한국농화학회지 Vol. 27, No. 3, Sept. 1984
J. Korean Agricultural Chemical Society

Preparation and Properties of Coimmobilized Glucose Oxidase-Catalase

Suk-Hee Lee, Tai-Boong Uhm[1], Sook-Ja Cho[2], and Si-Myung Byun

Department of Biological Science and Engineering, Korea Advanced Institute of Science and Technology, Seoul, Korea

[1]Department of Food Science and Technology, Jeonbug National University, Jeonju, Korea [2]Department of Food Science and Nutrition, Cheong-Ju College of Education, Cheong-Ju, Korea

(Received July 25, 1984)

Glucose Oxidase와 Catalase의 동시 고정화 제품과 성질

이석희 · 엄태붕[1] · 조숙자[2] · 변시명

한국과학기술원 생물공학과 [1]전북대학교 농과대학 식품공학과 [2]청주사범대학 식품영양학과

요 약

비교적 높은 역가의 glucose oxidase(GOD)와 catalase(CAT)를 세포의 효소로 생산하는 균주인 ***Penicillium spp.***, PS-8을 선별배지를 사용하여 액체 배양하였으며, 그 결과 배양액 1m*l*당 2.7units의 glucose oxidase와 2.0units의 catalase를 얻었다. Glucose oxidase와 catalase를 분리하기 위하여 60~90% $(NH_4)_2SO_4$ 분별침전을 행한후 DEAE-cellulose column을 사용하여 두 효소를 완전히 분리하였으며, 이들 효소의 회수율은 54%와 34%이었다. 분리된 glucose oxidase와 catalase는 PS-8 균주를 효소 고정 매개체로 하여 2.5% glutaraldehyde를 가교제로 12시간 동안 처리함으로써 효소를 고정시켰다. 이들 고정화 효소는 CAT/GOD 값이 서로 다르게 동시 고정, 고정후 혼합, glucose oxidase만의 고정 등의 형태로 만들었다.

pH에 따른 효소의 활성변화에서는 동시고정 및 고정후 혼합 방법이 수용성 효소보다 안정화됨을 보여 주었으며, 동시 고정에서는 CAT/GOD값이 높을수록 보다 완만한 pH 활성곡선을 나타내었다. GOD와 CAT/GOD=10의 Km′ 값은 각각 7.1×10^{-2} 및 5.1×10^{-2}M이었으며, 이들의 활성화 에너지값은 각각 3.97 및 2.98 kcal/mole/deg이었다.

Introduction

Glucose is oxidized to gluconolactone and hydrogen peroxide by glucose oxidase(GOD, E.C. 1.1.3.4.) and catalase(CAT, 1.11.1.6.) catalyzes the decomposition of hydrogen peroxide to molecular oxygen and water. The reaction systems are as shown:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2$$

$$2H_2O_2 \xrightarrow{\text{CAT}} 2H_2O + O_2$$

The gluconolactone undergoes rapid hydrolysis to gluconic acid in the aqueous system. The low solubility of O_2 in water at normal conditions of the GOD system would indicate that only a small amount of glucose could be oxidized before O_2 becomes rate limiting. Thus, O_2 must be continuously supplied by transfer either from air or from O_2. Furthermore, H_2O_2 produced in the GOD reaction system deactivates the GOD activity, specially the reduced form of the enzyme[1]. The catalase activity is also deactivated by H_2O_2 as shown in Fig. 1.

To minimize the H_2O_2 effect and to accelerate the oxidation of glucose by GOD, it takes advantages if the GOD reaction is coupled with the catalase reaction, since glucose is oxidized to yield H_2N_2 in the presence of limited amount of O_2 and the catalase regenerates the O_2 from H_2O_2. This approach has been described by several investigators [2~4], using the immobilized GOD and CAT. Bouin *et al*[3] immobilized GOD and CAT on nickel silica alumina particles and Prenosil[4] constructed the coimmobilized GOD and CAT on a copolymer of phenylenediamine and glutaraldehyde on pumice and titania carrier. They have studied mainly on the deactivation of the dual catalysts.

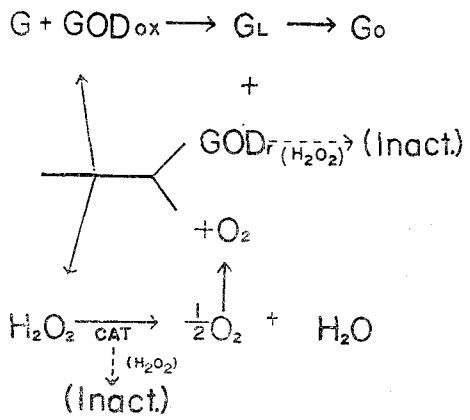

Fig. 1. The coupled reaction scheme of glucose oxidase and catalase
G : D-glucose
G_L : δ-gluconolactone
G_o : D-gluconic acid
GOD_{ox} : oxidized glucose oxidase
GOD_r : reduced glucose oxidase
CAT : catalase

In this study, we coimmobilized GOD and CAT on the microbial cell by a simple method which showed a good mechanical strength and flow rate in a column operation and their properties are presented.

Materials and Methoods

Materials

Glucose, yeast extract, malt extract, bactopeptone and agar flakes were purchased from Difco Lab. (Detroit, MI, U.S.A.); DEAE-cellulose from Bio Rad(Richimond, CA, U.S.A.); glutaraldehyde from Eastman Kodak Co. (Rochester, NY, U.S.A.); bovine serum albumin from Worthington Co. (Freehold, NJ, U.S.A.); 1,6-diaminohexane from Aldrich (Milwaukee, WI, U.S.A). All other chemicals were used of analytical grades.

Cell culture and enzyme purification

Penicillium spp., PS-8, which excretes large amounts of GOD and CAT and was screened by previous in estigators in our lab[5], was used a source of enzymes and also as a cell matrix. To increase the production of the enzymes and cell mass, a modified Czapek- Dox media was used by adding yeast extract in a concentration of 0.05%. After the cell culture was completed as described in the previous report[5], the culture media, which contained 2.7U of GOD, 2.0U of CAT and 26.7mg of cell mass per *ml* of

culture media, was centrifuged at 5000×g for 30 min. The cell harvested was saved and the clear supernatant was freeze-dried. A crude enzyme was prepared by saturation of the freeze-dried sample with $(NH_4)_2SO_4$. A 60~90% saturated fraction was obtained and dialyzed against dist. H_2O at 4°C. The crude enzyme preparation containing both GOD and CAT was dissolved in an adequate volume of 0.1M sodium phosphate, pH 6.8. The preparation was applied on a preequilibrated DEAE-cellulose column(1×10cm) for the separation of GOD and CAT sequentially and eluted with 300m*l* of gradient sodium acetate buffer, pH 4.65 from 0 to 2M. The active fractions were combined, dialyzed and freeze-dried.

Cell matrix preparation

The cell cake, which was harvested at the step of enzyme preparation, was well suspended in an adequate volume of deionized water. The slurry was sonified with Sonic Dismembrator Artek-300 for 20min at 4°C. The sonified cell suspension was filtered and treated with 2~3 volumes of acetone for 24hr at 0°C. After air dried, the cell debris was milled to powder form with Thomas Mill (Arthur H. Thomas Co.) and distributed the size.

Immobilization of glucose oxidase and catalase

Three kinds of the immobilized enzymes as shown in Fig. 2 were prepared, where a sole type means the immobilization of single enzyme of GOD or CAT, a dual type represents the immobilization of both GOD and CAT on the same cell matrix and a mixed type illustrates a mixture of the 2 kinds of sole type immobilized enzymes, GOD and CAT. Five grams of the cell matrix were suspended in enzyme solution containing either 200 units of GOD or CAT, and water was evaporated by preventillation at 4°C. After the adsorption of the enzyme on the cell matrix, a solution containing various concentrations of glutaraldehyde in 0.1M sodium phosphate buffer, pH 6.8 was added and placed at 4°C overnight. Residual glutaraldehyde was removed by several washing with dist. H_2O and 0.1M sodium phosphate buffer, pH 6.8. Finally the cell matrix was washed with 2M urea and 1M $NaHCO_3$, pH 9.5 to remove non-crosslinked adsorbed enzyme[6]. For the preparation of another type of the immobilized enzyme, 5g of the acetone treated cell matrix was activated with CNBr and coupled with 1,6-diaminohexane according to the method of March *et al.*[7] To the cell matrix treated with diaminohexane, 200 units of GOD or CAT added and immobilized.

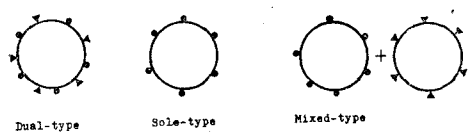

Fig. 2. Schematic representation of immobilized enzymes
●: glucose oxidase
▼: catalase
large circle: cell membrane

For the preparation of a dual type immobilized enzyme, an enzyme solution containing both GOD and CAT in various ratios was used instead of a solution containing a single enzyme.

Enzyme activity assay

The activities of both soluble and immobilized GOD were assayed by determining O_2 consumption in the reaction mixture by a Clark-type electrode (Gilson Medical Electronics, K-ICT-C Oxygraph) at 25°C. The reaction mixture contained 0.2m*l* of 13.9mM glucose as substrate and air saturated 0.1M citrate-phosphate buffer, pH 5.5(0.25mM O_2) in 1.2m*l*. The electrode was attached to a jacketed cell(1.8m*l* capacity) which was agitated using a 2×7mm magnetic stirring bar. The presence of catalase such as in the dual or mixed type immobilized GOD will produce O_2 from H_2O_2 produced by GOD. Thus, the observed rate of O_2 consumption is exactly one half of that of GOD produced. Therefore, the actual activity of the GOD was calculated by multiflying the observed O_2 con-

sumption 2 times in the presence of excess soluble catalase[3]. One unit of GOD activity was defined as an amount of enzyme which catalyzes the consumption 1μmole of O_2 for 1min at the above assay conditions.

The activity of catalase was determined by assaying O_2 production with the same Oxygenograph described in the GOD assay system. The assay was carried out at pH 5.5 using 0.1M citrate-phosphate buffer at 25°C and H_2O_2 concentration was 5×10^{-4}M. The presence of GOD in the CAT assay system had no effect on the measurement as long as glucose was not added to the reaction mixture. All solutions were aerated with N_2 gas before use. One unit of CAT was defined as an amount of enzyme which catalyzes the production of 1μmole of O_2 for 1min at the above assay conditions.

Characterization of various immobilized enzymes

Various immobilized enzyme preparations were characterized in terms of pH, temperature and substrate effect on the enzyme activities, according to the method of Byun *et al*[8].

Result and Discussion

Purification of glucose oxidase and catalase

Penicillium spp., PS-8 produced relatively higher GOD and CAT activities as well as higher cell mass. At about 20hr of the culture, 2.7 U of GOD and 2.0U of CAT per m*l* of broth were produced, which 2.67g of cell mass/100m*l* broth was obtained. Glucose oxidase and catalase were well separated by DEAE-cellulose column chromatography as shown in Fig. 3. The final preparations of GOD and CAT showed 40U and 31 U per mg protein, respectively and these preparation were used in next immobilization experiment. The overall yields of the enzyme activities by the DEAE-cellulose chromatography were 54% and 34% for GOD and CAT, respectively with respect to the sample loadings.

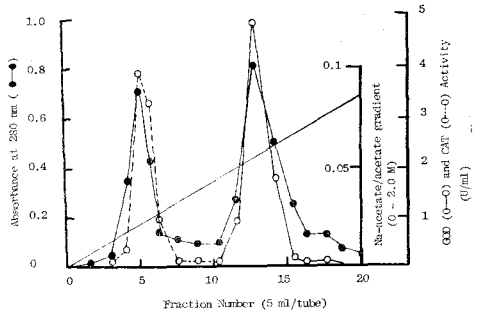

Fig. 3. Elution profile of glucose oxidase and catalase on DEAE-cellulose column. Column size; 1×10cm, flow rate; 20m*l*/hr

Immobilization of glucose oxidase and catalase

Since cell of PS-8 which was treated with acetone yielded good physical and mechanical strength because of granular particle formation and excellent flow rate when it was packed in the column. We decided that the whole cell will be used for the immobilization matrix by the covalent bonding. We tried two typical methods for the immobilization; (1) by CNBr activation-1,6-diaminohexane linked enzyme and (2) crosslinking with glutaraldehyde.

Fig. 4 shows the effect of the glutaraldehyde concentration on the crosslinking of GOD, where 2.5% was the optimal concentration. However, CNBr activation: 1,6-diaminohexane treated cell gave fairly low activity immobilized to compare the glutaraldehyde method. Specially

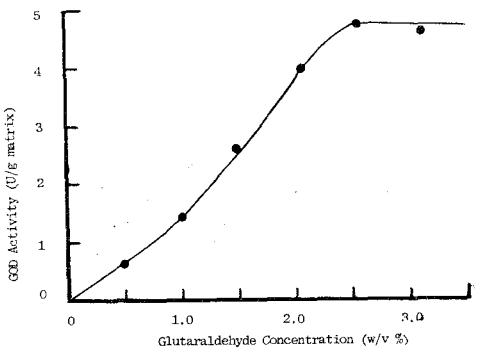

Fig. 4. Effect of glutaraldehyde concentration on the crosslinking of glucose oxidase on matrix cell

Table 1. Results of the immobilization of glucose oxidase

Type of immobilization	Activity immobilized (U/g of matrix)
CNBr activated cell only	0.071
CNBr activation-1,6-diaminohexane treated cell	0.554
Glutaraldehyde treated cell	2.67
Glutaraldehyde-albumin treated cell	4.84

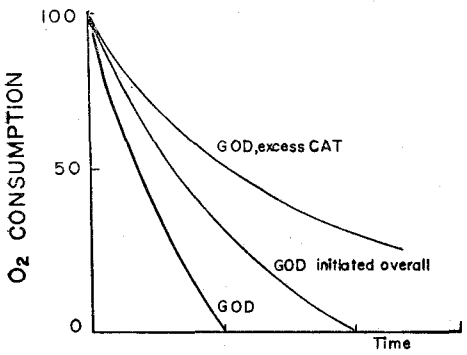

Fig. 5. Oxygen consumption by glucose oxidases
GOD, excess CAT: the trace of O_2 consumption due to GOD action on glucose in the presence of excess soluble CAT
GOD initiated overall: the depletion of O_2 in dual enzyme system
GOD: the O_2 consumption in the absence of any CAT activity

the immobilization of GOD or CAT by glutaraldehyde crosslinking method in the presence of bovine serum albumin(1%) with the enzyme yielded 2 folds higher GOD activity than the control (absence of albumin). This seemed to be that albumin assists the crosslinking of the enzyme. Table 1 shows the comparative results of immobilization. Generally the results of catalase immobilization were the same as those of GOD as well (data not shown).

Typical data used to calculate the percent "catalase efficiency" and "glucose oxidase efficiency" were shown in Fig. 5 and 6, respectively. For the mixed and dual immobilized enzymes,

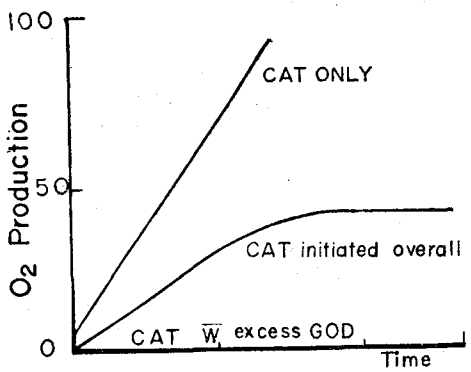

Fig. 6. Oxygen production by catalases
CAT only: the CAT activity only measured first with H_2O_2 as substrate
CAT initiated overall: the CAT-initiated overall reation when both CAT and GOD(not excess) are present along with H_2O_2 and glucose
CAT with excess GOD: the CAT activity in the presence of excess soluble GOD

typically the immobilized enzymes containing 3 different ratios of CAT/GOD were constructed and compared the relative efficiencies. The relative efficiency was calculated by the method of Bouin *et al.*[3] as follows: % catalase efficiency=

$$\frac{(K_{GOD\_excess\ CAT}) - (K_{GOD\_overall})}{0.5K_{GOD}} \times 100,$$ where K_{GOD} is the pseudo first-order rate constant of GOD reaction, $K_{GOD\_overall}$ is that of the GOD initial overall reaction and denominator equals to the rate of the GOD reaction in the presence of excess CAT, and % glucose oxidase efficiency=

$$\frac{(K_{CAT}) - (K_{CAT\_overall})}{K_{CAT}} \times 100,$$

where K_{CAT} is the first-order rate constant of the CAT reaction and $K_{CAT\_overall}$ is that of the overall reaction. One advantage of studying a cyclic enzyme system is that either enzyme can be used as the enzyme to catalyze the first reaction in the sequence depending on the substrate used. In either case, the first enzyme produced a product, P_1, which is then acted on by the second enzyme to produce the final product of the re-

action, P_2. It is defined that thee fficiency of the system as how well the second enzyme in the overall reaction sequence utilizes the product of the first reaction compared to utilization by an excess amount of soluble second enzyme.

Characteristics of the immobilized enzymes

In the comparison of pH profiles among dual, mixed and homogeneous soluble system as shown in Fig. 7, the dual and mixed type had broader maximum pH range than the soluble type, and that maximum pH shifted to the basic range for the mixed type and to the acidic range for the dual type relative to the soluble type were observed. From the above results, immobilized enzymes are more stable for the pH change than soluble enzyme.

Comparison of pH profiles with different CAT

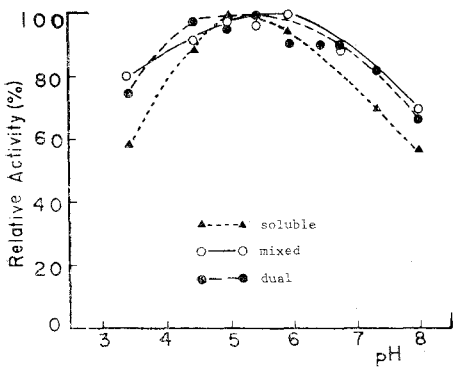

Fig. 7. Comparison of pH profile of dual, mixed and soluble system.

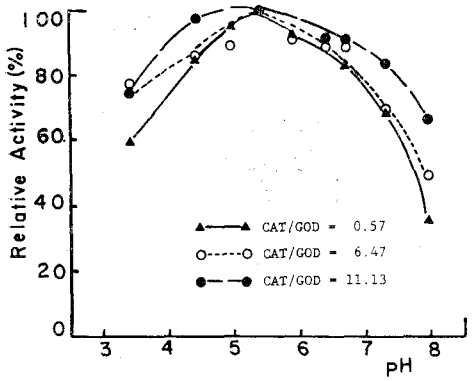

Fig. 8. Comparison of pH profiles with different CAT/GOD ratio in dual system.

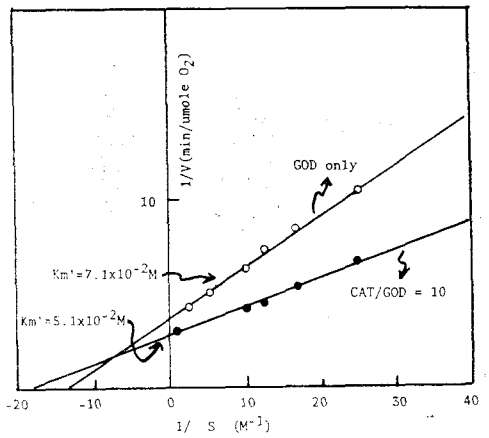

Fig. 9. Lineweaver-Burk plots for GOD only and CAT/GOD=10. (S)=Glucose

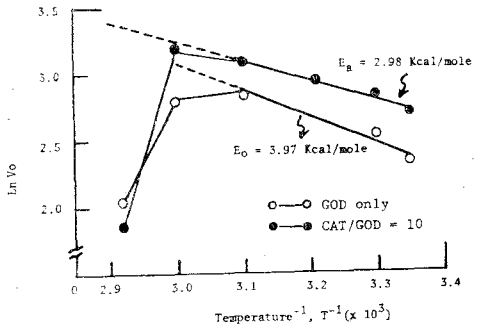

Fig. 10. Effect of temperature for GOD only and CAT/GOD=10.

/GOD ratio in the dual system was shown in Fig. 8. The higher the activity ratio of CAT/GOD shows the broader activity profile.

For the effect of glucose concentration on the GOD activity between two types of the immobilized enzymes (GOD only and CAT/GOD=10), Km of GOD only and CAT/GOD=10 were $7.1\times10^{-2}$and 5.1×10^{-2}M (Fig. 9).

Effect of temperature on the GOD activity between two types of the immobilized enzymes (GOD only and CAT/GOD=10) were shown in Fig. 10. The activation energy, Ea, obtained from measuring the reaction rate constant at different temperature and plotting *ln* Vo *vs* 1/T was 3.97 and 2.98 kcal/mole/deg for the reaction with GOD only and CAT/GOD=10 at the range from 20°C to 70°C.

As summary, we obtained a coimmobilized

preparation of glucose oxidase and catalase on a cell matrix. It showed good mechanical strength so that it is advantageous for utilization of the coimmobilized enzyme in continuous reaction system. For that we carried out to examine several characteristics of the coimmobilized enzyme. Using this result, we are planning to study the reactor performance of this enzyme system in the future.

Abstract

For the study of glucose oxidase(GOD) and catalase(CAT) coimmobilization system, the enzymes were obtained from *Penicillium spp.*, PS-8, and the strain itself was used as an immobilizing matrix.

To separate glucose oxidase and catalase after the ammonium sulfate fractionation of the culture broth, DEAE-cellulose column was used and its activity yield was 54 and 34%, respectively. Both enzymes were immobilized on the cell matrix, followed crosslinking with 2.5% glutaraldehyde for 12hr.

In the determination of efficiencies of GOD and CAT of dual, mixed and soluble enzyme systems, the dual immobilized one was superior to those of the soluble or mixed ones. In the comparison of pH profiles, the dual and mixed types showed broader maximum pH ranges than the soluble type. Varying CAT/GOD ratio of the dual system, the higher the ratio showed the broader activity profile.

In the comparison of apparent K_m of GOD only and CAT/GOD=10, they were 7.1×10^{-2} and 5.1×10^{-2}M. Their activation energies showed 3.98 kcal/mole/deg for GOD only and 2.98 kcal/mole/deg for CAT/GOD=10.

References

1. Kleppe, K.: Biochemistry, **5** : 139(1966).
2. Lawrence, R.L. and Okay, V.: Biotechnol. Bioeng., **15** : 217(1973).
3. Bouin, J.C., Atallah, M.T., and Hultin, D.: Biochem. Biophys. Acta, **438** : 23(1976).
4. Prenosil, J.E.: Biotechnol. Bioeng., **21** : 89 (1979).
5. Ko, J.H. and Byun, S.M.: J. Korean Chem. Soc., **23** : 165(1979).
6. Broun, G., Selegny, E., Avrameas, S., and Thomas, D.: Biochem. Biophys. Acta, **185** : 260(1969).
7. March, S.C., Parikh, I., and Cutrecasas, P.: Anal. Biochem., **60** : 149(1974).
8. Byun, S.M. and Wold, F.: Korean J. Food Sci. Technol., **8** : 253(1976).