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Preparation and Characterization of Coacervate Microcapsules for the Delivery of Antimicrobial Oyster Peptides

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Oyster peptides-loaded alginate/chitosan/starch microcapsules were prepared using external gelation method and internal emulsion gelation method. The solution of oyster peptides complexes was encapsulated into the microcapsules, which endowed the microcapsules with intestine passive targeting properties. The swelling behavior, encapsulation efficiency, and release behavior of oyster peptides from the microcapsules at different pH values were investigated. The microcapsules exhibited sustained release of the peptides in intestinal medium, and the release rate could be regulated by the pH value: in simulated gastric fluid, the release rate was greatly decreased, and in simulated body fluid and intestinal fluid, the microcapsules exhibited a sustained release in 24 h with different release rates. The microspheres were characterized by Fourier transform infrared. The results suggested that the alginate/chitosan/starch microcapsules could be a suitable copolymeric carrier system for intestinal protein or peptides delivery in the intestine.

Keywords microcapsule; oyster peptides; intestinal targeting; pH-responsive; alginate/chitosan/starch

INTRODUCTION

In view of dramatically increasing world population and the danger of overutilizing terricolous resource, there is a great urgency to use marine organisms, which are potentially an untapped source of bioactives and value-added food production. Oyster (known as "mu li" in Chinese) is Mollusca shellfish classified under the Pelecypoda class and Ostreidae family. Oyster has been honored as the "Milk of the Sea." Oyster peptides are typical representatives of biologically active peptides with high therapeutic potential, the antimicrobial peptide was named *CgPep33* that are inactive within the sequence of the parent protein. They can be isolated by the enzymatic hydrolysates of oyster. In the recent study, the antimicrobial peptides CgPep33 inhibited the growth of all studied bacteria (*Escherichia coli*, *Pseudomons aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus*) and fungi (*Botrytis cinerea* and *Penicillium expansum*). The IC_{50} (effective concentration for 50% growth inhibition) values of CgPep33 against all tested bacteria and fungi ranged from 18.6 to 48.2 μg/mL. Gram-positive bacteria were the most sensitive, with minimal inhibitory concentrations (MICs) values between 40 and 60μg/mL. (Liu, Zeng, Dong, Xu, & Song, 2007).

The most challenging task in the development of bioactive food industry is to deal with physical and chemical instabilities of bioactive peptides. Problems such as acid-catalyzed degradation in the stomach, proteolytic breakdown in the gastrointestinal (GI) tract, poor permeability across the gastrointestinal mucosa, and first-pass metabolism during transfer across the absorption barrier and in the liver must be overcome for the efficient delivery of bioactive peptides into the bloodstream. To achieve the successful oral delivery of protein or bioactive peptides, they should be protected from the harsh environment in the stomach.

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For designing oral dosage forms, one must consider that the natural pH environment of GI tract varies from acidic (pH ~ 1.2) in the stomach to alkaline in the intestine (pH ~7.5, 8.6).

In the design of oral delivery of bioactive peptides, pH-sensitive hydrogel microcapsules have attracted increasing attention. Swelling (Sw) of such hydrogel microcapsules in the stomach is minimal and thus the peptide release is also minimal. Owing to increase in pH, the extent of swelling increases as the hydrogel microcapsules pass down the intestinal tract. A variety of synthetic or natural polymers with acidic or basic pendant groups have been employed to fabricate pH-sensitive hydrogels (Kimura et al., 1993).

Alginate is an anionic linear polysaccharide with wide application in food and pharmaceutical industry (Gombotz & Wee, 1998). It was reported that alginate is nontoxic and biodegradable when given orally (Mumper, Hoffman, Puolakkainen, Bouchard, & Gombotz, 1994). Alginate can form gel in aqueous media with multivalent cations such as calcium ions. The relatively gentle gelation process has enabled it an excellent carrier for peptides. Alginate can coacervate with positively charged polyelectrolytes, such as poly-L-lysine, albumin, gelatin, and chitosan. In the alginate-chitosan system, a core-coating model was developed in the recent years (Acosta, Aranaz, Peniche, & Heras, 2003; Daly & Knorr, 1988; Lemoine, Wauters, Bouchend'homme, & Préat, 1998), in which alginate was dropped into chitosan solution. The electrostatic interaction of carboxylic groups of alginate with the amine groups of chitosan results in the membrane formation (Gåserød, Sannes, & Skjåk-Bræk, 1999; Gåserød, Smidsrød, & Skjåk-Bræk, 1998). Although alginate/chitosan microcapsules have been studied widely, the studies have been limited to a narrow pH region owing to the solubility of chitosan (Tapia et al., 2004). Chitosan has good biocompatibility and can be degraded by certain human enzymes (Muzzarelli, 1997). Chitosan has the special feature of adhering to mucosal surfaces owing to positive charges, which allows paracellular transport across the epithelium (van der Lubben, Verhoef, Borchard, & Junginger, 2001). Moreover, the resistant starch is a dietary component, and has an important role in colonic physiology and functions as well as a potential protective role against colorectal cancer (Cassidy, Bingham, & Cummings, 1994). The pharmaceutical applications of resistant starch are of interest to product developers and for achieving intestine targeting: the first being the potential physiological benefits and the second the high final quality of the products, which is not attainable with traditional insoluble fibers. The pharmaceutical applications of resistant starch are of interest to product developers and intestine targeting, which make it a functional ingredient that provides good handling and improves proper microstructure in the final microcapsular product.

The purpose of this study is to explore a oyster peptides' carrier to intestine, prepared in gentle environment based on microcapsules. The microcapsules were prepared with external or internal calcium sources, the so-called external gelation microcapsule or internal emulsion gelation microcapsule, respectively. The effects of properties of various processes, including alginate/chitosan ratio and percentage of starch as well as their swelling of these microcapsules and release behavior, were investigated to provide guidance for their preparation and application.

MATERIALS AND METHODS

Materials

Alginate (medium viscosity: 200–500 mPa s), Alcalase (Novo Nordisk, Denmark), and Bromelin were all procured from Biochemistry Reagent, Inc. (Shanghai, China). Chitosan (90% deacetylation, MW 80,000 Da) was obtained from Yuhuan Oceanic Biochemistry (Zhejiang, China). HighAmyloseCorn starch powder and olive oil were purchased from a commercial vendor. Coomassie Blue protein assay (G250) and bovine serum albumin (BSA) were purchased from Biochemistry Reagent Inc. Sorbitan monoleate (Span 80), polyoxyethylene sorbitan monoleate (Tween 80), iso-octane, isopropyl alcohol, and all other reagents were of analytical grade(Biochemistry Reagent Inc.)

Preparation of Oyster Peptides and Purification

Oyster muscles were homogenized and hydrolyzed with Alcalase 2.4 L (3% volume of enzyme weight of raw material at 50°C, pH 8.5 for 3 h); then the pH of the reaction mixture was adjusted to 5.5 and continuously hydrolyzed with bromelin for 3 h. The enzymes were inactivated by heating the resulting suspension to 100°C for 5 min. The suspension was centrifuged ($5{,}000 \times g$, 15 min) to pellet inactivated proteases and nonhydrolyzed oyster proteins. The supernatant, containing soluble peptides, was collected and ultrafiltered with membranes of 10 and 5 kDa; the 5–10 kDa samples with high antimicrobial activity were collected and concentrated. The supernatant fluid, the oyster liquid, was freeze-dried to obtain the coarse extract; the coarse extract was then subjected to gel column chromatography. The samples in the respective peak were collected and purified using ion-exchange column chromatography (DERE Sephadex A-25, 16×300 mm), and desalted in the gel column after freezing out to obtain the oyster natural purified activity peptide. They were further purified by high-performance liquid chromatography (HPLC) on a reverse-phase C_{18} column. Peptides were monitored by the measurement of the absorbance at 220 nm in UV spectrometer (Yuqian, Xiaomei, Zhiqiang, Xin, & Huashi, 2007).

Preparation of Alginate/Chitosan/Starch Microcapsule Formation

External Gelation Microcapsules

For the basal encapsulation protocol, 2% (wt/vol) alginate was dissolved in water and oyster peptides were added at 10%

loading rate (mass oyster peptides/mass alginate). Starch powder 20% (wt/vol) was used to prepare microcapsules. Chitosan was ground finely and dissolved in 2% (wt/vol) acetic acid for 4 h with gentle warming and filtered to remove any undissolved particles. The pH was adjusted to 5.5 with 4 M NaOH and $CaCl_2$ was added to a final concentration of 1.5% (wt/vol). Approximately 15 g of the alginate/oyster peptides solution was pipetted into a syringe fitted with a 7-gauge needle. Alginate/oyster peptides were extruded dropwise into 50 mL chitosan/starch/calcium chloride solution and allowed to react for 20 min, during which time samples of the gelation medium were taken at 0, 5, 10, 15, 20, 25, and 30 min following extrusion. Microcapsules were removed from the encapsulation medium via filtration under gentle vacuum and rinsed twice with 20 mL dilute 0.1 N HCl; a sample of the acid rinse filtrate was taken to quantify peptides loss at this step. A subsample of 20–30 microcapsules was taken to determine individual particle diameter. Microcapsules were observed under 4× magnification, and the diameter was noted using a microscope equipped with a calibrated side-mounted tracing device (Nikon Eclipse E-400), permitting an accuracy of ±50 μm. All microcapsules were then washed twice with 5 mL acetone under gentle vacuum, weighed, placed in an oven overnight to dry (30°C); the final dry weight was carefully recorded. Preliminary experiments showed that there was negligible peptides loss during the acetone washes (data not shown). Starting the above encapsulation protocol, individual parameters were evaluated separately, allowing a systematic evaluation of factors affecting oyster peptides retention.

The factors tested included concentrations of alginate (1.0, 1.5, 2.0%, wt/vol), chitosan (0.1, 0.2, 0.5%, wt/vol), starch (10, 15, 20%, wt/vol), and oyster peptides-loading rate (mass peptides/mass alginate [wt/wt], 10, 20, 30%). Gelation time was altered to determine its effects on peptides retention.

Internal Gelation Microcapsules

Another method of preparing internal gelation microcapsules with oyster peptides was adopted from an emulsification method described by Lemoine et al. (1998). To prepare much smaller microcapsules, surfactant type and few procedures were modified. The operation factors including the stirring rate and the rate of the addition of $CaCl_2$ were investigated. Freeze-dried oyster peptides were dissolved in the alginate solution at peptides/alginate ratios of 1:10, 2:10, and 3:10 (wt/wt), respectively. Span 80, a lipophilic surfactant, was dispersed in olive oil at a concentration of 5% (wt/vol), and then 40 mL of the oil phase (olive oil) was poured into 20 mL of the alginate aqueous solution. The mixture was emulsified for 10 min using a mechanical stirrer (FJ-200 Homogenizer, Shanghai sample model factory, Shanghai, China) at 12,000 rpm; 5.0 mL Tween 80 aqueous solution (40%, wt/wt) was added as the second emulsifier for attaining a proper hydrophile-lipophile balance (HLB) value; the mixture was further stirred at the same speed for 10 min. Then 10 mL of calcium chloride solution (5%, wt/vol) was added dropwise. This cross-linking process lasted for 10 min. Then 50 mL of isopropyl alcohol was added to harden the solidified microcapsules and to separate the microcapsules from the organic phase. After the mixture was stirred for another 10 min, the alginate microcapsules were collected by centrifugation and were dispersed into chitosan/starch solution at 0.2/10% (wt/vol) concentration, respectively (pH 4.5). Then the mixture was shaken gently for 30 min to form the alginate/chitosan/starch complex membrane. The microcapsules were centrifuged at $1{,}900 \times g$ for 10 min and collected, then washed once in the same chitosan solution, and twice in distilled water, and finally lyophilized and stored at 4°C.

Microcapsule Incubation and Peptides Determination

Approximately 10 mg of dried microcapsules were accurately weighed and incubated in 4.0 mL of HCl (0.1 N; pH 1.5) in a rotating agitator with sampling at 0, 5, 15, 20, 25, and 30 min. Microcapsules were subsequently transferred and incubated in 4.0 mL Tris (0.2 mol/L, pH 7.5) for 24 h in a rotating agitator with sampling at 0, 5, 10, 30, 60, 120, 240 min, and at 24 h. Samples were analyzed for peptides using a modified Coomassie Blue protein assay. The relative errors are smaller than 5%. The assay is performed at room temperature and no special equipment is required. The sample was added to the tube containing the reagent, and the resultant blue color was measured at 595 nm following incubation for a short time at room temperature. The Coomassie dye containing protein assay is compatible with most salts, solvents, buffers, thiols, reducing substances, and metal-chelating agents found in peptide samples.

Oyster Peptides Encapsulation Efficiency

The encapsulation efficiency (EE) of oyster peptides was determined by the an extraction method as described. Briefly, the microcapsules were dispersed in 5 mL buffered solution (0.05 mol/L Tris–HCl, pH 8.6) and incubated in a shaking water bath at 37°C, 100 rpm, for 2 h. Then the sample was centrifuged at 2,000 rpm, and the supernatant was collected. This extraction step was repeated three times before concentration of oyster peptides was determined. The content of oyster peptides in the microcapsules was calculated from the difference between the amount of oyster peptides added and the amount of oyster peptides in the external aqueous phase, which was determined by Coomassie Brilliant Blue protein assay, using nonloaded microcapsules as basic correction. The EE% of oyster peptides was calculated (actual content of oyster peptides/theoretical content of oyster peptides × 100).

In Vitro Release Study

To study the peptides release from the test microcapsules, the dried, oyster peptides-loaded microcapsules were immersed in solutions at pH 1.2, or 7.5 and 8.6 (Chen et al.,

2003). The in vitro release of oyster peptides from the microcapsules was carried out in 0.1 M HCl (pH 1.5), 0.2 mol/L Tris–HCl-buffered solution (pH 7.5), and 0.05 mol/L Tris–HCl-buffered solution (pH 8.6). Accurately weighed amounts (100 mg) of microcapsules were placed in a conical flask containing 100 mL of the buffer and incubated at 37°C at 100 rpm. At selected time intervals, the concentrations of oyster peptides in the supernatant were estimated by Coomassie Brilliant Blue protein assay for released oyster peptides, while the dissolution medium is replaced with the same amount of fresh buffer. The percentage of cumulative amount of released oyster peptides was calculated and plotted against time.

Characterization of the Peptide-Loaded Alginate/Chitosan/Starch Microcapsules

- The Fourier transform infrared (FT-IR) spectra: The FI-TR spectra of the samples were recorded with a Fourier transform infrared (Bruker VERTEX 70 FT-IR spectrometer)-attenuated total reflectance (ATR) spectroscopy in the range of 2,500–400 cm^{-1} using KBr pellets.
- Morphology observation: The morphologies and approximate sizes of the microcapsules were determined by means of an optical microscope (Nikon Eclipse E-400).
- Swelling study: Swelling experiments performed in pH 3.0 HCl solutions produced no significant changes and, hence, we studied the swelling of microcapsules in pH 8.6 buffer solution. To perform the swelling experiments, microcapsules were soaked in 8.6 buffer solution for 24 h at room temperature until a swollen equilibrium was reached. The swollen samples were collected by filtration, blotted with filter paper for the removal of the absorbed water on the surface, and then weighed immediately. The degree of Sw was calculated as follows:

$$\mathrm{Sw}\% = \frac{w - w_d}{w_d} \times 100$$

where w and w_d are the weights of the microcapsules in the equilibrium swelling state and in the dry state, respectively. Swelling experiments were repeated thrice for each sample, and average values were used in data analysis. The standard deviations (*SD*) in all cases were <3%.

RESULTS AND DISCUSSION

Microcapsule Incubation and Peptides Determination

The concentration of peptides were determined at 595 nm using Coomassie Brilliant Blue G-250. Its relative standard deviation is 3.6–4.9% and its recovery ratio is 93.2–108.7%. This assay is a simple, rapid, reproducible, and sensitive method for determining peptides in microcapsules.

Peptide Release In Vitro

Using the two basal encapsulation conditions, the typical release pattern of peptides from alginate/chitosan/starch microcapsules during synthesis and incubation in acid and neutral pH media is given in Figure 1. Under these conditions, peptides were encapsulated with high efficiency; 5% was lost during the external gelation method and washing processes (Figure 1A). Interpretation of the data is difficult as it is unclear whether the peptides loss represents simply an gelation-time loss or incorporates synthesis-related loss. Over the 12- and 24-h neutral medium incubation with external gelation microcapsules, approximately 41 and 75% of the oyster peptides diffused out of the microcapsules (Figure 1B1). Figure 1B1 shows that the production of external gelation microcapsules results in gradual release due to an outward diffusing gelling zone and reduces microcapsule permeability. The oyster peptides loading by internal gelation microcapsules diffused into the neutral medium by nearly 90% in 24 h (Figure 1B2). The results showed that the rates of oyster peptides release from the internal gelation microcapsules are higher than those found in the external gelation microcapsules. Many parameters influenced peptides retention during microcapsule synthesis and acid incubation. Both factors, methods of microcapsule synthesis and subsequent incubation, exert a strong

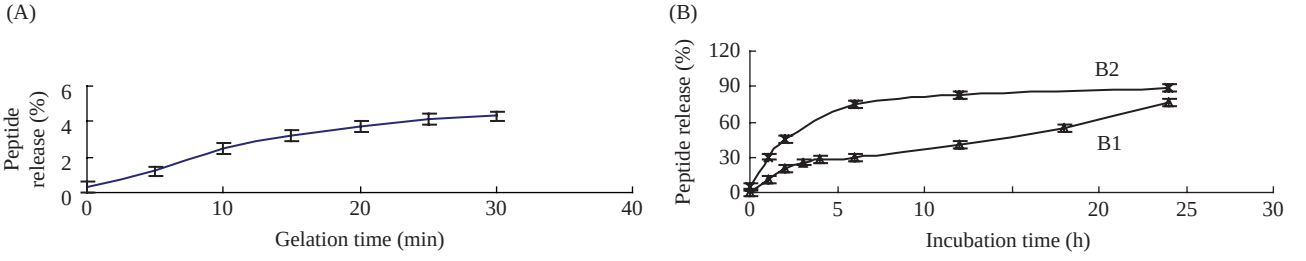


FIGURE 1. Typical release pattern of peptides from alginate/chitosan/starch microcapsules as a function of time during synthesis: (A) external gelation microcapsules in vitro release in an acid medium (0.1 N HCl, pH 1.5); (B) 1, external gelation microcapsules in vitro release in a neutral pH medium (0.2 mol/L Tris–HCl, pH 7.5); 2, internal gelation microcapsules in vitro release in a neutral pH medium (0.2 mol/L Tris–HCl, pH 7.5). Δ, B1; *, B2.

TABLE 1
Results of % of Encapsulation Efficiency, Mean Size, and Degree of Swelling (Sw) of Different Formulations (External Gelation Microcapsules)

Experiment No.	Microcapsules %A	%C	%S	% OP Loaded	EE% ± *SD*	Mean Particle Size (μm ± *SD*)	% Sw
1	1	0.2	10	10	91.2 ± 1.1	893 ± 12	456
2	1.5	0.2	10	10	93.3 ± 0.9	921 ± 15	425
3	2	0.2	10	10	94.2 ± 0.8	910 ± 10	398
4	2	0.1	10	10	93.7 ± 1.2	872 ± 17	410
5	2	0.2	10	0	0	903 ± 14	384
6	2	0.5	10	10	91.7 ± 0.8	1,186 ± 23	378
7	2	0.2	0	10	95.4 ± 1.1	840 ± 21	242
8	2	0.2	15	10	93.5 ± 1.3	1,228 ± 30	401
9	2	0.2	10	20	95.6 ± 0.9	942 ± 19	382
10	2	0.2	20	10	90.1 ± 1.4	1,328 ± 30	404
11	2	0.2	10	30	96.6 ± 1.5	955 ± 23	378
12[a]	2	0.2	10	10	76.34 ± 1.10	238 ± 5	256
13[a]	2	0.2	0	10	71.56 ± 1.21	210 ± 5	214

A, alginate (wt/vol); C, chitosan (wt/vol); S, starch (wt/vol); OP, oyster peptides (wt/wt).
[a]Internal emulsion gelation microcapsules.

influence on the retention of peptides. Figure 1 demonstrates the substantial influence of microcapsules on the retention of peptides during the 24-h acid and neutral medium. The ideal delivery system for oral delivery of peptides would be having a high encapsulation efficiency, provided maximal stability, and limited release in acidic pH ranges and rapid release in neutral pH conditions.

Encapsulation Efficiency and Peptide-Loading Percentage of Oyster Peptide Microcapsules

The EE% of oyster peptides-loaded microcapsules with different peptides/alginate ratios, chitosan, and starch concentrations was determined by the above method and the results are shown in Table 1. Three different concentrations of oyster peptides—that is 10, 20, and 30 wt%—were loaded during the external gelation microcapsules; EE% showed increasing trends with increasing peptides concentrations, which ranged from 94.2 to 96.6%. There was no significant difference in the EE within a definite range. The results showed that the EE% of internal gelation microcapsules was lower than that of the external gelation microcapsules. It was found that there were obvious differences in the microcapsule frame between external and internal microcapsules (photographs not shown). The pore size of the former was smaller and the gel structure of the former was denser than that of the latter. It is the structural difference of the microcapsules that contributes to the difference in the peptides loading. During the formation of external gelation microcapsules, peptides can diffuse easily into the core resulting in higher loadings (30%, wt/wt, mass peptides/mass alginate) than that of internal gelation microcapsules. During the formation of internal gelation, the ionotropy of gel core and subsequently phase change is much higher than that of external gelation; therefore, peptides can diffuse more on the outside of microcapsules than that in external gelation. Figure 1 shows that there was a burst release in the beginning in the internal gelation microcapsules, but it was not observed in the external gelation microcapsules. The internal gelation microcapsules also had a high capacity, and the EE% increased from 76.31 to 79.64% with the increase of peptides/alginate ratio from 10 to 30% (Table 2); there was no significant increase in peptide encapsulation. Such lower values are due to a lesser soluble peptides in the polymer solution, thus incorporating a lesser amount of peptides into microcapsules. The peptides-loading percentage of the internal gelation microcapsules prepared at a

TABLE 2
Influence of Peptide/Alginate Ratio and Chitosan Concentration on Encapsulation Efficiency of the Internal Emulsion Gelation Microcapsules (n = 3)

Peptide/Alginate (%, wt/wt)	Chitosan Concentration (%, wt/vol)	EE (%)
10	0.2	76.31 ± 1.10
20	0.2	78.10 ± 0.98
30	0.2	79.64 ± 1.23

$p > .05$ between the data (one-way analysis of variances test).

TABLE 3
Influence of Chitosan Concentration on Peptide-Loading Percentage of Internal Emulsion Gelation Microcapsules (Peptide/Alginate Ratio, 1:10; $n = 3$)

Chitosan Concentration (%, wt/vol)	0.1%	0.2%	0.5%
Peptide loading (%)[a] (±*SD*, wt/wt)	13.56 ± 0.30	15.32 ± 0.15	14.28 ± 0.24

[a]Peptide loading (%) = (weight of peptides in microcapsules/weight of microcapsules) × 100.

$p > .05$ between the data (one-way analysis of variances test).

peptide/alginate ratio of 1:10 and at different chitosan concentrations is shown in Table 3. The results show that there was no increase in peptide loading during microcapsule preparation when chitosan concentration was increased from 0.1 to 0.5% (wt/vol). The peptides loading in internal gelation microcapsules was about 14% (wt/wt). The significant peptide association most likely resulted from the electrostatic affinity between chitosan and sodium alginate (Rajaonarivony, Vauthier, Gouarraze, Puisieux, & Couvreur, 1993; Sezer & Akbuğa, 1999a). When alginate microcapsules were blended with chitosan, a polyelectrolyte complex membrane formed on the microcapsule surface (Sezer & Akbuğa, 1999b).

In Vitro Release Study

The release rates from the alginate/chitosan/starch microcapsules with various synthesis formations were studied (Figures 2–4). The release characteristics of microcapsules in different media differed largely from each other. The oyster peptides were completely and rapidly released from internal

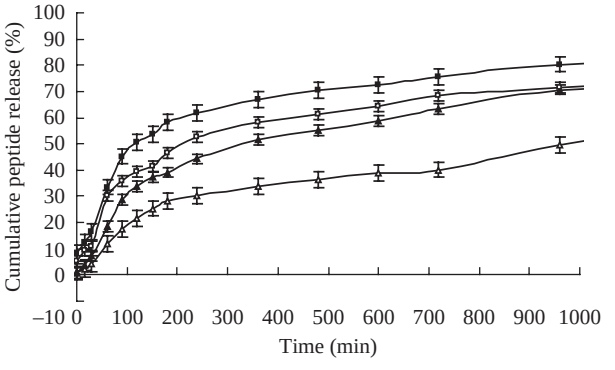


FIGURE 2. Typical release pattern of oyster peptides from alginate/chitosan/starch microcapsules by different methods as a function of time in a neutral pH medium (0.2 mol/L Tris–HCl, pH 7.5). ■, sample 12; □, sample 13; ▲, sample 3; Δ, sample 7 (the formulation of the samples are included in Table 1).

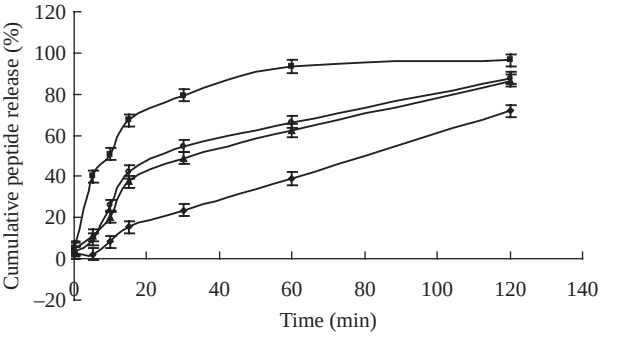


FIGURE 3. Typical release pattern of oyster peptides from alginate/chitosan/starch microcapsules by different methods as a function of time in an alkaline pH medium (0.05 mol/L Tris–HCl, pH 8.6). ■, sample 12; ❍, sample 10; Δ, sample 3; ✦, sample 7 (the formulation of the samples are included in Table 1).

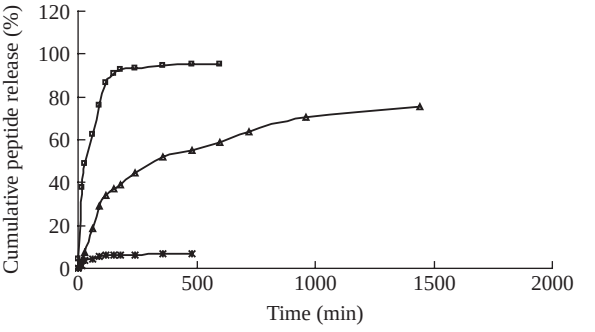


FIGURE 4. Typical release pattern of oyster peptides from alginate/chitosan/starch microcapsules by external gelation as a function of time at different pH values. □, pH 8.6; Δ, pH 7.5; *, pH 1.5.

gelation microcapsules in 0.05 mol/L Tris–HCl (Figure 3, pH 8.6), whereas in neutral medium (Figure 2, pH 7.5) it had a sustained release following a burst release and was blocked at low pH value of 1.5 (Figure 4). In other words, the peptide retention had maximal stability in acidic medium, such as in the stomach, by forming a surface gel cover, while peptide delivery is controlled at neutral pH, such as in the small intestine, by the swelling of starch blend matrix, and is accelerated at alkaline pH, in the large intestine.

The influence of starch concentration on the external gelation microcapsules release in alkaline medium was obvious; the data showed that formulations containing proper amount of starch (10%) displayed higher release rates than those formulations containing nonstarch microcapsules (Figures 2 and 3). Therefore, alginate/chitosan/starch showed a rapid and thorough peptide release in 0.05 mol/L Tris–HCl (pH 8.6). The microcapsules swelled in alkaline medium, and the peptide inside was released by dissolution, diffusion, and microcapsule erosion. However, the solubility of chitosan was less in neutral and alkaline medium so that the peptide release was largely

affected by the solubility of starch. Although Na^+ and K^+ ions could also exchange calcium associated with sodium alginate, their effect was not as strong as starch. Thus, the alginate/chitosan/starch microcapsules release in vitro was slower in neutral than in alkaline medium. The concentration change of chitosan had a slight effect on peptide release in alkaline medium. The alginate/chitosan/starch microcapsules release increased to some extent when the chitosan concentration decreased to 0.2% (wt/vol) (data not shown).

Figure 4 shows that the release rate could be regulated by the pH value: in simulated gastric fluid, the release rate greatly decreased, and in simulated body fluid and intestinal fluid, the microcapsules exhibited a constant release in 24 h with different release rates. In in vitro release tests, the microcapsules showed fine pH response properties, which provided possibility for pH targeting peptide delivery.

Characterization of the Oyster Peptides-Loaded Alginate/Chitosan/Starch Microcapsules

Figure 5 shows the FT-IR spectra of oyster peptides-loaded alginate/chitosan/starch microcapsules and plain physical blend formations. The introduction of oyster peptides into microcapsules was also investigated by FT-IR analysis of oyster peptides-loaded microcapsules. The spectrum of alginate showed a characteristic peak at 870 cm^{-1} for the associated carboxylic acid salt (Figure 5D). Additionally, plain oyster peptides peak was found at 1,408 cm^{-1} for the vinyl bond stretch on amide (Figure 5C). In contrast, it revealed two shoulders on the plain physical blend formations absorption bands, which were recognized as some peak changes in the amide (~1,408 cm^{-1}) and carbonyl (~870 cm^{-1}) bonds (Figure 5A). This suggested that the oyster peptides contain many ionizable groups, owing to amino acid residues that are capable of attaining a positive charge and attaching a negative charge (Brange, 1987). After the synthesis process, the absorption at 1,408 and 870 cm^{-1} corresponding to the vinyl bond (C=C) and carbonyl (C=O) bond of peptides and alginate disappeared as a result of the interaction between the negatively charged $-COO^-$ groups of alginate (cross-linked with $CaCl_2$), the positively charged $-NH_3^+$ groups of chitosan, and the curl spirality of the D-glucose group for high amylase corn. This suggests that interpolyelectrolyte complex alginate/chitosan/starch was well mixed to lead to significant changes in the molecular dynamics for the constituted components (Figure 5B). These properties are, therefore, possibly responsible for the entrapment of oyster peptides into alginate/chitosan/starch microcapsules. Observed changes in the absorption bands of the amino groups, carboxyl groups, and amide bonds can be attributed to an ionic interaction between the carbonyl group of alginate and the amino group of chitosan (Ribeiro, Silva, Ferreira, & Veiga, 2005). Similar observations were noted previously (Mitrevej, Sinchaipanid, Rungvejhavuttivittaya, & Kositchaiyong, 2001). These results suggest an effective interaction between polymers and seem to be in agreement with the stoichiometric ratios between them, indicating a prevalence of alginate/chitosan/starch in the final blend. Shifts in maximum infrared peaks observed between individual polyelectrolytes and final microcapsule carriers were thought to be due to ionic interactions, which led to the formation of new chemical entities with different absorption properties.

Microscopic Study

Particle size was measured alternatively by optical microscopy. These results along with EE%, % peptides loading, and mean particle size for different formulations are presented in Tables 1–3. In Table 1, the results showed that the size of particles depends on the amount of peptides present, % alginate content, % chitosan content, and the amount of starch used. External gelation microcapsules are generally spherical in shape with sizes ranging from 840 to 1,328 μm. Internal gelation microcapsules are usually microspheres with mean sizes 210 ± 5 and/or 239.07 ± 10 μm. The particle sizes of microcapsules without the starch are smaller than those of alginate/chitosan/starch microcapsules. Moreover, the size of microcapsules formed by external gelation increased from 872 to 1,186 μm by increasing the chitosan content for 10% oyster peptides-loaded microcapsules. This can be explained on the basis of hydrodynamic viscosity concept, that is, as the amount of chitosan in microcapsules increases, interfacial viscosity of the polymer droplets in the emulsion also increases. On the other hand, with increasing amount of chitosan, the number of free sites available for cross-linking is less so that the size of the microcapsules will also increase with increasing chitosan content of the microcapsules. A similar trend was also observed for starch matrix; the size of the microcapsules increased from 840 to 1,328 μm with increasing the starch content. This is attributed to the fact that starch curl spirality molecules might have occupied the free volume spaces within the matrix, thereby hindering the inward shrinkage of the cross-linked alginate/chitosan polymer matrix (Table 1, Figure 6A).

Using the internal emulsion gelation method, sodium alginate and calcium chloride developed a gelation reaction to form the microcapsule frame. The chitosan reacted with alginate through electrostatic interaction to form a stable polyelectrolyte complex on the frame (Hari, Chandy, & Sharma, 1996; Polk, Amsden, De Yao, Peng, & Goosen, 1994), then the starch curl spirality molecules might have occupied the free volume spaces within the matrix. Spherical and well-dispersed alginate/chitosan/starch microcapsules with a narrow size distribution were prepared (Figure 6B). The mean diameter of the microcapsules was 239.07 ± 10 μm, and the size distribution is shown in Table 4; it is evident that the distribution percentage of internal emulsion gelation microcapsules between 50 and 300 μm is nearly 80%.

Swelling Studies

It was noticed that there were obvious differences in Sw between external and internal gelation microcapsules (Table 1).

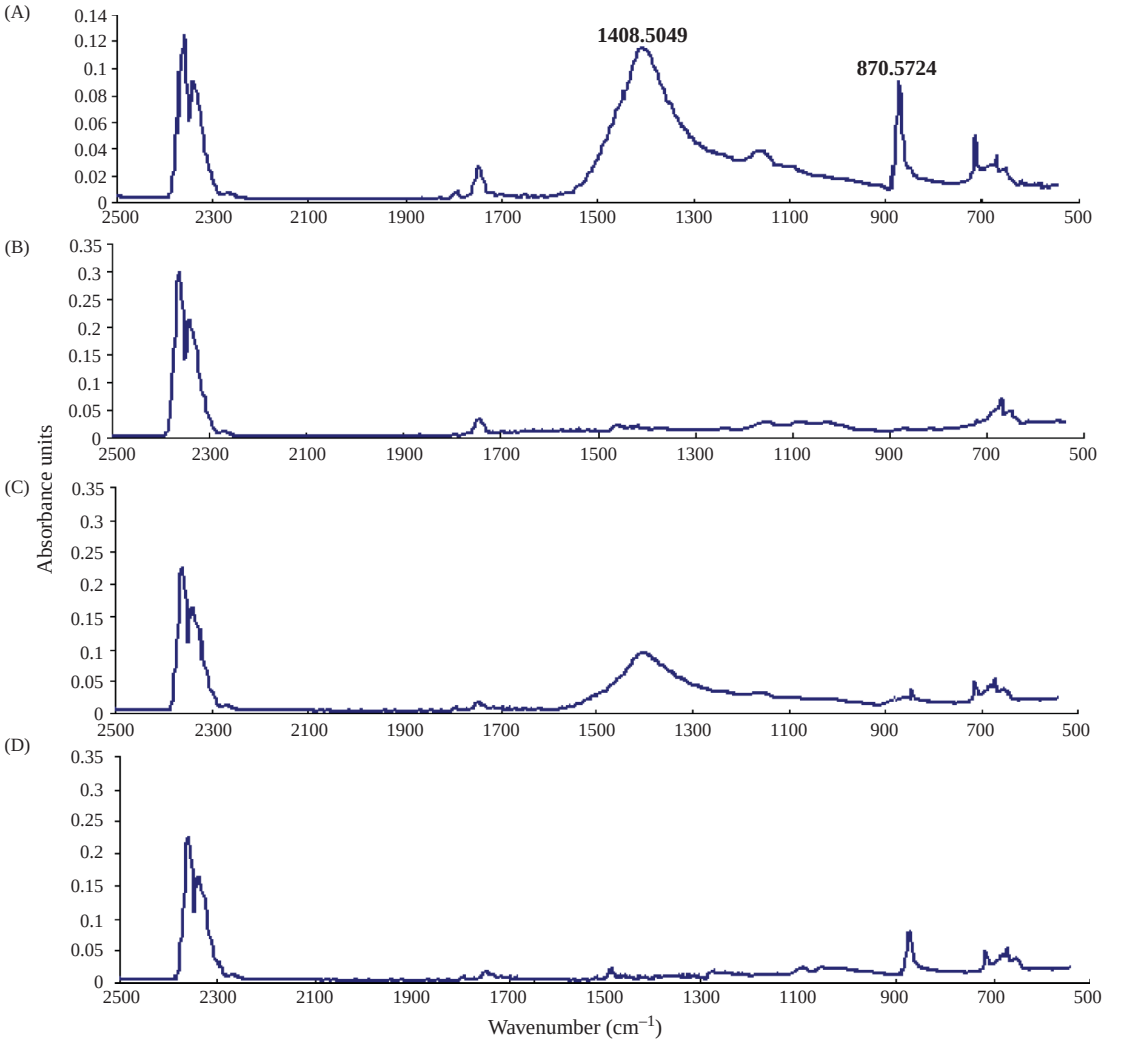
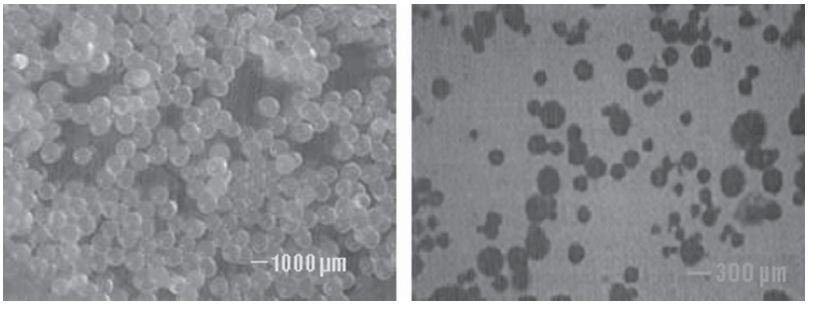


FIGURE 5. Infrared spectra of solid in the region 400–2,500 cm^{-1} for (A) oyster peptides/alginate/starch physical blend; (B) oyster peptides/alginate/chitosan/starch microcapsules; (C) plain oyster peptides; (D) plain alginate/chitosan/starch blend uncross-linked.



(A) (B)

FIGURE 6. Morphology of microcapsules. (A) Microscopic photograph of dried external gelation microcapsules re-dispersed in normal saline (×4). (B) Microscopic photograph of dried internal gelation microcapsules re-dispersed in normal saline (×300).

TABLE 4
The Size Distribution of Oyster Peptides-Loaded Internal Emulsion Gelation Microcapsules (n = 3)

Experiment No.	Diameter (μm)	Distribution Percentage (%) 50–300 μm	<400 μm
1	224.40	78.54	100.00
2	254.20	79.12	100.00
3	238.60	81.06	98.80
Average (±*SD*)	239.07 ± 10.1	79.57 ± 0.99	99.93 ± 0.12

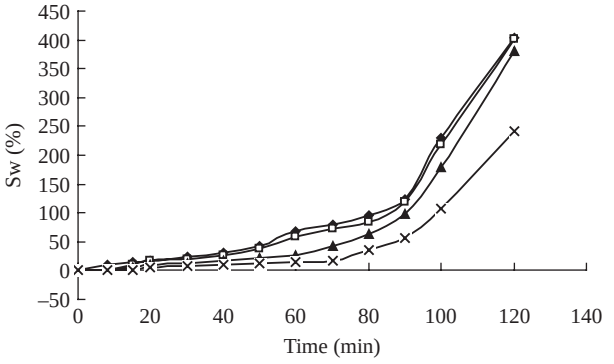


FIGURE 7. Changes of swelling of external gelation microcapsules with % starch at pH 8.6. ■, sample 10 (20%); □, sample 8 (15%); ▲, sample 3 (10%); ×sample 7 (0%).

Figure 7 shows the degree of swelling with the same formulation components and different amounts of starch by external gelation microcapsules. In this study, different amounts of starch were added to the blend microcapsules of alginate/chitosan containing 10% peptides, and these data are included in Table 1. The Sw values of external gelation microcapsules after 120 min were 242, 398, 401, and 404% with 0, 10, 15, and 20% starch, respectively. The amount of starch is dependent on equilibrium swelling. For instance, % equilibrium swelling increased from 242 to 398 with increasing amount of starch from 0 to 10%. This might be because of the decreased alginate/chitosan link density and increased pore volume of the blend matrix. As starch is a water-soluble polymer, it is readily miscible with alginate/chitosan in all proportions and, hence, blending of starch with alginate/chitosan will increase the matrix swelling because of their higher water uptake. However, % dynamic swelling of the blend matrix containing 10, 15, and 20% of starch are 398, 401, and 404%, respectively. The %Sw has not significantly changed with increasing amounts of starch in the blend matrix. This is due to the fact that starch with a high amylose content resulted in equilibrium swelling, where amylose is inaccessible because of the physical structure in which it is located, is less susceptible to

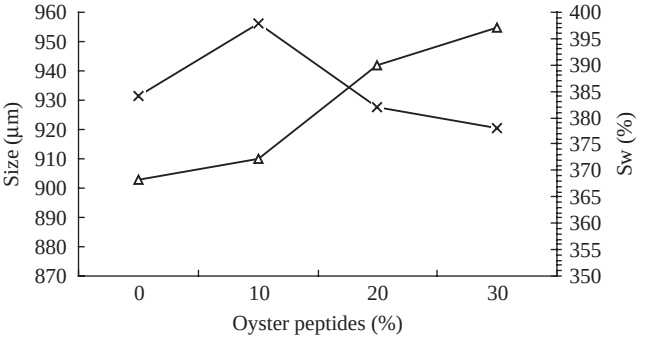


FIGURE 8. Changes of the size (△) and swelling (×) on external gelation microcapsules with % peptides at pH 8.6 (sample 11 [30%], 9 [20%], 3 [10%], 5 [0%] included in Table 1).

gelatinization and hence is more resistant to swelling. As the amount of starch increases in the blend matrix, hydrophobicity of the blend increases because of the presence of amylose groups in starch, which increases the hydrophobic character of the blend. The response of swelling behavior to amylose starch concentration during alkaline incubation has not been previously reported (Figure 7). The release of peptides from alginate/chitosan/starch microcapsules increased with increasing starch concentration between 0 and 15% (Figure 3). The size of microcapsules increased with increasing peptides concentration between 0 and 30% (Figure 8). The %Sw has changed with increasing amounts of peptides in the microcapsule synthesis (Figure 8), although interpretation of the data is difficult as it is unclear whether the peptides loadings represent simply a size–swelling relation or a synthesis-related process.

CONCLUSIONS

Oyster peptides alginate/chitosan/starch microcapsules were prepared using external or internal emulsion gelation process and characterized by FT-IR and particle size distribution. The FT-IR spectra have confirmed the uniform molecular distribution of the oyster peptides in the microcapsules. The optical micrographs exhibited a spherical morphology of the prepared microcapsules. The swelling studies of microcapsules have shown that within a certain amount of starch in the microcapsules, water uptake increased. This effect is correlated with the release rates of the oyster peptides although the microcapsules contain different amounts of starch. The oyster peptide was released in a controlled manner. It was demonstrated that the release behavior of these alginate/chitosan/starch microcapsules was affected by the properties of alginate, chitosan, and starch, including the concentration and pH, as well as the swelling behavior and erosion of the system gel matrix. Compared with external gelation microcapsules, the release of internal gelation microcapsules is faster. Considering the ease of scale-up, internal emulsion gelation microcapsule technology may be a better way for large-scale production of alginate/

chitosan/starch microcapsule as food delivery carriers. This study also proved that alginate/chitosan/starch microcapsules are a potential system for colonic delivery.

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