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친화성 크로마토그래피를 이용한 글루코오스 옥시다아제의 정제와 효소특성

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Purification of Glucose Oxidase by Affinity Chromatography and Its Characterizaton

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요 약. 토양중에서 분리한 *Penicillium* 속이 생산하는 글루코오스 옥시다아제를 친화성 크로마토그래피에 의해 정제하고, 이 효소의 특성을 알아보았다. D-Gluconyl-w-aminohexyl Sepharose 컬럼을 사용하여 친화성 크로마토그래피를 행한 결과 14.6배 정제되었고 수율은 79.7 % 였으나 카탈라아제가 소량 함유되어 있어서 Sepharose 6B 겔 여과를 행하여 이를 제거하였다. 이 결과 27.2배 정제되고 수율이 74.1 % 인 정제효소를 얻었으며 7 % polyacrylamide 겔 전기영동 결과 단일대를 보여주었고 비활성도는 단백질 mg 당 90.83 U 였다.

정제된 효소의 흡광스펙트럼과 기질에 대한 특이성을 조사하였으며 최적 pH 는 5.6~6.0, 최적온도는 40 °C, D-글루코오스에 대한 K_m 값은 $8.5\times10^{-3}M$, 활성화에너지는 3.43 kcal/mole 이었다.

ABSTRACT. A purification technique of glucose oxidase was developed. Using the gluconyl-w-aminohexyl Sepharose affinity chromatography, it was partially purified 14.6 folds with 79.7 % yield. With the combination of the affinity chromatography and Sepharose 6B gel filtration, the enzyme was purified 27.2 folds from the broth with 74.1 % yield. The final purified preparation showed 90.83 U of glucose oxidase activity per mg of protein and a single band by 7 % polyacrylamide gel electrophoresis.

The absorption spectrum and substrate specificity of the enzyme were studied and the final preparation showed the optimal pH between 5.6 and 6.0, the optimal temperature at 40 °C, 8.5×10^{-3} M of K_m for D-glucose, and 3.43 kcal/mole of the activation energy.

INTRODUCTION

Glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4.) is a flavin containing glycoprotein, which catalyzes the oxidation of

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glucose to δ-gluconolactone by transfering hydrogen directly to molecular oxygen[1]. The enzyme has been identified from extracts of fungi, *Aspergillus niger*[2,3], *Penicillium notatum*[4,5], *Penicillium amagasakiense*[6,7], and some of other sources[8].

In the recent studies the specificity, the kinetics, and the mode of action of glucose oxidase were reported[3,9~12]. Pazur *et al.*[13,14] found that this enzyme is a glycoprotein. It has generally assumed that glucose oxidases from different sources have shown the same properties.

Although glucose oxidases have been purified highly by the various conventional methods[3,6,7,15], Valyulis *et al.*[16] recently developed an affinity chromatographic technique of an antibody-Sepharose gel and purified glucose oxidase from *Penicillium vitale*. They obtained pure specific antibodies against glucose and attached it covalently to Sepharose for the purification of this enzyme.

Success of affinity chromatog aphy depends often on the selection of ligand (inhibitor/modifier) that is stable and highly specific. Though the method of immunosorbent for the affinity chromatography is powerful and promising to purify enzymes, the specific antibody should be used and this may cause more problems.

In this work we studied on the proper selection of ligand for the purification of glucose oxidase by affinity chromatography and characterization of the purified enzyme.

METHODS

Microorganism. Microorganisms producing high activity of glucose oxidase were screened from various soils and airs. A *Penicllium*, which was tentatively identified, produced high activity of glucose oxidase[17]. This was named as PS-8 and used throughout this work.

Enzyme Assays. Enzyme activities of glucose oxidase, catalase, xanthine oxidase, and galactose oxidase were determined by the Worthington manual[18]. Glucose oxidase was assayed by the method of the coupled-o-dianisidine system. One unit (U) of glucose oxidase activity is that amount of enzyme liberating one micromole of H_2O_2 per minute at 25 °C. Hexokinase and L-amino oxidase were assayed according to the methods of Bergmeyer *et al.*[19] and Boehringer mannheim[20] respectively. Protein was determined by the Folin-Lowry method using a bovine serum albumin as a standard[21].

Preparation of Affinity Adsorbents. Ligands were attached to Sepharose 6B by the two methods, expoxide coupling method[22] and aminohexyl coupling method[23]. The ligands tested were **D**-arabinose, α-methyl-**D**-glucoside and **D**-gluconic acid. It has been established that the primary alcohol at the C_6 position of the sugar is the primary site for the epoxide coupling[22] and the carbonyl group of gluconic acid is coupled to the amino group of ω-aminohexyl Sepharose for the aminohexyl coupling method[23].

For the preparation of the epoxide coupled Sepharose adsorbent, 15 m*l* of 1, 4-butanediol diglycidyl ether (oxirane) and 15 m*l* of 0.01 *N* NaOH containing 30 mg of $NaBH_4$ were added to 15 g of washed suction-dried Sepharose 6 B. The mixture was rotated mechanically at room temperature for 10 hr. The epoxide-activated Sepharose was subsequently collected on a sintered glass funnel and was washed with a large excess of distilled water. To 8 g of suction-dried epoxide-Sepharose, 2 g of ligand dissolved in 16 m*l* of 0.1 *N* NaOH was added and the mixture was rotated mechanically at 37 °C for 24 hr. The affinity adsorbent was collected by filtration and was washed with 500 m*l* of 0.1 *N* borate buffer pH 8.0, followed by a large volume of distilled water.

For the preparation of aminohexyl-coupled

Sepharose adsorbent, 7 g of a slurry of washed Sepharose 6B in 10 ml of distilled water was added to 10 ml of 2 *M* sodium carbonate and mixed by stirring slowly. A solution of 1g of cyanogen bromide in 0.5 ml acetonitrile was then added all at once in a well-ventillated hood. The slurry was stirred vigorously for 2 min and poured onto a course sintered-glass funnel, washed with 10 ml each of cold 0.1 *M* sodium bicarbonate, pH 9.5; cold water; and cold 0.1 *M* sodium borate buffer, pH 7.5 containing 50 m*M* $CaCl_2$. Ten ml of activated Sepharose 6B suspended in cold 0.1 *M* sodium bicarbonate, pH 9.0 was mixed with 10 ml of water containing 10 mmole of 1,6-diaminohaxane. The pH of the diamine solution was adjusted to pH 9.0 with 6*N* HCl before use and the coupling was performed at 4 °C overnight as described above. The resulted aminohexyl Sepharose was washed with cold water; 0.1 *M* $NaHCO_3$, pH 9.0; 0.05 *M* NaOH; water; 0.1 *M* CH_3COOH and water. The washed product was finally suspended in 10 ml of 0.1 *M* sodium borate-$CaCl_2$ buffer containing 40 mg of 1-cyclohexyl-3 (2-morpholinoethyl)-carbodiimide metho-*p*-toluene sulfonate and 2g of the ligand. After 30 min, the pH of the mixture was adjusted to pH 7.0 and the reaction was allowed to proceed 24 hr at 4 °C. The Sepharose gel was finally filtered and washed as above with cold water, 0.5 *M* NaCl, 2 *M* urea; suspended in distilled water and stored at 4 °C.

The presence of each sugar in the final product was estiblished as follows; D-gluconic acid was identified by a Silica gel thin layer chromatography (solvent; *n*BuOH: pyridine: H_2O, 9 : 5 : 7, v/v), after 4 hr of hydrolysis with 2 *N* HCl at 110 °C. The reducing powers of other sugars were tested qualitatively with 3,5-dinitrosalicylic acid after the hydrolysis as above.

Enzyme Purification. About 10 ml of the wet affinity adsorbent was packed into a column (1×10 cm) and washed thoroughly with distilled water. The enzyme solution was prepared as follows; the broth containing glucose oxidase was freeze-dried to reduce the volume. Subsequently this was dissolved in adequate volume of distilled water. This solution was either dialyzed against distilled water overnight at 4 °C or precipitated with ammonium sulfate (precipitate of 60~90 % saturation was used) and dialyzed as above. The crude enzyme preparation (4×10^3 U of glucose oxidase) was loaded on the top of the column and the flow rate was adjusted to 20 ml per 16 min. After the sample was applied the column was washed with 500 ml of distilled water and the enzyme adsorbed was eluted by the stepwise elution using 50, 100, 200 m*M* and 1*M* KCl solutions. Enzyme-active fractions were further chromatographed on a column of Sepharose 6B (1.85×45 cm) which was previously equilibrated with 0.1 *M* sodium acetate buffer, pH 5.6. Elution was carried out with the same buffer, the flow rate was adjusted to 8.0 ml per 30min, and 8.0 ml fractions were collected. Active fractions were combined, followed dialysis as above, and freeze-dried.

Characterization of the Enzyme. The purity of the enzyme preparation was determined by a 7 % polyacrylamide disc gel electrohporesis according to the method of Davis[24]. The purified enzyme was characterized with respects to the absorption spectrum, pH and temperature dependences, substrate concentration effect and substrate specificity. The absorption spectrum of glucose oxidase was measured by Beckman spectrophotometer Model 25 between the wavelengths of 200 and 450 nm. The 0.1 *M* sodium actate buffer solution (pH 5.6) was used. The pH dependence of the activity was determined using 50 m*M* sodium acetate buffers between pH

4.0 to 5.0, 50 m*M* Tris maleate buffers between pH 6.0 to 7.0, 50 m*M Tris* HCl buffers between pH 8.0 to 9.0 and 50 m*M* glycine-NaOH buffer at pH 10.0. The effect of temperature on the enzyme activity was examined at 6 different temperatures in the range from 20 to 60 °C. The substrate specificity was examined for the various monosaccharides.

RESULTS AND DISCUSSION

Crude Enzyme. The strain of PS-8 was very similar to *Penicillium amagasakiense* by the characteristics of colony and microscopic observation and hence classified *Penicillium* species tentatively[17]. The colonies grown on agar were velvety textured and white. The surface of the colony was smooth and the underside was cherry yellow. The penicillus was typically biverticillate and asymmetric under the microscopic analysis. PS-8 was grown with a submerged culture for 14 days under the conditions of pH 6.0, 30 °C, 1 VVM of aeration, and 500 rpm of agitation and this strain produced 108.1~152 U of glucose oxidase and 32~45mg of protein per 100 m*l* of broth. Specially it produced 6,865 U of catalase per 100 m*l* of broth and the activities of other oxidases were negligible. The broth was freeze-dried to reduce the volume and this was used as the crude enzyme. Treatment of ammonium sulfate at 60~90 % saturation showed that over 97 % catalase was removed while 90.5 % of glucose oxidase was recovered and therefore the ammonium sulfate precipitation was used in certain cases.

Affinity Chromatography. *Table* 1 shows the results of affinity of glucose oxidase to the various Sepharose affinity adsorbents. The results were confirmed by using either the crude or the purified enzyme preparations.

As the results, **D**-arabinose and α-methyl-**D**-glucoside coupled to Sepharose showed no affinity to the glucose oxidase of PS-8. This was also confirmed by using glucose oxidase of *Aspergillus niger* which was purchased from Sigma Chem. Co. (U. S. A) Only **D**-gluconyl-*w*-aminohexyl Sepharose 6 B showed the affinity to the enzyme. On the contrary **D**-gluconic acid coupled by the expoxide coupling showed no affinity at all to the enzyme. Hence **D**-gluconyl-*w*-aminohexyl Sepharose was used for the further purification works.

D-Arabinose is a competitive inhibitor to glucose oxidase[9] and Pazur *et al.*[3] studied the substrate specificities of the enzyme with various sugars. They suggested that equatorially oriented hydroxyl groups at C_1 and C_3 of the monosaccharide and the pyranose oxygen are possible sites of combination of the substrates with glucose oxidase. Furthermore, they found that hydroxyl group at C_6 is important to form the enzyme-substrate complex, since the enzyme can oxidize 6-deoxy-**D**-glucose (10 % refered to glucose) while it can not oxidize 6-*O*-methyl-**D**-glucose.

In view of above considerations, the reason why the **D**-arabinose-coupled adsorbent showed no affinity is not clear. This may be due to very weak binding affinity or the epimerization of arabinose at the alkaline condition of the coupling reaction. It is generallized that immobilization of a specific ligand can decrease its

Table 1. Affinity of glucose oxidase to various Sepharose affinity adsorbents.

Ligands	Method of coupling	Binding of the enzyme to the adsorbent column
D-Arabinose	Epoxide	(−)
D-Arabinose	Aminohexyl	(−)
α-Methyl-**D**-glucoside	Epoxide	(−)
D-Gluconic acid	Epoxide	(−)
D-Gluconic acid	Aminohexyl	(+)

(−) : no binding, (+) : binding.

affinity for the protein by upto three orders of magnitude. In practice, it has proved difficult to prepare adequate adsorbents for enzyme-ligand system whose dissociation constants in free solution are greater than $10^{-3}M$.[25] Therefore, further binding works of **D**-arabinose to glucose oxidase should be carried out to find the reason. It the case of α-methyl-**D**-glucoside, however, the hydroxyl group at C_6 is attached to the matrix by the epoxide coupling[22] and this may interfere the binding of enzyme to the ligand. With the same reason **D**-gluconic acid immobilized to the matrix by the epoxide coupling showed no binding at all.

For **D**-gluconyl-*w*-aminohexyl Sepharose, however, the primary site for the coupling reaction is the carboxyl group at C_1 of **D**-gluconic acid[23] and hence good affinity of the enzyme to the ligand seemed to obtain. In this case **D**-gluconic acid seemed to act as an analog of substrate (glucose), not as one of product (δ-gluconolactone). This was suggested by the result that all enzymes were not retarded on the column and washed out into the effluent, when the enzyme and 50 mM glucose solution were cochromatographed on the column of the **D**-gluconyl-*w*-aminohexyl Sepharose adsorbent. For a bisubstrate reaction of compulsory order, the immobilization of second reaction (B) as ligand will generate an incompetent adsorbent for the complementary enzyme unless first reactant (A) is included in the irrigating buffer. The presence of A is essential for the binding of the enzyme to the adsorbent, since it is the binary A-enzyme complex that binds B.[25] Duke *et al.*[11] showed the reaction mechanism of the glucose oxidase as follows: (1) $E+G \longrightarrow RL$; (2) $RL \longrightarrow R+L$; (3) $R+O_2 \longrightarrow E \cdot H_2O_2$; (4) $E \cdot H_2O_2 \longrightarrow E+H_2O_2$; and (5) $RL+G \longrightarrow R+L+G$, where E is oxidized enzyme, G is β-**D**-glucose, R is reduced enzyme, and L is δ-gluconolactone; RL and $E \cdot H_2O_2$ are complexes involving enzyme and products. According to this reaction mechanism, δ-gluconolactone shall have the affinity to R only and R shall be formed at the presence of **D**-glucose. The result, however, showed no binding of the enzyme in the presence of glucose, while it was retarded on the column in the absence of glucose.

Enzyme Purification. Using **D**-glucose-*w*-aminohexyl Sepharose 6B adsorbent, glucose oxidase was purified. The crude enzyme prepared by freeze-drying or 60~90 % ammonium sulfate saturation was applied on the column. The column was washed with 500 m*l* of distilled water and started to elute with 500 m*l* of each 50, 100, 200 mM, and 1M KCl solutions. The elution pattern is shown in *Fig.* 1.

Glucose oxidase was eluted at the concentration of 100 mM KCl solution. However, non-specifically adsorbed catalase was always contaminated at the glucose oxidase fraction in the small amount. This impurity of catalase was not separated from glucose oxidase even when a-specific elution with 50 mM glucose solution was used. With the combination of the ammonium sulfate saturation and the affinity chromatography over 98 % of the catalase activity of the broth was removed. By this affinity chromatography glucose oxidase was purified 14.6 folds and the resulted enzyme preparation showed 2 main and one very faint protein bands by the polyacryl amide gel electrophoresis with the specific activity of 48.7 U/mg protein.

To see the capacity of the affinity chromatographic column, the excess of the purified glucose oxidase was applied and the column was washed thoroughly with distilled water until no glucose oxidase activity was detected in the effluent. The retarded enzyme on the column was eluted with 1 M KCl solution and the enzyme activity was determined. The result showed 115

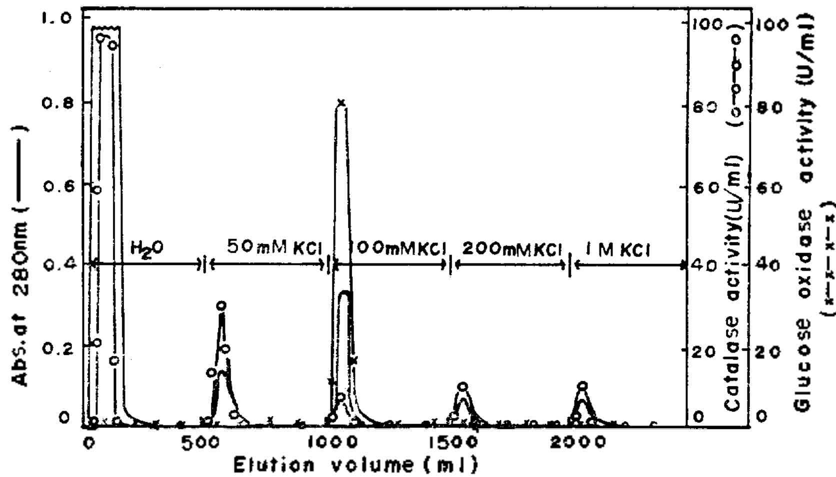

Fig. 1. Elution profile of D-gluconyl-ω-aminohexyl Sepharose 6B affinity chromatography. Flow rate: 20 m*l*/16 min, column size: 2.0×6.5 cm.

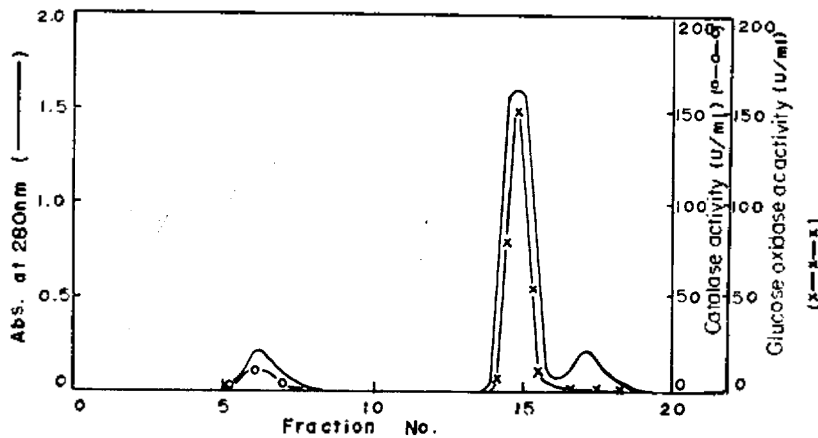

Fig. 2. Gel filtration of glucose oxidase on Sepharose 6 B. Column size: 1.85×45 cm, fraction vol.: 8.0 m*l*, flow rate: 8.0 m*l*/32 min

U of glucose oxidase was adsorbed per m*l* of the wet affinity adsorbent.

Although the enzyme preparation purified by the affinity chromatography showed no other oxidase activities, small amount of catalase, which was adsorbed nonspecifically, was always contained and this catalase was not separated with the specific elution as discussed previously. To remove catalase completely, Sepharose 6B gel filtration was carried out. *Fig.* 2 shows the result of the gel filtration and *Table* 2 summarizes the purification results.

The final preparation showed 90.8 U of glucose oxidase per mg of protein with 27.2 folds of overall purification and 74.1 % of final yield was obtained. It showed a single protein band on the 7 % polyacryl amide gel electrophoretogram as shown in *Fig.* 3 and no catalase activity was detected.

Valyulis *et al.*[16] purified glucose oxidase 5

Table 2. Purification results of glucose oxidase of PS-8.

Procedure	Vol. (m*l*)	Unit/m*l*	Total units	Protein (mg/m*l*)	Specific activity	Yield (%)	Purification	Catalase (U)
Culture broth	2,200	1.07	2,354	0.32	3.34	100	1.0	151,040
60~90% sat'd $(NH_4)_2SO_4$	50	42.6	2,130	4.16	10.24	90.5	3.1	3,630
Affinity chromatography (100 m*M* KCl eluate)	50	37.5	1,875	0.77	48.70	79.7	14.6	234
Gel filtration on Sepharose 6B	16	109.0	1,744	1.20	90.83	74.1	27.2	0

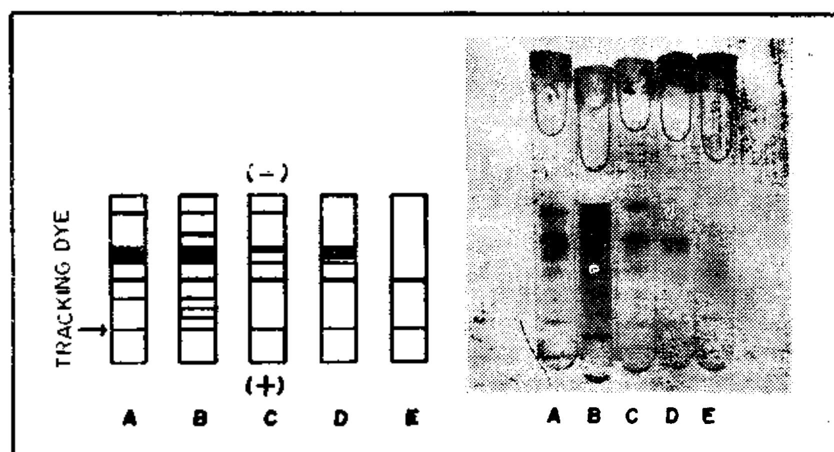

Fig. 3. Polyacrylamide gel electrophoresis of glucose oxidase of PS-8.
A: Commercial glucose oxidase (*A. niger*), B: PS-8 culture broth, C: 60~90% ammonium sulfate saturated fraction. D: preparation by D-gluconyl-*w*-aminohexyl Sepharose 6B affinity chromatography, E: preparation by Sepharose 6B gel filtration with the enzyme preparation obtained from the affinity chromatogaphy.

folds purification by an affinity chromatography on immunosorbent. They prepared the antibody by injecting a highly purified preparation of glucose oxidase to rabbit and purified the antibody by the combination of DEAE-Sephadex and an affinity chromatography in a column containing immobilized glucose oxidase. To prepare the immunosorbent the antibody was attached covalently to Sepharose 4B activated by cyanogen bromide.

Although Valyulis *et al.* purified glucose oxidase by the simple technique, the preparation of the specific antibody may cause more problems. Economic considerations show that the inexpensive ligands/adsorbents must be used and the resulted affinity adsorbents should be stable. In general, ligands can be a substrate, inhibitor, allosteric effector, cofactor, hormone or an analogue of any of these. Among them stable and inexpensive ligands are often used for a large purification. In view of above considerations we have selected D-gluconic acid as a ligand and conclude from this study that the affinity chromatography in the column containing the immobilized D-gluconic acid as ligand represents an effective and potentially very useful for the purification of glucose oxidase from PS-8.

Enzyme Characterization. *Fig.* 4 shows the adsorption spectrum of glucose oxidase of PS-8. It has been known that glucose oxidase is an enzyme containing two flavin adenine dinucleotide (FAD) moieties per molecule[3,7,10,13~15]. The oxidized enzyme of PS-8 also showed very similar adsorption spectrum to those of other sources whose maxima were at 278, 380 and 460 nm.

The pH optima of the enzyme appeared between pH 5.6 and 6.0 as shown in *Fig.* 5 and an assessment of the temperature effect on the activity of glucose oxidase revealed that a temperature transition was observed at 40 °C as shown in *Fig.* 6; and the activation energy obtained from Arrhenius plot covering the temperature range from 20 to 60 °C gave value close to 3.43 kcal/mol/degree with glucose as substrate.

In an attempt to test the effect of substrate concentrations on the activity of the enzyme, a kinetic assay was conducted with D-glucose as the variable substrate concentrations. The results are given in *Fig.* 7. From this result, the Michaelis constant, K_m, is $8.5\times10^{-3}M$. This value is lower than the value of $1.15\times10^{-2}M$ for the enzyme of *P. amagasakiense*[7] and higher than the value of $4.2\times10^{-3}M$ for the enzyme of *A. niger*[5].

Table 3 shows the substrate specificity of glucose oxidase of PS-8 and 1 *M* solution of each

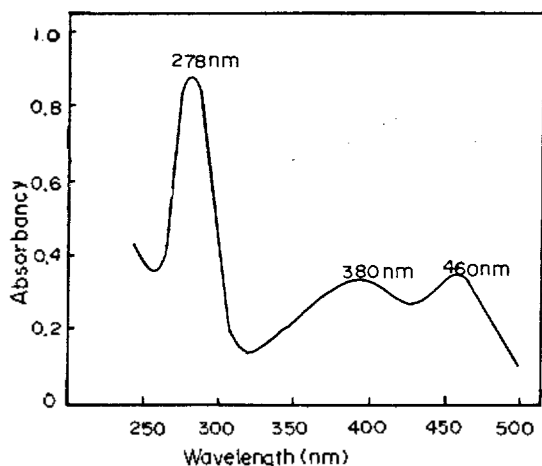

Fig. 4. Absorption spectrum of glucose oxidase of PS-8.

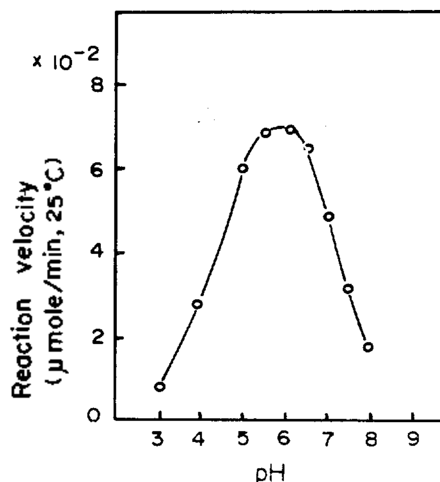

Fig. 5. Effect of pH on the glucose oxidase oxidase activity, 25°C.

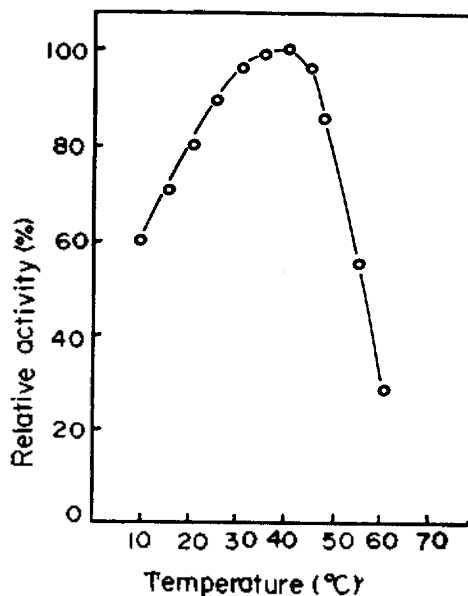

Fig. 6. Effect of temperature on the glucose oxidase activity.

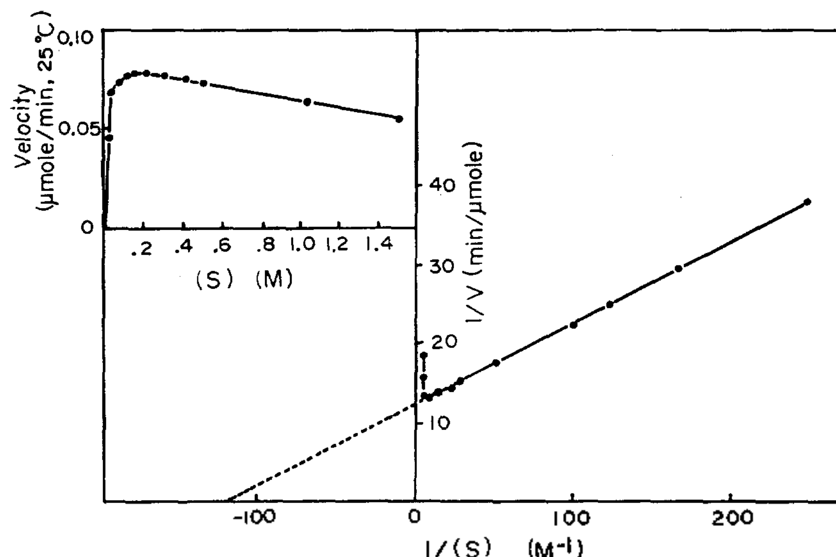

Fig. 7. Lineweaver-Burk plot of $1/V$ against $1/(S)$ for glucose oxidase of PS-8.

Table 3. Relative rate of oxidation of various sugars by glucose oxidase of PS-8.

Substrate	Activity of glucose oxidase (U)	Relative activity (%)
D-Glucose	19.2	100
L-Glucose	0	0
D-Glucosamine	0	0
D-Galactose	0.4	2.1
5-S-D-Glucose	0.02	0.1
D-Arabinose	0	0
L-Arabinose	0	0
α-Methyl-D-glucoside	0	0
D-Sorbitol	0	0
D-Mannose	0	0

monosaccharide was used. As the results the enzyme was highly specific to D-glucose as the enzymes of other sources were.

From the results of the characterization of the enzyme, glucose oxidase of PS-8 showed the similar properties with that of *P. amagasakiense.*

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