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An in vitro evaluation of a chitosan-containing multiparticulate system for macromolecule delivery to the colon

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Abstract

A multiparticulate system of chitosan hydrogel beads has been investigated for colon-specific delivery of macromolecules using fluorescein isothiocyanate–labeled bovine serum albumin as a model protein. The hydrogel bead was formed by polyelectrolyte complexation of chitosan with its counterion, tripolyphosphate (TPP). The protein release experiments were carried out in vitro under different conditions to simulate the pH and times likely to be encountered during intestinal transit to the colon. The results show that the hydrogel beads were degraded by rat cecal and colonic enzymes, resulting in a marked acceleration in the release of protein. The ability of rat cecal and colonic enzymes to degrade chitosan hydrogel beads was independent of pretreatment conditions. A commercial beta-glucosidase preparation containing a chitinase did not have a similar effect on the chitosan bead, even though it has been found to mimic the degradation function of rat cecal and colonic enzymes in vitro for chitosan in solution. Degradation of the chitosan–TPP hydrogel beads in the presence of rat cecal and colonic enzymes indicates the potential of this multiparticulate system to serve as a carrier to deliver macromolecules specifically to the colon.

Keywords: Chitosan; Colon-specific drug delivery; Hydrogel beads; Multiparticulates; Rat microbial enzymes; Almond emulsin beta-glucosidase

1. Introduction

Colon-specific delivery has the potential to deliver drug for the treatment of a variety of colonic diseases (Wakerly et al., 1996) and to deliver proteins and peptides to the colon for their systemic absorption (Lee, 1992). By employing the physiological parameters along the gastrointestinal (GI) tract, several methods for colon targeting have been proposed (Hovgaard and Brøndsted, 1996). As the human colon contains a large number of complex bacteria which are essentially absent from the stomach or the small intestine (Gorbach, 1971) and the colonic microflora can

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produce a large number of degrading enzymes (Scheline, 1973), a microbially controlled delivery system is the most appealing among the approaches proposed. This approach relies on the unique enzymatic ability of the colonic microbial flora and enables a more specific targeting, independent of pH variations along the GI tract, since the drug, peptide, or protein is only released upon the arrival of the dosage form at the colon (Rubinstein, 1990). Therefore, if a material is stable in the upper GI tract but degraded by the colonic bacteria, it could be used as a carrier for colon-specific targeting.

The design of biodegradable saccharidic delivery systems for colonic delivery has gained increasing interest (Rubinstein et al., 1997). These systems are based on the understanding that significant amounts of glycosidases and polysaccharidases, from microbial sources, are present in the human colon. Glycosidases are responsible for the hydrolysis of di- and oligosaccharides. Polysaccharidases, such as beta-D-glucosidase, beta-D-galactosidase, pectinase, and dextranase, are capable of hydrolyzing various polysaccharides. Many natural polysaccharides, such as chondroitin sulfate, pectin, dextran, and guar gum, etc., have been investigated for their potential to obtain colon-specific drug delivery. These polysaccharides are not digested in the upper GI tract, but are degraded by corresponding polysaccharidases produced by resident bacteria in the colon (Hovgaard and Brøndsted, 1996).

Chitosan is a functional linear polymer derived from chitin, the most abundant natural polysaccharide on the earth after cellulose, and it is not digested in the upper GI tract by human digestive enzymes (Furda, 1983; Ormrod et al., 1998). Chitosan is a copolymer consisting of 2-amino-2-deoxy-D-glucose and 2-acetamido-2-deoxy-D-glucose units linked with beta-(1→4) bonds. It should be susceptible to glycosidic hydrolysis by microbial enzymes in the colon since it possesses glycosidic linkages similar to those of other enzymatically-depolymerized polysaccharides. We recently reported that chitosan in solution was degraded by rat cecal and colonic enzymes, and that its susceptibility to degradation was dependent on both its molecular weight and degree of deacetylation (Zhang and Neau, 2002). Chitosan has drawn attention for its potential to achieve site-specific delivery to the colon. A few reports have been published on the investigation of the application of chitosan in colon targeting (Tozaki et al., 1997; Fernandez-Hervas and Fell, 1998; Macleod et al., 1999).

Due to the advantages of multiparticulate dosage forms over single unit preparations, such as more uniform dispersion in the GI tract, more uniform drug absorption, less inter- and intra-individual variability, and more flexible formulation process, interest in multiparticulates as oral drug delivery systems has been growing steadily (Bodmeier and Paeratakul, 1994). Because of their small particle size, multiparticulates can pass through the upper GI tract easily (Meyer et al., 1985; Davis, 1989), can reach the colon quickly and are retained longer in the ascending colon (Hardy et al., 1985). Therefore, a multiparticulate system of chitosan would be a desired dosage form for colon targeting.

In the present study, a multiparticulate system consisting of hydrogel beads formed by chitosan and tripolyphosphate (TPP) was investigated using fluorescein isothiocyanate–labeled bovine serum albumin (FITC–BSA) as the model compound. TPP was used as the counterion to positively charged chitosan to form gel beads. The suitability of such chitosan hydrogel beads as a multiparticulate system for the colon-specific delivery of macromolecules was evaluated in vitro by conducting the FITC–BSA release studies at different conditions to simulate the pH and times likely to be encountered during intestinal transit to the colon. In addition, a commercially available almond emulsin beta-glucosidase preparation capable of degrading chitosan and that mimicked the degradation function of rat cecal and colonic enzymes in vitro (Zhang and Neau, 2001, 2002) was used in one of the release media. The possibility of employing this enzyme preparation as a standardized in vitro system to substitute for microbial enzymes to limit animal use for assessing the suitability of chitosan based formulations for colon targeting was tested.

2. Materials and methods

2.1. Materials

Chitosan, labeled Sea Cure 242 from Protan Laboratories Inc. (Redmond, WA), was a gift from G.D. Searle (Skokie, IL). Almond emulsin beta-glucosidase was obtained from ICN Biomedical Inc. (Aurora, OH). Monobasic sodium phosphate and glacial acetic acid were purchased from Fisher Scientific (St. Louis, MO). FITC–BSA, the pentasodium salt of TPP, trizma base, pancreatin, porcine pancreas lipase, and the lipase diagnostic kit were obtained from Sigma Chemical Co. (St. Louis, MO). Micro BCA protein assay reagent and bovine albumin standard were purchased from Pierce (Rockford, IL).

Male Sprague–Dawley rats weighing 200–250 g were used (Harlan Sprague–Dawley, Indianapolis, IN). These rats were housed in the animal care facility and given free access to water and food.

2.2. Bead preparation

The chitosan solution was prepared by dissolving the desired mass of Sea Cure 242 in 1% (v/v) acetic acid and stirring for about 60 min. Meanwhile, 10 mg of FITC–BSA was dissolved separately in 2 ml of distilled water. Then, 8 ml of chitosan solution and 2 ml of FITC–BSA solution were mixed together to obtain 10 ml of 1.5% (w/v) chitosan and 1 mg/ml of FITC–BSA. The gelling medium was obtained by dissolving 2 g of TPP in 40 ml of 0.05 M Tris–HCl buffer, pH 7.0. The beads were formed by dropping the 10 ml of bubble-free chitosan solution with FITC–BSA present through a disposable plastic syringe with a 22-gauge needle into the gently agitated gelling medium using a KD Scientific (Boston, MA) Model 100 push–pull syringe pump at a speed of 70 ml/h. The beads were separated after 1 h of curing time by decantation and were rinsed twice with 3 ml of 0.05 M Tris–HCl buffer, pH 7.0, and the beads were used as such for further studies. The 40 ml of decanted solutions and two washes were collected for FITC–BSA loss measurements. Similar procedures were used to prepare placebo beads without FITC–BSA entrapped. All batches were prepared in triplicate.

2.3. Determination of the encapsulation efficiency

The FITC–BSA content in the beads can be calculated from the difference between the total amount of protein added and the amount of protein lost in the external aqueous phase. The concentration of FITC–BSA in the decanted TPP and two washing solutions was determined by measuring the absorbance at 490 nm using a Beckman DU 7400 UV–Vis spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Using a series of different concentrations of FITC–BSA as standards, the amount of FITC–BSA lost in these solutions was quantified. The encapsulation efficiency (EE) can be calculated from the following expression:

$$\mathrm{EE}(\%) = \left[\frac{\text{total amt. of BSA} - \text{amt. lost}}{\text{total amt. of BSA}}\right] \times 100\%$$

2.4. In vitro release conditions and release media

In the release studies, pH 7.0 was used to mimic the pH of the colon. The pH of the small intestinal fluid was 7.5 as described in the United States Pharmacopeia (1995). A duration of 6 and 14 h was used to simulate transit times in the small intestine and in the colon, respectively. Four release media were used. One of those media is simulated intestinal fluid (SIF), which was 0.05 M phosphate buffer, pH 7.5, with 1% (w/v) pancreatin present, as described in the United States Pharmacopeia (1995). Pancreatin is a mixture of digestive enzymes, containing lipase, amylase, and protease, obtained from porcine pancreas. Another medium was SIF without pancreatin, 0.05 M phosphate buffer, pH 7.5, and was used to compare the drug release behavior with that in SIF. Almond emulsin beta-glucosidase preparation, 0.5% (w/v), prepared in 0.05 M, pH 7.0 phosphate buffer was another release medium. The solution of rat cecal and colonic enzymes was prepared and was used as one of the release media.

2.5. Preparation of rat cecal and colonic medium

Since the extracelluar portion of the rat cecal and colonic enzymes was found to have more profound degradation effects on chitosan than did the cell-associated enzymes in the preliminary studies (Zhang and Neau, 2001), only extracellular enzymes were isolated and used. In the process, rat cecal and colonic contents were collected and then enzyme systems from them were isolated by a differential centrifugation technique (Prizont and Konigsberg 1981). Briefly, male Sprague–Dawley rats were lightly anesthetized under ether and then sacrificed by decapitation. After removal of the cecum and colon contents from the rat, bacterial enzyme preparations were generated at 0–4 °C by weighing the contents in centrifuge tubes, diluting with cold 0.05 M phosphate buffer, pH 7.0, to give a final cecal and colonic dilution of 33% (w/v), and centrifuging at $500 \times g$ for 15 min to remove debris. Supernatants were then recentrifuged at $15\,000 \times g$ for another 30 min in order to obtain a clear supernatant containing extracellular enzymes. The protein content in the supernatant was determined by a Micro BCA (bicinchoninic acid) protein assay with bovine serum albumin as the protein standard and was adjusted to 5 mg/ml. Such a rat cecal and colonic enzymes system was used as one of the release media.

2.6. In vitro release studies

Release of FITC–BSA from the chitosan beads was studied in sealed 25 ml conical flasks in a Magniwhirl® constant temperature shaker bath (GS Blue M Electric, Blue Island, IL) at 37 °C and 60 spm. Initially, an 8 mg amount of beads was added to 15 ml of SIF or SIF without pancreatin and release was conducted for 6 h. At predetermined time points, a 0.5 ml sample was withdrawn and the same volume of corresponding release medium was added back to the system to maintain a consistent volume. After 6 h, beads were then transferred either to 15 ml of almond emulsin beta-glucosidase preparation or to 15 ml of rat cecal and colonic enzymes medium. Samples were withdrawn over another 14 h period. In the study, 200 μl of sample withdrawn at each time point was transferred to a Nunc™ 96 Microwell plate and FITC–BSA released from the chitosan beads under the above conditions was quantified using a TECAN SPECTRA FluorPlus fluorometer (TECAN U.S. Inc., Research Triangle Park, NC) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Placebo beads containing no FITC–BSA were exposed to the above release media for the same duration so that their release profile can serve as the negative control. The fluorescence in the case of samples from the almond emulsin beta-glucosidase preparation or from rat cecal and colonic enzymes medium was compared with results for the release medium without these enzyme systems present. Each experiment was conducted at least in triplicate.

2.7. In vitro enzymatic degradation studies

Enzymatic degradations of chitosan by pancreatin and by porcine pancreatic lipase present in SIF were studied using a literature viscometric procedure (Nordtveit et al., 1994). The viscosity of a 0.5% (w/v) solution of chitosan dissolved in pH 5.0, 0.1 M acetate buffer in the presence of 1% (w/v) pancreatin, or in 0.14% (w/v) porcine pancreatic lipase that provides a lipase activity comparable to that in SIF with pancreatin, was monitored as a function of time using a Ubbelohde capillary viscometer held at 30 ± 0.05 °C. The reactions were monitored by viscosity determination after 1, 2, 3, 4, and 5 h. A decrease in viscosity as a function of time, when compared with the results for a 0.5% (w/v) chitosan solution without enzymes present, is evidence of enzymatic degradation of the chitosan.

2.8. Statistical analysis

In order to analyze the significance of the differences in the amount of FITC–BSA released in the system versus control under each of the protein release conditions, a one-way paired Student's *t*-test was conducted at necessary time points. Such statistical analysis was also conducted to examine the differences in chitosan

degradation by pancreatin and by porcine pancreatic lipase at each time point. In addition, a single factor ANOVA was also employed to compare the total amount of protein released in the rat cecal and colonic medium at the end of the experiment under three different conditions: 6 h in SIF or SIF without pancreatin then followed by 14 h in rat cecal and colonic enzymes medium, or the entire 20 h in rat cecal and colonic medium. A difference was considered statistically significant if the P value is less than 0.05.

3. Results and discussion

3.1. Bead characteristics

The hydrogel beads produced all had good spherical geometry. There was no significant variation in particle sizes among the different batches of beads, the mean diameter being 2.62 ± 0.28 mm ($n = 10$). The loading efficiency, expressed as a percentage of the amount of FITC–BSA entrapped in the beads, was around 68% for each batch of beads.

3.2. In vitro release studies

Identification of an enzyme system that can accomplish polysaccharide degradation in a manner similar to that found in the colon environment, such as pectinase for pectin (Ashford et al., 1993) and dextranase for dextran (Brøndsted et al., 1995), and conducting the enzymatic degradation test for the polysaccharide-based colon delivery device in that corresponding system would be beneficial for a more mechanistic investigation. In addition, the variables in the system can be easily controlled so that it is possible to have reproducible experimental conditions to allow comparison of results. Such a system would also be useful for initial screening purposes without requiring rat handling. Since commercially available almond beta-glucosidase preparation had the ability to degrade chitosan due to an existing chitinase in the enzyme preparation (Grassmann et al., 1934; Wadsworth and Zikakis, 1984; Zhang and Neau, 2001) and it was found to have a similar degradation function on chitosan as that of rat cecal and colonic enzymes (Zhang and Neau, 2002), therefore, such a beta-glucosidase preparation was used as one of the release media to investigate the possibility of its use as an in vitro enzyme system to substitute for rat microbial enzymes in the initial screening study for chitosan-based colon delivery systems.

In the present study, the in vitro investigation was conducted in a way not only to test the degradability of chitosan hydrogel beads by both rat cecal and colonic enzymes and the almond beta-glucosidase preparation but also to assess the resistance of such delivery system to drug release under conditions found in the small intestine.

Fig. 1 shows the percentage of FITC–BSA released as a function of time from chitosan–TPP hydrogel beads during conditions mimicking the pH and times likely to be encountered during intestinal transit to the colon (6 h in pH 7.5 enzyme-free SIF followed by 14 h in pH 7.0 rat cecal and colonic enzymes medium or beta-glucosidase solution). The release profiles reveal that, under each condition, there was only about 10% of FITC–BSA released during the first 6 h in enzyme-free SIF medium. The cumulative amount of BSA released over 20 h in the enzyme-free SIF, control group, was around 20%. As compared with the control, the release of BSA was increased

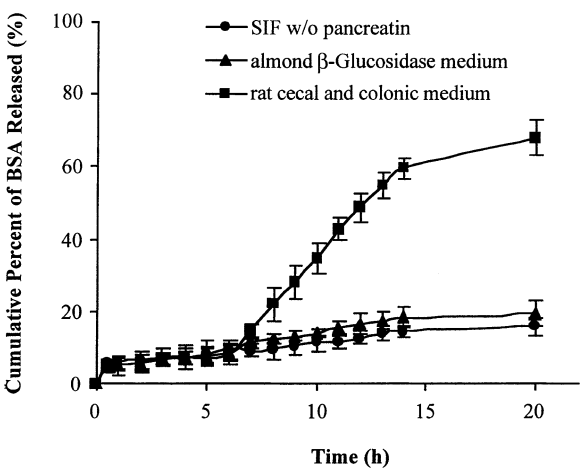

Fig. 1. The cumulative percentage of BSA released in pancreatin-free SIF for 6 h, then followed by three different conditions for another 14 h.

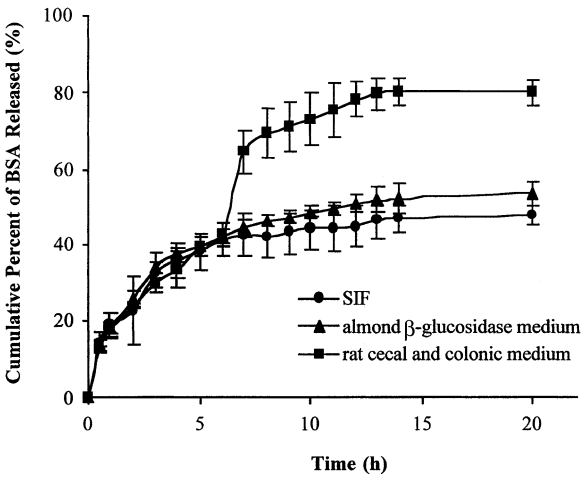

Fig. 2. The cumulative percentage of BSA released in SIF for 6 h then followed by three different conditions for another 14 h.

markedly when the beads were exposed to rat cecal and colonic medium with more than 60% protein released over 20 h, indicating that chitosan cross-linked with TPP in the hydrogel bead form was still available for degradation by rat cecal and colonic bacterial enzymes and, as a consequence, resulted in enhanced protein release. On the other hand, as results show in Fig. 1, the release profile associated with the almond beta-glucosidase preparation was parallel to that of the control group. No statistically significant difference was observed in BSA release between these two groups over 20 h time, suggesting that chitosan present in such a bead form was not susceptible to degradation by this commercially available almond beta-glucosidase preparation.

Similarly, the release profiles of BSA under three different conditions are illustrated in Fig. 2, 6 h in SIF (with pancreatin present), then followed by 14 h in the same SIF medium used as the control group, or in almond beta-glucosidase solution, or in rat cecal and colonic medium. The results reveal that, as compared with the control group, the rat cecal and colonic enzyme medium resulted in a marked enhancement in BSA release. However, there was no similar release behavior of BSA from chitosan hydrogel beads observed in the almond beta-glucosidase preparation medium.

Fig. 3 shows the release profile of BSA over 20 h under different conditions: 6 h in SIF or enzyme-free SIF followed by 14 h in rat cecal and colonic enzymes medium, or the entire 20 h in rat cecal and colonic enzymes medium. The total percentages of entrapped FITC–BSA that were released from the beads at the end of a total of 20 h under these conditions are as follows: after 6 h in SIF followed by 14 h in rat cecal and colonic enzymes medium, $80.0 \pm 3.63\%$ released; after 6 h in enzyme-free SIF followed by 14 h in rat cecal and colonic enzymes medium, $68.0 \pm 4.88\%$ released; and after 20 h in rat cecal and colonic enzymes medium, $74.6 \pm 6.68\%$ released. Although the appearance of the release profiles, as seen in Fig. 3, was varied due to the three different initial conditions, the cumulative percentage of BSA released over the entire experiment (20 h) was not significantly different among them, indicating that the ability of rat cecal and colonic enzymes to degrade chitosan formulated in the hydrogel beads was independent of pretreatment conditions.

Based on the above results, it is clear that protein release from the chitosan hydrogel bead was faster in rat cecal and colonic medium when compared with the enzyme-free SIF or SIF control medium. The cross-linked chitosan hydrogel bead can retain about 80 or 60% of its macromolecule content over 20 h in the enzyme-free SIF or SIF medium, respectively, which simulate the

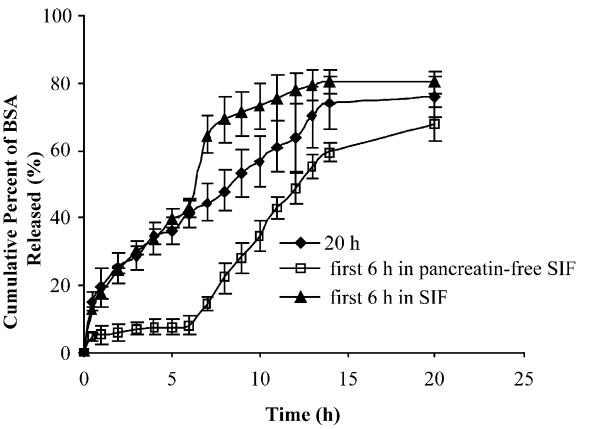

Fig. 3. Release profile of BSA in rat cecal and colonic medium under different conditions.

physiological conditions of the small intestine. On the other hand, the rat cecal and colonic enzymes medium, at a similar pH and over the same duration, significantly enhanced release of macromolecule, suggesting that this carrier can be used to deliver macromolecules to the colon.

3.3. Comparison of rat cecal and colonic enzymes and almond beta-glucosidase

The ability of the enzymes to penetrate into the hydrogel beads determines the extent of enzyme-catalyzed degradation and the mode of degradation, i.e. surface versus bulk degradation. It has been observed that, for hydrophilic materials, such as hydrogels, an increase in swelling after degradation indicates bulk degradation (Langer and Peppas, 1981). In this study, by visual observation, the beads in rat cecal and colonic enzymes medium were swollen to a large extent with bead size two to three times larger than that of control ones (results were not quantified). However, such a phenomenon was not observed for the beads in the almond beta-glucosidase preparation release medium. Thus, it may be concluded that rat cecal and colonic enzymes were more effective at diffusing into the beads than were enzymes from the beta-glucosidase preparation, enabling bulk degradation by the cecal and colonic enzymes. The degradable bond in chitosan that must be recognized and degraded by rat colonic microbial enzymes or chitinase in almond beta-glucosidase preparation to effect depolymerization is its beta-1,4 glycosidic bond. The BSA release from the beads is dependent on the enzyme-catalyzed hydrolysis of those bonds. Conformational and configurational changes in chitosan following cross-linking could influence the degree of enzyme penetration, and the ability of cross-linked chitosan to enter the active site of the chitinase from the beta-glucosidase preparation might have been hindered.

Table 1 summarizes the cumulative percentage of FITC–BSA released from the beads at the end of experiments under the different conditions. The results confirm that, compared with the corresponding control, protein release was faster in the rat cecal and colonic medium, but no difference was found in the almond beta-glucosidase preparation medium. The above results indicate that even though the positively charged amino groups of chitosan were cross-linked by TPP, chitosan was still able to be degraded by rat cecal and colonic enzymes. The accessibility of the degradable bonds of chitosan to rat colonic microbial enzymes was apparently not substantially hindered by TPP cross-linking. On the contrary, the almond beta-glucosidase preparation containing a chitinase did not accomplish the chitosan hydrogel bead degradation that was observed with the rat cecal and colonic enzymes.

Table 1
Cumulative percentage of BSA released at 20 h, the end of experiment, under different conditions[a]

Condition	Cumulative percentage released (%)
Control (SIF w/o pancreatin)	16.0 ± 2.25
Almond β-glucosidase (first 6 h in SIF w/o pancreatin)	19.5 ± 3.56
Rat cecal and colon medium (first 6 h in SIF w/o pancreatin)	68.0 ± 4.87[b]
Control (SIF)	47.8 ± 2.64
Almond β-glucosidase (first 6 h in SIF)	53.7 ± 2.89
Rat cecal and colon medium (first 6 h in SIF)	80.0 ± 3.63[b]

[a] Mean ± S.D., $n = 3$.
[b] Indicates that there is a statistically significant difference between this value and that of the respective control ($P < 0.01$).

3.4. Further in vitro enzymatic degradation studies

When the release profiles in the two control media, i.e. SIF with and without pancreatin, are compared (Fig. 4), a substantial difference in BSA release rates was observed. Since it is known that chitosan, like other dietary fibers, is not digested in the human small intestine, it was necessary to investigate whether chitosan was susceptible to degradation by the porcine intestinal digestive enzymes present in pancreatin. By using a previously described viscometric procedure (Zhang and Neau, 2001), the susceptibility of chitosan to

degradation by 1% (w/v) pancreatin present in SIF was studied and characterized by the decrease in the specific viscosity of the chitosan dispersion as a function of the reaction time. Pancreatin contains lipase, amylase, and protease from porcine pancreas. Since animal, wheat germ, and microbial lipases have been found to hydrolyze chitosan (Muzzarelli, 1999), it was suspected that porcine pancreas lipase was the enzyme component in pancreatin responsible for chitosan degradation. With a concentration of porcine pancreas lipase of 0.14% (w/v), whose lipase activity was the same as that in 1% (w/v) pancreatin as determined by a Sigma lipase diagnostic kit, the effects of pancreatin and porcine pancreas lipase on the viscosity of the chitosan dispersion were determined and the results are presented in Fig. 5. The results show that pancreatin and porcine pancreas lipase caused substantial loss in the specific viscosity of the chitosan dispersion over the reaction time, as compared with the control without enzymes present, suggesting that a certain extent of depolymerization occurred in chitosan. As the specific viscosity profiles of the porcine pancreas lipase group and of the pancreatin treatment group nearly overlapped each other, with no statistically significant difference observed over the reaction time period, the results in Fig. 5 substantiate that the porcine pancreas lipase preparation can degrade chitosan, which could be due to porcine pancreas lipase itself or an enzyme impurity present in the enzyme preparation.

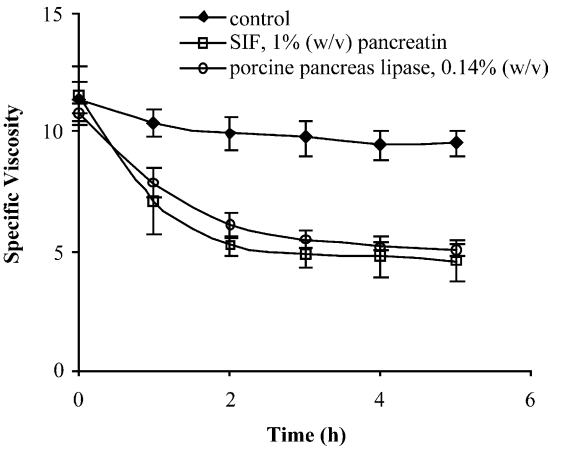

Fig. 5. Profiles of the change in specific viscosity of a chitosan dispersion following treatment with pancreatin or with porcine pancreas lipase.

Even though a larger amount of BSA was released from the beads in the presence of porcine pancreas digestive enzymes than in buffer alone, which would suggest premature protein release in the upper GI tract, such release can be attributed to the degradation function of porcine pancreas lipase. It has not been reported that human lipase shares this property with lipase from the porcine source, and chitosan is not known to be a substrate for human lipase in vivo.

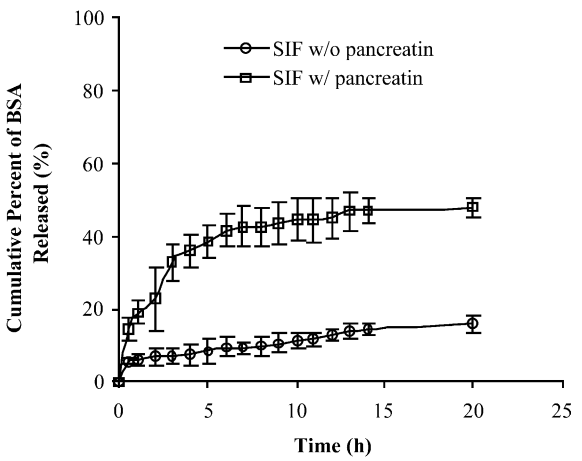

Fig. 4. Release profile of BSA in pancreatin-free SIF and SIF over 20 h.

4. Conclusions

The degradability of a chitosan hydrogel bead multiparticulate system by colonic bacterial enzymes has been confirmed. The results indicate that rat cecal and colonic enzymes still attacked chitosan even after it has been cross-linked and its solubility reduced, resulting in a greater protein release under conditions pertaining to the colon. If extrapolation can be made to the human colon, this chitosan-based delivery system could serve as a carrier to deliver macromolecules to the colon. On the other hand, the degradability of chitosan by human pancreatic lipase in vivo is worthy of investigation. The almond beta-glucosidase prepa-

ration cannot substitute for rat cecal and colonic enzymes in the in vitro assessment of the performance of chitosan-based colonic delivery systems.

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