Molecular characterization of Capra hircus lysosomal α-mannosidase and potential mutant site for the therapy of locoweed poisoning*

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Lysoosomal α-Mannosidase (LAM) belongs to the glyco-side hydrolyzing enzymes family 38 and is involved in the biosynthesis and turnover of N-linked glycoproteins process. Locoweeds, which contain swainsonine (SW) that inhibits LAM, are the main poisoning plants in many regions of the world, and thereby resulting in animal poisoning or even death. Based on regions of protein sequence conservation between LAM from Bos taurus and Homo sapiens, we cloned cDNA encoding Capra hircus LAM (chLAM). Expression of cDNA in Pichia pastoris resulted in the secretion of aLAM activity into the culture medium. The recombinant chLAM was activated 1.6 and 1.2-fold with Zn\(^{2+}\) and Ca\(^{2+}\), respectively. By homology modeling, molecular docking and mutant analysis, we obtained the probable binding modes of SW at the allosteric sites of chLAM, and the potential mutant sites for the resistance to SW. Prediction of SW sensitivity to A28 W/G, D58 Y/G mutant chLAM is lower than wild type chLAM. The obtained results lead to a better understanding of not only interactions between substrate/SW and chLAM, but also of a potential strategy for a novel therapy for locoweed poisoning.

Key words: lysosomal α-mannosidase; Capra hircus; locoweed poisoning; molecular docking; Pichia pastoris expression

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INTRODUCTION

Glycoside hydrolyzing (GH) enzymes have been classified into over 100 families on the basis of sequence similarities (Henrissat & Bairoch, 1993; Bourne & Henrissat, 2001; Cantarel & Coutinho, 2009). Lysoosomal α-mannosidase (LAM: EC 3.2.1.24) belongs to the sequence based GH family 38 (GH38) which are about 1000 residues long, and synthesized as a single polypeptide precursor, that is posttranslational cleaved into two to five polypeptide chains (Daniel et al., 1994). This cleavage pattern appears to vary in different organisms (Tollersrud et al., 1997). LAM is involved in N-glycan processing pathways and is associated with endoplasmic reticulum associated protein degradation process (Snaith, 1977; Uno et al., 2010). It is an exoglycosidase that hydrolyses all known α (axial)-mannosidic linkages on mannose-glycans originating from unfolded or endocyted proteins (Snaith, 1975; Merkle et al., 1997; Athanasopoulos et al., 2005). LAM have been isolated and well studied from various animals, plants, and microorganisms sources, such as rat, jack bean, and Aspergillus phoenicis etc (Snaith, 1975; Snaith, 1977; Athanasopoulos et al., 2005). In humans, cattle, cat and guinea pig, the lack of LAM activity causes the autosomal recessive disease α-mannosidosis (Michalski & Klein, 1999). Zn\(^{2+}\) is known to activate LAM and has even been suggested for a therapy of some forms of human α-mannosidosis (Bach et al., 1978). Locoweeds are plants of the Astragalus and Oxytropis genera from North America, South America, and China that contain the indolizidine alkaloid swainsonine (SW) (van Kampen & James, 1969). SW can inhibit LAM and Golgi mannosidase II (GAMII), which results in cellular vacuolation and degeneration similar to genetic α-mannosidosis (Haraguchi et al., 2003; Barbosa et al., 2006). Poisoned animals appear neurological changes, reproductive disturbance, and emaciation. It causes many animal death and huge economical loss every year (Dantas et al., 2007). Nowadays, the main methods of preventing the locoweed poisoning are such as exterminating locoweed by artificial excavated, incineration and herbicides, which easily cause destruction of vegetation, and lead to desertification of grassland (Li, 2003). However, in the light of its ecological perspective, such as drought and cold resistance, strong reproductive capacity, high distribution of density and nutrition value, locoweed is an important forage resource (Li, 2003). Therefore, the prevention and cure of locoweed poisoning should focus on the comprehensive utilization.

Although many LAMs gene sequences from various organisms have been reported (Costanzi et al., 2006), there is no detailed research about the characteristic of locoweeds sensitivity animal Capra hircus LAM (chLAM). Many domestic and foreign scholars have made great progress on prevention and therapy of locoweed poisoning (Balogh et al., 1999), but the mechanism about SW inhabiting chLAM has not been revealed yet. The therapy for most α-mannosidosis is providing normal enzyme to the lysosomal of abnormal cells (Ellinwood et al., 2004). The current approaches exist, or are under evaluation to deliver normal enzyme to abnormal cells by parental injection of purified recombinant enzyme (enzyme replacement therapy, ERT) (Gabrielli et al., 2010), bone

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Abbreviations: AMA, α-mannosidase; LAM, Lysoosomal AMA; chLAM, Capra hircus LAM; btLAM, Bos taurus LAM; hsLAM, Homo sapiens LAM; GAMII, Golgi mannosidase II; SW, swainsonine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ERT, enzyme replacement therapy; pNP-α-Man, p-nitrophenyl α-mannoside; Man5, D-Mannose5
marmot transplantation (Wagner et al., 2010), and substrate reduction therapy (Marshall et al., 2010). Drawing lessons from α-mannosidosis, to find out the mechanisms about SW inhabiting chLAM and to obtain a mutant chLAM which resistance to SW, appears very important.

Therefore, in this study, we described cDNA expression and molecular characterization of chLAM using Pichia pastoris as expression system. Using the technology of fluorescence quantitative PCR, chLAM gene expression profiling was obtained. Moreover, we generated a molecular 3-D model from the deuced amino acid sequence information using SWISS-MODEL web server, which showed that the amino acid residues located at the active site of chLAM are conserved between different species’ LAM. Molecular docking method was also performed for further prediction on the structure properties, the probable binding modes of SW at the allosteric sites of chLAM, and the potential mutant sites for the resistance to SW. All these methods applied on chLAM could not only help understanding of the ligand-receptor interactions, but may be also applied to the prevention and for the therapy of locoweed poisoning.

MATERIALS AND METHODS

Materials. pNP-α-Man was from Sigma Co., St. Louis, U.S.A. Restriction enzymes were purchased from New England BioLabs Beverly, MA, USA. All synthetic oligonucleotide primers and Tag polymerase used in this study were from Takara Bio Inc., Otsu, Japan. PCR TA cloning kit, RNA isolation kit, and the Pichia expression vector were from Invitrogen San. Diego, CA, USA. Experimental animals, nanny goat, supplied by Laboratory Animal Center of Northwest A&F University (Yangling, Shaanxi 712100, P. R. China). All other reagents were at least reagent grade and obtained from standard suppliers.

Nanny goat cDNA synthesis. Total RNA was isolated from ten 3-month-old nanny goats by the Trizol protocol (Invitrogen) and quantified by determining its absorbance at OD260/OD280. The animals were conducted according to institutional and ethical guidelines involving use of animals and the liver was quickly removed, preserved in liquid N2 and stored at –80°C. Poly (A)+ RNA was isolated using Oligotex-dT30 (Takara, Kyoto, Japan) in accordance with the manufacturer’s protocol. Total RNA (2 μg) was reverse transcribed with M-MLV reverse transcriptase (Life Technologies, Inc.) using the manufacturer’s protocols.

Polymerase chain reaction (PCR) and subcloning of PCR product. Degenerate oligonucleotide primers (F 5’-CTCGAGAGGGTGTGTTGCTG-3’ and R 5’-CGGTAGTGCAGATTTGA-3’) were designed based on Bos taurus LAM (chLAM, GenBank ID NM_174561.2) (Tollersrud et al., 1997). The amplification of goat liver LAM cDNA was carried out using PrimeSTAR HS DNA polymerase according to the manufacturer’s protocols. The sample were placed in a DNA thermal cycle programmed for temperature-step cycle of 92°C (45 s), 55°C (45 s), and 72°C (3 min) for 30 cycles. After the final cycle, the reaction was maintained at 72°C for 10 min. The final reaction products were resolved on a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Amplification products were purified from the gel using the DNA purification kit (Marathon, Clontech, Palo Alto, CA) and subcloned into pMD19-T simple vector. Recombinant plasmids were isolated from liquid bacterial cultures and subjected to DNA sequencing.

Transformation of Pichia pastoris. chLAM cDNA was amplified by PCR using the oligonucleotide primers, 5’-GGCTGAAAGTTCTATGGCGGATACAA-GACATGCCC-3’ (sense primer) and 5’-AAAGTGGCGGGCGCCGCGGTCTCCTTCCATTTGG-3’ (antisense primer). EcoRI and SacII restriction sites were designed into the sense and antisense primer, respectively. The primers permit the directional cloning of the amplified DNA in frame with the α-factor leader sequence in pPICZα A expression vector. The recombinant vector was linearized by Pme I and introduced into the Pichia host strain, X-33, using transformation procedures described in the Pichia expression manual (Hossain et al., 2010). pPICZα A plasmid without an insert was conducted as negative controls. Positive transformants were isolated by zeocin™ screening (Hossain et al., 2010). After methanol induction, the clarified culture medium from each of the transformants was collected by centrifugation at 5000 r/min for 5 min and assayed for LAM enzyme activity.

Assay system for LAM activity. Enzyme activity was measured by the chromogenic substrate pNP-α-Man (Liao et al., 1996). The incubation mixture contained 50 μl of sample, 50 μl of 0.5 M acetic acid/NaOH buffer (pH 4.5), and 20 μl of 10 mM pNP-α-Man. Incubation lasted 90 min at 37°C and was terminated by addition of 200 μl of 0.4 M glycine/NaOH buffer (pH 10.5). The released p-nitrophenol was measured by taking absorbance at 405 nm. One unit of enzyme activity was defined as releasing 1 nmol of p-nitrophenol in 1 second at 37°C.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant chLAM was analyzed by SDS-PAGE (7.5% polyacrylamide) in 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS buffer using slab gel apparatus. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

Effects of pH, temperature, and metal ions on the activity of chLAM. The effects of pH on the activity of recombinant chLAM were determined using pNP-α-Man as substrates at various pH levels (3.0–8.0). The effects of temperature were also examined at various temperatures (20–80°C) followed by standard assay method described in the earlier section. The enzymatic activities were calculated as percent (%) relative to the highest sample observed in this assay. The effects of metal ions on the activity of recombinant chLAM were investigated using Fe2+, Fe3+, Mn2+, Co2+, Ca2+, Zn2+, Cu2+, Mg2+, Al3+, and EDTA at a concentration of 5 mM.

Real-time quantitative PCR (qPCR). qPCR is a method for rapid and reliable quantification of mRNA transcription. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference to normalise mRNA levels between different samples for an exact comparison of mRNA transcription level. The sequence of the primers for GAPDH gene was F 5’-TGACCTGGACCTGGACAA-3’ and R 5’-AAGGAGGAGGATGGCGG-3’. The primer sequence for chLAM gene was 5’-GAGAAGGCGTTGCTACCTT-3’ and R 5’-GTTCACCGGCCATCAAAACA-3’. PCR reactions were performed on Bio-Rad iQ5 System. For each PCR run with SYBR Green I detection, a melting curve analysis was performed to guarantee the specificity in each reaction tube. The experimental procedure and calculation method was assayed according to Preter and cowokers (De Preter et al., 2002).
Bioinformatics analysis. The signal peptide was detected by SignalP 3.0 Server (Petersen et al., 2011). ExPASy Proteomics Server (Gasteiger et al., 2003) was used to determine the isoelectric point (pI) and theoretical molecular mass of dLAM. The secondary structure and N-glycosylation sites in the deduced residue sequence of dLAM were identified by PredicProtein online software (Rost et al., 2004). The most conserved domains of dLAM were identified by conserved domain database search at the National Center for Biotechnology Information (NCBI) web server (Bauer & Bryant, 2004). The phylogenetic reconstruction of dLAM and homologues sequences was carried out by ClustalX 1.83 (Thompson et al., 1997).

Homology modeling and docking analysis. 3D protein structure is an important source of information for better understanding the function of a protein. The 3D structure of dLAM was created by SWISS-MODEL server 3.5 (Kiefer et al., 2009). The template protein bLAM (PDB ID 1O7D chains A, B, C, D and E resolution of 2.7 Å) from Brookhaven Protein Database (http://www.pdb.org/pdb/home/home.do) identified by Blast Search (http://www.ncbi.nlm.nih.gov/BLAS) was employed here for the construction of the target proteins (Heikinheimo et al., 2003). In addition, the quality of the modeled structure was assessed by the protein structure verification Procheck module of the structural analysis and verification on-line server (Kiefer et al., 2009). In order to locate the appropriate binding orientations, and conformations of substrates (man3 and SW) into the enzyme (modeled wild type and mutant dLAM) binding pocket, molecular docking was carried out by AutoDock4.2. Detailed process can be easily available from other resources (Jiang et al., 2008).

RESULTS

Cloning, sequencing and identification of chLAM

We deposited the isolated nucleotide sequences which encoded chLAM by a PCR-based approach in the GenBank data base with accession number JN602369. The sequence of the longest clone contained a full length open reading frame as well as 10 bp of 5'UTR and 68 bp of 3'UTR sequences, respectively, based on a comparison with bLAM cDNA (Tollersrud et al., 1997).

Sequence and phylogenetic analysis of chLAM

A 3000 bp open reading frame (ORF) was obtained from cDNA sequence isolated from nanny goat. It coded for a polypeptide of 999 amino acid residues of which the first N-terminal 50 amino acid residues form the signal sequence. Therefore, mature chLAM is a polypeptide containing 949 amino acid residues with a predicted pI 8.01 and molecular mass of 107.56 kDa. Further analysis showed that mature chLAM amino acid sequence contained nine N-glycosylation sites. The positions of predicted N-glycosylation sites were 134, 347, 499, 755, and 919 in the deduced amino acid sequence.

The Class II α-mannosidase (AMA), which was classified under glycosyl hydrolase family 38 (GH38), conserved GH38 N-terminal domain, AMA middle domain and GH38 C-terminal domain and can cleave α1-2, α1-3, and α1-6 glycosidic linkages of glycoproteins (Hossain et al., 2010). The conserved domain database search program in NCBI web server showed that chLAM also has such three kinds of consecutive conserved domains in the deduced residue sequence. Therefore, these results demonstrated that chLAM belongs to GH38. To determine the evolutionary position of chLAM, a neighbor-joining phylogenetic tree was constructed (Fig. 1). We used the deduced amino acid sequence of chLAM as Blastp input to explore the neighbors or homologous sequences. For the phylogenetic reconstruction, we included 22 sequences, which were previously functionally characterized as LAM. The resulting neighbor-joining tree suggests that chLAM is very close to bLAM (NP 776986).

Protein expression in Pichia pastoris and enzymatic characterization

In order to demonstrate that chLAM cDNA encodes a functional LAM enzyme activity by expression in Pichia

Figure 1. Phylogenetic tree of amino acid sequence from LAM homologues found in NCBI/Blastp search

Multiple sequence alignment was performed using ClustalX 1.83 program. A total of 22 genes those characterized as GH38 were taken into consideration for the construction of phylogenetic tree. The analyzed LAM is very close to bLAM.

In order to demonstrate that chLAM cDNA encodes a functional LAM enzyme activity by expression in Pichia
Effects of pH, temperature, and metal ions on the activity of chLAM

The effects of pH and temperature on the activity of chLAM were analyzed by pNP-α-Man as substrates. chLAM shows optimum activity at pH 6.0 and the activity rapidly declines after pH 6.0 (Fig. 3), which suggests that chLAM could reside in the acidic region of cell. The optimum temperature of chLAM for substrate pNP-α-Man was found to be at around 55°C (Fig. 3). The metal ions screen showed that the recombinant chLAM was activated 1.6 and 1.2-fold with Zn^{2+} and Ca^{2+}, respectively (Fig. 3).

Tissue distribution of chLAM mRNA

The expression patterns of chLAM gene in different tissues were shown by qPCR. At first, the design and the pre-validation of the primers are essential to generate reliable data. As seen in Fig. 4A and 4B, the dissociation curve appears as a single “stacked” peak at the amplicon Tm, suggesting the absence of other nonspecific products and primer dimers. For each of the 3 replicates of a sample, the average cycle time (Ct), and the standard deviation (stdev) were calculated. The relative expression for each sample was calculated by 2-ΔΔCt method. As seen in Fig. 4A and 4B, the dissociation curve appears as a single “stacked” peak at the amplicon Tm, suggesting the absence of other nonspecific products and primer dimers. For each of the 3 replicates of a sample, the average cycle time (Ct), and the standard deviation (stdev) were calculated. The relative expression for each sample was calculated by 2-ΔΔCt method. The result is plotted as a histogram graph (Fig. 4C). The results denote that chLAM gene is generally expressed in various tissues, but at different levels. The greatest chLAM gene mRNA abundance is found in lung, and liver followed by cerebella, cerebrum, and ovary. chLAM transcript was less abundant in spleen, kidney, and heart.

Molecular 3-D modeling

An accurate three dimensional (3D) structure of the receptor is important for molecular docking (Ginalska, 2006). Due to the unavailability of X-ray structure of chLAM, the homology modeling for the protein structure from its primary sequence was performed. The template enzyme employed here is bLAM (PDB code 1O7D) (Heikinheimo et al., 2003), which exhibits a high Tm, suggesting the absence of other nonspecific products and primer dimers. For each of the 3 replicates of a sample, the average cycle time (Ct), and the standard deviation (stdev) were calculated. The relative expression for each sample was calculated by 2-ΔΔCt method. The result is plotted as a histogram graph (Fig. 4C). The results denote that chLAM gene is generally expressed in various tissues, but at different levels. The greatest chLAM gene mRNA abundance is found in lung, and liver followed by cerebella, cerebrum, and ovary. chLAM transcript was less abundant in spleen, kidney, and heart.

Effects of temperature, pH, and metal cation on the activity of recombinant chLAM protein

(Left) Effects of temperature on the activity of chLAM. pNP-α-Man was used as substrates to analyze the effects of temperature on the activity of chLAM. chLAM was mixed with pNP-α-Man in 1 mol/L acetic acid/NaOH buffer, pH 4.5 and incubated at various temperatures (20, 30, 40, 50, 60, 70 and 80°C). (Middle) The effects of pH on the activity of chLAM were examined using pNP-α-Man as substrates at various temperatures (20–80°C) and pH levels (3.0–8.0). The buffers used were 0.1 M Glycine-HCl (pH 3.0), 0.1 M Citrate (pH 3.5–5), 0.1 M MES (pH5.5–6.5) and 0.1 M HEPES (pH7.0–8.0). (Right) The effects of metal ions on the activity of recombinant chLAM were investigated using Fe^{2+}, Fe^{3+}, Mn^{2+}, Co^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+}, Al^{3+}, and EDTA at a concentration of 5 mM.

Figure 3. Effects of temperature, pH and metal cation on the activity of recombinant chLAM protein

Figure 2. chLAM expression vector construct and SDS/PAGE

(A) chLAM expression construct. chLAM cDNA (2850 bp) was expressed under AOX1 promoter with N-terminal His tag. (B) Purity recombinant P. pastoris expression product in X-33 was checked by 12% acrylamide and stained with Coomassie brilliant blue R-250. Lane M, Marker proteins; Lane 1, pPICZα A-chLAM-X-33 (10 times concentrated); Lane 2, pPICZα A-X-33 (20 times concentrated). The proteins used in the calculation of molecular masses were Precision Plus ProteinTM Standards (BioRad): 210 kDa, 140 kDa, 102 kDa, 75 kDa, 52 kDa and 20 kDa.

Figure 4. Real-time RT-PCR analysis of chLAM gene expression in different tissues

(A) Molecular structure of chLAM. The full-length cDNA (2850 bp) of chLAM was subcloned into the pPICZα A-expression vector. The signal peptide and N-terminal His tag were removed by NdeI and XhoI cleavage sites, respectively. The insert was confirmed by sequencing. The recombinant chLAM was expressed in P. pastoris X-33 by induction with methanol (1%). The expression patterns of chLAM gene in different tissues were analyzed by qPCR. (B) The effects of temperature on the activity of recombinant chLAM. The results are plotted as a histogram graph (Fig. 4C). The results denote that chLAM gene is generally expressed in various tissues, but at different levels. The greatest chLAM gene mRNA abundance is found in lung, and liver followed by cerebella, cerebrum, and ovary. chLAM transcript was less abundant in spleen, kidney, and heart.
Therefore, an overall quality of modeled structure was good as checked by the program.

An overall structural model of chLAM included 949 residues with five structural parts, A (1-298 aa), B (299-382 aa), C (383-541 aa), D (542-823 aa), and E (824-949 aa). An important secondary structural motif comprised 20 helices and 42 strands (Fig. 5). The α and β contents of the model protein were found to be 23.13% and 24.04%, respectively, as predicted by the PredictProtein program.

Docking and mutation analysis

Figure 6A and 6C show that D-Mannose 5 (man5) and SW nestles in the groove of wild type chLAM characterized by residues His A23 (α-peptide histidine acid residue 23), Asp A25, Trp A28, Asp A147, Asp A270, His C16, Asp C17, Tyr D58, and Arg D221. Figure 7 displays these key amino acids are conserved between different species’ LAM. The coordination bond formed between key residues and Zn²⁺, as well as H-bond and hydrophobic interactions strongly stabilize the complexes, suggesting the potent binding of man5 and SW to wild type chLAM. Figure 6A depicts that man5 is lodged in the active pocket surrounded by residues Asp A25, Asp A147, His C16, and Arg D221 via H-bonding and hydrophobic. Furthermore, it also forms a strong ionic bond with the catalytic Zn²⁺, which forms itself H-bond with residue His A23, Asp A25, and His C16 of the pocket. Figure 6C reveals that SW is anchored in the binding site of wild type chLAM via several H-bonds by residues Asp A25, Trp A28, Asp A147, Tyr D58, and Arg D221. A Zn²⁺ also stabilizes the binding by mediating the interaction between SW and His A23, and Asp A25. Man5 and SW adopt a similar global conformation.

Figure 4. chLAM expression profiling
Dissociation and amplification curve of GAPDH (A) and chLAM (B) gene. qPCR results of chLAM gene relative expression in nine tissues. The relative mRNA expression level of chLAM gene in different tissues (C).

Figure 5. Prediction for secondary of chLAM
The secondary structural motif of chLAM comprises 20 α-helices and 42 strands. Displays these nine key amino acids are conserved between different species’ LAM.

Figure 6. The molecular docked conformations of man5 (A/B) and SW (C/D) with wild type/mutant chLAM

Figure 7. The alignments of key amino acid sequence of each species’ LAM
Only key amino acids regions of 22 genes those characterized as GH38 are shown. *Represents the nine key amino acids which conserved between different species’ LAM.
locoweed poisoning. We closely inspect the difference in the wild type with man5, which nonetheless is inconsistent with other species’ LAM. Such as Zn$^{2+}$ are also required for the maximal activity of LAM from rat epididymis (Khan & Basu, 1982). For LAM from human placental tissue, Mn$^{2+}$, Cu$^{2+}$, and Co$^{2+}$ had no effect at 1 mM concentration (Snailth & Levy, 1968).

For tissue specific expression profile analysis, $\alpha$-LAM gene is generally expressed in various tissues. Such as SW-induced lysosomal storage disease in goats, cytoplasmatic vacuolation was observed in the central nervous system and pancreatic acinar cells, epithelial cells of the kidney (Novikoff et al., 1985; Dantas et al., 2007). The highest levels of mRNA were observed in lung, and liver. But for mouse, tissues with greatest LAM mRNA abundance are epididymis, spleen and testis (Becchari et al., 1997). Results display that various species of LAM expression levels are not conserved.

**Mutant prediction for the treatment of locoweed toxicity**

Locoweeds, which contain the toxic indolizidine alkaloid SW that inhibits LAM, cause goats, horses, sheep poisoning, and sometimes even death, as well as large economical loss every year (van Kampen & James, 1969). The action of $\alpha$-mannosidosis results in a lysosomal storage disorder which is caused by deficient activity of LAM closely mimics locoweeds poisoning, characterized by massive accumulation of mannose-containing oligosaccharides in affected individuals (Michalski & Klein, 1999). Therapy for $\alpha$-mannosidosis usually depends on providing normal enzyme to the lysosomal of abnormal bone marrow transplantation (Wagner et al., 2010), ERT (Gabrielli et al., 2010), and substrate reduction therapy (Marshall et al., 2010) et al. In this study, a potential therapy method for locoweeds poisoning, which is based on $\alpha$-mannosidosis, is to deliver mutant LAM, that is lower or not inhibited by SW, to toxic animal.

Crawley et al. have used the guinea-pig model to investigate efficacy of ERT as a treatment for $\alpha$-mannosidosis (Crawley et al., 2006). Intravenous recombinant *Homo sapiens* LAM (hLAM) was cleared by circulation with a half-life of 53 h, with 1.4-fold of normal enzyme activity levels detected one week post-injection (Crawley et al., 2006). However, multiple injections of recombinant hLAM in $\alpha$-mannosidosis guinea-pigs induced a very rapid hemorulal immune response precluding long-term intravenous treatment. In other studies, onset of ERT at birth in a feline mucopolysaccharidosis VI model using recombinant human protein resulted in lack of immune response compared with onset of therapy at a later age (Auclair et al., 2003). However, starting therapy at birth in the guinea-pig $\alpha$-mannosidosis model did not abrogate this immune response (Crawley et al., 2006). Further studies using recombinant guinea-pig $\alpha$-mannosidase would likely overcome immunological issues. Therefore, to obtain recombinant hLAM and find out a mutant hLAM which resistance to SW appears critically needed for goat locoweed toxicity.

Clearly, in vitro mutate remains a labor-intensive and time-consuming operation. Thus, more efficient and economical alternative methods should be employed, such as screening for resistance to SW.
as in silico molecular modeling approach that is used. Heikinheimo et al. have solved the structure of boLAM at 2.7 Å resolution by molecular replacement (Heikinheimo et al., 2003). It opens the way to build realistic structural model of chLAM through in silico modeling and provides a major breakthrough to learn the binding interaction between man5/SW and chLAM. By docking analysis, we confirm that the active site of chLAM is formed by nine key amino acids (His A23, Asp A25, Trp A28, Asp A147, Asp A270, His C16, Asp C17, Tyr D58, and Arg D221), which conserved between different species’ LAM. Studies have shown that D221 R/H and R/W mutations are associated with α-mannosidosis of cattle (Tollersrud et al., 1997) and guinea-pig (Berg et al., 1997), respectively.

After inspecting the difference binding modes of man5 and SW with the wild type chLAM, Trp A28 and Tyr D58 are only seen involved in H-bond interaction with SW. If Trp A28 and Tyr D58 of wild type chLAM mutate to Gly, it may contribute to the resistance to SW. Furthermore, it is no reported that this two amino acid mutations would affect the activity of LAM. The superimposition of molecular docked conformations of man5/SW with wild type and A28 W/G and D58 Y/G mutant chLAM show that SW stays ~4Å away from the active site. H-bond number of man5 does not appear to be significantly different, but the H-bonds with the His A23, Asp A25, Trp A28, Tyr D58, and Arg D221 are lost for SW. Overall, the mutation chLAM causes a shift to a lower binding affinity for SW, which nonetheless does not appear to be significantly different of the wild type with man5.

ERT associated with A28 W/G and D58 Y/G mutant chLAM could lead to a strategy for a novel therapy method in locoever poisoning. The next step is expressing the mutant chLAM with target characterizations. In another direction, the sequence of the mutant chLAM can also be combined with transgenic technology or gene therapy.

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