ELSEVIER

Carbohydrate Polymers 43 (2000) 163–169

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Swelling behavior and the release of protein from chitosan–pectin composite particles

K.L.B. Chang*, J. Lin

Department of Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 202, Taiwan

Received 3 November 1998; received in revised form 18 November 1999; accepted 25 November 1999

Abstract

Pectin solution gelled with chitosan and Ca^{2+} ion produced composite particles with a double-layer structure. Response surface analysis revealed that the particle swelling at pH 1.4 was influenced significantly by chitosan and Ca^{2+} concentrations. Chitosan and Ca^{2+} concentrations also significantly influenced the swelling in pH 7.4 buffer at 4 h, whereas chitosan and pectin concentrations significantly influenced the swelling in pH 7.4 at 12 h. Results from swelling experiment and statistical analysis suggested that complex formation between chitosan and Ca^{2+}, or between chitosan and pectin might have occurred. Negligible protein was released from the chitosan–pectin particles in pH 1.4 buffer. The chitosan–pectin particles could sustain the release of most loaded protein until after 24 h in pH 7.4 buffer. Pectin degrading enzyme increased the protein release from 39 to 63% within 12 h in pH 7.4 buffer. These characteristics of the chitosan–pectin particles would be useful for colon-specific delivery of bioactive macromolecules.

Keywords: Chitosan; Pectin; Swelling; Protein; Controlled release

1. Introduction

In recent years, the advances in biotechnology have made available many novel drugs consisting of proteins, peptide, or nucleotides. Researchers have been constantly searching for more effective ways of delivering these drugs to patients for various therapeutic purposes (Langer, 1990, 1998). Among various drug carriers for proteins, polysaccharide hydrogels have attracted a lot of interest because they are natural, biodegradable, and nontoxic after degradation (Chen, Jo & Park, 1995).

Chitosan is a cationic polysaccharide made from alkaline *N*-deacetylation of chitin, the second most abundant natural polymer on earth (Chang, Tsai, Lee & Fu, 1997; Knorr, 1984). Chitosan consists of *N*-acetyl-glucosamine and glucosamine residues. It has biocompatible, biodegradable, nontoxic, and mucoadhesive characteristics (Hirano, Seino, Akiyama & Nonaka, 1990; Lueßen et al., 1997). Pectin is an anionic polysaccharide that may provide colon-specific delivery (Ashford, Fell, Attwood, Sharma & Woodhead, 1993; Rubinstein, Radai, Ezra, Pathak & Rokem, 1993) for several drugs. The polymeric chain of pectin contains galacturonic acid, rhamnose, arabinose and galactose. The properties of interpolymeric complex gel beads (Meshali & Gabr, 1993; Munjeri, Collett & Fell, 1997), films (Yao, Liu, Cheng, Lu, Tu, & Silva, 1996) or laminated films (Hoagland & Parris, 1996) composed of chitosan and pectin have been reported recently. The complex formation changed the drug release behavior of hydrogel beads, modified their pH-sensitive swelling behavior, but retained the colon-specific characteristic of pectin. In addition, pectin improved the storage and loss moduli of laminated chitosan films. These features have proved the feasibility of combining chitosan and pectin to form new carriers for drug delivery. The swelling behavior of chitosan–pectin particles, however, has not been examined in detail. Moreover, the feasibility of using chitosan–pectin particles for protein delivery has never been explored.

The objectives of this study were to investigate the pH-sensitive swelling behavior of composite gel particles made of chitosan and pectin and to examine the release pattern of a model protein from these particles.

2. Materials and methods

2.1. Materials

Chitosan was purchased from Ohka Enterprise Inc. (Kaoshiung, Taiwan). The degree of *N*-deacetylation,

* Corresponding author. Tel.: + 88-62-2462-2192 ext. 5132; fax: + 88-62-2462-9781.

E-mail address: klchang@ntou66.ntou.edu.tw (K.L.B. Chang).

0144-8617/00/$ - see front matter
PII: S0144-8617(00)00145-4

Table 1
Central composite design conditions for the preparation of chitosan–pectin composite particles

Treatment	Variable 1 coded	Variable 2 coded	Variable 3 coded	X_1 (pectin concentration (%))	X_2, (Ca^{2+} Concentration (%))	X_3 (chitosan concentration (%))
1	−1	−1	−1	6.01	7.24	0.36
2	1	−1	−1	8.99	7.24	0.36
3	−1	1	−1	6.01	16.76	0.36
4	1	1	−1	8.99	16.76	0.36
5	−1	−1	1	6.01	7.24	0.84
6	1	−1	1	8.99	7.24	0.84
7	−1	1	1	6.01	16.76	0.84
8	1	1	1	8.99	16.76	0.84
9	−1.682	0	0	5	12	0.6
10	1.682	0	0	10	12	0.6
11	0	−1.682	0	7.5	4	0.6
12	0	1.682	0	7.5	20	0.6
13	0	0	−1.682	7.5	12	0.2
14	0	0	1.682	7.5	12	1
15–20	0	0	0	7.5	12	0.6

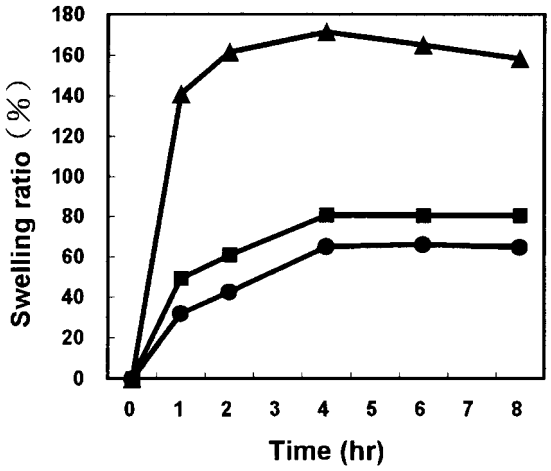

Fig. 1. The swelling ratio of chitosan–pectin composite particles in pH 1.4 buffer solution. Particle formulation (% chitosan/Ca^{2+}/pectin in solution): ● 0.84/16.76/8.99; ■ 0.6/12/7.5; ▲ 0.84/7.24/6.01.

determined by colloid titration (Chang et al., 1997) and IR spectroscopy (Baxter, Dillon, Taylor & Roberts, 1992), was 64%. The viscosity averaged molecular weight, determined by using the correlation of Wang, Bo, Li and Qin (1991), was $6.5–7.0 \times 10^5$. Low methoxyl pectin (nonamidated, containing 3.0–7.0% methoxyl group and 50–70% galacturonic acid) and reagent grade calcium chloride were obtained from Wako Pure Chemicals (Osaka, Japan) as received. Other chemicals used were reagent grades supplied by Merck (Darmstadt, Germany) or Wako Pure Chemicals. SIGMA (St Louis, MO, USA) supplied the pectinase (EC 3.2.1.15; from *Aspergillus niger*).

2.2. Preparation of chitosan–pectin composite particles

Chitosan and calcium chloride ($CaCl_2$) were dissolved in 5% acetic acid solution. Pectin was dissolved in deionized water. The wt.% of chitosan, $CaCl_2$, and pectin (Table 1) were determined by using a thee-variable central composite experimental design (Myers & Montgomery, 1995). The pectin solution was pumped by a metering pump (Pharmacia LKB Pump P-1, Sweden) and dropped through a plastic tubing (1.0 mm ID) into the chitosan–Ca^{2+} solution to form composite gel particles. The particles were cured in 0.2 N NaOH (in water/ethanol (v/v) = 8:2) solution for 15–30 min. The gel particles were then rinsed repeatedly with distilled water until neutrality and dried at ≤ 40°C in an air-circulated oven.

2.3. Measurement of pH sensitivity in vitro

The pH sensitivity of chitosan–pectin particles was determined under simulated gastric condition in pH 1.4 hydrochloric acid buffer solution or simulated intestine condition in pH 7.4 phosphate buffer solution (PBS) (Dong & Hoffman, 1991). The solution was maintained at 37°C in a thermostated shaker rotating at 50 rpm. The initial particle diameter (D_p) and the diameters after 1,2,6,8,12 and 24 h of immersion in test solution were measured with a micrometer. About 6–10 replicate measurements of diameter change were performed. In separate experiments, ~0.1 g (w_0) particles were weighed with an analytical balance. After the particles were immersed in test solution for selected periods of time, they were removed and the residual water on the surface was wiped off with a filter paper. The weight (w) after 1, 2, 4, 6, 8, 12 and 24 h of immersion were measured. Three independent experiments were performed. The diameter increase ratio was determined as $(D - D_p)/D_p$, whereas the swelling ratio was calculated as $(w - w_0)/w_0$.

2.4. Release of protein in vitro

A model protein, bovine serum albumin (BSA) was dissolved in distilled water to form a 3% (w/v) solution. Different amounts of pectin (Figs. 6 and 7) were added to the protein solution. Then the mixture solution was used to form composite gel beads with chitosan–Ca^{2+} solution as described previously. Dried particles of ~200 mg was measured into the test container. In order to simulate the physiological conditions in the digestion tract, the gel particles were first placed in 50 ml of pH 1.4 buffer for 6 h and the release of protein was determined. Then the particles were removed and the excess water on their surface was wiped off with a filter paper. The particles were subsequently placed in pH 7.4 PBS to test for the amount of protein released after different periods of exposure to PBS without or in the presence of 100 U of pectinase. The test solution was kept in a 37°C thermostated shaker rotating at 80 rpm. The amounts of protein released into the solution was determined by measuring the absorbance at 540 nm with an UV spectrophotometer (Hitachi U-2000, Japan). The reagents used was as described by Lowry, Rosebrough, Farr and Randall (1951). Total protein loading was determined by grinding the chitosan–pectin particles, placing them into the PBS in a 37°C thermostated shaker rotating at 80 rpm overnight, then measuring the total protein released into the solution.

3. Results and discussion

3.1. Time course study and response surface analysis of pH sensitivity

Microscopic examination revealed that the chitosan–pectin gel beads had two layers in their structure. Pectin formed the gelled core with calcium ion (Ca^{2+}), whereas chitosan constituted the majority of the outer layer. Ca^{2+} and chitosan had to diffuse into the pectin droplets to form the gel beads. As a consequence, their concentrations decreased towards the center of the gel beads. Due to the higher diffusivity of the smaller Ca^{2+} in solution, it is most likely that the concentration of chitosan in the inner layer

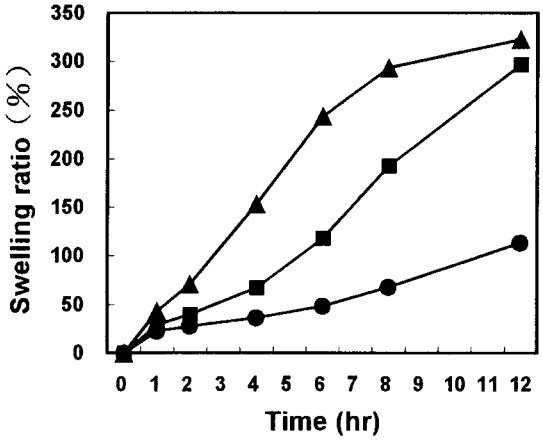

Fig. 2. The swelling ratio of chitosan–pectin composite particles in pH 7.4 phosphate buffer solution. Particle formulation (% chitosan/Ca^{2+}/pectin in solution): ● 0.84/16.76/8.99; ■ 0.6/12/7.5; ▲ 0.6/4/7.5.

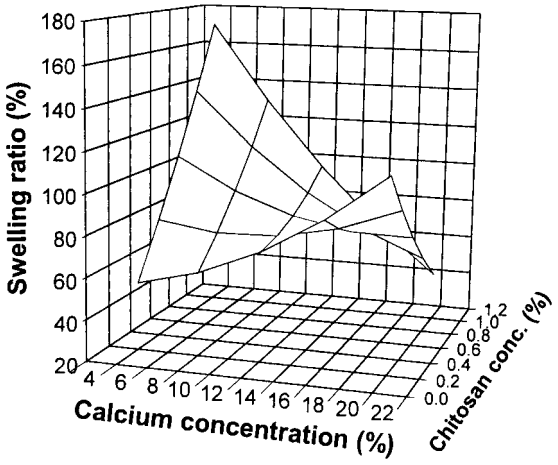

Fig. 3. The swelling ratio of chitosan–pectin composite particles after 4 h in pH 1.4 buffer solution (at a pectin concentration of 7.5%).

was lower than that of Ca^{2+}. The above factors led to a softer core and a more resilient surface layer for freshly formed gel beads.

When dried chitosan–pectin particles were put into the pH 1.4 buffer, the particles swelled considerably. The weight swelling ratio leveled off to ca 70–170% (Fig. 1) within 4 h. The diameter increased in parallel with the weight increase. However, the plateau of dimension increase occurred within ~2 h, before the weight increase plateau. This suggested that the particle expansion proceeded faster than the water uptake at pH 1.4. The phenomenon might be caused by the double-layer nature of the composite particles. Chitosan in the surface layer became positively charged and charge repulsion helped the swelling of the particles. The expansion in the positively charged chitosan surface layer might therefore be faster than water uptake. In addition, pectin became a polyacid at pH 1.4. The carboxyl groups of pectin were protonated and became neutral in charge. The hydrogen bonds and hydrophobic forces between or within pectin molecules increased the network elasticity. This would tend to make the inner core, composed mainly of pectin, harder to absorb water.

Dried chitosan–pectin particles swelled to a greater extent in pH 7.4 PBS (Fig. 2). In addition, both the weight and diameter increased continuously. Swelling ratio at 24 h ranged from 280 to 445% (data not shown). This indicated that water uptake occurred simultaneously with structure expansion at pH 7.4. These results indicated that the pH sensitivity of chitosan–pectin particles differed significantly in simulated physiological conditions (pH 1.4 and 7.4) due to the difference in the sample formulations. Several factors might influence the swelling behavior of prepared particles. Water diffusion from the solution into the gel and the electrostatic repulsion among the $-NH_3^+$ groups along chitosan chains contributed to the swelling in acidic solution. On the contrary, the complex interaction between chitosan and pectin, hydrogen bonding, hydrophobic interactions, and the network elasticity within the particles restricted them from further expansion. The balance of these phenomena led to a plateau in particle swelling after a short period of time in pH 1.4 buffer. In contrast, chitosan–pectin particles

Table 2
RSREG regression results of the swelling ratio (swelling ratio (%) $= C_0 + C_1 \times X_1 + C_2 \times X_2 + C_3 \times X_3 + C_{11} \times X_1 \times X_1 + C_{22} \times X_2 \times X_2 + C_{33} \times X_3 \times X_3 + C_{21} \times X_1 \times X_2 + C_{31} \times X_1 \times X_3 + C_{32} \times X_2 \times X_3$) for chitosan–pectin composite particles after 4 h in pH = 1.4 buffer

	Degrees of freedom	Parameter estimate C_I, C_{ii}, or C_{ij}	Probability $> \|T\|$
Intercept	1	137.3326	0.3874
X_1	1	− 30.3420	0.3405
X_2	1	− 5.0187	0.5307
X_3	1	361.0563	0.0407[a]
$X_1 \times X_1$	1	1.8757	0.3270
$X_1 \times X_2$	1	1.0524	0.1974
$X_2 \times X_2$	1	0.1598	0.3902
$X_3 \times X_1$	1	− 25.5348	0.1221
$X_3 \times X_2$	1	− 14.6435	0.0114[a]
$X_3 \times X_3$	1	30.8741	0.6725
R^2	0.7377		

[a] $P < 0.05$.

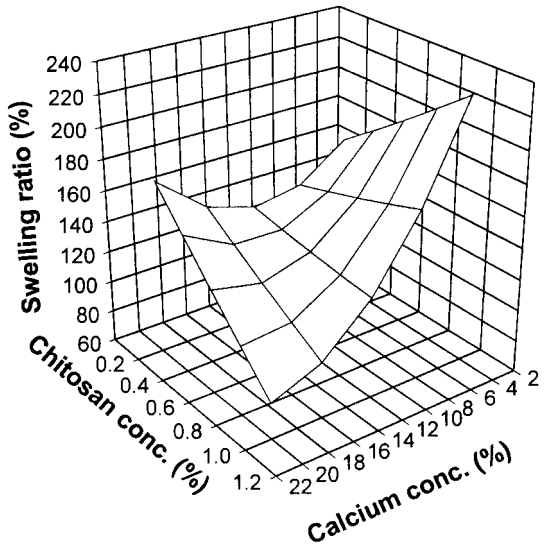

Fig. 4. The swelling ratio of chitosan–pectin composite particles after 4 h in pH 7.4 buffer solution (at a pectin concentration of 7.5%).

could swell to a larger extent at pH 7.4 because the fully negatively charged $-COO^-$ groups in pectin molecules (Zhong, Williams, Keenan, Goodal & Rolin, 1997) repelled one another. Apparently this repulsion force was greater than the attractive forces that tend to reduce the volume of the particles.

The swelling ratios of chitosan–pectin particles after 4 h at pH 1.4 were regressed with respect to the preparation condition using the RSREG procedure (SAS, 1985). The regression results were shown in Table 2. The regression equation was plotted in Fig. 3. These results indicated that the swelling of these particles at pH 1.4 were influenced significantly by the interaction term between chitosan and Ca^{2+} concentrations, and the first order term of chitosan concentration. At low Ca^{2+} concentration, the swelling ratio increased with increasing chitosan concentration. At high Ca^{2+} concentration, the interaction between chitosan concentration and Ca^{2+} concentration was more significant than the effect of chitosan concentration. Slightly more swelling occurred at the condition with high Ca^{2+} and low chitosan concentration. These results suggested statistically that chitosan might interact with Ca^{2+}.

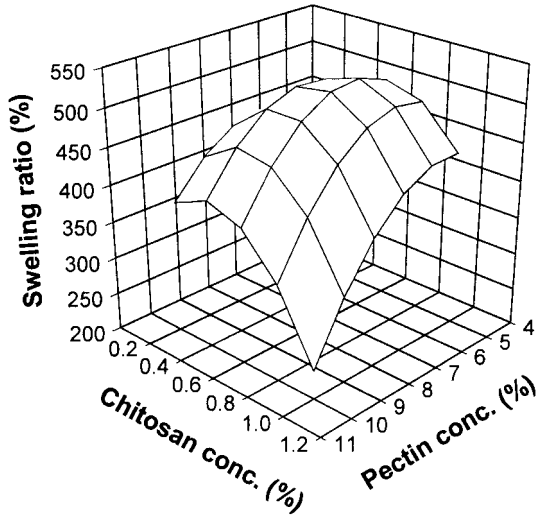

Fig. 5. The swelling ratio of chitosan–pectin composite particles after 12 h in pH 7.4 buffer solution (at a Ca^{2+} concentration of 12%).

The RSREG regression results for the swelling ratios of chitosan after 4 and 12 h in PBS (pH 7.4) could be obtained similarly. They are as follows:

$$\text{Swelling ratio (\%), 4 h in : pH 7.4}$$
$$= 133.2 + 1.546X_1 - 15.17X_2 + 272.4X_3 - 0.5418X_1 \times X_1 + 0.8391X_1 \times X_2 + 0.4328X_2 \times X_2 - 20.01X_3 \times X_1 - 12.02X_3 \times X_2 + 6.041X_3 \times X_3$$
$$R^2 = 0.7671$$

$$\text{Swelling ratio (\%), 12 h in : pH 7.4}$$
$$= -501.2 + 175.7X_1 + 19.63X_2 + 583.5X_3 - 9.549X_1 \times X_1 - 2.656X_1 \times X_2 - 0.7019X_2 \times X_2 - 40.12X_3 \times X_1 + 17.17X_3 \times X_2 - 469.9X_3 \times X_3$$
$$R^2 = 0.7512.$$

These regression equations corresponded to the 3D response surfaces in Figs. 4 and 5. The regression results indicated that the second order term of Ca^{2+} and the interaction term between chitosan concentration and Ca^{2+} concentration had more significant effect on the swelling of particles after 4 h in pH 7.4 PBS. The lower the Ca^{2+} concentration, the larger the swelling ratio. The second order term of chitosan concentration and the pectin concentration term had more significant influence on the swelling ratio after 12 h in pH 7.4 PBS. Higher swelling ratios occurred at intermediate to lower chitosan and pectin concentrations. These results suggested that chitosan might interact with Ca^{2+} in the gel matrix and influenced the initial swelling of the particles in pH 7.4 PBS. After 12 h of exposure to pH 7.4 PBS, the Ca^{2+} had mostly diffused out of the gel matrix. Therefore, the concentrations of chitosan and pectin that formed the polymeric matrix of the gel particles showed more significant influence on the swelling behavior. These statistical results revealed that complex might have been formed between chitosan and Ca^{2+}, and between chitosan and pectin. This is contrary to prior knowledge, that chitosan does not form complex with the ions of alkaline earth metals (Roberts, 1992). It is possible that complex formation occurred in this work because much higher Ca^{2+} concentration was used during the particle preparation.

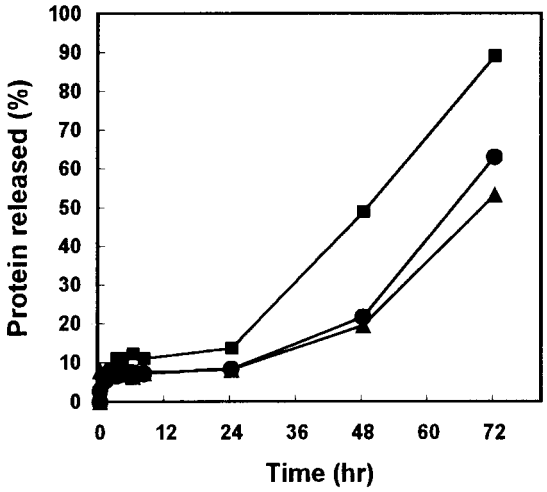

Fig. 6. Protein release from chitosan–pectin composite particles in pH 7.4 buffer solution. Particle formulation (% chitosan/Ca^{2+}/pectin in solution): ● 1/20/7.5; ■ 1/4/7.5; ▲ 0.6/12/6.

3.2. In vitro release of BSA from chitosan–pectin composite particles

A potential carrier of protein or peptide drugs for oral colon-specific delivery should preferably provide minimal swelling in stomach (pH ~1.4) and maximal swelling in colon (pH ~7.4). These optimal conditions for preparing protein-loaded gel particles should be close to the conditions for the minimum swelling in Fig. 3, or for the maximums in Figs. 4 and 5. These conditions were used to prepare chitosan–pectin particles containing BSA. Test results showed that practically no protein was released from the gel particles after 6 h in pH 1.4 buffer. The reason that there was a lack of release of protein might be that BSA molecules were denatured at pH 1.4. Presumably they could aggregate, became insoluble, or too bulky to diffuse out of the double-layered chitosan–pectin gel particle in the buffer solution.

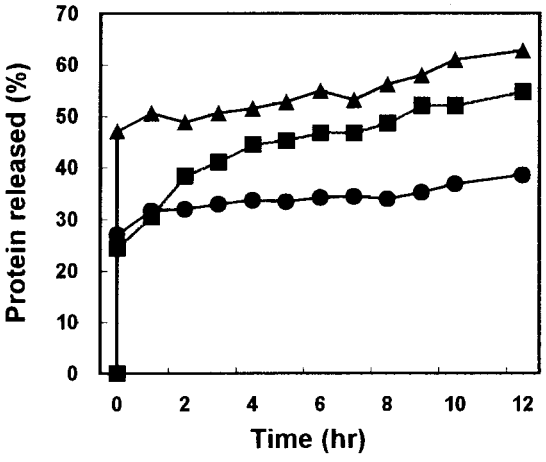

Fig. 7. Protein release from chitosan–pectin composite particles in pH 7.4 buffer solution in the presence of 100 U pectinase. Particle formulation (% chitosan/Ca^{2+}/pectin in solution): ● 1/20/7.5; ■ 1/4/7.5; ▲ 0.6/12/6.

Approximately 10% of the total protein were released after additional 24 h of exposure in pH 7.4 PBS (Fig. 6). This indicated a gradual diffusion of protein from the swollen particles. The rate of protein release increased dramatically after 24 h in pH 7.4 PBS. The particles prepared by chitosan to pectin ratio of ca 1–10 seemed to swell less and release less protein in pH 7.4 buffer. This ratio is the same as the ratio that yielded the lowest viscosity of chitosan–pectin solution as reported in Meshali and Gabr (1993). Thus it is probable that a weight concentration ratio of ca 1–10 could produce the best stoichiometric binding or complex between chitosan and pectin and form the most stable composite particles.

Total amounts of protein released after 72 h ranged from 53 to 90%. The sudden increase in the release rate corresponded with the erosion of the gel particles after prolonged exposure to the pH 7.4 PBS. The chitosan–pectin particles prepared with the lowest Ca^{2+} concentration released the largest amount of protein. This suggested that Ca^{2+} contributed to the cross-linked structure of the particles. The higher the Ca^{2+} concentration, the more the cross-linking bonds between Ca^{2+} and chitosan or pectin. More cross-linked particles would slow down the erosion process, make the diffusion of BSA from the swollen particles more difficult, and result in a slower rate of protein release. The release rate of BSA from chitosan–pectin composite particles was much lower than that from chitosan–alginate microcapsules (Okhamafe, Amsden, Chu & Goosen, 1996) under simulated gastric condition. This characteristic may help protect large biological molecules in stomach and make chitosan–pectin particles more effective as the oral delivery carrier of various macromolecular drugs under development.

Fig. 7 showed the release of protein from the chitosan–pectin particles into PBS containing pectinase. Pectinase dramatically increased the rate of release. In the presence of the enzyme, ~25–47% of total protein were released within 0.5 h. The amounts released in 2 h ranged from 32 to 50%. After 12 h of exposure to pH 7.4 PBS with pectinase, up to 39–63% of BSA could be released. The particles prepared with lower chitosan and pectin concentrations showed the fastest release rate. The release rate of protein from the particles prepared in this study was slower than that of indomethacin and sulphamethoxazole from chitosan coated pectin hydrogel beads (Munjeri et al., 1997). The difference in the release rate of encapsulated material could be attributed to the lower solubility and lower diffusivity of protein. Nevertheless, our data indicated that the protein release rate could be increased substantially by pectinolytic enzymes in vitro. It has been shown that calcium pectinate or pectin is degraded by commercial pectinolytic enzymes or microbes from rat cecal contents and human colon (Rubinstein et al., 1993; Salyers, Vercellotti, West & Wilkins, 1977). As a consequence, these results proved that chitosan–pectin

particles provided an enzyme controlled release system. This property would make these particles good potential colon-specific carriers for large molecules such as protein drugs.

4. Conclusion

Chitosan–pectin composite particles containing BSA could be prepared under mild temperature from aqueous solutions. The chitosan–pectin particles swelled slightly in pH 1.4 buffer solution; they swelled considerably in pH 7.4 PBS. The swelling of these particles under physiological pH could be adjusted by changing the concentrations of chitosan, Ca^{2+} and pectin. At a pH of 7.4, BSA gradually diffused through the swollen gel network in the first few hours. After ca 24 h of exposure, the swollen gel matrix started to erode. This could be due to the diffusion and dissolution of Ca^{2+}, the dissolution of pectin, or a breakdown of the cross-linked 'egg-box' structure between pectin and Ca^{2+}. Consequently, the release of protein was a combined result of swelling, diffusion, and erosion processes. In the presence of pectinase, the glycoside bonds in pectin were degraded. The cross-linked networks in the chitosan–pectin particles rapidly broke into pieces, releasing a considerable amount of protein in a short time. The release of protein was mainly the result of swelling and enzymatic degradation. This research suggested that chitosan–pectin particles were able to sustain the release of BSA under simulated gastric condition and release it under simulated condition in colon. These particles might be good oral controlled release carriers for large molecules such as vaccines, peptides, nucleotides, or proteins.

Acknowledgements

The research was supported in part by a Grant (NSC 86-2313-B-019-004) from the National Science Council of the Republic of China.

References

Ashford, M., Fell, J., Attwood, D., Sharma, H., & Woodhead, P. (1993). An evaluation of pectin as a carrier for drug targeting to the colon. *Journal of Controlled Release*, *26*, 213–220.

Baxter, A., Dillon, M., Taylor, K. D. A., & Roberts, G. A. F. (1992). Improved method for IR determination of the degree of *N*-acetylation of chitosan. *International Journal of Biological Macromolecules*, *14*, 166–169.

Chang, K. L. B., Tsai, G., Lee, J., & Fu, W. R. (1997). Heterogeneous *N*-deacetylation of chitin in alkaline solution. *Carbohydrate Research*, *303*, 327–332.

Chen, J., Jo, S., & Park, K. (1995). Polysaccharide hydrogels for protein drug delivery. *Carbohydrate Polymers*, *28*, 69–76.

Dong, L., & Hoffman, A. S. (1991). A novel approach for preparation of pH-sensitive hydrogels for enteric drug delivery. *Journal of Controlled Release*, *15*, 141–152.

Hirano, S., Seino, H., Akiyama, Y., & Nonaka, I. (1990). Chitosan: a biocompatible material for oral and intravenous administrations. In G. G. Gebelein & R. L. Dunn, *Progress in biomedical polymers* (pp. 283–289). New York: Plenum.

Hoagland, P. D., & Parris, N. (1996). Chitosan/pectin laminated films. *Journal of Agricultural and Food Chemistry*, *44*, 1915–1919.

Knorr, D. (1984). Use of chitinous polymers in food. *Food Technology*, *38* (1), 85–97.

Langer, R. (1990). New methods of drug delivery. *Science*, *249*, 1527–1533.

Langer, R. (1998). Drug delivery and targeting. *Nature*, *392*, 5–10.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry*, *193*, 265–275.

Lueßen, H. L., Rentel, C.-O., Kotzé, A. F., Lehr, C.-M., de Boer, A. G., Verhoef, J. C., & Junginger, H. E. (1997). Mucoadhesive polymers in peroral peptide drug delivery. IV. Polycarbophil and chitosan are potent enhancers of peptide transport across intestinal mucosae in vitro. *Journal of Controlled Release*, *45*, 15–23.

Meshali, M. M., & Gabr, K. E. (1993). Effect of interpolymer complex formation of chitosan with pectin or acacia on the release behaviour of chlorpromazine HCl. *International Journal of Pharmaceutics*, *89*, 177–181.

Munjeri, O., Collett, J. H., & Fell, J. T. (1997). Hydrogel beads based on amidated pectins for colon-specific drug delivery: the role of chitosan in modifying drug release. *Journal of Controlled Release*, *46*, 273–278.

Myers, R. H., & Montgomery, D. C. (1995). *Response surface methodology. Process and product optimization using designed experiments*. New York: Wiley (pp. 279–350).

Okhamafe, A. O., Amsden, B., Chu, W., & Goosen, M. F. A. (1996). Modulation of protein release from chitosan–alginate microcapsules using the pH-sensitive polymer hydroxypropyl methylcellulose acetate succinate. *Journal of Microencapsulation*, *13*, 497–508.

Roberts, G. A. F. (1992). *Chitin chemistry*. London: Macmillan (pp. 203–229).

Rubinstein, A., Radai, R., Ezra, M., Pathak, S., & Rokem, J. S. (1993). In vitro evaluation of calcium pectinate: a potential colon-specific drug delivery carrier. *Pharmaceutical Research*, *10*, 258–263.

Salyers, A. A., Vercellotti, J. R., West, S. E. H., & Wilkins, T. D. (1977). Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Applied and Environmental Microbiology*, *33*, 319–322.

SAS User's Guide: Statistics, Version 5; SAS Institute Inc.: Cary, NC, USA, 1985.

Wang, W., Bo, S., Li, S., & Qin, W. (1991). Determination of the Mark–Houwink equation for chitosans with different degrees of deacetylation. *International Journal of Biological Macromolecules*, *13*, 281–285.

Yao, K. D., Liu, J., Cheng, G. X., Lu, X. D., Tu, H. L., & Silva, H. A. L. D. (1996). Swelling behavior of pectin/chitosan complex films. *Journal of Applied Polymer Science*, *60*, 279–283.

Zhong, H.-J., Williams, M. A. K., Keenan, R. D., Goodal, D. M., & Rolin, C. (1997). Separation and quantification of pectins using capillary electrophoresis: a preliminary study. *Carbohydrate Polymers*, *32*, 27–32.