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Carboxymethyl high amylose starch for F4 *fimbriae* gastro-resistant oral formulation

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Abstract

The carboxymethyl high amylose starch (CM-HAS) was proposed as excipient able to protect F4 *fimbriae* oral vaccine against gastric acidity and pepsin, allowing its subsequent liberation in the intestinal fluid. Thus, F4 *fimbriae* formulated with CM-HAS as tablets displayed a markedly higher stability after 2 h of incubation in simulated gastric fluid (containing pepsin) than the free, non-protected F4 *fimbriae*, which, in these conditions, were almost completely digested after 120 min. In the presence of pancreatin (with alpha-amylase, lipase and proteolytic activities) in simulated intestinal conditions, the F4 *fimbriae* were liberated from CM-HAS tablets over a period of up to 5 h. The presence of pancreatin in intestinal medium did not affect the structural stability of the F4 *fimbriae* major subunits. Thus, F4 *fimbriae* formulated with CM-HAS would retain their receptor binding activity essential for the induction of an intestinal mucosal immune response.

Keywords: Carboxymethyl high amylose starch; F4 *fimbriae*; Vaccine; Oral administration; Tablet; Gastro-resistance

1. Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is an important cause of disease in mammals, and, in the case of neonatal and recently weaned pigs, is responsible for diarrhea, reduced growth rate and mortality, with subsequent economic losses. ETEC bacteria adhere to the small intestinal epithelium by means of fimbrial adhesins, F4 being the most prevalent type of *fimbriae* in ETEC strains ($F4^+$ETEC) causing neonatal and post-weaning diarrhea in pigs (Fairbrother and Gyles, 2006). F4 *fimbriae* are long filamentous polymeric surface proteins, mainly composed of FaeG subunits (fimbrial major subunit) and low amounts of the fimbrial minor subunits FaeC (located at the tip), FaeF and FaeH (situated along the fimbrial structure) and probably FaeI and FaeJ (Bakker et al., 1992a). These *fimbriae* are responsible for bacterial adhesion to F4-specific receptors present on brush borders of villous enterocytes. The binding site of F4 *fimbriae* is situated on the FaeG major subunit (Bakker et al., 1992b), permitting $F4^+$ETEC bacteria to withstand expulsion by intestinal peristalsis and to colonize the small intestine (Jones and Rutter, 1972). These bacteria produce enterotoxins which act on enterocytes, resulting in aqueous diarrhea (Nataro and Kaper, 1998). A prerequisite for ETEC infection is the presence of F4 receptors, piglets without this type of receptor being resistant to infection (Van den Broeck et al., 1999a).

One approach for the prevention of post-weaning diarrhea in pigs due to $F4^+$ETEC would be oral vaccination with an F4 vaccine. Orally administered, purified F4 *fimbriae* were found to induce an intestinal mucosal immune response in F4 receptor-positive ($F4R^+$) piglets (Van den Broeck et al., 1999a,b). This *in situ* mucosal immune response is not observed following parenteral immunization which activates the systemic immune system (Bianchi and Scholten, 1996). Thus, purified F4 *fimbriae*

Abbreviations: CM, carboxymethyl; CM-HAS, carboxymethyl high amylose starch (non-cross-linked); DS, degree of substitution; NMR, nuclear magnetic resonance; ETEC, enterotoxigenic *Escherichia coli*; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate buffer solution; TBS, Tris buffer solution; TTBS, TBS solution containing 0.2% Tween-20; IOD, integrated optic density

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can be considered as a suitable component of an oral vaccine for the blocking of subsequent adhesion of F4$^+$ETEC.

Oral administration of F4 *fimbriae* in suspension is not cost-effective because of possible fimbrial denaturation by acids, bile and enzymes present in the gastro-intestinal tract (Snoeck et al., 2004). Oral administration of a solid formulation of the F4 *fimbriae* may result in a more effective vaccine, due to protection of the F4 *fimbriae* from the gastric pH and digestion by pepsin in the stomach. The purpose of F4 formulation with polymeric excipients would be to confer such a protection and, consequently, to decrease vaccine dose and dosing frequency. Delivery of F4 *fimbriae* to a specific absorption window rather than slow release throughout their passage in the gastro-intestinal tract is also a desirable outcome. As the F4 *fimbriae* each consist of many hundreds of identical monomer subunits, it is important that they be formulated with an appropriate polymeric matrix type that would also permit their liberation. Some F4 *fimbriae* multi-particulate formulations have been reported (Snoeck et al., 2003; Huyghebaert et al., 2005). To ensure gastro-resistance, these pellets were enteric-coated with Eudragit® (an anionic polymethacrylic acid), an additional step which complicates the manufacturing process. In addition, the release of F4 *fimbriae* from these enteric-coated pellets was not optimal because of subsequent interactions between the F4 *fimbriae* and the coating polymeric material (Huyghebaert et al., 2005). Consequently, there is a need for simple formulations of F4 *fimbriae*, possibly eliminating the coating process.

Starch is an attractive, cost-effective, safe (biocompatible) and renewable material. It is composed of amylose, a non-branched polymer of glucopyranose units with α-D-(1,4) glucosidic bonds, and of amylopectin, a branched polymer with α-D-(1,4) glucosidic bonds, having periodic branches at the O-6 position (Pazur, 1965). Native starch characteristics can be substantially changed by physical modification (i.e. gelatinization) (Svensson and Eliasson, 1995) or by chemical (Fang et al., 2004) or enzymatic (Rajan et al., 2006) alteration, leading to the acquisition of interesting properties as excipients for drug delivery systems (Dumoulin et al., 1999; Mulhbacher et al., 2001). Previous studies have shown the important role of hydroxylic groups in the organization of the cross-linked high amylose starch matrices (Mateescu et al., 1994; Dumoulin et al., 1998; Ispas-Szabo et al., 2000). In addition, we have shown that the presence of carboxymethyl groups (CM-) can also ensure a good stability of non-cross-linked starch matrices (Calinescu et al., 2005). Starch carboxymethylation results in water-soluble derivatives whose properties are mainly determined by the total degree of substitution. Due to the presence of carboxylic functional groups, the swelling properties of carboxymethyl high amylose starch (CM-HAS) are influenced by the environmental pH (Mulhbacher et al., 2004). In an acidic simulated gastric fluid (SGF), the CM-HAS matrix will become protonated, thus ensuring gastro-resistance of bioactive agents to acidic/enzymatic media. In neutral or weak alkaline simulated intestinal fluid (SIF), the protonated form will exchange the protons for cations, facilitating the hydration and dissolution of the polymeric matrix with the gradual release of the bioactive agent. Matrices (e.g. tablet form) based on CM-HAS may represent a viable solution for the transport of bioactive agents in the gastro-intestinal tract. This excipient has already been successfully used *in vitro* for the gastric protection and delivery of large size bioactive agents such as *E. coli* microorganisms (Calinescu et al., 2005).

The aim of this study is to evaluate whether a matrix (tablet) based on CM-HAS as excipient can also confer gastric protection and permit intestinal delivery of bioactive agents smaller in size, such as peptide aggregates.

2. Materials and methods

2.1. Materials

High amylose corn starch (Hylon VII) was obtained from National Starch (NJ, USA); pepsin A from porcine gastric mucosa (Sigma–Aldrich Company, St. Louis, MO, USA); pancreatin (porcine pancreas) eight times strength (with alpha-amylase, lipase and proteolytic activities) from A&C American Chemicals Ltd. (Montreal, Quebec, Canada); acrylamide, *N*,*N*′-methylene-bis-acrylamide, protein molecular weight markers (broad range) from Bio-Rad Laboratories (Richmond, VA, USA); Western blotting membranes and ECL™ (enzymatic chemiluminescence) reagents from Amersham Pharmacia Biotech (NJ, USA); hyperfilm™, X-ray film cassettes, electrophoresis (Mini-Protean, Bio-Rad Laboratory) and Western blotting (W.E.P. Company, Seattle, WA, USA) systems.

Anti-F4 IgY primary antibody was produced by the Reference Laboratory for *Escherichia coli* (Saint-Hyacinthe, Quebec, Canada); horseradish peroxidase-labelled anti-IgY secondary antibody was obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Purified, freeze-dried F4 *fimbriae* were produced by the Reference Laboratory for *Escherichia coli* (Saint-Hyacinthe). The purity of the F4 *fimbriae* was assessed by electrophoresis on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the Bio-Rad Mini-Protean II electrophoresis system.

The derivatization agent (monochloroacetic acid) and the other chemicals were all reagent grade and used without further purification.

2.2. Synthesis of CM-HAS polymeric material

The CM-HAS was synthesized by etherification of the starch with monochloroacetic acid in an alkaline reaction medium, as described previously by Schell et al. (1978), Mulhbacher et al. (2001) and Calinescu et al. (2005), with slight modifications.

An amount of 70 g of high amylose starch (Hylon VII) was suspended in 170 mL of distilled water and warmed at 50 °C under continuous stirring in a Hobart planetary mixer. In order to obtain a better etherification, a volume of 235 mL of an aqueous 1.45 M NaOH solution was added and the reaction medium was homogenized for 20 min at 50 °C for gelatinization. Subsequently, 55 mL of 10 M NaOH solution were added to the reactional medium to activate the starch, transforming it into a more reactive alkoxide form and favoring the nucleophilic substitution for carboxymethylation. Then, 45.5 g of monochloroacetic acid

(dissolved in a minimum volume of water) were added and the starch was allowed to react (1 h at 50 °C) with monochloroacetic acid under continuous stirring. At the end of reaction, the gel-slurry was neutralized with an acetic acid solution and acetone was slowly added to the neutralized suspension. Washing with acetone:water (60:40, v/v) of polymeric derivative was done as previously described (Calinescu et al., 2005). After repeating the filtration and resuspension operations, the CM-HAS gel-slurry was dried with pure acetone, held overnight to air at room temperature, ground in a blender, and then sieved to obtain a powder with particles granulometrically smaller than 300 μm. This powder was used to prepare the tablets. The residual moisture of the polymer powder was calculated based on the product weight loss after the drying of the powder (100 °C).

2.3. Determination of CM-HAS substitution degree

The presence of carboxylic groups was first assessed by Fourier transform infrared spectroscopy (FTIR). FTIR spectral analysis of the CM-HAS powder was carried out in potassium bromide (KBr) pellets, using a BOMEM (Hartmann & Braun) spectrometer (MB-series, Quebec, Canada). Spectra were collected at a resolution of 4 cm^{-1} at 25 °C.

The degree of substitution of the CM-HAS was determined by direct titration with sodium hydroxide and by ^1H nuclear magnetic resonance (NMR).

2.3.1. Direct titration

The carboxymethyl groups of the CM-HAS were first activated by conversion into the acidic (protonated) form by treatment of the polymer with a 1 M HCl solution. The protonated CM-HAS was then precipitated with acetone, filtered, washed with acetone and finally dried in oven (50 °C). The protonated powder of CM-HAS was dispersed in water and the carboxyl groups were titrated with a 0.1 N NaOH solution.

2.3.2. ^1H NMR measurements

In order to determine the CM-HAS functionalization pattern, NMR analysis was performed on CM-HAS samples that had been previously hydrolyzed with perchloric acid as described by Heinze et al. (1999). Briefly, 0.1 g of CM-HAS were dispersed in 1 mL $HClO_4$ (70%) and after 10 min (room temperature), 9 mL of distilled water were added. The sample was heated at 100 °C for 16 h, and then neutralized with 2 M KOH and kept overnight at 4 °C to allow complete precipitation of $KClO_4$. The obtained solution was frozen and freeze-dried. The ^1H NMR analysis was carried out in deuterated dimethyl-d_6 sulfoxide (99.9 atom %D, CDN Isotopes, Quebec, Canada) at 90 °C. The NMR spectra were acquired on a 300 MHz Gemini-300 spectrometer (frequency of proton).

2.4. Tablet formulation of purified F4 fimbriae with CM-HAS

The tablets were produced by direct compression (Carver press) of mixed dry powders (CM-HAS and F4 *fimbriae*) at a compression force of 2.5 T/cm^2. Thus, 7 mg of lyophilized powder of *fimbriae* (containing 2 mg of purified F4 *fimbriae*) were incorporated into CM-HAS tablets (total mass: 200 mg).

2.5. Stability of F4 fimbriae in simulated gastric fluid

Samples of free, lyophilized F4 *fimbriae* (7 mg of powder containing 2 mg of purified F4 *fimbriae*) were individually incubated in 30 mL of SGF (pH 1.2) containing 0.32% pepsin, 1.17 U/mg (USP, 1990) for 30, 60, 90 and 120 min under gentle rotation at 50 rpm and 37 °C (simulating the gastric passage). At the end of incubation, the pH of each sample was increased to 7.5 ± 0.1 by addition of 240 μL of 10 M NaOH and 19.76 mL of phosphate buffer solution (PBS), pH 7.4 (total volume: 50 mL) and the stability of the F4 *fimbriae* in SGF was checked. For the CM-HAS formulated F4 *fimbriae*, the tablets were incubated for a duration up to 120 min in the same SGF conditions. Tablets were then transferred in 50 mL of PBS solution (pH 7.4), crushed, and dissolved for 4 h in this solution (under gentle agitation at 4 °C).

After each period of incubation indicated above, the F4 *fimbriae* integrity was evaluated by SDS–PAGE (12%) followed by Western blotting analysis (Sections 2.8 and 2.9).

2.6. Delivery of formulated F4 fimbriae in simulated intestinal fluid

The CM-HAS formulated *fimbriae* were incubated for 1 h in 50 mL SGF containing 0.32% pepsin (pH 1.2) and then for 5 h in 50 mL of SIF (pH 7.5 ± 0.1), with or without pancreatin (USP, 1990), at 50 rpm and 37 °C. After every hour of SIF incubation, liberated F4 *fimbriae* were evaluated by SDS–PAGE for pancreatin-free SIF samples (Section 2.8) and by SDS-PAGE followed by Western blotting analysis for SIF samples containing pancreatin (Sections 2.8 and 2.9).

2.7. Free F4 fimbriae stability in simulated intestinal fluid

Unformulated (free) lyophilized F4 *fimbriae* (7 mg preparation containing 2 mg of purified *fimbriae*) were incubated for 5 h in 50 mL of SIF containing pancreatin, pH 7.5 ± 0.1 (USP, 1990) at 37 °C and 50 rpm. The F4 *fimbriae* stability in SIF was then evaluated hourly by SDS–PAGE (12%) followed by Western blotting analysis (Sections 2.8 and 2.9).

For free F4 *fimbriae* controls, 7 mg of lyophilized F4 powder (2 mg purified F4) were dissolved in 50 mL PBS solution (pH 7.4). In the case of F4 formulated with CM-HAS (control), the tablets were crushed in 50 mL PBS solution (pH 7.4) and then kept 4 h under agitation at 4 °C for CM-HAS complete dissolution.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Degradation of F4 *fimbriae* was evaluated using SDS–PAGE. For each of the previously described experiments, 10 μL of F4 fimbrial sample was treated (1:1, v/v) with electrophoresis loading buffer (0.12 M Tris–HCl pH 6.8, 4% SDS, 20% glycerol,

10% β-mercaptoethanol and 0.004% bromophenol blue) and boiled for 2 min. A volume of 10 μL of treated F4 sample was loaded onto a 12% polyacrylamide gel prepared by the method of Laemmli (1970) and run at room temperature for 1 h (120 V) using an electrophoresis buffer (0.025 M Tris–Base pH 8.3 containing 0.192 M glycine and 0.1% SDS). Molecular weight protein markers (Broad Range, Bio-Rad Laboratories), containing bromophenol blue, were loaded on each electrophoresis gel. Proteins were stained with R-250 Coomassie blue.

2.9. Western blotting analysis

After SDS–PAGE migration, gels were separately equilibrated for 5 min in a transfer buffer (0.025 M Tris–Base, 0.192 M glycine and 20% methanol, pH 8.1–8.4) under gentle agitation. The gels were then blotted onto activated 0.45 μm polyvinylidene fluoride membranes by semi-dry transfer (1.25 h, 80 mA/gel). After the protein transfer process, the non-specific sites on the membrane were blocked (overnight, 4 °C, under gentle agitation) using 2.5% low fat dried milk dissolved in a Tris buffer solution (TBS: 0.015 M Tris–HCl, 0.5 M NaCl, pH 7.5). Membranes were then washed once with TBS solution containing 0.2% Tween-20 (TTBS) and once with TBS solution only. For detection of F4 *fimbriae*, membranes were incubated for 2 h (under agitation, at room temperature) in the 2.5% low fat milk TBS solution containing anti-F4 IgY primary antibody (dilution 1:700). Then, membranes were incubated for 1 h in a TBS solution containing horseradish peroxidase-labelled anti-IgY secondary antibody (dilution 1:3500) in the same conditions as above. Between and after the two antibody incubation steps, membranes were washed first in TTBS and then in TBS, both for 5 min. The F4 *fimbriae*–antibody complexes were detected using an enzymatic chemiluminescence test (ECL™) based on the oxidation of luminol by the horseradish peroxidase. The light produced by this chemiluminescent reaction was detected by a short exposure to a light-sensitive film, which was developed using an automated developer.

Densitometry analysis of bands was carried out using the Alpha Ease FC™ program (Chemilmager™ 5500). For each band on the film, the program evaluated its surface (mm^2) that was multiplied by its corresponding measured density, thus obtaining the integrated optic density (IOD). We determined the percentage of variation of band densities for every sample versus the standard, considering the standard (free or formulated F4 *fimbriae* in PBS, time zero) as 100%.

2.10. Water uptake and erosion of CM-HAS tablets

The water uptake and erosion of the CM-HAS matrices were determined under conditions similar to those described for the F4 *fimbriae* release study. Tracer-free CM-HAS weighed tablets (polymer only) were placed in flat-bottom dissolution vessels containing SGF medium (37 °C and 50 rpm). After 1 h in SGF, the CM-HAS tablets were transferred to 50 mL SIF medium with or without pancreatin (at 37 °C and 50 rpm). After each hour of incubation in SIF (with or without pancreatin), hydrated tablets were removed from the dissolution medium, blotted with tissue paper to eliminate the excess surface water, then weighed and placed for drying in an oven at 50 °C. The remaining dry weight was determined until constant mass. Three different tablets were used for each time point ($n = 3$).

The percentage of water uptake, erosion and water in remaining tablet were determined gravimetrically and calculated according to Freichel and Lippold (2000), Kavanagh and Corrigan (2004) and Sungthongjeen et al. (2004):

$$\%\ \text{Water uptake} = \frac{(W_{w(t)} - W_0)}{W_0} \times 100$$

$$\%\ \text{Erosion} = \frac{(W_0 - W_{d(t)})}{W_0} \times 100$$

$$\%\ \text{Water in remaining tablet} = \frac{(W_{w(t)} - W_{d(t)})}{W_{d(t)}} \times 100$$

where $W_{w(t)}$ is the weight of the wet tablet at time t, W_0 the initial dry weight of the tablet and $W_{d(t)}$ the remaining dry weight of the tablet at time t.

2.11. Statistical analysis

One-way Anova pair-wise comparisons test followed by Tukey honestly significantly difference (HSD) test were used. Statistical significance was assessed at $P \leq 0.01$.

3. Results and discussion

The CM-HAS properties are mainly related to the degree of substitution (DS), which is the average number of CM-groups bound per anhydroglucose unit with maximal (theoretical) DS = 3. In the present study, the total DS of the CM-HAS excipient was determined by two methods giving similar values: direct titration (DS 0.33) and ^{1}H NMR spectroscopy on a hydrolyzed CM-HAS (DS 0.38) (Table 1). ^{1}H NMR spectroscopy of a hydrolytically depolymerized sample of CM-HAS can also provide information on the partial substitution at the 2, 3 and 6 position of the repeating glucose unit and can be used for the direct quantitative evaluation of the DS. Our NMR analysis showed that a significantly preferred reaction occurred at O-2 in CM-HAS and that starch carboxymethylation proceeded in the order O-2 > O-6 > O-3 (Table 1), confirming the findings of Heinze et al. (2001) and Lazik et al. (2002). The assignments of peaks (Fig. 1) and the calculation of partial DS were carried out according to Lazik et al. (2002).

Table 1
Determination of the degree of substitution of non-cross-linked CM-HAS excipient

Method	DS_{CM}	x_2	x_3	x_6
^{1}H NMR[a]	0.38	0.2	0.076	0.111
Direct titration	0.33	–	–	–

x_2, x_3, x_6 = positions 2, 3, 6 of the glucose repeating unit.
[a] Functionalization pattern of CM-HAS determined by ^{1}H nuclear magnetic resonance spectroscopy after hydrolytic chain degradation.

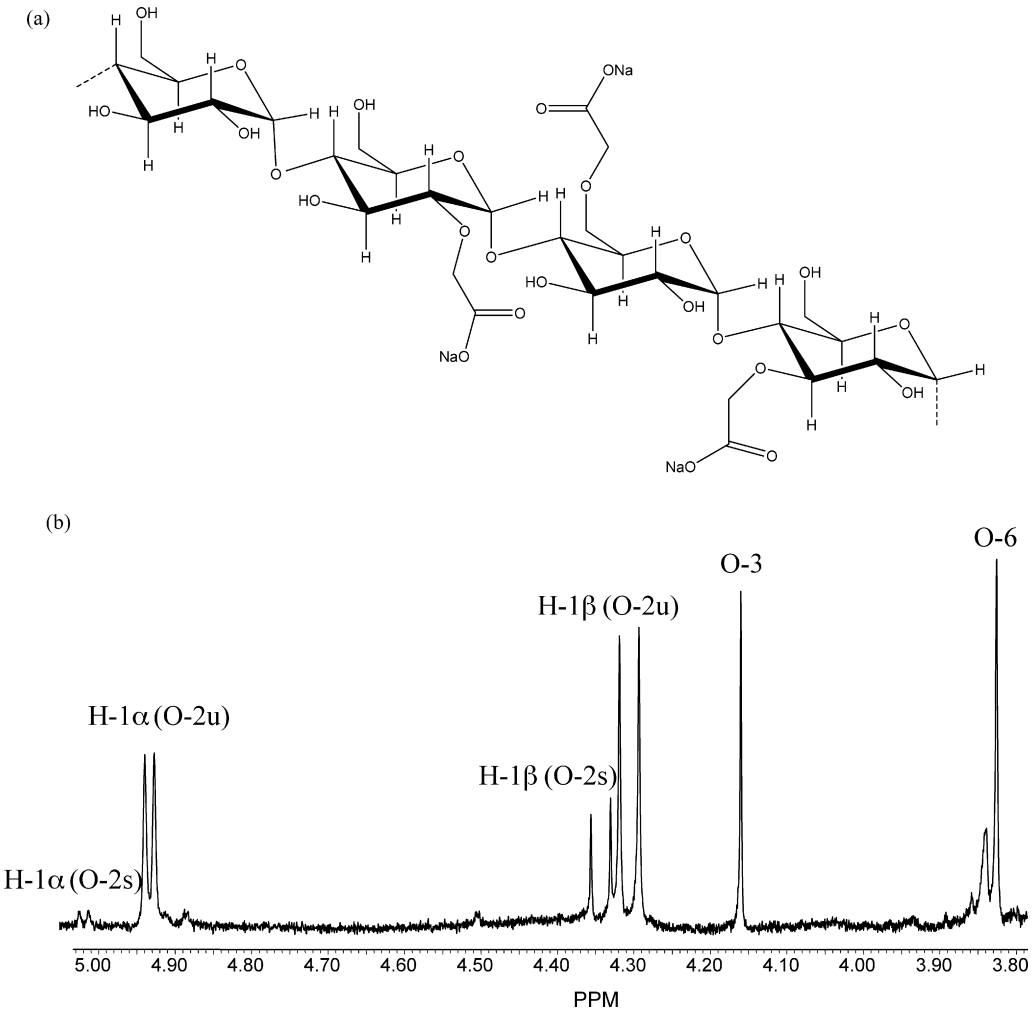

Fig. 1. ^{1}H NMR spectrum of CM-HAS after hydrolytic chain degradation. Schematical presentation of CM-HAS (a) and ^{1}H nuclear magnetic resonance spectrum (b). O is the oxygen atom at position *i* (*i* = 2, 3 or 6), H-1 is the hydrogen atom at the anomeric C (α, β-configuration of glucose), s means substituted, and u unsubstituted.

The residual moisture of the CM-HAS polymer powder was 11%, a value compatible with that of many starch derivatives. The FTIR spectroscopy in KBr yielded signals at 1607 and 1417 cm^{-1} specific for a carboxylate salt (–COONa), confirming the presence of CM- functions on the high amylose starch (data not shown).

Purified F4 *fimbriae* have polymeric structures mainly composed of FaeG subunits. A prerequisite for the induction of an intestinal mucosal immune response is the preservation of the proper FaeG conformation or at least of its binding site to ensure the adherence of F4 *fimbriae* on the villous enterocytes in the small intestine. In our study, since the SDS–PAGE was performed with previously boiled F4 fimbrial samples, and because of the effect of SDS, only the FaeG monomeric major subunit degradation could be observed. These free FaeG monomeric major fimbrial subunits were partially degraded after 30–90 min of incubation in SGF containing pepsin (with proteolytic activity at pH 1.2) and almost completely digested after 120 min of treatment (Fig. 2a).

Gastric resistance of F4 *fimbriae* is also a prerequisite for its antigenicity. Since free F4 *fimbriae* are destroyed by pepsin, the amount and/or integrity of antigen reaching the mucosal inductive sites could be reduced. When formulated with CM-HAS as tablets, the F4 *fimbriae* did not change markedly in their conformation since they were still recognized by their specific anti-F4 IgY antibodies. Additionally, SDS–PAGE and Western blotting tests showed that the CM-HAS polymeric matrix protected the *fimbriae* against gastric acid treatment and enzymatic denaturation (Fig. 2a and b), more than 90% of *fimbriae* being protected after 120 min of SGF treatment. This is a good protection efficacy when compared with that of the non-protected *fimbriae* (Fig. 2c). In this context, it is worth mentioning that is not necessary to apply an enteric coating on the CM-HAS matrix for a supplementary protection of the F4 *fimbriae*. The carboxylic groups are known for their ability to buffer and to create strong hydrogen bonds, ensuring thus a compact structure of the tablets in acidic conditions.

The delivery of the formulated F4 *fimbriae* was first observed after 2 h of treatment in pancreatin-free SIF (50 rpm, 37 °C) and continued for up to a further 3 h in the same SIF conditions (Fig. 3). In the presence of pancreatin (with alpha-amylase, lipase and proteolytic activities), the F4 *fimbriae* were liber-

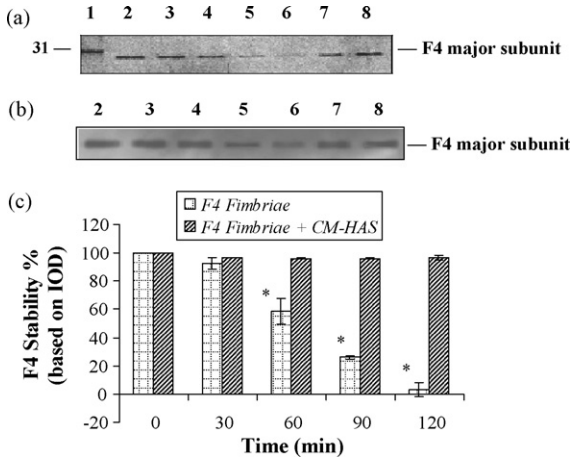

Fig. 2. Stability of F4 *fimbriae* in simulated gastric fluid (SGF/USP). (a) Sodium dodecyl sulfate—polyacrylamide gel electrophoresis; (b) Western blotting; (c) densitometry analysis of Western blotting films. Electrophoretic profiles: molecular weight protein marker (31 kDa) (lane 1); free (non-formulated) F4 *fimbriae* in phosphate buffer solution (0 min) (2) and after 30 min (3), 60 min (4), 90 min (5), 120 min (6) in simulated gastric fluid containing pepsin (SGF); F4 *fimbriae* formulated with CM-HAS – as tablet – in phosphate buffer solution (0 min) (7) and after 120 min in SGF (8). Volume of *fimbriae* sample: 10 μL/well; IOD, integrated optic density ($n = 3$). Asterisks stand for significant differences within the same group of samples ($P \leq 0.01$).

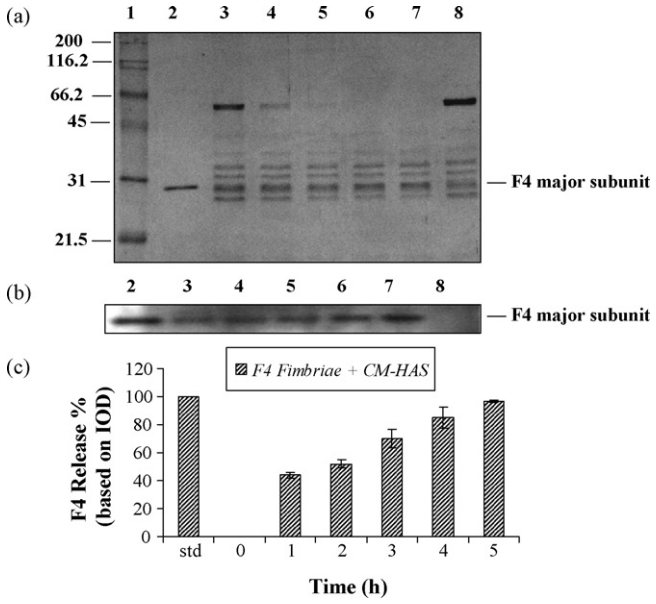

Fig. 4. Delivery of F4 *fimbriae* in simulated intestinal fluid containing pancreatin (SIF/USP). (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; (b) Western blotting; (c) densitometry analysis of Western blotting films. Electrophoretic profiles: molecular weight protein markers (kDa) (lane 1); free (non-formulated) F4 *fimbriae* in phosphate buffer solution (0 min) (2); F4 formulated with CM-HAS after 1 h in simulated gastric fluid (pepsin) and 1 h (3), 2 h (4), 3 h (5), 4 h (6), 5 h (7) in simulated intestinal fluid containing pancreatin (SIF); SIF containing pancreatin only (8). Volume of *fimbriae* sample: 10 μL/well; IOD, integrated optic density ($n = 3$).

ated more rapidly (Fig. 4a–c). In order to understand the release mechanisms of formulated *fimbriae*, water uptake and erosion of CM-HAS matrices (containing the polymer only) were evaluated. It is known that starch gelatinization and derivatization generate a loss of crystalline order of starch grains (Dumoulin et al., 1998; Ispas-Szabo et al., 2000). Because of this loss of crystallinity, the water will enter more easily into the CM-HAS polymeric tablet. Thus, the contact of the non-cross-linked CM-HAS matrix with the dissolution medium results in hydration of the system. As the carboxymethylation process results in a water-soluble starch, hydration and dissolution of the matrix can occur at the same time as the erosion. The percentage of CM-HAS matrix water uptake and of erosion as a function of time, in the presence and absence of the pancreatin, are presented in Fig. 5. Initially, an increase in water uptake was observed during the first hour of SIF treatment, followed by a decrease

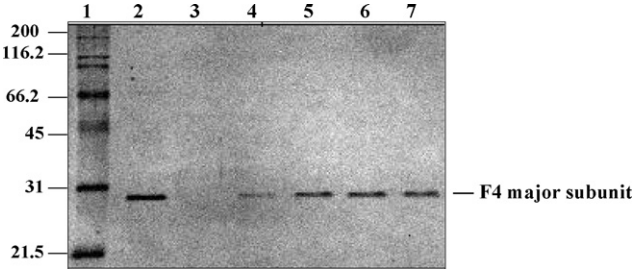

Fig. 3. Delivery of F4 *fimbriae* in pancreatin-free simulated intestinal fluid (SIF/USP). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Electrophoretic profiles: molecular weight protein markers (kDa) (lane 1); F4 *fimbriae* formulated with CM-HAS – as tablet – in phosphate buffer solution (0 min) (2), after 1 h in simulated gastric fluid (pepsin) and 1 h (3), 2 h (4), 3 h (5), 4 h (6), 5 h (7) in pancreatin-free simulated intestinal fluid. Volume of *fimbriae* sample: 10 μL/well ($n = 3$).

of water uptake after 2 h of treatment (Fig. 5a). This could be due to dissolution and erosion (Fig. 5b) of the polymeric matrices. The presence of pancreatin (with alpha-amylase enzymatic activity) in SIF significantly increased the degradation rate of the modified starch compared with that in SIF in the absence of pancreatin, when the erosion rate of the matrix was lower (Fig. 5b). The fact that CM-HAS can act, within certain limits, as an alpha-amylase substrate is important since the delivery can be modulated by duodenal alpha-amylase. This behavior differentiates CM-HAS from other excipients (i.e. CM-cellulose). Therefore, due to erosion of polymeric matrix in the presence of pancreatin, the F4 *fimbriae* are liberated faster than in the SIF medium in the absence of pancreatin. Fig. 5c shows the water regain normalized as a percentage of water remaining in the CM-HAS tablets in function of time, with and without pancreatin. This normalization is required, considering the tablet erosion. In both cases, the plateau corresponds to the real time water content (%), taking into account the loss of tablet mass due to erosion. The hydration is very fast in the first hour (SIF), but after 2–3 h, the erosion became determinant, explaining the apparent decrease of water uptake (Fig. 5a) and plateau after normalization (Fig. 5c). At this stage, the water uptake by the tablet was accompanied by a water loss and matrix dissolution and erosion, keeping the water percentage in the remaining tablets constant. The CM-HAS matrices underwent hydration, dissolution and bio-erosion at the same time, thus explaining the negative values obtained at 4–5 h in the study of CM-HAS water uptake (Fig. 5a) in the presence of pancreatin.

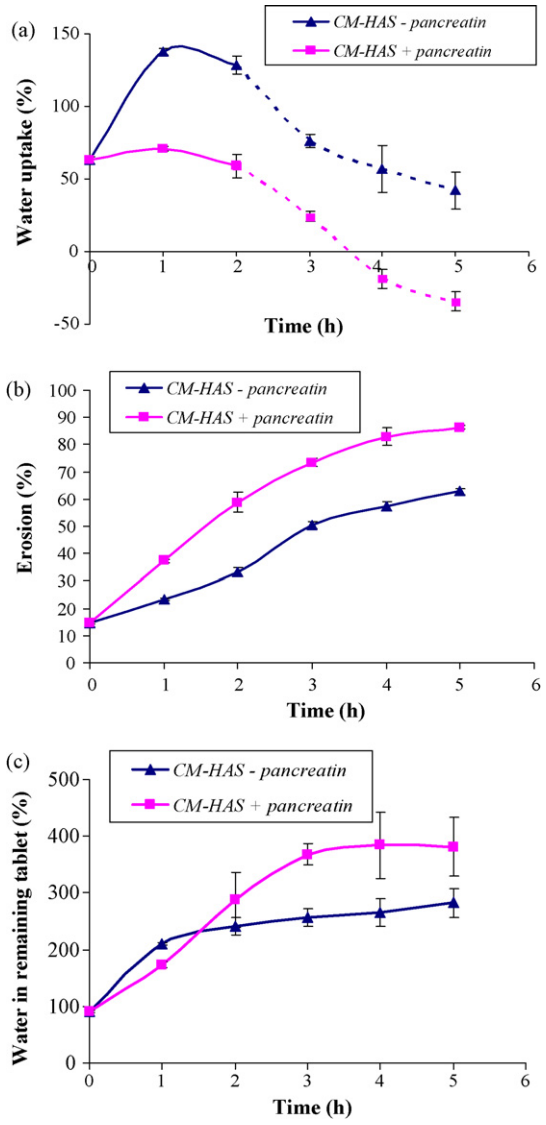

Fig. 5. Water uptake and erosion of CM-HAS tablet matrices. (a) Water uptake (%), (b) erosion (%) and (c) water normalized (%) for the mass of remaining eroded tablet. Tablets were treated for 1 h in simulated gastric fluid, followed by 5 h in simulated intestinal fluid with or without pancreatin ($n = 3$). Dashed line: decrease of water uptake due to dissolution and erosion of the polymeric matrices, which are faster than the swelling.

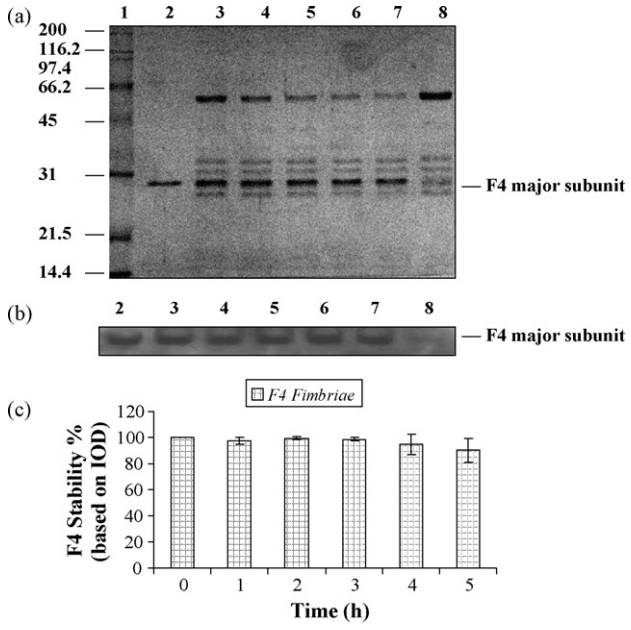

Fig. 6. Stability of F4 *fimbriae* in simulated intestinal fluid (SIF/USP). (a) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis; (b) Western blotting; (c) densitometry analysis of Western blotting films. Electrophoretic profiles: molecular weight protein markers (kDa) (lane 1); free (non-formulated) F4 *fimbriae* in phosphate buffer solution (0 min) (2) and after 1 h (3), 2 h (4), 3 h (5), 4 h (6), 5 h (7) in simulated intestinal fluid containing pancreatin (SIF); SIF containing pancreatin only (8). Volume of *fimbriae* sample: 10 μL/well; IOD, integrated optic density ($n = 3$).

It was found that the presence of pancreatin in SIF does not affect the structural stability of the F4 *fimbriae* major subunits after 4–5 h of SIF treatment as shown by SDS–PAGE (Fig. 6a) and Western blotting (Fig. 6b). Statistical analysis using a one-way Anova pair-wise comparisons test followed by a Tukey HSD test showed that there were not statistically significant differences in terms of IOD between F4 *fimbriae* incubations at time 0 and after 4 and 5 h of treatment in SIF medium containing pancreatin (Fig. 6c). Therefore, the formulated *fimbriae*, once liberated in SIF, would retain their antigenicity and their receptor binding activity which are essential for the induction of an intestinal mucosal immune response. We will test the effect of this F4 formulation on the ability of orally administered F4 *fimbriae* on the induction of a localized mucosal specific IgM and IgA antibody response in the jejunum and ileum of weaned pigs, which are the sites where most F4-positive ETEC colonization and induction of the physiological changes of diarrhea occur.

In conclusion, the CM-HAS hydrophilic polymer protected the F4 *fimbriae* against degradation by pepsin and conferred to the tablet formulation an appropriate combination of hydration, dissolution and erosion mechanisms allowing the delivery of a functional bioactive agent. The protection of F4 *fimbriae* by the CM-HAS biodegradable matrix could result in a more effective piglet vaccination than for the free F4 *fimbriae*, possibly due to a higher F4 antigen load in the jejunum of the pig. Further *in vivo* studies on piglets will give more answers on the conditions in which these formulations can protect the immunization capacity of F4 *fimbriae*.

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