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Biodegradable cross-linked starch/protein microcapsules containing proteinase inhibitor for oral protein administration

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Abstract

The objective of this study is to demonstrate the feasibility of microcapsules containing a protein and a proteinase inhibitor in order to allow the oral administration of proteic or peptidic drug. Starch/bovine serum albumin mixed-walled microcapsules were prepared using interfacial cross-linking with terephthaloyl chloride. The microcapsules were loaded with native or amino-protected aprotinin by incorporating protease inhibitors in the aqueous phase during the cross-linking process. Microcapsules can be degraded in the presence of α-amylase. The influence of the formulation parameters on the in vitro release of the inhibitor activity and the protein was studied. The protective effect of microcapsules with aprotinin for bovine serum albumin was revealed in vitro. The presence of the native bovine serum albumin was demonstrated after incubation of the microcapsules with aprotinin in a mixture of α-amylase (5.4 U/ml) and trypsin (900 spectrophotometric BAEE units/ml) for 3 h at 37°C, whereas the protein was completely degraded in the release medium of the microcapsules without aprotinin.

Keywords: Aprotinin; Proteinase inhibitor; Biodegradable microcapsules; Starch microcapsules; Oral protein drug delivery

1. Introduction

In the present day, it is easily admitted that proteic or peptidic drugs can be absorbed through the gastro-intestinal mucosa and enter the blood circulation in an intact form on the condition that they are protected from enzymatic degradation up to the absorption level (Langguth et al., 1997). Drug delivery to the colon is a way to circumvent the very drastic enzymatic degradation taking place in stomach and small intestine (Duchêne and Ponchel, 1993; Watts and Illum, 1997). Protection of the drug can be achieved by specific capsules (Brøndsted et al., 1998) or coatings (Milojevic et al., 1995; Kalala et al., 1996; Fernández-Hervás and Fell, 1998), prodrugs (Friend,

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1995) or matrix systems (Rubinstein and Gliko-Kabir, 1995; Kakoulides et al., 1998; Lorenzo-Lamosa et al., 1998) degraded in the colon environment.

More recently, it was proposed to co-administer the proteic or peptidic drugs with a specific enzyme inhibitor. Among the inhibitors investigated are: soybean or basic pancreatic trypsin inhibitor (Esposito et al., 1996), puromycin (Langguth et al., 1997), chymostatin or elastatinal (Bernkop-Schnürch and Scerbe-Saiko, 1998), and antipain (Bernkop-Schnürch and Scerbe-Saiko, 1998). These products can be incorporated into biodegradable microparticles (Esposito et al., 1996) or Eudragit S 100 coated pellets (Langguth et al., 1997). In order to a prevent possible toxic effect, Bernkop-Schnürch et al. (1998) and Bernkop-Schnürch (1999) proposed the immobilization of inhibitors on an unabsorbable carrier matrix system such as a mucoadhesive polymer: chitosan.

For their part, the authors worked on aprotinin (Larionova et al., 1999), a basic pancreatic proteinase inhibitor of bovine origin with a broad inhibitory specificity with respect to trypsin, chymotrypsin, and also plasmin and kallikrein. Aprotinin has been used for over three decades as an intensive-care drug for acute pancreatitis (Larvin et al., 1988), and it is recommended for shock of traumatic or septic origin, hyperfibrinolytic hemorrage (Robert et al., 1996). Due to its low toxicity and strong inhibition of luminally secreted and brush-border membrane-bound proteases, aprotinin has already been used to overcome the enzymatic barrier of perorally administered therapeutic peptides and proteins (Yamamoto et al., 1994; Bernkop-Schnürch, 1998). For these reasons, it was chosen to work with biodegradable starch microparticles rather than with an immobilizing matrix system (Larionova et al., 1997, 1998; Tiourina et al., 1998).

The aim of the present work is to demonstrate the feasibility of microcapsules loaded with both aprotinin as a proteinase inhibitor and bovine serum albumin as a proteic (drug) model without degradation of the latter.

2. Materials and methods

2.1. Materials

Soluble starch was donated by Roquette Freres (France). Bovine serum albumin was purchased from Sigma (France). Terephthaloyl chloride was purchased from Aldrich-Chimie (France). Surfactants were sorbitan trioleate (Span® 80) and polyoxyethylenesorbitan trioleate (Tween® 85) from ICI (Germany). Chloroform, cyclohexane and ethanol, analytical grade, from Prolabo (France) were used without further purification. The enzymes used were trypsin from the bovine pancreas (10 000 BAEE U/mg), α-amylase (27 U/mg) and pancreatin from the porcine pancreas (USP grade), and esterase (19 U/mg) from the porcine liver, all from Sigma (France). Aprotinin (Gordox®) was obtained from Gedeon Richter (Hungary). Benzoyl arginin-*p*-nitroanilide was from Sigma (France).

2.2. Preparation of microcapsules

Microcapsules were prepared using the interfacial cross-linking method (Levy and Andry, 1990) with some modifications (Larionova et al., 1997). Briefly, varying amounts of soluble starch and bovine serum albumin were dissolved in the selected buffer. This aqueous phase was emulsified under mechanical agitation in cyclohexane (ratio 1:3 v/v) containing 5% (v/v) Span® 80. A terephthaloyl chloride solution in chloroform was then added to the emulsion (ratio 1:2.4 v/v) and stirring was continued for 30 min. The microparticles were washed with cyclo-hexane (twice), with 2% (v/v) Tween® 85 solution in 95% ethanol (once), with 95% ethanol (three times), and with water (twice). Finally, the microcapsules were resuspended in water and lyophilized.

Variations were introduced in the composition of the aqueous phase (pH, soluble starch and bovine serum albumin concentrations), in the stirring speed, and in the terephthaloyl concentration. All batches were prepared at least three times.

2.3. Encapsulation of aprotinin

Aprotinin was used either in the native form or in the modified form, where amino groups were protected by citraconic anhydride (Larionova et al., 1977).

Aprotinin or protected aprotinin was incorporated into the aqueous phase during the cross-linking process. The microcapsules were isolated as described above.

2.4. Further treatment of microcapsules

The microcapsules (10 mg) were soaked for 8 h in 10 ml of a pH 8.0 buffer solution at room temperature. When protected aprotinin was encapsulated, the microcapsules were additionally incubated 5 h at pH 2.0. The microcapsules were finally rinsed three times with distilled water and lyophilized.

2.5. Microcapsule characterization

Microcapsule morphology was studied by optical microscopy and scanning electron microscopy. Microcapsules were sized using special equipment: a Coulter Multisizer II, Sampling Stand II A (UK). Size distributions were displayed in terms of volume against particle size.

2.6. In vitro release studies

Lyophilized microcapsules (10 mg) were rehydrated in a test tube with 5 ml of carbonate buffer solution (pH 8.0), containing either a suitable amount of α-amylase or a mixture of esterase, α-amylase and pancreatin. The tube was incubated at 37°C with constant agitation at 40 rpm. At appropriate time intervals, the sample was centrifuged and a 400 μl aliquot of the supernatant was withdrawn for protein content and antiprotease activity determinations. Four hundred μl of fresh enzymatic solution was replaced to maintain sink conditions. Studies were repeated three times to determine represented means ($\pm$ S.D.).

2.7. Determination of protein content

The supernatant protein content was determined using Lowry's method (Lowry et al., 1951).

2.8. Determination of antiprotease activity

Antiproteinase activity in the release media was determined by assaying the remaining amidase activity of bovine trypsin, using benzoylarginine-*p*-nitroanilide as the substrate (Kakade et al., 1969).

A 0.2 ml sample of the supernatant of the release medium was mixed with 0.2 ml trypsin solution (600 spectrophotometric BAEE U/ml). Then 2 ml 0.05 M Tris–HCl buffer pH 8.0 was added. After an incubation period of 10 min at room temperature, 0.1 ml of benzoyl-L-arginine-*p*-nitroanilide solution (10.8 mg/ml) in dimethylsulfoxide was added. The enzymatic reaction was allowed to proceed at room temperature for 15 min. Then the hydrolysis was stopped by adding 0.5 ml of 5 M acetic acid. Optical densities were read at 405 nm with a Shimadzu UV-265 FW spectrophotometer (Japan). The inhibitory activity in the supernatant of the release medium was then calculated by interpolation from the calibration curve, where a probe without the inhibitor was used as an initial activity. All experiments were performed at least in triplicate.

2.9. HPLC of the supernatant after degradation of microcapsules

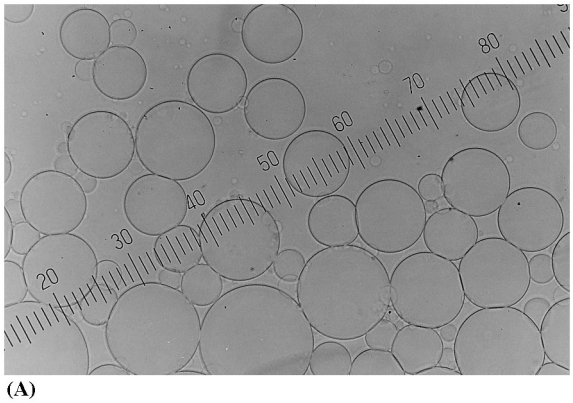
The supernatants were obtained as described in Section 2.6 after 3 h enzyme degradation with a mixture of α-amylase (0.2 mg/ml) and trypsin (900 spectrophotometric BAEE U/ml). The supernatant 50 μl was analysed by HPLC (QC-PAC GFC 200, column 7.8×150 mm, USA). The column was eluted with 10 mM sodium phosphate/sulphate buffer, pH 6.8. α-amylase, bovin serum albumin, trypsin and aprotinin were used as standards.

3. Results and discussion

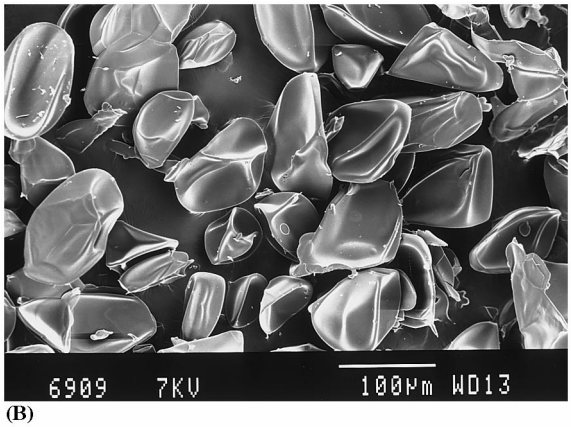
3.1. Morphology and size of microparticles

3.1.1. Influence of cross-linking pH on microparticle morphology

Influence of cross-linking pH on microcapsule morphology was first studied by carrying out a series of experiments in phosphate buffer solutions (pH 6.0 and pH 7.0) and in carbonate buffer solution (pH 8.0). Microcapsules were prepared from a 10% starch solution, with 5% (w/v) terephthaloyl chloride concentration, and 30 min reaction time. Microcapsules were obtained in small amounts, they formed aggregates and appeared fragile. Increasing the reaction time to 60 min did not improve the results.



(A)



(B)

Fig. 1. Cross-linked starch/protein microcapsules (10% starch, 5% bovine serum albumin, 0.4% aprotinin in buffer pH 9.8; 2% terephthaloyl chloride). (A) Optical microphotograph. (B) Scanning electron microphotograph.

In a second attempt, cross-linking was carried out in 0.5 M carbonate buffer solution of pH 9.8. Microcapsules were prepared from 5 and 10% starch solutions, with a terephthaloyl chloride concentration of 2.0% (w/v). In optical microscopy, they appeared as transparent and well-individualized spheres (Fig. 1A). An increase in the cross-linking agent concentration up to 5% (w/v) resulted in more distinct walls, and remarkably improved the stability of the microcapsules on storage. Microcapsule lyophilization gave free-flowing powders, from which the microcapsules could be easily rehydrated in buffer. Their walls were smooth and continuous, as shown by scanning electron microscopy (Fig. 1B).

3.1.2. Influence of stirring speed and soaking in alkaline buffer on microparticle size

The particle size could be adjusted by varying the stirring speed. For example, with a 10% starch solution and a 2.0% terephthaloyl chloride concentration, the size range was 10–30 μm for agitation at 1500 rpm (Fig. 2a) and 50–100 μm when lowering the stirring speed to 500 rpm (Fig. 2b).

Soaking of microcapsules in a slightly alkaline buffer solution (pH 8.0) resulted in a significant increase in size (Fig. 2b). For example, treated microcapsules prepared from a 10% starch solution and 2.0% (w/v) terephthaloyl chloride concentration had a size range of 90–180 μm for agitation at 500 rpm.

3.2. Obtention of degradable microcapsules releasing bovine serum albumin and active aprotinin

The membrane of microcapsules prepared as described above, can be formed upon cross-linking of hydroxy groups of the soluble starch involved in esters bonds, and amino, hydroxy and carboxy groups of bovine serum albumin and aprotinin involved in amide, ester and anhydride bonds, respectively. This was demonstrated in a series of papers concerning microcapsules prepared from cross-linked human serum albumin (Levy et al., 1991) and proteins (gelatin and human serum albumin) and various polysaccharides (Levy and Andry, 1991).

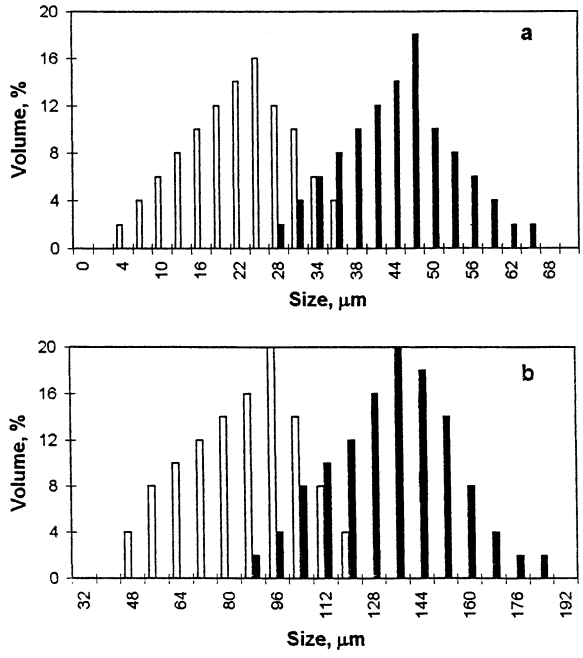


Fig. 2. Particle size distribution of starch/protein microcapsules (10% starch, 1% bovine serum albumin, 0.4% aprotinin in buffer pH 9.8; 2% terephthaloyl chloride). (a) Stirring speed: (open columns) 1500 rpm, (shaded columns) 1000 rpm. (b) Stirring speed 500 rpm: (open columns) without treatment, (shaded columns) treatment by pH 8.0 buffer, 8 h.

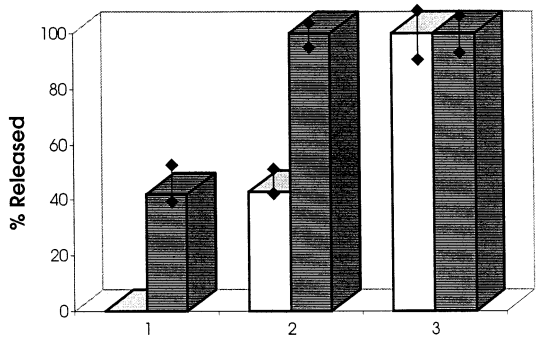


Fig. 3. Protein content (shaded columns) and antiproteinase activity (open columns) after degradation of microcapsules by amylase 0.2 mg/ml, 37°C, 6 h. Ten percent starch, 1% bovine serum albumin with (1,2) 0.4% native aprotinin, (3) protected aprotinin in buffer pH 9.8. Microcapsules treated before enzyme degradation (2,3). Each bar represents the mean of at least three experiments (± S.D.).

Degradation by different enzyme solutions was studied. The microcapsules were resistant to digestion by 19 IU/ml esterase solution. Meanwhile, α-amylase (0.2–1 mg/ml) was the main enzyme for dissolution of the microcapsules, and the use of a mixture of α-amylase (1 mg/ml), esterase (19 IU/ml) and pancreatin (1 mg/ml, corresponding to 280 spectrophotometric BAEE U/ml) only slightly enhanced the degradation rate. This can be explained by the relatively low concentration of the proteins (1–5%) in the aqueous phase during the microcapsule preparation and in the microcapsule wall.

Protein release was dependent on the enzymatic degradation of the capsules, as shown in Fig. 3. Unfortunately, antiproteinase activity of aprotinin was not detected in the dissolution medium (Fig. 3, sample 1). Both lack of the inhibitor activity and the resistance of the mixed-walled microcapsules to enzymatic degradation could be the consequence of extensive cross-linking between the proteins and the soluble starch which created the steric hindrance for substrate/inhibitor-enzyme reaction. To break the anhydride bonds formed during acylation of carboxylate groups with terephthaloyl chloride, and to decrease the degree of cross-linking, the microcapsules were treated with a slightly alkaline buffer solution, as described by Levy et al. (1991). The release of protein and antiproteinase activity was shown to increase after such treatment (Fig. 3, sample 2). In addition, modification of the reactive site amino group (Lys 15) of aprotinin during the process of encapsulation can be the reason for the activity loss. Therefore, to prevent acylation by terephthaloyl chloride, a reversible protection of aprotinin amino groups was used in most of the experiments. It resulted in total retention of antiproteinase activity of aprotinin after degradation of microcapsules by amylase (Fig. 3, sample 3).

3.3. Factors affecting aprotinin release

The cumulative release of aprotinin was dependent on the microparticle size. The aprotinin release rate could be increased by a decrease in particle size. However, there was no significant difference between microcapsules 25–55 μm in diameter and those 8–25 μm in diameter.

The influence of treating microcapsules before and after lyophilization was studied. There was an influence of the treatment sequence for microcapsules prepared with native or protected aprotinin. But an increased release of aprotinin was shown when microcapsules with native aprotinin treated in slightly alkaline buffer were additionally incubated 5 h at pH 2.0 (Fig. 4).

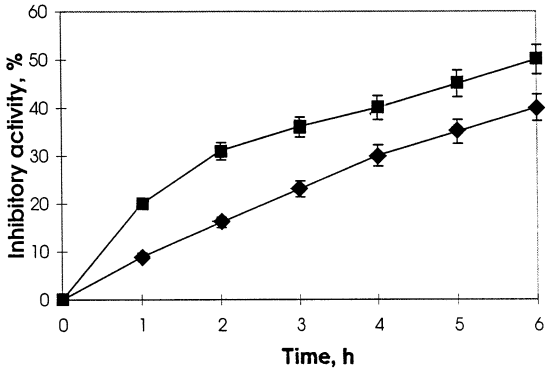


Fig. 4. Effect of a further treatment of microcapsules on the in vitro release of inhibitor activity after degradation by α-amylase. Ten percent starch, 1% bovine serum albumin, 0.4% aprotinin in buffer pH 9.8, 2% terephthaloyl chloride, α-amylase 0.2 mg/ml. (◆) 8 h at pH 8.0 and (■) 8 h at pH 8.0, then 5 h pH 2.0. Each bar represents the mean of at least three experiments (± S.D.).

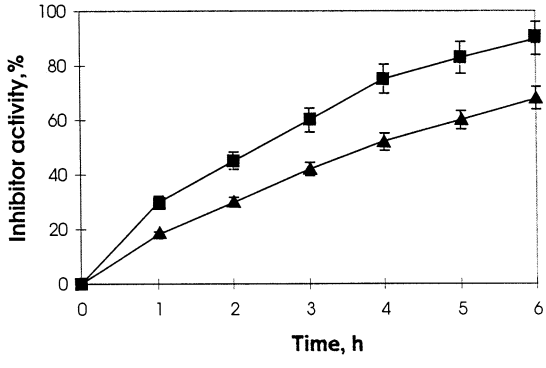


Fig. 5. Influence of terephthaloyl chloride concentration on the in vitro release of the inhibitor activity after degradation by α-amylase. Ten percent starch, 1% bovine serum albumin, 0.4% aminoprotected aprotinin in buffer pH 9.8, 500 rpm, α-amylase 0.2 mg/ml. (■) 2% terephthaloyl chloride and (▲) 5% terephthaloyl chloride. Each point represents the mean of at least three experiments (± S.D.).

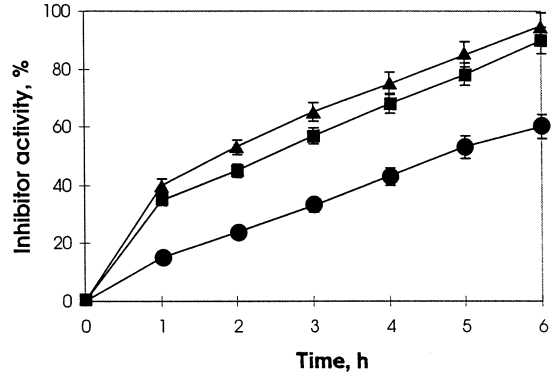


Fig. 6. In vitro release of the inhibitor activity after degradation by α-amylase as a function of bovine serum albumin concentration. Ten percent starch, 0.8% amino-protected aprotinin in buffer pH 9.8, 2% terephthaloyl chloride, 500 rpm, α-amylase 0.2 mg/ml. (●) without bovine serum albumin, (■) 1% bovine serum albumin, (▲) 5% bovine serum albumin in aqueous phase. Each point represents the mean of at least three experiments (± S.D.).

The cross-linking density of the microcapsule wall influenced aprotinin release profiles (Fig. 5). The least cross-linked microcapsules released at a faster rate, while the highly cross-linked capsules released at a slower rate. Increased cross-linking impeded microcapsule swelling in the dissolution medium as well as diffusion of the inhibitor, which resulted in a lower release rate.

Fig. 6 demonstrates the influence of bovine serum albumin concentration during microcapsule preparation on aprotinin release. Microparticles prepared from starch alone under the same cross-linking conditions released markedly less inhibitor activity. It can be caused by the participation of aprotinin in building the microcapsule membrane.

3.4. Demonstration of the protective effect of the encapsulated aprotinin for the incorporated model protein

The protective effect of the microcapsules with the proteinase inhibitor was studied towards trypsin for 3 h, corresponding to small intestinal transit time (Davis et al., 1986).

To evaluate the integrity of the encapsulated model protein, HPLC was used. Fig. 7 represents the elution profiles of supernatants obtained after

degradation of the microcapsules prepared without and with proteinase inhibitor by α-amylase and trypsin. Microcapsules containing aprotinin demonstrated a presence of the native bovine serum albumin in the release medium in contrast to a total trypsinic degradation of the protein for 3 h in the absence of the proteinase inhibitor. The protective effect was evaluated at a trypsin activity of 900 spectrophotometric BAEE U/ml, which is only 1.5 times lower than its presumed physiological concentration (Bernkop-Schnürch et al., 1998).

An increase of aprotinin content could result in a more successful protection of the encapsulated model protein. Furthermore, it is well known that the cross-linking makes the protein globule more stable to denaturation and resistant to proteolysis (Berezin et al., 1976).

Moreover, the cross-linking of a protein antigen is a routine method for the enhancement of the immune response. The controlled release of encapsulated vaccine components on the enzymatic degradation of the microcapsules could elicit a higher antibody titre.

Therefore, the microparticulate delivery system described here should be feasible not only for the given oral vaccine, but also for proteins whose amino groups are essential for biogical activity.

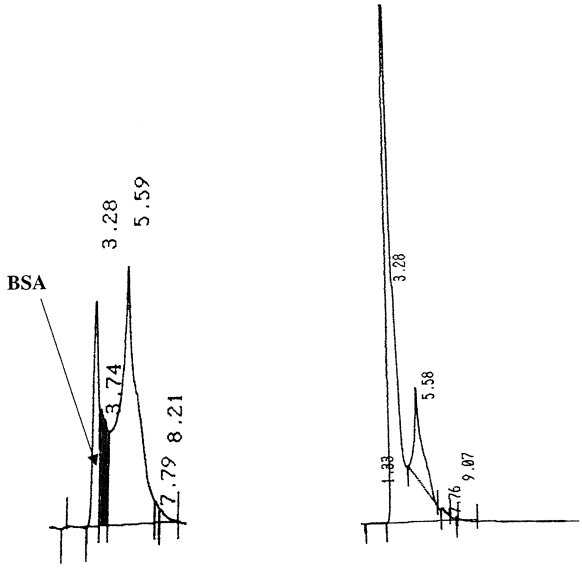


Fig. 7. Gel filtration HPLC (QC-PAC GFC 200, column 7.8 × 1500 mm) of supernatants obtained after 3 h degradation of starch microcapsules prepared with (left) and without (right) aprotinin by a mixture of α-amylase (0.2 mg/ml) and trypsin (900 spectrophotometric BAEE U/ml) in carbonate buffer pH 8.0 at 37°C. (Microcapsules prepared from 10% starch, 1% bovine serum albumin, 0.8% aprotinin in buffer pH 9.8, 2% terephthaloyl chloride, 500 rpm).

4. Conclusion

This study demonstrated the feasibility of mixed-walled starch/bovine serum albumin (model protein) microcapsules loaded with a protease inhibitor, aprotinin. The protein release is dependent on the enzymatic degradation medium and is primarily dependent on the presence of α-amylase. In vitro release of aprotinin can be modulated by manufacturing conditions. This system can be used for the preparation of oral administration systems intended for the delivery of a therapeutically active peptide or protein drug containing aprotinin as the protease inhibitor adjuvant.

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