Chitosan Reinforced Alginate Microcapsules Retained The Release of Papain in Simulated Gastric Fluid

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ABSTRACT

Alginate microcapsules were obtained by emulsification/internal gelation technique was chosen as carrier papain and the potential use as an oral controlled release system investigated. Chitosan was then applied as a membrane coat to increase the mechanical and stabilize the microcapsules in simulated gastric fluid. Papain microcapsules consisted of particle size distribution, morphology, entrapment efficiency of papain in the microspheres and release study. The release study was performed in various pHs with or without proteolitic enzyme. Narrow particle size distribution with average of 315-560 μm was obtained from microcapsule prepared with water/oil ratio of 30 : 70, acid/$CaCO_3$ molar ratio of 6 : 1 and agitation speed of 400 rpm. Papain microcapsule with that composition has entrapment efficiency of 95.79%. The release of papain from the alginate-chitosan microcapsule took place trough diffusion and relaxation of polymer at pH 1.2-4.5. The in vitro release studies showed that alginate-chitosan complex formation reduce erosion of alginate-chitosan matrix at pH 6.8. Both pepsin and pankreatin increased release of papain. Alginate-chitosan polyelectrolyte complex occurred on the surface of papain microcapsule controlled the release of papain from microcapsule. The presence of chitosan on the surface of microcapsule retained the release of papain in undesired site (gastric). In conclusion, papain microcapsule developed in this study is suggested to be appropriate approach for oral delivery of papain.

Keywords : papain, microcapsules, alginate, chitosan, pepsin, pankreatin,

INTRODUCTION

Peptide and protein normally administered by the parenteral route. However complication such as the trombophlebitis or tissue necrosis and poor patient compliance have simulated the investigation af alternative nonparenteral route. Among this, the most convenient nonparenteral route for patient remains the peroral application. Unfortunately, the oral bioavailability of peptidic drugs is generally very low, owing the acidic conditions of stomach, the proteolytic activity of the gastrointestinal tract, poor permeability across the intestinal mucosa, rapid clearance after absorption as well as chemical and physical instability during the process of manufacture and storage [26]

Papain is a protease with sulfidril group derived from Carica papaya which degraded by pepsin at pH 1.2 and temperature 37^0C into small peptides [8] To minimize protein denaturation and the loss of its biological activity, a mild microencapsulation methods, avoiding exposure to elevated heating and organic solvent, should be adapted [3]. Among this emulsification/internal gelation method could be considered proper for protein encapsulation[19]

Howefer, entrapment efficiency of internal gelation method is very low caused by several things, such as alginate microcapsules surface is not homogeneous, the protein diffuses from the internal phase to external phase and the loss of microcapsules in the collection phase. Moreover, the cation cross-linked alginate network can degraded by removal of the calcium ions by chelating agents such as lactate, citrate and phosphate. As calcium ions are removed, the cross-lingking in the gel decreases and gels are destabilized, leading to fast drug delivery rates [3]. To overcome the weaknesses of the method emulsification-internal gelation above, is necessary to modify the surface texture of alginate microparticles. Chitosan, a polysaccharide known to be used to achieve this goal through the formation of complex polyelectrolytes modified with Ca-alginate. Interaction between amino group of chitosan and carboxylic groups of alginate will strengthen the surface structure and reduce porosity so as to prevent the release of proteins in unwanted places [17]. Based on the expected interaction between alginate

and chitosan can produce a stable surface structure in acidic media.

Thus, the aims of this work was to investigate the feasibility of emulsification/internal gelation method to produce alginate and chitosan in order to obtain an oral formulation of papain which is physically and chemically stable.

MATERIALS AND METHODS

Materials

Sodium alginate, papain (with 3.08 U/ mg protein enzymatic activity), chitosan ≥ 95% deacetylation, casein from cow's milk, pepsin derived from bovine gastric mucosa, 800-2500 units/mg protein, pankreatin 4x USP specification, cysteine hydrochloride monohydrate, potassium dihydrogen phosphate, calcium carbonate, paraffin liquid , glacial acetic acid, Span® 80, Tween® 80, citric acid, trichloroacetic acid, sodium hydroxide, hydrochloric acid, trifloro acetic acid, sodium acetate, sodium chloride, acetonitrile, and water

Methods

Microcapsules preparation

Microcapsules are made using the method of emulsification/internal gelation [19].

Preparation of chitosan-coated alginate microcapsules

Chitosan-coating of microspheres obatained by internal gelation was performed based on Ribeiro [21] with stirring rate and time modification

Morphological and particle size analysis

The particle size distribution of microcapsules was determined by a sieve method with various mesh. The shape and surface texture of microcapsules was examined by scanning electron microscopy (SEM) JEOL JSM-6360LA, 10 kV

Determination of encapsulation efficiency

A number of microcapsules equivalent to 50 mg of papain crushed in a mortar and then dissolved in magnetically stirred phosphate buffer pH 6.8 for 1 hour. The resulting solution was centrifuged at 2500 rpm for 10 min and supernatant was assayed (n = 3) for enzyme content by casein digestion method [6].

Determination of Proteolytic Activity Papain by Casein Digestion Method

The ability of papain proteolysis determinate by casein digestion methods [6].The solution was then filtered using Whatman paper No. 42. Proteins are soluble in trichloroacetic acid were analyzed using HPLC. HPLC column used was C_{18} (250x4, 6 mm), 5 µm, flow rate 0.5 mL / min, UV detector 220 nm with a column temperature of 25^0C. The mobile phase used was a gradient elution using aquabidestilata/acetonitrile (each acidified with 0.1% v / v trifloroasetat acid) gradient was applied under the following conditions: 100% water, 2 min; 0-70% acetonitrile, 10 min; 70% acetonitrile, 4 min, 70% acetonitrile-100% water, 4 min; 100% water, 10 min (equilibration). The injection volume of the samples was 20 µL. The total peak area of the peptides was used to quantify the extent of papain digestion.

Effect of pH and proteolytic enzyme on release profile

To study the effect pH and enzyme proteolytic on papain release profile in vitro dissolution study was carried out using USP XXVI dissolution apparatus 2 (Erweka DT 6) in 500 ml of different pH media (buffer pH 1.2, buffer pH 4.5, buffer pH 6.8, buffer pH 4.5 plus pankreatin, simulated gastric fluid (USP) and simulated intestinal fluid (USP) on optimized batch of at 37 $\pm 0.1^0$ C with paddle speed of 75 ppm each for 2 hours. Accurately weighed samples (n =3) equivalent to 50 mg of papain were introduced to dissolution media and samples of 5.0 ml, were collected at 0, 5, 10, 15, 30, 60, 90, and 120 minutes respectively for the analysis of proteolytic activity of papain as before.

Curve fitting

The in vitro release pattern was evaluated to check the goodness of fit to the zero-order release kinetics, first-order release kinetics, Higuchi's square root of time equation, Korsmeyer-Peppas'power law equation and Hixson-Crowell's cube root of time equation. The goodness of fit was evaluated using the r (correlation coefficient) values.

Statistical Analysis

The digestion and analysis were repeated three times and analyzed

by means of standard deviation (SD). Statistical difference were analysed by using one-way analysis of variance (ANOVA) followed by a Bonferroni post test. For a value of P less than 0.05 the difference was considered significant

RESULTS

Encapsulation efficiency

Papain-dissolved alginate aqueous phase was emulsified into paraffin oil and the ionized alginate carboxylic groups were cross-linked by an internal source of calcium resulting in oil-dispersed gelled alginate microspheres. A high encapsulation efficiency, above 95.79%. was obtained for uncoated microsphere (Table 1). In respect to formulation parameters, values of encapsulation efficiency varied from 61.27 to 95.79% and especially affected by parameters influencing stirring speed, water/oil ratio and acid/$CaCO_3$ molar ratio.

Table 1. Effect of stirring speed, water/oil ratio and acid/$CaCO_3$ to encapsulation efficiency

Stirring speed (rpm)	Water/oil ratio (v/v)	Acid/$CaCO_3$ molar ratio	Encapsulation Efficiency
200	30 : 70	6 : 1	61,27 $\pm$ 0,37
300	30 : 70	6 : 1	80,83 $\pm$ 2,81
400	30 : 70	6 : 1	95,79 $\pm$ 2,21
400	20 : 80	6 : 1	93,12 $\pm$ 10,81
400	40 : 60	6 : 1	63,85 $\pm$ 1,48
400	30 : 70	3 : 1	70,10 $\pm$ 0,74
400	30 : 70	9 : 1	66,02 $\pm$ 2,91

Morphological and size characteristics

Scanning electron micrograph of alginate microcapsules showed spherical microspheres with a smooth external surface (Fig. 1A) while clumping observed during coating of microspheres as can see through the existence of alginate microsphere surrounded by chitosan coaservate (Fig. 1B). Papain-alginate microcapsules which produsedused with emulsification/internal gelation method more porous that can be seen in the Fig.

1D. Clumping on coated micracapsules indicated the presence of electrostatic interactions between alginate-chitosan which is a two polyelectrolytes of opposite charge.

Microspheres diameter ranged from 200-800 μm and particle size distribution at various stirring speeds are shown Fig. 2. Narrow particle size distribution with a peak of about 315-560 μm obtained from the microcapsules prepared with 30: 70 water/oil ratio, 6 : 1 acid/$CaCO_3$ molar ratio and 400 rpm stirring speed. A broader size was seen at microspheres prepared by stirring speed 200 and 300 rpm. Acid/$CaCO_3$ molar ratio, parameter influencing the gelation characteristics of the polymer, influence particle size distribution of microsphere. A broader size distribution was seen when acid/$CaCO_3$ ratio increased from 3: 1 to 6: 1

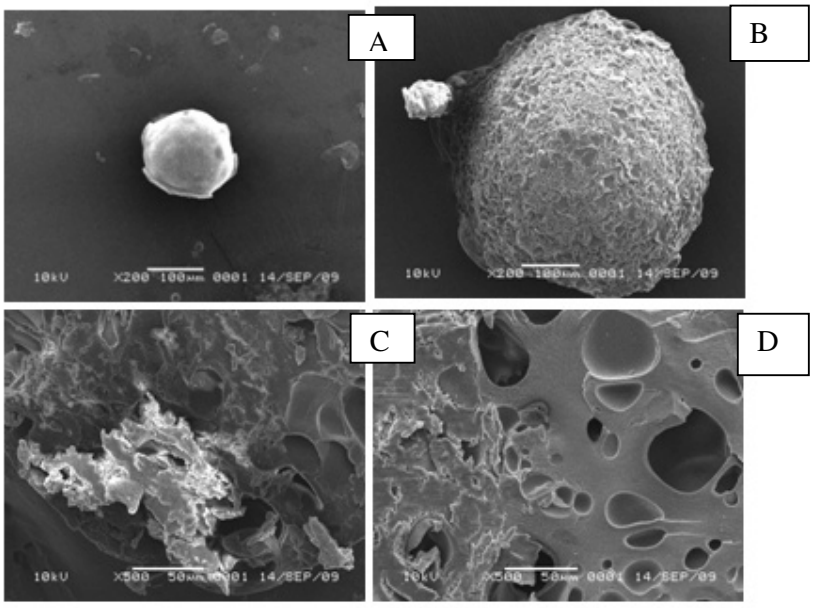

Fig 1. Scanning electron migrograph of : (A) papain loaded alginate microsphere (B) papain loaded alginate microsphere and coated with chitosan for 60 min; (C) papain was entrapment within the alginate microsphere; (D) large pore in alginate microsphere which produced by internal gelation

Papain release study

Papain release profile from microspheres, in gastrointestinal simulated pH conditions, is plottes in Fig.2. Both microspheres presented a low protein release at pH 1.2, reaching 7.72% and 7.06%. In other hand, it showed that rapid release of papain in the initial minutes (5 min) followed by a decline which indicated degradation of papain at pH 1.2 (Figure 2.A). Papain is stable at pH 4.5 as indicated by the constancy of the percentage amount of papain during study. The increased of papain release from the alginate microspheres which reached 75.03%. Howefer, papain release from of alginate-chitosan microspheres at pH 4.5 is still low at 17.23% for 2 hours of testing (Fig. 2.B). At pH 6.8, papain-alginate release at 9.77% and reached 100% after 2 hours of testing. Lower results occur in alginate-chitosan microspheres, the number of papain release into media was 4.96% at 5 minutes early and reached 85.38% at 2 hours of testing (Fig. 2.C).

Under simulated gastric fluid, in the presence of pepsin, papain release from alginate-chitosan is greater than the alginate microspheres respectively for 12.07% and 8.44%. When compared with the amount of papain release on the dissolution media pH 1.2 without pepsin, protein release from alginate-chitosan microspheres showed a significant increase ($p \leq 0.05$). When compared with alginate, alginate-chitosan complexes provide weaker protection to the activity of pepsin in increasing the percentage release of papain into the media. Chitosan which is a cation can compete with Ca^{2+} ions thus weakening the bond Ca-alginate and caused papain releases into (Silva et al, 2006) Then when tested in higher pH, at pH 4.5 with pankreatin, showing the amount of papain that release in to media was also higher. At 5 minutes beginning, papain releases from alginate microspheres and alginate-chitosan respectively 84, 10% and 50. 96% and reached 91.67% and 71, 54% after 2 hours of testing. Papain release from the alginate microspheres showed a significant increase when compared with the release in media pH 4.5 without pankreatin. This shows that alginate microspheres matrix erosion is increasing. Different profile was seen at release profile of alginate-chitosan

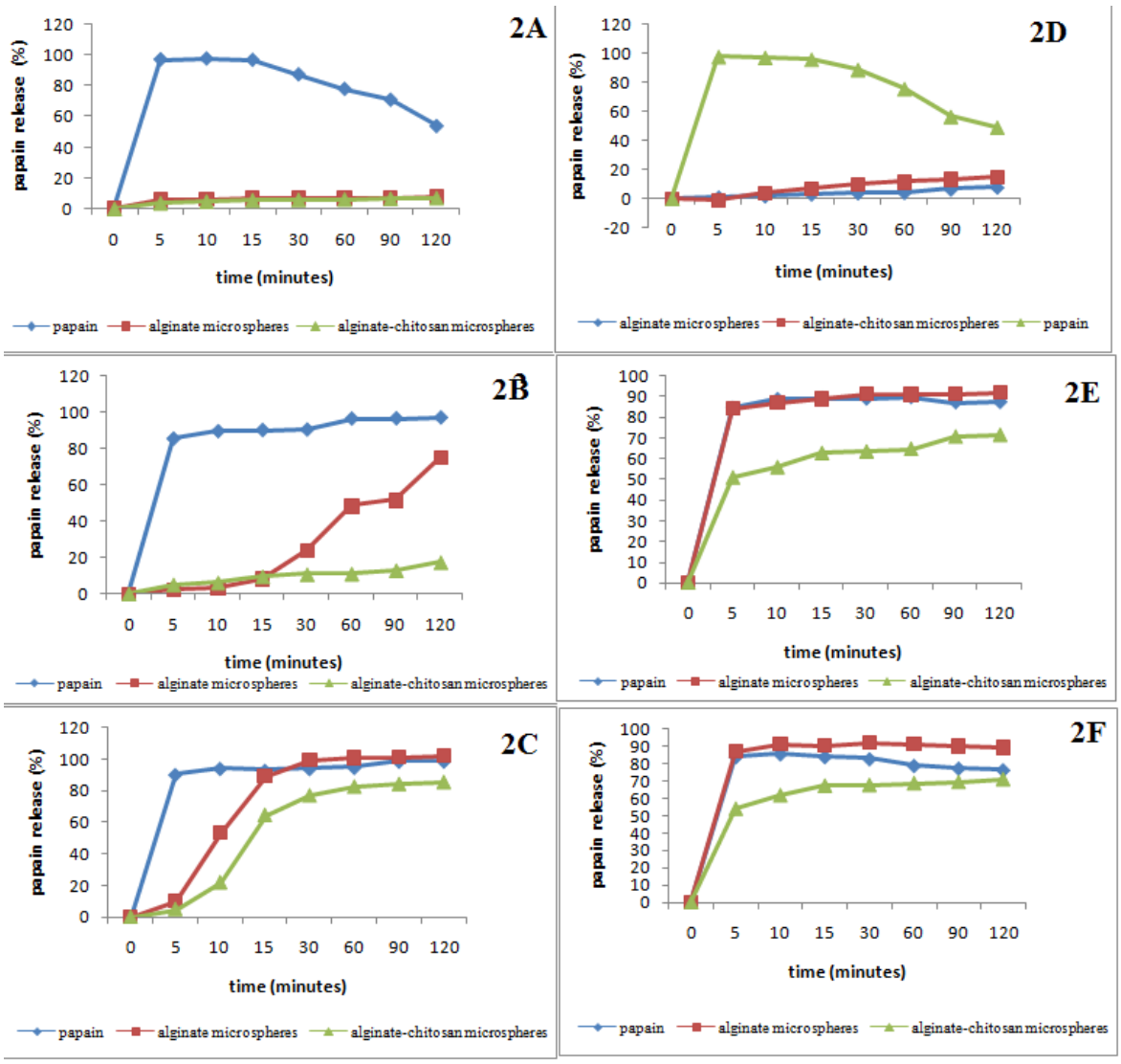

Fig 2. Papain release profile in various pH (pH 1.2 (2A), pH 4.5 (2B), pH 6.8 (2C) and in the dissolution media with proteolytic enzymes (simulated gastric fluid (2D), buffer pH 4 ,5 + pankreatin 0.25% (2E), pankreatin buffer pH 6.8 + 0.25% (2F)

microspheres. At pH 4.5 with and without pankreatin showed similar profile. These results confirm the research results of Coppi et al stating that the alginate-chitosan complex damaged slowly at pH 3.7 to 4.7

Curve fitting and release mechanism

The enzyme release data in dissolution media (pH 1.2, 4.5 and 6.8) show a good fit to the Korsmeyer-Peppas'power law release kinetics which can confirmed by comparing the values of the correlation coefficient (*r*) with those of the other models. The values of the Korsmeyer-Peppas' release exponent (*n*) determined for the various formulations studied ranged from 0.442-0.503, suggesting probable release by diffusion and polymer relaxation.

DISCUSSION

The aimed of this study was to develop papain-loaded microspheres prepared by emulsification/internal gelation capable retaining the protein in the mastrix during the gastric passage and sustaining the papain release at simulated intestinal pH. The necessary conditions for successful encapsulation of papain chitosan coated alginate microspheres prepared by emulsification/internal gelation with high encapsulation efficiency have been established.

A previous study comparing the encapsulation efficiency of bovine serum albumin in alginate microspheres showed that internal gelation of proteins may result in reduced encapsulation efficiency when compared to external gelation due to important loses during formulalation [21]. Microspheres formed via internal gelation are more homogeneous but more porous which lead to higher protein losses during manufacturing of DNA microspheres [20]. Macromelecular interaction between negatively alginate and positively papain were used to increase protein retention in alginate matrices during production and microspheres isolation. Specially, given papain 's isoelectric point of approximately 8.75 and hence a net positive charge at pH below 7.0, its complexes with alginate were explored. The high encapsulation efficiency of papain is consistent with the view that stronger protein-polyanion complex, can retain protein within the alginate matrix [21]. Higher papain losses in chitosan-coated alginate microspheres may be explained by an incomplete recovery of miscrospheres during phase partitioning and by the agitation destabilizing effect during emulsification in both microspheres networks.

Increasing the stirring speed increases the efficiency of papain

entrapment in microcapsules. Stirring speed affects the gelation process. The higher the speed of stirring, the calcium ions is more homogeneously distributed, so the more papain can be hooked into the microcapsules. The increase in viscosity produces emulsion droplet sizes are larger but the same amount of Ca ions resulted in fewer gelation so that encapsulation efficiency is lower. One mole of $CaCO_3$ reacted with 2 moles of acetic acid. Increasing acid/CaCO3 molar ratio, more calcium release to form a stronger microspheres that can prevent the losses of papain. Howefer, Very high on this parameter resulting a decrease in encapsulation efficiency of papain.

The size distribution of the recovered microspheres obtained by emulsification/internal gelation is correlated with the size distribution of the emulsion droplet. The average size of droplets is determined by various parameters such as apparatus design, viscosity of two immisible phases and speed of mixing [26]. In This study, parameters related to the vessel and stirrer characteristics were made constant.

The effect of higher stirring speed on microspheres mean diameter, contributing to a higher heterogeneity of the batch may caused by the distribution of turbulent forces through-out the emulsion [26]. Since, shear stress is higher at the tip of the propeller that at the centre, a faster stirring speed increased this difference, thereby providing a less uniform distribution of energy and giving rise to microspheres of a wider size distribution. Thus , strring speed of 400 rpm was chosen to allow the achievement of small microspheres with a narrow size distribution. Moreover, protein drugs are susceptible to denaturation when they are submitted to high shear forced

Increasing water/oil ratio from 20 to 40% led to increased on microspheres mean size where it was verified that a higher proportion of dispersed phase produce an overall increase in mean size, justified by an increase in the frequency of collisions during emulsification, resulting in the formation of aggregates [2]. Thus 30 : 70 ratio was chosen for the standard formulastions, because the effect of water/oil ratio on encapsulation efficiency was considered more

important comparatively to the mean size of microspheres.

Parameters directly related the gelation chsracteristics of the polymer, such us acid/$CaCO_3$ molar ratio influenced on the mean size of microspheres. Acid is responsible for calcium release. Considering the reaction between acetic acid and calcium carbonate, each mole of $CaCO_3$ react with 2 mol of CH_3COOH. When an acid/$CaCO_3$ molar ratio of 3 : 1 was used the amount of acetic acid was insufficient to dissolve all the amount of $CaCO_3$ added to alginate solution. But in acid/$CaCO_3$ ratio is too high (in this study (9: 1) causes the shringking of alginate gel so that the particle size will be smaller [29].

Carbon dioxide was liberated from calcium carbonate in the presence of an acid. The above observation could be due to the carbon dioxide that was rapidly liberated. This might result in a porous microspheres matrix. Scanning electron micrograph observation of microspheres confirms aggregation phenomena during chitosan-coating which can explained by strong electroctrolytes interaction between alginate and chitosan, two polyelectrolytes of opposite charge. The spherical shape of microspheres may be attributed to a high degree of cross-lingking occurring in each case.

Papain release profile of microcapsules depends on the pH. Alginate shrinks at low pH and the encapsulated drugs are not released. Microspheres incubation at pH 1.2, causes calcium ions to be displaced from the polymer network and the calcium-alginate gel is converted to the unionized form of alginic acid. At neutral pH (pH 6.8) alginate carboxylate groups almost entirely deprotonased, adhesion intermoleculer is reduced which causes increased water adsorption and swelling of alginate polymer and then papain was released into the dissolution media.

Release of papain from the microcapsules can occur through a process of diffusion or matrix relaxation. At a acid pH, erosion does not occur in the matrix because the alginate microspheres are stable in the environment so that the release of papain in the least amount of both alginate microsphere and chitosan coated alginate microspheres may be caused by diffusion. Whereas at pH 6.8, in addition to due process of diffusion of phosphate ions in the dissolution

media that has a high affinity for Ca^{2+} ions cause the erosion of the matrix so that the papain release. This study showed that chitosan can increase the strength of microspheres through ionic bonding (NH^{3+}) with alginate carboxy ion (COO-).

Level of deacetylation is an important factor that determines the strength of alginate-chitosan complex. The interaction may be weak or strong depending on the magnitude of residual amide groups on chitosan. The higher level of deacetylation the amide residues will be smaller so that the power complex will be increasingly weaker [10]. In this study deacetylation degree of chitosan was 95%. The level of deacetylation causes the residual amide group which is bonded to the carboxylic group alginate is limited.

In conclution, papain loaded alginate microspheres formulation was optimized in order to prevent papain release at gastric pH. Coated alginate microspheres wih chitosan achieving sustained release of papain at simulated gastric. Nevertheless, chitosan-coated alginate microspheres may good candidates for oral delivery of papain, since the presence of chitosan may promote papain absorption through intestinal epithelia and this needs further confirmation by in vivo studies

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