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Carboxylated high amylose starch as pharmaceutical excipients Structural insights and formulation of pancreatic enzymes

Louis Philippe Massicotte, Wilms Emmanuel Baille, Mircea Alexandru Mateescu *

Department of Chemistry and Centre BioMed, Université du Québec à Montréal, CP 8888, Succursale A, Montreal, Quebec H3C 3P8 Canada

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Abstract

Carboxymethyl high amylose starch (CM-HAS) and succinate high amylose starch (S-HAS) were proposed as pharmaceutical excipients for oral drug delivery, providing a significant gastroprotection to dosage forms of pancreatic enzymes (alpha-amylase, lipase and trypsin) compared to unprotected enzymes. In acidic medium, carboxylic groups are protonated (at least in tablet surface) ensuring local buffering properties and giving a compact shape of the tablets. The enzymes were formulated individually or in association as three enzymes formulation. After the first hour of incubation (over a 2 h experiment) in simulated gastric fluid (SGF), the three pancreatic enzymes retained an overall (average of the three enzymes) activity of 72% when formulated as tablets with CM-HAS excipient and 77% when formulated with S-HAS excipient. Furthermore, after incubation in SGF, the delivery of 75% of the total remaining enzymatic activity in the simulated intestinal fluid (SIF) taken 180 and 170 min for CM-HAS and S-HAS, respectively. Both formulations with carboxylated starch as excipient have a high loading capacity (up to 70–80% enzymes), which is of interest for pancreatic enzymes replacement therapy of pancreatitis. An advantage of these formulations is that gastroprotection is afforded by the carboxylated matrices (carboxylic groups), without enteric coating.

Keywords: Carboxymethyl high amylose starch; Succinate high amylose starch; Alpha-amylase; Lipase; Trypsin; Oral drug delivery

1. Introduction

The interest in drug delivery systems (DDS) able to release active agents over long periods (controlled delivery) or at a precise location and at a precise time (chrono delivery) is continuously growing. Such delivery, triggered by the environment or other external events makes possible a desired therapeutic effect at the appropriate site and with a drug concentration within the therapeutic window.

A wide range of polymeric matrices, natural or synthetic, is now used as excipients in DDS. Various synthetic matrices such as polyamides, polyamino acids, polyacrylamides, polycyanoacrylates, polyesters, polyurethanes and others have been used for various drug formulations (Jain, 2000). Cross-linked high amylose starch (HAS-CL) has been previously introduced as excipient for monolithic dosages forms able to control drug release over 18–24 h (Lenaerts et al., 1991, 1998). Furthermore, acetate (Ac-HAS-CL), aminoethyl (AE-HAS-CL) and carboxymethyl high amylose starch (CM-HAS-CL) derivatives of HAS-CL were proposed as matrices for drug sustained release, allowing a high drug loading (more than 60%) of tablets (Mulhbacher et al., 2001). Starch derivatives, including uncross-linked CM-starch and succinate starch (S-starch) are known from more than 40 years, being used particularly in food industries (Wurzburg, 1986). There are other commercial carboxylated starch excipients (Explotab™, Primojel™ and Vivastar P™) but they are cross-linked and used as disintegrants (Edge et al., 2002). Thus, they are clearly differing to our carboxylated starch which are not cross-linked and proposed as matrixes for delayed release. The CM-starch is not new as excipient for pharmaceutics. Its sodium salt form is commercialized under different brand names such as Explotab™, Primojel™ and Vivastar P™ mostly as disintegrants (Edge et al., 2002). The CM-HAS prepared in our conditions does not possess disintegrant properties, as previously shown (Calinescu et al., 2005).

The novelty of the carboxylated uncross-linked starch consists in their use as excipients for gastro-resistant coating-free oral formulations. Recently, carboxymethyl high amylose starch (CM-HAS) was proposed as a novel excipient for oral tablet for-

* Corresponding author. Tel.: +1 514 987 4319.
E-mail address: mateescu.m-alexandru@uqam.ca (M.A. Mateescu).

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mulation (Mulhbacher et al., 2004). This excipient allowed, for instance, oral formulations of *Escherichia coli*, ensuring protection of bioactive agent in the stomach and its delivery in the intestine (Calinescu et al., 2005, 2007). CM-HAS was thought to afford resistance in gastric fluid because of the presence of the carboxylate groups. In gastric (acidic) medium, tablets are protonated (at least in surface) ensuring thus local buffering properties and giving a compact shape of the tablets. Protonated carboxylic groups from neighboring chains can be dimerized by dipole and hydrogen association, stabilizing thus the network (Calinescu et al., 2005). In intestinal medium, protons are changed with sodium ions and tablets swell, releasing the active agent. As for the succinate starch, at our knowledge it was not used until now as pharmaceutical excipient. Since CM-HAS and succinate HAS (S-HAS) share similar but not identical carboxylated compositions, these matrices were both investigated as novel excipients for oral tablet formulation of pancreatic enzymes in pancreatitis therapy.

The pancreas secretes digestive enzymes (amylase, lipase and trypsin) that help digestion of carbohydrates, fats and proteins in food. Patients with severe pancreatic exocrine dysfunction cannot properly digest food. Steatorrhea and malabsorption due to pancreatic insufficiency are among the important features of chronic pancreatitis. Steatorrhea occurs prior to malabsorption because impaired synthesis and secretion of lipase, its shorter intraluminal survival or the lack of lipase activity which are not compensated by non-pancreatic mechanisms (Layer et al., 2001). Therefore, for these patients, replacement pancreatic enzymes are required. There are some commercial pancreatic enzymes, most of them formulated with enteric coating such as Cotazyme-S® (Organon Inc., West Orange, NJ), Entolase HP® (A.H. Robins Co. Inc., Richmond, VA), and Zymase® (Organon Inc., West Orange, NJ).

In this study, pancreatic enzymes (α-amylase, lipase and trypsin) were incorporated into CM-HAS and S-HAS matrices, without enteric coating. An advantage of our carboxylated starch matrices is that the whole monolithic device is pH sensitive, not only the coating. Moreover, the erosion of these modified starches can be modulated by duodenal α-amylase, enhancing thus the release of bioactive agents. In addition, such coating-free tablet formulations are also easier to produce, particularly at industrial scale. Furthermore, this type of formulation can be advantageous for certain patients with cystic fibrosis, with poor neutralization of gastric acid in the duodenum (Choi et al., 2001). In these cases, enteric coatings are not always suitable for pancreatic enzyme formulations. The aim of the study was to evaluate *in vitro* whether CM-HAS and S-HAS can ensure gastric protection of therapeutic agents against acidic/enzymatic denaturation and to determine the pattern of their liberation once the tablets are in the intestinal neutral pH.

2. Materials and methods

2.1. Materials

High amylose starch (Hylon VII) was obtained from National Starch (USA). Microbial α-amylase from *Bacillus* species (EC 3.2.1.1, with a specific activity of 839 units/mg solid), microbial lipase from *Candida rugosa* (EC 3.1.1.3, with 1180 units/mg solid), porcine pancreatic trypsin (EC 3.4.21.4, with 16,000 units/mg solid) and porcine stomach mucosa pepsin (EC 3.4.23.1, with 378 units/mg solid), as well as the other reagents, were obtained from Sigma–Aldrich Corp. (USA) and were used without further purification.

2.2. Carboxymethyl high amylose starch synthesis

The excipient was synthesized following Mulhbacher et al. (2001) with minor modifications. A quantity of 70 g of Hylon VII (HAS) was suspended in 150 mL of H_2O, stirred at 50 °C and then 200 mL of 1.7 M NaOH were added continuing the stirring for another 20 min at 50 °C. A volume of 45 mL of 10 M NaOH and then 36 g of monochloroacetic acid (MCA) dissolved in minimal H_2O volume, were added to the mixture and stirred for 1 h. The mixture was neutralized with acetic acid and cooled at room temperature. Salt ions were removed by washing the slurry with a solution of acetone/H_2O (7:3, v/v) followed by repeated filtration and resuspension. The slurry was then precipitated with acetone, filtered and the CM-HAS was recovered. The material was dried at air overnight.

2.3. Succinate high amylose starch synthesis

An amount of 70 g of HAS was suspended, as for CM-HAS, in 150 mL of H_2O (under stirring at 50 °C). Then a volume of 200 mL of 1.7 M NaOH was added to the mixture, keeping the stirring at 50 °C for 60 min. The pH was then adjusted to 8.0 with acetic acid, the mixture was cooled-down to room temperature and the medium was adjusted to 1.3 L with H_2O. A quantity of 11 g of succinic anhydride was then added to the mixture keeping the pH for reaction between 8.0 and 8.4 with 1 M NaOH. Once the pH is stabilized, the stirring was continued for another 10 min. Salt ions removing, washing the slurry, S-HAS recovery and powder drying were done as for CM-HAS.

2.4. Fourier transform infrared spectroscopy (FTIR)

FTIR spectroscopic analysis was carried out using a BOMEM (Hartmann & Braun) spectrophotometer (Model MB-series, Quebec City, Canada). Before analysis, HAS, CM-HAS and S-HAS powders were dried and stored in a desiccator prior to prepare KBr discs. Samples of 10 mg of HAS, CM-HAS or S-HAS were respectively mixed with 90 mg of KBr and about 20 mg of the mix was used to compress (6 T) a thin disc of 9 mm diameter. Spectra from 4000 and 400 cm^{-1} were recorded at 4 cm^{-1} resolution with a total of 48 scans for each sample.

2.5. Determination of the degree of substitution

2.5.1. Potentiometric titration of carboxylic groups

The degree of substitution (DS) was determined by potentiometric titration of carboxyl groups with 0.2 M NaOH solution and expressed in milliequivalents of functional groups per gram of polymeric powder (meq./g). A quantity of 1.5 g of matrix was

suspended in 40 mL 0.1 M HCl solution for 20 min under stirring (for conversion of carboxylate groups into carboxylic groups), then filtered and washed with acetone/H_2O (7:3) to eliminate HCl excess. The powder was then washed with acetone and dried. A mass of 1 g was dissolved in 50 mL H_2O and titrated. This value was converted in DS (as percentage of the hydroxyl groups of the polymer that have been substituted from the total number of available hydroxyl groups).

2.5.2. Sample hydrolysis and determination of the degree of substitution by ^1H NMR

The CM-HAS sample was hydrolyzed for determination of the degree of substitution. A quantity of 0.1 g of CM-HAS was dispersed in 1 mL of perchloric acid (70%), and after 10 min at room temperature it was diluted with 9 mL of distilled water. This mixture was kept at 90 °C overnight. The obtained solution was neutralized with 2 M KOH and kept at 4 °C for 4 h until a complete precipitation of the $KClO_4$. The salt was filtered off and the solution obtained was freeze-dried. The dry product was dissolved in D_2O and ^1H NMR spectrum was measured at 90 °C on a Varian Gemini 300 operating at a frequency of 300.0 MHz for protons. Measurement was performed using 128 scans with a repetition time of 30 s.

2.6. Atomic absorption spectroscopy

Solutions containing 200 mg/L of pregelatinized and retrograded (R)-HAS, 10 mg/L of CM-HAS or 10 mg/L of S-HAS were dissolved in nanopure water and filtered on a 0.45 μm filter before they undergo desolvation and vaporization in a high-temperature acetylene–air flame [SpectrAA 220FS atomic absorption spectrometer (Varian)]. A standard curve was made using NaCl (0.2 to 1 mg/L) dissolved in nanopure water. Sodium in the solutions was determined at 589 nm (Na lamp).

2.7. Differential scanning calorimetric (DSC) analysis

Heat properties of starch and of its carboxymethyl and succinyl derivatives were studied using a DSC analyzer [TA Instrument, Model 2910, New Castle (Delaware), USA], at a heating rate of 10 °C/min between 25 and 240 °C. The instrument was first calibrated with two empty compacted aluminum pans (baseline calibration). A second step of calibration was done with an empty compacted aluminum pan as reference and a compacted aluminum pan containing indium as standard. Each sample was placed in an aluminum pan, compacted, and scanned under helium flow, with an empty pan as reference.

2.8. Thermogravimetric analysis (TGA)

Thermogravimetric decomposition analysis was performed with a TA Instrument [Model 2950 Hi-Res, New Castle (Delaware), USA] under nitrogen atmosphere at a heating rate of 20 °C/min, between 25 and 800 °C. Before analysis, the instrument was calibrated with calcium carbonate as reference material.

2.9. Direct compression of tablets

The excipient powder (CM-HAS or S-HAS) was mixed manually with the bioactive agent powder(s) [enzyme(s)] until complete homogenization. No other component (lubricant, glidant, etc.) or coating was added. Tablets (9 mm diameter, 2 mm thickness) of 200 mg (total weight) were obtained by direct compression at 3.0 T (Carver hydraulic press) of the corresponding mixed powders of excipient and bioactive agent.

2.10. Determination of the enzymatic activities

The α-amylase activity was determined by the reductimetric method of Noelting and Bernfeld (1948) using dinitrosalicylic acid (DNS) to reveal (535 nm) the reducing products of amylolysis (maltose and dextrins). The enzyme was incubated 3 min at room temperature with 1% soluble starch as substrate and stopped with DNS reagent.

The lipase activity was determined following Cherry and Crandall (cited by Henry et al., 1957), using an emulsion of olive oil as substrate. Practically, 4.5 mL olive oil, 18 mL H_2O, 2.5 mL 0.2 M Trizma buffer (pH 7.7), 2.5 g gum arabic, 1 mL of solution containing 0.015 M $CaCl_2$, 1.2 M NaCl and 0.011 M Sodium taurodexoycholate. A volume of 2.5 mL of emulsion was incubated for 2 h at 37 °C with 200 μL of enzyme solution. Two drops of thymolphthalein 0.9% (w/v) pH indicator was added to the emulsion and the enzymatic activity was determined by the titration of the product (oleic acid) with 0.2 M NaOH until the solution turned to a light blue color.

The trypsin activity was determined by the method of Bergmeyer et al. (1974) using a 0.67 mM sodium phosphate buffer (PB) solution (pH 7.6) containing 0.25 mM *N*α-benzoyl-L-arginine ethyl ester (BAEE). A volume of 200 μL of the enzymatic solution was placed in 3 mL of BAEE solution. The increase of absorbency at 253 nm was recorded at room temperature for 5 min and the ΔA_{253nm}/min rate was used to determine the enzymatic activity.

2.11. Stability of pancreatic enzymes formulated with CM-HAS and S-HAS in simulated gastric fluid (SGF)

Tablets (200 mg) based on CM-HAS or S-HAS and were formulated to contain 10, 15 or 20 mg of each enzyme (depending of the enzyme activities and the sensibility of the enzymatic assays). Tablets were placed individually in 50 mL of SGF (pH 1.2 containing 2560–8000 units/mL of pepsin) (U.S. Pharmacopeia & National Formulary, 2000), for different times at 37 °C, under constant agitation at 50 rpm in a controlled environment incubator shaker (New Brunswick Scientific Co. Inc., N.J., USA) After each chosen period of incubation in SGF, the tablets were transferred into 50 mL of 50 mM PB (pH 7.2) and crushed in order to release the enzymatic activities to be evaluated. Native free (non-formulated) pancreatic enzymes and the enzymes formulated with retrograded HAS (non-substituted) in form of tablets (200 mg) were incubated in SGF in similar conditions.

2.12. Delivery of enzymes in the simulated intestinal fluid (SIF)

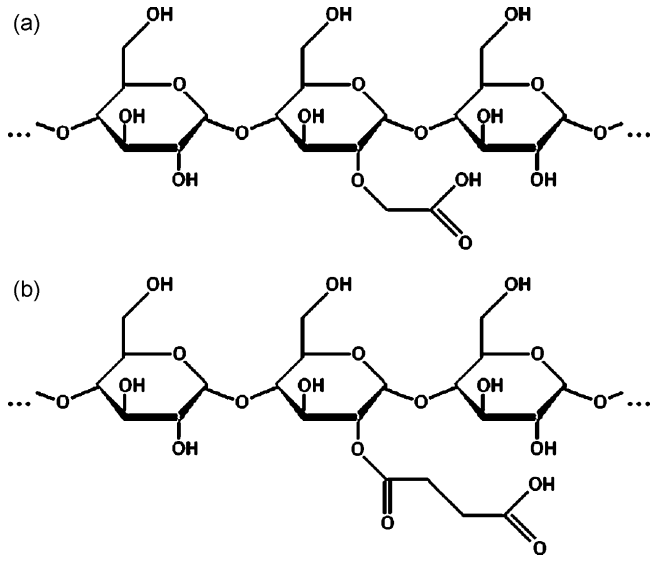
Tablets based on CM-HAS or S-HAS and containing 10–20 mg of each enzyme, were placed individually in 50 mL of SGF for 1 h at 37 °C under constant agitation (50 rpm) in the controlled environment incubator shaker. The tablets were then transferred in 50 mL SIF (50 mM PB) in the same conditions (50 rpm, 37 °C) and small aliquots (10–200 μL) were taken at regular intervals to determine the enzymatic activity released in function of time. The values were normalized, considering the maximal enzymatic activity released from SGF treatment and total tablet dissolution in SIF, as 100%.

2.13. Loading capacity of CM-HAS and S-HAS with active agent

Tablets of 200 mg (total weight) based on CM-HAS or S-HAS and containing 10, 20, 40, 80, 120, 160 or 200 mg of each enzyme, were placed individually in 50 mL of SGF (1 h at 37 °C under 50 rpm agitation) using the incubator shaker. After the incubation period in SGF, the tablets were transferred into 50 mL of 50 mM PB (pH 7.2) and then crushed. Each enzymatic activity was determined by the methods described above.

2.14. Polyacrylamide gel electrophoresis

For SDS-PAGE (Laemmli, 1970), gels (10–12%, 0.75 mm spacers and 10 wells combs) were loaded with: (i) 30 μg of individual native free pancreatic enzyme; (ii) 30 μg of the enzyme formulated with CM-HAS or S-HAS; (iii) 30 μg of free enzyme after 120 min of incubation in SGF (37 °C, 50 rpm) and (iv) 30 μg of the enzyme formulated with CM-HAS or S-HAS after 120 incubation in SGF (37 °C, 50 rpm). The tablets (200 mg) loaded with 20 mg α-amylase, lipase or trypsin and each free enzyme were separately maintained (incubator shaker) at 37 °C and 50 rpm for 2 h in 25 mL of SGF (pH 1.2) containing pepsin or, as a positive control, dissolved in 25 mL of 50 mM PB (pH 7.2). After the incubation period, the SGF was brought to pH 7.2 with 1 M NaOH solution. After a complete disintegration of the tablets, a volume of 400 μL of each enzymatic solution was precipitated with acetone (1:5 v/v) at −20 °C, centrifuged, dried and dissolved in 125 μL of a standard electrophoresis solution containing bromophenol blue. The gels were loaded with: standard broad range molecular weight (lanes 1 and 8), native enzyme (lane 2), enzyme formulated with CM-HAS incubated in PB but not in SGF (lane 3), enzyme formulated with S-HAS and incubated in PB but not in SGF (lane 4), native free enzyme incubated in SGF (lane 5), enzyme formulated with CM-HAS and incubated in SGF (lane 6), enzyme formulated with S-HAS and incubated in SGF (lane 7). The gels for α-amylase and lipase (10% bis-acrylamide) and for trypsin (12% bis-acrylamide) were run at 120 V until the migration front (bromophenol blue) reaches the end of the gel. The gels were stained with Coomassie brilliant blue R-250 and patterns photographed with a digital camera.



Scheme 1. Structures of (a) carboxymethyl high amylose starch and (b) succinate high amylose starch.

3. Results

The CM-HAS is a starch derivatized with a two-carbon carboxymethyl group linked as an ether (Scheme 1a) whereas S-HAS is derivatized with a four carbons group via an ester bond (Scheme 1b).

3.1. Structural aspects

The FTIR spectra of HAS and of its carboxymethyl (Fig. 1a) and succinyl (Fig. 1b) derivatives indicate the presence of the carboxyl functions (a new peak at 1618 cm^{-1} which corresponds to a carboxylate salt). As expected, the succinylation of starch shows (Fig. 1b) two characteristic absorptions, an ester peak at 1738 cm^{-1} and a carboxylate peak at 1580 cm^{-1}. These results (Table 1) confirm that carboxymethylation and succinylation occurred, as expected.

Both excipients are carboxylated starch and were synthesized in conditions chosen to give DS close to each other (i.e. 5.1% for CM-HAS and 5.6% for S-HAS). It is known that cellulose and starch carboxymethylation occurs, as a general rule, at various rates for different hydroxylic groups to be substituted. Using NMR data, Zhang et al. (1992) reported that carboxymethylation of starch yields a hydroxyl substitution preference decreasing in order: –OH in C2 > –OH in C6 > –OH in C3.

Table 1
FTIR of HAS and its carboxymethyl and succinyl derivatives

Samples	Wavenumber (cm^{-1})	
	Ester group	Carboxyl group
HAS	–	–
CM-HAS	–	1618[a]
S-HAS	1738[b]	1580[a]

[a] Predicted values are between 1610 and 1550 for carboxylate function.
[b] Predicted values are between 1750 and 1725 for ester function.

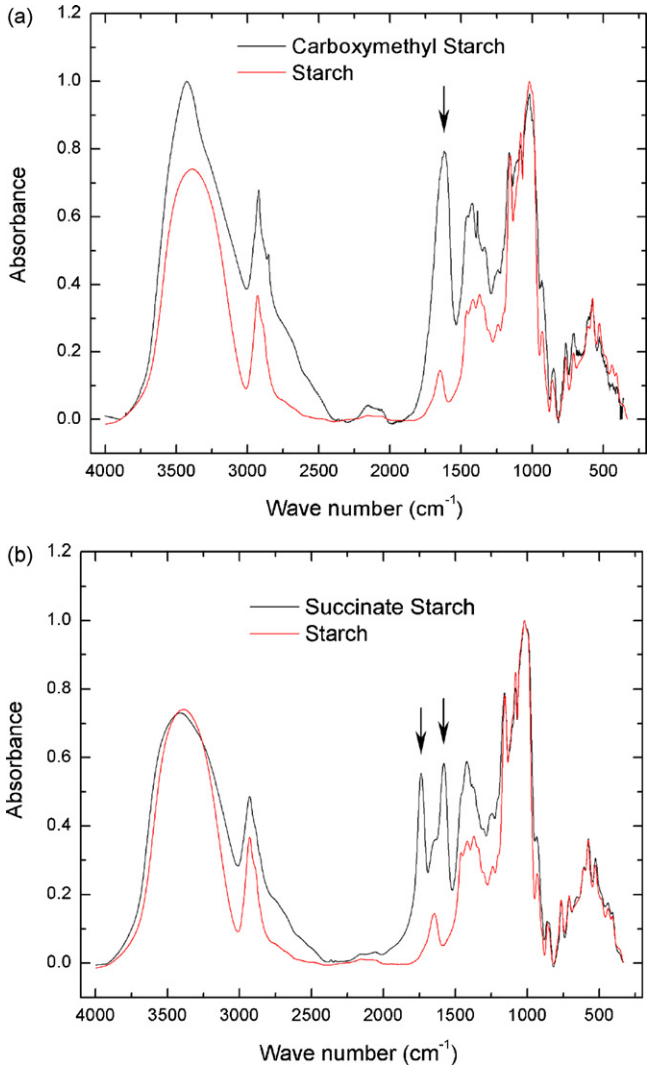


Fig. 1. FTIR spectra of HAS and its (a) carboxymethyl and (b) succinyl derivatives. Samples of 10 mg of HAS, CM-HAS or S-HAS were respectively mixed with 90 mg of KBr and about 20 mg was used to compress (6 T) a thin disc of 9 mm diameter. Arrows indicate peaks generated by derivatization.

The DS was also detected by ^{1}H NMR of the hydrolyzed CM-HAS (perchloric acid) (Heinze et al., 2000) which also allows to evaluate the distribution of the CM functional group within the glucose units. Fig. 2 shows the assignment of the peaks for CM-HAS and the DS values corresponding to the peaks were determined using the equations of Heinze et al. (2000). The results obtained show that mostly the positions 2 (DS 5.7%) and 3 (DS 2.3%) on the glucose units have been substituted. This suggests that the reactivity of position 3 is at least three times lower than position 2 of glucose units of starch due to the overall conformation. The total DS value (DS 8.0%) obtained by ^{1}H NMR spectroscopy fits well the one determined by titration (DS 5.1%).

As expected, a quantity of salt remained in the excipients even after washing the polymer. The atomic absorption data showed that the CM-HAS and S-HAS powders contained respectively, 4.82% and 6.25% sodium (w/w), whereas retrograded HAS (partly recrystallized high amylose starch) contained only 0.31%. The difference in the sodium content between the two carboxylated excipients is rather limited to affect their properties.

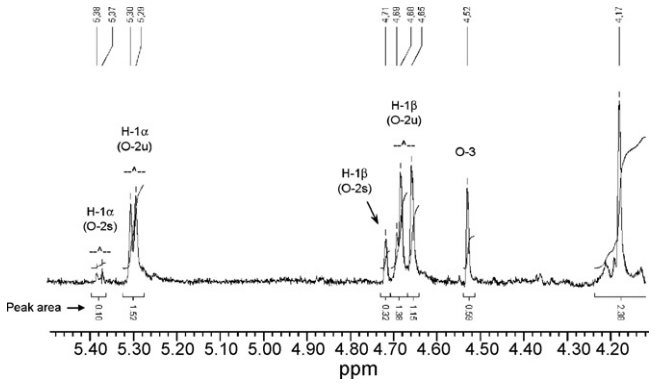


Fig. 2. ^{1}H NMR spectrum of CM-HAS. Peak assignment: H-1 (hydrogen atom at the anomeric carbon); α, β (configuration of glucose); S (substituted position); u (unsubstituted position) and O (oxygen atom at positions 2 and 3).

Table 2
DSC analysis of HAS and of its carboxymethyl and succinyl derivatives

Samples	DSC characteristics of peaks		
	T_{onset} (°C)	T_{peak} (°C)	ΔH_f (J/g)
HAS	180	191	204
CM-HAS	173	177	218
S-HAS	91	124	350

3.1.1. Differential scanning calorimetric (DSC) analysis

DSC analyses of HAS and of its carboxymethyl and succinyl derivatives show that the amount of crystalline domains of succinyl derivative is more important than that of HAS and of carboxymethyl derivative (Fig. 3a). The change in enthalpy (ΔH) obtained for the succinyl derivative is 1.7 times that of HAS, whereas values of ΔH close each other were found for starch and its carboxymethyl derivative (Table 2). However, the temperatures of fusion suggest that the overall crystalline domain of starch is larger than that of the two derivatives because for HAS the temperature needed to breakup these domains, is higher (Table 2).

3.1.2. Thermogravimetric analysis (TGA)

The water and volatile content of 10% (Table 3) fits well with the known 5–10% water content of starch. TGA profiles illustrated in Fig. 3b show that thermal stability of CM-HAS derivative is higher in comparison to that of native HAS, as

Table 3
TGA of HAS and its carboxymethyl and succinyl derivatives

Samples	TGA characteristics of peaks		
	Volatile (water) content (%)	$T_{10\%}$ (°C)	T_{peak} (°C)
HAS	10	244	262
CM-HAS	10	272	286
S-HAS	10	232	259

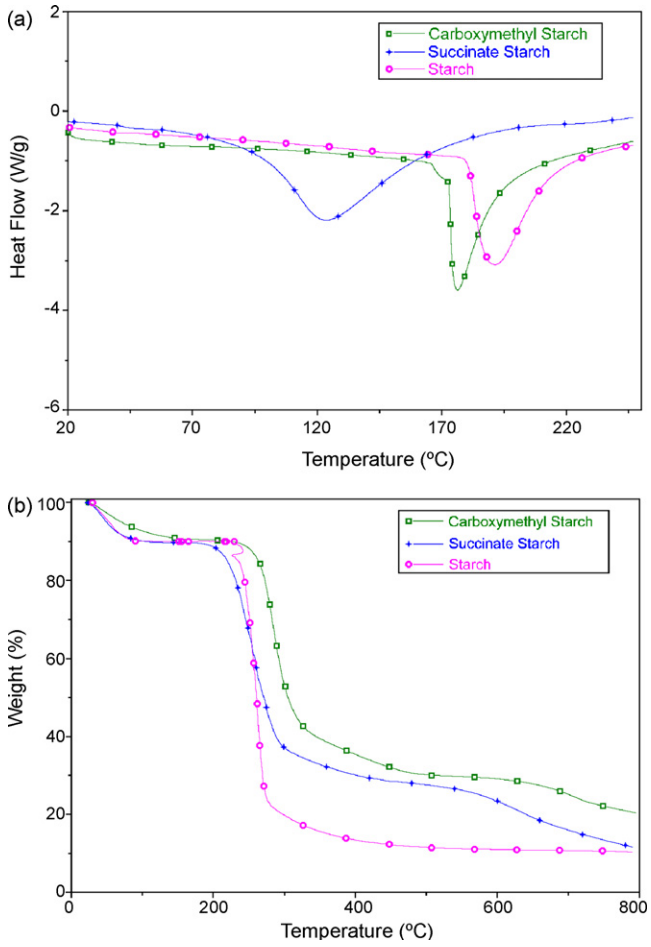


Fig. 3. DSC (a) and TGA (b) of HAS and its carboxymethyl and succinyl derivatives. For each DSC run, samples (5–10 mg powder) were heated at a rate of 10 °C/min between 25 and 240 °C ($n = 2$). For, thermogravimetric decomposition the heating rate was of 20 °C/min, between 25 and 800 °C ($n = 2$).

clearly illustrated by the temperature corresponding of 10% of mass loss (degradation of 10% of the mass of the polymer, excluding water and volatile) (Table 3). For instance, $T_{10\%}$ for HAS was 244 °C whereas for CM-HAS derivative, $T_{10\%}$ increases to 272 °C. However, when compared with HAS, the $T_{10\%}$ of S-HAS shows a moderate decreased effect on the degradation profile.

3.2. Stability of pancreatic enzymes formulated with CM-HAS and S-HAS in simulated gastric fluid (SGF)

The free (non-formulated) enzymes were, each of them, practically inactivated in less than 30 min of incubation in SGF (with pepsin): α-amylase lost 98% of its initial activity in 5 min, lipase 97% of initial activity in less than 10 min and trypsin 92% of activity within 30 min (Fig. 4).

When 10 mg α-amylase as active agent was formulated with the CM-HAS excipient in tablets of 200 mg, the enzyme conserved after 60 min incubation in SGF, 75% of the initial activity (Fig. 4a) and when formulated with S-HAS excipient, it retained 51% of activity (Fig. 4b). The lipase (20 mg) conserved 69% of its initial activity when formulated with CM-HAS (Fig. 4a) and only 37% with S-HAS as excipient (Fig. 4b). Trypsin (10 mg) retained 66% of initial enzymatic activity when formulated with CM-HAS (Fig. 4a) and 56% of its activity when formulated with S-HAS (Fig. 4b). Therefore, when the enzymes are formulated individually, CM-HAS affords a better protection than S-HAS, for the same incubation time in SGF.

The loading of tablets for this stability study was deliberately low (10–20 mg enzyme) in order to have a clear effect of the carboxylated starch matrices (thus associative effects protein–protein that can occur at high loading and altering stability data, are avoided).

The protection afforded by CM-HAS and S-HAS to the pancreatic enzymes was also evaluated by SDS-PAGE (Fig. 5). The free α-amylase incubated in SGF (Fig. 5a, line 5) was clearly irreversibly denatured (with two distinct bands of lesser molecular weight), whereas when formulated, was not (lines 6 and 7). The free lipase was also totally denatured (Fig. 5b, lane 5) whereas when formulated with CM-HAS or S-HAS the enzyme is only in part denatured. Lastly, the gel containing trypsin (Fig. 5c) shows that the free enzyme incubated in SGF did not exhibit any band characteristic to the native enzyme whereas when formulated with CM-HAS or S-HAS, the electrophoretic patterns are similar with those of native enzyme. The enzymes formulated with CM-HAS and S-HAS and incubated

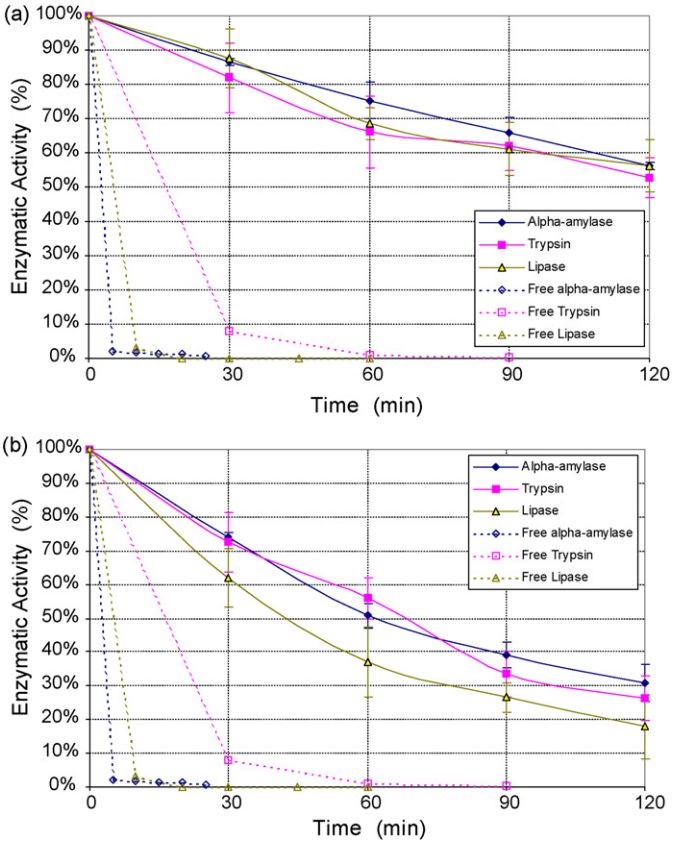


Fig. 4. Stability in SGF of α-amylase, lipase or trypsin formulated with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) loaded with 10 mg α-amylase or 20 mg lipase or 10 mg trypsin, were incubated in 50 mL of SGF containing pepsin in an incubator shaker (37 °C, 50 rpm). The tablets were then transferred in 50 mL of 50 mM PB (pH 7.2), crushed and the liberated enzymatic activity was determined ($n = 3$).

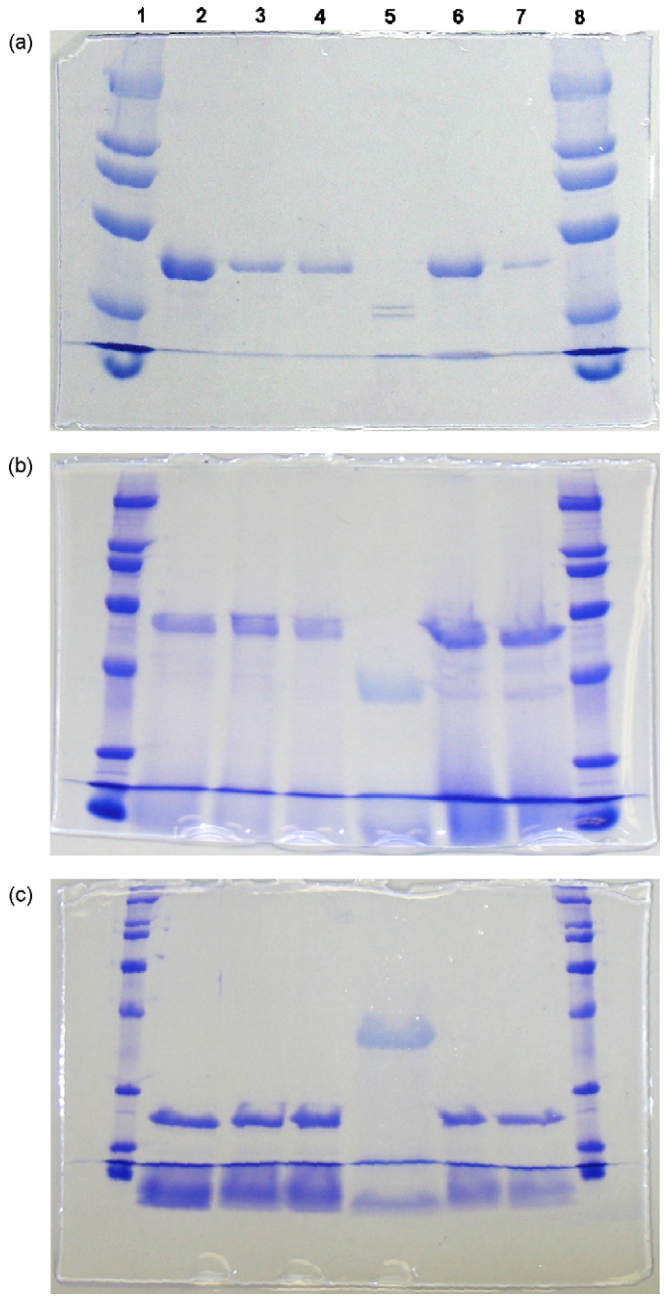


Fig. 5. SDS-PAGE electrophoretic patterns of (a) α-amylase, (b) lipase and (c) trypsin, in native form or in tablets formulated with CM-HAS or S-HAS. The gels were loaded as: lanes 1 and 8 standard broad range molecular weight, lane 2 native enzyme, lane 3 enzyme formulated with CM-HAS, incubated in PB but not incubated in SGF, lane 4 enzyme formulated with S-HAS, incubated in PB but not in SGF, lane 5 native free enzyme incubated in SGF, lane 6 enzyme formulated with CM-HAS and incubated in SGF, lane 7 enzyme formulated with S-HAS and incubated in SGF.

for 120 min in SGF migrate at the same level as the native, non-denatured enzymes, showing a good protection afforded by the two carboxylic excipients. Therefore, both carboxylated excipients afford gastroprotection as shown by lanes 6 and 7, each with a prominent band characteristic of native enzyme. This is in agreement with the results displayed on Fig. 4 showing that the excipients did not provide an absolute protection against gastric denaturation. On the other hand, compression at 3 T did not affect the electrophoretical mobility (lines 3, 4, 6 and 7 of the three enzymes).

When all three enzymes (15 mg each) are formulated together with CM-HAS, the enzymes conserved after 60 min incubation in SGF an overall pancreatic activity (conventionally considered as an average of contribution of each enzyme in the enzyme formulation) of 72% (60.6% α-amylase, 87.4% lipase and 68.6% trypsin) from the initial activity (Fig. 6a). When formulated with S-HAS, they conserved an overall pancreatic activity of 77% (74.0% α-amylase, 86.9% lipase and 70.6% trypsin) from the initial activity (Fig. 6b). (R)-HAS serves as a control to determine the effect of the carboxylation of the matrices thus comparing non-substituted matrices versus carboxylated matrices. The stability of enzymes formulated with (R)-HAS was definitely lower than that of enzymes formulated with CM-HAS or S-HAS (Fig. 6), but higher than that observed with the free, non-formulated enzymes. The S-HAS provides a slightly higher protection than CM-HAS formulations, mostly because α-amylase receives a markedly better protection when formulated with S-HAS than with CM-HAS. These results suggest possible interactions (ionic or hydrogen bonds) between the car-

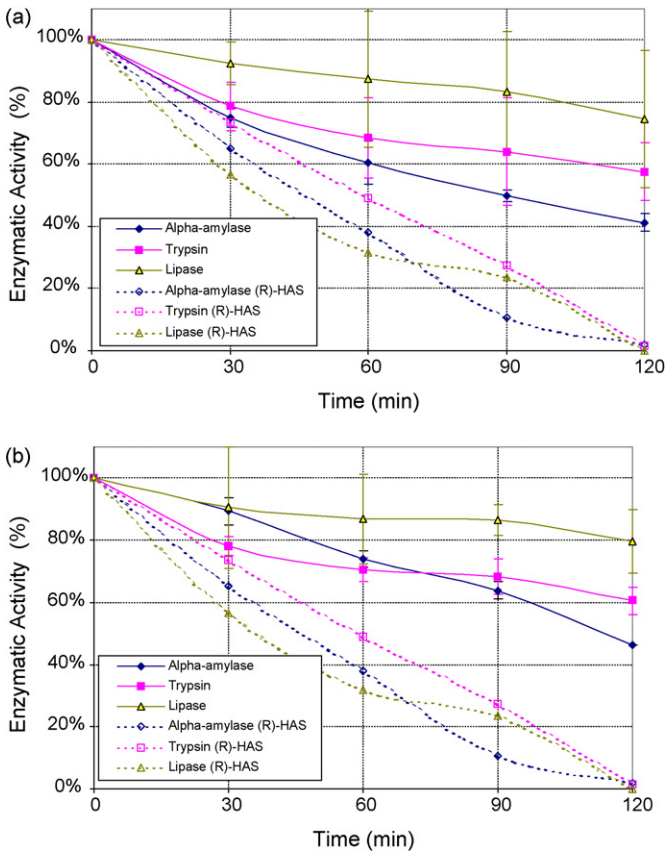


Fig. 6. Stability in SGF of α-amylase, lipase and trypsin formulated together with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) formulated with 15 mg α-amylase, 15 mg lipase and 15 mg trypsin, were incubated in 50 mL SGF containing pepsin in the incubator shaker (37 °C and 50 rpm). The tablets were then transferred in 50 mL of 50 mM PB (pH 7.2), crushed and each enzyme activity was measured ($n = 3$). Tablets (200 mg) containing 15 mg of each enzyme, formulated with (R)-HAS as control, were incubated following the same experimental method above.

boxylic matrices and the formulated proteins, affecting thus the tablet properties and the delivery kinetics.

Variations of DS of CM-HAS matrices (5.1% and 3.3%) and of S-HAS (5.6% and 6.6%) generate some differences in terms of enzyme protection against gastric denaturation. When 15 mg of each of the three enzymes are all together formulated with CM-HAS (DS 3.3%) and incubated for 1 h in SGF, an overall activity of 76% (62.3% α-amylase, 85.0% lipase and 81.7% trypsin) was preserved (figure not shown), maintaining lipase and α-amylase and moderately increasing the recovered trypsin activity. When 15 mg of each enzyme are formulated all together with S-HAS with a slightly higher DS (6.6%), after 1 h incubation in SGF, the overall activity preserved was moderately increased at 85% (73.9% α-amylase, 98.3% lipase and 83.3% trypsin) (figure not shown) mostly due to better protection provided to lipase and trypsin activities.

3.3. Delivery of the pancreatic enzymes in the simulated intestinal medium

After 1 h in SGF, formulations of 10 mg of α-amylase with CM-HAS released 75% of the enzyme after 140 min (Fig. 7a) while the enzyme formulated with S-HAS needs 84 min to achieve the same release (Fig. 7b). Formulation of 20 mg of lipase with CM-HAS gives a release particularly long: about 10% of the total enzymatic activity released after 630 min (Fig. 7a) while with S-HAS excipient 75% of the total enzymatic activity of lipase was released after 295 min (Fig. 7b). Formulation of 10 mg of trypsin with S-HAS excipient released 75% of the total enzymatic activity after 90 min (Fig. 7a), while formulations with CM-HAS, give again very long release times (the structures of tablets remain intact, showing very little degradation) with less than 10% of the total trypsin enzymatic activity liberated after 405 min (Fig. 7b). It appears that the active agent remains trapped inside the tablets since once the tablets are manually broken down, the enzyme activity released is high (Fig. 4). This behavior of CM-HAS tablets suggests some kind of interaction between the excipient and lipase and between the excipient and trypsin.

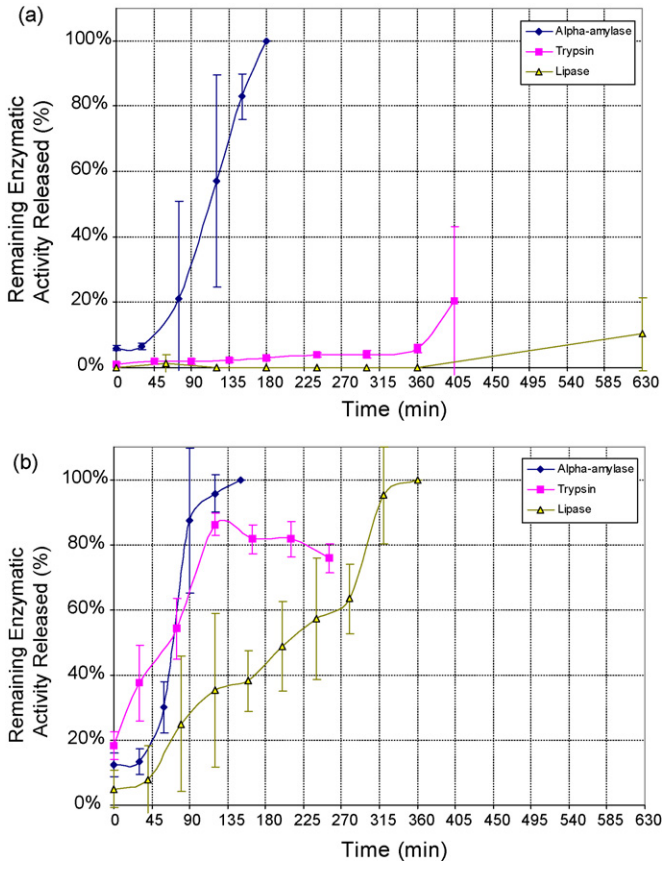


Fig. 7. Delivery in SIF of α-amylase, lipase or trypsin formulated individually with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) formulated with 10 mg α-amylase, 20 mg lipase or 10 mg trypsin were first incubated 1 h in SGF containing pepsin and then transferred in 50 mL of 50 mM PB (pH 7.2) at 37 °C and 50 rpm, withdrawing samples at various intervals to determine their enzymatic activities ($n = 4$).

When all three enzymes (15 mg each) are formulated together with either CM-HAS or S-HAS, the liberation pattern showed substantial differences in comparison with the patterns of enzymes formulated individually. For CM-HAS, 75% of the overall enzymatic activities is released in about 180 min (Fig. 8a) and when formulated with S-HAS, the same percentage of the initial enzymatic activity is released after 170 min (Fig. 8b).

In order to better understand certain disparities between the results with enzymes formulated individually (10–20 mg per tablet) and with the three enzymes combined together (45 mg per tablet), various combinations with only two enzymes were formulated in a tablet. When only α-amylase and lipase (15 mg each) are formulated with CM-HAS, 75% of their total enzymatic activities were released within 98 min (Fig. 9(1a)), while it takes 145 min when the two enzymes are formulated with S-HAS (Fig. 9(1b)). When combining only α-amylase and trypsin (15 mg each) with CM-HAS, 75% of the total enzymatic activity is released in 125 min (Fig. 9(2a)) and in 130 min for the two enzymes formulated with S-HAS (Fig. 9(2b)). These formulations of two enzymes show minor differences between CM-HAS and S-HAS in terms of the release time. Furthermore, for both excipients the release from these bi-enzyme formulations was faster than that of the formulation with all the three enzymes.

The results are different with formulations combining trypsin and lipase (15 mg each). When these two enzymes are formulated together with CM-HAS, 75% of the total enzymatic activity is released at 405 min (Fig. 9(3a)) and when formulated with S-HAS, it takes 295 min for the same level of liberation (Fig. 9(3b)).

3.4. Loading capacity of CM-HAS and S-HAS with active agent

It was found a linear dependency between the number of α-amylase units released and the number of α-amylase units initially contained by the tablets of CM-HAS or S-HAS, up to 40% and almost linear up to 70% loading with enzyme (Fig. 10). Moreover, it was found that although gradually loosing linearity, tablets were in good shape and preserved the enzymatic activities against gastric acidity (1 h incubation in SGF) even when loaded with 80% active agent.

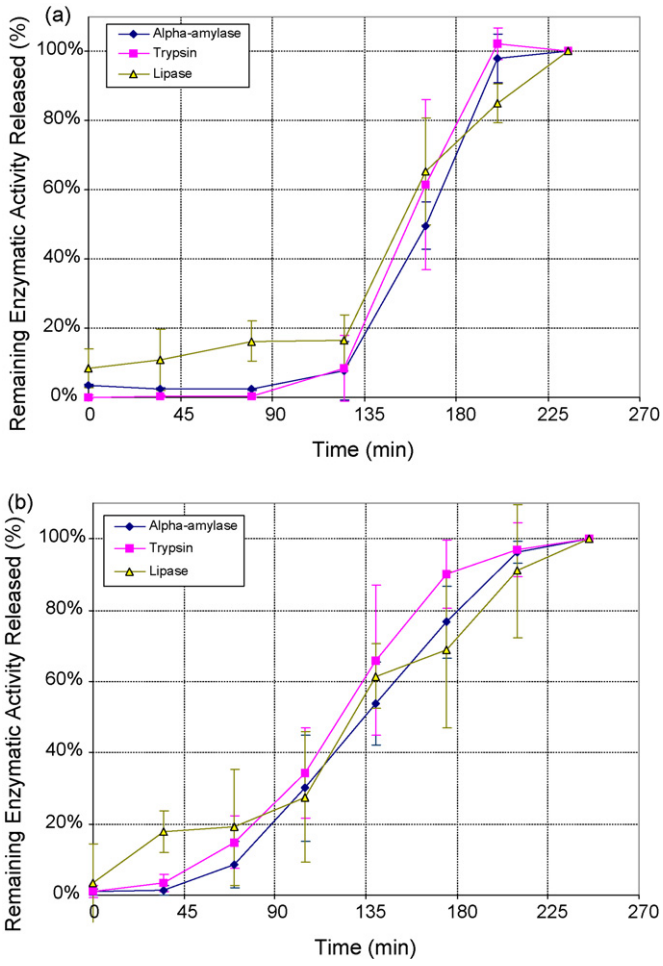


Fig. 8. Delivery in SIF of α-amylase, lipase and trypsin formulated together with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) formulated with 15 mg α-amylase, 15 mg lipase and 15 mg trypsin were incubated (37 °C and 50 rpm) for 1 h in 50 mL of SGF containing pepsin and then transferred in 50 mL of 50 mM PB (pH 7.2) at 37 °C and 50 rpm, withdrawing samples at various intervals to determine their enzymatic activities ($n = 4$).

For tablets formulated with lipase, the enzymatic activity released was in a linear dependency with the enzyme content up to 20% loading for both CM-HAS (Fig. 10a) and S-HAS (Fig. 10b), with the mention that the release of enzymatic activity continued to increase up to 80% loading. As a control, tablets made entirely of lipase (200 mg), after 1 h incubation in SGF, possess a remaining enzymatic activity lesser than that of tablets with 40% enzyme (80 mg) with CM-HAS or S-HAS as excipients, in the same conditions.

Tablets loaded with trypsin showed a linear dependency with the enzyme content up to 80% for CM-HAS (Fig. 10a) and up to 60% for S-HAS (Fig. 10b). High drug loading formulations have also been obtained with CM-HAS-CL (Mateescu et al., 2006).

4. Discussion

4.1. Structural aspects

The larger crystalline domain of HAS rather than of CM-HAS and S-HAS as shown by DSC, is in line with the tendency of HAS to generate ordered V (simple helix) structure at gelification, stabilized by hydrogen bonding. For derivatives, the formation of starch V structure is supposed to be, in part hindered by the presence of carboxymethyl or succinyl functions. The carboxyl function can contribute at stabilization by hydrogen or by dipole association despite less V order of the macromolecule.

TGA indicates a higher thermal stability of CM-HAS than that of HAS whereas S-HAS presents a lower stability than HAS. This opposite tendency between HAS and its carboxymethyl and between HAS and its succinyl derivatives can be explained by the difference in the type of substitution (ether for CM-HAS and ester for S-HAS) and in the nature of functional groups (hydroxyl and carboxyl). It is well documented that the thermal stability of starch increases with increasing degree of substitution (in the case of succinyl derivative with low DS versus high DS (Heinze et al., 2000; Thiebaud et al., 1997; Fang et al., 2002), the greater stability of the esters was explained by the lower amount of remaining hydroxyl groups after esterification (Rudnik et al., 2005). Since the main decomposition mechanism of starch is the dehydration reaction between starch hydroxyls, the lower the amount of hydroxyl groups remained, the better is the thermal stability of the starch esters (Rudnik et al., 2005). When starch contains functional groups (i.e. carboxyl or hydroxyl), it is expected that the polymer degradation would be facilitated (due the formation of CO_2 or H_2O). When formulated as tablets, if the functional groups (hydroxyl, carboxyl) of neighboring chains of the starch are close enough to allow hydrogen bonding, this can help to stabilize the tablet.

4.2. Stability of pancreatic enzymes formulated with CM-HAS and S-HAS in simulated gastric fluid (SGF)

The gastric resistance afforded by CM-HAS was higher than that obtained with S-HAS. An explanation can be a hydrolytic partial esterolysis of succinate protective function in gastric acidity. In fact, the added carboxymethyl group on starch in the case of CM-HAS is linked by an ether bond which is more resistant than the succinate group of the S-HAS (the ester bond is susceptible to esterolysis). By being susceptible to lose its substitute, the stability is also partly lost.

There is a certain loss of enzyme activities following the gastric passage. However, this loss of enzyme activity within the tablets is definitively lower than that observed in the case of free, non-formulated enzymes. It is not excluded that the enzymatic activity loss come in part from the slight tablet dissolution and enzyme release in SGF and in part from the surface swelling of the tablet and consequently a slow and progressive acidification of the outer layer generating a loss of enzymatic activity. In fact, previous studies (Calinescu et al., 2005) showed using pH indicators, that the tablets core is well protected against acidity.

It is worth to mention that the shape of the tablets [a relatively thin disc with a high surface/volume (*S*/*V*) ratio] can be considered as a particular case to evaluate the gastroprotection. Tablets with a different shape (oval shape caplet with a lower *S*/*V*

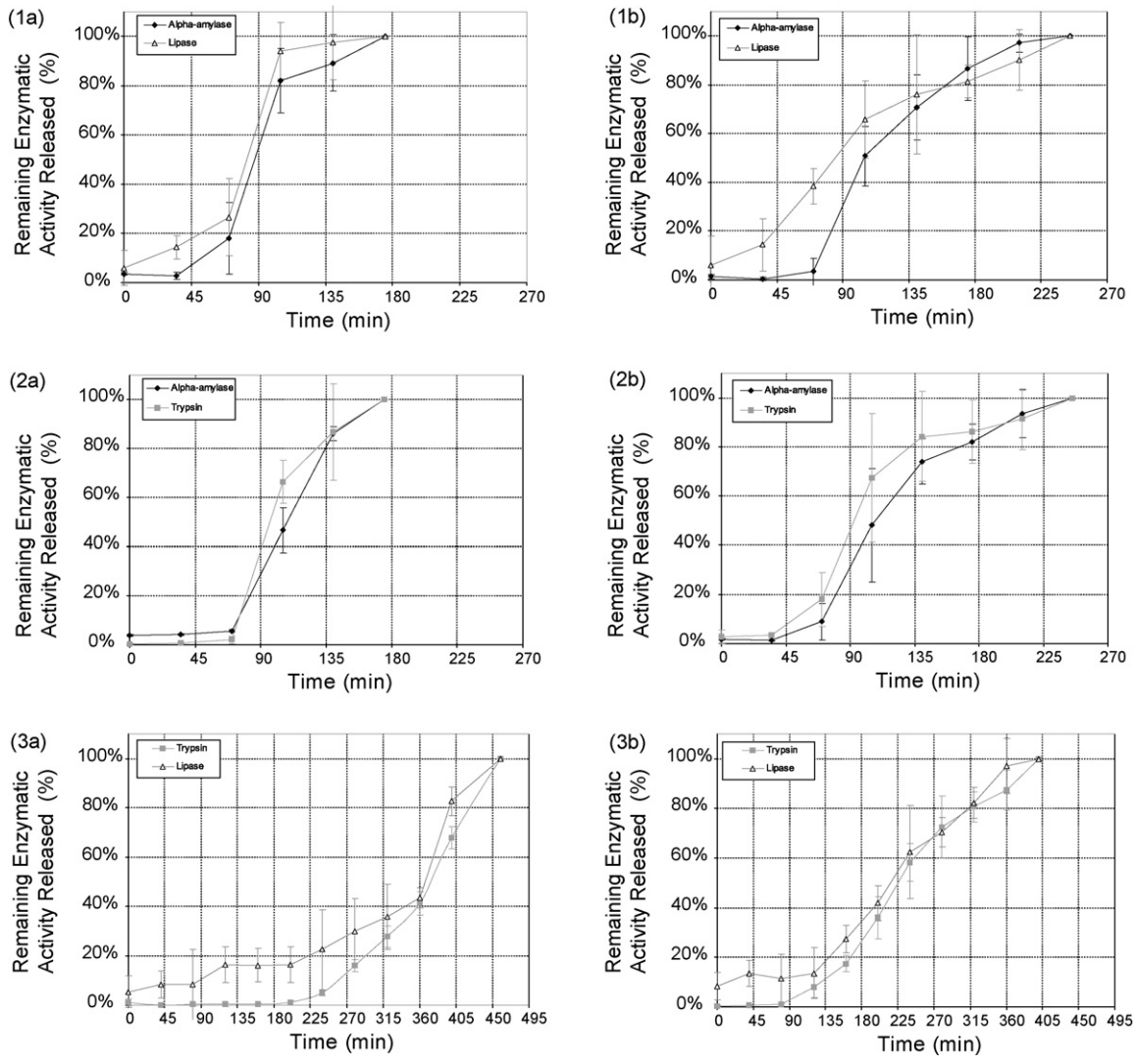


Fig. 9. Delivery in SIF of bi-enzymatic formulations with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) formulated with (1) 15 mg α-amylase and 15 mg lipase, or (2) 15 mg α-amylase and 15 mg trypsin, or (3) 15 mg lipase and 15 mg trypsin, were incubated (37 °C and 50 rpm) for 1 h in 50 mL of SGF containing pepsin and then transferred in 50 mL of 50 mM PB (pH 7.2) at 37 °C and 50 rpm, withdrawing samples at various intervals to determine their enzymatic activities ($n = 4$).

ratio) would probably afford a better protection to the enzymes. Our matrices can also be used in double core tablets were the external dry coating can be done with the same carboxylated starch excipient, without additional coatings (Ispas-Szabo et al., 2006). Eliminating the need for enteric coating allows a wider variety of tablet shapes, since the behavior of the tablets in the fluidized bed coating pans do not have to be taken into consideration for the tablet design. It is also important to note that the loading capacity of our formulation is high and the partial loss of activity, due to the gastric acidity, can be compensated by a higher loading within the tablet. Another interesting property of these starches is that they can be slowly hydrolyzed by the duodenal alpha-amylase, modulating thus the drug delivery not only by pH but also enzymatically.

Further studies are still needed for optimization of DS, considering that minor chemical alterations of starch macromolecules (Lenaerts et al., 1991) can generate modifications that can be of interest for stability of the formulated enzymes.

4.3. Delivery of the pancreatic enzymes in the simulated intestinal medium

The differences in the delivery patterns between CM-HAS and S-HAS tri-enzymatic formulations (Fig. 8) can be explained by the ways in which the tablets release their bioactive agents. CM-HAS tablets tend to slowly swell followed by disintegration. This provides first a period with no significant release of active agent and then a relatively fast release. On the other hand, S-HAS tablets control the release of the bioactive agents in two ways: swelling and tablet erosion. These two processes allow for S-HAS release that occurs sooner but over a longer duration. Both formulations achieve a complete release of the bioactive agents more or less after the same period of time.

The longer release times for the lipase–trypsin bi-enzyme combination are, for both excipients, between the release times of individual and of the three enzymes formulations. This behavior suggests some kind of association trypsin–lipase explaining

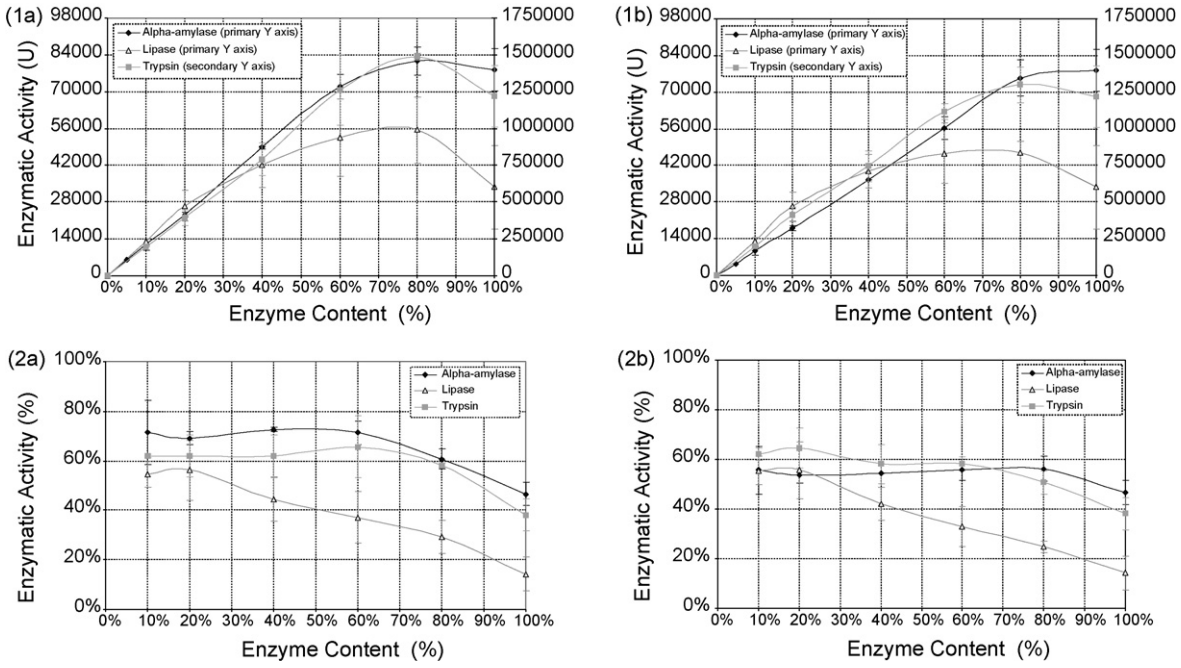


Fig. 10. Tablet loading of α-amylase, lipase or trypsin formulated with (a) CM-HAS or (b) S-HAS. The tablets (200 mg) formulated with increasing percentages of α-amylase ($n = 2$), lipase ($n = 3$) or trypsin ($n = 2$) were incubated (37 °C and 50 rpm) for 1 h in 50 mL of SGF containing pepsin and then transferred in SIF, crushed and enzymes activities measured and expressed in (1) total enzyme units and (2) percentage of total enzyme units recovered.

thus a shorter release time than for these enzymes individually formulated with CM-HAS (with particularly long release times, probably due to interactions between these enzymes and the matrix). It cannot be excluded that the association lipase–trypsin can compete with the individual interaction lipase–CM-HAS or trypsin–CM-HAS. Such interactions can be related to the cohesive properties of the two enzyme preparations.

Another aspect of interest is that both starch excipients, although modified, are poor substrates for α-amylase. Further experiments (data not shown) indicated that CM-HAS and S-HAS are about 3.2 and 4.6 times poorer (respectively) as substrate than soluble native starch. However, it is not surprising that in presence of water, the enzyme still degrades the polymeric matrix (hastening the tablet degradation) and plays a role in the faster release from CM-HAS compared to S-HAS (Fig. 9(1 and 2)).

When using CM-HAS with a lower DS (3.3% instead of 5.1%), the release time of 75% was linearly decreased (from 180 to 118 min) for the overall enzymatic activity of the three enzymes formulation (15 mg of each enzyme combined all together), suggesting a role of DS in the release properties of the excipient (figure not shown). In case of S-HAS, the differences were less important (155 min for DS 6.6% and 170 min for DS 5.6%).

4.4. Loading capacity of CM-HAS and S-HAS with active agent

The decrease of activity at high loading can be explained by a lower ratio of carboxylated matrix: enzyme(s). At this low ratio the matrix cannot afford enough gastric protection. However at a moderate loading, the experimental data clearly shows that the presence of carboxylated starch excipients is important for formulation.

Mulhbacher et al. (2001) showed that carboxymethylation drastically increases the loading capacity of cross-linked HAS. In the present formulations, although CM-HAS and S-HAS are not cross-linked, the loading capacity is high, probably due to interactions of proteins with the excipients. The good stability of formulations with high enzyme loading can be explained by certain interactions between free amino groups (from lysine) and carboxylic groups (from aspartic and glutamic acid) of enzymes and those of CM-HAS or S-HAS. These interactions can generate a kind of matrix stabilization also ensuring a local buffering protection. However, the role of CM-HAS or S-HAS is important, since the stability at 100% enzyme (excipient free) formulation is lower than that with 80% enzyme (Fig. 10). An advantage of these carboxylated high amylose starch excipients is that they afford gastroprotection without additional enteric coating. The high loading capacity and stability are another important advantage, considering the high dosage often required in an enzymatic treatment and the elevated cost of the enzymes.

In conclusion, the carboxylated starch derivatives ensuring good shape of tablets in acidic medium and gastroresistance afforded by the matrices (not by coating) even at high drug loading, represent interesting excipients for pharmaceutical formulations.

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