Preparation and Characterization of Alkaline Phosphatase Encapsulated in Gellan-Chitosan Hybrid Capsules

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Summary: Alkaline phosphatase (ALP) was encapsulated in gellan-chitosan polyion complex (PIC) capsules using a convenient procedure. The recovery of ALP was about 50% when the capsules were prepared by dropping a solution of ALP and gellan mixture (ALP/gellan) into a chitosan solution. When *p*-nitrophenyl phosphate (*p*-NPP) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were incubated with ALP/gellan–chitosan capsules as substrates for ALP, the transparent colorless capsules changed to yellow and blue, respectively. The encapsulation of ALP into the PIC capsules was also confirmed by SDS-PAGE and immunoblot analyses. The ALP and polypeptides of more than 30 kDa was retained without release even after incubation at 4 °C for 14 days. The biochemical properties of the encapsulated ALP activity in the capsules were similar to those of the intact enzyme. When the solution containing *p*-NPP was loaded on a column packed with ALP/gellan-chitosan capsules at 27 °C, approximately 75% of *p*-NPP was hydrolyzed by passing through the column. Any significant leakage of ALP was not observed during the procedure, indicating that the capsules were resistant to pressure in the chromatographic operation. Furthermore, 70% of the hydrolytic activity of the packed capsules remained after storage at 4 °C for one month. The present results suggest that the polyion complex capsules will be useful materials for protein fixation without chemical modification.
Key words: alkaline phosphatase; bioreactor; encapsulation; gellan-chitosan; polyion complex capsule

Introduction

The encapsulation system containing biological components, such as enzymes, proteins, and detoxicants, was originally proposed by Chang as an "artificial cell".[1] The concept of the artificial cell is now developed into diverse biological encapsulated formulations, for examples, microencapsulated invertase,[2] glucose odxidase,[3] cytochrome C,[4] fungal proteases,[5] asparaginase, and catalase.[6] The benefits for encapsulating and releasing a therapeutic agent from a polymer matrix are protection and sustained release of the encapsulated substances.[7] The microcapsules and microspheres are very promising and are designed as small size particles that enable repetitive administration as a therapeutic bolus via both injection or oral route. Biocompatible and biodegradable materials, including natural polysaccharides such as

chitin, chitosan,[8] alginate,[9] xanthane,[10] and synthetic biodegradable polymers such as poly(lactic-*co*-glycolic acid) and poly(lactic-*co*-amide), have been thoroughly investigated for this purpose.

To achieve biological encapsulation in which the enzymes or peptides fully retain their catalytic activity or biological function, selection of the methodology for encapsulation is rather important. A preparative method via complex coacervation is based on polyionic complexation[11, 12] through electrostatic interactions between cationic and anionic polymers, resulting in the formulation of insoluble spherical capsules. Recently, we have reported the preparation and characterization of several kinds of polyion complex (PIC) capsules, including gellan–chitosan,[13] poly(L-lysine)–gellan,[14] chitosan–poly(L-glutamic acid),[15] and poly(L-lysine)–poly(L-glutamic acid).[16] More recently, the membrane permeability and drug-releasing properties of the gellan–chitosan capsule were reported in detail.[17] The gellan–chitosan capsule retained proteins inside, while released low molecular weight substances across the capsule membrane. Thus, the gellan–chitosan capsule is expected to be a promising carrier material for active enzymes inside.

In the present study, we report (i) encapsulation of alkaline phosphatase (ALP) into the gellan–chitosan PIC capsules, (ii) biochemical characterization of the encapsulated ALP, and (iii) application of the ALP-encapsulating PIC capsule as a bio-preparative reactor.

Materials and Methods

Materials

Chitosans-10, -100, -500, and -1000, gellan, *p*-nitrophenyl phosphate (*p*-NPP) and horseradish peroxidase-conjugated goat anti-mouse antibodies were purchased from Wako Pure Chemical Industry. Average molecular weights of chitosans-10, -100, -500 and -1000 were 2.1×10^5, 13.1×10^5, 15.8×10^5, and 18.0×10^5, respectively.[13] Purified and crude ALP from porcine intestine and anti-alkaline phosphatase monoclonal antibody were purchased from Sigma. 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) was from KPL. Low molecular weight protein standard was from Pharmacia. All other chemicals used were reagent grade.

Encapsulation of ALP into Gellan–Chitosan Capsules

The gellan–chitosan capsules were prepared as we described previously in detail.[17] Briefly, 1% (weight per volume percentage; w/v) gellan solution (in H_2O) containing 0–2 mg · mL^{-1} ALP was dropped into 0.5% (w/v) chitosan solution (in 150 mM acetic acid, pH 2.5–3.0). After standing for 5–10 min at room temperature, the formed capsules were washed with TBS (Tris-buffered saline; 20 mM Tris-HCl, 137 mM NaCl, pH 7.5) three times and stored at 4 °C prior to use.

Assay of ALP Activity

In the standard assay, the ALP-encapsulating capsules in 1.8 mL of a buffer solution (40 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.5) were pre-incubated at 37 °C for 5 min, and the reaction was initiated by adding 200 µL of the substrate solution (100 mM *p*-NPP). The ALP began to hydrolysis of *p*-NPP to *p*-nitrophenol (*p*-NP). After incubation at 37 °C for 0–60 min, 300 µL of liquid was pipetted out, transferred to a test tube, then the enzyme reaction was terminated by the addition of 100 µL of 3 M NaOH. After flash centrifugation, absorbance of the supernatant was measured at 405 nm. One unit of ALP was defined to be that hydrolyzing 1 µmole of *p*-NPP per min at 37 °C.

Electrophoresis and Immunoblot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli[18] with a 13.5% slab gel. Tricine/SDS gel electrophoresis was carried out on 10/20% discontinuous polyacrylamide gel as described by Schagger.[19] Proteins in the gel were stained with 0.1% Coomassie brilliant blue R-250, 10% acetic acid and 40% ethanol for 1 h and destained in 10% acetic acid and 40% ethanol. For immunoblotting, proteins were electrophoretically transferred from the SDS-polyacrylamide gel onto nitrocellulose membrane. The membrane was blocked with a 1% bovine serum albumin solution in 10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and incubated with a 1:1000-fold dilution of anti-ALP monoclonal antibody, then treated with a 1:3000-fold dilution of horseradish peroxidase-conjugated goat anti-mouse antibodies. After immunoreaction, the membrane was washed with a buffer solution (10 mM Tris-HCl, 150 mM NaCl, 0.5% Tween-20, pH 7.4). The immunoreactive bands were visualized using 4-chloro-1-naphthylphosphate and H_2O_2 as the substrate for the horseradish

peroxidase.[20,21]

Results and Discussion

Encapsulation of ALP into Gellan–Chitosn Capsules

The encapsulation procedure is illustrated in Scheme 1. The ALP was mixed with the dropping solution and added dropwise into the receiving solution. Immediately, the polyionic complexation reaction between chitosan and gellan occurred at the interface, thus producing the true spherical capsules which retain the ALP inside. The encapsulated ALP activity was defined as the recovered percentage of the ALP activity in the capsules, towards the total ALP activity in the dropping solution.

At first, we examined the recovery of the ALP activity at the different encapsulating procedures. The molecular weight of chitosan is thought to be important for the formation of the PIC capsules.[17] We prepared gellan–chitosan PIC capsules incorporating ALP using two kinds of experimental combinations by exchanging cationic chitosan and anionic gellan as the dropping and receiving solutions. When the ALP-gellan mixed (ALP/gellan) solution was added dropwise into the chitosan-10 and -100 solutions, transparent spherical capsules having 3–5-mm diameters were immediately formed as we reported earlier.[13,17] After standing for 20–30 min and washing with TBS, the ALP activity of the capsules was measured (Table 1). The recovery of ALP activity was 53.8 ± 6.2% and 49.4 ± 10.1% for chitosan-10 and -100 as the receiving solutions, respectively. The number of capsules was 30–40 per ml of the dropping solution in both cases of gellan and chitosan. The opposite addition order of chitosans-10 or -100 (dropping) and gellan (receiving) was not effective for the formation of capsules under the present conditions used, because of their different densities.

When ALP/gellan mixture was added to the chitosan solution, the recovered ALP activities were higher than those when ALP/chitosan mixture was added to the gellan solution. This may be due to exposing the enzyme to low pH solution (150 mM acetic acid), resulting in the partial denature of ALP.

Characterization of Encapsulated ALP

The recovery of ALP activity into the gellan-chitosan PIC capsules as a function of ALP concentrations is shown in Figure 1a. For this experiment, crude and purified ALP

were employed. When the crude ALP (0.1–3.0 units) was mixed with a 1% gellan solution (1 mL) then dropped into the chitosan-10 solution, the recovery of the encapsulated ALP activity was 50–60%. On the other hand, the value was became lower (15–25%) when purified ALP (0.1–3.0 units) was used. Both in the cases of crude and purified ALP, the recovery percentages were the highest at 0.6 unit then decreased with increasing the ALP concentrations. The spherical capsules thus obtained in all ALP concentrations tested are stable to mechanical stimuli by magnetic stirring. Hence in the following experiments, we used the combination of 1% gellan/ALP (0.6 units per mL of gellan solution) and 0.5% chitosan-10.

Addition of *p*-NPP and BCIP, substrates of ALP, produced bright yellow-color and blue-color capsules, respectively (Figure 1b), indicating that the encapsulated ALP remained to be active. Time courses of *p*-NPP hydrolysis by intact and encapsulated ALP are represented in Figure 1c. The initial lag of *p*-NP release was apparently found in the encapsulated ALP. This delay will be spent in the diffusion of *p*-NPP (molecular weight = 263) and *p*-NP (molecular weight = 139) across the membrane of the gellan–chitosan PIC capsules. After incubation for 5 min at 37 °C, the hydrolysis of *p*-NPP proceeded at a constant rate for at least 60 min.

The biochemical properties of the encapsulated ALP activity were compared with those of the intact enzyme (Table 2). The intact and encapsulated ALP activities showed an absolute requirement for divalent cations such as Mg^{2+} and Ca^{2+}, while chelation of the divalent cations by EDTA significantly suppressed the ALP activities. Both intact and encapsulated ALP activities were observed with similar sensitivities over wide pH and temperature ranges. These data suggest that ALP behaves like free enzyme after encapsulation into the capsules.

Identification of Encapsulated ALP by Immunoblotting

Encapsulated proteins in the PIC capsules were re-extracted with sample buffer (31 mM Tris-HCl, 1% SDS, 5% glycerol, 0.15% 2-mercaptoethanol, 0.0005% pyronin Y, pH 6.8), and the protein compositions were analyzed by SDS-PAGE (Figure 2a). As for the crude ALP preparation, several protein bands with molecular masses of 14-100 kDa were stained with Coomassie-blue (Figure 2a, lanes 1,2). Purified ALP showed a single polypeptide with a molecular mass of 67 kDa (Figure 2a, lanes 3,4). Immunoblot analysis using anti-ALP monoclonal antibody confirmed that the 67kDa

bands in both crude and purified ALP preparations are actually porcine intestine ALP (Figure 2b, lanes 1–4). The pre-encapsulated protein (Figure 2b, lane 1) and the re-extracted protein (post-encapsulated; Figure 2b, lane 2) exhibited the identical patterns in their immunoreactive bands. The immunoreactive bands of the crude preparation were found mainly at 67 kDa and 95 kDa, as reported previously (Figure 2b).[21] The 67 kDa polypeptide of the crude ALP strongly cross-reacted with the antibody. These results indicate that ALP was successfully encapsulated into gellan–chitosan PIC capsules in the stable state. Furthermore, proteins with low molecular weights were also entrapped in the PIC capsules. The release of the proteins from the PIC capsule was examined in the following experiment.

Permeability of Gellan–Chitosan Capsules

The permeation of the encapsulated inner materials will be mainly dependent on their molecular masses, molecular structures, and electrical charges. In order to examine the molecular weight dependence on the protein release from the PIC capsules, the following proteins were encapsulated into the gellan–chitosan capsules; phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). After incubation for 0–10 days at 4 °C in TBS, the remaining proteins in the capsules were analyzed by Tricine/SDS-PAGE (Figure 3a). The proteins with molecular masses of more than 30 kDa were retained in the capsules even after 10 days. On the other hand, α-lactalbumin (14.4 kDa) and soybean trypsin inhibitor (20.1 kDa) were gradually released from the capsules, and the remaining proteins were decreased to 50% after 5–6 h for α-lactalbumin and after 1 day for soybean trypsin inhibitor. Similar results were observed over a wide range of pH 5–9 and at high salt concentration (1 M NaCl) in TBS (data not shown). These results suggested that the exclusion limit of the gellan/chitosan capsules used was about 30 kDa, which value was almost in accord with that obtained with the gellan–chitosan capsules as we reported previously.[17]

Application of ALP/Gellan–Chitosan Capsules as a Bioreactor

We prepared a column (ϕ 1 × 16.5 cm) packed with ALP/gellan–chitosan capsules, each of which contains 50 units of ALP. The experiments were performed after 1 and 30 days since the capsules have been prepared. The column was equilibrated with a

buffer solution (40 mM Tris-HCl, 1 mM $MgCl_2$, pH 8.5) at 27 °C, then 80 mL of the buffer containing 2 mM *p*-NPP was applied to the column at a flow rate of 18 mL · h^{-1}. As shown in the results of Figure 1b, the color of the column changed from transparent to light yellow (Figure 4a). The yellow color lasted for 2.5 h until the *p*-NPP containing buffer was replaced by the substrate-free buffer. This elution profile is represented in Figure 4b. More than 70% of total *p*-NPP applied was hydrolyzed in the fraction numbers from 15 to 60. No protein was detected in the collected fractions by means of SDS-PAGE and immunoblot analyses (data not shown), indicating that the proteins never leaked out of the capsules during the chromatographic procedure. After storage of the column at 4 °C for 30 days, the same experiment was carried out. Approximately 50% of total *p*-NPP applied was hydrolyzed, and the hydrolyzing activity corresponds to 70% of the original level (after 1 day) of the column. These results indicated that the gellan–chitosan PIC capsules encapsulating ALP and other enzymes will function as a bioreactor.

Conclusion

Isolation of biopolymers, tissues, and cells from the host body system is necessary to protect the implanted polymers from protease and immune systems. Encapsulation is thought to be an effective technique for solving these problems. Both gellan and chitosan are natural biopolymers and are widely utilized in food and medical science, because of their safety and low price. The enzyme-encapsulating procedure described in the present study does not need any of chemical modifications and is conveniently performed in the aqueous solution. The encapsulated enzyme thus prepared fully retains its biological catalytic activity. In conclusion, the chitosan–gellan PIC encapsulating system will further inspire a broad spectrum of application and will be a powerful tool in the field of biotechnology.

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Tables and Scheme

Table 1. Recovery of Alkaline Phosphatase Activity during Encapsulation.

Dropping solution (containing ALP)[a)]	Receiving Solution[a)]	Recovery of ALP Activity (%)[b)]
Gellan	Chitosans-10	53.8 ± 6.2
	-100	49.4 ± 10.1
	-500	n.f.[c)]
	-1000	n.f.
Chitosans -10	Gellan	n.f.
-100		n.f.
-500		34.2 ± 7.1
-1000		35.1 ± 6.5

[a)] Defined in Scheme 1.

[b)] Encapsulated alkaline phosphatase activity value calculated from the total activity in the dropping solution (100%).

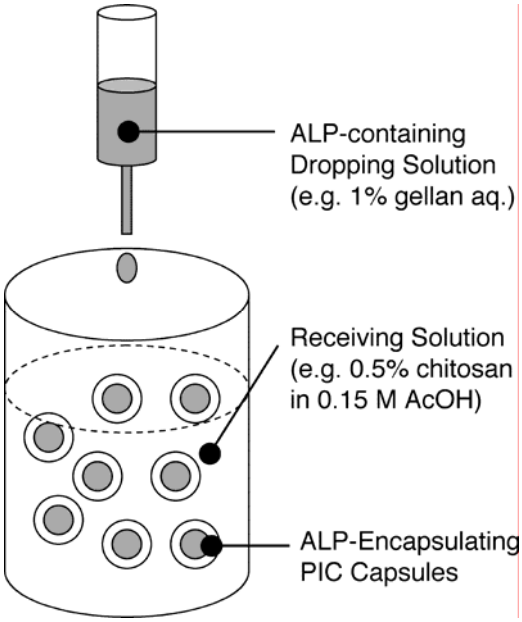
[c)] Capsules were not formed.

Table 2. Effects of Various Factors on Alkaline Phosphatase Activities in Intact and Encapsulated States.

Factors		Intact	Encapsulated[a)]
None (control) [b)]		100.0	100.0
10 mM EDTA		3.7 ± 1.8	3.7 ± 1.8
3 mM $MgCl_2$		104.1 ± 4.3	114.2 ± 14.6
$CaCl_2$		98.1 ± 7.5	96.5 ± 6.4
pH	8.0	65.3 ± 4.8	74.5 ± 5.8
	9.0	169.0 ± 8.5	144.2 ± 10.8
	10.0	171.6 ± 4.0	153.7 ± 10.5
Temperature (°C)	25	74.0 ± 1.7	78.5 ± 7.6
	35	97.2 ± 3.5	96.0 ± 6.5
	45	150.4 ± 5.6	141.7 ± 9.5
	55	118.5 ± 9.7	141.7 ± 5.1

[a)] Encapsulated in gellan–chitosan PIC capsule.

[b)] Measured in the standard assay conditions, as described in the Materials and Methods section.



Scheme 1. Encapsulation of alkaline phosphatase (ALP) into gellan-chitosan polyion complex (PIC) capsules.

Figures and Captions

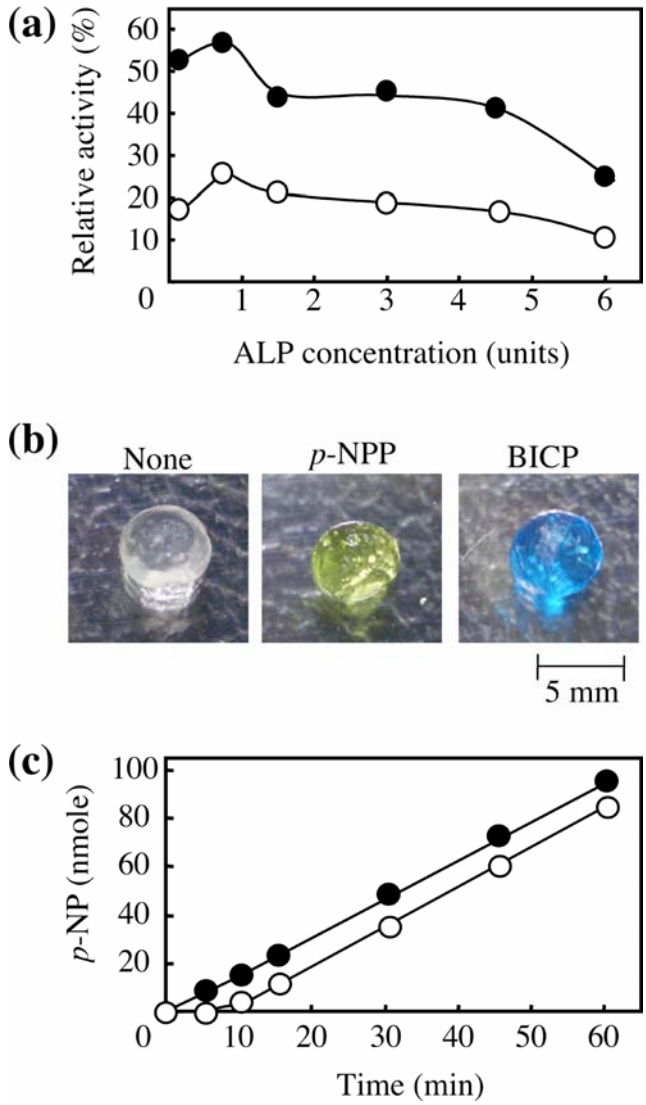


Figure 1. Characterization of ALP-encapsulating gellan-chitosan PIC capsules. (a) Relationship between the ALP concentration and the recovery of ALP activity in the PIC capsules: ●, crude ALP; ○, purified ALP. (b) Morphological observation of ALP-encapsulating gellan-chitosan PIC capsules in the absence (left) of the ALP substrate and in the presence of *p*-NPP (middle) and of BICP (right). (c) Time course of *p*-NP liberation from the ALP-encapsulating PIC capsules: ●, intact ALP; ○, ALP/gellan-chitosan capsules.

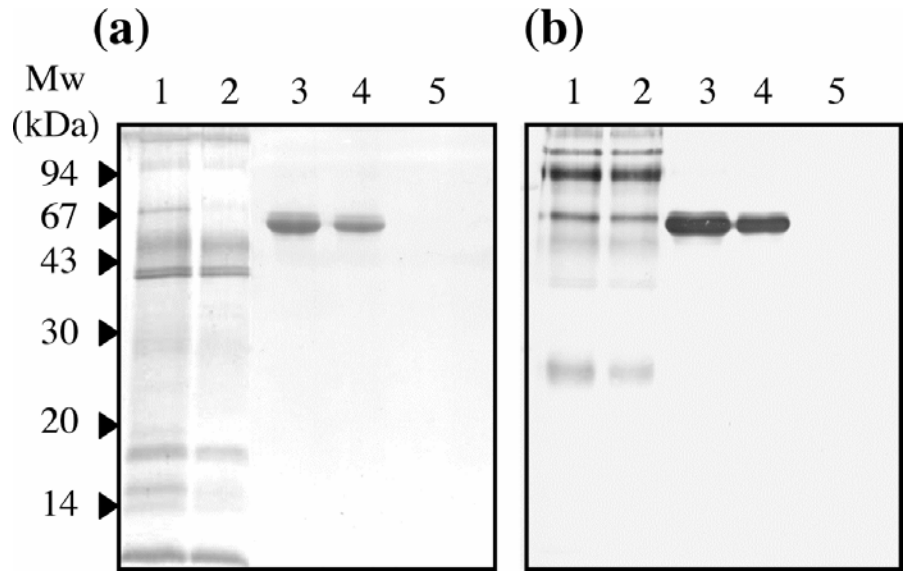


Figure 2. Identification of ALP in gellan-chitosan PIC Capsules. PIC capsules were incubated with an equal volume of sample buffer at 95 °C for 2 min. After centrifugation at 1000 × *g* for 3 min, the obtained supernatants were subjected to 13.5% SDS-PAGE. The separated proteins were transferred onto a nitrocellulose membrane: (a), Coomassie brilliant blue staining; (b), immunostaining with anti-ALP monoclonal antibody. Lanes 1, crude ALP; 2, crude ALP in the gellan-chitosan capsules; 3, purified ALP; 4, purified ALP in the gellan-chitosan capsules; 5, ALP-free gellan-chitosan capsules.

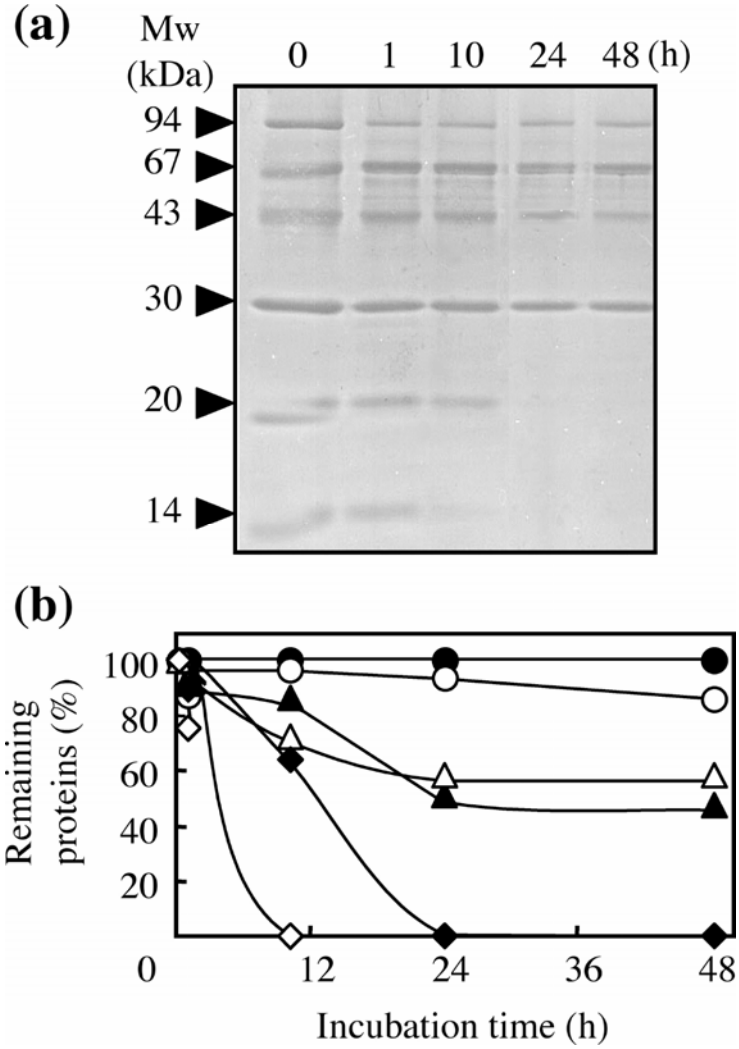


Figure 3. Relationship between the molecular weights of the encapsulated proteins and the releasing kinetics from gellan–chitosan capsules: (a), SDS-PAGE of the remaining protein in the gellan–chitosan PIC capsule; (b), Time course of the remaining protein (%), which was determined by densitometry of the protein bands: ●, phosphorylase b (94 kDa); ○, bovine serum albumin (67 kDa); △, ovalbumin (43 kDa); ▲, carbonic anhydrase (30 kDa); ◆, soybean trypsin inhibitor (20.1 kDa); ◇, α-lactalbumin (14.4 kDa).

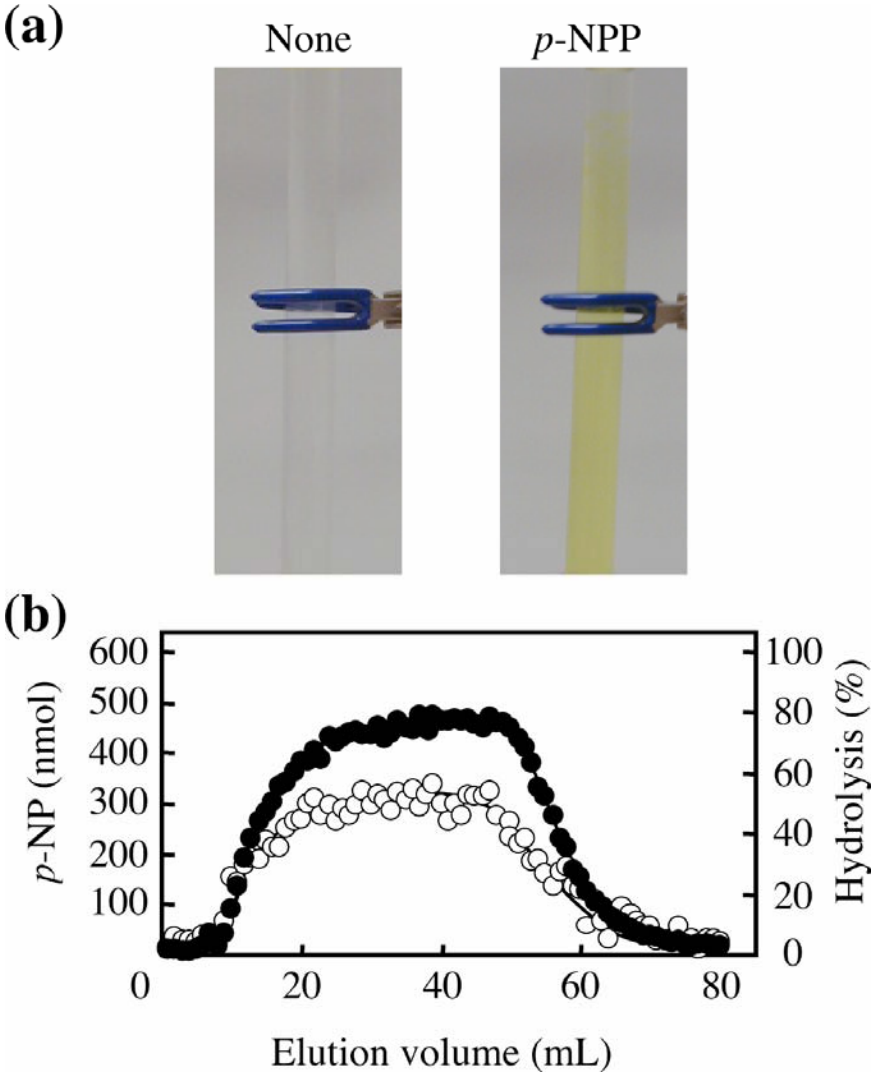


Figure 4. Preparation of the bioreactor column packed with the ALP/gellan–chitosan capsules: (a), before (left) and after (right) loading the substrate (*p*-NPP) solution; (b), elution profile of *p*-NP, the hydrolysis product of *p*-NPP. The chromatography was done using the same column stored for; ●, 1 day; ○, 30days. Fractions (1 mL) were collected and the absorbance (405 nm) were measured.

Table-Of-Content Graphic and Text

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Full Paper: Alkaline phosphatase (ALP) was encapsulated in gellan-chitosan polyion complex (PIC) capsules using a convenient procedure (Figure). When *p*-nitrophenyl phosphate (*p*-NPP) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were incubated with ALP/gellan–chitosan capsules as substrates for ALP, the transparent colorless capsules changed to yellow and blue, respectively. Application of the ALP-encapsulating PIC capsule as a bio-preparative reactor was examined. The present results suggest that the polyion complex capsules will be useful materials for protein fixation without chemical modification.