Open Access
Asian Australas. J. Anim. Sci.
Vol. 27, No. 4 : 574-579 April 2014
http://dx.doi.org/10.5713/ajas.2013.13529
www.ajas.info
pISSN 1011-2367 eISSN 1976-5517

# Expression, Purification, and Characteristic of Tibetan Sheep Breast Lysozyme Using *Pichia pastoris* Expression System

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**ABSTRACT:** A lysozyme gene from breast of Tibetan sheep was successfully expressed by secretion using a-factor signal sequence in the methylotrophic yeast, *Pichia pastoris* GS115. An expression yield and specific activity greater than 500 mg/L and 4,000 U/mg was obtained. Results at optimal pH and temperature showed recombinant lysozyme has higher lytic activity at pH 6.5 and 45°C. This study demonstrates the successful expression of recombinant lysozyme using the eukaryotic host organism *P. pastoris* paving the way for protein engineering. Additionally, this study shows the feasibility of subsequent industrial manufacture of the enzyme with this expression system together with a high purity scheme for easy high-yield purification. (**Key Words:** Clone, Lysozyme, *Pichia pastoris*, Purification, Secretory Expression, Tibetan Sheep)

## INTRODUCTION

Lysozyme (LZM, EC 3.2.1.17), a crucial enzyme in both innate immunity and digestion (Prager and Joliès, 1995; Leippe, 1999), can hydrolyze β-1,4-linkages between N-acetyl-D-glucosamine and N-acetylmuramic acid residues of the peptidoglycan layer of bacterial cell walls (Salton and Ghuysen, 1959). LZM has been found existing in various species where reports shown that LZM has anti-bacterial, anti-viral and anti-tumor activities (Ferrari et al., 1959; Hughey and Johnson, 1987; Sava et al., 1989). Futhermore, changes on LZM concentration in serum or urine can be used as a diagnostic marker for certain diseases (Peeters et al., 1978). Thus, LZM is under study as a potentially useful material for industrial use such as in medicinal feed, baby formula, and various food products (Yang et al., 2011).

The *P. pastoris* expression system has several advantages over other eukaryotic and prokaryotic expression systems such as the ease of manipulation, diverse post-translational modifications i.e. polypeptide folding, acylation, glycosylation, methylation, proteolytic adjustment, rapid growth rate and high expression levels (Li et al., 2007).

Tibetan sheep LZM is a C-type LZM, a single polypeptide composed of 130 amino acid residues. LZM widely existed in Tibetan sheep tissues or body fluids and also plays an important role as an anti-inflammatory factor (Ogundele, 1998). LZM, in sheep milk, can help prevent disease infection in infants and improve non-special immunity. However, until now, research on Tibetan sheep breast LZM (SLZ) has not been reported. Thus, we cloned the *SLZ* gene and successfully expressed it in *P. pastoris* GS115 using the expression vector pPICZαA at first time. We also optimized a method for purifying recombinant SLZ (rSLZ) from yeast culture for potential large-scale production in the future.

## MATERIALS AND METHODS

### Materials

Tissue samples of Tibetan sheep breast were collected

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Submitted Aug. 23, 2013; Accepted Nov. 17, 2013; Revised Nov. 30, 2013

from a 4-yr-old healthy female Tibetan sheep from Ruoergai county of Sichuan province. *E. coli* DH5α (Invitrogen, Carlsbad, Calif., USA) was used for subcloning and yeast vector amplification. *P. pastoris* GS115 (Invitrogen) was used as the host for rSLZ. All reagents were from commercial sources.

**Growth media and conditions**

Yeast extract peptone dextrose medium (YPD) contains 1% yeast extract, 2% peptone, 2% dextrose and 2% agar. Buffered glycerol-complex medium (BMGY) contains 1% yeast extract, 1% glycerol, 2% peptone, ($4\times10^{-5}$)% biotin, 1.34% yeast nitrogen base and was supplemented with 100 mmol/L potassium phosphate (pH 6.0). Buffered methanol-complex medium (BMMY) was prepared in accordance with BMGY but containing 0.5% methanol instead of 1% glycerol.

**Screening and amplification of SLZ cDNA**

Total RNA was extracted from Tibetan sheep breast using Trizol RNA extraction kit (life technologies, USA). The oligonucleotides with sequences of 5'-TCTCTCGAGAAAAGAGAGGCTGAAGCTAAGGTCTTTGAGAGATGTGA-3' and 5'- CTGTCTAGATCACACTCCACAACCCTGAATG-3' were designed based on the open reading frame of the SLZ (GenBank accession No. KF871070) without its signal sequence and used as specific primers for PCR reactions. Restriction sites at the 5' ends of the primers for *Xho*I and *Xba*I (underlined) were incorporated to facilitate subcloning of the PCR product. Amplification was performed using pfu DNA polymerase by PCR reaction for 30 cycles (denaturation: 94°C for 30 s; annealing: 54°C for 30 s; and extension: 72°C for 30 s). After, the inserted cDNA fragment was sequenced.

**Construction of expression plasmids and transformation of *Pichia pastoris* GS115**

The PCR product of the specific primer was purified and cloned into pMD19-T.The recombinant plasmid was digested with *Xba*I and *Xho*I and the small fragment was separated and ligated into the expression vector pPICZαA. This recombinant plasmid was named pPICZαA-SLZ and finally confirmed by sequencing. The plasmid pPICZαA-SLZ was linearized with *Sac*I and transformed into *P. pastoris* GS115 using lithium chloride transformation method according to the manufacturer's protocol (Invitrogen, *Pichia* Expression Kit). The transformants were cultivated at 30°C for 1 to 4 h and then spread over YPDZ (YPD medium plus $2.5\times10^{-3}$ % Zeocin) plates cultured at 30°C for 2 to 3 d. An enhanced Zeocin selection with a 5 to 20 fold concentration (12.5 to $50\times10^{-3}$ % Zeocin) of Zeocin was performed to screen multicopy recombinant colonies. One of transfortmants with a high Zeocin resistance was selected for expression.

**Cultivation of *Pichia pastoris* GS115 and expression of pPICZαA-SLZ**

The transformant was inoculated into 10 mL of BMGY medium and cultured at 28°C on a rotary shaker at 200 rpm for 2 days until OD600 nm value was 2-6. The yeast cells were harvested and resuspended in 20 mL of BMMY medium at an OD600 nm value of 1 ($0.5\times10^{8}$ cells/mL). The culture was grown at 28°C with constant shaking on a rotary shaker at 220 rpm for 168 h. The culture was supplemented every 24 h with 0.5% (v/v) methanol to maintain the induction of transformant expression.

**Purification of rSLZ from *Pichia pastoris* culture media**

The culture was centrifuged for 20 min at 5,000 g at 4°C. The supernatant was collected and ammonium sulfate was added to the crude extract to 65% saturation. The protein was precipitated and dissolved in PBS (phosphate buffer solution, pH 6.0) buffer and finally dialyzed in a regenerated cellulose membrane for 36 h and replaced dialyzate every 12 h. The solution was loaded onto a DEAE-Sepharose Fast Flow column (GE Healthcare) equilibrated with Tris buffer (pH 9.0, ionic strength = 10 mM, 50 mM Tris and 4.3 mM NaCl) and eluted with a linear gradient of Tris buffer (pH 9.0, ionic strength = 10 mM, 50 mM Tris and 4.3 mM NaCl), -1 M NaCl. Afterwards, the main peak was determined. Fractions with high rSLZ activity were pooled and stored at −20°C. After freeze-drying (Telstar*LyoQuest, Spain), rSLZ was purified by gel-filtration on a Sephadex G-75 (Pharmacia) HR column on a BioLogic LP chromatography system (Bio-RAD, USA). The flow rate was 0.2 mL/min, and the column was equilibrated and eluted using Tris buffer (pH 9.0, ionic strength = 10 mM, 50 mM Tris and 4.3 mM NaCl). The active fractions were pooled, desalinated and referred to as the purified enzyme preparation. The rSLZ fraction was identified by SDS-PAGE.

**Enzyme assay and molecular properties**

rSLZ activity was measured using *Micrococcus lysodeikticus* as substrate at a concentration of 0.25 mg/mL and recording the change in percent transmittance at 450 nm with time in a double beam UV-visible light spectrophotometer (Parry et al., 1965). One unit of rSLZ activity was defined as a 0.1% change in transmittance per minute. The protein concentration was determined by the Bradford method with bovine serum albumin as the standard (Bradford, 1976). SDS-PAGE was performed with 15% (v/v) separating gel and 5% (v/v) stacking gel. Protein

bands were visualized using Coomassie blue staining method.

## RESULTS

### Cloning of SLZ cDNA

The DNA fragment encoding LZM was amplified by PCR reaction. From the data shown (Figure 1), a 429 bp sequence was obtained, which is completely consistent with the expected result, and then the sequence was purified and inserted into a pMD19-T vector. The recombinant plasmid was extracted and digested by *Xba*I and *Xho*I, the result revealed that restriction enzyme digestion produced a 417 bp length DNA fragment (Figure 1). Finally the fragment was linked into pPICZαA for extracellular expressions.

### Expression of rSLZ in *Pichia pastoris*

As a basis for functional and structural studies, the SLZ cDNA was successfully expressed in *P. pastoris* under the control of the AOX1 promoter with the a-factor signal sequence for secretion. The cell density and activity of rSLZ increased until 144 h, where it proceeded at a constant value ($14\times10^8$ cells/mL and 1,800 U/mL).

### Purification of rSLZ

rSLZ was purified to homogeneity from culture supernatant after 168 h of induction and it was purified by a two-step chromatographic procedure as outlined in Table 1. Anion exchange chromatography was followed by a gel-filtration chromatography step resulting in three fractions containing active rSLZ. Fractions were pooled and loaded on a Sephadex G-75 HR column where a homogenous enzyme preparation as judged by SDS-PAGE (Figure 2). After induction of 168 h, concentration of rSLZ exceeded 500 mg/L. Finally, 109% of the initial activity was found in this enzyme preparation with a specific activity of 4,000 U/mg representing a 10.2-fold purification from culture supernatant (Table 1). Recombinant protein from this pool was used for further analyses.

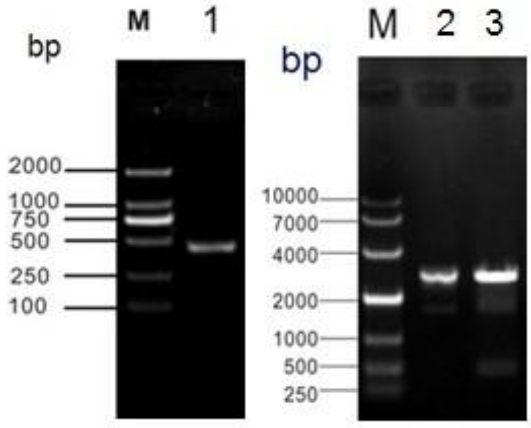


**Figure 1.** Agarose gel electrophoresis of PCR product and recombinant plasmid digested with *Xho*I and *Xba*I. Lane M = DNA marker; lane 1 = PCR product; lane 2 = recombinant plasmid; lane 3 = recombinant plasmid digested with *Xho*I and *Xba*I.

### Molecular properties

The molecular mass of rSLZ expressed in *P. pastoris* GS115 was corresponded very well to the theoretical molecular mass of 14.5 kDa based on the cDNA sequence of SLZ (Figure 2).

The optimal pH of the purified rSLZ varied with salt concentration (Figure 3). The highest activity of rSLZ was at pH 8 when the salt concentration of the buffer was 0.01 M; the highest activity of rSLZ was at pH 7 when the salt concentration was 0.05 M of the buffer; the highest activities of rSLZ were at pH 6 and 5 when the salt concentrations of the buffers were 0.1 and 0.2 M, respectively. When using a buffer with a salt concentration of 0.01 M, rSLZ tested have a broader region of optimum

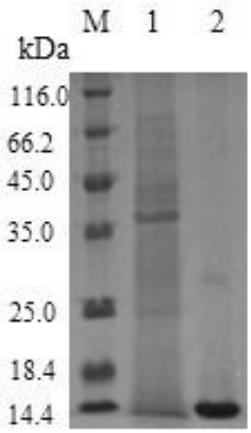


**Figure 2.** SDS-PAGE analysis of rSLZ purified from yeast culture. Lane M = protein molecular mass marker; lane 1 = culture supernatant; lane 2 = purified rSLZ.

**Table 1.** Purification of rSLZ from yeast culture

| | Total activity (U) | Total protein (mg) | Volume (mL) | Specific activity (U/mg) | Purification (-fold) | Activity yield (%) |
|---|---|---|---|---|---|---|
| Culture supernatant | 5,500 | 14 | 3 | 393 | 1.0 | 100 |
| DEAE Sepharose FF pool | 5,100 | 1.6 | 12 | 3,188 | 8.1 | 92.7 |
| Sephadex G-75 | 6,000[a] | 1.5 | 4 | 4,000 | 10.2 | 109[a] |

[a] rSLZ activity is higher after desalinated.
[b] Determination of molecular properties was done with protein from this fraction.

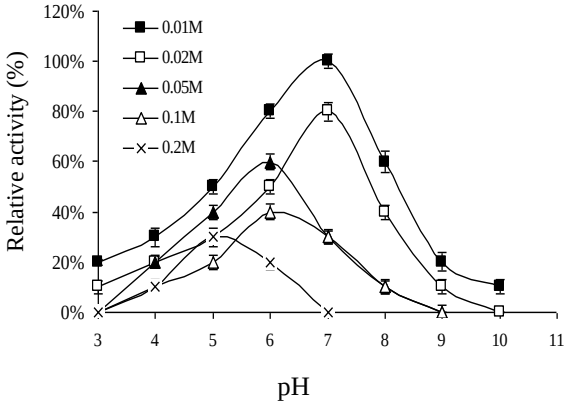


**Figure 3.** Dependence of activities of rSLZ on pH and ionic strength. The rSLZ activity assay was measured in different pH (3 to 10) buffer with different salt concentration (0.01 M to 0.2 M), with *Micrococcus lysodeikticus* as substrate. The lytic activity was measured in phosphate buffer of pH 3, sodium acetate buffers of pH 4 to 5, potassium dihydrogen phosphate buffer of pH 6, MOPS buffers of pH 7 to 8, Tris buffer of pH 9, and carbonate bicarbonate buffer of pH 10. The experiment for each group was done in duplicates and the error was always below 5%.

activity extending from pH 4 to 9 than salt concentrations of buffer from 0.1 M to 0.2M, and the activity of rSLZ declined sharply at extreme pH values in ionic-strength buffers. We also found that the lytic activities of the rSLZ varied with pH. The optimal pH assay revealed that rSLZ had a high activity at an acid pH and an alkaline pH (pH 4 to 8) (Figure 4).

The optimal temperature of the rSLZ was 45°C (Figure 5). The relative activity of rSLZ was compared under three different heating conditions (50°C, 60°C, and 70°C) after incubation for different times to reveal its thermostability (Figure 6). rSLZ maintained nearly 90% of its activity at 50°C after 40-min incubation. The relative lytic activity of rSLZ decreased when incubated at 60°C or 70°C within 40

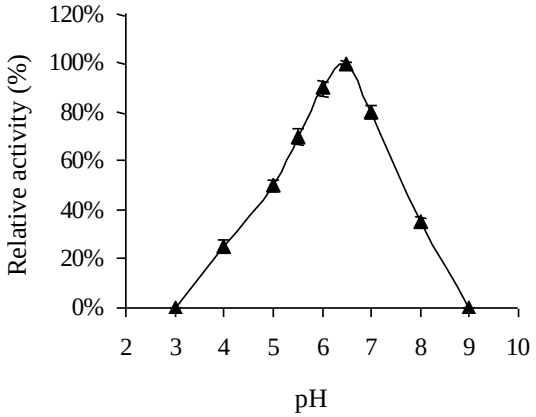


**Figure 4.** Dependence of lytic activity on pH for rSLZ. The rSLZ activity assay was measured at an ionic strength of 0.133 with *Micrococcus lysodeikticus* as substrate. The experiment for each group was done in duplicates and the error was always below 5%.

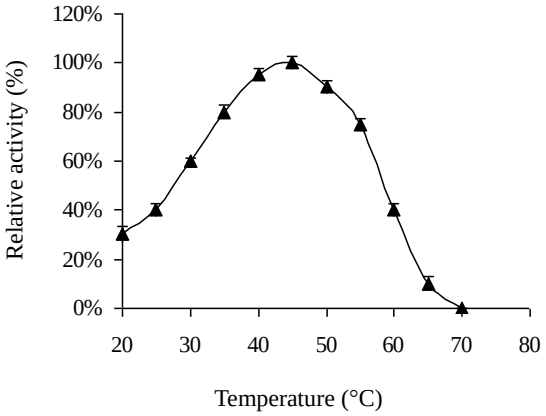


**Figure 5.** Dependence of lytic activity on temperature for rSLZ. The rSLZ activity assay was measured against *Micrococcus lysodeikticus* in 66 mM potassium phosphate buffer, pH 6.24, at different temperature (20°C to 70°C) after incubation for 30 min. Recombinant SLZ activity measured at 45°C represented 100% activity. The experiment for each group was done in duplicates and the error was always below 5%.

min. After 10-min incubation at 70°C, the lytic activity was nearly 50% lower than that at 50°C, and lytic activity was very low after a 30-min treatment.

## DISCUSSION

Production of SLZ in its natural source, Tibetan sheep, is extremely tedious and time consuming to be performed on high yields (Digan et al., 1989). The final yield and activity could be increased remarkablely with *P. pastoris* as expression host compared to native sheep milk. The first characterization and cDNA cloning of SLZ was reported by Irwin and Wilson (1990). Our work is the first to report the heterologous expression of rSLZ in *P.pastoris* and

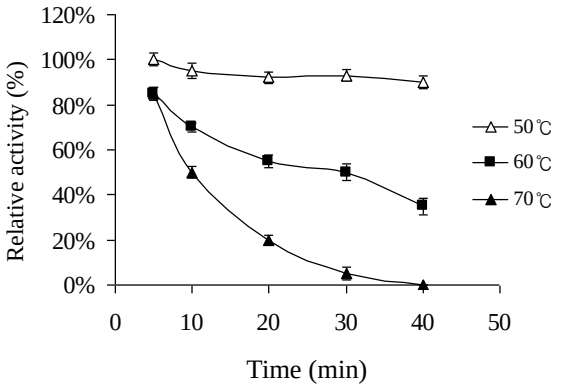


**Figure 6.** Thermostability of purified rSLZ at 50°C, 60°C, and 70°C. The rSLZ activity assay was measured against *Micrococcus lysodeikticus* in 66 mM potassium phosphate buffer, pH 6.24, at 25°C after incubation for different times at 50°C, 60°C, and 70°C. The experiment for each group was done in duplicates and the error was always below 5%.

additionally, the first to purify rSLZ by an experimental design protocol. The purification protocol with experimental design is a very useful tool to gain high yield and high levels of enzyme activity.

To extract rSLZ of high purity, we established a two-step chromatographic procedure for the purification of rSLZ from yeast culture. This purification procedure provides a new, high efficiency method for the extraction of high purity rSLZ from culture supernate. Using affinity chromatography to separate LZM can manufacture a high recovery and concentration (Vasstrand and Jensen, 1980). However, the coupling procedure used in affinity chromatography is complex and time consuming. Thus, this method has limited the possibility on an industrial scale. Ion-exchange chromatography has several advantages over affinity chromatography and it used to be as a common method in other expression systems for the purification of LZM (Iwata et al., 2004; Wilken and Nikolov, 2006). We selected Tris buffer for purification, and we optimized the pH and salt concentration. The best elution condition was with a buffer of 0.25 M sodium chloride and 50 mM Tris buffer at a pH of 9.0. Gel-filtration chromatography is a popular and versatile method that pursued the effective separation of proteins and high yield (Ó'Fágáin et al., 2011). In order to obtain high purity rSLZ and apply in possible subsequent industrial manufacture, gel-filtration chromatography was selected for the last step to purify rSLZ.

The optimal pH of the purified rSLZ varied with salt concentration in this study. For example, when the ionic strength gone up from 0.01 M to 0.2 M, the lytic activities gone down by a factor of 3 (at the optimal pH) just as has been reported before for oyster LZM (Xue et al., 2004) and hen egg white LZM (Davies et al., 1969). When the ionic strength declined to 0.01 M, rSLZ exhibited a remarkable increase in activity for optimal pH, a similar phenomenon was observed with bird and human LZMs (Saint-Blancard et al., 1970; Maurel and Douzou, 1976). We also found that the lytic activities of the rSLZ varied with pH. This phenomenon might be cause by the different solutions change the negative charge of the cell wall and the positive charge at the surface of the LZM, which reacts with rSLZ (Muraki et al., 1988; Kirby, 2001). The optimal pH assay of the purified rSLZ revealed that rSLZ had a high activity at an acid pH and an alkaline pH (pH 4 to 8). The pH of sheep milk is around 6.5-6.8 (Raynal-Ljutovac et al., 2008), which is optimal for the lytic activity of rSLZ. We infer that rSLZ will prolong the shelf-life of Tibetan sheep milk, and we are currently studying this possibility.

The optimal temperature and thermostability of the rSLZ were assayed because storage of rSLZ is an important consideration for commercial production. The US Food and Drug Administration requires that Grade A pasteurized milk undergoes a minimum heating 72°C for 15 s (Ranieri et al., 2009). For rSLZ to be processed into production, about 90% of the enzyme activity remained. We conclude that post-processing procedures will have fewer effects on rSLZ activity.

The specific activity of rSLZ was 4,000 U/mg, which was lower than egg white LZM (Su and Chiang, 2006). However, the expression yield of rSLZ exceed 500 mg/L which could supplement the lower specific activity and if rSLZ use as a industrial material in the future that will have no toxin and no residue for human and animals due to its animal source.

In conclusion, we successfully cloned *SLZ* gene that expressed in *P. pastoris* GS115 using pPICZαA as expression vector and finally obtained the purified rSLZ. In addition, this study could provide an inexpensive and industrial-scale method for the extraction of high purity rSLZ. Futhermore, we have shown that the enzymatic properties and physicochemical characteristics of rSLZ.

## ACKNOWLEDGEMENTS

This research was supported by the Key Projects in the National Science and Technology Pillar Program during the Twelfth Five-Year Plan Period (No. 2012BAD13B06), the Key Demonstration Project of Science and Technology Innovation Industrial Chain in Sichuan province (No. 2011NZ0003), the Fundamental Research Funds for the Central Universities (No. 12ZYXS73).

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