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Research paper

Encapsulation of Cwp84 into pectin beads for oral vaccination against *Clostridium difficile*

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ABSTRACT

We have designed an oral vaccine against *Clostridium difficile* infection. The virulent factor Cwp84, that is a cystein protease highly immunogenic in patients with *C. difficile*-associated disease, was entrapped within pectin beads. Beads encapsulating Cwp84 were shown to be stable in the simulated intestinal medium and to release the cystein protease once in the simulated colonic medium. Three groups of hamsters were immunized, the first receiving pectin beads encapsulating Cwp84, the second unloaded beads and the third one free Cwp84. After three immunizations by the intragastric route, all groups received clindamycine. Post-challenge survival with a strain of *C. difficile* showed that 2 days after infection, all hamsters treated with unloaded beads and all hamsters treated with free Cwp84 have deceased after 7 days, whereas about 40% of hamsters administered with Cwp84-loaded beads survived 10 days after challenge, proving that oral vaccination provides partial protection. These first data obtained with an oral vaccine against *C. difficile* appear promising for preventing this infection.

1. Introduction

Clostridium difficile is a Gram-positive anaerobic bacterium responsible for many nosocomial infections. The spectrum of diseases caused by *C. difficile* infection varies from antibiotic-associated diarrhoea to life-threatening clinical manifestations such as pseudomembranous colitis [1]. Antimicrobial therapy is the main risk factor responsible for the infection. Indeed, in susceptible individuals, disruption of the normal colonic microbiota by antibiotic treatment leads to colonization by *C. difficile* [2]. Pathogenicity of *C. difficile* is mediated by the release of two exotoxins, toxins A and B, which are potent cytotoxic enzymes inducing severe damages to the human colonic mucosa [3]. Other factors such as proteases were recently shown to be involved in the intestinal damages by degradation of the basement membrane which contributes to the intestinal epithelium necrosis. Among these factors, Cwp84 is *a C. difficile* surface protein that has recently been shown to possess cysteine protease activity and which may have such behaviour [4,5].

The current treatment regimens as well as the important rate of relapse make the eradication of the organism from infected patients difficult to achieve. In addition, the increasing prevalence of patients with *C. difficile* in elderly care wards or nursing homes suggests that a more rational strategy would be to design a vaccine against the infection. This is the reason why several studies have focused on the use of toxin preparations to stimulate active immunization in animal models infected by *C. difficile* inducing the secretion of serum antibodies against toxins [6–8]. In healthy volunteers, this vaccine induced high levels of specific neutralizing IgG. Initial studies have been conducted with promising results in a few patients with recurrent CDI [9]. However, if a toxin-based vaccine prevents toxin binding and neutralizes inflammatory effects, it is unable to affect colonization. This type of strategy therefore does not allow preventing patient-to-patient transmission. More recent studies have shown that surface proteins from *C. difficile*, such as flagellar proteins: FliC, FliD and the Cwp84 protease were able to induce an immune response that could play a role in the host defense mechanism [10,11]. The possibility of mucosal immunization against *C. difficile* by intra-nasal, rectal and oral route through the administration of FliD, and Cwp84 was tested [12]. Only the rectal route was able to induce an increase in IgA antibody specific to *C. difficile*. Although the rectal route is interesting, an orally administered vaccine would be vastly superior because of the increased patient compliance, patient

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comfort and low cost. Unfortunately, delivery of vaccine antigens by the oral route is plagued with challenges such as gastrointestinal destruction of labile molecules, poor immunogenicity of orally delivered soluble antigens and inefficient uptake of the antigens [13–16]. Much of the research directed at overcoming these barriers has focused on the development of microparticles containing antigens, as oral vaccine delivery vehicles [17]. Biocompatible and biodegradable polymers, either natural or synthetic, have become popular as materials for encapsulation. Moreover, since the lower GI-tract may provide an advantageous absorption site for such molecules and may induce an effective immune response in animals [18,19], it was interesting to deliver the above-mentioned virulence factor of *C. difficile* Cwp84 to the colon. Since the development of the *C. difficile* infection is localized at the colonic level, a colonic immune response could be appropriate to limit the development of the infection. Several colon-specific drug delivery systems are based on an enzyme-triggered degradation of the polymer they are made of [20–22]. Among these, pectin, a polysaccharide naturally present in plant cell walls have clearly shown interesting potentialities for protein encapsulation and delivery to the colon [23–26]. Indeed, pectin is non-toxic, not digested by gastric or intestinal enzymes and almost totally degraded by pectinolytic enzymes produced by the colonic microbiota. Low methoxylated pectin, amidated or not, can form a gel in the presence of divalent cations, such as calcium or zinc [25–31]. Beads can therefore be obtained by ionotropic gelation in very mild conditions favourable to the encapsulation of proteins [26,32,33], such as the *C. difficile* protease Cwp84 and afterwards to deliver it to the colon, promoting a vaccination against *C. difficile*. The aim of this study was to confirm the suitability of the chosen matrix to deliver the protein to the colon and to evaluate Cwp84, encapsulated into pectin beads as an oral vaccine candidate against *C. difficile* in a hamster model.

2. Materials and methods

2.1. Materials

Infusion broth was obtained from Difco Laboratories (France). Colombia agar plates supplemented with 4% horse blood were obtained from Biomérieux (France). Unipectine™ OG 175C (degree of esterification from 22% to 28% and degree of amidation from 19% to 23%) was a gift from Cargill (France). All the following compounds were purchased from Sigma–Aldrich (USA): Zinc acetate, Calcium chloride, Azocasein, EDTA, Sodium Citrate, NaCl, HEPES, Pepsine, Pancreatin, the mixture of pectinases from *Aspergillus aculeatus*, di-sodium hydrogen phosphate, sodium di-hydrogen phosphate, 5-(6)-carboxy-X-rhodamine *N*-succinimidyl ester, bovine serum albumin (BSA), rabbit anti-hamster IgG, IgM, IgA (H&L) conjugated to biotin (Biovalley), 3,3′-5,5′ tetramethylbenzidine (TMB), ammonia buffer solution and 1-(2-pyridylazo)-2-naphthol (PAN). TRIS was provided by from Prolabo (France), and hydroxy methylcellulose phthalate (HPMCP), type HP55S, was a gift from SEPPIC (France). Acetonitrile and ethanol were purchased from Carlo Erba Reagents (Italy), the Bradford reagent from BioRad (France), streptavidin-horseradish peroxidase conjugate from Amersham and Maxisorp™ 96-well microtiter plates from Nunc (France). All other chemicals used were of reagent grade.

2.2. Bacterial strain and culture

The *C. difficile* toxigenic strain 79–685 was isolated from a patient affected by pseudomembranous colitis and was a gift from the Department of Microbiology of the University of Strasbourg, France. This strain was grown under anaerobic conditions in Tryptone Glucose Yeast (TGY) infusion broth (Difco Laboratories, France) at 37 °C for 24 h unless indicated otherwise, and onto Columbia agar plates supplemented with 4% horse blood (Biomérieux, France). The *Escherichia coli*/pET-28a(+)_*cwp84* strain was grown on LB (lysogeny broth) agar or in broth (Difco Laboratories, France) supplemented with 50 μg/mL kanamycin to maintain the pET plasmid.

2.3. Purification of recombinant protein Cwp84

Recombinant Cwp84 was purified as previously described [10]. Briefly, Cwp84 was obtained from the *E. coli*/pET-28a(+)_*cwp84* clone by the induction of protein expression with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) (Sigma–Aldrich, France) and subsequent purification by single-step affinity chromatography employing BD TALON cobalt affinity resin (BD Biosciences, France) as described in the protocol supplied by the manufacturer. The eluted fraction containing the recombinant protease was dialysed overnight against phosphate-buffered saline and then frozen at −20 °C for storage. The molecular weight of Cwp84 is 84 kDa [4].

2.4. Cwp84 fluorescent labelling

Five milligrams of 5-(6)-carboxy-X-rhodamine *N*-succinimidyl ester, dissolved in 300 μL of acetonitrile, were added to the purified protein solution (phosphate buffer 0.1 M pH 8.5) and kept under magnetic stirring at 4 °C for 2 h [34]. The excess of reagent was eliminated by dialysis against phosphate buffer, and subsequently, a last dialysis was carried out against TRIS buffer (25 mM pH 7.5) to exchange buffer. The labelled protease was frozen at −30 °C for storage.

2.5. Preparation of pectin beads

Unipectine™, with or without Cwp84 (720 μg in a final volume of 2.7 mL), was dissolved in TRIS buffer (25 mM, pH 7.5) at the concentration of 6% (w/V). After agitation for 2 h at room temperature, the solution was left undisturbed overnight and then dropped from a syringe into a cross-linking solution of Zn acetate (12% w/V), using a syringe pump (Havard Apparatus, 11 PLUS, France) at a rate of 1 mL/min. The nozzle diameter was 0.8 mm and the dropping distance 8 cm. Beads (about 50) formed instantaneously by contact with zinc ions were left in the cross-linking solution for 30 min at room temperature under magnetic stirring. Free zinc ions in excess were removed by filtration and three washings with distilled water for 1 min. Finally, beads were dried at 37 °C for 0, 1, 2 or 3 h.

2.6. Optimization of the formulation

Preliminary studies were carried out to optimize Cwp84 encapsulation within pectin beads. The drying time was investigated mainly because beads stability and protease enzymatic activity could be influenced by the water content and the thermal process. The drying temperature was fixed at 37 °C, and four drying times (0, 1, 2 and 3 h) were tested. Beads were then characterized by thermo-gravimetric analysis, stability in various media, and Cwp84 enzymatic activity was also assayed.

2.6.1. Determination of weight loss upon drying

Beads weight loss during drying was determined by thermo-gravimetric analysis. It was expressed as percentage according to the following equation:

$$\text{Weight loss} = \frac{w_0 - w_t}{w_0} \times 100$$

with w_0 and w_t representing the weight of beads before and after the drying process, respectively, at 150 °C. The water content was determined in triplicate for 4 groups of beads, dried at 37 °C during 0, 1, 2 and 3 h.

2.6.2. Bead stability in simulated digestive media

Characterization of bead stability was carried out in three different media under the following conditions:

- Simulated gastric medium (SGM, NaCl/HCl, pH 1.2, 0.1 M) containing pepsin (0.3% w/V).
- Simulated intestinal medium (SIM, HEPES/NaCl buffer, pH 6.8, 10^{-3} M) containing pancreatin (1% w/V). The medium composition is different from the one indicated in pharmacopoeia and was chosen following to previous studies, performed in our laboratory and specific for the Zn-pectinate beads [30].
- Simulated colonic medium (SCM, HEPES/NaCl buffer, pH 6, 10^{-3} M) containing 1.9 IU/mL of pectinolytic enzymes. A pH of 6 was chosen for colonic medium as a compromise between colonic pH (6.4–7.0) and the optimum pH for pectinolytic enzyme activity (3–5) [29].

In this experiment, 3 beads were placed in test tubes containing 4.5 mL of SGM under oscillating agitation at 650 rpm (Heidolph Tritamax 1000, France) and 37 °C. After 2 h, beads were transferred in the same preheated volume of SIM for 5 h. Subsequently, the medium was replaced by 4.5 mL of SCM for other 5 h of incubation. The amount of intact beads was monitored during incubation, and results were determined in triplicate.

In order to protect beads from exposure to the gastric acid environment, capsules of gelatin (size 9, Harvard Apparatus) were coated with a hydroxypropyl methylcellulose phthalate (HPMC) film. Coating was performed by dipping capsules into a 10% w/V solution of HPMCP in acetone/ethanol (50:50) and drying them at room temperature. The process was reiterated three times. The efficacy of the gastro-resistant coating was checked by a disintegration test according to European pharmacopoeia recommendations (capsules undamaged after 2 h in HCl 0.1 M, $n = 3$).

2.6.3. Enzymatic activity of Cwp84 after encapsulation

The proteolytic activity of encapsulated Cwp84 was quantified with azocasein, after bead degradation in TRIS buffer (25 mM, pH 7.5) containing EDTA (5 mM). Two-hundred microliters of sample were added to 500 μL of azocasein solution (5 mg/mL in TRIS buffer, 25 mM pH 7.5). After 16 h of incubation at 37 °C, intact azocasein was removed by 5% trichloroacetic acid precipitation and the amount of released dye was measured spectrophotometrically at 336 nm. The caseinolytic activity of encapsulated Cwp84 was compared with free protease, assuming a 100% activity. A control was obtained by the same method without the addition of protease to the sample. The drying time (0, 1, 2 and 3 h), the effect of cross-linking agent (Zn^{2+}) and the disintegrating agent (EDTA 5 mM in TRIS buffer) on Cwp84 proteolytic activity were investigated. Experiments were carried out in duplicate.

2.7. Determination of zinc ions content in beads

The amount of residual zinc ions in beads after ionotropic gelation and washings was determined as reported [35,36]. Briefly, beads, dried 3 h at 37 °C, were completely degraded into a dilute pectinase solution (1:25 v/v) at the concentration of 1 mg/mL. After filtration on a 0.22 μm filter, 100 μL of degraded bead solution were placed into 10 mL volumetric flasks, followed by 1 mL of ammonia buffer solution (pH 10), 3 mL of ethanolic stock PAN solution (5×10^{-4} M) and completed with ethanol. Afterwards, samples were shaken and left in a thermostated water bath (RM6 Lauda, France) at 40 °C for 10 min before analysis. The amount of zinc ions, complexed with PAN, was monitored, in triplicate, by UV–VIS spectrophotometry (Shimadzu UV-2101PC, France) at 550 nm. The UV standard absorbance curve for zinc ions was established in the concentration range between 0.02 and 0.30 mg/mL [30].

2.8. Determination of the encapsulation efficacy

Beads were disintegrated in TRIS buffer (25 mM, pH 7.5) added with EDTA (5 mM). The encapsulation efficacy (EE) of Cwp84 was calculated according to the following equation:

$$EE = \frac{\text{Actual Cwp84 content } (\mu g)}{\text{Theoretical Cwp84 content } (\mu g)} \times 100$$

Several methods were used for Cwp84 assay:

(a) The Bradford dye-binding method was applied, and the amount of encapsulated Cwp84 was measured spectrophotometrically at 595 nm (Shimadzu UV-2101PC, France), using BSA as reference protein according to the micro assay protocol of BioRad.
(b) Encapsulated Cwp84 was assayed by spectrofluorimetry (Perkin Elmer, LS50B, France) following the intrinsic tryptophan fluorescence [37]. The excitation wavelength was set at 280 nm and the emission intensity monitored at unitary wavelength of 338 nm. The calibration curve was carried out using a solution of free Cwp84. Samples were centrifuged at 10.000 rpm for 2 min (MiniSpin Eppendorf) prior to measurements.

For both methods, a solution of unloaded beads, degraded in the same conditions as loaded ones, was used for calibration curves.

2.9. Cwp84 release from beads and swelling studies

Standard dissolution methods described in different pharmacopoeias utilize large volume vessels, and therefore, relatively large amounts of dosage forms are needed. In our study, beads could not be prepared in large amounts due to the low amount of Cwp84 produced. Therefore, in order to quantify the protein released in SIM, one bead was placed in a test tube containing 0.8 mL of SIM (HEPES/NaCl, pH 6.8) without pancreatin at 37 °C under oscillating agitation at 650 rpm (for 5 h). One test tube was assigned to each time point. The Bradford method was used to determine both the protein released in SIM and the entrapped one, after bead degradation into 2 mL of TRIS buffer added with EDTA 5 mM. The Cwp84 amount was determined, in triplicate, spectrophotometrically at 595 nm according to the following equation:

$$\text{Cwp84 release } (\%) = \frac{\mu g \text{ released}}{(\mu g \text{ released} + \mu g \text{ entrapped})} * 100$$

Calibration curves were extrapolated using solutions of BSA (reference protein) in SIM and TRIS buffer plus EDTA, respectively. The percentage of Cwp84 released in SIM was also determined by intrinsic fluorescence as described above.

Cwp84 released in SCM (HEPES/NaCl pH 6) could not be determined using the Bradford assay or the intrinsic tryptophan fluorescence, due to pectinolytic enzymes interferences. Loaded beads were therefore prepared using rhodamine-labelled Cwp84, and the amount of Cwp84 released was determined by fluorimetry. In this experiment, after 5 h of incubation in SIM, the medium was

replaced with SCM containing the pectinolytic enzymes at the concentration of 1.9 IU/mL. One test tube was assigned to each time point. At desired time, the supernatant was withdrawn, centrifuged at 10.000 rpm for 2 min, and the amount of Cwp84 was determined by fluorescence.

Bead swelling behaviour was followed by measuring the weight of dry beads after exposure to SIM (HEPES/NaCl pH 6.8), using the same conditions as for the release studies. One test tube was assigned to each time point. Beads were removed at every time point, blotted on filter paper to remove the excess water and weighed. The swelling ratio was calculated by:

$$\text{Swelling ratio} = \frac{w_t - w_0}{w_0} \times 100$$

where w_t is the bead weight at the given time point and w_0 is the initial weight of the dry bead. The swelling ratio was calculated in triplicate and expressed as a percentage.

2.10. Vaccination study

2.10.1. Animals

Adult *Mesocricetus auratus* female hamsters (weight, 80–100 g) were obtained from Janvier Laboratories and were housed in polypropylene isolator cages fitted with filter covers holding disposable polyester air filters. All water, cages, wire lids and filter covers were sterilized before being used. Animals were caged in groups of five during the immunization period and then caged individually during *C. difficile* challenge. All animal studies were carried out according to EC Directive 86/609/EEC for animal experiments.

2.10.2. Preparation of C. difficile spores

Confluent cultures of each strain were grown anaerobically at 36 °C for 5–7 days, on blood/agar plates, to promote spore formation. The cultures were harvested with disposable loops into 10 mL of PBS, were washed in PBS and then were heat-shocked at 56 °C for 10 min, to kill surviving vegetative cells. The spores were centrifuged, resuspended in Dulbecco's modified Eagle medium (DMEM), aliquoted, and frozen at −80 °C. The frozen spores were quantified by 10-fold serial dilutions of the spores onto Columbia agar plates supplemented with 4% horse blood. Spores were diluted in TGY, for orogastric inoculation into hamsters.

2.10.3. Vaccination protocol

An oral regimen of immunization was tested. Three groups of animals were used. Hamsters were anaesthetized by intraperitoneal and intramuscular routes with of a cocktail composed of Ketamin 1000® (1 mL/kg) and Rompun® 2% (0.25 mL/kg). One group (12 animals) was immunized with beads encapsulating Cwp84 (corresponding to about 200 μg of Cwp84 administered), a control group (10 animals) had received similarly unloaded beads and finally a group (6 animals) was immunized directly with 200 μg of the recombinant protein Cwp84 diluted in 400 μL of PBS by gavage (Fig. 1).

All groups received a total of three doses of the vaccine (or control) on days 0, 14 and 30. Fifteen days after the last immunization (d45), the hamsters were administered by gavage clindamycine (Dalacine®) at a single dose of 50 mg/kg to disrupt the barrier microbiota. Five days after (d50), the hamsters were challenged by i.g. administration of 350 spores of *C. difficile* 79–685.

2.10.4. Evaluation of specific antibody level after immunization

To evaluate antibody response in sera, blood samples (200–400 μL) were withdrawn before the first immunization and 15 days after the last immunization. The blood was left to clot for 1 h at room temperature and 3 h at 4 °C. Serum was obtained by centrifugation and frozen at −20 °C until use. Indirect ELISA was used to detect antibodies in the serum. Wells of 96-well microtiter plate were coated with 100 μL of a 3 μg/mL solution of recombinant, purified Cwp84 in 0.1 M sodium carbonate – 0.1 M sodium bicarbonate, pH 9.6 for 1 h at 37 °C and then overnight at 4 °C. Wells were subsequently washed five times with PBS, pH 7.4, containing 0.1% (v/v) Tween 20 (PBS-T). Blocking of remaining sites on the plastic was achieved by an overnight incubation with 1% (w/v) BSA in PBS-T followed by five washings. For detection of IgG, 100 μL of serum samples diluted in PBS-T-BSA were added to the wells and incubated at 37 °C for 30 min. Sample dilutions tested were 1:10; 1:50; 1:100; 1:500; 1:1000; 1:5000; 1:10,000; 1:50,000. After washings, positive reactions were detected by successive incubations with 100 μL of anti-mouse IgG conjugated to biotin (1:25,000 dilution) for 30 min at 37 °C and with a streptavidin-horseradish peroxidase conjugate (1:1000 dilution) for 30 min at 37 °C. The final reaction was visualized by the addition of 3,3′-5,5′ tetramethylbenzidine (TMB, Sigma). After 10 min, the reaction was stopped with 100 μL of 1.2 M H_2SO_4. Absorbance values were measured at 450 nm using a micro plate ELISA (Anthos II, Labtech Instruments).

All samples in this study were tested in duplicate and treated simultaneously to avoid interassay variation. Assays with sera in the absence of antigen served as negative controls.

2.10.5. Statistical analysis

The survival of animals following infection was analysed using Kaplan–Meier estimates. Survival rates across groups were compared using log rank tests. p values of <0.05 were considered to be statistically significant. Statistical analysis was performed using Stata 8.0 (Statacorp, College Station, TX).

3. Results and discussion

The goal of the present work was to encapsulate the Cwp84 protease from *C. difficile*, in order to deliver it to the colon and to induce an immune response by oral administration.

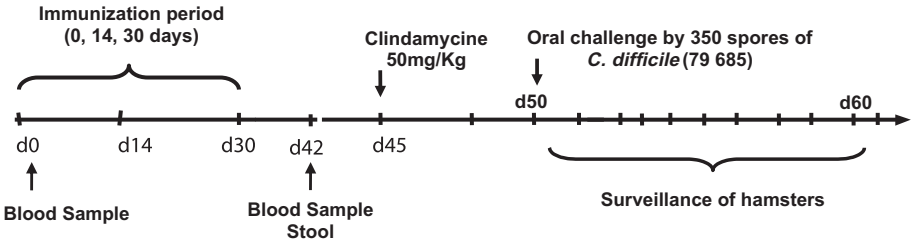

Fig. 1. Oral immunization scheme.

3.1. Bead preparation

Ionic interactions between the negatively charged carboxyl groups of pectin and the positively charged divalent zinc ions were used to prepare zinc-pectinate gel beads. The mechanism of gelation is not explained only by dimer associations described in the 'egg-box' model [38], but also by a combination of hydrogen bonding and hydrophobic interactions between polymeric chains [29,39–41]. Pectin solution, at the concentration of 6% w /V, was very viscous and Cwp84 addition apparently increased its viscosity. Even 12 h after mixing, little bubbles remained included in the pectin solution. Neither the high viscosity nor the presence of bubbles influenced bead formation and characteristics. Beads appear rather spherical with a diameter of about 2–3 or 1–2 mm, for the undried and dried (3 h at 37 °C) beads, respectively (Fig. 2). Protein and polymer dissolution in TRIS buffer (25 mM, pH 7.5) were considered necessary to preserve the stability and the activity [5] of Cwp84 after encapsulation into beads. Other characteristics of the process, such as the zinc acetate concentration, the flow-rate of the solution, the nozzle diameter, the dropping distance, the permanence time in the cross-linking solution, the number and duration of washings, were chosen following previous studies [30].

3.2. Influence of beads drying time on weight loss and bead stability

Preliminary studies were carried out to determine the effect of bead drying on weight loss on one hand and on the other hand on bead stability in simulated digestive media. Bead weight loss was therefore measured by thermo-gravimetric analysis as a function of the drying time. Data on Table 1 show that undried beads lose about 90% of their weight which is logical since the concentration of the pectin solution is 6%. After 1 and 2 h drying, the weight loss decreases down to around 85% and 70%, respectively. After 3 h drying, the weight loss decreases dramatically down to 15%, the remaining weight corresponding most probably to pectin and associated moisture due to the hygroscopic properties of zinc ions. Weight loss results are in agreement with bead size decrease observed optically (Fig. 2).

The stability of the zinc-pectinate matrix in simulated digestive media was then investigated by determining the disintegration time macroscopically. All groups of beads, dried different times at 37 °C, showed the same resistance to the acidity of the simulated gastric medium (pH 1.2) for 2 h. However, subsequently to SGM incubation, beads disintegrated rather quickly in SIM (pH 6.8). Indeed, when beads are placed in SGM and afterwards in SIM, ion-exchange reactions occur. The exchange of Zn^{2+} retained by beads with H^+ or Na^+ from the gastric medium and with Na^+ from the intestinal medium occurs in the beads via diffusion. As a result, the egg-box structure is depleted of cross-linkers. Beads keep apparently their structure intact since the pectinic acid, product of the ion exchange, is insoluble in the acidic environment (the pH of SGM is below the p*K*a of pectin 3.5). Afterwards, upon medium change and entrance into pH 6.8 of the SIM, the COOH groups of the pectinic acid turn into COO^-, which repel each others. Since beads are depleted of cross-linkers, the structure is loose and a dramatic disintegration occurs [28,30]. Therefore, gastro-resistant capsules, coated with HPMCP, will be used to protect beads from exposure to harsh gastric environment and prevent their later disintegration in the intestinal medium [30].

Following studies were therefore carried out avoiding the acidic exposure and analysing bead stability directly in SIM and after their transfer in SCM with pectinolytic enzymes. In SIM, beads kept their spherical shape for the 5 h of incubation, showing, however, a dramatic and constant swelling without erosion, starting after a few minutes of incubation (Fig. 3). After transfer in SCM, their disintegration by pectinolytic enzymes was observed after 1 h or 2 h of exposure: the drier the beads, the longer the disintegration.

Therefore, since all groups of beads showed more or less the same behaviour in SIM and SCM, the 3 h of drying time was chosen for further studies. The low water content measured for this group may ensure a better stability for the protein. In addition, the smaller bead size is more convenient for capsule filling: up to 18–20 beads could be loaded within a size-9 capsule.

3.3. Enzymatic characterization of Cwp84

The drying times investigated for weight loss and beads stability in simulated intestinal fluids had no influence on the proteolytic activity of encapsulated Cwp84 (Table 1) Results indicate that encapsulation may cause the loss of about 80% of original activity, independently of the drying time (Table 1). We have investigated whether this loss of activity was due to the encapsulation process or to the influence of the disintegration process and

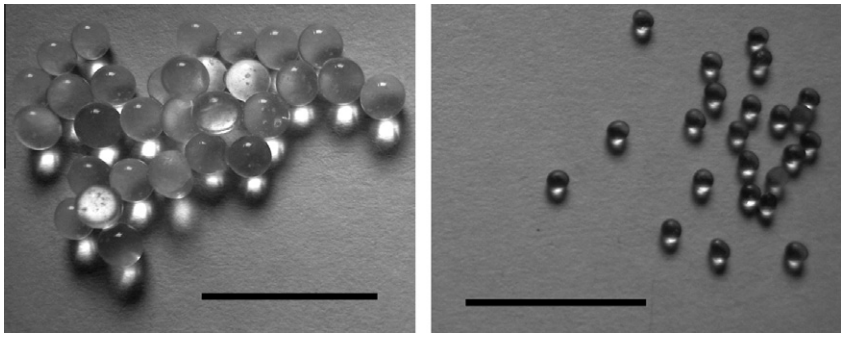

Fig. 2. Zn-pectinate beads, either undried (left) or dried for 3 h at 37 °C (right). Scale bars represent 1 cm on both images.

Table 1
Bead weight loss and Cwp84 activity as a function of the drying time.

Drying time (h)	0	1	2	3
Bead weight (mg ± SD) $n = 30$	9.45 ± 0.77	6.22 ± 0.30	2.60 ± 0.90	1.23 ± 0.14
Weight loss (% ± SD) $n = 3$	89.38 ± 2.06	84.80 ± 1.84	70.39 ± 1.56	14.55 ± 0.90
Enzymatic activity (%) $n = 2$	17 ± 2.3	20.4 ± 3.5	16.1 ± 0.9	18.5 ± 2.6

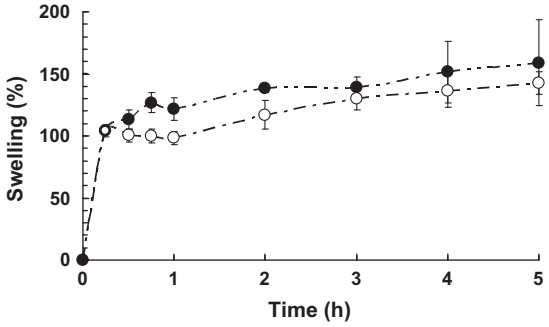

Fig. 3. Comparison of swelling ratio of Zn-pectinate beads encapsulating Cwp84 (●) or not (○) in SIM for 5 h (bars indicate SD; $n = 3$).

protein extraction from beads. Controls were carried out using the same quantity of Cwp84 that the one found in beads which was added to a solution of unloaded beads disintegrated in EDTA. The protein activity was tested immediately and after 90 min with or without stirring at room temperature. Ninety minutes is the time needed to obtain complete bead disintegration. Results show that 30% of the initial activity is lost when Cwp84 is added to disintegrated bead solution in EDTA. This effect may be linked to the presence of zinc ions coming from beads. Moreover, 90 min of incubation without stirring induced the same activity loss. Nevertheless, adding stirring the whole mixture induce an additional loss of activity showing that about 40% of the protease activity is lost in the extraction process from beads.

The encapsulation efficacy (EE) of Cwp84 was estimated using both the Bradford method and the intrinsic fluorescence of tryptophan. For both methods, a solution of unloaded and disintegrated beads was used for calibration curve background. For the Bradford method, this was necessary because of the immediate pectin gelification following the addition of the Bradford reagent at pH = 1.2. Samples resulted clear but with some particles remained in suspension. However, their presence did not cause any deviation from linearity, and the method was reproducible. For the tryptophan method, the solution of unloaded beads was employed to minimize the interference with protein impurities coming from pectin. Both techniques give an EE around 40%. The low EE is most probably linked to the encapsulation method. Moreover, protein loss may also happen during gelation by diffusion from the concentrated gel to the less concentrated gelation bath as suggested by Bourgeois et al. [25] who have found similar EE. Several authors suggest other methods involving the immersion of gelled polymers in protein solutions. A quick encapsulation method performed post-synthesis would be more effective, minimizing protein loss and increasing encapsulation efficiency [42,43]. However, this method involves the use of concentred soaking solutions of protein, which is not feasible given the low quantities of Cwp84 available.

3.4. In vitro bead swelling and Cwp84 release

Fig. 3 shows the swelling behaviour of beads encapsulating or not Cwp84, during 5 h of incubation in SIM, at pH 6.8 and 37 °C. The swelling ratio increases steeply during the first 15 min of incubation and then slowly during the remaining 5 h. It finally reaches about 140% for the unloaded beads and about 160% for the loaded ones, after 5 h of incubation. The difference of swelling is not significant. Even if bead swelling is immediate and of great extent, Cwp84 release in SIM is rather low around 5%. This result indeed shows the suitability of zinc-pectinate beads to prevent protein release in SIM without pancreatin. Pancreatin may be omitted from the composition of most dissolution testing applications in order to avoid potential interference in drug determination. However, the absence of the intestinal enzyme may give a different profile of drug release [44]. The low amount of Cwp84 released is most probably due to Cwp84 size, which is larger than the diameter of hydrated pores of the zinc-pectinate matrix and therefore unable to diffuse through them.

By contrast, as soon as beads are placed in SCM containing pectinolytic enzymes, 80% of the encapsulated Cwp84 is released after 2 h of incubation and 90–95% after 5 h (Fig. 4). The protein release arises from the enzymatic degradation of the matrix [45]. The pectinolytic enzymes consist of pectin methylesterase and two types of pectin depolymerases. Pectin methylesterase removes the methoxy groups from the pectin while polygalacturonase (or hydrolase) hydrolyses glycosidic bounds next to the free carboxyl groups of the galacturonan chain. The pectate lyases degrade the glycosidic linkages next to the free carboxyl group by β elimination, resulting in the formation of double bonds between C4 and C5 monomer [24].

3.5. Determination of zinc ions content in beads

Zinc is an essential micronutrient with recommended dietary allowance (RDA) 11 and 8 mg, for human male and female, respectively (NIH's Office of Dietary Supplements). It is an integral part of numerous enzymes, so a deficiency or an excess of this metal ion may lead to disturbing effects. To estimate the amount of zinc ions ingested by hamster after each immunization, zinc ions retained in beads were measured and found to be about 0.14 mg in each bead. Therefore, hamsters have ingested up to about 25–35 mg/kg zinc ions after each immunization (1 capsule filled with 18–20 beads). Although this dose is a 10-fold higher than what is considered safe for human daily, since immunizations are only performed three times, the amount of zinc ions ingested may be surely safe [46].

3.6. Vaccination results

Fig. 5 presents the rate of survival after vaccination and challenge by *C. difficile*. Hamster is a model very sensitive to *C. difficile* infections. On one hand, 2 days after spores administration, all the control hamsters immunized with unloaded beads have deceased. On the other hand, about 40% of hamsters administered with Cwp84-loaded beads survived 10 days after challenge. The difference observed between these two groups is statistically significant ($p < 0.039$). This result proves that an oral vaccination with encapsulated Cwp84 provides partial protection.

Even if the survival results clearly indicate that protection is better with encapsulated Cwp84, the statistical difference with the free Cwp84 is not significant on the whole Kaplan–Meier analysis (Fig. 5). Degradation by gastric and intestinal secretions, dilution in the intestinal fluids, poor sampling via Peyer's patches, may

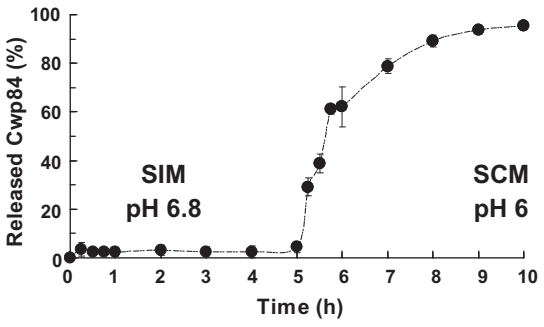

Fig. 4. *In vitro* Cwp84 release from Zn-pectinate beads in SIM (for 5 h) and SCM (for other 5 h) (bars indicate SD; $n = 3$).

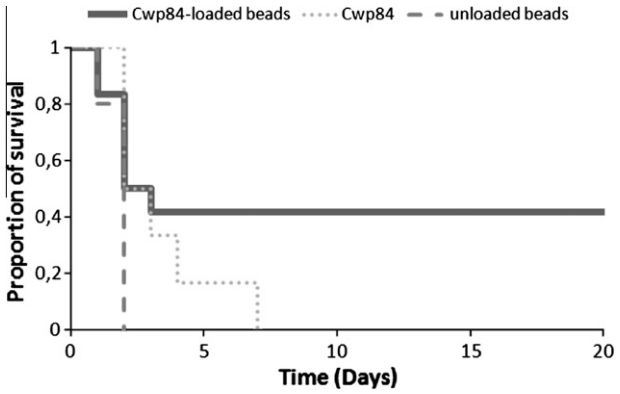

Fig. 5. Kaplan–Meier plot for hamster survival after challenging with *C. difficile* spores.

all be parameters that contribute to the limited efficacy of the oral route.

Cwp84 is a cystein protease which is highly immunogenic in patients with *C. difficile*-associated disease, suggesting that Cwp84 could play an important role in the physiopathology of *C. difficile* [11]. Cwp84 exhibit degrading activity with fibronectin, laminin and vitronectin which are important components of the basement membrane and are involved in the cellular adhesion process. In vivo, this proteolytic activity could contribute to the degradation of the host tissue integrity and to the dissemination of the infection. This direct proteolytic degradation of the basement membrane could contribute to the intestinal epithelium necrosis [5]. Kirby et al. demonstrated that this cystein protease plays a key role in the maturation of the surface proteins layer [47,48]. Results obtained in this study confirm the important role of this protease in the pathogenic process. Further *in vitro* studies should be performed in order to precise the mode of action of Cwp84.

The seric immune response analysis shows highly variable response between animals (data not shown). However, the seric immune response does not reflect the mucosal response. IgA levels should be measured, but unfortunately reagents are not available commercially. This seriously hampers a more precise determination of the specific immune response at the intestinal level. Usually, for mucosal immunization, adjuvants are needed to enhance the immune response. Pectin might not be enough to stimulate the immune response, and antibody levels could be improved by co-encapsulation of an adjuvant such as cholera toxin. These preliminary results are encouraging and should be further confirmed. In the future, the vaccination protocol could be optimized by varying the dose administered or the vaccination schedule. In addition, since *C. difficile* colonization is a multifactor process, it could be interesting to use zinc-pectinate beads to deliver simultaneously several antigens, and therefore enhance the level of protection.

4. Conclusion

We have optimized the encapsulation of the *C. difficile* protease Cwp84 into zinc-pectinate beads. The mild process of encapsulation allows to partially preserve the enzymatic activity and the immunogenicity of Cwp84. The chosen delivery system is enzymatically triggered to release Cwp84 in the colon only, where this antigen could stimulate the mucosal immune response to *C. difficile*. Although they only show partial protection preliminary oral vaccination results are promising. Zinc-pectinate beads appear interesting vehicles for colonic delivery of candidate proteins for oral vaccination.

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