Kor. J. Appl. Microbiol. Bioeng.
Vol. 15, No. 6, 402-407 (1987)

Purification of Glucose Oxidase from *Aspergillus niger* KUF-04

Yang, Ho-Suk[1]*, Han-Chul Yang[1] and Yoshiki Tani[2]

[1]Department of Food Technology, Korea University, Seoul 132, Korea
[2]Department of Agricultural Chemistry, Kyoto University, Kyoto, JAPAN

Aspergillus niger KUF-04가 생산한 Glucose Oxidase의 정제에 관한 연구

양호석[1]*·양한철[1]·다니 요시기[2]

고려대학교 식품공학과 [2]일본 경도대학 농화학과

Glucose oxidase from *Aspergillus niger* KUF-04 was purified homogeneously by the procedure of seven steps including crystallization. The ball-like crystalline enzyme was obtained from the 23-fold purified enzyme solution. The glucose oxidase was found to be composed of two identical subunits and the molecular weight of the enzyme and its subunit were estimated to be about 210,000 and 110,000 by HPLC and SDS-acrylamide gel electrophoresis, respectively.

Glucose oxidase catalyzes the oxidation of glucose to gluconic acid, simultaneously with the reduction of molecular oxygen to hydrogen peroxide. Catalase decomposes hydrogen peroxide into water and oxygen. Since the existence of glucose oxidase in *Asp. niger* and *Pen. glaucum* was first reported by Muller (1,2) in 1926, properties of the enzyme from various molds have been made to be the subject of investigation by several workers(3-7).

In 1952, the first commercial glucose oxidase was obtained from *Asp. niger* by Takamine Laboratory. At present, glucose oxidase is commercially prepared from *Asp. niger* and *Pen. amagasakiense.*

The enzyme is applied to various fields, including the removal of glucose from egg albumin and whole egg to prevent to development of off-flavor, removal of oxygen from canned food to prevent flavor and color change, and determination of glucose concentration in blood or urine(8). Tsuge *et al.* (9) reported that the enzyme from *Asp. niger* contains 74.0±2.8% protein, 16.4±0.3% neutral sugar, 2.4±0.5% amino sugar, 2 moles of iron per 160,000 daltons and 2 moles of flavin-adenine dinucleotide. Hayashi and Nagamura(10) also reported that glucose oxidase. From *Asp. niger* might contain several microheterogeneous species. Recently, kusai(11) succeeded in crystallizing of glucose oxidase from a culture medium of *Pen. amagasakiense* using a technique of ion exchange chromatography. But the crystallization of the glucose oxidase from other organisms has not been published. Therefore it was studied on the crystallizing conditions and properties of glucose oxidase from *Asp. niger* KUF-04.

Materials and Methods

Chemicals

Reference proteins for molecular weight determination by SDS-slab gel electrophoresis were purchased from pharmacia Fin Chemicals and those for high performance liquid chromatography (HPLC) were purchased from Oriental Yeast Company. All of other chemicals used in this work were obtained from the usual commercial sources and used without further purification.

Key words: Glucose oxidase, *Aspergillus niger*

*Corresponding author: 410 shindorim-dong, Guro-Gu, Seoul, Korea

Microorganism and cultivation

Aspergillus niger KUF-04(12) isolated from a soil sample from Seoul area was used for the production of glucose oxidase.

Culture medium used for sporulation was Czapek-Dox agar.

Seed culture medium contains 30.0 glucose, 2.0 KH_2PO_4, 1.0 $MgSO_4 \cdot 7H_2O$, 10.0 $CaCO_3$, 9.0 NH_4Cl and 1.0 g corn steep liquor (50% w/v) in 1,000 m*l* of tap water, pH 6.5. One loop of spore after cultivation at 28 °C for 4-6 days on Czapek-Dox agar was inoculated into the 300-m*l* flask containing 50 m*l* of seed medium. The cultivation was carried out at 28 °C for 1 day with a reciprocal shaker at 120 rpm.

Main culture medium contains 100.0 glucose, 0.2 KH_2PO_4, 1.0 $MgSO_4 \cdot 7H_2O$, 30.0 $CaCO_3$ and 0.2 g $(NH_4)_2SO_4$ in 1,000 m*l* of tap water, pH 7.0.

After incubating the seed culture at 28 °C for 1 day, the seed culture was inoculated by 5% (v/v) into 500 m*l* of the main medium in the 2-liter shaking flask. Then cultivation was carried out at 28 °C for 2 days with a reciprocal shaker at 120 rpm.

Enzyme purification

I) Preparation of cell-free extract

Mycelia harvested after 2-day cultivation, washed with distilled water and suspended in 50 mM potassium phosphate buffer, pH 7.0. The suspension was treated with Dyno-Mill for 5 min. The cell-free extract was obtained by centrifugation at 8,000 × g for 20 min after the treatment. All the purification steps were carried out at 5-8 °C.

II) Purification procedure

Cell-free extract was fractionated with ammonium sulfate. Active precipitate obtained by 60-90% saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialyzed enzyme solution was applied to DEAE-cellulose column (2.5 × 55cm) which was previously equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The enzyme was eluted by stepwise increasing the concentration of the buffer from 50 to 150 mM potassium phosphate. Active fractions were combined and fractionated again with ammonium sulfate. Active precipitate formed by 60-90% saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer. The dialyzed enzyme solution was, then, applied to hydroxyapatite column (2.3 × 13.5cm) equilibrated with 50 mM potassium phosphate buffer, pH 7.0. The adsorbed enzyme was eluted by stepwise increasing the strength of the buffer solution from 50 to 150 mM potassium phosphate. Active fractions were combined and fractionated with ammonium sulfate. Active precipitate formed by 60-90% saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.0, and dialyzed against the same buffer solution. The dialyzed enzyme solution was applied finally to the gel filtration on sephadex G-200 column (1.2 × 85cm) which was equilibrated with 50 mM potassium phosphate buffer, pH 7.0.

Determination of enzyme activity

I) Colorimetry

The activity of glucose oxidase was determined by measuring the amount of quinoneimine dye based on the formation of hydrogen peroxide. The standard reaction mixture (3.0 m*l*) containing 0.82 mole of 4-aminoantivpyrine, 5 moles of phenol, 73.3 moles of glucose and 4.5 units of peroxidase in 50 mM sodium phosphate buffer, pH 6.0 was incubated at 35 °C for 5 min. The reaction was started by addition of the enzyme solution (0.1 m*l*).

The velocity of quinoneimine dye formation was followed by measuring the absorbance at 505 nm in a cuvette of 1-cm path (Shimdzu MPS-2000) at 35 °C. One unit of glucose oxidase activity was defined as the amount of the enzyme which catalyzed the formation of 1.0 mole of hydrogen peroxide per min. The molar absorptivity for dye formed under these conditions was $4.2 \times 10^3\ M^{-1}\ cm^{-1}$.

Molecular weight determination

I) The molecular weight was determined by HPLC, using TSK-Gel G3000SW column with the running solution of 0.1M potassium phosphate buffer containing 0.2M of NaCl, pH 7.5. Reference proteins were prepared by Oriental yeast Company.

II) The molecular weight of subunit was deter-

Table 1. Summary of glucose oxidase purification.

Fraction	Total[a] Activity (units)	Total[b] Protein (mg)	Total Activity (units/mg)	Yield (%)	Fold
Cell-free extract	24,646	2,532	9.7	100.0	1.0
Ammonium sulfate (60-90%)	18,976	261	72.0	77.0	7.5
DEAE-cellulose	16,976	105	162.3	69.0	16.7
Hydroxyapatite	10,674	54	198.0	43.0	20.4
Sephadex G-200	6,319	28	226.0	25.6	23.2

a) Activity was measured by spectrophotometry at 505 nm.
b) The amount of protein was estimated by the absorbance at 280 nm, assuming $E_{cm}^{1\%} = 10.0$.

mined using SDS-slab gel electrophoresis according to the method of Weber and Osborn (13).

Determination of protein concentration

Protein concentration was determined by the methold of Lowry *et al*(14), or by measuring absorbance at 280 nm. Bovine serum albumin was used as a standard protein.

Results and Discussion

Enzyme purification

I) Enzyme production

The glucose oxidase production by *Asp. niger* KUF-04 was optimized according to the cultural conditions established for gluconate fermentation (15). About 650g of mycelia obtained by filtration was destroyed as described in MATERIALS AND METHODS section. The resulting cell- free extract was used as the crude enzyme solution for the purification of intracellular glucose oxidase.

II) Purification result

Table 1. Shows a summary of the purification. The yield of the purified enzyme was about 25% and the purity increased 23-fold as compared with the cell-free extract. Among the purification procedures the ammonium sulfate fractionations and DEAE-cellulose were increased the specific activity of glucose oxidase to 7.5-and 16.7-fold, respectively, as compared with cell-free extract. The active fractions obtained by the first ammonium sulfate fractionation contained mainly glucose oxidase and catalase. The colour of the above solution was yellowish-brown. When the enzyme solution was applied to a DEAE-cellulose column, glucose oxidase was completely adsorbed at the upper part of the column as distinct yellowish-brown band. The glucose oxidase absorbed was eluted by 50 mM potassium phosphate buffer, pH 7.0. The active fractions were collected and precipitated with ammonium sulfate and its fraction was applied to Hydroxyapatite column which was eluted by same buffer, pH 7.0.

When the noble yellow enzyme solution obtain-

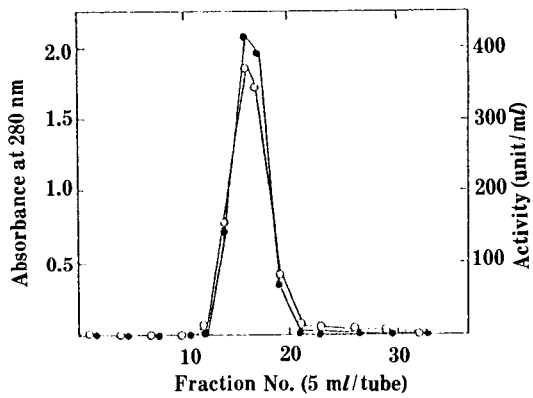

Fig. 1. Chromatography of glucos oxidase on Sephadex G-200.

Approximately 37 mg of protein in 7ml of 50mM postassium phosphate buffer (pH 7.0) was applied to a column (1.2×82cm). The enzyme was eluted by 50mM potassium phosphate buffer (pH 7.0). Five-milliliter fractions were collected at flow rate of 2ml per hour.
● – ●, absorbance at 280 nm, ○ – ○, glucose oxidase activity.

ed from the 2nd ammonium sulfate fractionation was again subjected to a Sephadex G-200 column, the resultant elution pattern of the enzyme activity coincided almost exactly with that of the protein as presented in Fig. 1.

III) Crystallization

The enzyme solution collected from the gel filtration was concentrated to about 20 mg/ml of protein using a collodion bag. The purified enzyme obtained from Sephadex G-200 gel filtration was used for crystallization procedure. To the concentrated enzyme solution, solid ammonium sulfate was added gradually until faint turbidity appeared. When the enzyme solution obtained was allowed to stand at 5 °C for 2 or 3 days, ball-like crystals were formed there as shown in Fig. 2. Whereas Kusai(11) reported that crystalline glucose oxidase from *Pen. amagasakiense* was needle-like one.

IV) Homogeneity of the purified enzyme

The purified enzyme migrated toward the anode

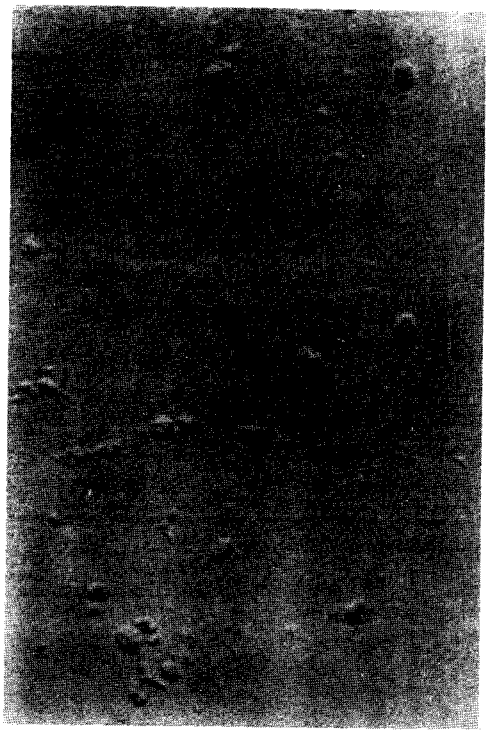

Fig. 2. Photograph of crystalline glucose oxidase from Asp. *niger* (×400)

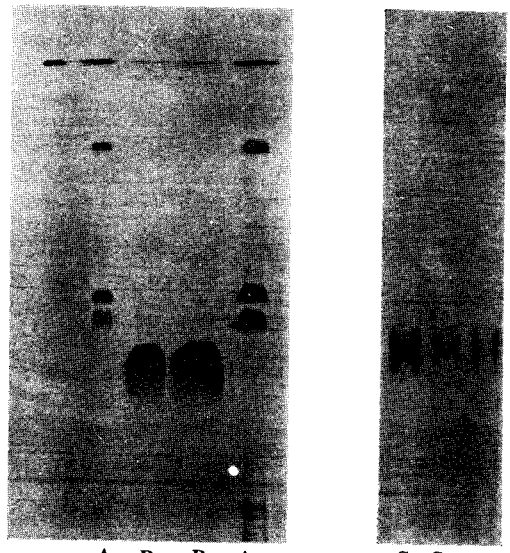

Fig. 3. Polyacrylamide gel electrophoresis of purified enzyme.

A. Reference protein in the presence of SDS
B. Sample in the presence of SDS
C. Sample in the absence of SDS

The SDS-slab electrophoresis was performed in 8.2% polyacrylamide gel at pH 8.8 with a current of 50 mA per slab. About 20 ug of enzyme was applied and the gel was stained with brillant blue.

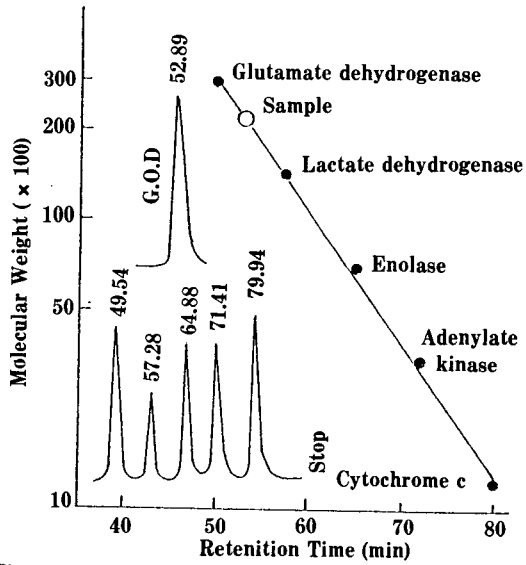

Fig. 4. Estimation of molecular weight of glucose oxidase HPLC conditions.

Column : TSK-Gel G3000SW (0.75 × 60 cm)
Running Solution : 0.05M K-PO_4 + 0.2M NaCl (pH 7.5)
Standard : Lot MW-Marker 03101
Injection volume : 20 ul
Detection : UV 280 nm

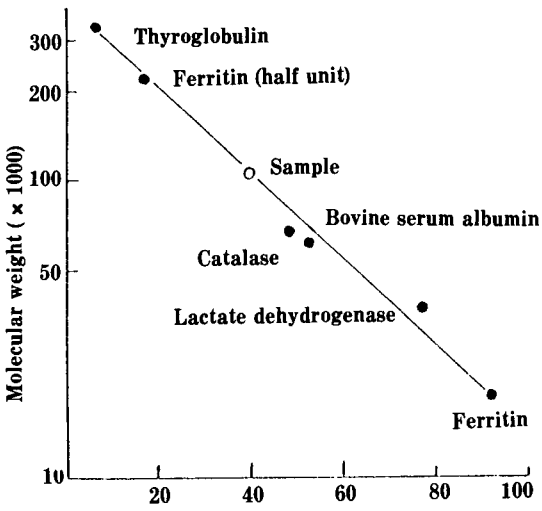

Fig. 5. Estimation of molecular weight of glucose oxidase by SDS-polyacrylamide gel electrophoresis.
The SDS-slab electrophoresis was performed in 8.2% polyacrylamide gel, pH 8.8 with a current of 40 mA per slab. About 20 ug was applied and the gel was stained with brillant blue.

and gave a single protein band on SDS-polyacrylamide gel electrophoresis at pH 8.8 (Fig. 3). The enzyme was also applied to HPLC with TSK-Gel G 3000SW column and gave a single peak (Fig. 4.)

V) Molecular weight

The molecular weight of the purified enzyme was determined to be about 210,000 by HPLC (Fig. 4.). The molecular weight of the subunit was determined to be about 110,000 by SDS-slab gel electrophoresis (Fig. 5.) Judging from these results, the molecular weight of glucose oxidase of *Asp. niger* KUF-04 was estimated as 210,000 composing of two subunits of the molecular weight of 110,000. The number of subunit coincided with the result of Tsuge *et al*(9).

요 약

Aspergillus niger KUF-04로 부터 얻은 glucose oxidase는 결정화를 포함하여 7단계의 공정을 거쳐 순수하게 정제되었다. 공모양의 결정은 23배 정제된 효소액에서 얻어졌다. 분자량은 HPLC에 의해 210,000, SDS-polyacrylamide electrophoresis에 의해 110,000을 나타냈으므로 본 효소는 동일한 2개의 subunit로 이루어진 단백질임을 알 수 있었다.

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(Received October 16, 1987)