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Covalent Coupling of β-Fructofuranosidase on Microbial Cells

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미생물 세포에 공유결합으로 고정화시킨 β-Fructofuranosidase에 관한 연구

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Abstract

β-Fructofuranosidase was immobilized covalently on the oxidized microbial wall of a *Penicillium spp.* "PS-8", which is totally different from the conventional whole cell immobilization in concept. The immobilization of β-fructofuranosidase by a series of treatments; oxidation of microbial cells with sodium metaperiodate, enzyme loading on the oxidized cells, extrusion, and crosslinking induced by glutaradehyde, were carried out. The final product had a good mechanical strength and showed 26% of the applied enzyme activity. The specific activity was 750 units per *g* of the dry cell product. The immobilized enzyme showed the kinetic parameters as follows; optimum pH at 5, optimum temperature at 55°C, activation energy of 19 $kJ\ mol^{-1}$, and apparent Km of 55 *mM*.

Introduction

Various immobilization techniques have been developed to resue expensive enzymes for various industrial purposes. Among them, whole cell immobilization, by which intracellular enzymes are immobilized, is advantageous than other techniques, because it is not necessary to isolate enzyme and relatively easy to immobilize. However, final products often show relatively low yields of enzyme activity, or show diffusional problem or problems of mechanical strength as final product.

Therefore, we planned to adapt different techniques. In concept, we utilize whole cells as a exogeneous matrix instead of other organic or inorganic polymers. Using this materials, we aimed to develop the techniques to activate the cell wall chemically and reacted covalently with exogeneous enzymes which are not included in microbial cell itself.

In fungi, cell walls are composed of polysaccharides to a great extent. In various polysaccharides, cellulose is the most ubiquitous substance of all the cell wall carbohydrates.[1-3] By the characteristics of chemical components in fungi the cell wall can be activated with an oxidant to produce reactive aldehyde groups. Periodate is a selective oxidant capable of cleavage of the anhydroglucose units between carbon atom 2 and 3. Aldehydes in microbial cell walls by oxidation with periodate react rapidly with amino groups of the enzyme under mild condition to form Schiff's base. It was found that *Penicillium spp.*, which has been screened by our laboratory, yielded higher biomass and fine microbial cell granules which resulted in good flow rates and mechanical strength. Therefore, we immobilized ß-fructofuranosidase on this microbial cells by covalent bonds and crosslinking by glutaraldehyde.

In this study, the purpose was focused on two points of view such as to screen the optimum condition

possessing high enzyme activity and to examine the reaction properties and knietic behaviors of immobilized enzyme.

Materials and Methods

Materials

Yeast extracts, peptone, and glucose were purchased from Difco Lab. (Detroit, Michigan, U.S.A). ß-Fructofuranosidase (44 units/mg protein) was obtained from Sigma Chemical Co. (St. Louis, U.S.A). Glutaraldehyde (50% in water) from Kanto Chemical Co. (Tokyo, Japan), sodium metaperiodate from Wako Pure Chemical Co. (Osaka, Japan), and all other chemicals used were of analytical grade.

Cultivation of Microorganism

The strain used in this experiment was *Penicillium spp.* which has been screened and used by the previous workers in our laboratory.[4] This strain named as *Penicillium spp.* "PS-8" was cultured in the same conditions described previously.[4]

Enzyme Assay

ß-Fructofuranosidase activity was measured by determining the reducing sugar released from sucrose by 3,5-dinitrosalicylic acid method.[5]

To measure the activity of the immobilized anzyme, a mixture of 3 *ml* of 0.1 *M* sucrose in 0.1 *M* sodium acetate buffer, pH 5 and 0.1 *g* of the immobilized enzyme was incubated at 30°C for 3 min. After incubation was completed, 0.3 *ml* of 1% sodium hydroxide solution was added and shaked to inactivate the activity of enzyme. The released reducing sugar of 1 *ml* aliquot of the supernatant was assayed by 3,5-dinitrosalicylic acid reagent.

One unit of ß-fructofuranosidase is defined as one micromole of sucrose hydrolyzed per min at 30°C.

Immobilization

Treatment of sodium metaperiodate: One gram of dry cell was slurried in 6 *ml* of water. With stirring an aqueous solution containing 1.2 *g* of sodium metaperiodate in 16 *ml* of water was added dropwise to the cell slurry over a period of one hr and the reaction was continued with stirring for 18 hr at room temperature. The oxidized cells were filtered on a Büchner funnel and washed at least 10 times by agitation in 1,000 *ml* of water with filtration until essentially free of iodate. Finally, cell debris was washed with 100 ml of acetone and dried. Carbonyl groups produced were determined by the method of Mehltretter *et al.*[6]

Enzyme loading and extrusion: Periodate treated cells were then milled by 60 mesh filter equipped milling machine. An enzyme solution (1,152 units/*ml*) was applied to 1 *g* of the dried cell debris and the mixture was well homogenized. The enzyme mixture was incubated at 30°C for 4 hr and then 2 *ml* of 5% starch solution as a thickening agent was added and homogenized. This slurry was extruded through a syringe with 1.0 *mm* of the needle diameter onto the glass plate. The preparation was dried for 24 hr at room temperature and the resultant extruded fiber was cut into pellets (1 *mm* x 2 *mm*).

Treatment of glutarladehyde: For the immobilization of enzyme physically adsorbed on the surface of dried pellets, cell pellets were treated with 0.5% (w/v) of a glutaraldehyde solution in 0.1 *M* sodium acetate buffer, pH 5 for 1 hr. Immobilized cell pellets were washed with water several times and dried at room temperature for 24 hr. Fig. 1 shows the general flow sheet of immobilization of ß-fructofuranosidase. The final products were stored at 4°C. A photo of the final products prepared by a series of treatment is shown in Fig. 2.

Penicillium spp. "PS-8" culture
↓
Heating at 100℃
↓
Harvest and washing
↓
Treatment with acetone
↓
Treatment with periodate
↓
Washing and drying
↓
Enzyme loading
↓
Spinning by syringe (diameter 1 *mm*)
↓
Drying
↓
Treatment with glutaraldehyde
↓
Final product

Fig. 1. General scheme of immobilization of β fructofuranosidase

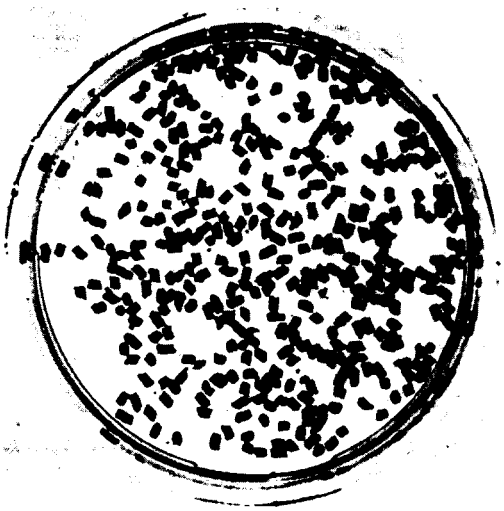

Fig. 2. **Final product of β-fructofuranosidase immobilized on microbial cells**
Two *ml* of 5% starch solution was added to 1*g* of the enzyme-cell mixture. This slurry was extruded through the syringe (diameter; 1*mm*). The preparation was dried for 24hr at room temperature and the resultant extruded fiber was cut into small pellets whose size was 1×2 *mm*.

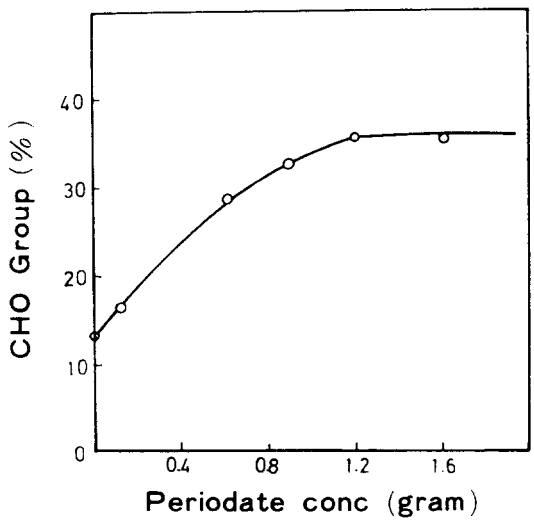

Fig. 3. **Effect of periodate concentration on the aldehyde group formation of microbial cells.**
One gram of dry cell was slurried in 6 *ml* of water. With stirring each aqueous solution containing various concentrations of sodium metaperiodate in 16 *ml* of water was added to the each cell slurry and the reaction was continued with stirring for 18 hr at 25℃.

Kinetic Studies

Optimum pH was determined under the standard assay condition by use of 0.1 *M* sodium acetate buffer having different pH values. Temperature effect on enzyme activity was determined at the various temperatures from 35° to 75°C. For the temperature stability, immobilized preparation was suspended in water and heated for 15 min at various temperatures. The preparation was cooled rapidly and equilibrated at 30°C. Three *ml* of 0.1 *M* sucrose in 0.1 *M* sodium acetate buffer, pH 5 was added and stirred at 30°C for 3 min. The reducing sugar formed was determined. To determine the apparent Michaelis constant of the immobilized enzyme, 0.2 to 160 *mM* sucrose in 0.1 *M* sodium acetate buffer, pH 5.0 were used as substrate.

In the batch reactor system, stability of the immobilized enzyme during inversion of sucrose was studied. A mixture of 0.1 *M* sodium acetate buffer was stirred for 120 min at 30°C. After stirring, the mixture was filtered and the pellets were suspended in the substrate solution for the next inversion.

Results and Discussion

Effects of Periodate Treatment

There are many literatures on the periodate oxidation of carbohydrates including various polysaccharides, starch, and cellulose. Such factors as the effects of pH, temperature, time, solvent, and concentration of oxidant during periodate oxidations have been studied.[6-9] In this experiment, periodate concentration and temperature effects on aldehyde formation of microbial cells were studied. From Fig. 3, 1.2 *g* of periodate per *g* of dry cell was the optimum treating concentration. It also showed that cell debris contained 35% glucosyl units which is convertable into dialdehyde units. When investigated on the aldehyde formation of microbial cells against temperature, temperature did not affect the aldehyde formation at the oversaturated concentration of periodate.

Effects of Glutaraldehyde Treatment

After the immobilization of enzyme on the periodate-treated microbial cells, glutaraldehyde was treated for the higher retention of enzyme through crosslinking. Fig. 4 and 5 show that the concentration and treating time of glutaraldehyde are important factors in the enzyme immobilization.

As shown in Fig. 4, 0.5% (w/v) glutaraldehyde concentration was adapted as an optimal condition to immobilize ß-fructofuranosidase. The highest activity was

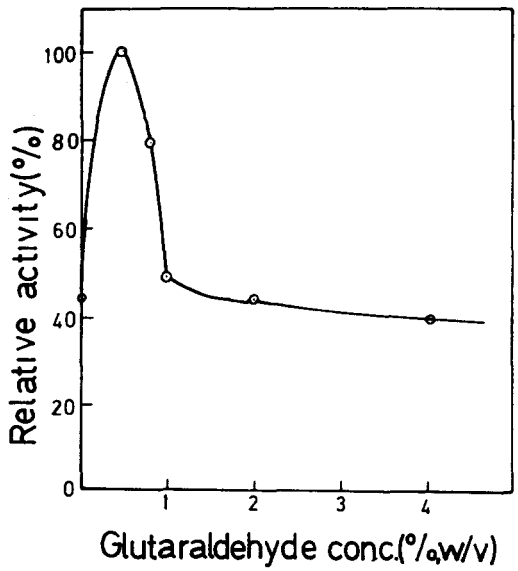

Fig. 4. Effect of glutaraldehyde concentration of the immobilization of β-fructofuranosidase on periodate treated microbial cells
One gram of periodate treated cell pellets was treated with various concentrations of glutaraldehyde solution in 0.1 *M* sodium acetate buffer, pH 5, for 1 hr.

observed when immobilized for 30 min at 0.5% (w/v) concentration of glutaraldehyde. From these results, it was assumed that treatment with this concentration for 30 min was the optimal condition both to immobilize the enzyme adsorbed around the cell surface and to minimize plugging of pores and loss of activity of enzyme through crosslinking.

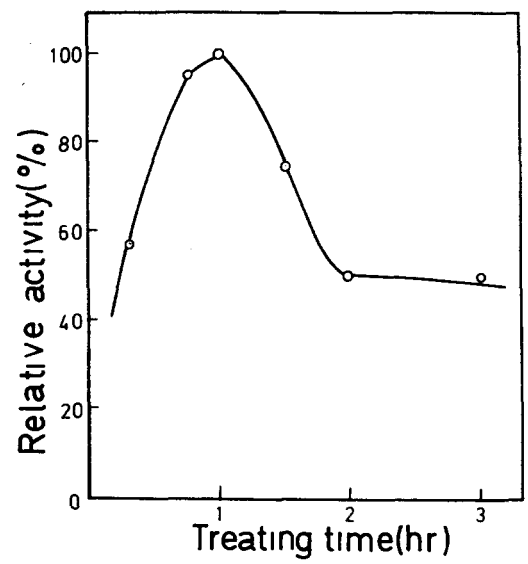

Fig. 5. Effect of treating time of glutaraldehyde on the immobilization of β-fructofuranosidase on periodate treated microbial cells

Table 1. Recovery of the enzyme activity during immobilization steps

Treatment	Enzyme activity (units/*g* cell debris)	Percentage of enzyme activity retained
Cell only	90	3
Periodate treated cell	350	12
Glutaraldehyde crosslinking after immobilization on periodate treated cell	750	26

The overall recoveries of enzyme activity during immobilization steps are summarized in Table 1. The retention yield of ß-fructofuranosidase increased from 3 to 12% when cell debris was treated with periodate and from 12 to 26% by glutaraldehyde crosslinking after the immobilization on periodate treated cell.

Kinetic Behaviors of the Immobilized Enzyme

Using the enzyme immobilized pellets, several kinetic behaviors were studied. Effects of pH on the reaction rate are shown in Fig. 6. Optimum pH was 4.8–5.0 both in the soluble and immobilized enzymes. But the immobilized enzyme showed broader otpimum pH than that of the soluble enzyme.

The temperature activity profile (Fig. 7) showed that the optimum temperature for sucrose inversion by the immobilized enzyme was 55°C, which was shifted to lower temperature than that of the soluble enzyme. Ef-

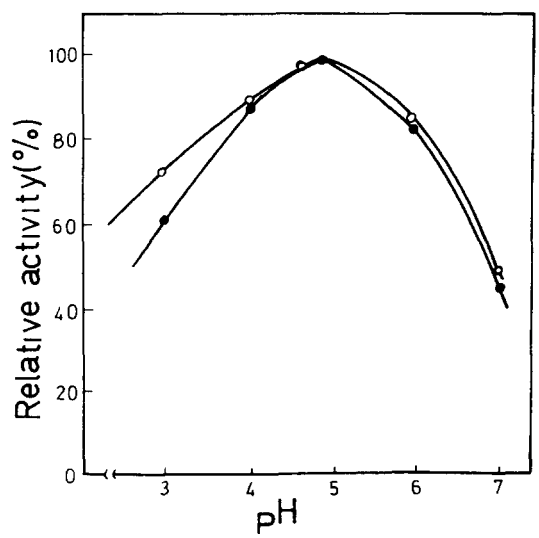

Fig. 6. pH-activity profiles for the soluble(●) and immobilized(○) β-fructofuranosidase

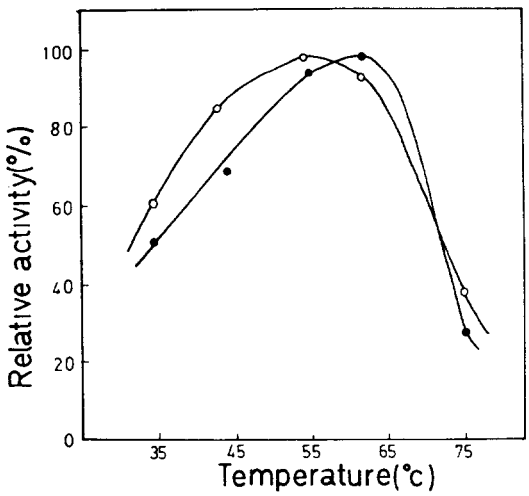

Fig. 7. Effect of temperature on the activities of the soluble(●) and immobilized (○) β-fructofuranosidase

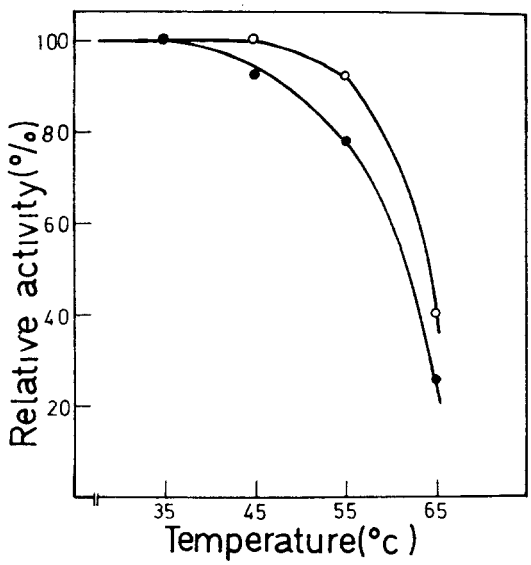

Fig. 8. Temperature stability on the reaction rate of the soluble(●) and immobilized(●) enzyme

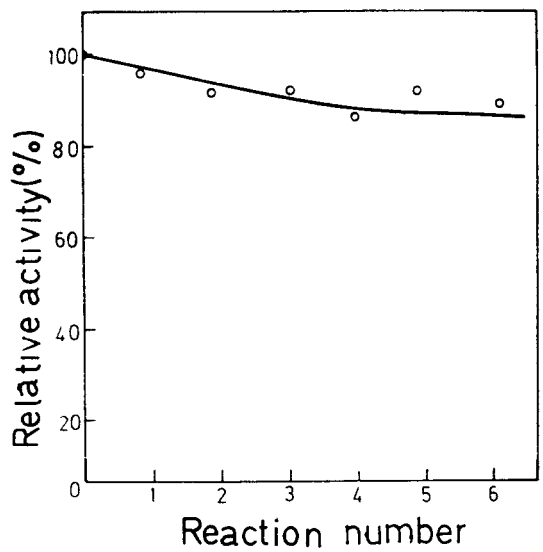

Fig. 9. Stability of the immobilized β-fructofuranosidase to continuous inversion of sucrose by batch system
A mixture of 0.1g of the immobilized enzyme and 30ml of 0.1M sucrose solution was stirred for 120 min at 30℃.

fect of temperature on the enzyme reaction was plotted according to Arrhenius equation to calculate the activation energy. The activation energy of the soluble enzyme for the hydrolysis of sucrose was 21.8 *kJ mol*$^{-1}$ and that of the immobilized enzyme was 19 *kJ mol.*$^{-1}$ Thermal stability of the immobilized enzyme was measured as shown in Fig. 8. Both soluble and immobilized enzymes became increasingly unstable above 55°C. When incubated above 55°C, the immobilized enzyme was more stable than the soluble enzyme. Although the precise chemical nature for the increased thermal stability is not known, presumably crosslinks between the anzyme and the cell matrice could be responsible for the observed result. Appropriately positioned intramolecular crosslinks in the soluble enzyme derivatives can affect an enhanced conformation stability.(10)

The apparent Michaelis constant of the immobilized enzyme was determined under the standard assay conditions with sucrose concentration in the range of 20 to 100 *mM*. As calculated from Lineweaver-Burk plot, the apparent Km of the immobilized enzyme was 55 *mM* and that of soluble enzyme was 50 *mM*. The difference of Km values between the immobilized and soluble enzyme was not significant. Several factors affecting Km values such as steric, microenvironmental, and diffusional effects can be considered in this experiment. Because the steric limitation of enzyme activity was especially observed when the immobilized one is catalyzing a reaction involving a high molecular weight

Table 2. Several kinetic parameters of the soluble and the immobilized β-fructofuranosdase

Parameter	Immobilized enzyme	Soluble enzyme
Apparent Km	55*mM*	50*mM*
Optimum temperature	55℃	65℃
Optimum pH	5.0	5.0
Activation energy	19 *kJmol*$^{-1}$	21.8 *kJmol*$^{-1}$

substrate, this immobilization system would not be affected by steric factor. Therefore, it was assumed that electrostatic and/or diffusional factor can effect on the alteration of Michaelis constant. Comparative kinetic parameters of soluble and immobilized enzyme are summarized in Table 2.

In the batch reactor system, the stability of the immobilized enzyme for hydrolysis of sucrose was studied. During 5 times of repeated continuous reaction in a batch reactor, there was slightly decrease in the first reaction, but was not significant decreases in enzyme activities during the remaining reactions as shown in Fig. 9. From this result the immobilized enzyme was a stable one during the operation in a batch system, though the operation time was within 12 hr.

요 약

미생물 세포를 효소 고정화 담체로 사용하여 β-fructofuranosidase를 고정화 시켰다. *Penicillium spp.* "K-8"로 명명된 곰팡이를 배양한 뒤 균체의 세포벽에 존재하는 다당체를 periodate와 반응시켜 활성화된 알데히드기를 얻을 수 있었다. 이 때 건조 세포 g당 periodate, 1.2g이 최적 농도이었고, 이 농도하에서 온도가 알데히드 형성에 미치는 영향은 거의 없었다. 활성화된 균체에 β-fructofuranosidase를 공유결합에 의하여 고정화시켰다. 더 높은 효소의 고정화를 위하여 이를 glutaraldehyde를 처리한 바 0.5%의 농도와 1시간의 반응조건에서 최대 효소 고정화율 26%를 나타내었다. 이 조건에 의해 제조한 고정화 효소는 kinetic parameters로서 최적온도가 55℃, 최적 pH가 5, Km값이 55*mM*, Ea가 19*kJ mol*$^{-1}$이었다. 회분식 반응조내에서는 6번의 반복된 반응기간동안 고정화 효소 활성에 약간의 감소가 있었으나 비교적 좋은 역가의 안정성을 보여주었다.

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