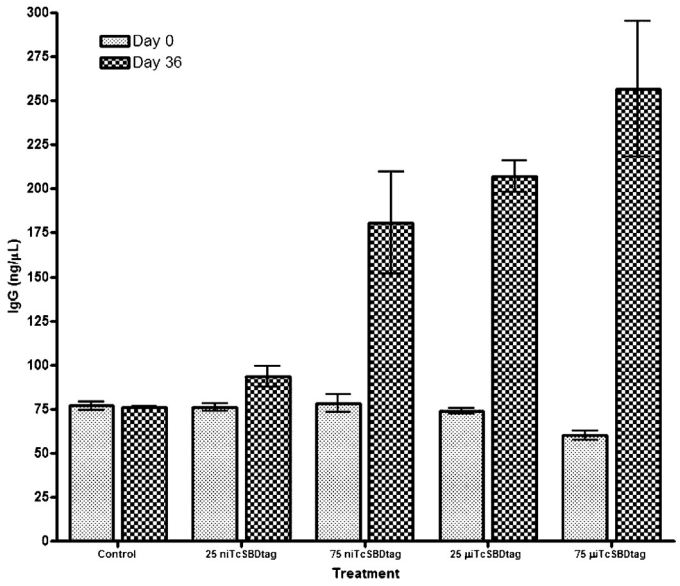

Fig. 4. A comparison of the specific IgG response against antigen TetC at day 0 and day 36 of the dose administration, before the tetanus toxin challenge.

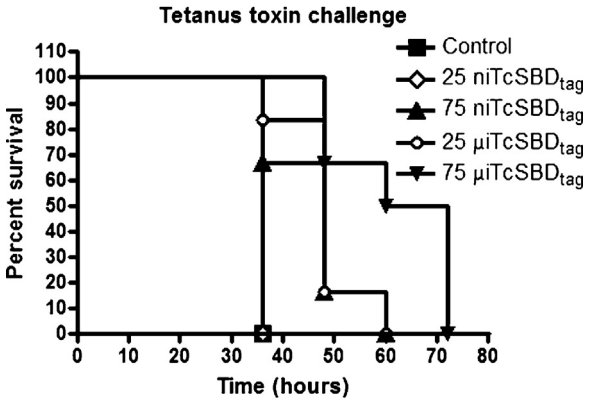

Fig. 5. A comparison of mice survival following a tetanus toxin injection seven days after their last $TcSBD_{tag}$ administration.

mucosal antigen delivery. To prove that the proposed SBD_{tag}-starch system can act as a vehicle for the oral administration of therapeutic proteins and antigens, the induction of specific antibodies against the protein/antigen was evaluated. This induction could indicate that the proteins were properly stabilized and able to reach the sites of immune response induction, located in the gut associated lymphoid tissue (GALT).

Microparticulated systems for antigen delivery enhanced both the antigen's stability in the gastrointestinal tract and the capture of the desired proteins or peptides (O'Hagan, 1998). Frequently, these systems use chemical cross-linking to attach or trap proteins to the microparticle's surface (Andrianov & Payne, 1998). We used a SBD_{tag} to bind an antigen to natural starch granules, thus avoiding chemical reagents or conditions that could affect the immunogenicity of an antigen (Andrianov & Payne, 1998; O'Hagan, 1998; Rajapaksa & Lo, 2010). Therefore, we are proposing this starch complex as a simple and inexpensive carrier of peptides or proteins of interest.

The starch granule, loaded with the fusion protein $TcSBD_{tag}$, was used as a carrier for intragastric TetC delivery. Oral administration must manage two important aspects to succeed. First, the antigens must pass through the barriers of the digestive tract (containing proteases and at a pH range of 1–4) to reach the intestinal environment. Second, the antigens must be captured by specialized cells located in the intestinal epithelium and transported to the GALT, where the antigen presenting cells and other immune system cells are located (Andrianov & Payne, 1998; O'Hagan, 1998; Wikingsson & Sjöholm, 2002). The harsh conditions of pH and proteases could be detrimental to the fusion proteins or their adsorption to starch affecting thus the antigen delivery, however, preceding work had already demonstrated that proteins fused to the SBD_{tag} and previously immobilized onto the surface of starch granules present improved stability when incubated *in vitro* with gastric and intestinal fluids, compared to the same proteins in soluble form, even using the simulated fluids that usually result in more drastic conditions than those presented in natural gastric or intestinal fluids (Moreno-Mendieta, Guillén, Sánchez, Espitia, & Rodríguez-Sanoja, submitted for publication).

Regarding antigen capture, microparticles up to 10 μm in diameter are transported by specialized M cells from the intestinal epithelium and remain into the Peyer's patches, while microparticles up to 5 μm can be transported through the efferent lymphatics to the other mucosal associated lymphoid tissue (MALT) and the spleen (Eldridge et al., 1990). Depending on the botanical source, starch granules have different sizes. In this study, rice starch was selected as the matrix for fusion protein adsorption and delivery. The average size of the rice starch granule is 4–5 μm (Hossen et al., 2011), a dimension that allows effective capture and transportation to MALT.

The immune response can also be affected by factors such as the nature of the vehicle, the use of an adjuvant, the antigen dose and the administration protocol (Raghuvanshi et al., 2002). To evaluate the ability of the system to withstand the low gastric pH and intestinal enzymes, which allows the bound protein to reach the specialized cells responsible for eliciting an immune response in the intestinal epithelium, different protein doses were employed using a natural microparticulated substrate. No adjuvant was used because its addition could enhance the antibody response and then the measured induction effect could be unrelated to the system's ability to carry the antigen.

With the administration protocol used in this study, we observed that the mice receiving the larger dose of starch-adsorbed antigen (75 μg of $\mu iTcSBD_{tag}$) developed the most important response. Doses of 25 μg of $\mu iTcSBD_{tag}$ and 75 μg of $niTcSBD_{tag}$ elicited similar IgG responses against TetC. However, the group that was administered 25 μg of $niTcSBD_{tag}$ did not develop any response against the antigen. This result indicates that the devised system allows the antigen to be recognized and presented to MALT.

The humoral response enhancement is also related to the stability conferred by the protein's immobilization and the size of the particle. In this instance, the starch granules loaded with $TcSBD_{tag}$ allowed and facilitated antigen capture in the sites of immune induction. Another important factor is the mucoadhesive properties of natural starch since bioadhesive polymers increase the residence time (Saraswathi, Balaji, & Umashankar, 2013). In this particular case, starch may retard the clearance time in the intestine, allowing the microparticles to be more effectively captured, with the advantage of using a natural substrate that do not have adverse effects on the mucosal tissue that other synthetic systems do (Adriaens, Ameye, Dhondt, Foreman, & Remon, 2003).

The mice that were challenged with lethal doses of tetanus toxin responded differently depending on their received treatment. The animals given $\mu iTcSBD_{tag}$ developed symptoms later than the mice administered $niTcSBD_{tag}$ or starch alone. These results are related to those observed in the ELISA assays, in which the group that presented the greatest increase in specific IgG production (75 μg $\mu iTcSBD_{tag}$) also showed a delay in intoxication.

Although no total protection against the toxin was observed, it is remarkable that the system stimulated some protection, as shown by the delay in symptom development. No further assumption may

be made regarding the degree of protection because the mice were euthanized just after the first symptoms occurred. No adjuvant was used in this study, as opposed to other systems tested for the generation of a protective response against tetanus, which besides the use of adjuvants; use more responsive immunization routes, such as the subcutaneous route (Charles et al., 1991; Fairweather, Lyness, & Maskell, 1987). Regarding oral administration systems, live microorganisms such as *Salmonella*, *Lactobacillus* strains and *Bacillus subtilis* spores have been tested as potential carriers (Abd El Ghany et al., 2007; Duc, Hong, Fairweather, Ricca, & Cutting, 2003; Reveneau, Geoffroy, Locht, Chagnaud, & Mercenier, 2002), with good antibody response induction against TetC; although no tetanus challenge has been performed (Abd El Ghany et al., 2007; Reveneau et al., 2002). Regrettably, one of the problems encountered is the need to use attenuated strains when pathogens are employed as carriers. Additionally, there are safety concerns with the release of genetically modified strains into nature, even strains considered GRAS.

Non-living vehicles have also been tested to induce a humoral response against tetanus; for example, chitosan microparticles demonstrated that the oral administration of microparticles with immobilized antigens is an adequate strategy to induce a good humoral response (Ahire, Sawant, Doshi, & Ravetkar, 2007). Furthermore, microparticles generated with acryloylated starch and chemically conjugated to human serum albumin have been reported to induce a strong humoral response in mice after oral administration (Wikingsson & Sjöholm, 2002).

Concerning the efficiency of different systems and immunization routes to generate protection against tetanus, in recent years, lots of work has been done specially with microorganisms expressing TetC with different results obtained. The efficiency of the systems depend of factors like the immunization route which includes the intranasal (Lee et al., 2010; Reveneau et al., 2002), sublingual (Amuguni et al., 2011), subcutaneal (Reveneau et al., 2002) and intragastric (Grangette et al., 2002; Lee et al., 2010; Reveneau et al., 2002), although immunization by different routes was able to induce an immune response, for intragastric administration no reproducibility has been reported (Lee et al., 2010) or the need of high cell quantities (10^8–10^9) of high expression TetC strains to achieve antibody induction (Grangette et al., 2002; Reveneau et al., 2002).

Biodegradable particles, of Poly lactide-co-glycolide (PLGA) and polylactide (PLA), entrapping tetanus toxoid (TT) were efficient to induce antibodies when they were administered to rats intramuscularly with an adjuvant (Raghuvanshi et al., 2002), however the protection capacity of these antibodies was not reported. Subcutaneously administration of TT-PLGA microspheres with trehalose or TT-chitosan microspheres with trehalose to mice and guinea pigs also induced antibodies when the adjuvant alum was employed (Alonso, Gupta, Min, Siber, & Langer, 1994; Jaganathan et al., 2005). As far as we know, there are no works that demonstrate any degree of protection to tetanus by oral or intragastric administration of TetC.

Finally, comparing the various systems available, the set-up proposed in this work simplifies antigen immobilization and eliminates the need for a microparticle production process, thus reducing the production costs. In addition, because starch is part of the daily diet and is a non-immunogenic substrate, reduces the background response that could be elicited against the vehicle, as occurs with genetically modified living organisms.

5. Conclusions

The efficiency of starch granules as vehicles for protein delivery *via* a starch-binding domain was demonstrated. The system allows for and facilitates antigen capture in the sites of immune induction, as measured by the humoral response induction. In addition, the system allows the fusion proteins to reach the intestinal environment, which suggests that the system could be used not only as a vehicle for antigens but also as a carrier for therapeutic proteins and peptides to benefit gastrointestinal health, a perspective that is currently under evaluation.

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