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Immobilization of β-Galactosidase from *E. coli* K-12 CHS36 Using Tannin-Activated Cellulose Beads

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β-Galactosidase의 Tannin활성화 섬유소 입자에의 고정화

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초 록

Tannin 및 *p*-benzoquinone으로 활성화시킨 다공성 섬유소 입자를 사용하여 대장균 변이주(*E. coli* K-12 CSH36)로 부터 추출한 β-galactosidase를 고정화시켜 효소학적 성질과 그 응용성에 대하여 연구하였다. β-galactosidase는 pH 11.0으로 맞춘 tannin과 *p*-benzoquinone을 사용하여 6시간 동안 활성화시킨 섬유소 입자담체에 고정화 시켰을 때 그 잔여 활성도가 가장 높았다. β-galactosidase의 최적작용 pH는 5.5였으나 고정화 시켰을 때 pH 6.0으로 변화하였으며 최적작용 온도는 고정화 전이나 후에도 일정하였다. β-galactosidase의 Km 값은 4.0×10^{-4}M으로 나타났으며 고정화 효소의 경우에는 7.5×10^{-4}M이었다. 고정화 효소의 재사용성을 검토한 결과, 담체 활성화 물질로 tannin과 *p*-benzoquinone 용액을 사용하였을 때 20회 사용 후에 초기 효소 활성도의 80% 이상이 유지되는 결과를 나타내었다.

Introduction

Up to now, β-galactosidase was investigated intensively for biochemical and industrial application. β-Galactosidase catalyzes the splitting of glycosidic linkage of lactose, as natural substrate, to the product glucose and galactose. Therefore, using β-galactosidase, it is possible to obtain glucose and galactose from whey which contains high content of lactose. The application could solve the problems of lactose intolerance which is common in Asian and African populations.

Until now, various types of artificial and natural matrices for enzyme immobilization have been explored. Giacin et al.[1] and Jakubowski[2] investigated collagen bound β-galactosidase, Okos et al.[3] reported on β-galactosidase immobilized on porous glass, as did Wondolowski[4]. While, Chales et al.[5] studied various enzymes immobilized on alumina and stainless steel supports, Hustad et al.[6] reported on immobilization of

β-galactosidase on an insoluble carrier with a polyisocyanate polymer, Hannibal-Friedrich et al.[7] immobilized β-galactosidase, albumin and γ-globulin on epoxy-activated acrylic beads. And Beddows et al.[8] investigated the immobilization of β-galactosidase and other enzymes onto p-amino-carbanilated cellulose derivatives, Ono et al.[9] studied the preparation and properties of immobilized naringinase using tannin-aminohexyl cellulose.

In this experiment, β-galactosidase extracted from *E. coli* K-12 CSH36, which is a constitutive mutant for β-galactisidase production, was immobilized on porous cellulose beads which were previously activated by tannin and *p*-benzoquinone. Tannin, or synonyme tannic acid, is the general term for substances which produce multivalent phenols by hydrolysis, It exsists in nature widely. It has astringent taste, and is easy soluble in water. Albumin, starch, gelatin and most alkalodidal metallic salts become insoluble by tannin treatment[10]. Owing to these properties, tannin was attempted to be one of the coupling reagents in this work.

p-Benzoquinone, the second activation reagent in this experiment, is very reactive with hydroxyl polymer. Goldstein and Manecke suggested the coupling reaction mechanism of *p*-benzoquinone as arylation between an amino group of enzyme and p-benzoquinone bound to a matrix[11]. In conventional method, BrCN has been generally used as activation reagent for a cellulose matrix, but since BrCN is a strong toxic compound, the procedure of enzyme immobilization using BrCN has caused problems during operations or food processing. *p*-Benzoquinone does not provide these problems. Moreover, during immobilization, it becomes a non deleterious reaction product, namely with 2, 5-substituted hydroquinone structure[12].

Materials and Methods

Materials

Difco nutrient broth was used for cultivation of *E. coli.* ONPG(o-nitrophenyl β-D-galactopyranoside) and bovine serum albumin were purchased from Sigma Co. (U.S.A.). Tannic acid was obtained from Hayashi Pure Chemical Co. Ltd. (Japan), cellulose acetate was obtained from Shinyo Pure Chemicals Co. Ltd. (Japan). Other reagents used in this experiment were of the highest grade.

Culture

Escherichia coli K-12 CSH36, which is constitutive mutant for β-galactosidase production, was used as enzyme source. This strain was obtained from Cold Spring Harbor Laboratory (New York, U.S.A.). Cells were grown in liquid culture containing 0.8% nutrient broth and 0.5% NaCl in distilled water in a batch system. Growth was continued for 18 hours at 37°C.

Enzyme assay and protein determination

ONPG was used as synthetic substrate for estimation of the enzyme activity. The absorbancy was measured at 405nm against a reagent blank. One unit of enzyme activity was defined as one micromole of OPN(o-nitrophenol) produced per minute at 37°C. Protein was determined by the method of Lowry et al. with bovine serum albumin as standard[13].

Preparation of galactosidase

Cells were harvested by centrifugation. The precipitated cells were collected by adding a small volume of phosphate buffer and distrupted with ultrasonic disintegrater. The disrupted cell suspension was centrifuged at 10,000×g for 20 minutes. The clear supernatant solution containing β-galactosidase was fractionated by adjusting to 60% saturation with solid ammonium sulfate followed by centrifugation at 13,000×g for 15minutes. The pellet was dissolved in 15m*l* of phosphate buffer and dialyzed in a large volume of the same buffer. The dialyzed extract was centrifuged again, to remove insoluble materials. The supernatant was used as a crude β-galactosidase preparation and stored at 4°C until used. This preparation was adjusted to an activity of 8 units/m*l*.

Immobilization of galactosidase

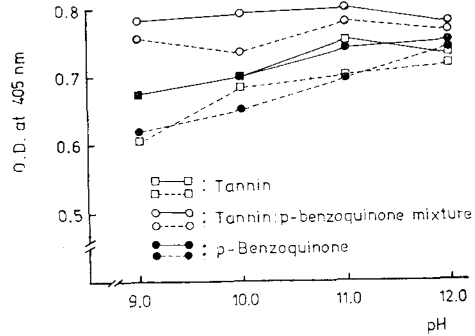
Porous cellulose beads were prepared by modification of the method of Chen et al. method[14]. 12.5g of cellulose acetate was dissolved in 100ml of dimethylsulfoxide-acetone mixture(4 : 6, v/v). The cellulose suspension was dropped in 30% acetone solution with vigorous stirring to increase the surface area. Manufactured cellulose beads were washed with distilled water, and dried at room temperature.

3% (w/v) tannic acid solution and 0.1M p-benzoquinone were used as activation reagents. To investigate the effect of pH on activation reagents these reagents were adjusted to pH 9.0, 10.0, 11.0, 12.0 with 1M NaOH solution, respectively. These activation reagents were filtered to remove insoluble materials in them. Beads were activated with tannin, with p-bezoquinone, with tannin and p-benzoquinone together respectively to determine the most effective method. 8ml of activation reagent was reacted with 20mg of cellulose beads for 6 hours at room temperature with vigorous shaking. After activation, beads were washed with 20ml of 0.1M Na_2CO_3, 100ml of distilled water and 50ml of 0.2M phosphate buffer to remove remaining salts in the activated beads.

0.2ml of enzyme solution was added to 1.3ml of potassium phosphate buffer, pH 7.6, and filled in a 5ml test tube containing cellulose beads activated by the methods mentioned above. The mixture was shaken in a rotary shaker for 16 hours at 4°C. After immobilization, the beads were washed in the same way as the activated cellulose beads. The rate of enzyme immobilization was calculated by measurement of the residual enzyme activity.

Results and Discussion

The manufactured cellulose beads had a mean diameter of 230μm were nearly spherical and their water content was about 91.5%. Figure 1 shows the effect of activation reagents and pH on β-galactosidase immobilization. The most effective enzyme immobilization was obtained when tannin and p-benzoquinone were used together as activation reagents which were adjusted to pH 11.0, and 6 hours of activation.



Fg. 1. The effect of activation reagentis and pH on β-galεctosidase immobilization. Activation time was 6 hours and immobilization was continued for 16 hours.
—— : activity of 1st run
...... : activity of 5th run.

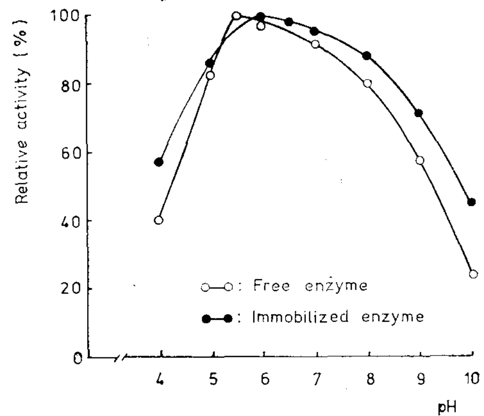


Fig. 2. The effect of temperature on free enzyme and immobilized enzyme.
○—○ : Free enzyme
●—● : Immobilized enzyme

The activity of the immobilized enzyme and free β-galactosidase as function of temperature is shown in figure 2. Optimum temperature for the free and immobilized enzyme were both 50°C. Figure 3 shows the pH dependence of the relativerate of hydrolysis of ONPG with immobilized and free enzyme. The optimum pH was increased from 5.5 to 6.0 after immobilization. In Figure 4, the ONPG hydrolysis by native and

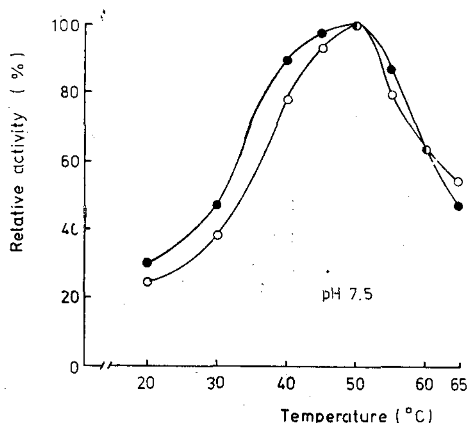


Fig. 3. The effect of pH on β-galactosidase immobilization. Enzyme assay condition: 5mM ONPG, at 37°C

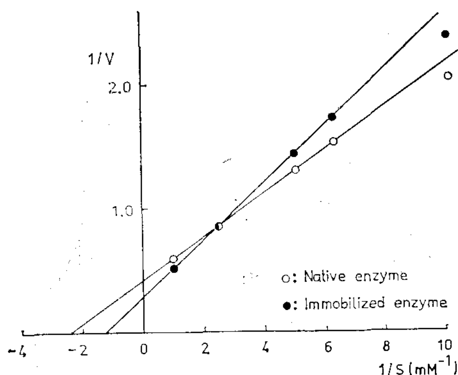


Fig. 4. Lineweaver-Burk plots for hydrolysis of ONPG by soluble and immobilized β-galactosidase.

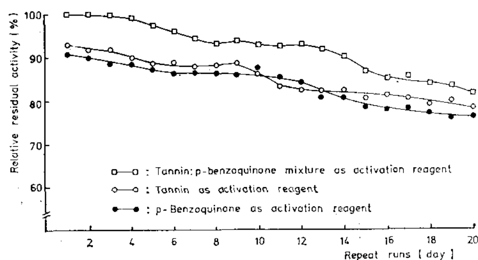


Fig. 5. Reuse profile of immobilized β-galactosidase bound to porous cellulose beads. All activation reagents were adjusted to pH 11.0. Relative residual activity was stannin : *p*-benzoquinone activated immibilized enzyme.

immobilized enzyme is shown for Michaelis-Menten constants. Kms of native β-galactosidase for ONPG were 4.0×10^{-4}M and 7.5×10^{-4}M respectively.

In practical use, operational stability and reusability of immobilized enzyme are important for the application of enzyme immobilization. As shown in Figure 5, the residual enzyme activity of tannin : *p*-benzoquinone activated cellulose beads was significantly dominant among the three types of preparations for a long time. They retained over 80% of the initial enzyme activity after 20 runs. There were no remarkable differences between tannin activated and *p*-benzoquinone activated preparations. Since tannin forms insoluble complexes with proteins, we believe that this property could be used for enzyme immobilization. In fact, β-galactosidase was immobilized very effectively to cellulose beads when tannin was used as activation reagent. The chemical mechanisms of the interactions between tannin, enzyme, and cellulose matrix could not be identified since tannin is not a defined substance. The result was that the tannin : *p*-benzoquinone activated cellulose beads show the highest immobilized enzyme activity among that of other cellulose beads. It is considered that the chemical interaction between tannin and *p*-benzoquinone displays more effective capacity for a activation reagent to cellulose beads.

We also investigated whole cell immobilization using tannin and *p*-benzoquinone as activation reagents to cellulose beads. 50mg of lyophilized *E. coli* K-12 CSH36 cells were used and the other processes were as same as in enzyme immobilization, the activity and reusability of immobilized whole cell were considerably lower than that of immobilized β-galactosidase, but it revealed the possibility of whole cell immobilization with tannin and *p*-benzoquinone.

As in β-galactosidase immobilization, the most effective result for whol ecell immobilization was obtained when tannin and *p*-benzoquinone were used together as activation reagents which were

adjusted to pH 11.0.

Abstract

β-Galactosidase(β-D-galactoside galactohydrolase, E.C. 3.2.1.23) from *E. coli* K-12 CSH 36 was immobilized on porous cellulose beads which were previously activated with tannin and *p*-benzoquinone. Their general properties and applicational possibities were investigated.

The most effective, enzyme immobilization was obtained when tannin and *p*-benzoquinone, pH 11.0, were used together as activation reagents and a period of 6 hours of activation. The optimum pH of β-galactosidase was 5.5 for free enzyme and 6.0 for the immobilized enzyme, the optimum temperatures for native and immobilized enzyme were both 50°C. Kms of native β-galactosidase and immobilized enzyme for ONPG(o-nitrophenyl galactopyranoside) were about 4.0×10^{-4}M and 7.5×10^{-4}M, respectively. In the case of tannin : p-benzoquinone activated cellulose beads, the immobilized enzyme retained over 80% of the initial enzyme activity after 20 runs, which is very promissing result for a possible industrial application.

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