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Siripran Tidiarat

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Siripran Tidjarat[1], Tanasait Ngawhirunpat[1], Praneet Opanasopit[1] and Theerasak Rojanarata[1,*]

[1]*Pharmaceutical Development of Green Innovations Group (PDGIG), Faculty of Pharmacy, Silpakorn University, Nakhon Pathom 73000, Thailand (*corresponding author email: teerasak@su.ac.th)*

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INTRODUCTION

Chitosan, a natural polysaccharide, is an N-deacetylated derivative of chitin obtained from crustaceans, insects and fungi. It possesses interesting features i.e. film forming ability, gelation and bioadhesive characteristics. Moreover, it is biodegradable and biocompatible[1)]. Because of the polymeric cationic characteristics, chitosan can interact with negatively charged molecules or polymers such as tripolyphosphates (TPP) leading to interpolymer linkages and the formation of beads or micro/ nanoparticles. In pharmaceutical fields, these particulate systems have been used for encapsulation of drugs and biological substances[2)]. In addition, they can be applied to enzyme immobilization, a practical approach to attach the enzyme to an inert, insoluble material in order to facilitate continuous and recycle use of enzyme. Apart from such ionic crosslinking, chitosan which contains primary amine groups can undergo covalent crosslinking with certain crosslinkers such as genipin. This compound is an aglycone derived from an iridoid glycoside called geniposide present in fruit of *Gardenia jasminoides*[3)]. Compared with glutaraldehyde and many other commonly used synthetic cross-linking reagents, genipin is known as a natural crosslinker with much lower toxicity and more environmental friendliness

D-phenylglycine aminotransferase (D-PhgAT; EC 2.6.1.72) is an enzyme firstly discovered in Thailand from *Pseudomonas stutzeri* ST-201[4)]. It catalyzes a reversible stereo-inverting transamination of D-phenylglycine or D-4-hydroxyphenylglycine and 2-oxoglutaric acid to yield benzoylformic acid or 4-hydroxybenzoylformic acid (HBZF) and L-glutamic acid. Currently, D-PhgAT has been applied for the synthesis of optically pure D-phenylglycine, an intermediate for the synthesis of ampicillin[5)]. In addition, it has been used for the analysis of L-glutamate in foods[6)] and the assay of amoxicillin formulations[7)]. Since D-PhgAT employed for the aforedmentioned purposes were as solution or immobilized form using expensive matrix for immobilization, the use of low cost and green carriers such as natural polymers especially chitosan is an interesting alternative.

In this study, doubly crosslinked chitosan beads were prepared by using two safe and environmentally friendly crosslinkers TPP and genipin and aimed to use for immobilizing D-PhgAT. The influence of some conditions of the crosslinking reaction (pH of TPP, genipin concentration) on the resulting beads was studied. Chitosan beads were studied in terms of size, solubility under various pH conditions and preliminarily tested for the reaction catalyzed by immobilized enzyme.

MATERIALS AND METHODS

Materials Chitosan powder (high molecular weight with the degree of deacetylation > 75%) and sodium TPP were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Genipin was supplied by Challenge Bioproducts Co. (Taichung, Taiwan). D-PhgAT was purified in the laboratory from recombinant *Escherichia coli* expressing the cloned gene. All other chemicals were of analytical grade from Merck (Darmstadt, Germany). Distilled water was used throughout the experiments.

Ionic crosslinkink of chitosan beads Briefly, 3% (w/v) chitosan powder was dissolved in 2% (v/v) acetic acid. and mechanically stirred for 3 h to obtain clear chitosan solution. Sodium TPP was dissolved in deionized water to prepare 10% (w/v) TPP aqueous solutions. The pH of TPP aqueous solutions were adjusted from pH 8.1 (original pH value) to pH 3.0, 5.0, 7.0, 8.0, 9.0 and 11.0, respectively. The chitosan solution was directly dropped through a syringe needle (20 G) into the solution of TPP, and the chitosan droplets were stood in the solution for 12 hours to crosslink the gel beads. After crosslinking, the solidified gel beads were separated and washed thoroughly with deionized water to remove residual ionic crosslinking agents.

Covalent crosslinking of chitosan beads The previously ionic-crosslinked beads prepared using TPP at pH 8 were immersed in genipin solution with various concentrations including 2.5, 5, 10, 25 and 50 mM. The beads were shaken at 40 °C for 12 hours and then washed three times with distilled water and blotted with tissue paper to remove adsorbed water on the surface.

Study of solubility of beads The solubility of wet beads was examined by shaking them in acidic and alkali media e.g. hydrochloric acid (pH 2, 3, 5), distilled water (pH 7) and sodium hydroxide solution (pH 9, 11, 14) at 30 °C for 12 hours.

Immobilization of D-PhgAT on crosslinked chitosan Enzyme immobilization was carried out on crosslinked chitosan matrix which was dried at 40 °C for 12 hours and then grounded and sieved. To 100 mg of the dry grounded matrix, 1 mL of enzyme solution prepared in 20 mM Tris buffer pH 7.6 was added. The immobilization reaction was shaken at 25 rpm, 25 °C for 1 hour. After then the enzyme solution was removed from the reaction and the resulting enzyme-immobilized matrix was washed with water.

Tests of the reaction catalyzed by immobilized enzyme To study the catalytic performance of D-PhgAT immobilized on the crosslinked chitosan, transmination reaction was tested. The reaction involved the conversion of 5 mM D-4-hydroxyphenylglycine in the presence of 10 mM 2-oxoglutaric acid at pH 8.6 to HBZF and L-glutamic acid. After setting up the reactions by using about 100 mg of D-PhgAT-immobilized matrix per total reaction volume of 1 mL and incubating the reactions at 37 °C for 15 mins, the products of interest i.e. HBZF was assayed by high performance liquid chromatography (HPLC). The chromatographic system consisted of HPLC apparatus equipped with ODS column (250 mm x 0.4 mm, 5 μm) and UV detector (340 nm for HBZF), a mobile phase containing 50 mM potassium phosphate buffer (pH 6.8) and the flow rate set at 1.0 $mL.min^{-1}$. The experiments were performed five rounds to see the effect of repeated use.

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RESULTS AND DISCUSSION

Properties of ionic-crosslinked chitosan beads It was found that ionic crosslinked chitosan beads were generally round and white with the average diameter of about 2 mm (Figure 1, left). However, the use of TPP solutions with different pH could affect some appearance of the bead and their durability under different acid-base conditions. From the experiments, TPP solution with lower pH produced opaque beads while more clear beads were obtained from neutral or alkaline TPP. In term of the dissolution, beads prepared by using TPP solution at pH 3 resisted to the dissolution at all pH , but beads prepared by using TPP solution adjusted to pH 5, 7, 8, 9 and 11 dissolved at pH 2 (Table 1). These results suggested that the ionic crosslinking occurred at the higher extent under acidic condition because amino groups of chitosan were positively ionized and thus crosslinked well with the negative charge of phosphate ion. As the result, the beads prepared under acidic conditions were more durable than the beads prepared under neutral or alkaline conditions. To further improve the durability, the ionic crosslinked beads were subsequently subjected to covalent crosslink using genipin.

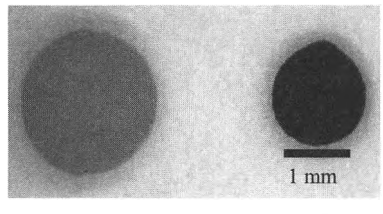

Figure 1 Wet chitosan beads prepared by ionic crosslinking with TPP solution (left) and chitosan beads prepared by ionic crosslinking followed by genipin-mediated covalent crosslinking (right).

Table 1 Solubility of the beads prepared via ionic crosslinking in the medium with different pH.

IONIC CROSSLINK							
pH of TPP	pH of medium						
	2	3	5	7	9	11	14
3	✓	✓	✓	✓	✓	✓	✓
5	×	✓	✓	✓	✓	✓	✓
7	×	✓	✓	✓	✓	✓	✓
8	×	✓	✓	✓	✓	✓	✓
9	×	✓	✓	✓	✓	✓	✓
11	×	✓	✓	✓	✓	✓	✓

Table 2 Solubility of the beads prepared via ionic crosslinking, followed by covalent crosslinking in the medium with different pH.

IONIC + GENIPIN							
[Genipin]	pH of medium						
	2	3	5	7	9	11	14
0	×	✓	✓	✓	✓	✓	✓
2.5	✓	✓	✓	✓	✓	✓	✓
5	✓	✓	✓	✓	✓	✓	✓
10	✓	✓	✓	✓	✓	✓	✓
25	✓	✓	✓	✓	✓	✓	✓
50	✓	✓	✓	✓	✓	✓	✓

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Properties of doubly (ionic and covalent) crosslinked chitosan beads Since beads prepared by using TPP solutions at pH 8 showed similar pH-solubility to those prepared at other pH wheras the preparation was easier and more economical since they required minimal pH adjustment of TPP solution, this type of beads was chosen for the subsequent covalent crosslinking. Upon the crosslink reaction with colorless genipin solution, the beads changed the color from white to dark purple. In addition, the size of beads became smaller with the average diameter of about 1.5 mm (Figure 1, right). Interestingly, the resulting beads increased their strength and durability as they did not dissolve at all pH (Table 2). However, the use of higher concentration of genipin than 25 mM yielded brittle beads.

Catalytic performance of enzyme immobilized on crosslinked chitosan Since fine particles hold higher surface area which is useful for enzyme immobilization and they are more practical for column packing for the future use, doubly crosslinked chitosan beads were finely ground prior to use as enzyme carrier. The evaluation of the catalytic performance of immobilized enzyme as determined by formation of HBZF product revealed that both TPP-crosslinked chitosan and doubly crosslinked chitosan could adsorb the enzyme with some extent. In general, the enzyme activity as evidenced by the amount of HBZF formed declined upon the repeated uses of the enzyme, probably due to the release of enzyme from the carrier. Despite the activity of enzyme immobilized on doubly crosslinked chitosan was lower that on TPP-crosslinked chitosan in the first round of reaction, both of them were not significantly different in the subsequent rounds. Therefore, it may imply that the covalent crosslink step which improved the pH-stability of chitosan matrix did not drastically reduce the capability of chitosan to adsorb the enzyme as well as the resulting catalytic performance of enzyme.

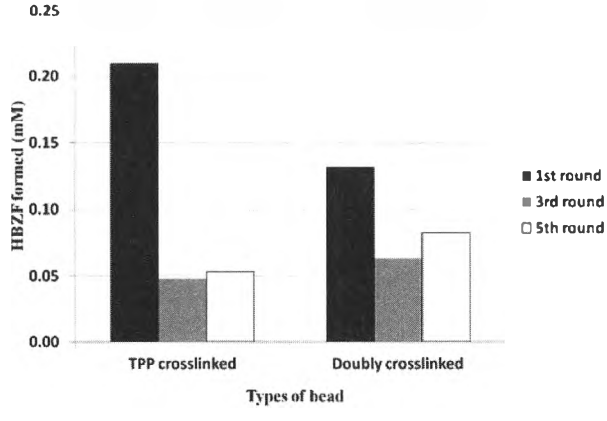

Figure 2 The formation of HBZF (mM) after round 1, 3 and 5 of the catalytic reaction using ground ionic-crosslinked chitosan versus doubly crosslinked chitosan.

CONCLUSION

In this study, chitosan beads were prepared with ease by the use of safe and environmentally friendly crosslinkers namely TPP and genipin. The procedures and conditions of the crosslinking method influenced on the properties of the resulting beads. In overall, double crosslinking process could increase the durability of the beads over the broader pH compared with those crosslinked only by TPP. Also, it did not drastically reduce the capability of chitosan for enzyme immobilization as well as the catalytic

performance of enzyme. However, since the enzyme activity was found to decrease upon repeated uses, some improvement strategies should be applied in the future study in order to anchor the enzyme more tightly onto the matrix for more effecient use.

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