Improving the thermostability of *Penicillium amagasakiense* glucose oxidase based on molecular modification to replace feeding antibiotics

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Glucose oxidase (GOD) is a new type of green enzyme products. GOD uses molecular oxygen as an electron acceptor to specifically catalyze the production of gluconic acid and hydrogen peroxide from β-D-glucose. This feature makes it widely used in feed, food, textile, medicine, and many other fields (Bankar et al., 2009; Rasiah et al., 2005). GOD has received generally recognized safety status from the U.S. Food and Drug Administration (Wong et al., 2008). GOD has an inhibitory effect on many kinds of bacteria and fungi, which is not only limited to the food field but also has applications in other fields (Liang et al., 2022). Bacteria, bacterial toxins, and mycotoxins may cause contamination of feed, feed under the action of microorganisms, protein decomposition into ammonia, hydrogen sulfide, mercaptan, fecal odorant, etc., and fat decomposition to produce acid, and aldehyde, these substances are harmful to the animal organism. GOD added to feed, on the one hand, can inhibit animal intestinal pathogenic microorganisms (Wu et al., 2019), on the other hand, can maintain a weak acid environment conducive to enhancing animal digestive enzyme activity, regulating the intestinal flora, improving feed digestibility, improve reproductive performance, etc. (Yang et al., 2014). It has great potential for market application in saving feed resources, replacing antibiotics, and improving animal performance.

The glucose oxidase genes that have been commercially exploited are mainly from *Aspergillus niger* and *Penicillium amagasakiense* (Liu et al., 2001; Todde et al., 2014), but they lose their activity quickly at high temperatures and are difficult to resist the high-temperature process in the granulation process. In this study, PaGOD 1GPE, a glucose oxidase from *Penicillium amagasakiense*, was used as the material. The aim was to improve thermal stability while maintaining catalytic activity, to remain active after the high-temperature process of feed pelleting, and to inhibit pathogenic microorganisms when added to feed. Based on molecular dynamics simulations and rational design methods such as virtual saturation mutations, we identified 27 mutation sites that may affect the thermal stability of PaGOD 1GPE and finally confirmed that the thermal stability of mutants A263P and K424F was significantly improved by constructing mutant gene expression vectors and plate screening procedures.

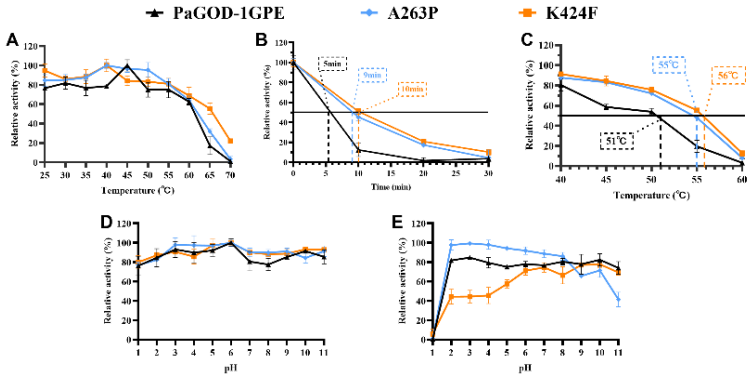

Fig.1. (A) Optimal temperature of wild-type PaGOD 1GPE and its mutants; (B) Time course of thermal inactivation of wild-type PaGOD 1GPE and its mutants at 60°C; (C) T_{50} of wild-type PaGOD 1GPE and its mutants; (D) Optimal pH of wild-type PaGOD 1GPE and its mutants; (E) pH stability of wild-type PaGOD 1GPE and its mutants

Table 1. Kinetic parametersa of wild-type PaGOD 1GPE and its mutants.

	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}min^{-1}$)	V_{max} (U/mg)
PaGOD 1GPE	31.85±2.39	815.26±31.53	25.74	74.93±2.90
PaGOD 1GPE_A263P	66.59±5.35	869.19±45.43	13.07	78.32±3.15
PaGOD 1GPE_K424F	22.69±4.47	834.40±25.44	38.01	75.94±2.32

Fig. 1A shows that the optimum temperature of PaGOD 1GPE was 45°C, the optimum temperature of PaGOD 1GPE_K424F, PaGOD 1GPE_A263P was 40°C, and the relative enzyme activity was significantly higher at high

temperature (65℃) compared with WT. Fig. 1B shows that the $t_{1/2}$ of PaGOD 1GPE_A263P and PaGOD 1GPE_K424F was 9 min and 10 min, respectively, which were 1.8-fold and 2-fold longer than that of wild-type PaGOD 1GPE (5 min). The mutant PaGOD 1GPE_K424F had the best thermal stability, which might be due to the change of Lys to Phe, which made the enzyme structure more stable. From Fig. 1C, The T_{50} of wild-type was determined to be 51°C, while the T_{50} values of the two mutants were increased by 4°C and 5°C, respectively. Fig. 1D and 1E show that the pH stability of PaGOD 1GPE_A263P was better than that of wild-type in a pH 2-7 environment. In addition, the mutant PaGOD 1GPE_K424F exhibited a 1.47-fold increase in catalytic efficiency (k_{cat}/K_m). As a result of improved thermostability for PaGOD 1GPE_K424F, the balanced relationship of activity and stability was achieved again by protein engineering.

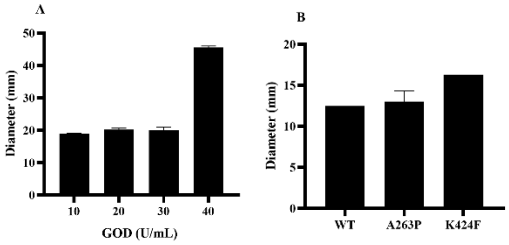

Fig.2. (A) Diameter of inhibition circle of *Pseudomonas aeruginosa* by different concentrations of GOD; (B) Inhibition circle diameter of PaGOD 1GPE and its mutants against *Pseudomonas aeruginosa*

In addition, in vitro, *Pseudomonas aeruginosa* inhibition assays were performed using different concentrations of GOD to evaluate the inhibition effect of glucose oxidase on Gram-negative bacteria at different concentrations. Afterward, in vitro inhibition experiments were conducted against *P. aeruginosa* using WT and the fermentation supernatants of mutants A263P and K424F to evaluate the inhibition effect of the fermentation enzyme solution. The results in Figure 2 showed that the inhibition circle expanded with the increase of glucose oxidase concentration, and the inhibition circle reached 20 mm at 20 U/mL. This is mainly due to the enzymatic reaction between GOD and glucose to produce hydrogen peroxide(Liang et al., 2022). Compared with the wild type, the inhibition effect of mutant K424F was higher than that of A263P and higher than that of WT.

In conclusion, Mutant K424F had improved thermal stability, viability, and inhibition effect compared with the wild type. This study successfully improved the thermal stability of glucose oxidase and increased the catalytic activity of the enzyme, providing a theoretical basis for the molecular improvement of glucose oxidase for feeding and a reference for the industrial production of glucose oxidase and the application of feed additives.

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