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Original Article

Proteolytic and Biocatalytic Activities of Animal Spleen Extracts

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Abstract

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Proteolytic activity and biocatalytic properties of spleen extracts from six animals were studied. Among tested spleens, extracts from the camel's spleen showed the highest activity, followed by bovine, horse, caprine, porcine and ovine extracts. Also the effects of NaCl and $(NH_4)_2SO_4$ on proteolytic activity was investigated. Spleen extracts contained heat stable acid protease with optimum pH of 2.6 and Topt at 40ºC. Thermal inactivation kinetics was described as second order model.

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Introduction

Meat and meat processing industry is one of the leading industries in food production of Mongolia. According to the data of Mongolian Ministry of Agriculture for 2015, about 17.5 thousand tons of meat was processed in this country, which increased by 0.7 thousand tons in comparison with the previous year (https://www.mofa.gov.mn). For 2015, about 5.6 thousand tons of meat and meat products were exported and the meat processing industry is increasingly and becoming one of the export income generators for economy. Animal viscera are a potential source of enzymes as proteases. Digestive proteases have been extensively studied. The most important digestive enzymes are gastric pepsin, pancreatic trypsin and chymotrypsin. Mongolian scientists isolated and purified trypsin and chymotrypsin from cattle pancreas, conducted a comparative studies with porcine and bovine enzyme (Alimaa *et al*., 1985a; 1985b), and developed pancypsin production technology from the ovine + caprine pancreas (Alimaa *et al.*, 1989). However, no information regarding the characteristics and properties of the pasture animals' spleen proteolytic enzymes as the source for protease, has been reported. This study aimed to characterize some proteolytic activities and biocatalytic properties of spleen extracts of six animals as cattle, horses, camels, sheep, goat and pig, commonly used in meat processing industry in our country.

Material and Methods

General. All chemicals used in this study were chemically pure. Reagents for buffer solution, acids and bases were purchased from Tsetsuuh Trade Co Ltd. (Mongolia). Albumin was obtained from Sigma-Aldrich (Korea). All used reagents were of analytical

grade. Experiments were carried out with a 3-5 repetitions and average results were taken.

Spleen material. Spleens from six animals as cattle, horses, camels, sheep, goat and pig were collected in 2016 from the local slaughter-houses. Samples were packed in polyethylene bags, kept in ice, transported to the laboratory and stored in a freezer until further studies. For extraction, crushed spleen was mixed with cold distilled water at a ratio of 1:6 (w/v) stirred continuously for 10 min, centrifuged for 15 min at 3000 *rpm*. The supernatant was collected and used for the next procedures.

Enzyme assay and study of biocatalytic properties. Protease activity was estimated according to the method of Kunitz (1947) and Kochetov (1980), using 0.2% BSA solution as substrate. The assay mixture contained 1 ml of spleen extract and 1 ml of BSA solution, which was incubated for 20 minutes at 37ºC and color development rate was measured at 750 nm. The 0.2% of BSA was prepared in 0.04 M Britton-Robinson buffer (pH=3-7) for estimation of protease activity, Topt, temperature stability, effect of NaCl and $(NH_4)_2SO_4$, and in 0.1 M Britton-Robinson buffer (pH=2.1-6.2 and 3-7) for pHopt and effect of pH. Protein concentration was measured by the Lowry method (Lowry *et al*., 1951; Waterborg, 2002) and calculated using standard BSA solutions of calibration. Effect of NaCl and $(NH_4)_2SO_4$ on proteolytic activity was estimated with 0.25%, 0.5%, 1% and 2% solutions of these salts. For estimation of Topt, incubation was carried out at 20-60ºC. Enzyme activity was expressed in unit. One unit (U) of enzyme activity was taken as the amount of 1 mg enzyme protein, which forms 1 μg of tyrosine in 1 hour.

Results

Soluble protein and enzyme activity of spleen extracts were measured. Among tested spleens extracts of camels showed the highest activity, followed by bovine, horse's, caprine, porkine and ovine extracts. But the soluble protein content was less in the camel's spleen extract followed by porcine, bovine, horse's and ovine, caprine extracts on ascending order (Table 1).

In order to evaluate the pHopt and pH stability of spleen protease incubations were carried out with Britton-Robinson buffer (pH=2.1-6.2 and 3-7), and the activity was measured. Results show that pH range of spleen protease was narrow (pH=2.1-4.6) and optimal activity of spleen extract from all animals was at pH=2.6 (Fig. 1). Press *et al*. (1960) evaluated the pHopt of the bovine spleen cathepsin D using purified enzyme with bovine haemoglobin as substrate and Britton-Robinson buffer (pH=2-8). It was at pH 3.0 and pH range was 2-4.5. With BSA as substrate pHopt was at 4.2 and pH range was 2.5-5. Gunningham & Tang (1976)

Table 1. Soluble protein and enzyme activities of spleen extracts

№	Spleen	Soluble protein, %	Proteolytic activity, U
1	Bovine	11.8	78.0
2	Horse's	12.5	73.7
3	Camel's	3.2	134.7
4	Ovine	16.5	59.7
5	Caprine	16.5	61.9
6	Porcine	7.4	61.2

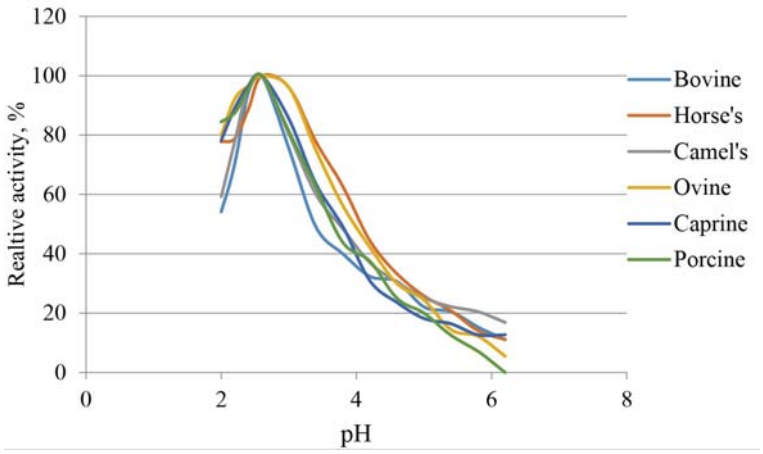

Figure 1. pH_{opt} of spleen proteases from different animals

estimated the pHopt of porcine spleen cathepsin D using hemoglobin as substrate. pH range was 2-5 and two optima at 3.4 and 3.8 were evaluated with 0.5 M sodium acetate buffer.

In our study, the spleen protease was more stable at acid than in neutral pH. At the neutral pH, the activity begins to lose except for horse's spleen protease, which has a relative activity of about 13.5% at pH=7. As for the bovine, camel's and ovine spleen protease, it already inactivates at pH=6, and at pH=5 shows the relative activity of 14.9%, 16.5% and 20.3% respectively. Caprine and porcine spleen protease activities at pH=6 have 6.4% and 4.4% of relative activity after 80 minutes of incubation (Table 2).

Next, we studied the optimum temperature value and thermal stability of spleen protease. To confirm the optimal temperature for the spleen protease, we measured protease activities following the incubation of the spleen extract at different temperatures (20–70ºC) for 20 min. Optimal activity of spleen extracts from all animals was at 40ºC (Fig. 2). Temperature range was 20-60ºC for bovine and porcine spleen protease. Horse's, camel's, ovine and caprine spleen protease showed an interval of activity that varied between 20-50ºC.

The effect of temperature on the stability of protease activity was tested by incubating the spleen extract proteases at 50ºC and 55ºC for 80 min, followed by the measurement of residual activity.

The results show that spleen proteases are stable to temperature effects, after 80 minutes of incubation at 50ºC, the residual activity for bovine, ovine, caprine and porcine spleen proteases were more than 50%. The most stable one was ovine spleen enzyme, which shows 41.9% of residual activity after 80 minutes of heating at 55ºC (Table 3). Hayes *et al*. (2001) heated the commercial preparation of cathepsin D from bovine spleen enzyme at 55, 58, 60, 62, 63 and 68°C for 0-30 min, and studied thermal inactivation kinetics. Their results show that the enzyme was stable to heat and inactivation kinetics described as first order model.

Our results show that the enzyme was stable to heat and the thermal inactivation kinetics

Table 2. Effect of pH on spleen proteolytic activities

pH	Relative activity, % (incubation period 80 minutes)					
	Bovine	Horse's	Camel's	Ovine	Caprine	Porcine
3	75.6	96.1	80.4	96.1	85.5	81.3
4	36.2	53.6	42.7	49.2	40.0	40.6
5	14.9	42.3	16.5	20.3	15.0	18.5
6	0	32.8	0	0	6.4	4.4
7	0	13.5	0	0	0	0

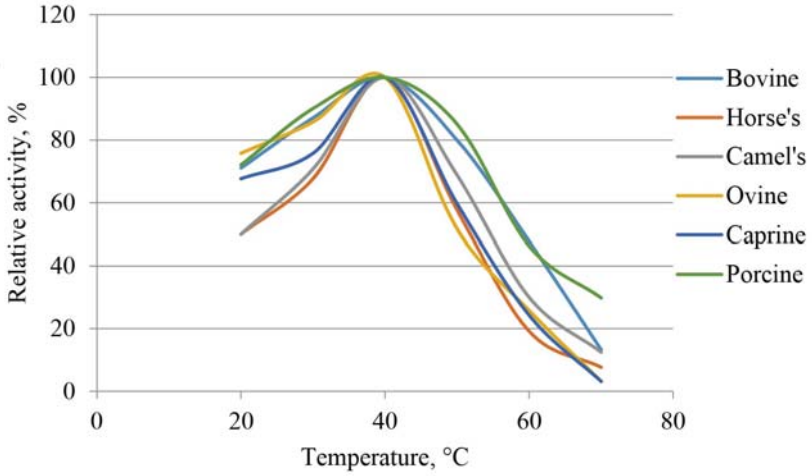

Figure 2. T_{opt} of spleen proteases from different animals

Table 3. Temperature stability of animal's spleen proteases (relative activity)

Time, min.	Bovine		Horse's		Camel's		Ovine		Caprine		Porcine	
	50°C	55°C	50°C	55°C	50°C	55°C	50°C	55°C	50°C	55°C	50°C	55°C
0	100	100	100	100	100	100	100	100	100	100	100	100
20	83.3	52.5	76.5	44.5	72.7	48.7	84.1	79.2	83.7	35.4	73.3	45.1
40	73.2	37.5	53.4	26.2	59.1	35.0	70.2	60.1	75.5	19.6	60.1	28.1
60	65.6	32.5	40	19.0	48.2	28.6	61.4	45.1	67.3	15.2	53.3	20.5
80	59.5	29.8	30.1	11.9	42.7	23.1	54.1	41.9	65.0	14.9	50.1	18.8

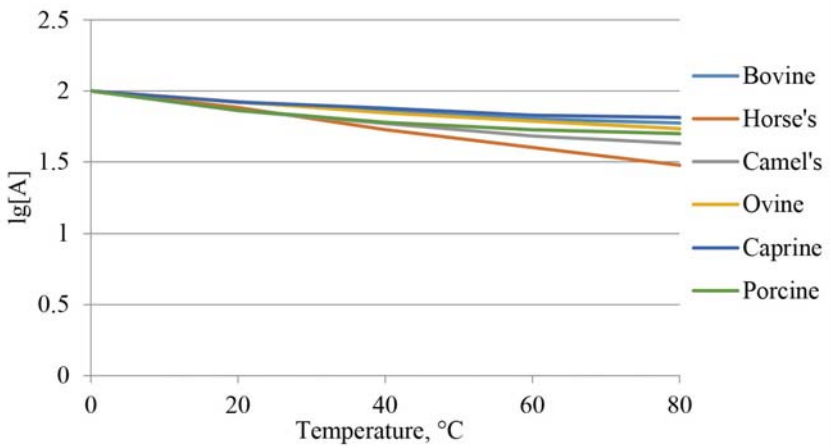

Figure 3. Thermal inactivation of spleen extracts at 50°C (up to 80 min)

was described as second order model, which is especially clear seen from the Fig. 4. This indicates it is not cathepsin D isoform.

Also, we investigated the effect of NaCl and $(NH_4)_2SO_4$ on the spleen protease activities. After incubation with different (0.25-2%) solutions of these salts for 20 minutes, the relative activities were measured and results shown below.

Results show that bovine spleen protease was more stable in NaCl, while the horse's spleen protease was the least stable. In $(NH_4)_2SO_4$, porcine and caprine spleen protease were more stable, followed horse's, camel's, bovine, whereas ovine spleen protease showed the less stability. These salts do not activate the spleen protease.

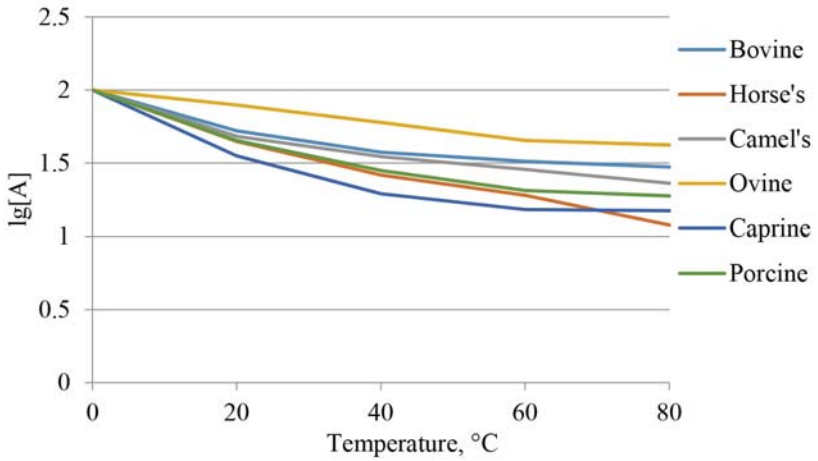

Figure 4. Thermal inactivation of spleen extracts at 55°C (up to 80 min)

Table 4. Effect of NaCl and $(NH_4)_2SO_4$ on spleen proteolytic activities

Spleen	Relative activiy, %									
	Concentration of NaCl, %					Concentration of $(NH_4)_2SO_4$, %				
	0	0.25	0.5	1.0	2.0	0	0.25	0.5	1.0	2.0
Bovine	100	100	100	100	100	100	100	93.3	93.3	90.0
Horse's	100	100	100	95.0	85.0	100	100	100	95.0	94.7
Camel's	100	100	100	100	93.8	100	100	100	100	94.1
Ovine	100	100	100	97.0	93.3	100	100	95.2	92.3	71.4
Caprine	100	100	100	92.5	90.9	100	100	100	92.5	100
Porcine	100	100	100	100	93.3	100	100	100	100	100

Conclusion

Spleen extracts contained heat stable acid protease with optimum pH of 2.6 and Topt at 40ºC, respectively. Thermal inactivation kinetics was described as second order model. NaCl and $(NH_4)_2SO_4$ are not activators of the spleen protease. Spleen proteases can be potential enzymes as the source for protease for future applications. That is why further investigations of protease inhibitors and activators, isolation and purification of spleen protease are currently in progress.

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