Biochemical and mutational analyses of a *Trametes* pyranose oxidase and comparison of its mutants in breadmaking

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Abstract
Pyranose oxidase (POx) is a homotetrameric flavoprotein that catalyzes the oxidation of pyranose-configured sugars at position C-2 to corresponding 2-ketoaldoses. The wide substrate specificity makes POx potential for application in various biotechnological industries. In the present study we reported the gene cloning and heterologous expression of a POx from the basidiomycete *Trametes* sp. and functionally expressed the gene in *Escherichia coli* BL21(DE3). Based on sequence alignment, three residues were chosen for site-directed mutagenesis to obtain two single mutants (K312E and E539K) and two double mutants (T166A/E539K and K312E/E539K). In comparison to the wild-type, K312E shifted its optimal pH to 5.5 while the optimal temperature of E539K and K312E/E539K increased by 10 °C. The mutants retained more activities over broader pH ranges and higher temperatures and catalyzed *d*-glucose at higher efficiency (5800–12,667 M⁻¹ s⁻¹ for the mutants versus 5083 M⁻¹ s⁻¹ for the wild-type). The recombinant POx and its mutants were all useful in gluten agglomeration and enlarging the loaf volume, which depends on the amounts of enzymes added. Interestingly, adding the same amount (0.5 nkat/g of flour) of wild-type and mutant enzymes differed in the change of loaf volumes, pinpointing that the catalytic activity is not the sole determinant in applying POx in breadmaking.

Keywords: Pyranose oxidase, *Trametes*, Breadmaking, Gluten agglomeration

Introduction
Pyranose oxidase (POx) (EC 1.1.3.10) is a large and homotetrameric protein that contains covalently bound flavin adenine dinucleotide (FAD) as a co-factor (Hallberg et al. 2004). It is widely distributed in wood-decaying basidiomycetes but less frequently found in ascomycetes like *Aspergillus* (Pisanelli et al. 2012a; Takakura and Kuwata 2003; Volc et al. 1985). POx catalyzes the oxidation of aldopyranoses at position C-2 to generate corresponding 2-ketoaldoses and releases H₂O₂ (Giffhorn et al. 2000). In some cases, it can catalyze the oxidation of C-3 of a few sugars to form 3-keto and 2,3-diketo sugars (Freimund et al. 1998; Volc et al. 2003). The monomeric constituents of plant cell wall polysaccharides such as glucose, xylose, mannose, and galactose and other naturally occurring monomer sugars like sorbose are all substrates of POx (Freimund et al. 1998). POx genes are commonly found in the genomes of lignocellulose-degrading fungi, which have lignin-degrading peroxidases such as lignin peroxidases and manganese peroxidases (Ander and Marzullo 1997; Daniel et al. 1992; Forney et al. 1982; Highley and Murmanis 1985). The released H₂O₂ can activate the lignin peroxidases, manganese peroxidase and versatile peroxidase, which in turn are able to oxidize the lignin components in the plant cell wall.

In addition to its important roles in microbial physiology, the unique properties of pyranose oxidase make it potentially useful in many areas including carbohydrate biotransformation (Haltrich et al. 1998), biosensing (Jürgens et al. 1994), and biosynthesis of rare sugars (Granström et al. 2004). POx can also be used in synthetic
biology due to its capability to generate a fungal pyrone-
antiobiotic cortalcerone from glucose (Baute et al. 1987).

In breadmaking industry, POx performs a role similar
to glucose oxidase: by introducing disulfide bonds into
gluten proteins, it improves the dough strength, rheology,
and bread loaf volume (Decamps et al. 2012a, b). With the increasing evidence that POx could be used
to improve bread dough stability, it is noticed that many
pyranose oxidases tested in breadmaking are limited to
those from *Trametes multicolor* (Decamps et al. 2012a,
2013, 2014). In another aspect, although there have been achievements in engineering POx for better thermosta-

tibility and catalytic efficiency (Bastian et al. 2005; Heck-

mann-Pohl et al. 2006; Masuda-Nishimura et al. 1999), it
is not known how the mutations would affect the behav-
ior of the pyranose oxidase in breadmaking.

To address these issues, in the present study, we cloned
a POX-encoding gene from *Trametes* sp., a basidiomy-
cete white rot filamentous fungus that can efficiently uti-

lize lignocellulose. The gene product was heterologously produced in *Escherichia coli*, purified, and biochemically
characterized. The gene was further subjected to site-
directed mutagenesis and the mutants were compared for
their activity and stability and evaluated for their per-
formance in breadmaking.

**Materials and methods**

**Strains, plasmids, and reagents**

The *E. coli* strains Trans I-T1 and BL21(DE3) from
Transgen (Beijing, China) were used for plasmid amplifi-
cation and expression, respectively. The white-rot fungus
*Trametes* sp. is a strain isolated from Shennongjia Nature
Reserve (Hubei province, China) (Yang et al. 2011, 2012).
The plasmid pET-28a (+) (Invitrogen, Carlsbad, CA) was
used for gene expression in *E. coli*. The LA *Taq* DNA
polymerase and restriction endonucleases were pur-
ed from TaKaRa (Otsu, Japan). The T4 DNA ligase
was obtained from New England Biolabs (Hitchin, UK).
The Pfu DNA polymerase was purchased from Tiangen
(Beijing, China). The substrates d-glucose, d-xylose,
l-sorbose, d-mannose, and d-galactose were purchased
from Sigma-Aldrich (St. Louis, MO). All chemicals were
of analytical grade and commercially available.

**Gene cloning**

*Trametes* sp. was grown at 28 °C in 100 mL of medium
containing 20 g/L of glucose, 5 g/L of yeast extract, 5 g/L
of peptone, 1 g/L of MgSO4 and 2 mg/L CuSO4 with con-
stant agitation at 160 rpm. The mycelia were collected
on the 5th day for RNA extraction when the *Trametes*
sp. culture produced maximum POX activity. The total
RNA was extracted using the TRIzol reagent (Invit-
rogen, Carlsbad, CA) according to the manufacturer's
instructions. The first strand cDNA was synthesized
using the First Strand cDNA Synthesis kit (Fermentas,
Ontario, Canada). The cDNA of *TsPox* was amplified
using the primers *TsPOxf* and *TsPOxr* (Additional file 1:
Table S1) and inserted into the expression vector pET-
28a (+) between the EcoRI and NotI restriction sites to
obtain the expression plasmid pET-28a-*TsPox*.

**Sequence analysis**

Sequence comparisons with known sequences were con-
ducted with BlastP at NCBI (https://blast.ncbi.nlm.nih.
gov/Blast.cgi). A multiple amino acid sequence alignment
was carried out using the ClustalW program (http://
www.ebi.ac.uk/clustalW). The molecular mass of the
mature peptide was calculated using Vector NTI 10.0
software (Invitrogen, Carlsbad, CA). No signal peptide
was predicted for POX by using the SignalP server (http://
www.cbs.dtu.dk/services/SignalP/).

**Construction of mutants**

Two single mutants (E539K and K312E) and two double
mutants (T166A/E539K and K312E/E539K) were gener-
dated by site-directed mutagenesis using pET-28a-*TsPox*
as the template, which was carried out by using the Fast
Mutagenesis System (Transgen, Beijing, China) accord-
ing to the instructions of the manufacturer. Specific
primers (Additional file 1: Table S1) were used to intro-
duce residue substitution, which was confirmed by DNA
sequencing.

**Expression and purification of the POx wild-type
and mutants**

To express active POx and its mutants, all recombinant
plasmids constructed above were individually trans-
formed into the chemically competent cells of *E. coli*. Pos-
itive transformants were cultivated in 1 L-flasks at 37 °C
and shaken at 200 rpm. When the OD600 values reached
0.8–1.0, IPTG at a final concentration of 1 mM was
added to induce the protein expression. After cultivation
at 16 °C for an additional 16 h, the cells were harvested
by centrifugation at 12,000g for 10 min and then re-sus-
pended in a binding buffer (20 mM Tris–HCl, 500 mM
NaCl, 10% glycerol, pH 7.6). The cell wall was disrupted
by sonication followed by centrifugation at 10,000g for
15 min. The supernatant was separated and loaded into
a Nickle-NTA (nitrilotriacetic acid) chelating column (GE
Healthcare, Uppsala, Sweden). The bound proteins were
eluted from the resin with a linear imidazole gradient
(40–500 mM) in the binding buffer. Fractions with POX
activity were analyzed by the sodium dodecyl sulfate–
polyacrylamide gel electrophoresis (SDS-PAGE; 12% sep-
aration gel and 5% spacer gel) and the pure fractions were
collected and combined. The protein concentration was
determined using a Protein Assay Kit (Bio-Rad, Hercules, CA).

**Biochemical characterization**

The POx activity was determined spectrophotometrically at 420 nm and 30 °C by monitoring the formation of H₂O₂ for 3 min with a peroxidase-coupled assay using 2,2′-azinobis (3-ethylbenzthiazolinesulfonic acid) (ABTS) as the chromogen. The standard assay mixture (total volume, 1 mL) contained 1 mM of ABTS in 50 mM potassium phosphate buffer (pH 6.5), 2 U of horseradish peroxidase, 100 mM of d-glucose, and properly diluted POx sample. One unit (U) of POx activity was defined as the amount of enzyme to oxidize 2 μmol of ABTS per POx sample. The pH optimum for the POx activity was estimated at 30 °C in the 200 mM Tris–HCl (pH 2.0–5.0), McIlvaine buffer (200 mM sodium phosphate, 100 mM sodium citrate, pH 6.0–8.0), and 200 mM glycine–NaOH (pH 9.0–12.0). To determine the optimal temperature for activity, the assays were performed at temperatures from 20 to 70 °C at the pH optimum as determined above. For analysis of pH stability, the enzymes were pre-incubated in the 200 mM Tris–HCl (pH 4.0–5.0), McIlvaine buffer (pH 6.0–8.0), or 200 mM glycine–NaOH (pH 9.0) without substrate at 30°C for 1 h, and residual enzyme activities were measured under the standard condition (pH 6.0, 30 °C and 10 min). The thermal stability of the enzymes was determined by measuring the residual activities after incubation at 60 and 65 °C for various durations. The influence of metal ions and chemical reagents on the TsPOx activity was tested at concentrations of 5 and 10 mM, respectively.

**Determination of substrate specificity and kinetic parameters**

The substrate specificities of wild-type TsPOx and its mutants were determined by measuring the enzyme activities at pH 6.5 and 30 °C in the McIlvaine buffer containing 100 mM of d-glucose, d-xylose, d-mannose, l-sorbose or d-galactose as the substrate. Their kinetic parameters were determined in the McIlvaine buffer containing 1–50 mM of d-glucose under pH 6.5 and 30 °C for 5 min. The $K_m$ and $k_{cat}$ values were estimated by fitting the data to the Michaelis–Menten equation using the software GraphPad Prism 5.01 (GraphPad Softwares, La Jolla, CA). The experiments were carried out three times, and each experiment included triplicates.

**Determination of the wheat gluten content**

The wheat gluten was checked for residual starch by iodine solution (0.1 g of iodide and 1 g of potassium iodide in 250 mL water). The wheat gluten content was determined according to the State Standard of the People’s Republic of China GB/T 14608-93 (“Method for determination of wet gluten in flour”). The dough was prepared by mixing 100 g of wheat flour and 50 mL of water, followed by extensive washing with water until no gluten was present. The wet gluten was incubated at 105 °C for 12 h to eliminate the bound water. To investigate the effect of POx on gluten agglomeration, 0.25, 0.5, 0.75, and 1.0 nkat/g flour of wild-type TsPOx or its mutants was supplemented. The wet and dry gluten were then recorded.

**Application potentials of TsPOx and its mutants in breadmaking industry**

The dough was prepared by mixing 100 g of flour, 3.2 g of yeast, 3.0 g of salt, 12.0 g of sugar, 8.0 g of milk powder, 6.0 g of butter, and 120 g of water. The wild-type TsPOx or its mutants was supplemented at the dosage of 0.25, 0.50, 0.75 or 1.00 nkat/g flour. Dough mixing was performed at room temperature for 3 min. Mixed dough was then transferred to a lightly greased beaker and incubated at 37 °C in a fermentation cabinet. The fermentation in the breadmaking process continued for 90 min. After fermentation, the dough was punched, transferred to a greased metal baking plate, and incubated at room temperature for a final proof of 45 min. The proofed dough was subsequently baked at 180 °C for 30 min. The loaf volume and weight were measured after cooling down to room temperature for 60 min.

**Nucleotide sequence accession number**

The nucleotide sequence of the TsPOx gene was deposited in the GenBank database with the accession numbers MG344741.

**Results**

**Gene cloning and sequence analysis**

A putative POx gene was identified in the draft genome of *Trametes* sp. Its cDNA was predicted to contain 1866 bp and had a G+C content of 64.1%. The gene was named *TsPOx* and the deduced TsPOx protein had a calculated molecular mass of 69.2 kDa and a pl value of 6.45. TsPOx shared the highest identity (90%) to the POx from *Trametes hirsute* (GenBank accession number: P59097).

**Expression and purification of recombinant TsPOx**

The cDNA fragment coding for the mature TsPOx was ligated into pET-28a(+) to obtain the recombinant plasmid pET-28a-TsPOx, which was then transformed into *E. coli* competent cells. After IPTG induction at 16 °C for 16 h, the cells were disrupted and the crude enzyme was collected. Recombinant TsPOx was purified by immobilized metal affinity chromatography. All recombinant
proteins appeared to be composed of two tightly packed bands centering around 69 kDa on the SDS-PAGE gel (Fig. 1), corresponding to the predicted molecular mass. The slightly differing molecular weights of the two bands suggested that the recombinant TsPOx could be truncated when it was produced.

**Design, construction and production of mutant enzymes**
According to the previous studies on mutagenesis of POx for improved thermostability or broadened substrate specificity (Bastian et al. 2005; Heckmann-Pohl et al. 2006; Masuda-Nishimura et al. 1999), three residues (T166, K312, and E539) of TsPOx were selected for mutation (Additional file 1: Figure S1). Two of these mutations have been studied in the TsPOx homologs in Trametes spp. (Spadiut et al. 2009a, b). The substitutions T166A and K312E correspond to the mutants T158A of P2OxA1 from Peniophora sp. (Heckmann-Pohl et al. 2006) and K312E of P2OxB1 from Peniophora gigantean (Bastian et al. 2005), respectively, which had increased catalytic efficiencies on glucose, sorbose, and xylose. The substitution E539K is equivalent to the E542K of a POx from Trametes versicolor (and T. multicolor) with improved thermostability (Masuda-Nishimura et al. 1999; Spadiut et al. 2009a). Therefore, the double mutations T166A/E539K and K312E/E539K were designed in the hope of obtaining mutants with higher catalytic efficiency and better thermostability. By using the recombinant plasmid pET-28a-TsPOx as template, the four mutant plasmids bearing K312E, E539K, T166A/E539K and K312E/E539K substitutions were obtained. These plasmids were individually transformed into the E. coli BL21(DE3) competent cells for heterologous expression and purification. The purified recombinant mutant enzymes showed similar apparent molecular masses to the wild-type TsPOx on SDS-PAGE (Fig. 1).

**Effects of pH and temperature on TsPOx activities**
The TsPOx activities were assayed using d-glucose as the substrate. The purified recombinant TsPOx displayed an optimal pH of 6.0 at 30 °C (Fig. 2a), which is similar to other fungal POxes (Table 1). After incubation at pH 4.0–9.0 and 30 °C for 1 h, the enzyme retained more than 56% of its original activity (Fig. 2b). The optimal temperature of TsPOx was determined to be 50 °C (Fig. 2c), which falls within the optimal temperature range (40–60 °C) of most reported POxes (Table 1). The enzyme exhibited good thermostability at 60 °C for 1 h (data not shown) but rapidly lost its initial activity within 40 min at 65°C (Fig. 2d).

Among the mutants, K312E had a down-shifted optimal pH to 5.5, while WT and all other mutants had pH optima of 6.0 (Fig. 2a). Besides, the mutants were stable over the pH range of 5.0–9.0 as TsPOx, but generally retained greater activities (Fig. 2b). The temperature optima of E539K and K312E/E539K were both 60, 10 °C higher than that of the WT (50 °C) (Fig. 2c). At 65 °C, the mutants E539K, T166A/E539K, and K312E/E539K had 60.7, 64.2, and 71.9% activities respectively, which were higher than that of wild-type TsPOx (36.5% of maximal activity) (Fig. 2c). At the temperature of 70 °C, E539K and K312E/E539K showed above 40.3 and 66.7% of maximal activity, respectively, much higher than that of wild-type TsPOx (hardly any maximal activity) (Fig. 2c). E539K, T166A/E539K, K312E/E539K showed largely increased thermostability. T166A/E539K, E539K, and particularly K312E/E539K retained more activity than the wild-type when the enzymes were pre-treated at 65 °C for 1 h (Fig. 2d).

**Substrate specificity and kinetic analyses**
The wild-type TsPOx had the highest specific activity towards d-glucose (6.4 U/mg). When this value was set as 100%, TsPOx had moderate activity on d-xylose (55.8%) and l-sorbose (36%), and minor activity on d-mannose (19.7%) and d-galactose (6.5%).

The kinetic parameters of TsPOx and its mutants were compared in Table 1. In comparison to WT, the mutants, K312E, E539K, and K312E/E539K had decreased $K_m$
but increased $k_{cat}$ (turnover rate) values, which in combination resulted in elevated catalytic efficiencies by 2.1-fold to 2.5-fold. Although the $K_m$ value of mutant T166A/E539K against d-glucose was also decreased, its decreased $k_{cat}$ (from $6.1 \text{ s}^{-1}$ to $2.9 \text{ s}^{-1}$) accounted for the approximately nearly unchanged catalytic efficiency. The results demonstrated that mutations at positions 166, 312, 539 had effects both on the enzyme properties and catalysis of TsPOx.

Effects of metal ions and chemicals on TsPOx

The influence of a range of metal ions and chemical reagents on the TsPOx activity was also tested. Except for SDS and Fe$^{2+}$ that greatly inhibited the TsPOx activity, other chemicals had slightly or negligibly negative effects at both concentrations (5 and 10 mM) (Table 2). Some metals ($\text{Na}^+$, $\text{Co}^{2+}$, $\text{K}^+$, $\text{Ca}^{2+}$, $\text{Ni}^{2+}$, $\text{Mg}^{2+}$, $\text{Zn}^{2+}$, and $\text{Mn}^{2+}$) even had a more or less stimulatory effect on the activity of TsPOx. The great resistance to most metal ions and chemicals may widen the potential application spectrum of TsPOx in more industrial fields.

Effects of TsPOx on gluten aggregation in wheat flour

When different dosages of TsPOx (0–0.75 nkat/g flour) were added to the wheat flour dough, both the wet and dry weights of gluten more or less increased (Table 3). The best improvement was obtained at the concentration of 0.5 nkat/g flour, with the wet and dry weights of 34.2 and 12.6 g, respectively. However, when increasing the concentration of TsPOx to 1.0 nkat/g flour, no elevation in the wet and dry weights of wheat gluten was detected. This suggested that high concentrations of TsPOx might have a negative effect on gluten aggregation. Therefore, the dosage of 0.5 nkat/g flour was used to test the agglomeration effects of TsPOx mutants. Of the four mutants, the double mutant K312E/E539K had the greatest performance. The wet and dry gluten weights treated by K312E/E539K were up to 35.5 and 13.2 g, which were significantly higher than those treated by WT TsPOx. The results indicated that all POxes tested in this study had effects on gluten agglomeration, and the double mutant K312E/E539K was the best performer.

Fig. 2  Biochemical characterization of the purified recombinant TsPOx and its mutants. a pH profile; b pH stability; c temperature profile; d thermostability
Table 1: Biochemical properties of $TsPOx$, its mutants, and other pyranose oxidases

| Enzyme   | Organism     | Optimal pH | Optimal temperature (°C) | Specific activity (U/mg) | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (M$^{-1}$s$^{-1}$) | References            |
|----------|--------------|------------|--------------------------|--------------------------|----------------------|------------|---------------------------------|-----------------------|
| TiPOx   | Trametes sp. | 6.5        | 50                       | 6.4 ± 0.8                | 61.1 ± 0.2           | 1.2 ± 0.1  | 5083                            | This study            |
| E539K   |              | 6.0        | 60                       | 5.3 ± 0.2                | 7.6 ± 0.5            | 0.7 ± 0.0  | 10,857                          |                      |
| K312E   |              | 5.5        | 50                       | 10.2 ± 1.1               | 85.0 ± 0.0           | 0.8 ± 0.1  | 10,625                          |                      |
| K312E/ES39K |            | 6.0        | 60                       | 6.2 ± 0.1               | 7.6 ± 0.2            | 0.6 ± 0.0  | 12,667                          |                      |
| T166A/ES39K |            | 6.0        | 50                       | 2.4 ± 0.0               | 29.0 ± 0.0           | 0.5 ± 0.0  | 5800                            |                      |
| PROD    | Trametes versicolor | 7.0–7.5 | 50                       | 1.2                     | 59.9 ± 1.4           | 1.4        | 42,785                          | Nishimura et al. (1996) |
| E542K   |              | N/A        | 55                       | 13.3                    | 70.6 ± 0.7           | 0.7        | 100,857                         | Masuda-Nishimura et al. (1999) |
| AoPOx   | Aspergillus oryzae | N/A   | N/A                       | 0.58                   | 1.48 ± 2.86          | 2.86       | 517                             | Pisanelli et al. (2012a) |
| AnPOx   | Aspergillus nidulans | N/A   | N/A                       | 1.16                   | 35.44 ± 1.77         | 1.77       | 20,022                          | Pisanelli et al. (2012b) |
| Pyranose oxidase | Tricholoma matsutake | 7.7–8.0 | 50                       | 26.6                   | 111 ± 1.28           | 1.28       | 86,718                          | Takakura and Kuwata (2003) |
| P2Ox    | Peniophora gigantea | 4.5–6.0 | 44                       | 0.29                   | 11.9 ± 0.8           | 0.8        | 14,875                          | Bastian et al. (2005) |
| ES40K   |              | 4.5–6.0    | N/A                       | 13.4                   | 28.5 ± 0.6           | 0.6        | 47,500                          |                      |
| ES40K/312E |            | N/A        | 50                       | 107.6                  | 94.0 ± 5.0           | 0.4        | 269,000                         |                      |
| P2Ox/A  | Peniophora sp. | 5.0–6.5   | 50                       | 2.1                    | 9.4 ± 5.0            | 5.0        | 1880                            | Heckmann-Pohl et al. (2006) |
| E542K   |              | 5.0–6.5    | 58                       | 4.34                   | 19.4 ± 1.0           | 1.0        | 19,400                          |                      |
| T158A/E542K |          | 5.5–6.0    | 51                       | 29.54                  | 133.48 ± 0.47       | 0.47       | 284,000                         |                      |

Table 2: Effect of metal ions and chemical reagents on the activity of $TsPOx$

| Chemicals | Relative activity (%) | Chemicals | Relative activity (%) |
|-----------|-----------------------|-----------|-----------------------|
|           | 5 mM                  | 10 mM     |                       |                       |
| Control   | 100                   | 100       |                       |                       |
| Ni$^{2+}$ | 119.5 ± 2.3           | 125.4 ± 1.7 | 100.0 ± 0.6           | 61.6 ± 0.8            |
| Mg$^{2+}$ | 113.0 ± 2.6           | 120.6 ± 1.6 | 100.0 ± 0.6           | 61.6 ± 0.8            |
| Mn$^{2+}$ | 113.0 ± 1.3           | 123.3 ± 1.7 | 94.9 ± 1.2            | 51.4 ± 1.3            |
| K$^+$     | 110.3 ± 2.2           | 127.6 ± 1.3 | 93.0 ± 0.6            | 73.0 ± 1.1            |
| Na$^+$    | 108.1 ± 2.3           | 125.4 ± 1.7 | 116.0 ± 0.3           | 84.0 ± 0.3            |
| Ca$^{2+}$ | 106.5 ± 0.6           | 120.0 ± 0.8 | 85.3 ± 0.7            | 63.8 ± 2.4            |
| Co$^{2+}$ | 106.5 ± 2.3           | 122.2 ± 0.6 | 0.0                   | 0.0                   |

* Values represent mean ± SD (n = 3) relative to the untreated control samples

Application of $TsPOx$ in breadmaking

The wild-type $TsPOx$ and its four mutants were tested for their effect on enlarging the loaf volume in breadmaking. Addition of different dosage of $TsPOx$ caused change of the bread loaf volume (Fig. 3). For all proteins, adding 0.25 and 0.5 nkat/g flour increasingly enlarged the loaf volume. K312E and T166A/ES39K had a similar enlarging effect to that of the wild-type. E539K and K312E/ES39K performed better at both dosages. However, when increasing the POx dosages to 0.75 and 1 nkat/g flour, no more improvement was detected in the bread loaf volumes.

Discussion

POx is widely distributed in lignocellulose-degrading fungi. It catalyzes the oxidation of pyranoses to corresponding keto-sugars and provides hydrogen peroxide to class II peroxidases. With broad substrate specificity and $H_2O_2$-producing capability, POX has much application potential in many areas. However, unlike the other enzymes such as glycoside hydrolases or laccase involved in lignocellulose degradation, much less attention has been paid on POX regarding either its gene diversity or application. In this study, we explored the genome of a $Trametes$ sp. and discovered a pyranose oxidase gene with homology to other pyranose oxidases. The conservation of residues important for thermostability and catalytic ability enabled us to fast design, construct, and test the mutants with substantially improved characters in breadmaking.

For the $T. versicolor$ POX E542K mutant, an elevated optimal temperature and lower $K_m$ were observed, which is also the case for E539K of the $TsPOx$ (Table 1 and Fig. 2). As shown by the results, the mutant K312E had a decreased $K_m$ and increased $k_{cat}$, which resulted
in a ~2.5-fold improvement in the catalytic efficiency. Combining K312E and E539K further decreased the \( K_m \) to 0.6 mM, similar to that observed in the \( P. \) gigantea P2OxB2H (Bastian et al. 2005). However, this combination did not have much effect on the turnover number. Addition of another mutation T166A did not have a beneficial effect on the enzyme regarding catalytic ability and thermostability. The \( K_m \) of the T166A/E539K decreased to 0.6 mM, similar to that observed for the \( P. \) gigantea P2OxB2H (Bastian et al. 2005). It suggested that the additive effects of combinatorial mutations would depend much on the context of the mutated residues even in pyranose oxidase homologs with high amino acid sequence similarity.

Glucose oxidase and POx have a similar role in oxidizing glucose and producing hydrogen peroxide as one of the products, which is widely believed to be at least partially accounting for their roles in breadmaking (Bonet et al. 2006; Decamps et al. 2014). In the present study, low dosages of TsPOx and its mutants had significant enlarging effects on the bread loaf volume, but high dosages of enzymes didn’t. Similar results have been observed in glucose oxidase or POx in changing dough rheology (Bonet et al. 2006), extracting glutenin (Decamps et al. 2013), and enlarging the loaf volume in breadmaking (Decamps et al. 2012a). Unexpectedly, we observed that even when equal dosage (0.5 nkat/g of flour) of POx was added in breadmaking, the enlarging effect was quite different. Currently, the reason(s) are not known for the observed differences in breadmaking with equal dosages of POx. However, we noticed that, the best performers E539K and K312E/E539K in breadmaking were also most outstanding in gluten agglomeration. E539K and K312E/E539K were the two mutants with elevated optimal temperature (Fig. 2c) and enhanced thermostability (Fig. 2d). Therefore, these results collectively might suggest that, first, since breadmaking involved a heating step (baking), the improved thermostability would allow E539K and K312E/E539K in catalyzing more efficient crosslinking during this process, thereby enlarging the loaf volume to a higher extent. Second, enhanced thermotolerance is well-known to be positively related to increased resistance to stress conditions (Owusu and Cowan 1989). E539K and K312E/E539K could also be more stable in the dough environment than the wild-type and other mutants, which resulted in their excellent performance. Adding more enzymes may cause unwanted reactions which could mask the positive effect of the POx. These hypothesis, however, need to be explored in detail in the future. Our study for the first time pointed out that the biochemical properties of POx may affect its behavior in gluten agglomeration and breadmaking. It is also suggested that either wild-type pyranose oxidases identified from microbes or those artificially evolved require an evaluation before their real application in the bread industry.

### Additional file

**Additional file 1.** Amino acid sequence alignment of TsPOx with selected pyranose oxidases and primers used in this study.
Abbreviations
POx: pyranose oxidase; FAD: flavin adenine dinucleotide; H$_2$O$_2$: hydrogen peroxide; IPTG: isopropyl-β-D-thiogalactoside; ABTS: 2,2′-azinobis(3-ethylbenz-thiazolinesulfonic acid).

Authors’ contributions
ML and XS designed the work; ML performed the experiments and analyzed the data; HD, RM, HL, and BY reviewed and edited the manuscript; ML, RM, and XS wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data are shown in figures and tables within this article. Any material used in this study is available for research purposes upon request.

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