## Rational design of α-glucosidase for the synthesis of 2-*O*-α-d-glucopyranosyl-l-ascorbic acid

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# These authors contribute equally to this study.

## ABSTRACT

**Background**

α-Glucosidase (AG) is a bifunctional enzyme, it has a capacity to synthesize 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) from L-ascorbic acid (L-AA) and low-cost maltose under mild conditions, but it can also hydrolyze AA-2G, which leads to low synthesis efficiency of AA-2G.

**Main Methods and Major Results**

This study introduces a rational molecular design strategy to regulate enzymatic reactions based on inhibiting the formation of ground state of enzyme-substrate complex. Y215 was analyzed as the key amino acid site affecting the affinity of AG to AA-2G and L-AA. For the purpose of reducing the hydrolysis efficiency of AA-2G, the mutant Y215W was obtained by analyzing the molecular docking binding energy and hydrogen bond formation between AG and the substrates. Compared with the wild type, Isothermal Titration Calorimetry(ITC) results showed that the equilibrium dissociation constant (*K*D) of the mutant for AA-2G was doubled; the Michaelis constant (*Km*) for AA-2G was reduced by 1.15 times; and the yield of synthetic AA-2G was increased by 39%.

**Conclusions and Implications**

Our work also provides a new reference strategy for the molecular modification of multifunctional enzymes and other enzymes in cascade reactions system.

**Keywords：**2-*O*-α-d-glucopyranosyl-l-ascorbic acid; Bifunctional enzymes; Rational design; α-glucosidase

**Abbreviations**

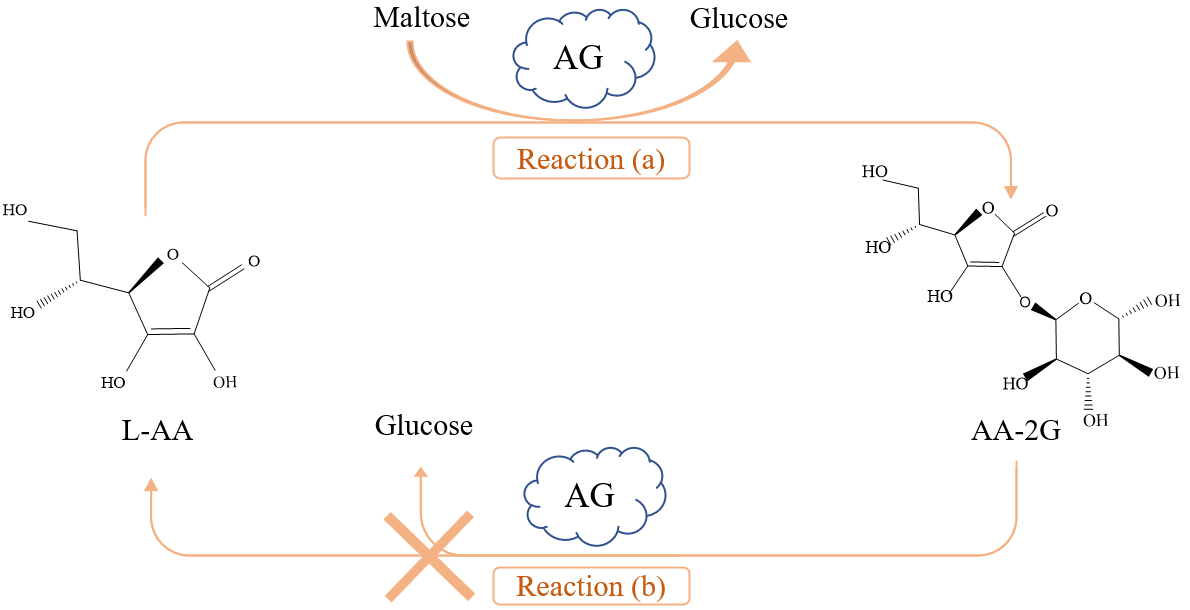
AA-2G, 2-O-α-D-glucopyranosyl-L-ascorbic acid; AAG, Aspergillus niger α-glucosidase; AG, α-glucosidase; JrAG, Japonica rice α-glucosidase; L-AA, L-ascorbic acid; rAG, recombinant α-glucosidase; RAG, Rat α-glucosidase

**1. Introduction**

α-Glucosidase (AG, EC 3.2.1.20) is a soluble glycoprotein that hydrolyzes the non-reducing terminal alpha-d-glucosidic linkages of α-glucoside, α-glucan, and maltogenic oligosaccharides, thereby releasing α-glucose (Nimpiboon, Nakapong, Pichyangkura, Ito, & Pongsawasdi, 2011; Chiba, 1997; Okuyama, Saburi, Mori, & Kimura, 2016). Earlier studies demonstrated that AG can catalyze the synthesis of 2-*O*-alpha-d-glucopyranosyl-l-ascorbic acid (AA-2G) via a specific transglucosylation reaction involving l-ascorbic acid (L-AA) and maltose (Muto, Suga, Fujii, Goto, & Yamamoto, 1990; Yamamoto, Muto, Nagata, Nakamura, & Suzuki, 1990; Ahn et al., 2015). According to a previous report, AG from rat intestinal (Yamamoto, Muto, Murakami, Suga, & Yamaguchi, 1990), *Oryza sativa* (Japonica rice, Muto, Suga, Fujii, Goto, & Yamamoto, 1990), and *Aspergillus niger* (Li et al., 2017)can synthesize AA-2G via a transglucosylation reaction.

AA-2G has attracted considerable attention in cosmetics, food, and medicine applications because of its stable and nonreducible properties (Takebayashi, Tai, & Yamamoto, 2002; Tai et al., 2010; Miura et al., 2017; Miura & Tai, 2017; Ichiyama et al., 2009; Yim et al., 2019). The substrate specificity of AG is better than that of cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19). AG can use maltose as a sugar donor. Maltose is easy to dissolve in water and is cheap. The AG-catalyzed synthesis of AA-2G produces few intermediates and by-products (Han, Liu, Li, Du, & Chen, 2012; Qi et al., 2021), but AG catalyzes the hydrolysis of AA-2G at the same time, resulting in low efficiency of AA-2G synthesis (Yamamoto, Muto, & Miyake, 1998). Therefore, it is an attractive challenge to obtain high-yield AA-2G using maltose as a glycosyl donor.

AG catalyzes the synthesis of AA-2G from L-AA and maltose via an enzymatic reaction system involving reaction (a) in which AG catalyzes the synthesis of AA-2G from L-AA and maltose, and reaction (b) in which AG catalyzes the degradation of AA-2G to L-AA and glucose (Fig. 1). This means that the total synthesis efficiency of AA-2G is the result of the dynamic balance of the two reactions (a) and (b) in the reaction microenvironment. As we know, the formation of ground state between enzyme and substrate is the first step of enzymatic reaction. If the enzyme and substrate cannot form a ground state, the catalytic reaction will be difficult to proceed. Therefore, when AG in the above system is not easy to form ground-state complex with AA-2G, that is, AG is hard to decompose AA-2G into L-AA and maltose. In this study, through rational molecular design of α-glucosidase (JrAG), reduce its affinity with AA-2G, reduce the formation of ground-state complex of α-glucosidase (JrAG) and AA-2G, and reduce the decomposition of AA-2G by AG, to improve the efficiency of synthesis of AA-2G by JrAG.



**Fig. 1.** The glycosylation and hydrolysis reaction catalyzed by AG, including reactions (a) and (b)

The molecular docking and biological experiment verification revealed that the mutation of Y215 affected the affinity of AG and AA-2G, thus directly affecting the substrate-binding conformations when AG hydrolyzed AA-2G. This study proved that the enzyme rational molecular improvement strategy is feasible based on the characteristics of inhibiting the formation of ground-state complex to regulate the enzyme reaction.

**2. Materials and methods**

**2.1 Strains, vectors, and chemicals**

The AG from rat intestinal (RAG, GenBank accession No. XM\_032912099.1), Japonica rice (JrAG, GenBank accession No. XM\_015787634.2), and *Aspergillus niger* (AAG, GenBank accession No. XM\_001402016.2) was synthesized for heterologous expression in *Komagataella phaffii* GS115 (Invitrogen, USA). pPIC3.5k (Invitrogen, USA) was used as the eukaryotic expression vector, thus obtaining the plasmid pPIC3.5k-AG (Supplementary material; Fig. S1). The plasmid pPIC3.5k-AG was synthesized with optimized codons from shanghai Generay Biotech Co., Ltd. Luria-Bertani medium was used to culture *E*. *coli* cells.Taq DNA polymerase, T4 DNA ligase, restriction endonuclease and other chemical reagents were purchased from New England Biolabs (Beverly, MA, USA). Standard AA-2G was purchased from Macklin Biochemical Co. (Shanghai, China). All other chemical reagents were of analytical grade.

**2.2 Expression and purification of AG in *K. phaffii***

The three AGs mentioned above from different biological sources(RAG, JrAG, AAG) were heterologously expressed. The recombinant *K. phaffii* GS115 with rAG were incubated in 250 mL of YPG medium at 30 °C and 250 rpm for 72 h. After centrifugation of the yeast cell culture, the supernatant was concentrated by precipitation with ammonium sulfate. The rAG was purified with Ni2+-NTA agarose affinity column (General Electric Company, USA). Then, the elute solution containing rAG were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and collected for further experiments. Protein concentration was measured via the Bradford method (Bradford, 1976).

**2.3** **Transglycosylation catalyzed by rAG**

The transglucosylation reaction was performed in separate experiments using each rAG as catalyst, reaction conditions were modified based on previous studies(Muto et al. 1990; Qi et al., 2021). The reaction mixture (1.0 mL) consisted of 500 mM maltose, 100 mM L-AA, 0.02 mg rAG, and 0.1 M citric acid with sufficient 0.2 M disodium hydrogen phosphate, and was incubated in the dark at 40℃ for 16h, unless otherwise specified. Three replicates were performed for each group. Before confirming the reaction conditions, the authors have respectively optimized the three rAGs conditions from temperature, pH, maltose and L-AA concentration to obtain the optimal transglycosylation reaction condition parameters (Data not provided). The yield of AA-2G at the end of the reaction was determined via high-performance liquid chromatography (HPLC). The concentration of AA-2G was calculated based on the peak area of standard samples.

**2.4** **Homology modeling and key amino acid analysis**

We used Discovery Studio 4.5 homology modeling to predict the three-dimensional structural model of JrAG. Molecular docking analysis was performed using the CHARMm-based docking program CDOCKER (Jiang, Wang, Zhang, & Zhang, 2009; Wu, Robertson, Brooks III, & Vieth, 2003), with JrAG as the receptor, and maltose, AA-2G, and L-AA as the substrate molecules (ligands). After docking, all amino acid residues in the binding zone of 5 Å between the enzyme and the substrate were analyzed by alanine scanning, and the key amino acids affecting the binding of AG and substrates were obtained.

**2.5** **Rational Design of AG for low binding affinity to AA-2G**

The key amino acid and then mutated it to the amino acid that was considered to reduce the binding affinity of JrAG to AA-2G while ensuring that its structure was similar to that of the wild-type AG (WT).During the mutation simulations, we considered the effects of temperature and pH. Three-dimensional models were constructed for all the mutants using the standard mutagenesis protocol provided by Discovery Studio 4.5 (Feyfant, Sali, & Fiser, 2007). The best mutants were identified via stability prediction and molecular dynamics simulations (Guerois, Nielsen, & Serrano, 2002). The structural stability of the mutants was assessed based on the free energy of folding (mutation energy), calculated as follows: ΔΔGmut = ΔΔGfold (mutant) − ΔΔGfold (WT). The mutant with the lowest ΔΔGmut value was selected for molecular dynamics simulations (simulated heating steps of 100 ps; simulated equilibrium steps of 500 ps; simulated production steps of 400 ps; time steps of 5.0 fs with the particle grid Ewald method for electrostatic and constant pressure thermostatic kinetics). The mutants with stable structures were identified via simulations and validated in subsequent bioassays.

**2.6 Isothermal titration calorimetry (ITC) determination of the binding affinity of WT, Y215W and AA-2G**

The ITC experiment was carried out on a MicroCal ITC200 system (Malvern Panalytical, Malvern, UK). The enzyme solution (0.2 mg/mL) was prepared as the titration solution using 0.1 M citric acid with sufficient 0.2 M disodium hydrogen phosphate buffer (pH 5.0). Prepare a 50 µM AA-2G standard solution using the same buffer as the ligand solution.. The operating parameters of the Auto iTC200 were as follows: cuvette temperature 40 °C; number of injections, 20; reference power, 5 μCal/s; titrant volume, 2 μL; stirring speed, 750 rpm; duration, 4 s; injection interval, 150 s; filtration cycle time of 5 s.

**2.7 Determination of enzyme activity and kinetic analysis**

Hydrolytic AA-2G activity: The 1.0-mL of 0.1 M citric acid with sufficient 0.2 M disodium hydrogen phosphate buffer (pH 5.0) comprised 2.0 mg/mL and 10 μL of enzyme protein, 40 °C for 15 min under dark conditions. An inactivated enzyme solution was used in the control experiment. Three replicates were performed for each group. Subsequently, the yield of L-AA at the end of the reaction was determined via high-performance liquid chromatography (HPLC). One unit of enzyme activity was defined as the amount of enzyme required for the release of 1 mM of L-AA/min under the assay conditions. The concentration of L-AA was calculated based on the peak area of standard samples.

The initial reaction rate was calculated from the slope of the zero-order plot of product concentration vs. reaction time, and the initial reaction rate was then plotted against the substrate concentration. The resulting curve was fitted to the hyperbolic equation v = Vmax × [S]/(*K*m + [S]) using Origin 7.0 (OriginLab, Northampton, MA, USA) to obtain the turnover number (*k*cat) and *K*m. All measurements were performed in duplicate with substrate concentrations ranging from 10 mM to 500 mM.

**2.8 HPLC analysis and liquid chromatography–mass spectrometry (LC-MS) identification of AA-2G**

The content of AA-2G in the transglucosylation reaction volume was determined using an HPLC system (UltiMate3000; Thermo Scientific, Bohemia, NY, USA) equipped with an Athena C18-WP column (4.6 mm × 250 mm; column temperature: 25 ℃) and an ultraviolet detector (detection wavelength: 238 nm). The mobile phase was 75 mM KH2PO4, pH adjusted to 2.0 with phosphoric acid, at a flow rate of 0.8 mL/min.

The products synthesized via the enzymatic reaction were characterized by LC-MS and compared with a commercial standard of AA-2G. Screening of target analytes was performed via liquid chromatography with tandem MS (LC-MS/MS) using a QTRAP 4500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo-V IonSpray electrospray ionization (ESI) source and an Agilent series 1260 Infinity HPLC instrument (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). Chromatographic separation was performed at 25 °C on a Zorbax Eclipse XDB-C18 column (2.1 × 50 mm; 3.5 µm; Agilent, Palo Alto, CA, USA).

ESI-MS/MS was performed in scheduled multiple-reaction monitoring mode in both positive and negative polarities by scanning two fragmentation reactions per analyte with the following settings: source temperature, 550 °C; curtain gas, 30 psi; ion source gas 1 (sheath gas), 45 psi; ion source gas 2 (drying gas), 50 psi; ion spray voltage, −4500 V and +5500 V for ion source gas 1 and 2, respectively; collision gas (nitrogen) medium. Two separate chromatographic runs per sample were performed. The multiple-reaction monitoring detection windows were 54 s and 96 s in the positive and negative ionization mode, respectively, and the cycle time was set to 1 s.

**3 Results**

**3.1 Transglycosylation catalyzed by rAG**

The highest yield of AA-2G was obtained using JrAG (Supplementary material; Table S1), which was selected as the research object in this study.

**3.2 Homology modeling and key amino acid analysis**

The homology modeling of JrAG was constructed based on maltase-glucoamylase from human intestine (Protein Data Bank ID: 3L4Y), which shares 55.5% identity with JrAG, as the structural template (Fig. S2) (Sim et al., 2010). Molecular docking (Fig. 2) and alanine scanning showed that Asp540 was identified as a key amino acid residue in the interaction of JrAG with L-AA; a mutation of Asp540 resulted in decreased binding affinity between JrAG and L-AA. We also found that Ile205, Phe450, Arg524, Asp540 and Tyr215 (Y215) were key amino acid residues affecting the binding of JrAG to maltose, and mutations of these amino acid residues resulted in decreased binding affinity between JrAG and maltose. Therefore, mutations to the above amino acid residues was avoided as much as possible in the subsequent experiments.

Excitingly, Y215 was identified via alanine scanning as a key amino acid residue affecting the binding of JrAG to AA-2G, and a mutation in this amino acid residue resulted in a decreased binding affinity between JrAG and AA-2G (Table S2). Therefore, Y215 was selected as the candidate amino acid site to introduce a mutation in the next phase of the study.



(b)

(c)

(a)

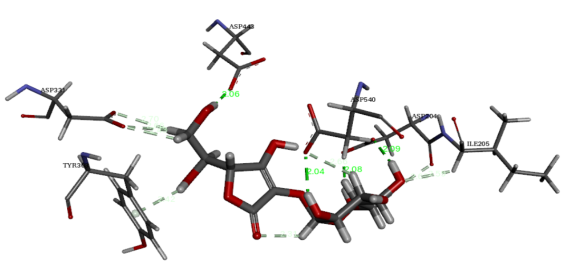
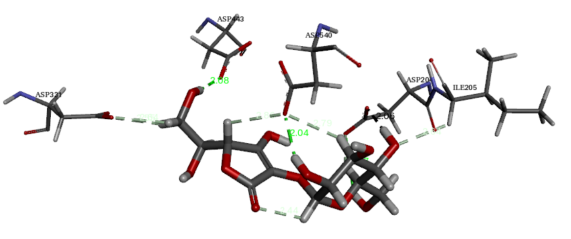
**Fig. 2.** Docking analysis of *Japonica rice* α-glucosidase (JrAG) and (a) 2-*O*-α-d-glucopyranosyl-l-ascorbic acid, (b) l-ascorbic acid, and (c) maltose. Substrates are shown as gray sticks, the interacting residues are shown as green circles, and hydrogen bonds are shown as green dashes

**3.3 Rational Design of AG**

Reduce the affinity of AA-2G to AG by site saturation mutation on Y215. Based on the docking results of JrAG with AA-2G and jrag with maltose, Y215 was successively replaced by the remaining 19 amino acids using Discovery studio 4.5. Stable mutants were selected according to the calculated Mutation Energy (Stability) protocol, with constraints of pH 5.0 and 40°C(Table S3). According to the principle of selecting stable enzyme-substrate complex mutants, the mutant with a large decrease in Mutation Energy at pH 5.0 and a small increase in Mutation Energy at 40 °C was selected. Therefore, Tyr215Phe, Tyr215Leu, Tyr215Trp, Tyr215His, Tyr215Ile and Tyr215Met were selected for further study.

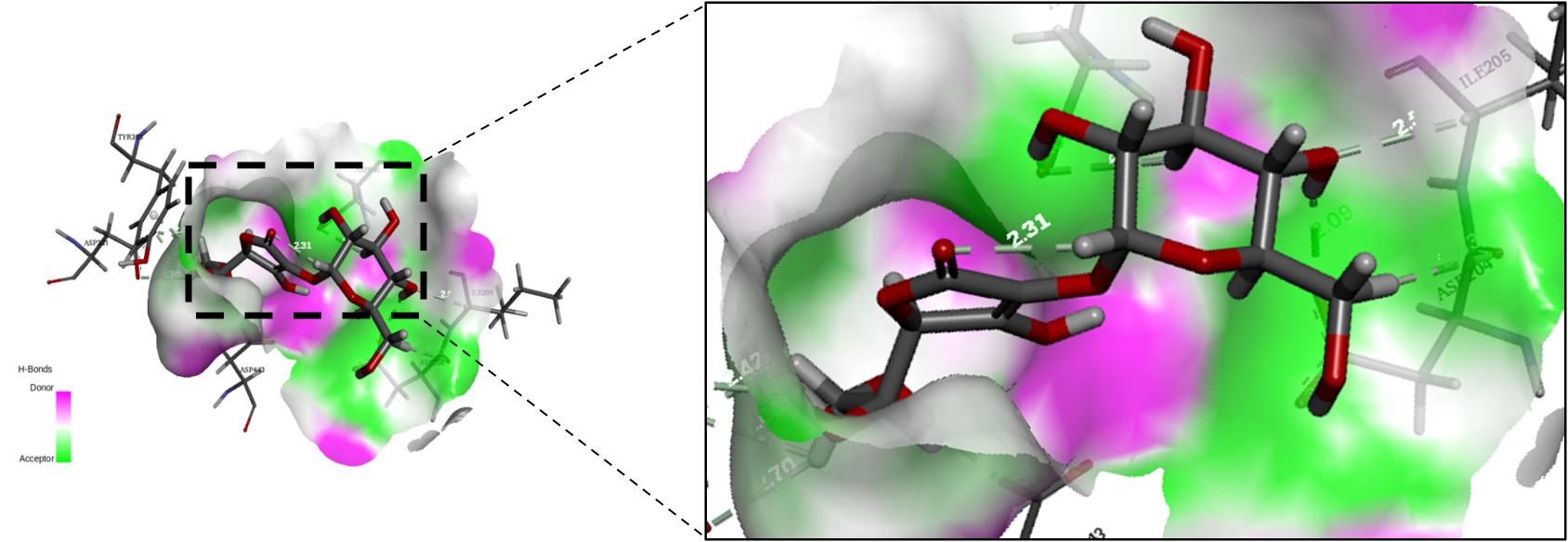
The binding affinity between stable mutants and substrates was calculated using the Calculate Mutation Energy (Binding) protocol. Among the six mutants, Tyr215Trp (Y215W) and Tyr215Ile (Y215I) showed increased binding energy to AA-2G (Table S4), i.e., there was decreased affinity of the enzyme to AA-2G at 40 °C and pH 5.0; however, under the same constraint, the Y215I mutant displayed decreased binding affinity to maltose. In comparison, although Y215W also displayed a decreased binding affinity to maltose at 40 °C, the increase was smaller; moreover, the decrease in the binding affinity of Y125W to AA-2G was greater. Therefore, the stable mutant Y215W with reduced affinity to AA-2G was selected for further study.

The molecular docking analysis of AA-2G with both WT and Y215W identified the binding active pockets, the bond formations (Fig. 3) and bond distances (Table 1). Furthermore, we examined the stability of the molecule via molecular dynamics analysis to simulate the movement of the mutant AG in the solution environment. We analyzed the root mean square deviation (Reva, Finkelstein, & Skolnick, 1998) of the WT and the mutant AGs after the simulation to identify the most stable mutant (Fig. S3).

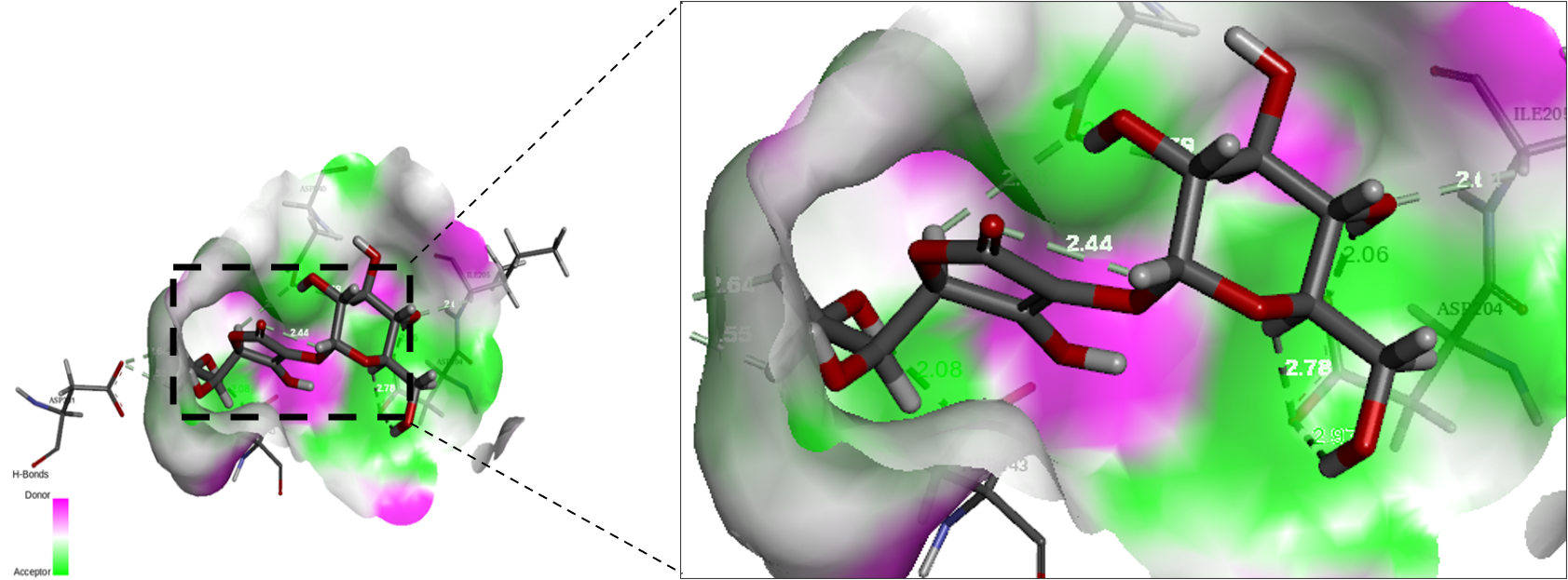
 

(a)

(b)



(c)



(d)

**Fig. 3.** Molecular docking of *Japonica rice* α-glucosidase (JrAG) and 2-*O*-α-d-glucopyranosyl-l-ascorbic acid (AA-2G). (a) wild-type JrAG (WT); (b) mutant JrAG (Y215W); solid surface representations of the molecular docking of AA-2G and JrAG: (c) WT; (d) Y215W. AA-2G is shown as thick gray and red sticks, interacting residues are shown as thin gray and red sticks, and hydrogen bonds are shown as gray-green dashes

**Table 1**   
Hydrogen bond formation between wild-type *Japonica rice* α-glucosidase (JrAG) or mutant JrAG (Y215W) and 2-*O*-α-d-glucopyranosyl-l-ascorbic acid (AA-2G).

|  |  |  |  |
| --- | --- | --- | --- |
| **AA-2G-amino acid residue** | **H-Bond distance (Å)** | | |
| **Wild-type** | **Y215W** |
| H34- Asp540:OD2 | 2.04 | 2.04 | |
| H41- Asp443:OD2 | 2.06 | 2.08a | |
| H36- Asp204:OD2 | 2.08 | 2.97a | |
| H33- Asp204:OD1 | 2.09 | 2.06b | |
| H40- Tyr303 | 2.42 | -a | |
| H38- Asp331:OD2 | 2.43 | 2.64a | |
| O4- Ile205: HA | 2.59 | 2.64a | |
| H30- Asp204:O | 2.63 | -a | |
| H24- Asp540:OD2 | 2.66 | 2.79a | |
| H37- Asp331:OD2 | 2.66 | 2.55b | |
| H37- Asp331:OD1 | 2.70 | -a | |
| H27-Asp204:OD2 | - | 2.78b | |
| H31- Asp540:OD2 | - | 2.86b | |

"-": The enzyme did not form a bond between AA-2G and amino acid residues;

a: compared with the wild-type, Y215W has a longer H-bond distance or no bond;

b: the H-bond distance of the mutant has become shorter compared with the wild type, or the new H-bonds exclusive to Y215W.

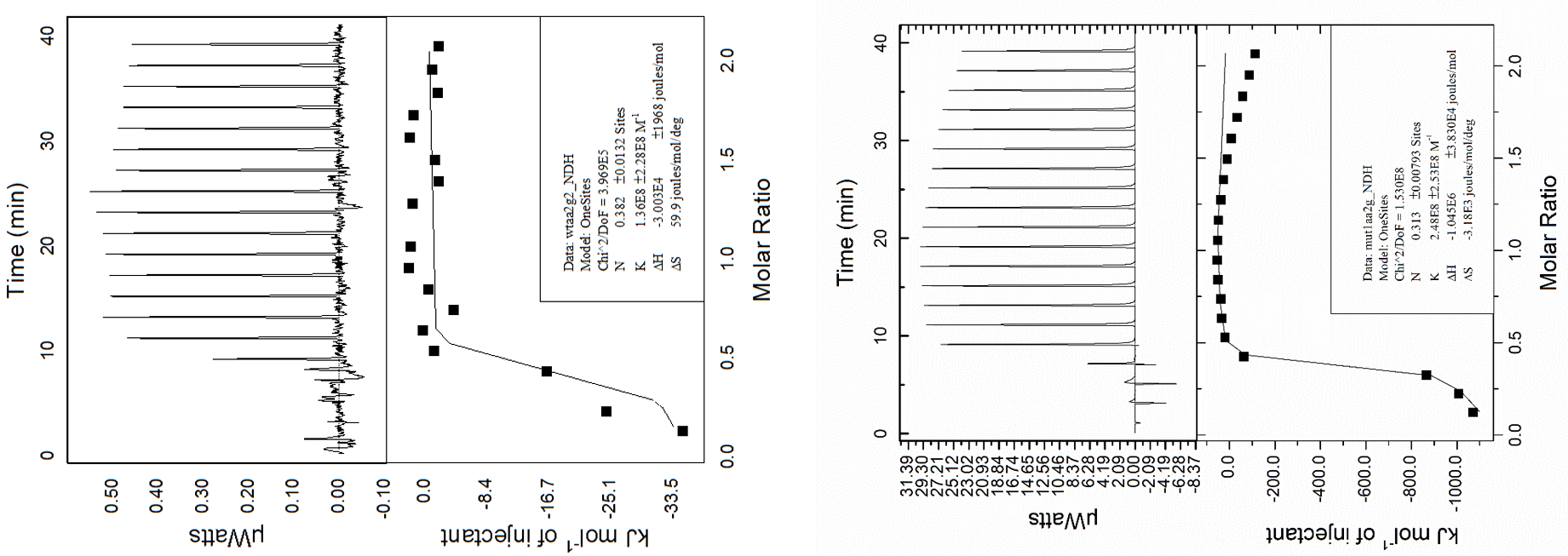
**3.4 Expression and purification of WT and Y215W**

Both WT and Y215W were expressed extracellularly and displayed detectable enzyme activity. WT and Y215W were His-tagged and purified via Ni-NTA affinity chromatography. SDS-PAGE showed purified bands of approximately 150 kDa (Fig. S4). The optimum pH and temperature of WT and Y215W were also determined (Fig. S5 and S6). The expression levels of WT and Y215W were 0.22 mg/mL and 0.17 mg/mL respectively.

### 3.5 Mutant Y215W has lower binding affinity for AA-2G

The equilibrium dissociation constant (*K*D) value obtained via ITC, which reflects the binding affinity of the compound to the target. Binding affinity is the strength of binding interactions between a biomolecule and ligand. The smaller the *K*D value, the stronger the binding affinity. Therefore, a larger *K*D indicates weaker binding that requires a high ligand concentration to saturate the binding site.

Both WT and Y215W were titrated with AA-2G to measure the binding affinity of the enzyme to AA-2G (Fig. 4). The heat flow was positive, i.e., all binding reactions were exothermic, which is consistent with the characteristics of hydrolysis reactions. The obtained data were fitted to the curve using Origin 7.0 to obtain the *K*D values. Excitingly, according to the comparison of the calculated *K*D values (WT [13.6 nM] < Y215W [24.8 nM]), WT displayed a stronger binding affinity to AA-2G than Y215W did.



(a)

(b)

**Fig. 4.** Isothermal calorimetry titration of 2-*O*-α-d-glucopyranosyl-l-ascorbic acid (AA-2G) and *Japonica rice* α-glucosidase (JrAG); (a) wild-type (WT) JrAG; (b) Mutant JrAG (Y215W).

### 3.6 Enzyme activity and kinetic parameters of Y215W and wild-type AG

As shown in Table 2. The *K*m (AA-2G) of Y215W was higher than that of wild-type, describing that the affinity of the Y215W to AA-2G decreased relative to the wild-type. The AA-2G-hydrolyzing activity (U/mg) of Y215W was one-fifth that of wild-type. The catalytic efficiency (*k*cat/*K*m) of Y215W in the AA-2G hydrolysis reaction was 1/7.3 that of wild-type; moreover, the *K*m (L-AA) of Y215W decreased, describing that the affinity of Y215W to L-AA increased relative to wild-type, contributing to the synthesis of AA-2G.

**Table 2**Kinetic parameters of wild-type and mutant AG.

|  |  |  |
| --- | --- | --- |
| **Parameters** | **Wild-type (WT)** | **Mutant (Y215W)** |
| *K*m (AA-2G) (mM) | 15.14 | 17.37 |
| *k*cat*/K*m (AA-2G) | 1.69 | 0.23 |
| Enzyme specific activity for hydrolysis of AA-2G (U/mg) | 146.73 | 30.52 |
| *K*m (L-AA) (mM) | 104.30 | 63.62 |
| *k*cat*/K*m (L-AA) | 0.934 | 0.910 |
| *K*m (maltose) (mM) | 73.6 | 88.3 |
| *k*cat*/K*m (maltose) | 0.67 | 0.34 |
| Transglucosylation rate (%)\*\* | 7.62 | 10.61 |
| Transglucosylation rate improvement ratio (%)\* | - | 39.23 |
| AA-2G yield (g/L) | 2.58 | 3.59 |
| Yield improvement ratio (%)\* | - | 39.26 |

*k*cat, turnover number; *k*cat*/K*m, catalytic efficiency; *K*m, Michaelis constant

\*: The ratio of mutant values changed compared with wild type

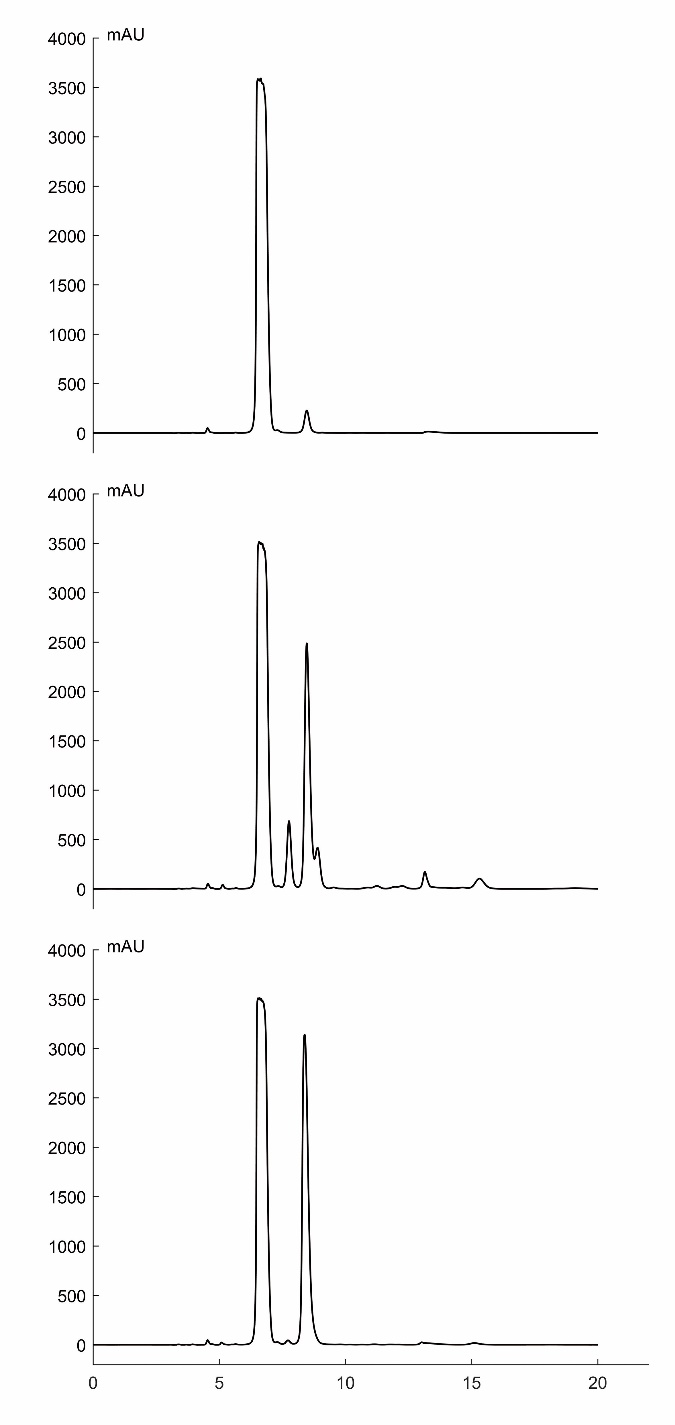
\*\*: The molar mass ratio of the added L-AA to the generated AA-2G

## 3.7 Analysis of AA-2G synthesized by Y215W

The commercial standard of AA-2G as well as the wild-type and Y215W transglucosylation reaction solutions were analyzed using HPLC (Fig. 5). The retention time of AA-2G was approximately 8.40 min, and the peak samples were collected for further identification via LC-MS/MS (Fig. 6). The mass-to-charge ratio (m/z) was calculated for the AA-2G standard: 337.0773 (M-1) and 337.0922 (M-1). The presence of AA-2G in the samples was determined based on the HPLC retention time (t = 8.46 min) using the optimal absorption wavelength of 238 nm for the AA-2G standard.

AA-2G was produced via the transglycosylation reaction using Y215W or wild-type with maltose as a glycosyl donor and L-AA as a glycosyl acceptor. Y215w had higher transglycosylation ability than WT, and the transglycosylation rate increased by 39.23%. The yields of AA-2G were 3.59 g/L and 2.58 g/L, respectively, with an increase of 39.26% (Table 2).

(a)



(c)

(b)

AA-2G

AA-2G

AA-2G

**Fig. 5.** High-performance liquid chromatography analysis of the transglucosylation reaction solutions containing wild-type *Japonica rice* α-glucosidase (JrAG) or the mutant JrAG, Y215W. (a) Authentic commercial standard of 2-*O*-α-d-glucopyranosyl-l-ascorbic acid (AA-2G); (b) Wild-type JrAG reaction solution; (c) Y215W reaction solution. The retention time of AA-2G was approximately 8.40 min.

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(a)

(c)

(b)

**Fig. 6.** Liquid chromatography with tandem mass spectrometry analysis of the transglucosylation reaction; (a) Authentic samples of 2-O-α-d-glucopyranosyl-l-ascorbic acid (AA-2G); (b) Wild type JrAG reaction solution; (c) Y215W reaction solution.

**Discussion**

Alpha-glucosidase was first discovered to transfer the glucosly group of maltose to L-AA to synthesize AA-2G. However, AG is a bifunctional enzyme that catalyzes the synthesis of AA-2G by transglucosylation and the hydrolysis of AA-2G, resulting in extremely low yield and efficiency of AA-2G synthesis from AG. Studies on the improvement of the yield of AA-2G synthesis through AG mainly focuses on improving the catalytic efficiency of AG synthesis, and many recombinant AGs have been studied. For example, *A. niger* AG was expressed in *Aspergillus nidulans* (Nakamura et al., 2017) and *Emericella nidulans* (Ogawa et al., 2006), with reported expression levels of 0.04 U/mg and 0.96 U/mg, respectively. Chen et al. (2010) expressed *A. niger* AG in *Komagataella phaffii*, and the enzyme activity in the culture supernatant of 3-L fermenters reached 2.07 U/mL.

Reaction (a) and (b) together constitute a cascade reaction system. According to the kinetic theory of enzymatic reactions, in multiple consecutive reactions, the priority pathway depends on the enzyme to the substrate (*K*m); the lower the *K*m value of the substrate in the reaction, the more favorably its reaction with the enzyme proceeds. Herein, Y215W had a higher *K*m (AA-2G) than wild-type (Table 2), indicating that Y215W prioritized reaction (a) over reaction (b). In addition, the lower *K*m (L-AA) value of Y215W also reflects this result.

The *K*m value is defined as the concentration of substrate at which the enzyme operates at half its maximum catalytic rate; it hence describes the affinity of an enzyme for a particular substrate (Kroll A et al., 2021). The *K*m values were not associated with improved transglycosylation activity, but changes in turnover numbers could explain the increased yield of AA-2G (Table 2). Rational design studies of substrate specificity and regioselectivity have also found a similar situation (Eser et al., 2020). A higher *k*cat indicates that the enzyme converts the substrate at a faster speed, and a lower *K*m describes that the affinity between the enzyme and the substrate is greater. The ratio of *k*cat to *K*m represents the catalytic efficiency of the enzyme. *k*cat/*K*m can compare the catalytic efficiency of different enzymes or different substrates of the same enzyme. A high *k*cat/*K*m value indicates a high catalytic efficiency of the enzyme. Herein, among the wild-type *k*cat/*K*m(AA-2G), *k*cat/*K*m(L-AA) and *k*cat/*K*m(maltose), *k*cat/*K*m (AA-2G) is the highest. However, among the *k*cat/*K*m(AA-2G), *k*cat/*K*m(L-AA) and *k*cat/*K*m(maltose) of Y215W, the highest is *k*cat/*K*m(L-AA).Moreover, *k*cat/*K*m (AA-2G) of Y215W is one seventh of that of wild-type (Table 2). These results show that compared with wild-type, the hydrolysis efficiency of AA-2G catalyzed by Y215W in reaction (b) is greatly reduced, and Y215W tends to synthesize AA-2G in reaction (a). Therefore, it is ultimately more favorable for the accumulation of AA-2G in the reaction system.

The hydrogen bond network of the active pocket of the substrate is also a key factor affecting the regioselectivity (Chen et al., 2021). The weakened hydrogen-bonding interactions have poorer substrate affinity (Fig. 3). Analyzing molecular docking, we found that Y215W forms fewer hydrogen bonds when docked with AA-2G and fewer amino acid residues form hydrogen bonds with AA-2G compared to WT, resulting in reduced interaction between Y215W and AA-2G. In general, the bond distance was longer in Y215W than in WT (Table 1). Moreover, Tyr303 of mutant Y215W was not involved in bonding, and the catalytic sites Asp540 and Asp443 amino acid residues had longer bond distances to H24 and H41 of AA-2G. Mutation of positions Y215 seemed to influence the H-bonds between AG and AA-2G, resulting in changed flexibility of the active pocket, as well as the substrate channel, therefore, it is speculated that the binding affinity between Y215W and AA-2G decreases, the equilibrium dissociation constant (*K*D) results can also confirm the inference.

Binding affinity is affected by non-covalent intermolecular interactions, such as hydrogen bonding, electrostatic interactions, hydrophobicity, and van der Waals forces between two molecules. The weaker binding affinity between Y215W and AA-2G makes it more difficult for [E]+[S]→[ES] in reaction (b), which is beneficial for AA-2G not to be hydrolyzed, thus achieving the purpose of improving the yield of AA-2G. The kinetic parameters measured in this study also confirm this idea.

However, there are two other substrates, maltose and L-AA, for the synthesis of AA-2G by AG. In the process of rational design, our principle was to reduce the binding affinity of AA-2G as much as possible and to minimize the interaction with maltose and L-AA. From the results of kinetic parameters, the affinity of Y215W to L-AA was enhanced, and the affinity to maltose was decreased. The molecular docking results (fig. 3) showed that Y215W formed less bonds with the glycoside part of AA-2G, and the distance between the glucoside and the active pocket became further. This change may result in a decrease in the ability of Y215W to hydrolyze to obtain glucosides, resulting in a decrease in the affinity of the enzyme for maltose and AA-2G. Current silico approaches are not capable of analyzing all three substrates simultaneously. This is a deficiency of this study, which does not take into account the effect of this side reaction of hydrolyzed glucoside on the whole cascade reaction system.

We followed a rational design approach to the molecular modification of JrAG. A key amino acid site of the substrate binding pocket was selected to introduce a mutation, resulting in mutant Y215W, which catalyzed the synthesis of AA-2G from L-AA and maltose with increased efficiency compared with WT. The JrAG with L-AA and Maltose system, as the reaction proceeded there existed simultaneous synthesis of AA-2G catalyzed by JrAG and hydrolysis of AA-2G by JrAG, In the reaction, the product AA-2G becomes the hydrolysis substrate of JrAG. The special feature of this reaction is that the synthesis and hydrolysis of AA-2G are catalyzed by the same enzyme (JrAG). In this study, a rational design approach was used to reduce the stability of ground-state complex structure of JrAG and AA-2G, change the catalytic characteristics of JrAG, thus reducing the hydrolysis efficiency of JrAG on AA-2G and increasing the yield of AA-2G in the reaction system. Fortunately, we also found that AA-2G was synthesized by Y215W with very few by-products compared with WT (Fig. 5b and c).

Finally, this study demonstrates the feasibility of a strategy to improve the yield of target products by regulating the characteristics of enzymatic reaction based on rational molecular modifications that inhibition of the formation of the ground-state complex of the enzyme and the substrate. Moreover, it provides a new reference strategy for the molecular modification of other enzymes such as multifunctional enzymes and enzymes in cascade reaction systems.

**Data availability**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Ethics declarations**

Declaration of Competing Interest

There are no conflicts of interest to declare

Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

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Contributions

ZS: Data curation, Writing - original draft. ZW: Data curation, Investigation, Writing - Final draft. LX: Investigation. LF: Investigation. XC: Supervision. LD: Supervision. YD: Conceptualization, Methodology, Validation, Supervision.

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