Biochemical Characteristics of Point Mutated Goat Lysosome A- Mannosidase

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Abstract

Background Locoweeds are widely distributed all over the world. Goats and other herbivores often get poisoning if they eat a large number of locoweeds by mistake. Swainsonine (SW), the main toxin in locoweeds, can inhibit lysosomes α -Mannosidase (LAM) competitively in animal cells. The insufficient activity of lysosomes α -Mannosidase can lead to abnormal metabolism of animals, forming alpha mannosidosis. However, the details of interaction between SW and LAM are not clear yet.

Methods In this study, molecular docking was used to predict the interaction points between SW and LAM. The effect of putative points was investigated by constructing mutated LAM (LAM^M) and analyzing its biochemical characteristics.

Results The results showed that the Trp at the 28th position and Tyr at the 599th position of the LAM were the candidates of interaction points. Both tryptophan at position 28 of A peptide residue and tyrosine at position 599 of D peptide residue of goat LAM were mutated into glycine, and goat lysosomes were obtained α - The mutant sequence of mannosidase; The recombinant yeast GS115 / pPIC9K LAM^M was successfully constructed; After SW induction, the sensitivity of goat LAM^M to SW decreased significantly, and the enzyme activity decreased about 3 times compared with wild-type LAM; The optimum temperature of LAM^M decreased from 55 °C to 50 °C, and the optimum pH value increased from 4.5 to 5.0; All the trivalent metal ions (Fe^{3+} , Al^{3+} , Cr^{3+}) had significant activation ($P < 0.05$), and EDTA had inhibitory effect on goat LAM before and after mutation.

Conclusions These results indicated that the sensitivity of goat LAM^M to SW was significantly reduced, but other characteristics changed little, which was consistent with the molecular docking results of goat LAM^M. This study provides help for the study of interaction mechanism between SW and goat LAM, and also provides a new idea for the development of animal locoweed disease control technology.

Introduction

lysosome α - Mannosidase (LAM) is a major exoglycosidase in the glycoprotein degradation pathway, which belongs to the glycosyl hydrolase 38 family (gh38). LAM is mainly involved in the biosynthesis and folding of N-linked glycoproteins and expressed in almost all tissues [1]. The studies on human [2], cattle [3], cats [4], guinea pig [5], goat [6, 7], and llamas [8] showed that insufficient activity of lysosomal α -mannosidase can lead to alpha-mannosidosis[9, 10]. This accumulation of α -Mannoside can have pleiotropic effects on cell functions, such as synaptic release, exocytosis and autophagy [11] with characteristics of vacuolation.

Swainsonine (SW) is also known as 1,2,8-trihydroxyoctahydro indolizidine. Plants containing SW include Swainsona, Oxytropis, Astragalus [12], Ipomoea [13], Turbina [6] and Sida [14], which are collectively referred to as locoweeds. Herbivores can get locoism after ingesting locoweeds, which mainly manifests as unstable gait, mild paralysis or paralysis of hind limbs, head and neck tremor and other neurological

symptoms. Herbivores, particularly goats, with severe locoism often lead to death [15]. Studies have found that SW and mannose have a similar cation space structure and can compete to inhibit intracellular lysosomal α -mannosidase [16, 17]. As a result, abnormal metabolism causes the accumulation of a large number of oligosaccharides in cells.

Several approaches have been evaluated for the treatment of alpha-mannosidosis. For most α -mannosidosis is providing normal enzyme to the lysosomal of abnormal cells [18]. Such as enzyme replacement therapy (recombinant α -mannosidase) [19], bone marrow transplantation [20], gene therapy and substrate reduction therapy [21]. Studies have shown that the production of recombinant lysosomal α -mannosidase and α -mannosidosis mouse models enables research on enzyme replacement therapy and the efficacy in this disease becomes possible [22]. The use of recombinase through cell internalization to reach the lysosome to replace the missing endogenous enzyme has become the most promising treatment method [23]. In view of this, whether this method can also be used for the prevention and treatment of animal loco-weed poisoning, and how to obtain recombinant lysosomal α -mannosidase is particularly important.

The lab cloned the lysosomal α -mannosidase (*Capra hircas* LAM) gene of goat-susceptible locoweed, and conducted bioinformatics analysis on it [24]. This study intends to reduce the sensitivity of lysosomal α -mannosidase to SW without affecting the properties of lysosomal α -mannosidase. Site-directed mutation of goat LAM was performed, and the characteristics of LAM before and after mutation and the sensitivity to SW were compared. To lay the foundation for further exploration of the mechanism of action of LAM and SW, in order to provide a new way for the prevention and treatment of locoism.

Materials And Methods

Material

The GenBank accession number of the sample goat LAM gene is JN602369; the plasmid pMD19T-LAM containing the goat lysosomal α -mannosidase gene fragment has been constructed by our laboratory [24].

The pEASY-Blunt Simple vector is preserved in our laboratory; the yeast expression strain *Pichia pastoris* GS115 and the *Pichia pastoris* protein expression vector pPIC9K were presented by Mr. Zhang Yanming, Northwest A&F University.

Software analysis

Goat LAM consists of 949 amino acid residues and five peptides: A (1-298 aa), B (299-382 aa), C (383-541 aa), D (542-823 aa) and E (824- 949 aa). SW and oligosaccharide D-Mannose 5 (D-Mannose 5, abbreviated as man5) were respectively molecularly docked with goat LAM before and after the mutation. According to Kong et al. [24], AutoDock software was used for molecular docking and mutation analysis of goat LAM genes. In this study, Adobe illustrator CC 2018 software was used to draw a two-dimensional

docking map to facilitate observation of the interaction between the two and key amino acids, and groups. The corresponding amino acid position is represented by peptide + colon + amino acid single letter symbol + amino acid sequence number (such as A: H23, which means A-peptide histidine acid residue 23).

Site-directed mutation of goat LAM gene

According to the goat LAM sequence provided in GenBank, Primer Premier 5.0 software was used to design and synthesize primers for wild-type and mutant LAM genes (Table 1). Using the plasmid pMD19T-LAM as the template, LAM F and A: 28 R as the upstream and downstream primers, amplify the DNA fragment containing the 28th tryptophan mutation site; use A: 28 F and D: 599 R as the upper the downstream primers amplify the middle DNA fragment containing two mutation sites; use D: 599 F and LAM R as the upstream and downstream primers to amplify the DNA fragment containing the 599th Tyrosine mutation site. Purify and recover the above DNA fragments, adjust the molar amounts of the three segments to be consistent, and use LAM F and LAM R as upstream and downstream primers to perform overlap extension PCR. Gel electrophoresis detection, recovery of the full-length target fragment of site-directed mutation.

Connect the purified and recovered mutant LAM target gene fragment to the pEASY-Blunt Simple vector (refer to TransGen instructions) to construct a mutant recombinant cloning plasmid, denoted as pEASY-LAM^M. With the wild-type recombinant plasmid pEASY-LAM as a control, it was transformed into DH5 α competent cells, and positive clones were screened by blue and white spots. Use vector universal primers to screen positive bacteria, extract the positive bacteria plasmids and send them to Shanghai Bioengineering Co., Ltd. for sequencing.

Screening and identification of recombinant *Pichia pastoris* expression strains

Use His-LAM upstream and downstream primers (see **Table 1**) to amplify the target gene fragment in pEASY-LAM^M. The target gene fragment recovered by *EcoRI* digestion and purification and the yeast expression vector pPIC9K were ligated overnight at 16°C with T4 ligase to construct the mutant recombinant yeast expression plasmid pPIC9K-LAM^M. Transform into *E. coli* DH5 α competent cells, pick a single colony, verify with the primer α -factor F' and the downstream primer His-LAM R of the target fragment, and extract the plasmid for sequencing.

Plasmids pPIC9K-LAM, pPIC9K-LAM^M and empty vector pPIC9K were electrotransformed into GS115 yeast competent cells [17]. The electric shock conditions were as follows: voltage 2 kV, time 6 ms. Immediately after the electric shock, 600 μ L of ice-cold 1 mol/L sterile sorbitol was added, spread on the RDB plate, and incubated at 30°C for 4-6 d. Pick single colonies and spot them in sequence on yeast extract peptone dextrose medium (YPD) plates containing G418 (concentrations of 50 μ g/mL, 100 μ g/mL, 150 μ g/mL, and 200 μ g/mL). Take a single colony grown on the transformation plate with high

concentration of G418, and use AOX1 primers to amplify and screen positive bacteria, which are recorded as GS115/pPIC9K-LAM, GS115/pPIC9K-LAM^M and GS115/pPIC9K.

Pick a single positive colony and inoculate it into YPD liquid medium, cultivate until OD₆₀₀ reaches 2-4, and inoculate it in buffered glycerol-complex medium at a ratio of 1%. When OD₆₀₀ reaches 4-6, centrifuge and add buffer methanol-complex medium to resuspend for bacterial cells, adjust the OD₆₀₀ of the bacterial solution to about 1.5. Incubate at 30°C and 200 r/min, and add methanol to the culture solution every 24 hours to make the final concentration 1%. Induced and cultured for 96 h, detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Activity determination and characteristic analysis of goat LAM

For the specific enzyme activity determination system and operation, see Kong's report [24]. Each sample was repeated 3 times, and 3 parallel controls were used to calculate the enzyme activity of α-mannosidase based on the absorbance value. The α-mannosidase activity unit (U) is expressed in nanomoles of p-nitrophenol produced by hydrolysis per liter of bacterial liquid per second, that is, nmol•L⁻¹•s⁻¹. The sensitivity of the recombinant yeast expression product LAM^M to SW is tested by adding 10% SW (m/v) on the basis of the above experiment. The positive control is the recombinant *Pichia pastoris* expression product wild-type LAM, and the negative control is the *Pichia pastoris* empty vector expression product. After reacting at 37°C for 1.5 h, the absorbance change was detected under the condition of 405 nm wavelength.

Add equimolar amounts of different metal ions or inhibitors (Ca²⁺, EDTA, Mn²⁺, Fe²⁺, Mg²⁺, Zn²⁺, Fe³⁺, Cu²⁺, Al³⁺, Co²⁺, Cr³⁺) to the enzyme activity determination reaction system to determine the number of ions with different charges and different species the effect of metal ions on the activity of expressed enzymes; set different reaction temperatures (20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C, 70°C, 75°C, 80°C), detect the optimal reaction temperature of LAM^M, compare the reaction temperature changes of wild-type and mutant enzymes; prepare different pH values (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0) reaction buffer, determine the optimal pH of LAM^M, and compare the pH changes of wild-type and mutant enzymes.

Results

Molecular docking between SW and LAM

Docking between goat LAM and D-Mannose 5 (D-man5) residues showed that D-man5 formed H-Bond interactions with LAM at ten docking sites (A: H23, A: D25, A: D147, A: R171, A: Y212, A: S213, A: S269, B: D334, C: H398, and D: R762) (**Fig. 1A**). By contrast, docking between goat LAM and SW showed that SW forms H-Bond interactions with LAM at another set of six docking sites (A: H23, A: D25, A: W28, A: D147, D: Y599 and D: R762) (**Fig. 1B**). Comparison of docking sites of LAM with D-man5 and SW revealed four common sites (A: H23, A: D25, A: D147 and D: R762). To be noted, the sites of A: W28 and D: Y599 in LAM

exclusively interact with SW in the form of H-bond and are not involved in domain sites (A: H23, A: D25, A: D147 and C: H398) in form of ionic bonds with Zn^{2+} . In summary, molecular docking indicates blocking interaction of SW with A: W28 and D: Y599 in LAM may detoxify SW but does not affect the normal physiological function of LAM.

Recombinant expression of wildtype and mutated LAM in yeast

Next, we mutated these two sites of LAM (A: W28 and D: Y599) into glycine because of considerations as follow: glycine has a small molecular weight; glycine does not have a chiral carbon atom; glycine is not optically active; The R group of glycine has no charge, just a hydrogen atom; and glycine is easily soluble in water. Therefore, site-directed mutations were made to the goat LAM gene (**Fig. 1C, D**) may reduce the binding affinity of LAM and SW. The wild type and the site-directed mutation of LAM were ligated to the pEASY vector, respectively and confirmed by digesting with restriction endonuclease *EcoRI* (**Fig. 2A, C**) and Sanger sequencing (**Fig. 2B**). Then we transformed GS115/pPIC9K-LAM and GS115/pPIC9K-LAM^M into the *Pichia pastoris* strain GS115 and successful transformation of single colonies were identified by PCR (**Fig. 2D**). After induction expression, SDS-PAGE electrophoresis confirmed that the size of the target proteins (LAM and LAM^M) was identical to 110 kDr (**Fig. 2E**).

Sensitivity of goat LAM to SW before and after mutation and analysis of its characteristics

Next, we tested the sensity goat LAM before and after mutation. According to the principle that α -mannosidase can hydrolyze p-nitrophenyl- α -D-mannosides into p-nitrophenol under alkaline conditions, and p-nitrophenol has a maximum absorption peak at 405 nm wavelength. When the concentration of p-nitrophenol is 0-100 $\mu\text{mol/L}$, the absorbance value (y) has a linear relationship with the concentration of p-nitrophenol (x). After testing, the linear equation is: $y = 0.0034x + 0.0002$, $R^2=0.9998$ (**Fig. 3A**). Then, the activity of LAM in the recombinant yeast expression supernatant was compared before and after the SW reaction, and it was found that the enzyme activity of the wild-type goat LAM before and after the SW reaction in the control group was reduced by 47.6 times, and the difference before and after the enzyme reaction was extremely significant ($P<0.01$). It shows that goat LAM is highly sensitive to SW. However, there was no significant difference in the experimental group goat LAM^M before and after SW response ($P>0.05$) (**Fig. 3B**), which shows that the sensitivity of goat LAM^M to SW after mutation is significantly reduced. Interestingly, the enzyme activity before and after the LAM mutation without SW added was reduced by about 3 times (In terms of enzyme activity unit, the activity of LAM was 324 U, and that of LAM^M was 107 U).

By measuring the effects of different metal ions and inhibitors on enzyme activity, it is found that Ca^{2+} , Zn^{2+} , Fe^{3+} , Al^{3+} , Cr^{3+} can promote enzyme activity, while Mg^{2+} , Cu^{2+} , and EDTA can inhibit enzyme activity (**Fig. 4A**). The effects of Mn^{2+} , Fe^{3+} , Al^{3+} , Co^{2+} , Cr^{3+} and EDTA on goat LAM enzyme activity before and after mutation were significantly different ($P<0.05$). The test of the influence of temperature on enzyme activity found that the optimum temperature for goat LAM enzyme activity is 55°C , while the optimum temperature for goat LAM^M enzyme activity is slightly lower (50°C) (**Fig. 4B**). The results of the effect of

pH on enzyme activity showed that the optimal pH for goat LAM enzyme activity was 4.5; while the optimal pH for goat LAM^M enzyme activity was 5, which was higher than the optimal pH for goat LAM enzyme activity (**Fig. 4C**).

Discussion

Lysosomal α -mannosidase is a member of Class 2 mannosidases (glycosyl hydrolase family 38, GH38). This type of enzyme has a relatively large molecular weight (110-135 kDa) and often requires divalent cations to exert its catalytic activity (such as the *Drosophila* enzyme contains a protein-bound Zn^{2+} in the active site [25], which can hydrolyze α -1, 2, α -1, 3 and α -1, 6-mannosidic bonds and aromatic substrates [26, 27]. In mammals, the activity of lysosomal α -mannosidase is related to the catabolism of oligosaccharides [11, 23], and its inhibitory effect on the furanose transition state analogue SW sensitive [26], high affinity [22, 28], and cleave glycosidic linkages by retention of anomeric configuration of the released monosaccharide [29]. It has been confirmed that lysosomal α -mannosidase of various animals has almost the same structure in a 10 Å radius around the Zn^{2+} ion cofactor. The Zn^{2+} ion cofactor is located at the bottom of the active site, which is essential for the catalytic activity of the enzyme and the strong binding of the inhibitor [25, 30].

In the early stage of this experiment, molecular docking was used to predict that A: W28 and D: Y599 on goat LAM could specifically bind to the hydroxyl groups at the 1 and 2 positions of the SW molecule [24]. Therefore, it is proposed to mutate the tryptophan of A: W28 and the tyrosine of D: Y599 on goat LAM into the simplest glycine (ie A: W28G and D: Y599G), which may reduce the sensitivity of the enzyme to SW. Results The overlap extension PCR method was used to carry out double-site mutations in the goat LAM gene, and successfully cloned the goat LAM^M gene sequence containing 2 amino acid mutation sites, which laid the foundation for the study of the characteristics of recombinant expression goat LAM^M.

We found that the enzyme activity of wild-type goat LAM expressed by yeast is 324 U, and the enzyme activity of mutant goat LAM^M is 107 U, which is about 3 times lower than that of wild-type LAM. However, the binding ability of LAM to SW was significantly reduced before and after the mutation (Reduced by about 47 times). Indicating that the sensitivity of goat LAM^M to SW decreased significantly.

Corresponding goat LAM^M docking with SW molecule found that A: W28G and D: Y599G of goat LAM no longer participated in the specific binding of the hydroxyl groups at the 1st and 2nd positions on the SW molecule (**Fig. 1D**). Interestingly, the Zn^{2+} and SW of goat LAM^M did not form an ionic bond again, and the binding site of Zn^{2+} of goat LAM^M with D-Mannose5 did not change much. It shows that these two sites are the key binding sites of goat lysosomal α -mannosidase and substrate SW, and these two sites have little effect on the activity of the enzyme itself. Studies have shown through crystallography that when the substrate (or inhibitor) binds to the active site of α -mannosidase, the mannose ring transforms from a stable chair conformation to an unstable boat conformation [31, 32], this active conformational

change is necessary for catalysis and strong interaction with the Zn^{2+} ion cofactor at the active site [33]. Therefore, we can speculate that the participation of the SW molecule leads to the conformational change of the mannose ring, and the hydroxyl group at the 1 and 2 positions of the SW molecule plays an important role, preventing the hydrolysis of mannosidase. Compared with the parent, the β -mannanases (EC3.2.1.78) mutant screened by Zhang et al. through random mutation has greatly improved specific activity and thermal stability [34]; Another example is the mutation of four sites (43, 44, 55, and 62) with methionine on α -amylase to alanine, the oxidative stability of the enzyme was significantly improved [35]. This further indicated that a slight conformational change appears to influence the substrate-binding and enzyme catalytic characteristics [36].

Lysosomal α -mannosidase is ubiquitous in eukaryotic cells and can be activated by Zn^{2+} [23]. Our study on the characteristics of mutant lysosomal α -mannosidase found that metal ions Mn^{2+} , Fe^{3+} , Al^{3+} , Co^{2+} , Cr^{3+} and EDTA have significantly higher enzymatic activity on LAM^{M} than wild-type LAM ($P < 0.05$). And Ca^{2+} , Zn^{2+} , Fe^{3+} , Al^{3+} , Cr^{3+} can promote the enzyme activity before and after the mutation (**Fig. 4A**). Interestingly, Zn^{2+} has no significant effect on the activity of goat LAM before and after the mutation, and it still has a promoting effect. Studies have shown that mutant human LAM H72L (His72 $\rightarrow$ Leu) can cause lysosomal α -mannosidase storage disease [37], which interferes with metal coordination, affects Zn^{2+} binding and leads to loss of enzyme activity [38]. The Class 2 mannosidases (GH38) mutant from *Sulfolobus solfataricus* showed increased activity against divalent metal ions such as Co^{2+} , Zn^{2+} , and Mn^{2+} [39]. Although the results we obtained also increased, they did not Significant (such as Zn^{2+}). Ca^{2+} plays a key catalytic role in helping distort the mannoside away from its ground-state 4C_1 chair conformation toward the transition state [40]. This study found that Ca^{2+} had a promoting effect before and after the goat LAM mutation, and the difference was not significant, indicating that the mutant goat LAM^{M} did not affect the conformational catalysis. It is worth noting that although the results of this experiment showed that the enzyme activity of EDTA on goat LAM^{M} was significantly higher than that of wild-type LAM, it was still lower than that of the blank control group, so it was still an inhibitory effect. This is consistent with the conclusion that EDTA has an inhibitory effect on the activity of α -mannosidase reported in previous studies [41, 42]. Class 2 mannosidases (GH38) The divalent metal ion bound to the active site is a prerequisite for substrate binding [39], and most studies only reported the relationship between the divalent metal ion and this enzyme, but for mutations there are no reports on the post- α -mannosidase and trivalent metal ions. The trivalent metal ions used in our research have a significant promoting effect on the enzyme activity. It is speculated that the above results may be caused by gene mutations that have changed the preference for different ions.

The optimal temperature of mutant lysosomal α -mannosidase (55°C) is slightly lower than that of the wild type (50°C). The plasticity of enzymes is a way to regulate enzyme activity. Psychrophilic enzymes can compensate for the reduced ambient temperature by reducing the number of ionic bonds, thereby increasing flexibility and catalytic turnover [43, 44]. The optimal pH value of wild-type goat LAM is 4.5, which is consistent with the optimal pH value of lysosomal α -mannosidase reported in the article [23].

The optimum pH value of goat LAM^M increased slightly (from pH 4.5 to pH 5.0, but it was still acidic). Similarly, after site-directed mutation of β -glucanase in *Penicillium verrucosa*, the optimal pH value after mutation is changed from pH 4.0 to pH 5.1[45]. These results may be that the mutation site causes the change in the hydrogen bond between the helical structure and the amino acid residues in the protein [46], and the hydrophobicity of the protein also changes. When the hydrophobicity increases, the thermal stability of the enzyme Sex will decrease [47, 48].

In this study, the sensitivity of goat α -mannosidase to swainsonine was changed by site-directed mutagenesis. Results The sensitivity of goat LAM^M to SW was significantly reduced. Generally speaking, D-mannosides is a structural part of α -mannosidase substrate and has no obvious inhibitory activity on α -mannosidase [49]. However, in order to better verify the effect of mutant LAM^M and D-mannosides on the activity, the next plan is to conduct more accurate calculations and enzyme activity verification. This result lays the foundation for in-depth research on the competitive inhibition mechanism of LAM and SW. Furthermore, it provides a new research direction for the prevention of loco-weed disease from the perspective of competition and inhibition.

Conclusion

This study found that after the 28th Trp of the A peptide residue and the 599th Tyr of the D peptide residue of goat LAM were mutated into simple glycine, the results of goat LAM^M and SW molecular docking are consistent with the changes in LAM^M activity detected after SW is added. The activity of goat LAM^M is reduced by about 3 times, while the sensitivity of goat LAM^M to SW is reduced by 47.6 times; metal Zn²⁺ still has a promoting effect on goat LAM before and after the mutation.

Declarations

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Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Zhang JY, Xiong HF and Li QF. The first draft of the manuscript was written by Wang Y and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing Interests

The authors declare that they have no conflict of interests.

Ethical approval

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

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Tables

Table 1. Relevant primers used in this study

Primer name	Gene sequence	Enzyme
LAM F	5'- <u>TACGTAAT</u> GGCGGGATACAAGACATGCC-3'	<i>SnaB</i> I
LAM R	5'- <u>GAATTC</u> GCCGTCCTCTTCCCATTGGA-3'	<i>EcoR</i> I
LAM 28 F	5'-CGATGATGTCGGC GGG CTCAAGACGGTGG-3'	
LAM 28 R	5'-CCACCGTCTTGAG CCC GCCGACATCATCG-3'	
LAM 599 F	5'-CAGGCCTCCGGCGCC GGC ATCTTCAGACCCAAC-3'	
LAM 599 R	5'-GTTGGGTCTGAAGAT GCC GGCGCCGGAGGCCTG-3'	
His-LAM F	5'- CCG <u>GAATTC</u> GCGGGATACAAGACATGCC-3'	<i>EcoR</i> I
His-LAM R	5'-GCG <u>GAATTCTCA</u> TGGTGATGGT GATGATGAT GGCC GTCCTCTTCCCATT-3'	<i>EcoR</i> I
α-factor	5'-TACTATTGCCAGCATTGCTGC-3'	
AOX1 F	5'-GACTGGTTCCAATTGACAAGC-3'	
AOX1 R	5'-GCAAATGGCATTCTGACATCC-3'	

Note: the dotted underline is His tag, and the bond before His tag means termination codon. Red and blue bold marks indicate the sequence of mutated amino acids, and the bold red marks the coding bases of mutated amino acids. The underline is the sequence of endonuclease.

Figures

Figure 1

Molecular docking 2D model **A** Goat wild-type lysosomal α-mannosidase and oligosaccharide D-mannose5 molecular docking 2D model **B** Goat wild-type α-mannosidase and swainsonine molecular docking 2D model **C** Goat site-directed mutant lysosomal α-mannosidase and oligosaccharide D-

mannose5 molecular docking 2D model **D** Goat site-directed mutant α -mannosidase and swainsonine molecular docking 2D model

Figure 2

Identification of recombinant *Pichia pastoris* expression bacteria **A** Site-directed mutagenesis pattern map of goat lysosome α -mannosidase gene. **B** Sequences alignment before and after mutation of goat lysosomal α -mannosidase. Note: mutation sites are shown in the red box. **C** Results of single restriction digestion of pEASY cloning vector *EcoRI* containing the target gene fragment. Lanes 1 and 2 represent the enzyme digestion results of pEASY-LAM and pEASY-LAM^M, respectively, and M represents DL10000 DNA Marker. **D** PCR identification results of positive recombinant *Pichia pastoris* GS115. M represents 2000 DNA Marker; lanes 1-7 represent GS115/pPIC9K-LAM recombinant bacteria, 8-14 represent GS115/pPIC9K-LAM^M recombinant bacteria, and 15-21 represent GS115/pPIC9K recombinant bacteria. **E** Sodium dodecyl sulfate polyacrylamide gel electrophoresis detection. M stands for standard protein; 1,2 stands for the expression of recombinant protein in GS115/pPIC9K-LAM and GS115/pPIC9K-LAM^M yeast supernatants. Note: The band in the red box is the target protein

Figure 3

Sensitivity of goat lysosome α -mannosidase to SW after mutation **A** Equation of linear egression. **B** Goat LAM^M to SW enzymatic activity detection, ** means the difference is extremely significant

Figure 4

Characteristic detection of goat LAM^M **A** The effect of metal ions and inhibitors on the activity of goat LAM^M protein. *Means significant difference. **B** The effect of temperature on goat LAM^M protein activity. **C** The effect of pH on the activity of goat LAM^M protein