Purification and Properties of Pyranose Oxidase from *Coriolus versicolor*

Yozo MACHIDA and Toru NAKANISHI

Tokyo Research Laboratory, Kyowa Hakko Kogyo Co., Machida-shi, Tokyo 194, Japan

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Coriolus versicolor KY2912 grown on a medium containing glucose, sucrose or glycerol produced pyranose oxidase. Pyranose oxidase (glucose-2-oxidase) was purified by HPA-75 chromatography, Sepharose 4B and Sephadex G-100 gel filtration, and hydroxyapatite chromatography. The purified enzyme preparation showed a single protein band on acrylamide gel electrophoresis. The highest activity was obtained when D-glucose was employed as substrate and molecular oxygen as electron acceptor. The enzyme was most active at pH 6.2 and 50°C, stable in the pH region between 5.0 and 7.4, and the activity was completely lost above 70°C. The activity was inhibited by Ag^+, Cu^{2+} and PCMB. The enzyme contained FAD covalently bound to the polypeptide chain. The enzyme consisted of identical subunits with a molecular weight of 68,000, and showed a total molecular weight of 220,000.

Glucose oxidase and glucose dehydrogenase are known as glucose oxidizing enzymes. These enzymes catalyze the oxidation of D-glucose at the first carbon atom to form gluconolactone.[1～3] On the other hand, pyranose oxidase (PROD) (EC 1.1.3.10) (glucose-2-oxidase) has been shown to catalyze the oxidation of several carbohydrates at the second carbon atom to yield 2-keto products and hydrogen peroxide.[4～7]

PROD was found in basidiomycetes and purified 17-fold from *Polyporus obtusus*,[4～6] but some properties of the enzyme remain unknown.

We screened for PROD activities in basidiomycetes grown on a medium containing glucose, and found *Coriolus versicolor* KY2912 to be prominent in the enzyme activity. The *Coriolus* PROD was purified to homogeneity. The enzyme revealed a high substrate specificity, *i.e.*, it was particularly active on glucose. This paper reports on the purification and some properties of PROD of *C. versicolor* KY2912.

MATERIALS AND METHODS

Cell cultivation and crude enzyme extract preparations. The basidiomycetes examined were culured aerobically on a medium containing 0.5% glucose, 0.4% yeast extract, 1.0% malt extract and 0.001% $FeCl_3 \cdot 6H_2O$ at pH 6.0 for 2～7 days at 30°C.

The grown cells were collected by filtration, suspended in 50 mM Tris-HCl buffer (pH 7.0), and disrupted with an Ultrasonic Disruptor Model UR-200P (Tomy Seiko Co., Ltd.) for 7 min. The disrupted mixture was centrifuged and the supernatant used as a crude enzyme extract.

Enzyme assay. PROD was assayed by determining H_2O_2 produced during oxidation of glucose.[8] The standard assay mixture contained 100 μmol Tris-HCl buffer (pH 7.0), 2 μmol 4-aminoantipyrine, 2 μmol phenol, 4 units peroxidase, 0.01 μmol FAD, 0.1 m mol glucose and enzyme in a total volume of 3 ml. The reaction was performed at 37°C for 10 min, and the production of H_2O_2 was determined from the resulting absorbance at 500 nm. A molecular extinction coefficient of 5.34×10^3 was used.[8] One unit of enzyme activity was defined as 1 μmol H_2O_2 production from glucose per min at pH 7.0 and 37°C.

Protein assay. The amount of protein was determined by the method of Lowry *et al.*[9] using bovine serum albumin as a standard. In chromatographic procedures, the absorbance at 280 nm was measured.

Electrophoresis. Analytical disc electrophoresis was

carried out according to the method by Davis[10] using columns of 7.5% polyacrylamide gel with a pH 8.3 buffer system. Protein samples were loaded on the columns and a constant current of 4 mA per column was applied at room temperature. The gel was stained for protein with Amido Black 10B.

SDS-Electrophoresis. SDS-electrophoresis was performed by a modification of the method of Weber and Osborn[11] with columns of 10% polyacrylamide gel containing 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA per column at room temperature. Cytochrome *c* (molecular weight, 12,400), myoglobin (17,800), chymotrypsinogen (25,000), ovalbumin (45,000), bovine serum albumin (68,000), and phosphorylase *b* (92,500) were used as molecular weight markers.

Molecular weight determination. The molecular weight was determined by gel filtration according to the method of Andrews[12] with a column of Sephadex G-200 (2.6 × 83.5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M KCl. Elution was performed with the same buffer. Ovalbumin (45,000), bovine serum albumin (68,000), alcohol dehydrogenase (148,000), γ-globulin (160,000), and milk xanthine oxidase (283,000) were used as molecular weight markers.

Isoelectric focusing. Isoelectric focusing was carried out according to Vesterberg and Svensson.[13] The enzyme protein was applied to a column (110 ml) of carrier ampholite with a pH range of 3.5 to 10.0. A constant potential of 900 V was applied to the column at 5°C for 40.5 hr and the column was fractionated (2 g per fraction).

Thin layer chromatography for the product during oxidation of glucose by the Coriolus enzyme. The reaction mixture contained 0.7 mmol potassium phosphate buffer (pH 7.5), 800 μmol glucose, 4,000 units of catalase and 25 units of the *Coriolus* enzyme in a total volume of 4 ml. The reaction was performed at 25°C overnight with shaking, and the precipitates formed during the incubation were removed by cenrifugation, then the supernatant was spotted onto silica gel thin layer plates. The plates were developed with two solvent systems of *n*-butyl alcohol–pyridine–water (6:4:3 by volume) and phenol–water (4:1 by volume). The plates were dryed and then sprayed with 0.5% potassium permanganate in 1 N NaOH for product detection.

Chemicals. Phosphodiesterase (from snake venom), phosphorylase *b* and xanthine oxidase (from milk) were obtained from Boehringer Mannheim. Peroxidase, ovalbumin and cytochrome *c* were obtained from Sigma. Alcohol dehydrogenase, γ-globulin (from human), and pronase E were obtained from Miles Laboratories, ICN Pharmaceuticals. Inc., and Kaken Chemicals Co., respectively. Other compounds were of reagent grade and from commercial sources.

RESULTS

Pyranose oxidase activity in various basidiomycetes

Pyranose oxidase activity was detected in the genera *Coriolus*, *Coprinus*, *Daedaleopsis*, *Gloephyllum*, *Trametes*, *Agaricus*, *Morchella*, *Pleurotus*, *Lenzites* and *Pholiota*. The basidiomycetes listed in Table I showed considerable activities. Among these basidiomycetes, *Coriolus versicolor* KY2912 showed a high activity with the lowest *Km* value after a short cultivation time. The following experiments were performed with *Coriolus versicolor* KY2912.

Enzyme production

The enzyme activity of the cells grown on

TABLE I. PROD ACTIVITY IN VARIOUS BASIDIOMYCETES

Cultivation of each strain was performed in a 2 liters flask containing 300 ml of the medium on a rotary shaker. Cultured broth in a test tube was inoculated with a 10% volume. PROD activity was measured by the standard assay method, and expressed as the activity in 1 ml of cultured fluid. The *Km* values were obtained from a Lineweaver–Burk plot using crude enzyme extracts as the enzyme source. The various low molecular substances such as glucose were previously removed from crude enzyme extracts with a Sephadex G-25 column for the *Km* determination.

Strain	PROD activity (m units/ml)	*Km* (mM)	Cultivation time (days)
Coriolus hirsutus KY2911	49.6	1.7	2
Coriolus versicolor KY2912	328	0.9	3
Daedaleopsis styracina KY2915	50.6	1.0	2
Gloeophyllum sepiarium KY2940	365	1.8	7

media containing various carbohydrates was compared (Table II). This enzyme activity was found in the cells grown on glucose, surcose, dextrin, starch or glycerol. *Coriolus versicolor* also grew on xylose, mannose or L-sorbose, and in these cases, too, showed fairly high activity. In all of these cases, however, the activity was lower than that of cells grown on glucose.

TABLE II. PYRANOSE OXIDASE ACTIVITY OF THE CELL ON VARIOUS CARBOHYDRATES

Each substrate was added instead of glucose to the medium described in MATERIALS AND METHODS at a concentration of 0.5%. *C. versicolor* KY2912 was cultured for 4 days. The seed broth was cultured on glucose and inoculated with a 10% volume. PROD activity was expressed as the activity in 1 ml of cultured fluid.

Carbohydrate	Cell mass[a] (g)	PROD activity (m units/ml)
Glucose	25.7	221
Galactose	1.5	0
Xylose	13.2	41
L-Sorbose	11.2	23
Mannose	10.2	75
Sucrose	18.3	217
Maltose	1.0	0
Lactose	0.8	8
Dextrin	12.3	131
Starch	17.0	176
Glycerol	29.3	135

[a] Wet cell weight.

Purification of the enzyme

All purification procedures were carried out at room temperature (15~20°C) in 50 mM Tris-HCl buffer (pH 7.0) and centrifugation was carried out at about 10,000 × *g* for 15 min.

The grown cells (wet weight 135 g) were harvested by filtration, suspended in the buffer and disrupted with an Ace Homogenizer (Nihon Seiki kaisha Ltd.). Homogenates were centrifuged and the supernatant solution was used as a crude extract.

After the crude extract was adjusted to pH 7.0 with NaOH, it was applied to a column of HPA-75 (500 ml anion exchange column) (Mitsubishi Kasei Co., Ltd.) previously equilibrated with the buffer. The column was washed with the buffer containing 0.1 M ammonium sulfate and then the enzyme was eluted with the buffer containing 0.25 M ammonium sulfate (Fig. 1). The active fractions were combined and precipitated with ammonium sulfate (0.8 sat).

The precipitates were dissolved in the buffer and applied to a column of Sepharose 4B (500 ml column) equilibrated with the buffer, and the column was eluted with the buffer.

The active fractions were collected and concentrated by ultrafiltration, and then applied to a Sephadex G-100 column (500 ml column). The eluted active fractions were loaded on a column of DEAE-Sephadex A-50 (200 ml col-

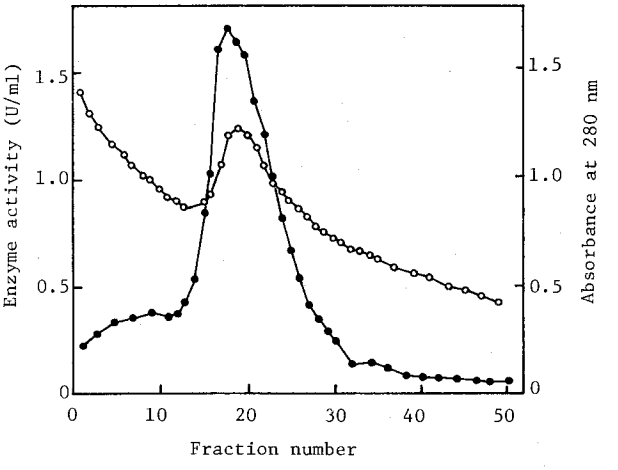

FIG. 1. Column Chromatography of Pyranose Oxidase on HPA-75.
Eluate was collected in 19 g fractions. (○), absorbance at 280 nm; (●), PROD activity.

umn) previously equilibrated with the buffer containing 0.2 M NaCl. The column was washed with the same buffer and then eluted with an increasing linear concentration gradient of from 0.2 ~ 0.6 M NaCl in the buffer. The active fractions were collected and concentrated by ultrafiltration.

The solution was loaded on a column of hydroxyapatite (50 ml column) equilibrated with 10 mM phosphate buffer (pH 6.8) and the enzyme was eluted with a linear buffer concentration gradient of from 10 ~ 100 mM phosphate buffer (pH 6.8). The active fractions were combined and used as the purified enzyme. The results of purification of the enzyme are summarized in Table III. The enzyme was purified by about 110-fold from the crude extract with a yield of 16.8%.

As shown in Fig. 2, the purified enzyme gave a single protein band on analytical disc electrophoresis and SDS-electrophoresis.

Molecular weight and subunit structure

The molecular weight of the purified enzyme was calculated to be about 220,000 by gel

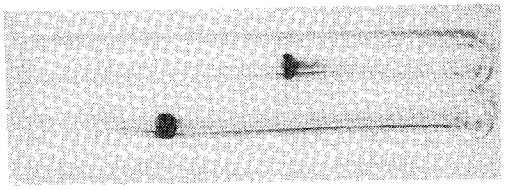

FIG. 2. Polyacrylamide Gel Electrophoresis of the Purified Pyranose Oxidase.

A, disc electrophoresis; B, SDS-electrophoresis. These gels were stained with Amido Black 10B.

TABLE III. PURIFICATION OF PYRANOSE OXIDASE FROM *Coriolus versicolor*

Step	Total activity (units)	Total protein (mg)	Sp. activity (units/mg)	Recovery (%)
Crude extract	955	3,389	0.28	100
HPA-75	738	148	5.0	77
Sepharose 4B	488	40	12.1	51
Sephadex G-100	414	23	18.0	43
DEAE-Sephadex A-50	228	15	15.2	24
Hydroxyapatite	161	5.2	30.9	17

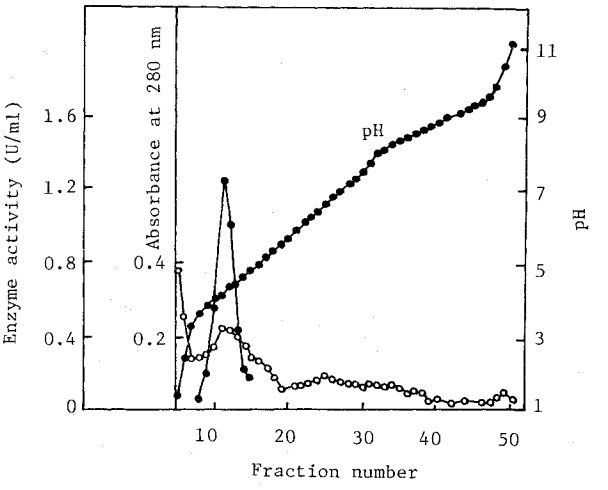

FIG. 3. Isoelectric Focusing of Pyranose Oxidase.

The experimental conditions were given in MATERIALS AND METHODS. (○), absorbance at 280 nm; (●), PROD activity.

filtration on Sephadex G-200. On SDS-electrophoresis (Fig. 2), a single protein band corresponding to a molecular weight of about 68,000 was obtained. These results suggest that the enzyme consists of identical subunits.

Isoelectric point

The isoelectric point of the enzyme was determined to be 4.2 by isoelectric focusing (Fig. 3).

Spectral characteristics

Absorption spectra of the oxidized and reduced forms of the enzyme are shown in Fig. 4. The oxidized enzyme exhibited absorption maxima at 360 and 458 nm in the visible region. Reduction of the enzyme by the addition of sodium hydrosulfite or glucose to the enzyme solution in the cuvette resulted in elimination of the absorption maximum at 458 nm. These spectral characteristics indicate that the enzyme is a flavoprotein.

Treatment of the enzyme in 5% trichloroacetic acid for 10 min at 100°C did not show any release of colored material in the soluble fraction. This shows the presence of a covalently bound flavin in the enzyme. For identification of the flavin, the enzyme was digested with pronase and then incubated with phosphodiesterase.[14] The released AMP was identified by paper chromatography with isobutyric acid–acetic acid–1 N ammonia (10:1:5 by volume) as solvent and from the UV-spectrum. The enzyme was shown to contain FAD, but not FMN, which was covalently bound to the polypeptide chain.

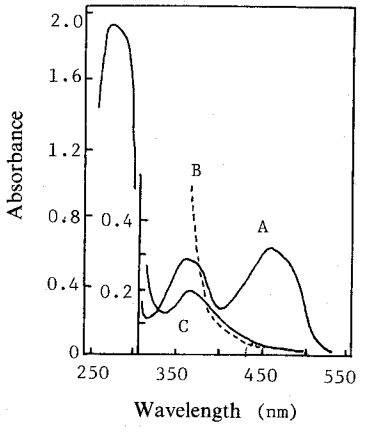

FIG. 4. Absorption Spectra of the Purified Pyranose Oxidase.

A, native enzyme solution; B, the reduced enzyme after the addition of sodium hydrosulfite; C, the reduced enzyme after the addition of glucose.

Effect of pH, temperature and various compounds on the activity

The effect of pH and temperature on the activity was studied. The enzyme was found to be most active at pH 6.2 and 50°C (Fig. 5).

The effect of various metal ions and other compounds on the activity was tested and the results are presented in Table IV. The activity was inhibited by Ag^+, Cu^{2+} and PCMB, and also by Ni^{2+}, Co^{2+}, *o*-phenanthroline, 8-hydroxyquinoline and H_2O_2.

Effect of pH and temperature on the stability

The enzyme was found to be stable in the pH range between 5.0 and 7.4, when kept at 50°C for 30 min. After holding of the en-

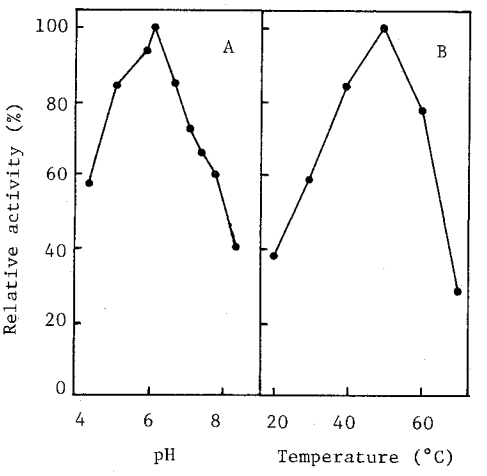

FIG. 5. Effect of pH and Temperature on the Activity of Pyranose oxidase.

(A): Relative activity was determined by measuring the decrease in dissolved O_2 with a Bioxygraph (Kyusui Kagaku Kenkyusho Co., Ltd). The reaction mixtures contained 250 m units of PROD, 50 μmol glucose, and 720 μmol Tris-maleate buffer of several pHs in a volume of 3.7 ml, and were incubated at 37°C.

(B): Enzyme reactions were performed with the standard assay mixture containing 100 m units of PROD.

TABLE IV. EFFECT OF METAL IONS AND VARIOUS COMPOUNDS ON THE ENZYME ACTIVITY

Inhibition was determined by measuring the rate of O_2 consumption during the oxidation of glucose with an oxygen electrode. The reaction mixtures contained 170 μmol Tris-HCl buffer (pH 7.0), 28 m units of PROD, 50 μmol glucose, and one of the compounds listed at 3.7 μmol in a volume of 3.7 ml, and were incubated at 37°C.

Compound	Inhibition (%)
$MnSO_4$	0
$MgSO_4$	0
$CoSO_4$	13.3
$CuSO_4$	35.7
$AgNO_3$	53.3
$NiCl_2$	28.6
$FeSO_4$	0
$ZnSO_4$	13.3
Diethyldithiocarbamate	0
EDTA	0
NaN_3	0
α,α′-Dipyridyl	6.1
o-Phenanthroline	13.3
8-Hydroxyquinoline	13.3
H_2O_2	20.0
PCMB*	50.0
L-Ascorbate	0
KCN	0

* *p*-Chloromercuribenzoate.

zyme at 60°C for 30 min, the residual activity was 90%, but it was lost above 70°C (Fig. 6).

Substrate specificity

The compounds listed in Table V were examined. D-Glucose, L-sorbose, D-xylose, D-mannose and D-galactose were oxidized, and the activity was highest with D-glucose. The activity with L-sorbose or D-xylose was lower than that of the enzymes already reported.[5] The enzyme did not oxidize glycoside, glucosamine or gluconic acid.

When the compounds listed in Table VI were examined as electron acceptors for the oxidation of D-glucose, the activity was highest with O_2. 2,6-Dichlorophenolindophenol or cytochrome *c* also served as an acceptor, but only slight activity was observed with NAD, NADP and ferricyanide.

The *Km* value obtained from a Lineweaver–Burk plot was 0.83 mM for D-glucose.

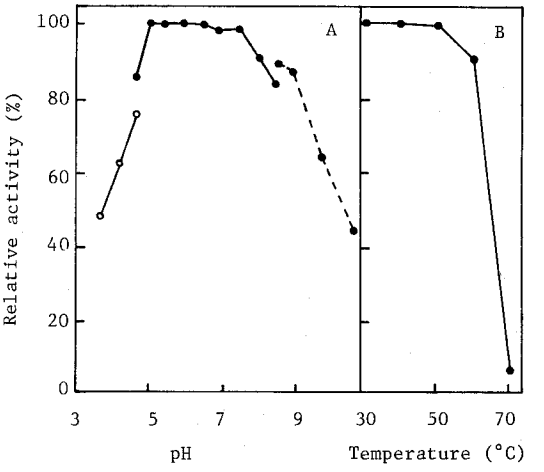

FIG. 6. Effect of pH and Temperature on the Stability of Pyranose Oxidase.

(A): The enzyme (100 m units) was kept at 50°C for 30 min at various pHs, and then the remaining activity was measured by the standard assay method. The following buffers were used: ○—○, sodium phosphate (dibasic)–citrate; ●—●, Tris-maleate–NaOH; ●----●, borate-KCl–sodium carbonate (dibasic).

(B): The enzyme (100 m units) in 40 mM Tris-maleate buffer (pH 6.0) was kept at various temperatures for 30 min, and then the remaining activity was measured by the standard assay method.

TABLE V. SUBSTRATE SPECIFICITY OF THE ENZYME

Enzyme reactions were performed in the standard assay mixture containing 2.5 units of PROD, and 10 μmol of the listed substrate for 5 min.

Substrate	Relative activity (%)
D-Glucose	100
D-Mannose	0.9
D-Galactose	0.9
L-Sorbose	3.4
D-Xylose	2.6
D-Fractose	0
Maltose	0
γ-D-Galactonolactone	0
Gluconic acid	0
D-Glucosamine	0

The difference between the reaction products with glucose oxidase and the glucose oxidizing enzyme from C. versicolor

We compared the product during oxidation of glucose by the *Coriolus* enzyme with gluconic acid or gluconolactone by thin layer chro-

TABLE VI. ACTIVITIES WITH VARIOUS ELECTRON ACCEPTORS

Enzyme reactions were performed anaerobically in mixtures (2.0 ml) containing 100 μmol of Tris-HCl buffer (pH 7.0), 50 μmol glucose, 250 m units of PROD, and one of the compounds listed at the indicated concentration at 37°C, and activities were determined by measuring the absorbance change at the wavelengths indicated between 30 and 90 sec.

Electron acceptor	Concentration (mM)	Wavelength observed (nm)	Relative activity (%)
O_2	Dissolved	500	100
2,6-Dichlorophenolindophenol	0.04	600	2.5
Nitroblue tetrazolium	0.1	540	0
Cytochrome *c*	0.1	550	0.3
NAD	0.1	340	0
Ferricyanide	0.5	420	0

matography. The chromatograms obtained with two solvent systems showed that the product during oxidation of glucose by the *Coriolus* enzyme has a different *Rf* value from those of gluconic acid or gluconolactone. Therefore, we inferred that the glucose oxidizing enzyme from *C. vercicolor* is a pyranose oxidase. Strict identification of the product will be described by other biochemists.

DISCUSSION

High PROD activity was found in the cells of *C. versicolor* grown on glycerol as well as glucose. This indicates that PROD is not an inducible enzyme, but is a constitutive enzyme.

Enzyme purification from a large amount of the crude extract of *C. versicolor* was readily performed by using an anion exchanging HPA-75 column. *Coriolus* PROD was purified to give a single band on electrophoresis by the method summarized in Table III. This is the first example of PROD purification.

Coriolus PROD consisted of identical subunits with a molecular weight of 68,000. The spectrum of this enzyme was similar to those of some oxidases.[15~17] The spectrum of the *Coriolus* enzyme included peaks at 360 and 458 nm. The peak at 458 nm disappeared upon reduction with sodium hydrosulfite or glucose. These spectral characteristics suggest that the *Coriolus* enzyme is a flavoprotein. The flavin was covalently bound to the polypeptide chain. The flavin was identified as FAD by paper chromatography after digestion with pronase and phosphodiesterase.

In comparison with glucose oxidase, PROD showed differences in molecular weight and conditions of binding of FAD to the enzyme.[1,18]

Inhibition by PCMB, Ag^+ or Cu^{2+} was observed, and this inhibition suggests that this PROD contains an essential SH group.

It has been reported that PROD catalyzes the oxidation of L-sorbose and D-xylose, in addition to D-glucose.[5] The *Coriolus* enzyme oxidized both the carbohydrates only at very low rates. Although xylose oxidase[19] and sorbose oxidase[20,21] have been reported as the carbohydrate oxidizing enzymes responsible, *Coriolus* PROD is different from these enzymes in substrate specificity.

With the *Coriolus* enzyme, O_2, 2,6-dichlorophenolindophenol and cytochrome *c* served as electron acceptors. Of these electron acceptors, O_2 was the most effective. The rate of glucose oxidation in the presence of 2,6-dichlorophenolindophenol was only 2.5% of that in the presence of oxygen. The specificity for electron acceptors was similar to that of glucose oxidase.[1] The *km* value for glucose was 0.83 mM, which is about one-twentieth of that of glucose oxidase from *Aspergillus niger*.[18]

Coriolus PROD showed a high substrate specificity and a low *Km* value for glucose.

These characteristics allow the enzyme to be used for the determination of glucose instead of glucose oxidase from *Aspergillus niger* or *Penicillium amagasakiense*.

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