A carbohydrate:acceptor oxidoreductase from *Paraconiothyrium* sp. was purified and characterized. The enzyme efficiently oxidized β-(1→4)-linked sugars, such as lactose, xylobiose, and cellobiose:quinasecarbohydrases. The enzyme also oxidized maltoligosaccharides, D-glucose, D-xylose, D-galactose, L-arabinose, and 6-deoxy-D-glucose. It specifically oxidized the β-anomer of lactose. Molecular oxygen and 2,6-dichlorophenol indophenol were reduced by the enzyme as electron acceptors. The *Paraconiothyrium* enzyme was identified as a carbohydrate:acceptor oxidoreductase according to its specificity for electron donors and acceptors, and its molecular properties, as well as the N-terminal amino acid sequence. Further comparison of the amino acid sequences of lactose oxidizing enzymes indicated that carbohydrate:acceptor oxidoreductases belong to the same group as glucooligosaccharide oxidase, while they differ from cellobiose dehydrogenases and cellobiose:quinone oxidoreductases. 

Key words: carbohydrate:acceptor oxidoreductase; lactobionic acid; glucooligosaccharide oxidase; *Paraconiothyrium*; lactose

Lactobionic acid (O-β-galactosyl-(1→4)-gluconic acid, LacA) is produced by oxidation of the reducing terminal of lactose. LacA is manufactured by a chemical process and is utilized as an ingredient of an antibiotic, erythromycin lactobionate, and as a component of a preservative solution for the transplantation of organs. Furthermore, it is expected to have nutritional usefulness, for example, in causing bifidobacteria proliferation and in promoting the intestinal absorption of minerals by forming highly-soluble salts with minerals such as calcium and magnesium. The enzymatic process is advantageous due to the avoidance of poisonous chemicals. Hence we conducted enzymatic oxidation of lactose to broaden the application of LacA. 

Most enzymes that effectively oxidize lactose also oxidize cellobiose:quinone oxidoreductase efficiently and are flavoproteins. These enzymes are separated into two groups depending on the presence of the heme molecule: Cellobiose dehydrogenase (CDH) with heme, and without heme. It is possible to subdivide the latter enzymes into three groups; cellobiose:quinone oxidoreductase (CBQ), carbohydrate:acceptor oxidoreductase (COX), and glucooligosaccharide oxidase (GOOX). It is difficult to distinguish the three enzymes, because the substrate specificities and molecular properties are almost the same. GOOX and COX, however, differ from CBQs in amino acid sequences. COX and GOOX show no sequence homology to CBQ or CDH, whereas the amino acid sequence of GOOX and CDH are similar. These results indicate that COX and GOOX are different enzymes from CBQ and CDH, and that CBQ and CDH belong to the same group. On the other hand, no homology between COX and GOOX has been proposed.

In our previous studies, we isolated a bacterium, *Burkholderia capacia*, that produced LacA efficiently. This enzyme, however, was not necessarily preferable to the manufacture of LacA, since the enzyme was a membrane-bound type that needed to be solubilized, and it is unstable after solubilization. In this study, we obtained a fungus, *Paraconiothyrium* sp. KD-3, that secreted a sugar-oxidizing enzyme. The enzyme was stable enough to be used in the oxidation of lactose. As a result of purification and characterization, the enzyme (PCOX) was identified as a member of COX.

Materials and Methods

Materials. Lactose monohydrate, purchased from Wako Pure Chemical Industries (Osaka, Japan), was used as the α-anomer of lactose. The purity of α-lactose was calculated to be above 95% from the specific rotation ([α]D = +86.7) at 30°C (Horiba polari meter SEPA-200, Kyoto, Japan). β-Anomer of lactose (purity,
95%) was obtained from Kanto Chemical (Tokyo), 2,6-Dichlorophenol indophenol (DCIP), FAD, and cytochrome c were purchased from Sigma Chemical (St. Louis, MO). Cellobioigosaccharides and maltooligosaccharides were from Seikagaku Corporation (Tokyo, Japan) and Wako Pure Chemical Industries respectively.

**Microorganism.** A fungal strain, KD-3, was isolated from soil. The strain was identified as *Paraconiothyrium* sp. by TechnoSuruga (Shizuoka, Japan), according to 18S rDNA and ITS-5.8 S rDNA analysis.

**Culture conditions.** *Paraconiothyrium* sp. strain KD-3 was precultured in 100 ml of medium (500 ml flask) consisting of 7% lactose, 0.5% polypepton (Nihonseiyaku, Tokyo, Japan), 0.1% dried yeast extract D-3 (Nihonseiyaku), 0.2% NH₄NO₃, 0.05% NaCl, 0.13% KH₂PO₄, 0.04% K₂HPO₄, 0.05% MgSO₄ (anhydrous), 0.001% FeSO₄·7H₂O, and 1.0% CaCO₃. The flasks were rotated at 120 rpm at 30 °C for 5 d. The cultured fungus was transferred to 1 liter of the same medium (3 liter flask), and cultivated for a further 9 d under the same conditions.

**Enzyme activity assay.**

**O₂ electrode method.** The reaction mixture, consisting of 1.4 ml of 100 mM lactose in 100 mM acetate buffer (pH 5.5), was vigorously shaken to saturate O₂ at 30 °C. The enzyme solution (100 μl) was added, and the dissolved O₂ concentration was measured with an O₂ electrode (Yellow Springs Instrument, Yellow Springs, OH) at 30 °C. The enzyme activity was calculated from the consumption rate of O₂. One unit of activity was defined as the amount of the enzyme that oxidized 1 μmol of lactose per min.

**DCIP method.** Substrate solution (1.35 ml) consisting of 0.075 mM DCIP and 5 mM lactose in 100 mM acetate buffer (pH 5.5) was mixed with 150 μl of the enzyme solution and incubated at 30 °C. The consumption rate of DCIP was measured by the absorbance at 530 nm (ε₅₃₀ = 7500 M⁻¹ cm⁻¹). One unit of activity was defined as the amount of the enzyme that oxidized 1 μmol of lactose per min.

**Purification procedure for PCOX.** Protein concentrations in chromatographic procedures were tentatively measured by the absorbance at 280 nm (light path, 1 cm). The concentration of purified enzyme was measured with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (Sigma Chemicals, St. Louis, MO) as a standard. A molecular weight value of 54 kDa was adopted for calculation of the mole concentration.

**Step 1. Preparation of crude enzyme solution.** Culture broth was centrifuged at 5,000 g for 30 min. The supernatant (7 liter, 0.11 U/ml) was concentrated to 1 liter by ultrafiltration (Minikros Sampler M21-S3000 module, Spectrum Laboratories, Rancho Dominguez, CA).

**Step 2. Ammonium sulfate precipitation.** Solid ammonium sulfate was added to the crude enzyme solution to 50% saturation. After storage at 5 °C for 5 h, precipitates were removed by centrifugation at 5,000 g for 30 min. Solid ammonium sulfate was added again to 75% saturation. After storage at 5 °C for 5 h, the precipitates were collected by centrifugation at 5,000 g for 30 min and were then dissolved in 10 mM acetate buffer (pH 5.5).

**Step 3. DEAE-Toyopearl column chromatography.** The active fraction was dialyzed against 10 mM acetate buffer (pH 5.5). The dialyzed solution was applied onto a DEAE-Toyopearl column (5.0 × 20 cm, Tosoh, Tokyo) equilibrated with 10 mM acetate buffer (pH 5.5). The enzyme was eluted by linear gradient from 0 to 0.5 M NaCl in 10 mM acetate buffer (pH 5.5) at a flow rate of 1.0 ml/min. The active fractions were combined and dialyzed against 10 mM acetate buffer (pH 5.5).

**Step 4. Pros-Q/EM column chromatography.** Pros-Q/EM column chromatography was done under the following conditions: system, fast protein liquid chromatography (FPLC, Pharmacia, Uppsala, Sweden); pump, P-500; controller, LCC-500; detection, absorbance at 280 nm; column, Pros-Q/EM column (0.8 × 10 cm, Mitsubishi Chemical, Tokyo); elution, linear gradient from 0 to 0.5 M NaCl in 10 mM acetate buffer (pH 5.5); flow rate, 0.5 ml/min. The active fractions were combined and dialyzed against 10 mM acetate buffer (pH 5.5). The dialyzed solution was used as a purified enzyme preparation.

**Electrophoresis.** Native polyacrylamide gel electrophoresis (PAGE) and SDS–PAGE were done by the methods of Davis and Laemmli respectively. The molecular mass standards were phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa) (Amasham Biosciences UK, Buckinghamshire, UK). The proteins were stained with Quick-CBB (Wako Pure Chemical Industries).

**Estimation of molecular mass by gel filtration.** The molecular mass of the enzyme was estimated by gel filtration under the following conditions: system, FPLC system; pump, P-500; controller, LCC-500; detection, absorbance at 280 nm; column, Superdex 200 (Pharmacia); solvent, 100 mM NaCl in 10 mM acetate buffer (pH 5.5). The molecular mass standards were alcohol dehydrogenase (150 kDa), albumin (66 kDa), and carbonic anhydrase (29 kDa) (Sigma Chemical).

**N-Terminal sequence of PCOX.** N-Terminal amino acid sequence of the purified PCOX (0.01 mg) was determined with a Protein Sequencer PPQS-21A (Shimadzu, Kyoto, Japan).

**Effects of pH and temperature on activity and stability.** (A) Optimum pH: The activities in 100 mM
acetate-HCl buffer (pH 4.0–6.0, DCIP) and 100 mM acetate buffer (pH 4.0–6.0, DCIP and O₂) were measured by the O₂ electrode and DCIP method. pH stability: The enzyme (0.2 U/ml, 200 µl) in 100 mM sodium acetate-HCl buffer (pH 2.0–4.0), acetate buffer (pH 4.0–6.0), phosphate buffer (pH 7.0–8.0), or glycine-NaCl-NaOH buffer (pH 9.0–11.0) was incubated at 30 °C for 24 h. The sample solutions were diluted with 1.8 ml of 10 mM lactose in 100 mM acetate buffer (pH 5.5), and the remaining activities were measured by the O₂ electrode method. (B) Effects of temperature on activity: Reaction mixtures (180 µl) consisting of the enzyme (0.02 U/ml), 10 mM lactose, and 100 mM acetate buffer (pH 5.5) were mixed with 20 µl of 0.75 mM DCIP and then incubated at various temperatures (20–60 °C). Thermostability: Enzyme solutions (180 µl), consisting of the enzyme (0.02 U/ml) in 100 mM acetate buffer (pH 5.5), were incubated at various temperatures (5–50 °C) for 1 h. To the enzyme solution, 100 mM lactose (20 µl) was added, and the remaining activities were measured at 30 °C by the O₂ electrode method.

Absorption spectrum. The absorption spectra of the native enzyme (1.5 µM) and FAD (1.2 µM) were measured by spectrophotometer UV-166 (Shimadzu). The spectrum of the reduced enzyme (1.5 µM) was measured in the presence of 10 mM lactose.

Kinetic parameters for various sugars. O₂ was removed from the reaction mixture by bubbling N₂ before the activity assay with DCIP. The enzyme (0.008 U/ml) and 0.075 mM DCIP in 100 mM acetate buffer (pH 5.5) were incubated with various concentrations of sugars at 30 °C. When O₂ was used as the electron acceptor, the enzyme activity was measured as follows: The substrate solutions, containing various concentrations of the sugars in 100 mM acetate buffer (pH 5.5), were vigorously shaken to saturate O₂, and the enzyme (0.008 U/ml) was added and incubated at 30 °C. The consumption rates of O₂ were measured with an O₂ electrode. The Kₘ and kₐₐ values were calculated from a Hanes-Woolf plot.¹⁹

Electron acceptor specificity and kinetic parameters for DCIP. The reaction mixtures (180 µl), consisting of the enzyme (0.008 U/ml) and 10 mM lactose in 100 mM acetate buffer (pH 5.5), were degassed by N₂ bubbling, and electron acceptor solutions (20 µl) containing cytochrome c (0.5 mM), potassium ferriyanide (10 mM), methylene blue (0.2 mM), and Fe(II)Cl₃ (10 mM) were added to the reaction mixtures and incubated at 30 °C for 10–60 min. Reductions of electron acceptors were monitored by the absorbance at 550 nm (cytochrome c), 420 nm (ferriyanide), 610 nm (methylene blue), and 340 nm (Fe(II)Cl₃). The Kₘ and kₐₐ values for DCIP were calculated from Hanes-Woolf plots, with various concentrations of DCIP and 10 mM of lactose as substrates.

Substrate specificity for α- and β-lactose. The purified enzyme solution was desalted by dialysis against distilled water. α-Lactose (0.2 mM) and β-lactose (0.2 mM) were reacted individually with the dialyzed enzyme (0.1 U/ml) at 25 °C, and the consumption rate of the O₂ was measured by the O₂ electrode method.

Production of LacA. A reaction mixture (3 ml), consisting of 10% lactose (w/v), 0.25 U/ml PCOX, and 1.46% CaCO₃ (w/v), was incubated at 40 °C with a reciprocal shaker (120 rpm). The amounts of LacA and lactose were measured by high performance liquid chromatography, done under the following conditions: column, Asahipak NH2P-50 (Showa Denko, Tokyo); solvent, 40 mM citrate buffer (pH 5.0)/CH₃CN (40/60, v/v); flow rate, 1.0 ml/min; temperature, 40 °C; detection, RI detector (Hitachi High Technologies, Tokyo, Japan).

Results

Purification

Paraconiothyrium sp. KD-3 began to secrete the enzyme into the liquid medium after 5 d of cultivation. The activity reached the highest level at 9 d (0.11 U/ml), and then gradually decreased under further cultivation. Thus, the enzyme was purified from the culture supernatant after 9 d. The results of the purification are summarized in Table 1. The enzyme was purified 226-fold in a yield of 59%. At the two-anion exchange chromatographic steps, a single active peak was observed. DEAE-Toyopearl column chromatography was so effective that the specific activity was increased 38-fold. Only one protein band was observed, when 10 µg of the purified enzyme was analyzed by native PAGE followed by staining with the CBB staining reagent. The molecular mass of the enzyme was estimated to be 52 kDa by gel filtration (data not shown), and 54 kDa by SDS–PAGE (Fig. 1). These results indicate that the enzyme was composed of a single subunit protein.

Absorption spectra

Figure 2 shows the absorption spectra of the purified PCOX in the oxidized and reduced states. The enzyme showed absorption maxima at 385 and 440 nm, which were also observed in FAD. The absorption maxima disappeared when lactose was added in order to reduce the enzyme. These spectral profiles suggest that the enzyme was a flavoprotein that contained FAD. PCOX probably contained one FAD molecule in an enzyme molecule based on the molar ratio of FAD/PCOX (1.1), which was calculated from the absorbance at 440 nm. PCOX had no absorption maxima at 420 or 500–600 nm, which probably originated from heme.

N-Terminal amino acid sequence

The N-terminal sequence of PCOX of 40 amino acid residues was determined and compared with those of
other lactose oxidizing enzymes (Fig. 3). The sequence was homologous to that of COX from M. nivale (55%) and of GOOX from A. strictum (25%). 10,12) PCOX showed no homology to those of CDH and CBQ from Fusarium oxysporum, as discussed below. 7,14,15,20)

Effects of pH and temperature
The effects of pH and temperature on the activity and stability of the enzyme were measured (Fig. 4). The enzyme was stable in a pH range of 2.0–7.0 at 30°C. When O₂ and DCIP were used as electron acceptors, the enzyme was most active at pH 5.5 and 4.5 respectively. The enzyme was stable up to 50°C after incubation for 1 h at pH 5.5, and showed highest activity at 50°C.

Electron acceptor specificity of PCOX
The specificity for electron acceptors other than that for O₂ was examined using lactose as an electron donor substrate. The reactions followed Michaelis-Menten kinetics in the substrate concentration range tested. The Kₘ and kₐₗₜ values determined for DCIP were 0.13 mM and 5.7 S⁻¹ respectively. The maximum reaction rate with DCIP was almost the same as that with saturated O₂. The enzyme did not reduce other acceptors, such as cytochrome c, ferricyanide, methylene blue, or Fe(III)Cl₃, which were favorable to other sugar oxidizing enzymes. 2–7,9)

Substrate specificity for saccharides
The substrate specificity of PCOX was investigated based on Kₘ and kₐₗₜ values determined for various oligosaccharides (Table 2) and monosaccharides (Table 3). When O₂ was used as an electron acceptor, the kₐₗₜ/Kₘ values for lactose, β-(1→4) xylobiose, and cellooligosaccharides were 57, 36 and 42–69.

---

**Table 1. Summary of Purification of PCOX**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total activity (Unit)</th>
<th>Specific activity (Unit/mg)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>13,000</td>
<td>690</td>
<td>0.053</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>2,800</td>
<td>570</td>
<td>0.20</td>
<td>3.7</td>
<td>82</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>53</td>
<td>410</td>
<td>7.7</td>
<td>145</td>
<td>60</td>
</tr>
<tr>
<td>Pros-Q/EM</td>
<td>33*</td>
<td>400</td>
<td>12*</td>
<td>226</td>
<td>59</td>
</tr>
</tbody>
</table>

*Measured by Bio-Rad protein assay kit.

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**Fig. 1. SDS-PAGE of Purified PCOX.**
SDS-PAGE was done with 8% polyacrylamide slab gel. The protein was stained with CBB staining reagent. Lane 1, purified PCOX. Ten μg of the purified enzyme was applied. Lane 2, molecular mass standards, including phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20 kDa).

**Fig. 2. Absorption Spectra of PCOX.**
The absorption spectrum of 1.5 μM of purified PCOX (solid line) was measured. The reducing enzyme (thick solid line) was prepared by mixing the enzyme with 2.5 mM lactose. The absorption spectrum of 1.2 μM FAD is inserted.

**Fig. 3. N-Terminal Amino Acid Sequence of PCOX.**
The N-terminal amino acid sequences of COX from M. nivale and GOOX from A. strictum are compared. The same amino acid residues with PCOX are boxed.
respectively. When the enzyme used DCIP as an acceptor, the $k_{cat}/K_m$ values were 23, 14, and 19–86 respectively. These results indicate that the enzyme effectively oxidized the oligosaccharides with the $\beta$-$(1\rightarrow4)$ glycosyl linkage. PCOX also oxidized maltooligosaccharides with $\alpha$-$(1\rightarrow4)$ glycosyl linkages, although the activities were considerably lower than those against $\beta$-$(1\rightarrow4)$ linked substrates: The $k_{cat}/K_m$ values for the maltooligosaccharides were about $10^2$ to $10^3$ times smaller than that for lactose. PCOX showed no activities on $\alpha$- and $\beta$-glucosides with $(1\rightarrow2)$, $(1\rightarrow3)$ or $(1\rightarrow6)$ linkages. The activities of the enzyme against monosaccharides were also much lower than that against favorable oligosaccharides such as lactose (Table 3): The $k_{cat}/K_m$ value for D-glucose was about $10^4$ times smaller than that for lactose. In addition to D-

![Fig. 4. Effects of pH (A) and Temperature (B) on Activity and Stability of PCOX.](image)

A. optimum pH ($O_2$, dotted line; DCIP, thick solid line): The relative activities of PCOX in 100 mM sodium acetate-HCl buffer (pH 2.0–4.0, ●) and 100 mM acetate buffer (pH 4.0–6.0, ○) are indicated. pH stability (solid line): PCOXs in 100 mM sodium acetate-HCl buffer (pH 2.0–4.0, ●), acetate buffer (pH 4.0–6.0, ○), phosphate buffer (pH 7.0–8.0, □), and glycine-NaCl-NaOH buffer (pH 9.0–11.0, △) were treated at 30 °C for 24 h. The residual activities were measured. B. effects on activity (solid line): PCOXs in 100 mM acetate buffer (pH 5.5) were reacted with 10 mM lactose at various temperatures (20–60 °C). Effects on stability (dotted line): PCOXs were incubated at various temperatures (25–70 °C) for 24 h. After treatment, substrate solution was added and the remaining activities were measured.

Table 2. $K_m$ and $k_{cat}$ Values of PCOX for Di- and Oligosaccharides

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (S$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ S$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}$ (S$^{-1}$)</th>
<th>$k_{cat}/K_m$ (mM$^{-1}$ S$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Disaccharide</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Lactose</td>
<td>0.11</td>
<td>6.3</td>
<td>57</td>
<td>0.19</td>
<td>4.4</td>
<td>23</td>
</tr>
<tr>
<td>Celllobiose</td>
<td>0.13</td>
<td>9.0</td>
<td>69</td>
<td>0.086</td>
<td>7.4</td>
<td>86</td>
</tr>
<tr>
<td>Xylobiose</td>
<td>0.096</td>
<td>3.3</td>
<td>33</td>
<td>0.21</td>
<td>3.0</td>
<td>14</td>
</tr>
<tr>
<td>Maltose</td>
<td>α-Glc (1→4) Glc</td>
<td>11</td>
<td>2.0</td>
<td>0.18</td>
<td>4.9</td>
<td>0.76</td>
</tr>
<tr>
<td>Kojibiose</td>
<td>α-Glc (1→2) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Sophorose</td>
<td>β-Glc (1→2) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Nigerose</td>
<td>α-Glc (1→3) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>β-Glc (1→3) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Isomaltose</td>
<td>α-Glc (1→6) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Gentiobiose</td>
<td>β-Glc (1→6) Glc</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Melibiose</td>
<td>α-Gal (1→6) Glc</td>
<td>—</td>
<td>—</td>
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<tr>
<td><strong>Oligosaccharide</strong></td>
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<td></td>
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<tr>
<td>Cellotriose</td>
<td>0.11</td>
<td>7.5</td>
<td>68</td>
<td>0.062</td>
<td>1.5</td>
<td>24</td>
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<tr>
<td>Cellotetraose</td>
<td>0.26</td>
<td>11.0</td>
<td>42</td>
<td>0.060</td>
<td>1.4</td>
<td>23</td>
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<tr>
<td>Cellopentaose</td>
<td>0.15</td>
<td>9.0</td>
<td>60</td>
<td>0.030</td>
<td>1.1</td>
<td>36</td>
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<tr>
<td>Cellohexaose</td>
<td>0.16</td>
<td>10.0</td>
<td>63</td>
<td>0.058</td>
<td>1.1</td>
<td>19</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>30</td>
<td>2.3</td>
<td>0.076</td>
<td>18</td>
<td>2.2</td>
<td>0.12</td>
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<tr>
<td>Maltotetraose</td>
<td>30</td>
<td>2.3</td>
<td>0.076</td>
<td>12</td>
<td>2.6</td>
<td>0.21</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>32</td>
<td>1.8</td>
<td>0.056</td>
<td>11</td>
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<td>0.082</td>
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<tr>
<td>Maltohexaose</td>
<td>13</td>
<td>0.57</td>
<td>0.044</td>
<td>30</td>
<td>0.35</td>
<td>0.012</td>
</tr>
</tbody>
</table>

—, Not reacted.
glucose, the enzyme oxidized d-galactose, d-xylose, l-arabinose, and 6-deoxy-d-glucose. D-mannose, D-glu-
cosamine, 2-deoxy-d-glucose, D-allose, and 3-deoxy-d-
glucose were not oxidized at all.

Substrate specificity for α-Lactose and β-Lactose
Anomeric specificity was examined by incubating α- and β-lactose with the enzyme (Fig. 5). β-Lactose
gave rise to much faster O₂ consumption, while the O₂ consumption rate on α-lactose was extremely slow.
The slow O₂ consumption was probably not due to oxidation of α-lactose by PCOX, but to oxidation of the
β-lactose formed by mutarotation. The oxidation started responding to the addition of β-lactose to the reaction.
These results support the thesis that PCOX specifically oxidized the β-anomeric form of the substrate.

LacA production by PCOX
Oxidizing activity at high lactose concentrations was investigated. By the addition of solid CaCO₃, the pH
of the reaction mixture was easily kept in a moderate pH range of 4.0–6.0. Lactose (10%) was oxidized to
produce a stoichiometric amount of LacA without by-products or hydrolysis of the products from lactose
(Fig. 6). Within 7 h, 10% of the lactose was completely converted to LacA. The enzyme was oxidized lactose in
the presence of 20% of LacA, the reaction was inhibited. Nevertheless, the lactose solution was probably oxidized
more than 10%.

---

Table 3. Kₘ and kₐₐ Values for Monosaccharides

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>Kₘ (mM)</th>
<th>kₐₐ (S⁻¹)</th>
<th>kₐₐ/Kₘ (mm⁻¹ S⁻¹)</th>
<th>Kₘ (mM)</th>
<th>kₐₐ (S⁻¹)</th>
<th>kₐₐ/Kₘ (mm⁻¹ S⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexoses</td>
<td></td>
<td></td>
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<tr>
<td>D-Glucose</td>
<td>25</td>
<td>2.1</td>
<td>0.084</td>
<td>20</td>
<td>0.46</td>
<td>0.0023</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>91</td>
<td>1.5</td>
<td>0.016</td>
<td>61</td>
<td>0.25</td>
<td>0.0041</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>D-Allose</td>
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<tr>
<td>D-Altrose</td>
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<tr>
<td>D-Gulose</td>
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<tr>
<td>2-Deoxy-d-glucose</td>
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<tr>
<td>3-Deoxy-d-galactose</td>
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<td>3-Deoxy-d-glucose</td>
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<tr>
<td>6-Deoxy-d-glucose</td>
<td>52</td>
<td>0.45</td>
<td>0.0087</td>
<td>75</td>
<td>0.81</td>
<td>0.011</td>
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<tr>
<td>D-Glucosamine</td>
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<td>—</td>
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<tr>
<td>D-Galactosamine</td>
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<tr>
<td>D-Mannosamine</td>
<td>—</td>
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<tr>
<td>D-Glucuronic acid</td>
<td>—</td>
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<tr>
<td>L-Glucose</td>
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<tr>
<td>Pentose</td>
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<tr>
<td>D-Xylose</td>
<td>23</td>
<td>1.7</td>
<td>0.073</td>
<td>6.7</td>
<td>0.58</td>
<td>0.087</td>
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<tr>
<td>L-Arabinose</td>
<td>47</td>
<td>0.73</td>
<td>0.016</td>
<td>85</td>
<td>1.1</td>
<td>0.013</td>
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<tr>
<td>D-Ribose</td>
<td>—</td>
<td>—</td>
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<tr>
<td>D-Arabinose</td>
<td>—</td>
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</tr>
<tr>
<td>—, Not reacted.</td>
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</tbody>
</table>

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Fig. 5. O₂ Consumption of PCOX in the Reaction of α-Lactose or β-Lactose.
PCOXs were incubated with 0.2 mM β-lactose (●) or α-lactose (○) to measure O₂ consumption. After 2 min, 1/100 volume of
20 mM β-lactose was added (⊗) to the reaction mixture of 0.2 mM
α-lactose. O₂ saturation at 30°C was defined as 100%. The arrow
indicates the addition of β-lactose.

Discussion
PCOX showed oxidation activity against various oligosaccharides, such as lactose, cellooligosaccharides,
and maltooligosaccharides, as is common for other
related enzymes, COX, GOOX, CDH, and CBQ. However, COX, GOOX, and CBQ differ from CDH in the following properties: no activity against cytochrome c, a smaller molecular mass (50–60 kDa), and the absence of heme (Table 4). COX, GOOX, and CBQ had very similar properties; for example, a molecular mass of 55–60 kDa, broad substrate specificities, and the presence of FAD as a prosthetic group. So far, however, these enzymes have not been distinguished strictly. The homology between COX and CDH, we propose a new classification that divides the enzymes into two groups: the COX and GOOX to COX group, and the CDH and CBQ to CDH groups.

PCOX oxidized oligosaccharides having α- and β-(1→4) glycosyl linkages (Table 2) and several monosaccharides (Table 3). In the presence of O₂ as an electron acceptor, the \( K_m \) values for the substrates with the β-(1→4) linkage were almost the same. The structure of the sugars at the nonreducing ends, the presence of C-6 hydroxymethyl residue, and the position of C-4 hydroxyl residue did not affect the affinity of the enzyme for the substrates. On the other hand, the \( k_{cat} \) values of these substrates indicated that the turnover rates of these substrates were faster, in the following order: celllobiose > lactose > xylobiose. These results indicate that the enzyme is applicable in the production of oxidized cellooligosaccharides and xylooligosaccharide in addition to LacA.

The structures at C-6 position of d-glucose, 6-deoxy-d-glucose, d-glucuronic acid, isomaltose, and gentiobiose were hydroxymethyl residue, methyl residue, carboxyl residue, α-glucosyl residues, and β-glucosyl residues respectively, and d-xylose did not have the C-6 carbon. The activities of PCOX against these sugars

Table 4. The Properties of PCOX and Other Sugar Oxidizing Enzymes

<table>
<thead>
<tr>
<th></th>
<th><em>P. nival</em></th>
<th><em>S. pulverolentum</em></th>
<th><em>A. strictum</em></th>
<th><em>P. chrysosporium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Optimum pH</strong></td>
<td>( 5.5 (O_2), 4.5 ) (DCIP)</td>
<td>( 5.5 (O_2) )</td>
<td>( 6.0 (O_2) )</td>
<td>( 10.0 (O_2) )</td>
</tr>
<tr>
<td><strong>Anomeric specificity</strong></td>
<td>( \beta )-from</td>
<td>N.D.</td>
<td>N.D.</td>
<td>( \beta )-from</td>
</tr>
<tr>
<td><strong>Absorption derived from</strong></td>
<td>FAD</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Molecular mass (kDa)</strong></td>
<td>54 (SDS-PAGE)</td>
<td>55</td>
<td>60</td>
<td>61</td>
</tr>
<tr>
<td><strong>Substrate specificity (( k_{cat}/K_m ))</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactose (mM⁻¹ S⁻¹)</td>
<td>57 ( (O_2), 23 ) (DCIP)</td>
<td>N.D.</td>
<td>17–15 (DCIP)</td>
<td>210 ( (O_2) )</td>
</tr>
<tr>
<td>Celllobiose (mM⁻¹ S⁻¹)</td>
<td>69 ( (O_2), 86 ) (DCIP)</td>
<td>0.21, 0.20( (O_2) )</td>
<td>350–330 (DCIP)</td>
<td>110 ( (O_2) )</td>
</tr>
<tr>
<td>Maltose (mM⁻¹ S⁻¹)</td>
<td>0.18 ( (O_2) ), 0.16 (DCIP)</td>
<td>0.55* ( (O_2) )</td>
<td>0.26–0.27 (DCIP)</td>
<td>3.6 ( (O_2) )</td>
</tr>
<tr>
<td>d-Glucose (mM⁻¹ S⁻¹)</td>
<td>0.084 ( (O_2) ), 0.0023 (DCIP)</td>
<td>0.095* ( (O_2) )</td>
<td>0.0030–0.0032 (DCIP)</td>
<td>1.1 ( (O_2) )</td>
</tr>
</tbody>
</table>

| **Reference** | This study | 10 | 9 | 12, 13 | 6, 26 |

N.D., not determined; --, not detected

*values obtained with recombinant enzyme.

**Fig. 6.** Time Course of LacA Production.

Lactose (10%) was oxidized with PCOX. Half mol equivalent of CaCO₃ was added to suppress pH decline. Lactose (●) and LacA (○) were measured by HPLC.
were different, although the configurations of the sugars were identical, except for the C-6 positions. PCOX oxidized D-glucose, 6-deoxy-D-glucose, and D-xylose. Furthermore, D-galactose (the C-4 epimer of D-glucose) and L-arabinose (the C-4 epimer of D-xylose) also acted as substrates. The enzyme, however, showed no activity against D-glucuronic acid or glucobioses with an α- or a β-(1→6) linkage. The enzyme might recognize substrates having a residue smaller than hydroxymethyl groups at the C-6 position. Bulky residues at that position might not be preferable.

PCOX did not oxidize D-mannose (the C-2 epimer of D-glucose), D-glucosamine having the –NH₂ residue at the C-2 position, 2-deoxy-D-glucose, β-allose (the C-3 epimer of D-glucose), or 3-deoxy-D-glucose. Furthermore, glucobioses with the α-(1→2), α-(1→3), β-(1→2), or β-(1→3) glucosyl linkage were not oxidized. The structures of the C-2 and C-3 positions at reducing ends of these sugars are different from those of D-glucose, maltose, or cellobiose. Based on this selectivity, PCOX was assumed to recognize strictly the C-2 and C-3 positions of the substrates. As for the anomeric configuration, the enzyme specifically oxidized β-lactose (Fig. 5), indicating strict recognition of the C-1 position at the reducing end.

As summarized in Table 4, PCOX and GOOX effectively oxidized lactose and cellobio-oligosaccharides, while the activities against maltooligosaccharides and D-glucose were not very high.\(^2\) Cellobio-oligosaccharides are the most preferable substrates for CBQ and for most CDHs, while the activities against lactose, maltoligosaccharide, and D-glucose are lower in general.\(^2\)–\(^9\) COX from \(M. \text{nivale}\) oxidizes not only cellobio-oligosaccharide but also maltoligosaccharide, and D-glucose at similar rates, which differ from those of PCOX: the relative activity of COX against lactose is 52% of that against cellobiose.\(^10\)

\(O₂\) and DCIP were favorable electron acceptors of PCOX and COX from \(M. \text{nivale}\), but these enzymes show no or very low activity against other compounds that are favored electron acceptors for CDH and CBQ, such as methylene blue, ferricyanide, and cytochrome c.\(^2\)–\(^7,9\) GOOX also oxidized substrate using \(O₂\) as an electron acceptor. The high activity against \(O₂\) is a preferable property for LA production, since the electron acceptor is supplied by aeration, while the rates of lactose oxidation by CBQ and CDH in the presence of \(O₂\) are much slower than those in the presence of other favorable electron acceptors.\(^2\)–\(^3,21\) Hence CDH and CBQ may require the addition of electron acceptors other than \(O₂\) to the reaction mixture for effective production of LacA from lactose. The activity against \(O₂\) might distinguish COX group (PCOX, COX, and GOOX) from CBQ.

The optimum pH of PCOX against DCIP and \(O₂\) were pH 4.5 and pH 5.5 respectively (Fig. 4). CDH from \(P. \text{chrysosporium}\) showed almost the same optimum pH against these electron acceptors.\(^2\)–\(^3\) The absorption spectra of PCOX indicate that the enzyme contained one molecule of FAD (Fig. 2). COX, GOOX, CDH, and CBQ show the same absorption maxima (385 and 440 nm), and are reported to be flavoproteins, containing the FAD molecule.\(^5\)–\(^12\) When CDH oxidized the substrates, FAD was reduced to FADH₂.\(^2\) The FAD molecule in PCOX might take part in the same oxidation/reduction process as in CDH.

As reported above, PCOX successfully converted a high concentration of lactose (over 10%) to calcium lactobionate in the presence of \(\text{CaCO}_3\) (Fig. 6). CDHs, lactose dehydrogenase from \(\text{Pseudomonas graminicola}\), and glucose-fructose oxidoreductase from \(\text{Zymomonas mobilis}\) have been investigated as to LacA production.\(^22\)–\(^25\) Compared with PCOX, however, the activities against lactose of these enzyme are not high enough, and the addition of an electron acceptor is often needed to attain effective production. Electron acceptors are not only expensive, but also unfavorable with respect to foods. Therefore, PCOX, COX from \(M. \text{nivale}\), and GOOX, which oxidize lactose using \(O₂\) as an electron acceptor, might have advantages in LacA production. The optimum pH of GOOX was 10, while PCOX reacted under acidic conditions (pH 4.0–5.5). In the oxidation of lactose, pH drops to the acidic range during the reaction, and often to an unstable range. Therefore control of pH in the reaction mixture is requisite in the production of LacA. It might be easier to maintain the pH level at pH 4.0–5.5, which was a suitable pH for PCOX with the addition of \(\text{CaCO}_3\) (Fig. 6). It is impossible to keep a favorable pH level for GOOX (pH 10) with \(\text{CaCO}_3\). Furthermore, in the alkaline region, undesirable isomerization of lactose to lactulose can occur. High activity against lactose at acidic pH, as well as utilization of \(O₂\) as an electron acceptor, might permit PCOX to be a useful and advantageous biocatalyst in the production of LacA.

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