**INTRODUCTION**

Diabetes is a metabolic disease characterized by hyperglycemia, which can lead to eye damage, kidney disease and increased susceptibility to infections, and it is predicted to be the 7th leading cause of death in the world by 2030. Control of blood glucose level is critical for diabetes treatment, and considerable efforts have been made to identify biomarkers that reflect blood glucose levels. 1,5-anhydro-D-glucitol (1,5-AG), the 1-deoxy derivative of glucopyranose, is one of the principal polysaccharides in human blood, and 99.9% of it is reabsorbed by the kidney. In diabetic hyperglycemia, the renal absorption of 1,5-AG is competitively inhibited by glucose, and so the 1,5-AG concentration in blood is decreased. The concentration of 1,5-AG in human blood can be determined by biochemical methods, e.g., by using an oxidase to generate hydrogen peroxide from 1,5-AG, coupled to horseradish peroxidase to provide a measurable absorbance change, but few oxidases exhibit sufficient activity or selectivity for 1,5-AG, because this compound lacks reducing-end.

However, pyranose 2-oxidases (POXs, EC 1.1.3.10) show high reactivity towards 1,5-AG and are candidates for monitoring hyperglycemia. POXs oxidize substrates at the C2 position, producing 2-ketoaldose and hydrogen peroxide as shown in Fig.1. However, they recognize not only 1,5-AG, but also glucose, so enzymatic depletion of glucose is used in a commercial bioassay (e.g., GlycoMark Assay) to increase performance, though this makes the assay more complex. Therefore, there is a need to create or discover new POXs with higher specificity for 1,5-AG.

Here, we focused on POX from the basidiomycete *Phanerochaete chrysosporium* (PePOX), as its structure has already been reported, the protein can be expressed in *Escherichia coli* and this enzyme has thermal stability.
enough for clinical application. P. chrysosporium POX is a tetrameric flavoprotein that contains a binding site for flavin adenine dinucleotide (FAD), and shows activity towards various kinds of aldose, using molecular oxygen or various quinones as electron acceptors. Based on a consideration of the three-dimensional structure of the substrate-binding site of P. chrysosporium POX and the structural difference between glucose and 1,5-AG, we selected alanine 551 of P. chrysosporium POX as a target residue for mutation with the aim of improving the 1,5-AG/glucose activity ratio, since this residue is likely to be important for recognition of the C1 hydroxyl group of glucose. We used site-directed mutation to replace this residue with 19 other amino acids. The obtained P. chrysosporium POX mutants were simply purified by use of a Histidine-tag, and their substrate specificity and kinetic values were determined.

MATERIALS AND METHODS

Cloning of cDNA encoding pyranose 2-oxidase. P. chrysosporium strain K-3 was grown on the Kremer and Wood medium containing 2% cellulose (CF11, Whatman Laboratory Products, Fairfield, USA) as a sole carbon source, based on a previous report. After 3 days of cultivation, mycelia were separated from the culture filtrate on a glass filter membrane (ADVANTEC GA-100, Toyo-Roshi Kaisha Ltd., Tokyo, Japan) and frozen with liquid nitrogen. Total RNA was extracted from the mycelial powder in the same way as described for wild-type P. chrysosporium POX. The amplified gene was ligated into pET-27b(+) vector. The obtained fragment 15 bases (underlined) that correspond to the multicloning site sequence of pET-27b(+) vector. The obtained fragment was ligated into pET-27b(+) digested with NdeI and HindIII by using an In-Fusion® HD Cloning Kit (Takara Bio, Otsu, Japan). The construct was used for BL21(DE3) heat-shock transformation, and the cells were cultured in 200 µL of LB for 60 min at 37 ºC after the heat shock. The recovered cells were transfected on LB Agar plates containing 50 µg/mL kanamycin. Selected transformants were picked up after 16 h and used for sequence analysis.

For protein expression, the selected colony was inoculated into 5 mL liquid LB medium containing 0.02 mM riboflavin, and induced for 24 h at 16 ºC with 0.1 mM IPTG. Cells were harvested by centrifugation at 8,000 × G, 4 ºC for 3 min, and the pellet was disrupted with Bugbuster (Merck Millipore, Tokyo, Japan). The lysate, making up the total cell fraction, was centrifuged (16,000 × G, 20 min, 4 ºC) to isolate the soluble fraction. The cleared lysate supernatant was applied to a His-spin column (GE Healthcare, Tokyo, Japan) pre-equilibrated with binding buffer (pH 8.0), containing 0.5 M NaCl, incubated on ice for 60 min, and centrifuged at 300 × G, 4 ºC for 30 seconds. The column was washed with wash buffer, 50 mM sodium phosphate buffer (pH 8.0), containing 0.5 M NaCl and 20 mM imidazole. The target protein was eluted with elution buffer, 50 mM sodium phosphate buffer pH 8.0, 0.5 M NaCl, 0.5 M imidazole. Eluate fractions were subjected to activity assay.

Preparation of P. chrysosporium POX mutants. The QuikChange method was used for site-directed mutagenesis, with plasmid extracted from the selected transformant as a template and degenerate primers, F3: GACAACGGCGCTCNNNNTGGGCACCTGGCCGCCCGCACACACGACCAAACTTCG, to introduce 15 bases (underlined) that correspond to the multicloning site sequence of pET-27b(+) vector. The obtained fragment was ligated into pET-27b(+) digested with NdeI and HindIII by using an In-Fusion® HD Cloning Kit (Takara Bio, Otsu, Japan). The construct was used for BL21(DE3) heat-shock transformation, and the cells were cultured in 200 µL of LB for 60 min at 37 ºC after the heat shock. The recovered cells were transfected on LB Agar plates containing 50 µg/mL kanamycin. Selected transformants were picked up after 16 h and used for sequence analysis.

RESULTS AND DISCUSSION

Because of their broad substrate specificity, P. chrysosporium POXs
have been considered for the detection of various sugars or glucose-related compounds. However, for quantitative detection of 1,5-AG in blood, the high activity of POXs towards glucose is a critical problem. According to the crystal structure of Pc-POX-glucose complex (PDB ID: 4MII), the C1 hydroxyl group of glucose is hydrogen-bonded to the main-chain carbonyl group of Ala551 in addition to the side-chain of catalytic histidine (His553), and no direct interaction can be found with other amino acid residues (Fig. 2A). Although Ala551 is located close to the catalytic residue His553 and is highly conserved among known fungal POXs, it is substituted with valine or other amino acids in some other enzymes (Fig. 2B), suggesting that mutation of Ala551 would not necessarily eliminate POX activity. Therefore, we generated 19 Pc-POX mutants with mutation of Ala551 and compared their activities towards 1,5-AG and glucose.

Fortunately, all the Pc-POXs including WT were successfully produced as active recombinant proteins in E. coli (Fig. 3A), and were easily purified on a His-tag column, while the specific activities of these enzymes were generally low comparing to WT as shown in Fig. 3B with log scale y-axis. Since WT has an alanine, one of smallest amino acids, at the position, the specific activity towards glucose, 1,5-AG, maltose, xylose and galactose is retained when A551 is mutated to amino acids with smaller side chain such as proline (P), serine (S), cysteine (C), and threonine (T). Arginine (R) mutation also retains activity, possibly because of its flexibility in conformation of the side chain. On the other hand, other mutations showed significant loss of specific activity towards glucose (approximately 1/50). For instance, when A551 was mutated to negatively charged amino acids glutamic acid (E) and aspartic acid (D), amphipathic amino acid methionine (M), and bulky hydrophobic amino acids, such as phenylalanine (F), tyrosine (Y), and tryptophan (W), the specific activities were decreased significantly. In addition, we evaluated kinetic assays of A551L mutant and A551N mutant for determining their enzymatic parameters, and found out that their $K_m/K_c$ decreased significantly (Table 1). This experimental outcome indicates a lowering of mutants enzymatic activity, which coincides with the result of Fig. 3B. The A551 is located around the catalytic histidine (His553). Pc-POX structure may be distorted by the interaction between negatively charged amino acid introduced by A551 mutation and positively charged amino acid His553 or bulky hydrophobic amino acid insertion.

Then, we calculated relative activity to confirm substrate specificity towards 1,5-AG of WT and mutant Pc-POXs. Relative activity is the ratio between the specific activity towards 1,5-AG and glucose, specific activity towards glucose of each specimen is taken as 100. Relative activity towards xylose, maltose, xylose and galactose were also expressed, as was the case in 1,5-AG. As results of specific activity assay and calculation of relative activity, notably, the A551V, A551N, and A551L mutants showed higher relative activity towards 1,5-AG; in particular, A551N and A551L showed 1.5 and 3.6 times higher relative activity towards 1,5-AG, respectively, as shown in Fig. 3C.
value for 1,5-AG to that for glucose was approximately 2.5 and 3.6 times higher for A551N and A551L, respectively, compared to WT. As stated previously, A551L mutant also showed 3.6 times higher relative activity towards 1,5-AG compared to glucose as shown in Fig. 3C, the value of relative activity was almost the same as the ratio of $k_{cat}/K_m$ value for 1,5-AG to that for glucose as shown in Table 1. These results suggest that the experimental results obtained

from Table 1 and Fig. 3C do not contradict each other and the mutations effectively decreased the affinity for glucose relative to that for 1,5-AG. When homology models of A551N and A551L were compared with the template PcPOX crystal structure (Fig. 5), these mutations appeared to exclude a water molecule interacting with the main-chain amide group of Ala551 and thus affect ligand binding. When we calculated the distance between the catalytic residue His553 and both ligands based on the WT crystal structure, we found that the distances between His553 and C1 or C2 of glucose and between His553 and C2 and C3 of 1,5-AG were about 3Å. Thus, we concluded that PcPOX might oxidize the C1 and C2 positions of glucose and the C2 and C3 positions of 1,5-AG. On the other hand, C2 of both ligands in the A551N mutant was located near His553, whereas C1 of glucose and C2 of 1,5-AG in the A551L mutant were located near His553. The results may

Table 1. Kinetic parameters for glucose and 1,5-AG.

|       | Glucose | 1,5-AG |       |
|-------|---------|--------|-------|
|       | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$) | $K_m$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (s$^{-1}$mM$^{-1}$) |
| WT    | 0.97 ± 0.156 | 115 ± 2 | 120 | 18.9 ± 4.4 | 82.5 ± 10.3 | 4.4 | 19 | 0.037 |
| A551N | 12.1 ± 0.3 | 0.496 ± 0.021 | 0.041 | 65.7 ± 4.5 | 0.253 ± 0.006 | 0.0038 | 5.4 | 0.094 |
| A551L | 3.44 ± 1.15 | 0.00659 ± 0.00129 | 0.0019 | 26.9 ± 3.5 | 0.000696 ± 0.00027 | 0.00026 | 7.8 | 0.135 |

Fig. 3. Expression and activity comparison of WT (Ala551) and mutant PcPOXs (A551X).

(A) SDS-PAGE gel electrophoresis of crude PcPOXs. STD, Standard molecular weight marker proteins. The upper alphabets are single letter abbreviations for amino acids and they indicate WT (A) or 19 PcPOX mutants with mutation of Ala551. The black arrowhead points to PcPOXs band. (B) Specific activities for substrates (glucose, green; 1,5-AG, red; xylose, blue; maltose, yellow; galactose, purple). The y-axis is logarithmic scale. (C) Relative activities for 1,5-AG, xylose, maltose, and galactose. These expressed the ratio of specific activity for 1,5-AG, xylose, maltose, and galactose, as compared specific activity for glucose of each specimen, which is taken as 100.

Fig. 4. Substrate dependence of oxidation rate by WT and mutant PcPOXs.

Oxidation rates of glucose (blue) and 1,5-AG (red) estimated by measuring hydrogen peroxide formation are shown in the left and right sections, respectively (WT, upper; A551N, middle; A551L, lower).
indicate that the A551N mutant oxidizes the C2 position of glucose and 1,5-AG, while the A551L mutant oxidizes the C1 position of glucose and the C2 position of 1,5-AG.

In conclusion, we selected the A551 residue of PcPOX as a target and created a series of mutants. Among them, the A551N and A551L mutants were less active towards glucose and 1,5-AG, while the A551L mutant oxidizes the C2 position of glucose and more specific for 1,5-AG, relative to WT. Al-C1 position of glucose and the C2 position of 1,5-AG.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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