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Immobilization of β-Glucosidase from *Exiguobacterium* sp. DAU5 on Chitosan Bead for Improved Enzymatic Properties

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Glutaraldehyde was used to cross-link chitosan beads to immobilize the crude enzyme β-glucosidase from *Exiguobacterium* sp. DAU5. The conditions for preparing cross-linking chitosan beads and immobilization such as concentration of glutaradehyde, cross-linking time, immobilization pH and time were optimized. The chitosan beads were cross-linked with 1.5% glutaraldehyde for 1.5 hr. The immobilized β-glucosidase had an overall yield of 20% and specific activity of 5.22 U/g. The optimized pH and temperature were 9.0 and 55°C, respectively. More than 80% of its activity at pH 7.0-10.0, 80% at 40°C for 2 hr and 48% at 50°C for 1 hr, were retained. However, the immobilization product showed higher pH and thermal stabilities than free enzymes. It also showed high hydrolyzing activity on soybean isoflavone glycoside linkage. These results suggest the broad application prospects of immobilization enzymes.

Key words : β-glucosidase, chitosan bead, cross-link, immobilization, isoflavone

Introduction

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) is a key enzyme for carbohydrate metabolism in many organisms and it was involved in rate control of cellulose hydrolysis, functioning primarily to hydrolyze cellobiose to two glucose [7,10,16]. On the basis of sequence homology, β-glucosidases have been divided into Glycoside Hydrolase Family 1 and Glycoside Hydrolase Family 3 [19]. In general, β-glucosidases from Family 1 catalyze the hydrolysis of β-glycosidic bonds between a monosaccharide and a moiety, which may or may not be a carbohydrate. Thus, those enzymes have broad substrate specificity and can be applied to hydrolysis glycoside products to improve bioavailability and pharmacokinetics (e.g.,isoflavones from soybeans) [11]. Numerous studies have shown that the aglycone isoflavones are highly bioactive due to their unimpeded intestinal absorption, unlike their related glycosides, which are not absorbed across enterocytes because of their higher hydrophilicity and molecular weight [3,17,21].

In the past few years, a number of studies have been reported on immobilization of various enzymes by different supports and methods. Immobilization of enzyme can offer many advantages, such as activity and stability of enzyme at a broad pH and temperature. Therefore immobilized enzyme has been widely used in the production of food, pharmaceuticals and other biologically important fine products [18].

Chitosan, a partly acetylated or nonacetylated counterpart (4-linked-2-amino-2 deoxy-β-D-glucopyranan) of chitin, is present in the mycelial and sporangiophore walls of fungi and the exoskeletons of insects and crustacean [6,13]. It is usually obtained by the artificial deacetylation of chitin in the presence of alkali. Conservative estimates of the amount of crustacean shells produced worldwide fall in the range of 1.5×10^5 metric ton per annum [2]. Chitosan is the non-toxic and not harmful immobilization support of biological macromolecules. It has many advantages such as cheap price, excellent hydrophilicity and high porosity, both of which lead to a low steric hindrance to the enzymes and thus a low mass transfer resistance [4,8]. Chitosan has hydroxyl (-OH) and amino ($-NH_2$) groups, which easily link with enzymes [20] and can be cross-linked with glutaraldehyde to prevent dissolution in acidic solutions (pH < 2) [15]. Chitosan is natural linear polyglucosamine chains with high molecular weight, which is one of few alkaline sugar found in nature [14]. It can be soluble in aqueous acidic media at pH < 6.5. Chitosan adheres to negatively charged surfaces because it possesses high positive charge on $-NH_3^+$.

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Unique biological properties of chitosan such as biocompatibility, nontoxicity, physiological inertness and remarkable affinity to proteins offer an extraordinary potential in a broad applications [9]. In the last decade, chitosan has been applied widely as enzyme immobilization supports materials.

In this work, we report immobilization of recombinant β-glucosidase from *Exiguobacterium* sp. DAU5 on chitosan bead for improved enzymatic properties. The immobilization product showed higher pH and thermal stabilities than free enzymes. It also showed high activity to hydrolyze soybean isoflavone glycoside linkage. This will be of practical importance for further applications.

Materials and Methods

Chemicals

p-Nitrophenyl-β-D-glucopyranoside (*p*-NPG), chitosan, genistein, daidzein, genistein-7-*O*-glucoside (genistin), daidzein-7-*O*-glucoside (daidzin) were purchased from Sigma-Aldrich Co. (St. Louis, MO). 25% Glutaraldehyde solution was purchased from Junsei chemical Co., (Tokyo, Japan).

Preparation of cross-linked 2.5% chitosan beads

2.5% (w/v) Chitosan solution was prepared by suspending 0.25 g of chitosan powder in 10 ml of 2% (v/v) acetic acid solution and stirred during 2 hr. The viscous solution was centrifuge for 15 min at 6,000 rpm to remove air bubbles, and then sprayed drop-wise through a syringe, at a constant rate, into 7.5% NaOH and 95% ethanol in a volume ratio of 4:1 to solidify bead form. The beads were placed in NaOH-ethanol solution for over night at 4°C. The prepared beads were washed with deionized water until the chitosan beads were neutral, and then stored in deionized water at 4°C. The optimal condition for preparation of chitosan beads was determined by different concentration of glutaraldehyde and cross-linking time. The reaction solution was shaken at 180 rpm and 37°C and then the cross-linked chitosan beads were washed thoroughly by 0.2 M sodium phosphate buffer (pH 6.5).

Enzyme immobilization

In this study, crude β-glucosidase, BglA-DAU5 was expressed in *E. coli* BL21 (*trxB*) and enzyme extract (26 U/ml) was prepared by the methods of previous report [5]. An amount of cross-linked chitosan beads were in contact with β-glucosidase in a shaker at 150 rpm and 20°C. For immobilization of enzyme, optimal pH and incubation time were determined.

Enzyme assay

The routine assessment of free β-glucosidase activity was performed using a reaction mixture containing 150 µl of 5 mM *p*-NPG in 200 mM sodium phosphate buffer (pH 7.0) and the free β-glucosidase (final volume of 200 µl) was incubated at 45°C for 5 min in a shaker, before adding 200 µl of 2 M Na_2CO_3 to stop the reaction. The absorbance of the product *p*-NP was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to produce 1 µmol of *p*-NP per min. *p*-NP was used as a standard. The protein concentration was determined via the method described by Lowry *et al.* [12], using bovine serum albumin as the standard.

Activities of the immobilized β-glucosidase were similarly determined by the above condition. Reaction mixture containing 200 µl of 5 mM *p*-NPG in 50 mM glycine-NaOH buffer, pH 9.0, and a given amount of immobilized enzyme was incubated at 55°C for 5 min and then 200 µl of 2 M Na_2CO_3 was added to stop the reaction.

Effect of pH and temperatures on enzyme activity

To determine the effect of various pH, 50 mM citric acid-citrate (pH 4.0-6.0), 200 mM sodium phosphate (pH 6.0-8.0), and 50 mM glycine-NaOH (pH 9.0-11.0) were used. The pH stability on activity of immobilized β-glucosidase was tested by incubating immobilized enzyme at different pH 9.0-11.0 for 2 hr on ice. The remaining activity was assayed at intervals of 30 min under the standard assay conditions.

The effect of temperature on activity of immobilized β-glucosidase was determined at various temperatures in the range of 35-65°C. Thermal stability was tested by incubating immobilized enzyme at different temperatures, 40 or 50°C for 2 hr without substrate. The remaining activity was measured at intervals of 30 min under the standard assay conditions.

Analysis of isoflavone hydrolysis

The hydrolysis products for soybean flavonoids, daidzin and genistin were analyzed by silica gel thin layer chromatography (TLC). Aliquots (1 µl) of the reaction mixtures were analyzed on a silica gel plate (Dieselgel 60: Merck Co., Berlin,

Germany) with ethyl acetate-methanol-water (8:1:1, v/v/v), and the products were detected at 254 nm short-wave ultraviolet.

Results and Discussion

Effect of concentration of glutaraldehyde and cross-linking time on enzyme activity

The effects of glutaraldehyde concentration for enzyme immobilization were shown in Fig. 1. To study effect of glutaraldehyde concentration on β-glucosidase immobilization, enzyme was immobilized on chitosan beads cross-linked with different concentrations of glutaraldehyde ranging from 1% to 3%. The immobilized enzyme has maximum activity at 1.5% glutaraldehyde under the concentration range investigated. But cross-linking of chitosan beads with lower concentration of glutaraldehyde have poor mechanical strength and that with high concentration may denatured of enzyme. The effect of cross-linking time on enzyme activity was determined. The activity of immobilized enzyme for cross-linking was high at 1.5 hr, and was selected as an optimal condition (Fig. 2).

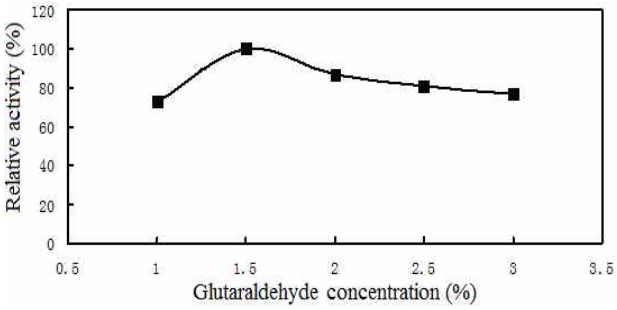

Fig. 1. Effect of glutaraldehyde concentration on enzyme immobilization. Chitosan beads were prepared by shaking at 180 rpm and 37°C for 2 hr.

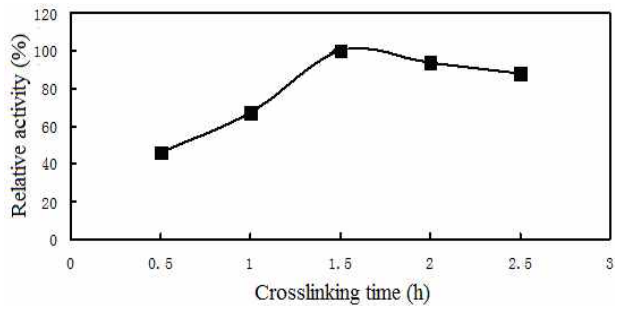

Fig. 2. Effect of cross-linking time of glutaraldehyde for preparation of chitosan beads. Chitosan beads were prepared by shaking at 180 rpm and 37°C with solution containing 1.5% glutaraldehyde.

Effect of immobilization pH and time on enzyme activity

The chitosan beads were cross-linked with 1.5% glutaraldehyde and thoroughly washed by 0.2 M sodium phosphate (pH 6.5) for immobilization of crude β-glucosidase. Aliquots of chitosan beads were incubated with crude enzyme at various pH and different immobilization time.

Fig. 3 shows the optimum pH of immobilization solution during immobilization process. For immobilization of crude β-glucosidase, various buffers at ranging from pH 5.0 to pH 7.0 were used. The immobilized enzyme has highest activity when crosse-linked beads were immobilized with enzyme in 0.2 M sodium phosphate (pH 6.5). The effect of immobilization time on enzyme activity was shown in Fig. 4. In the period of 4-24 hr, the activity of immobilized enzyme reached a maximum at 12 hr. The summary of activity of immobilized β-glucosidase under the optimal condition was given in Table 1. The immobilized enzyme has an overall yield of 20% and specific activity of 5.22 U/g.

Effect of pH and temperatures on enzyme activity

To study effect of pH on activity of immobilized enzyme,

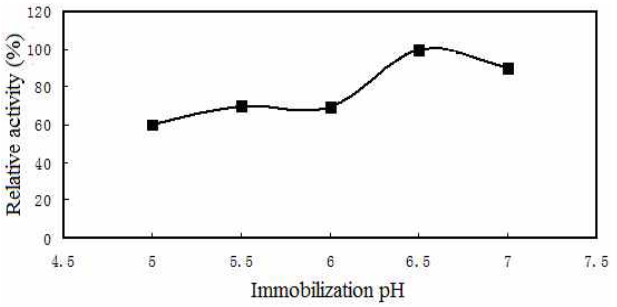

Fig. 3. Effect of pH on enzyme immobilization. Cross-linked chitosan beads with crude β-glucosidase were shaken at 150 rpm and 20°C for 24 hr.

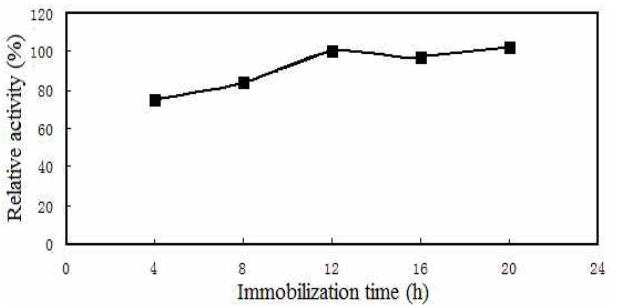

Fig. 4. Effect of immobilization incubation time for immobilizing enzyme on chitosan beads. Cross-linked chitosan beads were incubated with crude β-glucosidase by shaking at 150 rpm and 20°C in 0.2 M sodium phosphate buffer (pH 6.5).

Table 1. Yield of immobilized β-glucosidase

	Total activity	Specific activity	Yield (%)
Crude enzyme solution	5.2 U	26 U/ml	100
Chitosan composite	1.04 U	5.22 U/g	20

the activity of immobilized enzyme was measured using various pH buffers under the standard assay conditions. The activity of free enzyme was also determined under the standard assay condition. Both the free and immobilized β-glucosidase showed the highest activity at pH 7.0 and pH 9.0, respectively. Free β-glucosidase activity was approximately 8% and 54% of the maximum activity at pH 5.0 and pH 10, respectively. The immobilized β-glucosidase has higher activity than free enzyme at a broad range of pH (Fig. 5). The immobilized enzyme retained more than 80% of maximal activity at pH 7.0-10.0.

The immobilized β-glucosidase was stable in the pH range from 9.0 to 11.0. The immobilized enzyme maintained up to 86%, 70% and 45% of maximum activity at pH 9, 10 and 11, respectively (Fig. 6).

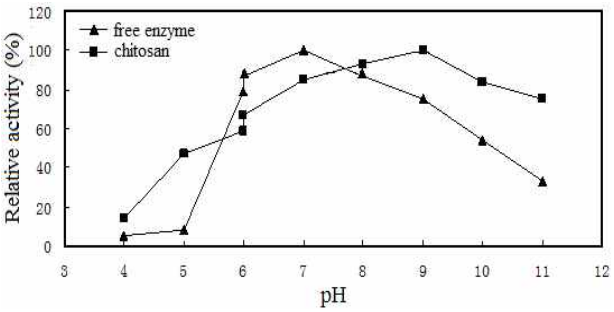

Fig. 5. Effect of pH on the activity of free and immobilized enzyme. Various buffers such as 50 mM citric acid-citrate (pH 4.0-6.0), 0.2 M sodium phosphate (pH 6.0-8.0), and 50 mM glycine-NaOH (pH 9.0-11.0) were used.

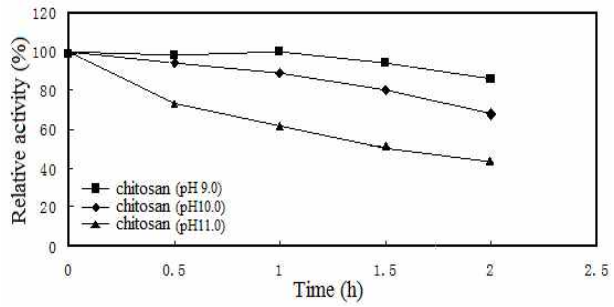

Fig. 6. Effect of pH on the stability of immobilized enzyme. The pH stability was tested by incubating with aliquots of enzyme at different pH 9.0-11.0 for 2 hr on ice and the remaining activity was assayed.

The optimal temperature of the free and immobilized β-glucosidase was determined at different temperatures ranging from 35°C to 65°C under standard assay conditions. The free enzyme and immobilized enzyme showed the maximum activity at 45°C and 55°C, respectively. The optimal temperature was shifted 45°C to 55°C by immobilizing enzyme. The activity of free enzyme exhibited 87% and 61% of its maximal activity at 40°C and 50°C, respectively whereas that of the immobilized β-glucosidase showed more than 80% of maximal activity at 45-65°C (Fig. 7). Thermal stability was determined by incubating at 40°C and 50°C for 2 hr. The variation of the residual activity of free and immobilized β-glucosidase was shown in Fig. 8. The immobilized enzyme has 48% of initial activity at 50°C for 1 hr while free enzyme has less

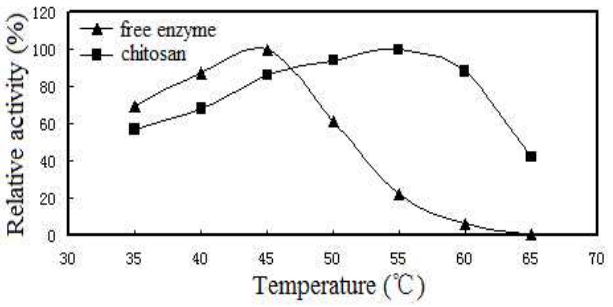

Fig. 7. Effect of temperature on the activities of free and immobilized enzyme.

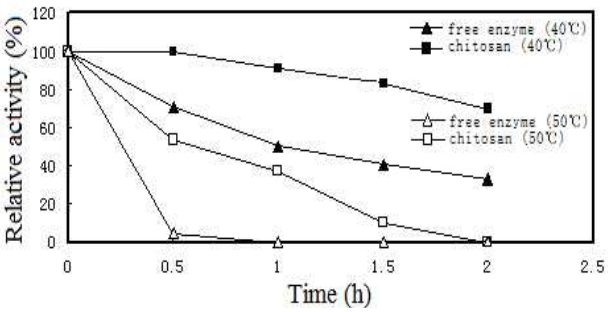

Fig. 8. Effect of temperature on the stability of free and immobilized enzyme. Thermal stability of free enzyme and immobilized enzyme were tested by incubating aliquots of enzyme at 40 °C and 50 °C for various times and the remaining activity was assayed under the standard conditions.

than 5% of initial activity at 50°C for 0.5 hr. At 40 and 45°C, the immobilized β-glucosidase maintained 80% and 48% of its original activity after 2 hr, respectively, however the free β-glucosidase retained 33% of initial activity after incubation of 2 hr at 40°C. In general, the immobilization support has a protecting effect at high temperatures when enzyme deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. The immobilization step causes an increase in enzyme rigidity, commonly reflected by an increase in stability towards denaturation by raising the temperature [1].

Analysis of hydrolysis isoflavone glycoside from soybean

Hydrolysis products of the immobilized enzyme were examined using isoflavone glycosides such as daidzin and genistin from soybean as substrates. As shown in Fig. 9, daidzin and genistin were incubated with immobilized enzyme, and the resultant products were analyzed on the TLC plate. Daidzin and genistin were converted to daidzein (Fig. 9A) and genistein (Fig. 9B), respectively. The free enzyme also hydrolyzed daidzin and genistin (data not shown). These results indicated that isoflavone aglycones such as daidzein and genistein which are hydrolyzed of isoflavone glycosides by immobilized β-glucosidase.

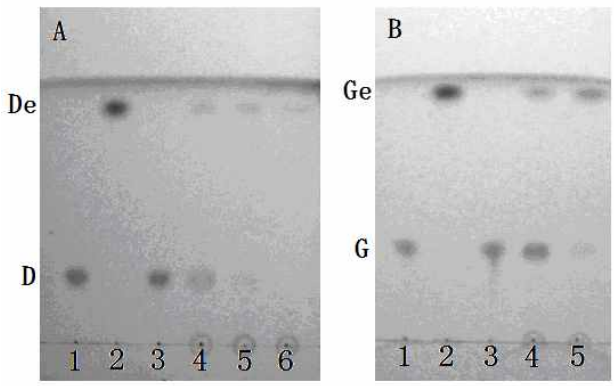

Fig. 9. Analysis of hydrolysis products of daidzin (A) and genistin (B) by immobilized enzyme. (A): Lane1: standard sample of daidzin (D); Lane 2: standard sample of daidzein (De); Lane 3, Lane 4, Lane 5 and Lane 6: products by incubation of daidzin with immobilized β-glucosidase for 0, 15, 30 and 45 min, respectively; (B): Lane1: standard sample of genistin (G); Lane 2: standard sample of genistein (Ge); Lane 3, Lane 4 and Lane 5: products by incubation of genistin with immobilized β-glucosidase for 0, 15 and 30 min, respectively.

Conclusion

In this work, the chitosan beads were cross-linked with glutaraldehyde as the supports for immobilization of crude β-glucosidase from *Exiguobacterium* sp. DAU5. Based on optimal condition for β-glucosidase immobilization, immobilized enzyme has an overall yield of 20% and specific activity of 5.22 U/g. The optimal pH and temperature were 9.0 and 55°C, respectively. The immobilized β-glucosidase showed higher pH and thermal stabilities than free β -glucosidase. The immobilized β-glucosidases could hydrolyze isoflavone glucosides such as daidzin and genistin into isoflavone aglycones such as daizein and genistein.

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초록 : 효소 특성 개선을 위한 *Exiguobacterium* sp. β-glucosidase의 키토산 비드에 효소 고정화

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Exiguobacterium sp. 유래의 β-glucosidase 고정을 위하여 글루타르알데하이드를 사용한 키토산 비드를 조제하였다. 키토산 비드의 교차결합 및 고정화의 조건을 최적화하였다. β-glucosidase 고정화의 최적생산 조건에서 20%의 수율과 5.22 U/g의 효소활성을 나타냈다. 최적 pH 와 온도는 9.0과 55°C를 나타냈다. 고정된 효소의 안정성은 pH 7.0-10.0에서는 80%, 40°C 2시간 반응에서는 80% 및 50°C 1시간 반응에서는 48%의 활성을 보유하였다. 이러한 결과는 높은 pH와 고온에서 비고정 효소보다 안정성을 보여주었다. 고정된 효소를 가지고 대두 이소플라본 배당체의 높은 가수분해능을 확인하였다. 이상의 결과는 고정화 효소의 다양한 이용 가능성을 시사하였다.