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Release of albumin from chitosan-coated pectin beads in vitro

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Abstract

The release behavior of albumin from chitosan-coated pectin beads in vitro was investigated. The factors, such as concentration of $CaCl_2$, molecular weight of chitosan, pH of chitosan solution, and pH of release medium, which can have a significant effect on the release behavior from the beads, were discussed in this study. The loading efficiency (LE) of albumin showed maximum value when the concentration of $CaCl_2$ and the weight ratio of pectin to albumin were 2 wt.% and 2, respectively. The release of albumin from pectin beads could be retarded by coating with chitosan at various pH medium. The increase of the concentration of $CaCl_2$ induced the decrease of albumin release for uncoated-pectin beads, but not much difference of release for coated-pectin ones. The higher molecular weight of chitosan showed less albumin release than the lower one. The release of albumin from the chitosan-coated pectin beads was dependent on pH of coating solution and release medium, which might affect the degree of swelling of pectin beads.

Keywords: Pectin bead; Chitosan coating; Albumin; Swelling; Release behavior

1. Introduction

Protein drugs are increasingly becoming a very important part of therapeutic agents with the rapid advances in the field of biotechnology (Sriamornsak, 1998). However, delivery of protein drugs is highly challenging due to the low permeability, short circulatory half-life, rapid proteolysis, low stability, and immunogenicity of the protein drugs. Recently, these drugs are mostly delivered by parenteral administration. According to their short-acting, repeated injections are often required and this can lead to another side effects. To overcome these problems, non-parenteral route of administration as well as the controlled delivery systems are in urgent need of studying (Tomlinson and Livingstone, 1989).

Oral delivery is the most popular and convenient method for drug delivery. However, protein drugs are easily denatured in the stomach and degraded by enzymes present in the gastrointestinal tract (GI). Therefore, it is in need of developing new drug delivery systems which can be used for non-parenteral administration and other controlled release protein delivery systems.

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Hydrogel systems have been used broadly in the development of delivery systems for protein drugs (Chen et al., 1995). Hydrogels of natural polymers, especially polysaccharides have been widely used because of their unique advantages such as non-toxic, biocompatible, biodegradable and abundant properties (Pitt, 1990).

Pectin is a heterogeneous anionic polysaccharide present in the cell of most plants. It consists mainly of linearly connected α-(1→4)-D-galacturonic acid residues, which have carboxyl groups. Recently, pectin has attracted attention as a carrier for colon targeting since it is almost totally degraded by colonic bacteria and is not digested by gastric or intestinal enzymes (Cummings et al., 1979; Ashford et al., 1993). In addition, pectin can form the gelation by cross-linking with calcium ions. Intermolecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules, called an 'egg-box' conformation with interstices in which the calcium ions may pack and be coordinated (Grant et al., 1973). Pectin beads were prepared by ionic gelation method. Amidated pectin hydrogel beads were also prepared as a delivery matrix for chloroquine (Munjeri et al., 1998). But the pectin beads have several drawbacks such as fast breakdown of the beads and difficulty of the controlled release of drugs.

Chitosan which consists of *N*-acetyl-glucosamine and glucosamine residues is a cationic polysacchride made from alkaline *N*-deacetylation of chitin. It forms polyelectrolyte complexes with polyanionic polymers such as alginate (Takahashi et al., 1990; Huguet and Dellacherie, 1996). Albumin and alginate microspheres, for example, have been treated with chitosan solution to improve the properties of microspheres. It was reported that cisplatin albumin microcapsules coated with chitosan showed higher antitumor activity than untreated microcapsules. Furthermore, cisplatine release could be controlled by the use of chitosan (Nishioka et al., 1989; Kyotani et al., 1992). A modified method was used to prepare chitosan-reinforced alginate gel beads (Murata et al., 1993) and the encapsulating processes based on the electrostatic interaction in the formation of a reinforced gel have also been described (Knorr and Daly, 1998; Pandya and Knorr, 1991). The properties of such amidated pectin beads may be altered by the formation of a polyelectrolyte complex membrane around the bead using cationic polymers such as chitosan in a similar manner to that used with alginate gel beads (Munjeri et al., 1997; Chang and Lin, 2000). In addition, the cationic polyelectrolyte nature of chitosan could provide a strong electrostatic interaction with mucus or a negatively charged mucosal surface (Fibrig et al., 1994, 1995). And these mucoadhesive properties of chitosan can be expected to prolong the residence time of protein drug carrier in GI (Gåserød et al., 1998). Liposomes coated with chitosan have also been shown in vivo to have a prolonged residence time in the GI tract of rats relative to uncoated liposomes (Takeuchi et al., 1996; Yamamoto et al., 2000).

In this study, the pectin beads prepared by using gelation of calcium chloride with pectin were coated with chitosan to enhance the mechanical property and mucoadhesiveness of pectin beads, and to control the release of albumin from the chitosan-coated pectin beads for potential use as an oral delivery system for protein, using bovine serum albumin (BSA) as a model protein. The factors such as concentration of $CaCl_2$, molecular weights of chitosan, pH of chitosan solution and release medium, which have a significant effect on the release behavior from the beads, were discussed in this study.

2. Materials and methods

2.1. Materials

Esterified low-methoxy pectin (potassium salt from citrus fruit, degree of esterification DE = 26; total galacturonic acid = 66%) was purchased from Sigma (St. Louis, MO, USA). High molecular weight chitosan (MW: 310 000–380 000) and medium molecular weight chitosan (MW: 190 000–310 000) were provided by Aldrich (Mil-

waukee, WI, USA). The degree of *N*-deacetylation, determined by IR spectroscopy (Baxter et al., 1992), was about 84%. BSA (MW = 66.400) and bicinchoninic acid (BCA) solution was obtained from Sigma.

2.2. Preparation of albumin-loaded pectin bead

The preparation of pectin bead is similar to that of Sriamornsak's method (Sriamornsak and Nunthanid, 1998). Low-methoxy (LM) pectin and albumin were dissolved in distilled water at a concentration of 5% (w/v), respectively. Solution of albumin was added to aqueous solution of pectin at the specified pectin/albumin ratios (w/w) of 4/1, 2/1, and 1/1, and mixed thoroughly by stirring. Ten milliliter mixture of pectin and albumin solutions was dropped into 500 ml agitated calcium chloride solution (0.5, 1, 2, 5% w/v) by peristaltic pump (Watson–Marlow, London) through the 30 G needle connected with a tube of 1 mm diameter. After 30 min of gelation with stirring, the beads were filtered, washed, treated with acetone and then vacuum dried at 50 °C for 12 h.

2.3. Preparation of chitosan-coated pectin beads

After filtering the pectin beads prepared previously, the pectin beads were transferred into 100 ml chitosan solution and coated for 2 h with stirring. The chitosan-coated pectin beads were rinsed, filtered, acetone-treated, and then vacuum dried at 50 °C for 12 h. The chitosan solution (0.25% w/v) was made by dissolving the polymer in 1% (w/w) acetic acid and the pH value of chitosan solution was adjusted to the various pH (3, 4, and 5) by adding 0.1 N HCl or 0.1 N NaOH solution.

2.4. Evaluation of albumin-loading efficiency of pectin beads

The loading of albumin in pectin beads was determined using an indirect procedure. The albumin-loaded pectin beads were separated from suspension medium by filtering and the non-loaded free albumin in the medium was measured by UV spectrophotometry at $\lambda = 562$ nm using BCA protein assay (Smith et al., 1985). The loading efficiency (LE) was calculated from equation indicated below (Calvo et al., 1997). All experiments were performed in triplicate.

$$\mathrm{LE}(\%) = \frac{\text{Total amount albumin} - \text{Free amount albumin}}{\text{Total amount albumin}} \times 100$$

2.5. Swelling studies and release of albumin in vitro

The swelling behavior of the beads was studied by measuring the diameter of the beads by the digital camera (NikonE990, Japan). For the pH dependent swelling studies, beads were immersed in 0.2 M hydrochloric acid–potassium chloride buffer solution for pH 1.2 simulated gastric condition, 0.2 M sodium phosphate buffer solution for pH 7.4 simulated intestine condition and 0.2 M acetic acid–sodium acetate buffer solution for pH 5, the middle range of both conditions. The solutions were thermostated at 37 °C. The initial mean diameter (D_p) of 20 dried beads and the diameters (D) after specified time intervals of immersion in test solution were calculated by image analysis program after images were taken through digital camera. The magnitude of swelling was presented by the ratio of the mean diameter of swollen beads to the mean diameter of the dried beads before the test and the diameter increase was determined as $(D - D_p)/D_p \times 100$.

The albumin-loaded chitosan-coated pectin beads (0.01 g) enclosed in teabags were placed into 10 ml of buffer solution at 37 °C under mild

agitation in a water bath. At predetermined time intervals, samples were taken and replaced into equal volume of fresh buffer solution. And the amount of albumin released from the beads collected at each time intervals was taken and measured by UV spectrophotometry at $\lambda = 562$ nm using BCA protein assay. All experiments were performed in triplicate.

2.6. Observation of scanning electron microscopy (SEM)

The surface and cross-section morphologies of the albumin-free pectin beads, albumin-loaded beads and chitosan-coated pectin beads were observed using a scanning electron microscope (JSM-5410LV, JEOL, Japan). Before gold coating, the beads were sectioned and freeze-dried to observe the inner structure.

2.7. Measurement of Fourier transform infrared spectrometry (FT-IR)

Infrared (IR) spectra of pectin, pectin–chitosan complex, and chitosan were recorded with FT-IR spectrometer (M series, Midac Co., USA). Pectin beads and chitosan-coated pectin beads were squashed and spread onto silicon wafers with spatulas, and dried at room temperature for 1 day and then dried in a vacuum oven for 2 days to remove the solvent completely. Chitosan film was cast from 0.25% (w/v) chitosan solution onto glass petridish and dried in the same method indicated above.

3. Results and discussion

3.1. Preparation of albumin-loaded pectin beads

The ionic interaction between the negatively charged carboxyl groups of LM pectin and the positively charged counter ion, calcium, was used to prepare pectin beads. Aqueous solution of LM pectin containing albumin was dropped into calcium chloride solution and a gelled sphere was formed instantaneously by ionotropic gelation (Sriamornsak, 1998). The weight ratio of pectin to albumin and the concentration of $CaCl_2$ were selected by the loading efficiencies (LE) of albumin to set the optimum condition for the preparation of pectin bead. The percent LE of albumin in pectin beads was calculated from the fractional amount of drug remaining in the beads. The albumin loading efficiencies of various beads prepared are given in Table 1. The loading efficiencies were dependent on $CaCl_2$ concentration and the weight ratio of pectin to albumin. The results indicated that the optimum conditions for the preparation of pectin beads were 2 wt.% of $CaCl_2$ and 2 of the weight ratio of pectin to albumin. So these conditions were chosen for the chitosan coating.

Table 1
Loading efficiency (%) of albumin

$CaCl_2$ concentration (%)	P:A*		
	4:1 ± S.D.	2:1 ± S.D.	1:1 ± S.D.
0.5	58.2 ± 4.1	63.2 ± 1.3	61.6 ± 2.1
1	63.0 ± 5.0	70.5 ± 1.8	64.6 ± 4.3
2	70.5 ± 3.5	73.8 ± 3.0	67.1 ± 1.0

P:A* = pectin:albumin weight ratio.

Fig. 1 shows S.E.M. of the pectin bead (a), and albumin-loaded pectin one(b). The results indicated that the pectin bead had spherical shape and smooth surface, while the fibril-like structure by the exposure of the albumin was observed on the surface of the albumin-loaded pectin beads. Some distorted shapes could be observed during filtering and freeze-drying of the beads.

3.2. Effect of chitosan coating on the physicochemical properties of the pectin beads

The extent of swelling of the pectin beads upon hydration can play an important role on the release of albumin. Fig. 2 shows the effect of chitosan coating on the swelling behavior of pectin beads at various pH buffer solutions. When dried pectin beads were put into the pH 1.2 buffer solution (a), the carboxyl groups of pectin ($pK_a = 3.5$) were protonated and became neutral. So the hydrogen bonds and hydrophobic forces between or within pectin molecules increased the network

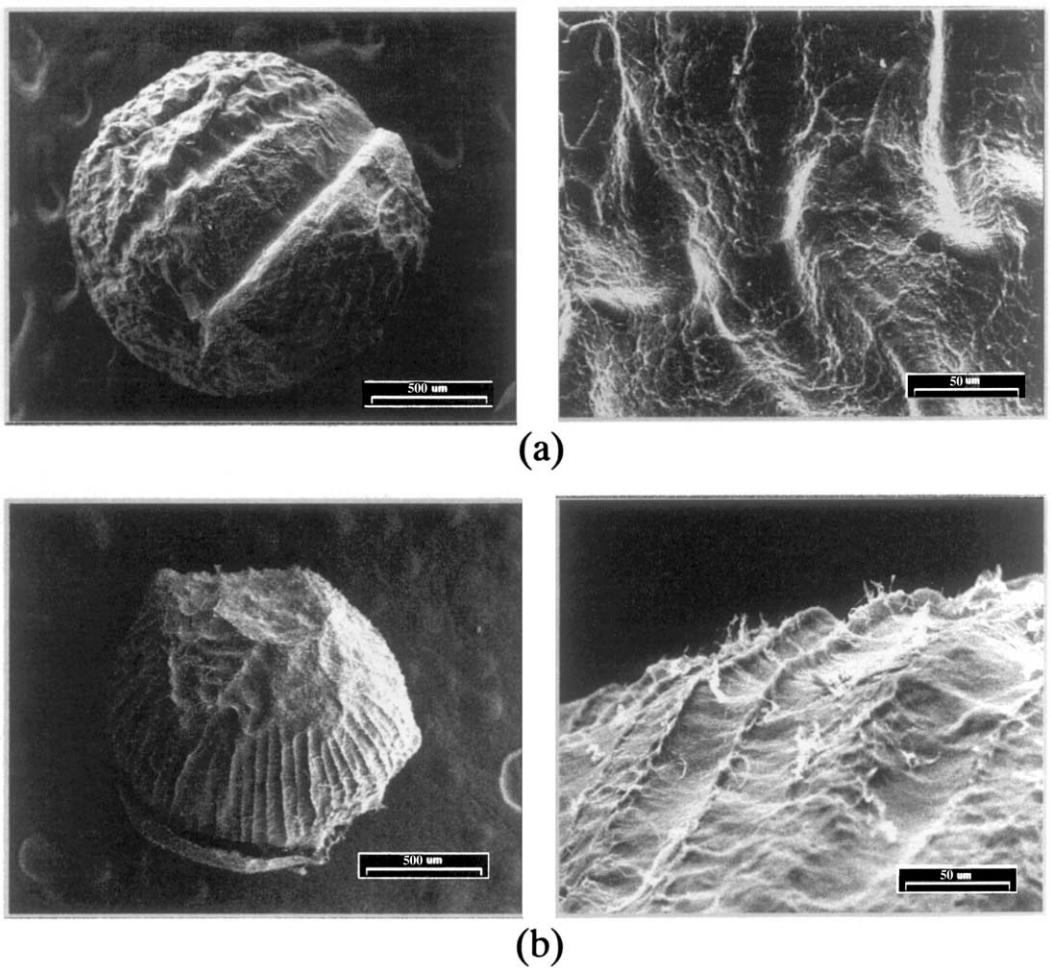

Fig. 1. Scanning electron micrographs of (a) surface of the pectin bead without albumin; and (b) surface of the albumin-loaded pectin bead.

elasticity and the pectin beads rarely swelled. Besides, the chitosan-coated pectin beads swelled less than the uncoated ones, which was due to the formation of skin layer with chitosan. Dried pectin beads and chitosan-coated pectin beads swelled much greater extent at pH 5 buffer solution (b) than at pH 1.2 buffer solution. This indicated that the carboxyl groups of pectin got partly negative charge, but the chitosan-coated one still had an effect on restriction of swelling compared with uncoated one. As shown in Fig. 2, the initial extent of swelling is higher for coated ones at pH 1.2, and 5, indicating that the initial swelling might be influenced by the chitosan coating membrane which had strong positive charge repulsion between $-NH_3^+$ groups. When the dried pectin beads were put into pH 7.4 buffer solution, the uncoated one swelled much faster than previous cases and then rapidly broke into pieces, and precipitated even within 1 h, so it was impossible to measure the swelling ratio. This indicated that a small number of charges present on the phosphate buffer solution might allow the gel beads to swell more strongly due to greater solvent penetration into the calcium pectinate network, followed by greater ion exchange between calcium and potassium ions. However, after coating of pectin beads with chitosan, the beads kept their spherical shapes

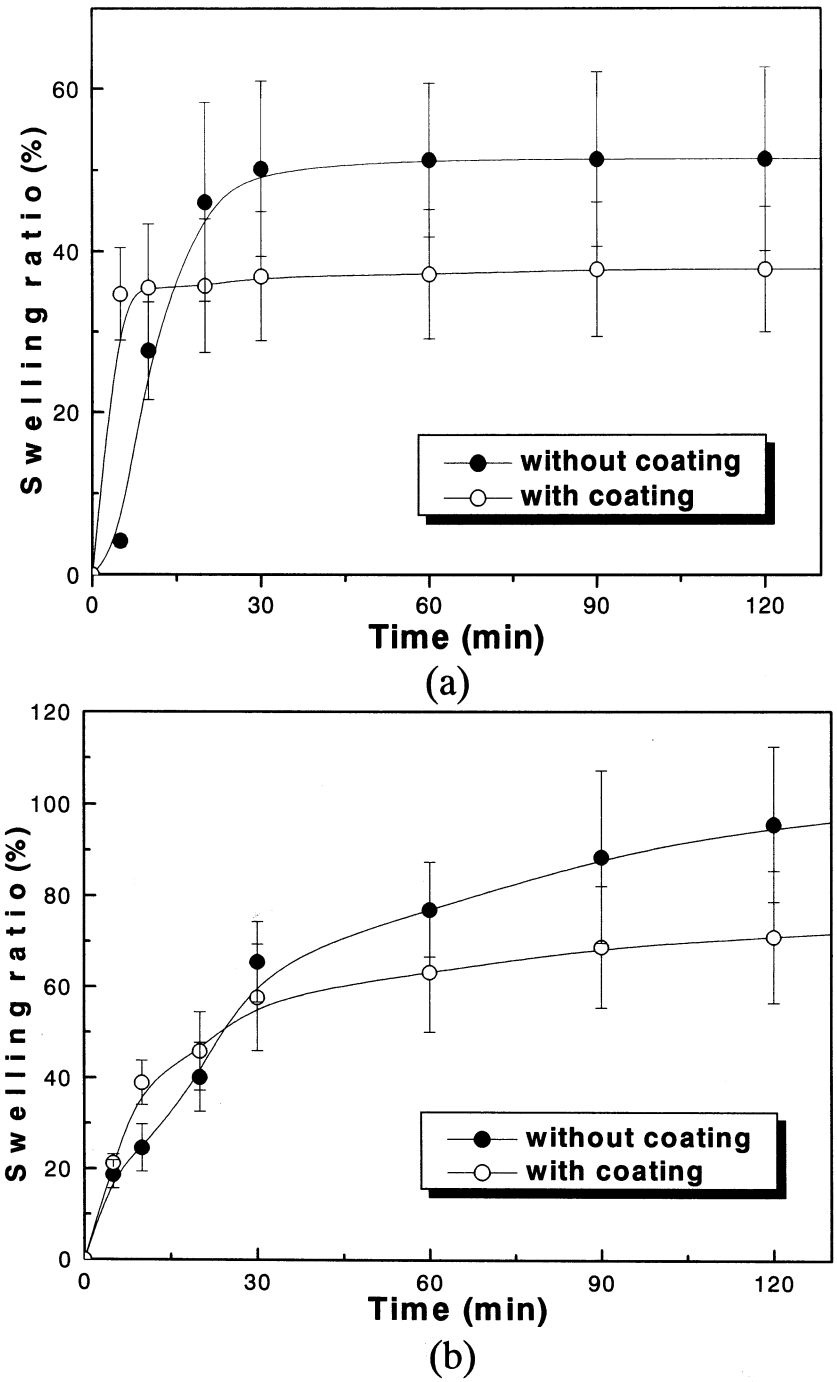

Fig. 2. The effect of chitosan coating on the swelling behavior of pectin beads at (a) pH 1.2; (b) pH 5 buffer solution (bars indicate S.D.; $n = 3$).

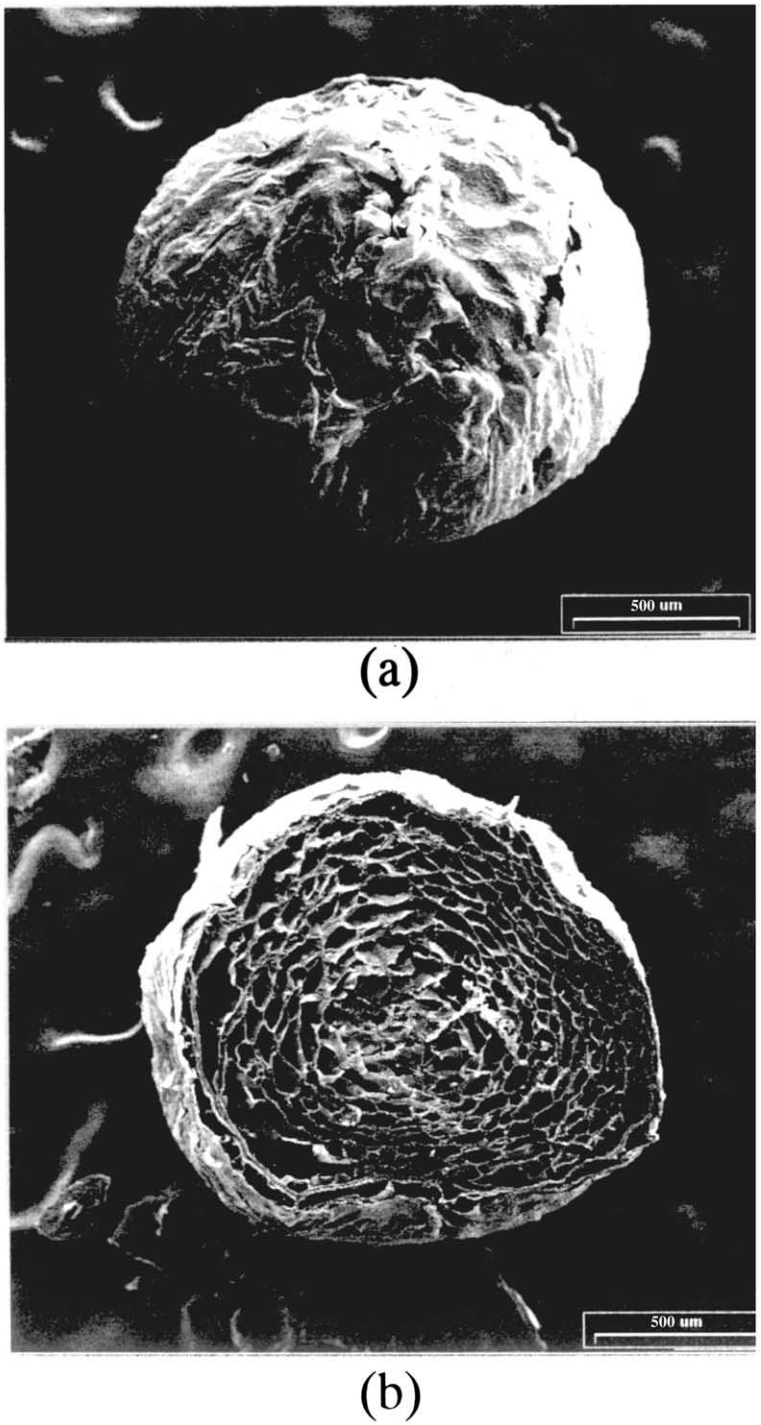

Fig. 3. Scanning electron micrographs of the chitosan-coated pectin bead (a) surface; and (b) cross-section.

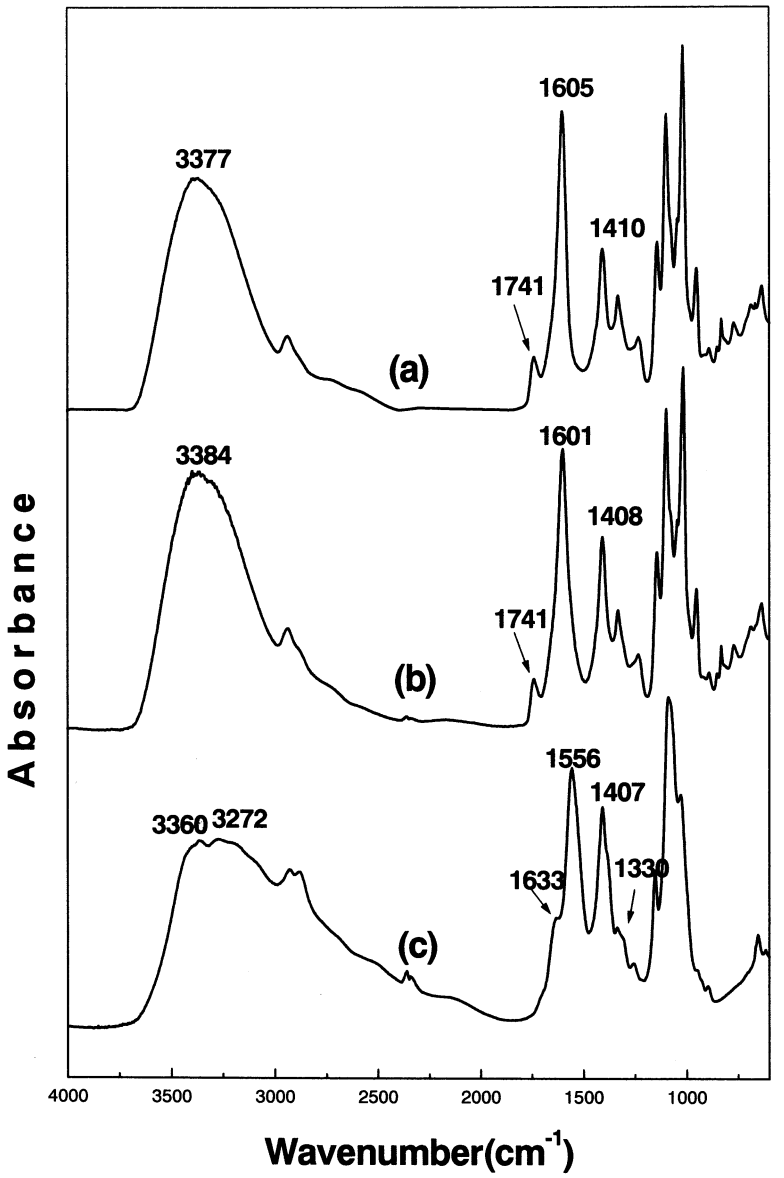

Fig. 4. FT-IR spectra of (a) pectin; (b) pectin–chitosan composite; and (c) chitosan.

more than 3 days at pH 7.4 buffer solution, and the mechanical strength was enhanced (data not shown).

Fig. 3 shows S.E.M. photographs of the surface (a) and the cross-section (b) of the chitosan-coated pectin bead. The surface of the pectin bead was covered with the thin layer and the albumin revealed on the surface (Fig. 1(b)) was not seen after coating with chitosan.

In order to confirm the pectin–chitosan interaction, and its role in the release mechanism, samples were analyzed by IR spectroscopy. Fig. 4 shows FT-IR spectra of pectin(a), pectin–chitosan complex (b), and chitosan (c). Pectin

showed the characteristic band of C=O vibration of COOH groups at 1741 cm^{-1}, strong absorption band around 1605 cm^{-1} belonging to the asymmetric stretching vibration of COO^-, and the band around 1410 cm^{-1} of –COO symmetric stretching vibration (Yao et al., 1997; Gnanasambandam and Proctor, 2000; Sinitsya et al., 2000). Chitosan showed the characteristic band of amide at 1663 cm^{-1} and band of amine at 1556 cm^{-1}. In the case of the pectin–chitosan complex, the band of COO^- at 1605 cm^{-1} was shifted to 1601 cm^{-1}. However, band shift of the chitosan was not shown due to the overlapping with bands of the pectin. Consequently, it seems that ionic interaction between chitosan and pectin is the main force of the formation of membrane formed by chitosan coating.

3.3. Release of albumin from chitosan-coated pectin beads

The study of albumin release profiles from chitosan-coated pectin beads at pH 7.4 is critical matter for prolonged release in the intestine. However, due to the rapid dissolution of pectin beads at pH 7.4 buffer solution, it was impossible to compare the release profiles of albumin from pectin beads and chitosan-coated ones. Therefore, the release study for the effect of chitosan coating compared with uncoated ones were performed at pH 5 buffer solution, but, release studies in other experimental conditions were performed at pH 7.4 buffer solution.

The effect of chitosan coating on the representative release profiles of albumin from pectin beads at pH 5 buffer solution is shown in Fig. 5. This indicated that the release of albumin from pectin beads was retarded by coating with chitosan. And the retardation of albumin release was also obtained at other pH buffer solutions such as pH 1.2 (data not shown).

Albumin release from uncoated-pectin beads was decreased at pH 5.0 with an increase of $CaCl_2$ because the more intermolecular cross-links were formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules and resulted in the decreased swelling ratio of the pectin beads. But albumin release from pectin beads coated with chitosan was not much changed with an increase of $CaCl_2$, although swelling ratio of the pectin beads was decreased owing to the polymer complex formation between chitosan and pectin (data not shown). Therefore, it can be said that the release pattern is affected by both the structure of the

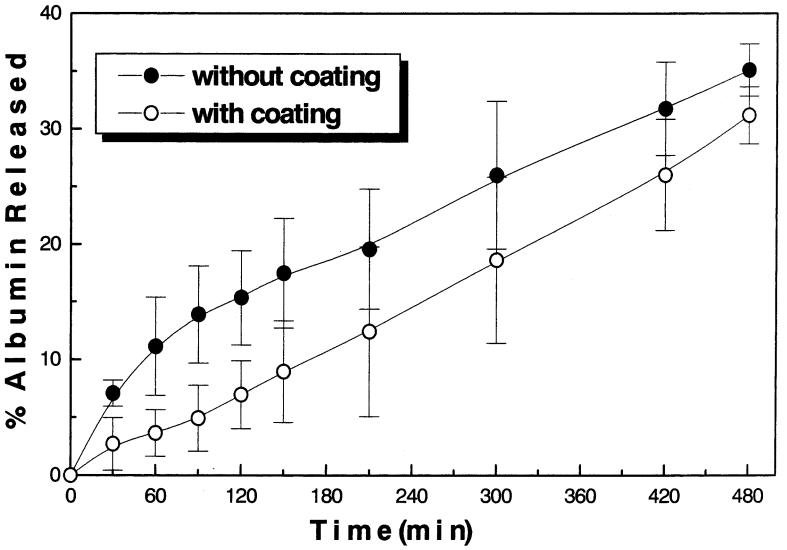

Fig. 5. The effect of chitosan coating on the release profiles of pectin beads at pH 5 buffer solution (bars indicate S.D.; $n = 3$).

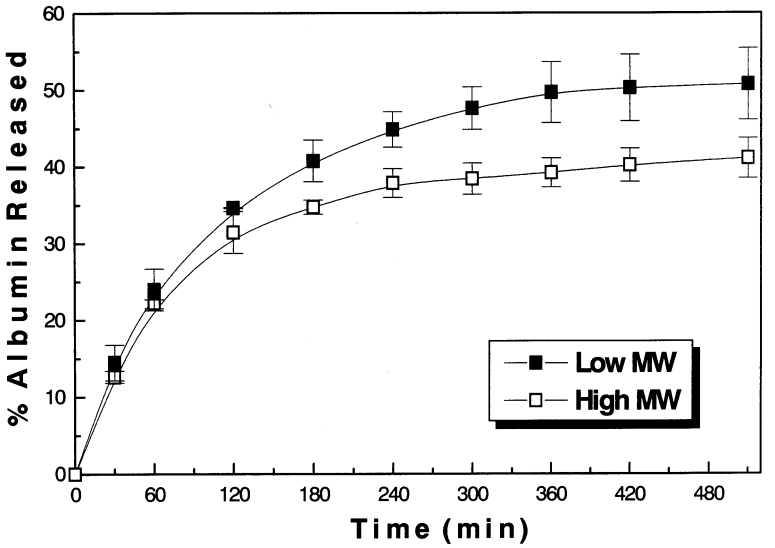

Fig. 6. The effect of chitosan molecular weight on the release of albumin from chitosan-coated pectin beads at pH 7.4 buffer solution (bars indicate S.D.; $n = 3$).

chitosan coating membrane and the inner core intermolecular structure of pectin.

Fig. 6 shows the effect of chitosan molecular weights on the release of albumin from chitosan-coated pectin beads at pH 7.4 buffer solution. The higher molecular weight of chitosan showed less albumin release than the lower one. This may be due to the fact that larger molecules of chitosan formed more entanglements between chitosan and pectin during formation of coating layer, and resulted in stronger outer coating membrane than those of smaller molecules.

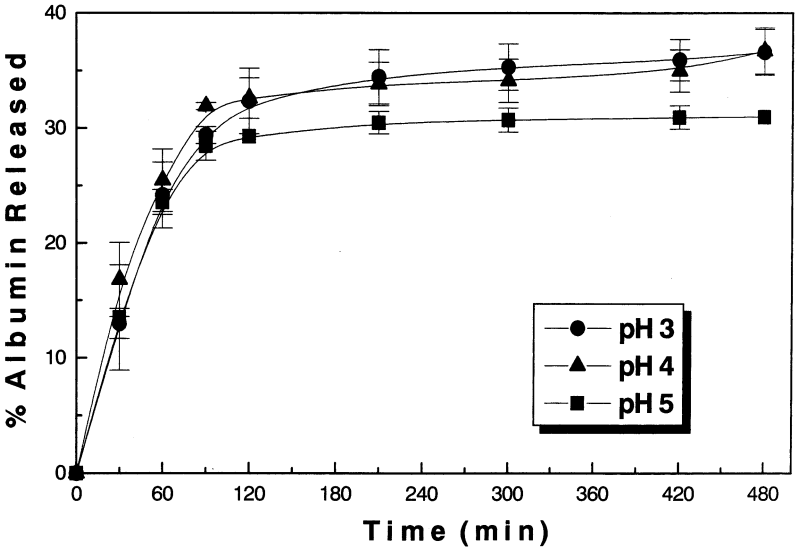

Fig. 7. The effect of pH of the coating solution on the release of albumin from chitosan-coated pectin beads at pH 7.4 buffer solution (bars indicate S.D.; $n = 3$).

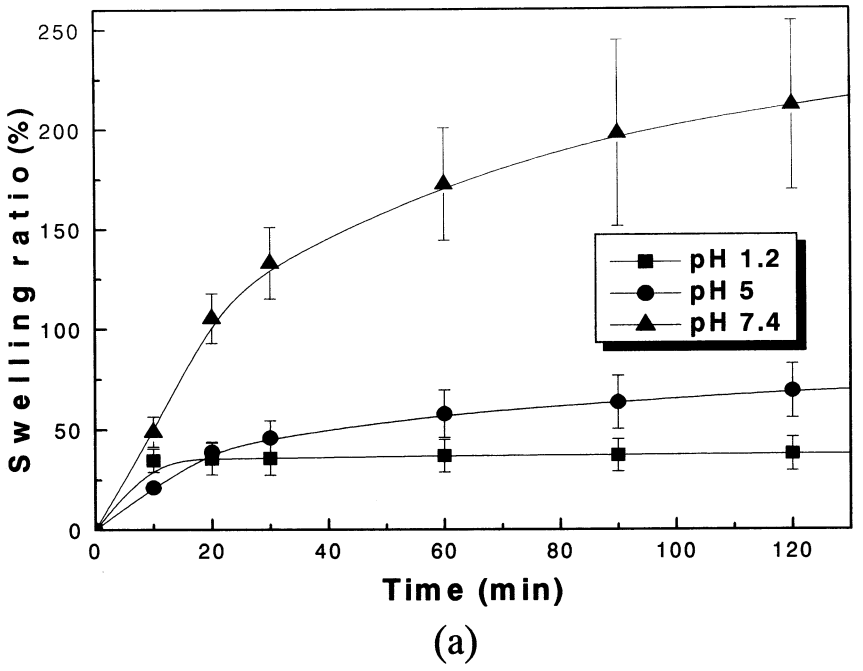

Fig. 8. The swelling behavior (a) and the release profiles; (b) of chitosan-coated pectin beads at various pH medium (bars indicate S.D.; $n = 3$).

Effect of pH at chitosan coating on the release of albumin from pectin beads was shown in Fig. 7. The results indicated that the least of albumin was released at pH 5 than pH 3 and 4. It is thought that the strongest ionic interaction between $-NH_3^+$ of chitosan and $-COO^-$ of pectin was formed at pH 5 coating solution and resulted in more dense skin layer formation by chitosan coating.

Fig. 8 shows the swelling behavior (a) and the release profiles (b) of chitosan-coated pectin beads at various pH medium. The swelling degree of the chitosan-coated pectin beads decreased in the acidic range and increased in the alkaline one, indicating that the swelling degree is much more influenced by the pectin inner structure than by the chitosan coating layer, which would get more hydrogen bonding in the acidic condition. But it showed less albumin release in pH 5 buffer solution than in pH 1.2, and the most albumin release in pH 7.4 buffer solution. These results suggest that the release pattern is influenced by the chitosan coating layer, which would get the strongest ionic interaction between $-NH_3^+$ of chitosan and $-COO^-$ of pectin in pH 5 buffer medium.

4. Conclusions

In this paper, chitosan-coated pectin beads for a novel oral delivery of protein drug were successfully prepared. The release of albumin from pectin beads could be retarded by coating with chitosan in various pH medium and it was dependent on pH of coating solution and release medium. And the swelling behavior was much influenced by the inner structure of pectin whereas the release pattern of albumin was mainly dependent on the outer coated membrane.

This research suggested that chitosan-coated pectin beads were able to sustain the release of albumin under simulated gastric condition and release it under simulated condition in colon. And the release of albumin could be regulated by the appropriate choice of experimental conditions for the preparation of chitosan-coated beads. Hence, these may be used in oral controlled-release systems for protein drugs. However, additional experiments, including their biological activity of the released drug, would be required for a more reliable confirmation of this conclusion.

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