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Comparative study of catalytic properties in three Tibetan ruminant stomach lysozymes

Guo Xia[1,†], Yi Li Liu[2,†], Wei Hua Jiang[1], Jung Nam Lee[3] and Ming Feng Jiang[2,3,*]

[1]The Research Institute of Qinghai-Tibet Plateau, Southwest Minzu University, Chengdu 610041, China; [2]College of Animal & Veterinary Sciences, Southwest Minzu University, Chengdu 610041, China; [3]Key Laboratory of Animal Genetics & Breeding, State Ethnic Affairs Commission and Ministry of Education, Chengdu, Sichuan 610041, China
†These authors contributed equally to this work
*Corresponding author's e-mail: mingfengjiang@vip.sina.com

The stomach lysozymes of ruminants play a paramount role in digestion and offer the chance to probe evolutionary changes in complex organisms on a biochemical basis. In this paper, we focus on the characterization of the catalytic properties of Tibetan ruminant (TR) stomach lysozymes and provide a comparative study of TR stomach lysozymes and nonplateau ruminant stomach lysozymes. The stomach lysozymes were purified with a Carboxymethyl (CM) Sepharose Fast Flow (FF) column and a Bio-Gel P-100 column. The purified stomach lysozymes were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analyses. The antimicrobial activity was evaluated using an agar diffusion method, and the pH, ion strength, temperature, pepsin and trypsin effects on enzymatic activities were evaluated by normal biochemistry methods. The stomach lysozymes of TRs (cattle:TC, yak:TY, and sheep:TS) exhibit maximum activity at pH 5 and ionic strengths below 0.02. TC and TS stomach lysozymes are more resistant to higher temperatures than nonplateau lysozymes. Stomach lysozymes of TRs exhibit greater enzymatic activity than nonplateau lysozymes, in the presence of pepsin and trypsin. Stomach lysozymes from TS retain 86% of their initial enzymatic activity against pepsin inactivation. After trypsin treatment, the stomach lysozymal activities of TRs were significantly affected by trypsin inactivation, with the exception of TS, whose enzymatic activities in the presence of trypsin were 5- and 10-fold higher than those of nonplateau lysozymes. The stomach lysozymes of TRs are more resistant to environmental factors such as pH, temperature, pepsin, and trypsin than nonplateau lysozyme C.
Keywords: Stomach lysozyme, Tibetan ruminants, antimicrobial activity, pepsin, trypsin, thermal stability.

INTRODUCTION

Tibetan ruminants (TRs), such as Tibetan cattle (TC) (*Bos Taurus*), Tibetan yak (TY) (*Bos grunniens*) and Tibetan sheep (TS) (*Ovis aries*), are iconic symbols of livestock on the high-altitude plateau throughout the Qinghai-Tibetan Plateau and adjacent regions.

As a digestive enzyme, stomach lysozymes enable ruminants to use bacterial protein for nutritional purposes (Domínguez-Bello *et al.*, 2004; Mackie, 2012; Flint, 2020). Lysozymes are robustly secreted in the stomach of ruminants and function as catalytic enzymes to help ruminants digest cellulose and other dietary factors leading to more efficient absorption of nutrients (Abdel -Latif *et al*., 2017). Stomach lysozymes from nonplateau ruminants have adaptively evolved to include certain enzymatic properties such as thermal stability, diacetylation resistance, and inactivation from pepsin or trypsin that originates from the digestive tract (Jollès *et al.*, 1984; Irwin *et al*., 1992; Qasba *et al.*, 1997). Previous research on stomach lysozymes has provided evidence for the evolution of lysozymal proteins, as well as, a structural basis for the altered protein function (Jollès *et al.*, 1984; Wen *et al.*, 1999; Irwin, 2015). Prior studies have also revealed that the catalytic properties of nonplateau ruminant stomach lysozymes differ from those of nonplateau lysozyme C. However, as a representative ruminant, TRs are a source of livelihood for millions of Tibetan people, and the enzymatic characteristics of TR stomach lysozymes have seldom been demonstrated. Thus, it is imperative to understand the biological characteristics of TR stomach lysozymes.

Through this research, we expect to provide a more thorough understanding of the biochemical features of Tibetan ruminant stomach lysozymes to approach animal physiology

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in high plateau areas and apply it to the enzyme engineering industry.

MATERIALS AND METHODS

***Stomach lysozyme extraction*:** This study was approved by the Southwest Minzu University Institutional Animal Care and Use Committee (permit number: 2013-3-1). Stomach tissues were collected from TC, TY and TS, which were slaughtered. All samples were frozen at -20 °C for at least 24 h, thawed at 4 °C, and washed with 0.9% (w/v) NaCl. The mucosal linings of the stomach samples were stripped away from the outer muscle wall, weighed, minced, and stored at -80 °C. To prepare a crude extract, frozen linings were thoroughly thawed at 4 °C and homogenized with 2 volumes (v/w) of 10 mM ammonium acetate (pH 6.0). After centrifugation at 2,7000×g for 15 min, the supernatant was collected, and the pH was adjusted to 4.0 using acetic acid (> 99.9%). The sample was bathed at 100 °C for 2 min, adjusted to pH 5.0 using ammonium hydroxide (> 98%), and finally filtered using a 0.22 µm membrane. Chicken lysozyme (Amresco Inc., Ohio, USA) and human lysozyme (Sigma–Aldrich Inc., LA, USA) were used in the experiments.

To purify the stomach lysozyme, the supernatant was applied to a Carboxymethyl (CM) Sepharose Fast Flow (FF) column (GE Healthcare Life Sciences, USA) equilibrated with equilibrium buffer (10 mM ammonium acetate, pH 5.8). After the target protein was bound to the column, we washed the column with equilibrium buffer, washed it again with an elution buffer (0.3 M ammonium acetate, 1 M NaCl, pH 8.0) and desalted using dialysis. The eluted solution was subsequently applied to a Bio-Gel P-100 column (BIO-RAD, USA), which was equilibrated with 0.2% (v/v) acetic acid. The fractions were collected, lyophilized, and stored at -20 °C.

To determine the specific lysozyme activity units in the purified protein, an antimicrobial assay was performed, and the specific activity units were determined in accordance with the change in % transmittance over time using a Lysozyme assay kit (Nanjing Jiancheng Bioengineering Institute, China). Furthermore, *M. lysodeikticus* (0.25 mg/mL) was used as an indicator organism based on the manufacturer's protocol. The purified stomach lysozyme samples (200 µL) and standard lysozyme provided (200 U/mL) were separately applied to 2 mL *M. lysodeikticus* suspension (0.25 mg/mL). The change in % transmittance at 530 nm was measured every 30 sec over 15 min at 37 °C using a double-beam UV/V spectrophotometer. The purified lysozyme units were calculated as follows:

Purified lysozyme unit =

$$\frac{T_{2\ min}(\text{sample})-T_{0\ min}(\text{sample})}{T_{2\ min}(\text{standard})-T_{0\ min}(\text{standard})} \times \text{standard units}$$

The total purified protein concentration was determined using Bradford Protein Assay Kit (Tiangen Biotech, Beijing, China). All measurements were performed in triplicate.

***Characterization using SDS–PAGE and MALDI-TOF analyses*:** To determine the molecular weight of the purified protein, the eluted samples were used for SDS–PAGE (15%) under denaturing conditions. Protein dissociation and reduction were performed by heating for 15 min at 100 °C with 0.1% SDS and 2-mercaptoethanol (0.1%). A mini VE electrophoretic system (GE Healthcare Life Sciences, USA) was used for electrophoresis. The loaded protein was visualized through staining with 0.04% Coomassie Brilliant Blue R250. The molecular mass was estimated by comparing the electrophoretic mobility of the sample against a protein molecular weight standard (Tiangen Biotech, Beijing, China). After SDS–PAGE, the protein bands were excised, and the samples were treated for compatibility with MALDI-TOF analyses. MALDI-TOF mass spectrometry was performed using an AB SCIEX TOF/TOF 5800 system (Applied Biosystems, USA) to identify the purified protein. The proteins were identified using Protein Pilot version 3.0 with the Paragon and Pro Group algorithm (SCIEX, USA).

Antimicrobial activity: First, the antimicrobial activity of the purified protein was evaluated using an agar diffusion method (Grossowicz and Ariel, 2006; Liu *et al*., 2013). The agar plates were composed of LB medium and *S. aureus* (5×106-107 CFU/mL, Qingdao Haibo Biotechnology, China) was used as an indicator organism and cultured on an agar plate.

Second, the antimicrobial activity was determined based on the change in % transmittance over time using the Lysozyme assay kit as previously described. The reaction solution was prepared as follows: for TC, TY, and TS, 0.022 M sodium acetate and 0.117 M NaCl (pH 5.0, ion strength = 0.133); for chickens and humans as references, 0.055 M sodium phosphate and 0.05 M NaCl (pH 6.2, ion strength = 0.133) as optimal condition buffers. The change in % transmittance at 530 nm was measured over 12 min of incubation at 37°C.

***pH and ionic strength effects on lysozyme activities*:** We evaluated the effect of pH on the enzymatic activity using phosphate buffer at pH 2 to 3, sodium acetate buffers at pH 4-5, potassium dihydrogen phosphate buffer at pH 6, MOPS buffers at pH 7-8, Tris buffer at pH 9, and carbonate bicarbonate buffer at pH 10. The total concentration of the components in each buffer was maintained at 10 mM, and the ionic strength was 0.133.

To assess the effect of the ionic strength, we prepared the following standard ionic buffer: 0.022 M citric acid/sodium hydroxide buffer (pH 5.0) with a 0.007-0.5 ionic strength using NaCl.

Both pH and ionic strength effects were evaluated using the Lysozyme assay kit with *M. lysodeikticus* as previously mentioned. The antimicrobial activity was calculated based on the change in % transmittance at a wavelength of 530 nm every 5 min during 1 h of incubation at 37 °C.

***Temperature sensitivity*:** The purified lysozymes were exposed to different temperatures (4-100 °C) for 15 min. The treated lysozymes (150 µL) were added to 2 mL *M.*

lysodeikticus suspension and incubated at 25°C for 1 hour. The antimicrobial activity change in % transmittance was detected at 530 nm using the Lysozyme assay kit as stated in the previous paragraph.

***Sensitivity to pepsin and trypsin*:** To simulate gastric and intestinal fluids, we prepared pig gastric and intestinal fluid mimic solutions as provided by a United States Pharmacopeia protocol (Xie, 2021). Each 100 µL purified lysozyme was digested by 100 µL mimicked pig gastric and intestinal fluid at 37 °C for different reaction times up to 1 hour. The reaction was terminated by adding 25 µL of 0.168 M Na_2CO_3 to the solution at a defined time. The antimicrobial activity change in % transmittance was detected at a wavelength of 530 nm using a lysozyme assay kit as previously mentioned.

RESULTS

Characteristics of the purified stomach lysozymes from TRs: Stomach lysozymes from three TR species (TC, TY, and TS) were purified using previously reported protocols (Jollès, *et al.*, 1984). The stomach lysozyme purification efficiency was evaluated in steps (Table 1). Each specific lysozyme activity (unit per mg purified protein) was assessed. The TC and TY lysozymes exhibited similar specific activity yields, but the TS lysozyme activity was lower. Based on a previous study, the TS lysozyme has similar specific activity to a cow lysozyme in units per mg of lysozyme, and TC and TY lysozymes have 3 times higher specific activity than a cow lysozyme (Jollès, *et al.*, 1984).

SDS–PAGE was used to detect the purified protein (Fig. 1). Based on the electrophoresis results, all three species exhibited homogeneous purified protein sizes at approximately 15 kD and were identified as lysozymes based on MALDI-TOF mass spectrometry (Fig. 2). The types were 2D for TC, C for TY and 4A for TS. Because they are mixed lysozymes, MALDI-TOF analysis may be biased in determining the type. Therefore, the MALDI-TOF analysis data suggest that the purified proteins are lysozymes.

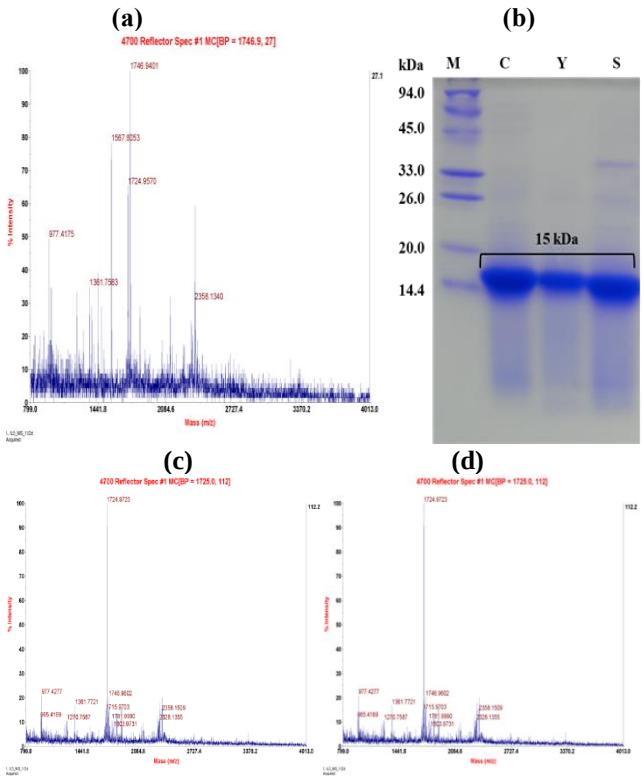

Figure 1. Characterization of the extracted stomach lysozymes using SDS–PAGE and

Antimicrobial activity of TR stomach lysozymes: As shown in Fig. 2a, the purified protein exhibited lysozyme activity; *S. aureus* was cultured as an indicator microbe, and the enzyme

Table 1. Summary of Tibetan ruminant stomach lysozyme purification.

Process	Total activity (U)	Protein content (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Tibetan Cattle					
Crude extract	735060	3555	207	1	100
IP precipitation[a]	520000	176	2955	14	71
CM Sepharose FF	496587	101	4941	24	67
Bio-Gel P100	271059	2	112941	182	37
Tibetan Yak					
Crude extract	1197817	1929	621	1	100
IP precipitation	780606	309	2530	4	65
CM Sepharose FF	720000	101	7111	11	60
Bio-Gel P100	632727	17	37219	60	53
Tibetan Sheep					
Crude extract	82000	774	106	1	100
IP precipitation	67902	149	456	4	83
CM Sepharose FF	59875	11	5280	50	73
Bio-Gel P100	50591	6	7795	74	62

Note: Isoelectric point precipitation

activity zone expanded with the quantity of purified protein. The negative control did not exhibit lysis activity. Additionally, we compared the lysozyme activity with other species (i.e., Chicken and human) as a reference using the antimicrobial activity under each optimal buffer condition (Fig. 2b). The TR lysozyme activities increased with time and were lower than those of the reference lysozymes.

MALDI-TOF analyses. (a) SDS–PAGE result. M: Protein molecular weight marker, C: Tibetan cattle, Y: Tibetan yak, S: Tibetan sheep. MALDI-TOF results for Tibetan cattle (b), Tibetan yaks (c) and Tibetan sheep (d).

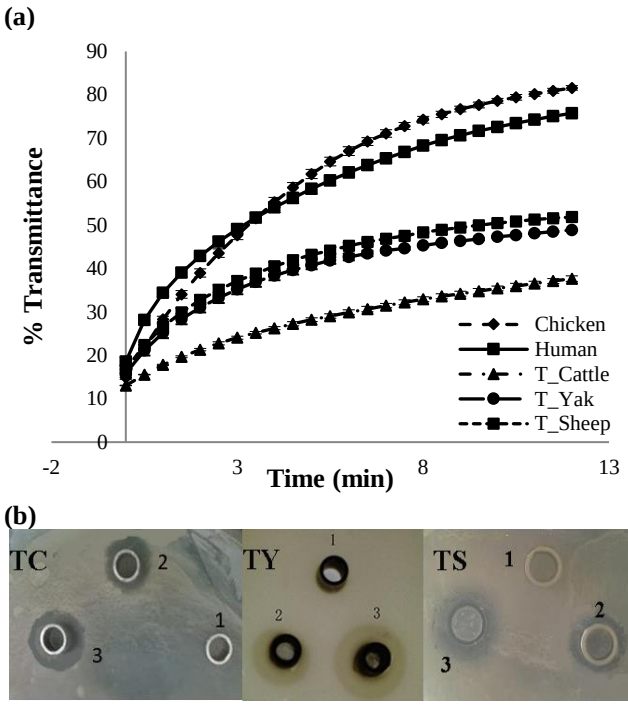

Figure 2. Antimicrobial activity of Tibetan ruminant stomach lysozymes. (a) Agar diffusion assay. 1: 0 μg (negative control), 2: 2.7 μg, and 3: 3.6 μg of purified TC, TY and TS stomach lysozymes. (b) Antimicrobial activity test as a function of time using *M. lysodeikticus*.

Catalytic properties of TR stomach lysozyme: The optimal pH for TR stomach lysozymes was pH 5.0, which provided the highest catalytic efficiency at a constant ionic strength. The optimal pH for chicken lysozyme and human lysozyme was 7.0. The optimal ionic strength was 0.05 for TR lysozymes compared with 0.125 for chicken lysozyme and human lysozyme (Fig. 3a and 3b). The results show that TR lysozymes exhibit the highest activity near pH 6 and at lower ionic strengths (0.02 in our results) (Fig. 3c). The results for chicken and human stomach lysozymes are shown in Additional File 3. These results for TR stomach lysozymes are comparable to cow stomach lysozyme, which exhibited the highest activity at pH 7.0 and 0.005 ionic strength (Jollès *et al.*, 1984).

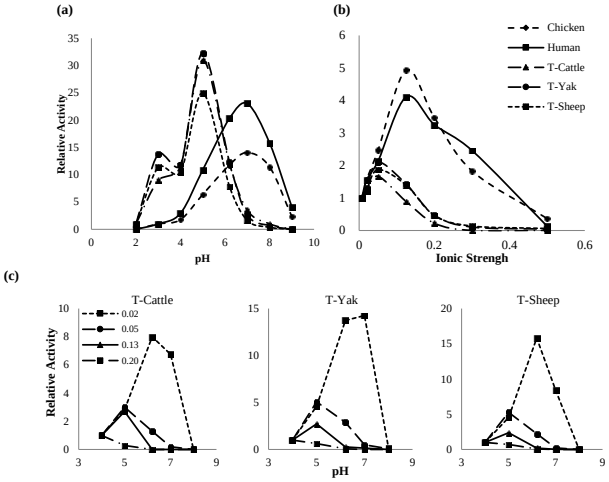

Figure 3. Stomach lysozyme activity is associated with pH and ion strength. (a) pH effect on the lysozyme activity (pH 2~9) at an ionic strength of 0.133 and with an initial substrate concentration of 0.25 mg/mL. (b) Effect of the ion strength on the lysozyme activity (ion strength = 0.007~0.5) at pH 5 and with an initial substrate concentration of 0.25 mg/mL. (c) Tibetan ruminant lysozyme activity at various pH values and ion strengths. Chicken and human lysozymes were used as references. The chicken and human lysozyme results are shown in Additional File 3. The relative activity was calculated by dividing by the initial lysozyme activity value.

Environmental sensitivity (temperature, gastric, and intestinal fluids) of stomach lysozymes from TRs: In our study, Tibetan cattle and Tibetan sheep stomach lysozymes were not significantly affected by temperature ($p>0.05$). The data were analyzed with temperature using the Glimmix procedure of SAS (v 9.2, SAS Institute Inc., USA). The TC and TS stomach lysozymes retained their activities at approximately 75% and 100% of their initial activities, respectively. Tibetan yak stomach lysozyme was affected by high temperatures, which exhibited significant effects above 80 °C. However, the TY stomach lysozyme was approximately 2-fold more resistant to temperature than nonplateau lysozymes, and the lysozyme retained 50% of its initial activity compared with chicken lysozyme (25% of its initial activity), which is similar to humans (50% of the initial activity).

In stomach conditions mimicking pepsin, the lysozymal activity of five species were significantly different from pepsin treatment times ($p<0.05$). As shown in Fig. 4b, chicken lysozymes and human lysozymes were sensitive to pepsin, and their activities rapidly decreased within 5 min of reaction time. However, the TR lysozymes were more resistant to pepsin; the lysozyme maintained over 50% of their initial activity after 60 min of reaction time with pepsin, i.e., 55% for TC, 52% for TY, and 86% for TS. This resistance to

pepsin is consistent with a previous study on nonplateau ruminant stomach lysozymes (Jollès *et al.*, 1984).

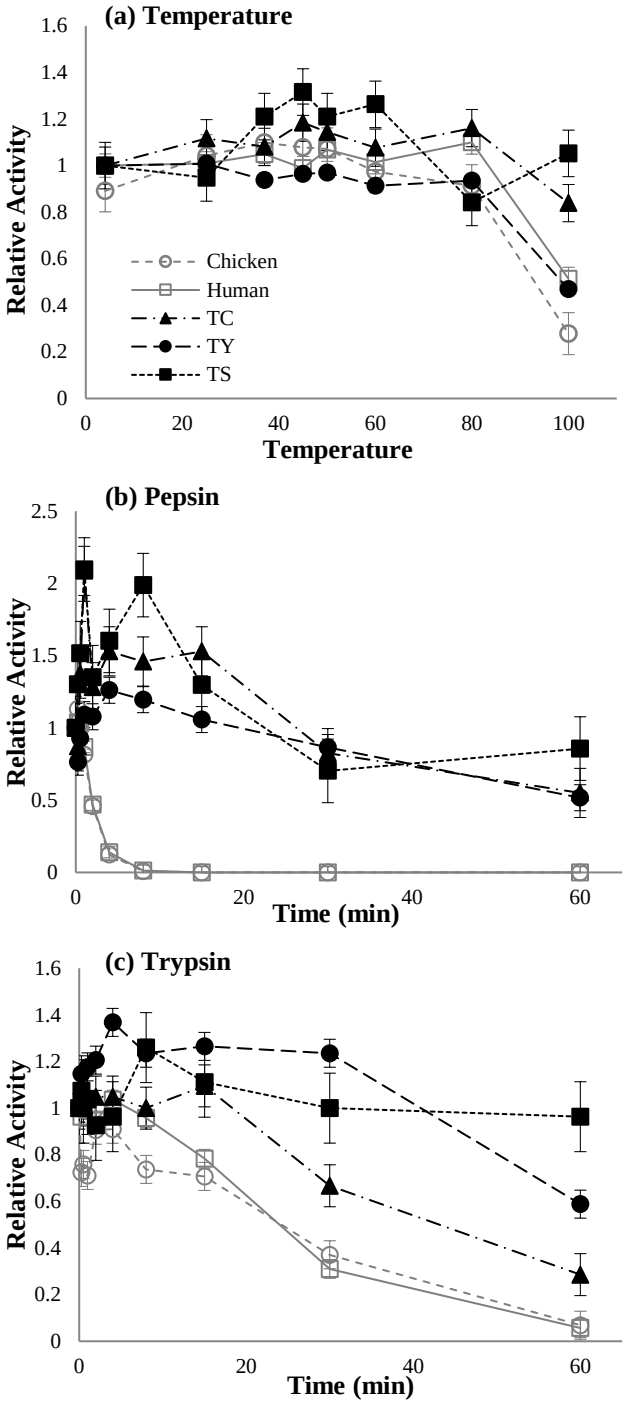

Figure 4. Environmental effect on Tibetan ruminant stomach lysozyme activity. (a) Sensitivity to temperature, (b) sensitivity to pepsin (simulating gastric fluids) and (c) sensitivity to trypsin (simulating intestinal fluids). Chicken and human lysozymes were used as references.

To evaluate trypsin resistance as an intestinal fluid mimic for TR lysozyme activity, lysozymes from five species were treated with trypsin for 1 h (Fig. 4c). The results indicate that TR lysozymes were more resistant to trypsin inactivation than chicken and human lysozymes. TC, TY, and TS retained 29%, 59%, and 96% of their initial lysozyme activities, respectively. Chicken and human lysozymes retained 7% and 6% of their initial activities, respectively. Furthermore, TS lysozyme was not effectively influenced upon treatment with trypsin for 1 hour ($p>0.05$). The lysozymes of the remaining four species were significantly influenced by trypsin ($p < 0.05$).

DISCUSSION

In our study, we purified lysozymes from TC, TY, and TS stomachs, investigated their catalytic activity, and characterized their activities under various conditions, including different pH values, ionic strengths, heat levels and gastrointestinal fluid mimics. All purified lysozymes from the 3 TR species exhibited catalytic activities in an antimicrobial assay similar to cow stomach lysozyme (Jollès *et al.*, 1984). Furthermore, an amino acid structural analysis using MALDI-TOF identified the purified lysozymes as lysozymes from TRs. Stomach lysozymes from TC, TY, and TS exhibited maximum activities at pH 5 and a constraint high ionic strength. The results are consistent with the optimal pH in ruminant stomach lysozymes, which lose enzymatic function at pH 7 (Jollès *et al.*, 1984). At variable pH values and ionic strengths, the TC, TY, and TS lysozymes exhibited maximum activities at higher pH values and lower ionic strengths, which shows a similar pattern to non-TR stomach lysozymes. At a low ionic strength, TR stomach lysozymes are similar to non-TR stomach lysozymes, and both are highly stimulated to lysing bacteria (Jollès *et al.*, 1984).

Lysozyme activities are typically evaluated based on temperature resistance (Wang, *et al.*, 2005; Saurabh,*et al.*, 2008; Huang *et al.*, 2021), but there are few published studies on the effects of temperature on the enzymatic activity of ruminant stomach lysozyme. In our study, the TC and TS stomach lysozymes were more resistant to temperature than nonplateau lysozymes, especially the TS stomach lysozyme, which was nearly uninfluenced by temperature. According to a recent report, temperature tolerance is due to disulfide bond stabilization in wild-type lysozyme, and the lysozyme disulfide bond stabilizes the molecule (Hildebrand *et al.*, 2018; Pu *et al.*, 2018). Consequently, stomach lysozymes of TRs may differ in their amino acid sequence to generate disulfide bonds in lysozymes. To clearly demonstrate this hypothesis, further investigation is necessary.

To determine TR stomach lysozyme activity in the digestive tract, we mimicked a gastrointestinal environment by using pepsin and trypsin and analyzed the lysozyme resistance to pepsin and trypsin. TC, TY, and TS stomach lysozymes

Table 2. Summary of the stomach lysozyme enzymatic properties

	Unit	Chicken	Human	Tibetan Cattle	Tibetan Yak	Tibetan Sheep
Optimal pH (ionic strength=0.133, pH 2~9)	pH	7	7	5	5	5
Optimal ionic strength (pH=5, ion 0.007~0.5 M)	M	0.125	0.125	0.05	0.05	0.05
Both pH and ionic strength (pH 2~8, ion 0.02~0.2)	pH/ ion	8/0.020	7/0.020	6.2/0.020	6.2/0.020	6.2/0.020
Temperature effect	%	27	51	75	47	111
(4~100 °C)	P	0.0002	<0.0001	0.3302	<0.0001	0.077
Pepsin effect	% [a]	0	0	55	52	86
	P	<0.0001	<0.0001	0.0002	0.0008	0.0028
Trypsin effect	% [a]	7	6	29	59	96
	P	<0.0001	<0.0001	<0.0001	<0.0001	0.9189

Note: Calculated using the % lysozyme activity efficiency from the initial activity after 1 hour.

exhibited higher enzymatic activities in the presence of pepsin and trypsin than lysozymes from chickens and humans.

In the presence of pepsin, all five species were effectively influenced by pepsin. Moreover, chicken lysozymes and human lysozymes rapidly decreased to zero within 5 min of reaction time. However, the 3 TR lysozymes retained activity for 30 min of reaction time, and the TS lysozyme retained enzymatic activity at 86% of the initial activity after 1 h. Our result is consistent with other ruminant stomach lysozyme activities in the presence of pepsin, where ruminant stomach lysozymes retain 60-90% of their initial activity (Jollès *et al.*, 1984).

The effect of trypsin on lysozymes in the ruminant stomach has not been verified. To learn more about the impact on TR stomach lysozymes in the gastrointestinal tract, lysozymes were treated with trypsin. Upon treatment with trypsin, the stomach lysozyme activities of four species, not TS, were significantly affected by the trypsin inactivation. Although the TC and TY lysozymes are sensitive to trypsin, their enzymatic activities in the presence of trypsin were 5-fold and 10-fold greater than chicken and human lysozymes, respectively. The TS lysozyme was especially resistant to trypsin inactivation; it nearly maintained 96% of the initial activity even after 1 hour. The results are summarized in Table 2.

Conclusion: In this trial, we purified stomach lysozymes from Tibetan cattle, Tibetan yaks, and Tibetan sheep and characterized their enzymatic activities. TR stomach lysozymes are more resistant than nonplateau lysozyme C against environmental factors (pH, temperature, pepsin, and trypsin), and the result is almost identical to that of non-TRs. Moreover, in our study, Tibetan sheep stomach lysozyme exhibited especially strong resistance to temperature, pepsin, and trypsin. Demonstrating the basis for this increased resistance requires more detailed research. These results will help build a better understanding of the high-plateau-area animal physiology and can be applied to further develop the enzyme engineering industry.

***Conflicts of interest*:** The Authors declare that there is no conflict of interest.

***Authors' Contribution Statements*:** GX and YL write the manuscript, WH and JNL executed research, MJ conceived the idea and supervised the work.

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***Availability of supporting data*:** The datasets supporting the results are included in the article and additional files.

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