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Short communication

Immobilization of chitinases from *Streptomyces griseus* and *Paenibacillus illinoisensis* on chitosan beads

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ABSTRACT

To investigate the enzyme activity of the chitosan beads immobilized with chitinases this study was carried out. Protein content of the *Streptomyces griseus* enzyme in supernatant was not detected in test tubes including chitosan beads treated with GA 1, 2, 3, 4, 5, and 10% at 6 h after the reaction except for glutaraldehyde (GA) 0%. Also, chitinase activity of the supernatant was highest (2.68 unit/mL), in GA 0%. Regarding enzyme stability of *S. griseus*, total chitinase activities were 1.4, 0.4, and 0.3 units/2 mL in GA 0%, 3%, and 5%, respectively, at the initial reaction time. The supernatant protein from *Paenibacillus illinoisensis* KJA-424 was bound at the highest level of 42% in chitosan beads treated with GA 10% after reaction at 4 °C for 24 h. Immobilized enzyme with GA 10% showed the highest activity level for degradation of hypha from *Rhizoctonia solani*.

1. Introduction

Chitosan having linear polyglucosamine chains of high molecular weight possesses distinct chemical and biological properties. Therefore, chitosan has been used as a swollen bead support for preparation of immobilized enzyme (Juang, Wu, & Tseng, 2001; Sakai, Uchiyama, Matahira, & Nanjo, 1991; Zubriene, Budriene, Lubiene, & Dienys, 2002). Enzymes may be immobilized by a variety of methods such as adsorption (physical, ionic) on a water-insoluble matrix, covalent attachment to a water-insoluble matrix, and cross-linking with use of a multifunctional, low molecular weight reagent. Many studies for immobilization of enzymes have been worked using chitin and chitosan (Elnashar, 2005; Jollès and Muzzarelli, 1999; Muzzarelli, 1980). In industrial applications, immobilized enzymes are mainly used to produce fructose and glucose from glucose and lactose by glucose isomerase and β-galactosidase, respectively (Woodley, 1992). In analytical applications, immobilized enzymes are mainly used in biosensors for inhibition-based determination of heavy metal ions and pesticides using horseradish peroxidase (Guilbault, 1983). Chitin powder and glutaraldehyde were used as carrier and cross-linking agents to immobilize chitosanase (Zeng & Zheng, 2002).

In this study, chitosan beads and glutaraldehyde were used as carrier and cross-linking agents, respectively, to immobilize chitinase from *Streptomyces griseus* and *Paenibacillus illinoisensis*. The utility value of this cross-linking agent is due to its ease of use and the availability of amino groups for reaction with glutaraldehyde not only on enzymes, but also on chitosan. The application of immobilized chitinase on swollen chitosan beads has not been extensively studied. The present work was designed to investigate the activities of immobilized chitinases on swollen chitosan beads against phytopathogen *Rhizoctonia solani*.

2. Experimental

2.1. Materials

Chitosan powder [90% degree of deacetylation, 10 cps (in 0.5% acetic acid + 0.5% chitosan solution, at 20 °C)] was purchased from Keumho Chemical Co., Ltd., Seoul, Korea. Chitinase (EC 3.2.1.14) from *S. griseus* (4.2 unit/mg, glycanohydrolase) was purchased from Sigma (C-6137) (St. Louis, MO, USA), and partially purified chitinase obtained from *P. illinoisensis* KJA-424 (Jung, Kuk, Kim, Kim, & Park, 2005) was used for enzyme immobilization in chitosan beads. Glutaraldehyde ($C_5H_8O_2$, 25% aqueous solution) was purchased from Sigma (G 6257, EEC No. 203-856-3). *R. solani* KACC 40111 was obtained from the Korea Agricultural Culture Collection (KACC), Suwon, Korea. All other chemicals were of analytical grade.

2.2. Preparation of enzymes

The chitinase producing *P. illinoisensis* KJA-424 was grown aerobically in 1 L of broth medium containing 0.5% swollen chitin in a 2 L flask at 30 °C while shaking at 150 rpm for 5 days. The culture

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supernatant was collected after centrifugation at 10,000 × g for 15 min. The culture filtrate was fractionated with ammonium sulfate to 80% saturation. After storage overnight, the protein precipitate was obtained by centrifugation at 6000 rpm for 1 h. The precipitate was dissolved in small volumes of distilled water. The solution was dialyzed overnight at 4 °C against 3 changes of 1 L each of distilled water. The dialysate was concentrated with polyethylene glycol (# 6000) and used for enzyme immobilization of chitosan beads.

2.3. Preparation of chitosan beads

The chitosan beads were prepared using chitosan powder (7 g) dissolved in 100 mL of 7% acetic acid. The resultant viscous solution was de-gassed under vacuum, and dropped into 200 mL of an alkali coagulating solution (H_2O–MeOH–NaOH = 4:5:1, w/w/w) to prepare highly swollen spherical beads of 3.5 mm in average diameter. The beads were collected and thoroughly washed with distilled water (Mitani, Fukumuro, Yoshimoto, & Ishii, 1991).

2.4. Immobilization of enzymes

A 10 mL volume of wet chitosan beads was prepared by crosslinking in tubes containing 10 mL of 0.1 M phosphate buffer (pH 7.04) with different concentrations of glutaraldehyde (GA 0, 1, 2, 3, 4, 5, and 10%) and shaken at 120 rpm, at 25 °C for overnight (Yi et al., 2007). The glutaraldehyde crosslinked chitosan was washed several times with distilled water until the washings were free of glutaraldehyde. Then, 1 mL of enzyme solution (238 μg/mL, chitinase from *S. griseus*, in 0.1 M phosphate buffer, pH 6.45) was added to 1 mL of glutaraldehyde treated wet chitosan beads (20 beads) and shaken for 0, 3, 6, 12 h at 25 °C (125 rpm). One milliliter of enzyme solution (200 μg/mL, chitinase from *P. illinoisensis* KJA-424, in 0.1 M phosphate buffer, pH 6.45) was added to 1 mL of glutaraldehyde treated wet chitosan beads (20 beads) and allowed to stand for 24 h at 4 °C (125 rpm) with occasional stirring. The immobilized chitinase beads were then collected and stored at 4 °C. The immobilization yields and supernatant protein were calculated from the protein concentrations as measured by the Bradford method (1976), with the enzyme solutions before and after the immobilization process.

2.5. Stability and activity of enzymes

S. griseus enzyme in chitosan beads immobilized with different concentrations of glutaraldehyde (0, 3, and 5%) was added to 2 mL sodium acetate buffer (pH 5.5) including 0.5% swollen chitin in a water bath set at 37 °C, and incubated for 2 h, and the reaction was repeated 10 times. The chitinase assay mixture consisted of 100 μL of sample, 500 μL of 0.5% swollen chitin (Lingappa & Lockwood, 1962) and 400 μL of 50 mM sodium acetate buffer (pH 5.0). Following incubation at 37 °C for 1 h, 200 μL of 1 N NaOH was added. For *P. illinoisensis* KJA-424 enzyme in chitosan beads immobilized with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) was added to 2 mL sodium acetate buffer (pH 5.5) including a piece of hypha from *R. solani* KACC 40111 (5 mm × 5 mm) which was incubated on potato dextrose agar (PDA) at 27 °C for 3 days, and incubated in a water bath at 37 °C for 2 h. Next, the sample was briefly centrifuged (10,000 × g, 5 min), after which 500 μL of supernatant was mixed with 1 mL of Schales' reagent and then heated in boiling water for 15 min. The absorbance was then immediately measured at 420 nm using a spectrophotometer (uQant Bio-Tek, USA). One unit of chitinase activity was defined as the amount of enzyme that liberated 1 μmol of GlcNAc per hour.

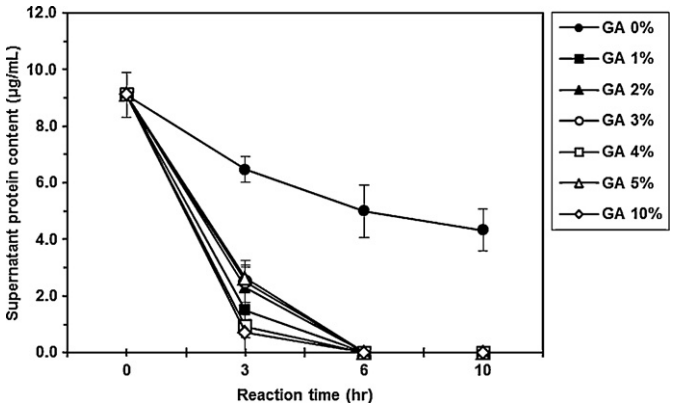

Fig. 1. Protein content of *S. griseus* in supernatant of enzyme immobilized with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) and incubated at 25 °C (125 rpm) for 0, 3, 6, and 10 h.

3. Results and discussion

To investigate the degree of enzyme immobilization, chitosan beads were treated with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%). Chitinase from *S. griseus* was added to chitosan–glutaraldehyde complex and shaken for 0, 3, 6, or 12 h at 25 °C. The protein content of the supernatant in test tubes was measured at 0, 3, 6, and 10 h after the reaction (Fig. 1). Protein content was not detected in the supernatant of test tubes containing chitosan beads treated with various glutaraldehyde concentrations (1, 2, 3, 4, 5, and 10%) at 6 h after reaction except for the tube containing GA 0%. Protein content was decreased by 45.1% at 10 h after reaction in GA 0%. Chitinase activity of the supernatant on enzyme immobilization of *S. griseus* with different concentrations of glutaraldehyde was measured at 0, 3, 6, and 10 h after reaction (Fig. 2). Chitinase activity of the supernatant was rapidly decreased in glutaraldehyde treated-chitosan beads for an initial 3 h after reaction, and was slightly increased in 0% glutaraldehyde chitosan beads until 6 h after reaction. Chitinase activity of the supernatant was 2.68 units/mL, which was the highest value in GA 0% of chitosan beads at 6 h after reaction. The effect of chitinase immobilization from *S. griseus* is presented in Fig. 3. Chitosan beads immobilized with different concentrations of glutaraldehyde (0, 3, and 5%) were added to 2 mL sodium acetate buffer (pH 5.5), including 0.5% swollen chitin in a water bath set at 37 °C for 2 h, and the reaction was repeated 10 times. The enzyme activity was determined after each reaction. As shown in Fig. 3 the immobilized enzyme showed a different activity between incubation with glutaraldehyde (3 and 5%) and

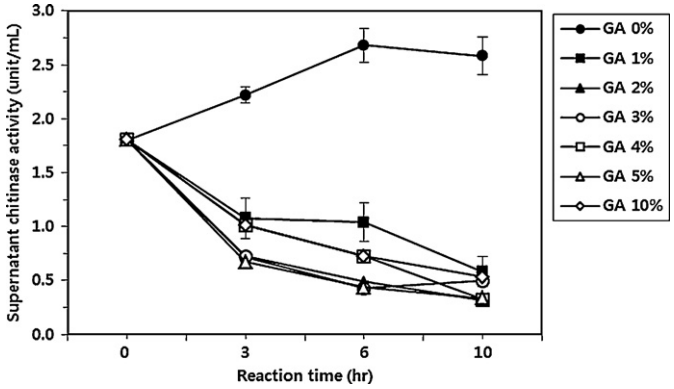

Fig. 2. Chitinase activity of *S. griseus* in supernatant of enzyme immobilized with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) and incubated at 25 °C (125 rpm) for 0, 3, 6, and 10 h.

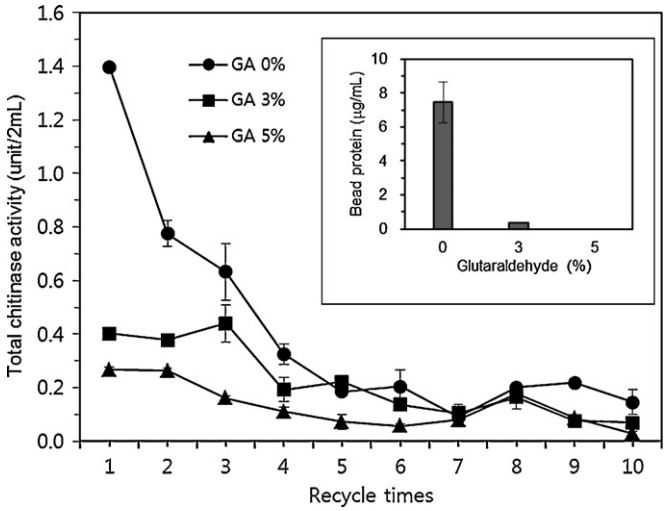

Fig. 3. Total chitinase activity of *S. griseus* after enzyme immobilization with different concentrations of glutaraldehyde (0, 3, and 5%) at 37 °C for 2 h, including 0.5% swollen chitin. The reaction was repeated 10 times.

without glutaraldehyde. Total chitinase activities were 1.4, 0.4 and 0.3 units/2 mL in GA 0%, GA 3%, and GA 5%, respectively, at the first recycle time. Total chitinase activity in 0% GA was rapidly decreased until 4 recycle times. At the second recycle time, relative activities were 55.5, 27.1, and 18.9% in GA 0%. GA 3%, and GA 5%, respectively, compared with first recycle time. Protein content in chitosan beads was measured at GA 0%, GA 3%, and GA 5% (Fig. 3, insert). The protein content in beads was 7.45 μg/mL and 0.39 μg/mL in GA 0% and GA 3%, respectively. The activities of immobilized chitinase and NAHase on tannin–chitosan beads remained at 47.2% and 32.5% of the initial activities, respectively (Sakai et al., 1991). Operational stability of immobilized chitosanase loss was <20% after 10 reaction cycles (Zeng & Zheng, 2002). Immobilized lysozyme retained ~75% of its stability after 9 days (Crapisi, Lante, Pasini, & Spettoli, 1993). The immobilized ω-transaminase from *Vibrio fluvialis* JS17 on chitosan bead retained 77% of its initial conversion after five repetitive uses (Yi et al., 2007).

Immobilization of enzyme (200 μg/mL) from *P. illinoisensis* KJA-424 on chitosan beads treated with different concentration of glutaraldehyde was carried out for 24 h at 4 °C (125 rpm) with occasional stirring, and then the supernatant protein was measured in each tube (Table 1). The supernatant protein decreased with increasing glutaraldehyde concentration. The supernatant protein concentrations were 168.4, 167.9, 167.7, 159.1, 156.2, 156.8, and 115.9 μg/2 mL in GA 0%, 1%, 2%, 3%, 4%, 5%, and 10%, respectively. The protein binding was 42% in chitosan beads treated with GA10%. When chitin was immobilized with 5% glutaraldehyde and the partially purified chitosanase from *Penicillum* sp. ZDZ1, relative enzyme activity was indicated the highest level (Zeng & Zheng, 2002). Also, it was demonstrated that the optimal amount

Table 1
Protein content of *P. illinoisensis* KJA-424 in supernatant of enzyme immobilized with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) and incubated at 4 °C for 24 h. Values given in the column are the mean ± SD for $n = 3$.

Glutaraldehyde (%)	Supernatant protein (μg/2 mL)
0	168.4 ± 4.8
1	167.9 ± 2.9
2	167.7 ± 2.6
3	159.1 ± 5.1
4	156.2 ± 0.3
5	156.8 ± 1.9
10	115.9 ± 1.0

Table 2
Chitinase activity of *P. illinoisensis* KJA-424 after enzyme immobilization with different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) and reaction for 2 h using *R. solani* KACC 40111 as a substrate. Values given in the column are the mean ± SD for $n = 3$.

Glutaraldehyde (%)	Chitinase activity (unit/mL)
0	3.48 ± 0.05
1	3.59 ± 0.11
2	3.74 ± 0.11
3	3.14 ± 0.02
4	3.41 ± 0.15
5	3.37 ± 0.05
10	4.18 ± 0.04

of added chitosanase was 2.0 mg. Enzyme activity was examined after immobilization of α-L-arabinofuranosidase (EC 3.2.1.55) from *Aspergillus niger* on chitosan with GA 0 ~1.0% (Spagna, Andreani, Salatelli, Romagnoli, & Pifferi, 1998). For activity of immobilized chitinase from *P. illinoisensis,* KJA-424 in different concentrations of glutaraldehyde (0, 1, 2, 3, 4, 5, and 10%) was prepared at 37 °C for 2 h, including a piece of hypha from *R. solani* KACC 40111 (Table 2). Chitinase activity of chitosan beads were 3.48, 3.59, 3.74, 3.14, 3.41, 3.37, and 4.18 unit/mL in GA 0%, 1%, 2%, 3%, 4%, 5%, and 10%, respectively. Chitinase activity of chitosan beads was 1.2-fold higher in GA10% than in GA 0%.

4. Conclusions

In this study, we investigated an enzyme immobilization process for commercial chitinase from *S. griseus* and crude enzyme from chitinase producing-bacterium on chitosan beads with different concentrations of glutaraldehyde. After immobilization of chitinase from *S. griseus*, chitinase activity of the supernatant was the highest level at 6 h after reaction in GA 0% including 0.5% swollen chitosan. Regarding enzyme stability of *S. griseus*, chitinase activity on chitosan beads reached its highest level during the initial reaction in GA 0% treatment. This enzyme activity on chitosan beads was the opposite of results expected to be obtained with glutaraldehyde treatment. Therefore, we suppose that the inactivation of chitinase could be occurred by some factors such as cross-linking of glutaraldehyde with enzyme and activation of chitinase and chitosan in beads. The supernatant protein from *P. illinoisensis* KJA-424 was bound at the highest level in chitosan beads treated with GA10%. This immobilized enzyme in chitosan beads showed the highest level of activity for degradation of hypha from *R. solani* KACC 40111. We found different results for enzyme immobilization using commercially purified chitinase of *S. griseus* and crude enzyme of *P. illinoisensis* KJA-424 on chitosan beads.

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