Milk-clotting mechanism of *Dregea sinensis* Hemsl. protease

Yali Zhang,¹ Hongyan Wang,¹ Liang Tao, and Ai-xiang Huang²

College of Food Science and Technology, Yunnan Agricultural University, Kunming 650201, Yunnan, China

ABSTRACT

Dregea sinensis Hemsl. is used as a milk coagulant to produce goat milk cakes in Yunnan, China. However, the composition of milk-clotting compounds and the related mechanism have not been reported. Crude protease was extracted from the stem, purified, and then separated with a Millipore ultrafiltration centrifuge tube. Cysteine protease (procerain B) was identified as the main milk-clotting protein through electrospray ionization mass spectrometry, and its molecular weight was 23.8 kDa. The protease can partially degrade α -casein (CN) and completely degrade β - and κ -CN, and κ -CN degradation resulted in milk clotting. The molecular weight and AA sequence of the peptide fractions were determined through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and a peptide sequencer, respectively. The enzyme cleaved κ -CN at Ala90-Gln91 and produced deputy κ -CN and caseinomacropeptide with molecular weights of 12 and 6.9 kDa, respectively. This cleavage site differed from the majority of chymosins cleaved at Phe105-Met106.

Key words: *Dregea sinensis* Hemsl. protease, separation and identification, κ -casein, enzyme cleavage site

INTRODUCTION

Dregea sinensis Hemsl. var. *corrugata* (Schneid.) Tsiang and P. T. Li, which belongs to *Asclepiadaceae* (*Dregea* E. Mey., nom. cons.), is a woody vine commonly known as milk slurry vine. This species is a variety of *D. sinensis* [*Wattakaka sinensis* (Hemsl.) Stapf] (Wu, 2003) and grows in mountain forests or chaparral at elevations of 500 to 3,000 m, particularly in Yunnan, Dali, and Kunming. In the Chinese northwest region of Yunnan Province, a warm aqueous solution of *D. sinensis* stems, instead of acid water, is used as a coagulant to process milk cakes (Xue, 1994).

Dregea sinensis contains 11 types of milk-clotting compounds, which could be extracted, separated, and identified using petroleum ether, ethyl acetate, *n*-butyl alcohol, and other organic solvents (He, 2010). Ma (2013) inferred that a protease could be the milk-clotting component of an aqueous solution of *D. sinensis*. Tao (2014) used salt to extract crude enzyme from *D. sinensis* and investigated the characteristics of the protease. Nevertheless, no study has reported the separation and identification of proteases and their milk-clotting mechanism in *D. sinensis*.

Many studies have separated and identified proteases and determined their milk-clotting mechanisms in several species. Generally, the proteolytic degradation of κ -CN could result in milk clotting. Calf rennet enzymes specifically hydrolyze the Phe105-Met106 peptide bond of κ -CN during milk clotting (Carles and Dumas, 1985; Carles and Martin, 1985; Galán et al., 2008). Aspartic protease, which exhibits a strong proteolytic activity (PA), can cleave the peptide bonds at Phe105-Met106 in κ -CN extracted from cardoon (*Cynara cardunculus*) and at Lys116-Thr117 in κ -CN of goat milk (Galán et al., 2008). Chymosin (36 kDa), which can hydrolyze casein, was separated from glutinous rice wine through ion exchange chromatography; its degradation site in κ -CN was Thr94-Met95 as determined through time-of-flight mass spectrometry (TOF-MS; Jiang et al., 2007). Egito et al. (2007) reported that protein extracts from *Albizia julibrissin* and sunflower seeds also exhibited milk-clotting activity (MCA) because they can hydrolyze casein and decompose α -, β -, and κ -CN; their κ -CN decomposition sites were Lys116-Thr117 and Phe105-Met106, respectively, and the activity of *A. julibrissin* extract was 15-times higher than that of sunflower. Huang et al. (2011) separated and extracted a 31-kDa protease from fresh ginger. The protease reached its maximum MCA at 70°C and could hydrolyze α -, β -, and κ -CN; the degradation degree of α -CN was the highest, and κ -CN exhibited higher specificity than α - and β -CN. The sites for κ -CN hydrolysis by the ginger protease were Ala90-Glu91 and His102-Leu103. Furthermore, Kumari et al. (2010, 2012) and Ahmed et al. (2009a,b) purified and obtained a new serine protease from the Bodhi tree and named it religiosin

Received May 22, 2015.

Accepted September 2, 2015.

¹These authors contributed equally to this work.

²Corresponding author: aixianghuang@126.com

B. The molecular weight of the enzyme was 63 kDa, and de novo sequencing proved that it was a newly discovered protease. The enzyme also exhibited high MCA and could be used in cheese processing and other food industries, as well as in biological industries. This study aimed to separate and identify protease in *D. sinensis*, determine casein and κ -CN degradation by the protease in enzyme cleavage sites, and investigate the milk-clotting mechanism.

MATERIALS AND METHODS

Experimental Materials

Dregea sinensis (Guan Jin Teng, GJT) was collected from a mountain at an elevation of 2,000 to 3,000 m in Jianchuan County, Dali City, Yunnan Province. Goat milk was purchased from YiXingHeng Animal Husbandry Science and Technology Limited Company (Kunming, China). Casein, α -CN, β -CN, and κ -CN were purchased from Sigma Co. (St. Louis, MO). All other reagents were of analytical grade.

Extraction, Separation, and Identification of Protease in *D. sinensis*

Extraction of Protease. *Dregea sinensis* stems with high MCA were selected and divided into 15- to 20-cm pieces. Running water was used for rapid rinsing of the stems to remove surface dust. Each stem was divided into 20 pieces, soaked in warm water at 55°C for 30 min, cooled, filtered, and then vacuum concentrated to 20% of the original volume at 55°C. The concentrate was allowed to stand for 24 h at 4°C before salt fractionation. The salting-out protein (40% ammonium sulfate) with the highest MCA was selected, dialyzed, and purified for 4 h with a MW3500 dialysis bag (dialysis tubing 3500, Sigma Co.) at 0 to 4°C. This process was carried out 3 times. The purified protein was vacuum freeze-dried to produce powder and preserved at -20°C.

Separation and Purification of Protease. The molecular weights of the protease in *D. sinensis* were 24 and 10 kDa. The protease were further separated, purified, and then subjected to the milk-clotting experiment. *Dregea sinensis* protein powder was dissolved in 100 mL of phosphate buffer solution (100 mmol/L, pH 6.5). The resulting solution was heated at a low temperature, dissolved through vortex oscillation for 3 min, and then centrifuged ($5,000 \times g$, 2 min at 4°C). The dissolved sample was transferred into 3K Millipore ultrafiltration centrifuge tubes (Amicon Ultra-4, Ultracel-3 Membrane, Beckman Coulter Inc., Brea, CA) and centrifuged at 4°C ($1,300 \times g$, 40 min) to separate the

24-kDa protein from the 10-kDa protein. The 15- μ g sample was subjected to SDS-PAGE to determine its purity and then to milk-clotting experiments. Equal amounts of 24- and 10-kDa protease samples were added to goat milk in the following ratios: 1:10, 1:5, 1:1, 5:1, and 10:1. The samples were placed in a water bath for 30 min at 85°C. We observed the milk-clotting phenomenon; then we could find which protein has MCA.

Assay of Enzyme Activity. The method of MCA was modified from Iwasaki et al. (1967). One Soxhlet unit of MCA was defined as the amount of enzyme required to clot 1 mL of goat milk in 40 min at 85°C. The PA was detected with the modified method reported by Mohanty et al. (2003) with the substrate, 1.5% solution of bovine whole casein (pH 6.0 with 0.1 M phosphate buffer). The preheated substrate (1 mL) was mixed with 0.1 mL of purified enzyme solution, and the sample was incubated at 85°C for 60 min, followed by the addition of 1 mL of 12% TCA. The optical density of soluble peptides was read at 280 nm with a UV-visible spectrophotometer (Cintra20, GBC Scientific Equipment Pty. Ltd., Dandenong, Victoria, Australia), and 1 unit of PA was defined as the amount of enzyme required to increase optical density 0.001 in 1 min.

Identification of Main Components in Protease. The SDS-PAGE was conducted to separate and purify *D. sinensis* proteases with MCA (12% separation gel, 4% concentration gel). Enzymatic gel digestion was performed (trypsin, 20 h), and the peptide was extracted for electrospray ionization mass spectrometry. For comparison, data were retrieved from the uniprot database with Mascot2.2 software (http://www.matrix-science.com/mascot_support_v2_2.html) to identify the main milk-clotting protein.

Hydrolysis of Casein by *D. sinensis* Protease

Casein, α -CN, β -CN, and κ -CN (1.0 g each) were dissolved in 100 mL of 100 mmol/L phosphate buffer solution (pH 6.5) and added with 0.1% NaN_3 . *Dregea sinensis* protease (1.0 g) was dissolved in 100 mL of ultrapure water. The solution was vortexed for 3 min, centrifuged ($5,000 \times g$, 2 min at 4°C), and then preserved.

The proteases were reacted with casein, α -CN, β -CN, or κ -CN at 1:1 ratio and then treated in a water bath for 1 h at 85°C. The SDS-PAGE was performed to determine casein hydrolysis by the protease. The protease was added to α -CN, β -CN, and κ -CN at 1:20 ratio and allowed to react at 85°C for 5, 15, 25, and 35 min and 1, 2, 3, 4, and 12 h. Degradation degree was also analyzed through SDS-PAGE.

Mass Spectrometry Analysis of the Cleavage Site on κ -CN by *D. sinensis* Protease

The protease was reacted with κ -CN for 1 h in a water bath at 85°C until complete milk clotting was observed. The clots were desalinated at 4°C in a Millipore 3K ultrafiltration centrifuge tube to obtain the protein solution. The molecular weight of the κ -CN peptide was determined through matrix-assisted laser desorption/ionization (MALDI) TOF-MS (Autoflex MALDI-TOF-MS; speed, Bruker, Billerica, MA, Flex-Control, Flex Analysis, Biotools). The protease was reacted with κ -CN at a ratio of 1:20 in a water bath for 1 h at 85°C until complete milk clotting. The SDS-PAGE was then conducted, and the target band on the polyvinylidene fluoride film was cut after transmembrane. The AA sequence on the N-terminal of the peptide was determined, and the κ -CN site was detected using a protein polypeptide sequence detector (Shimadzu Co., Kyoto, Japan; PPSQ-31A).

RESULTS AND DISCUSSION

Separation and Identification of Proteases in *D. sinensis*

Enzyme Property. The specific milk-clotting activities of extracts increased more than the PA during purification, which is shown in Table 1. The MCA of purified enzyme is 20-times higher than filtrates. The purified enzyme gave the highest ratio of MCA:PA, 27.31, which showed the enzyme was a kind of rennet-like enzyme and had a great potentiality to be developed as a milk-coagulating enzyme in the future. Previous studies that separated and purified plant protease, included the following: Jiang et al. (2007), separated a chymosin from glutinous rice wine that the highest ratio of MCA:PA was 6.7, and the highest ratio of

Figure 1. Electrophoresis mapping of purified proteases. Lane 0 is the marker. Lanes 4 and 5 are *Dregea sinensis* proteases before separation. Lanes 1, 2, and 3 are 10-kDa proteases after separation. Lanes 6, 7, and 8 are 24-kDa proteases after separation. Color version available online.

MCA:PA of protease from ginger was 43.8 (Huang et al., 2011). *Dregea sinensis* protease is a new source of proteases with potential use for cheese production. Figure 1 shows the separation and purification of 24- and 10-kDa *D. sinensis* proteases. The 2 bands representing 10 and 24 kDa emerged on lanes 4 and 5, respectively, and lanes 1, 2, and 3 showed the 10-kDa band. Only one 24-kDa protein band emerged on lanes 6, 7, and 8, which indicated that the 2 proteases were completely separated and reached electrophoresis purity. The performance of these proteases was confirmed through milk-clotting experiments.

Milk-Clotting Experiment Results. As shown in Table 2, enzyme A was the 10-kDa protease and enzyme B was the 24-kDa protease. Milk clotting did not occur when enzyme A was added at ratios of 1:10, 1:5, and 1:1. Fine flocculation was initiated at 5:1 addi-

Table 1. Purification and enzymatic assays of extracts from *Dregea sinensis*

Item	Purification step		
	Filtrates	Crude enzyme	Purified enzyme (enzyme B)
Volume (mL)	500	30	15
Milk-clotting activity (Soxhlet units/mL)	15.38	221.2	307.6
Total Soxhlet units	7,690	6,636	4,614
Protein (mg/mL)	30.2	18.6	6.4
Total protein (mg)	15,100	558	96
Specific activity (Soxhlet units/mg)	0.51	11.89	32.72
Relative purification fold	1.0	23.31	64.16
Proteolytic activity (U/mg)	0.12	0.48	1.76
Milk-clotting activity:proteolytic activity	4.24	24.78	27.31

Table 2. Milk-clotting experiment results

Enzyme:goat milk (vol/vol)	Enzyme A (10 kDa)	Enzyme B (24 kDa)
1:10	Reaction for 30 min, noncondensing	Reaction for 11 min, large clot, loose structure
1:5	Reaction for 30 min, noncondensing	Reaction for 7 min, large clot, even and smooth structure
1:1	Reaction for 30 min, noncondensing	Reaction for 6 min, large clot, even structure
5:1	Reaction for 28 min, finely flocculent	Reaction for 6 min, large clot, even structure
10:1	Reaction for 23 min, finely flocculent, emulsion	Reaction for 6 min, large clot, even structure

tion ratio, and fine flocculation and an extremely loose flocculent structure were detected at 10:1. The mixture was an emulsion, indicating that the protease exhibited a low clotting activity and had no practical application value. When enzyme B was added at a ratio of 1:10, a whole clot with slightly loose structure was rapidly generated. At 1:5 addition ratio, large clots were produced after 7 min. The surface of the clots was fine, smooth, and elastic, with a satisfactory tissue state. With increasing protease ratio, milk-clotting time stabilized at 6 min. The resulting clot was large, with an even structure and satisfactory tissue structure. These findings indicated that the 24-kDa protease was the main milk-clotting compound with a strong MCA. Previous studies that separated and purified plant protease

included the following: Jiang et al. (2007) separated a 36-kDa chymosin from glutinous rice wine; Huang et al. (2011) extracted a 31-kDa protease from ginger; and Kumari et al. (2012) purified and obtained a novel 63-kDa serine protease from the Bodhi tree and named it religiosin B.

Identification of Protease in *D. sinensis*. The contrasting uniprot protein database showed that the protein with the highest peak intensity was procerain B, with a relative molecular mass of 23,802.6 Da (Figure 2). As the protein exhibited cysteine protease activity and thiol catalyzing hydrolysis, it could be categorized as a hydrolase, protease, and thiol protease; hence, the protease could be related to the milk-clotting property of *D. sinensis* and was the major milk-clotting protein.

Figure 2. Electro-spray ionization mass chromatogram of 24-kDa protein. m/z = mass:charge ratio. Color version available online.

Nandana et al. (2014) identified 127 AA in procerain B through N-terminal sequencing, and these AA belonged to the I29 inhibitory peptide. The tertiary structure was determined through x-ray crystal diffraction, and the recombinant active site of the enzyme was obtained by activating prokaryotic cells at pH 4.0 *in vitro*. Procerain B is a plant cysteine protease that effectively promotes milk clotting and could be used as a substitute milk-clotting agent in the cheese industry. The enzyme may also be used as a dietary supplement to improve digestion (Singh and Shukla, 2010). The ratio of MCA and PA is an important indicator of cheese production efficiency (Arima et al., 1970). The serine protease religiosin B extracted from grapefruit exhibited high MCA and could be used as a milk-clotting agent to improve cheese processing and dairy industry (Kumari et al., 2012).

Milk-Clotting Mechanism of Protease in *D. sinensis*

Degradation of Casein by *D. sinensis* Protease. As shown in Figure 3, the protease can degrade casein, α -CN, β -CN, and κ -CN. The degradation de-

gree of κ -CN was more pronounced than of β -CN and α -CN. Lane 7 represented casein, and lane 8 denoted the drawing effect after the reaction of *D. sinensis* with casein and indicated that casein was completely degraded. The corresponding bands on lanes 2, 4, and 6 became significantly pale, implying that α -CN, β -CN, and κ -CN were also degraded. The different thinning degrees indicated the different degradation degrees. The milk-clotting mechanism showed that the protease can specifically degrade κ -CN into deputy κ -CN and caseinomacropeptide, which further degrade α -CN and β -CN and result in milk clotting (Home and Banks, 2004). The specific degradation of κ -CN by *D. sinensis* protease was considered the main factor that affects milk clotting. A previous study showed that protease separated from glutinous rice wine can induce milk clotting and demonstrate different degradation degrees for α -CN, β -CN, and κ -CN (Jiang et al., 2007). The ginger protease also exhibited different degradation degrees for α -CN, β -CN, and κ -CN as temperature changed (Huang et al., 2011).

Relationship Between Degradation Degree and Milk-Clotting Time in α -CN, β -CN, and κ -CN.

Figure 3. Degradation of protease in *Dregea sinensis* (Guan Jin Teng, GJT) on casein. Note: 15% SDS-PAGE. Lanes 0 to 8 represent the markers, α -CN, GJT + α -CN, β -CN, GJT + β -CN, κ -CN, GJT + κ -CN, casein, and GJT + casein, respectively. Color version available online.

Figure 4. Electrophoresis (15% SDS-PAGE) of the enzyme reacted with (a) α -CN, (b) β -CN, and (c) κ -CN. Lane 0 is the marker. The reaction times were 5, 15, 25, and 35 min and 1, 2, 3, 4, and 12 h from left to right, represented by lanes 1 to 9, respectively. Color version available online.

As shown in Figure 4, *D. sinensis* protease produced different degrees of casein degradation. The degradation specificity could be arranged as κ -CN < β -CN < α -CN. Figure 4a shows that α -CN was digested into many small peptide products. At a reaction time of 5 min, most proteins were degraded into specific prod-

ucts. With increasing reaction time, the product types were constant. β -Casein was mainly degraded into a large-molecule product when the reaction time was 25 min. With further increase in reaction time, β -CN was gradually degraded into many small molecules. When the enzyme reacted with κ -CN for 35 min, β -CN was

degraded into 2 products. With increasing time, the large-molecule products also gradually degraded until only one product with a single molecular weight was generated.

Hydrolysates of κ -CN by *D. sinensis* Protease. Milk solidifies because of chymosin through casein modification. Degradation of κ -CN is the main factor attributed for milk clotting. The molecular weight of κ -CN was approximately 19 kDa, and the molecular weight of its κ -CN peptide was obtained through MALDI-TOF-MS analysis.

Figure 4c shows that the reaction between *D. sinensis* protease and κ -CN produced a final degradation product with a molecular weight between 10 and 15 kDa. Two major protein peaks with molecular weights of 12 and 6.9 kDa emerged in Figure 5. The molecular weight of κ -CN was approximately 19 kDa, indicating that the *D. sinensis* protease could digest κ -CN into 2 peptides. The peptides presented molecular weights of 12 and 6.9 kDa and contained enzyme cleavage sites. Figure 5 shows a peak with a relatively weak signal strength ranging from 14 to 15 kDa; this peak represented the product formed in the early reaction stage. However, with increasing reaction time, the peak gradually and completely degraded according to the electrophoresis mapping shown in Figure 4c.

Enzyme Cleavage Sites of κ -CN. Figure 6 shows the AA sequence Gln-Pro-Thr-Thr-Met as determined using a polypeptide sequence detector. In the contrasting κ -CN AA sequence alignment, the κ -CN cleavage

site of *D. sinensis* protease was Ala90-Glu91 as shown in Figures 4c and 5. Previous study showed that chymosin from glutinous rice wine degraded κ -CN at Thr94-Met95 (Jiang et al., 2007), whereas the protease from ginger cleaved κ -CN at His102-Leu103 and Ala90-Glu91 (Huang et al., 2011). The milk-clotting mechanism of chymosin involved κ -CN degradation on the surface of casein micelles; thus, α -CN and β -CN lost the colloidal protective effect of κ -CN because of the formation of a gelatin system (Anema, 1997).

CONCLUSIONS

The separated and identified *D. sinensis* protease was cysteine proceraïn B, with a molecular weight of 23.8 kDa. The *D. sinensis* protease can degrade κ -CN, α -CN, β -CN, and casein, with Ala90-Gln91 as the enzyme cleavage site of κ -CN. In food processing, *D. sinensis* protease is a novel chymosin and could be used to produce milk cake and cheese because it exhibited higher degradation rate for κ -CN than that for α -CN.

ACKNOWLEDGMENTS

We thank the National Natural Science Foundation of China (Grant No. 31160331) and the Key Laboratory of Food Processing and Safety Control University in Yunnan for their financial support. We are also grateful to teachers Shi Chongying and Wang Xuanjun (Engineering Center, Yunnan Agricultural University),

Figure 5. Matrix-assisted laser desorption/ionization time-of-flight spectrum of protease in *Dregea sinensis* reacted with κ -CN. m/z = mass:charge ratio.

Figure 6. N-Terminal sequencing spectrum. (a) The AA sequence of Gln-(Q); (b) AA sequence of Pro-(P); (c) AA sequence of Thr-(T); (d) AA sequence of Thr-(T); (e) AA sequence of Met-(M). DTT = dithiothreitol; DMPTU = *N*-dimethyl-*N*-phenylthiourea; DPTU = *N,N*-diphenylthiourea; DPU = *N,N*-diphenylurea.

and student Shi Yanan (College of Food Science and Technology, Yunnan Agricultural University) for their help in this project.

REFERENCES

- Ahmed, I. A. M., I. Morishima, E. E. Babiker, and N. Mori. 2009a. Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. *Food Chem.* 116:395–400.
- Ahmed, I. A. M., I. Morishima, E. E. Babiker, and N. Mori. 2009b. Dubiumin, a chymotrypsin-like serine protease from the seeds of *Solanum dubium* Fresen. *Phytochemistry* 70:483–491.
- Anema, S. 1997. The effect of chymosin on κ -casein-coated polystyrene latex particles and bovine casein micelles. *Int. Dairy J.* 7:553–558.
- Arima, K., J. Yu, and S. Iwasaki. 1970. Milk-clotting enzyme from *Mucor pusillus* var. Lindt. Pages 446–459 in *Methods in Enzymology*. Vol. 19. G. Perlmann and L. Lorand, ed. Acad. Press, New York, NY.
- Carles, C., and P. Martin. 1985. Kinetic study of the action of bovine chymosin and pepsin A on bovine κ -casein. *Arch. Biochem. Biophys.* 242:411–416.
- Carles, C., and B. R. Dumas. 1985. Kinetics of the action of chymosin (rennin) on a peptide bond of bovine α_{s1} -casein: Comparison of the behaviour of this substrate with that of β - and κ -caseins. *FEBS Lett.* 185:282–286.
- Egito, A. S., J. M. Girardet, L. E. Laguna, C. Poirson, D. Mollé, L. Miclo, G. Humbert, and J.-L. Gaillard. 2007. Milk-clotting activity of enzyme extracts from sunflower and albizia seeds and specific hydrolysis of bovine κ -casein. *Int. Dairy J.* 17:816–825.
- Galán, E., F. Prados, and A. Pino. 2008. Influence of different amounts of vegetable coagulant from cardoon *Cynara cardunculus* and calf rennet on the proteolysis and sensory characteristics of cheeses made with sheep milk. *Int. Dairy J.* 18:93–98.
- He, S. 2010. Preliminary studies on milk-clotting property and functional components of *Dregea sinensis* (Hemsl.). MS Thesis. Yunnan Agricultural University, Yunnan, China.
- Home D. S., and J. M. Banks. 2004. Rennet-induced coagulation of milk. Pages 47–70 in *Cheese: Chemistry, Physics, and Microbiology*. Vol. 1. 3rd ed. Elsevier Appl. Sci., Amsterdam, the Netherlands.
- Huang, X. W., L. J. Chen, Y. B. Luo, H. Y. Guo, and F. Z. Ren. 2011. Purification, characterization, and milk coagulating properties of ginger proteases. *J. Dairy Sci.* 94:2259–2269.
- Iwasaki, S., G. Tamura, and K. Arima. 1967. Milk clotting enzyme from microorganisms. II. The enzyme production and the properties of crude enzyme. *Agric. Biol. Chem.* 31:546–551.
- Jiang, T. L., J. Chen, and L. Xue. 2007. Study on milk-clotting mechanism of rennet-like enzyme from glutinous rice wine: Proteolytic property and the cleavage site on κ -casein. *J. Dairy Sci.* 90:3126–3133.
- Kumari, M., A. Sharma, and M. V. Jagannadham. 2012. Religiosin B, a milk-clotting serine protease from *Ficus religiosa*. *Food Chem.* 131:1295–1303.
- Kumari, M., A. Sharma, and M. V. Jagannadham. 2010. Decolorization of crude latex by activated charcoal, purification and physico-chemical characterization of religiosin, a milk-clotting serine protease from the latex of *Ficus religiosa*. *J. Agric. Food Chem.* 58:8027–8034.
- Ma, Y. 2013. Studies on protease in *Dregea sinensis* (Hemsl.) and its milk-clotting property. MS Thesis. Yunnan Agricultural University, Yunnan, China.
- Mohanty, A. K., U. K. Mukhopadhyay, J. K. Kaushik, S. Grover, and V. K. Batish. 2003. Isolation, purification and characterization of chymosin from riverine buffalo (*Bubalus bubalis*). *J. Dairy Res.* 70:37–43.
- Nandana, V., S. Singh, and A. N. Singh. 2014. Procerain B, a cysteine protease from *Calotropis procera*, requires N-terminus pro-region for activity: cDNA cloning and expression with pro-sequence. *Protein Expr. Purif.* 103:16–22.
- Singh, A. N., A. K. Shukla, and M. V. Jagannadham. 2010. Purification of a novel cysteine protease, procerain B, from *Calotropis procera* with distinct characteristics compared to procerain. *Process Biochem.* 45:399–406.
- Tao, L. 2014. Preliminary studies on protease in *Dregea sinensis* (Hemsl.) characteristics and its milk-clotting mechanism. MS Thesis. Yunnan Agricultural University, Yunnan, China.
- Wu, Z. 2003. *Flora of Yunnan*. Sci. Press, Beijing, China.
- Xue, L. 1994. New Biological Records of Dali. Pages 356–357 in *Monograph 4 Yunnan People's Publ. House*, Kunming, Yunnan, China.