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# Proteomic analysis of *Moringa oleifera Lam.* leaf extract provides insights into milk-clotting proteases

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ABSTRACT

As a new food source, *Moringa oleifera Lam.* leaves containing large amounts of proteins attract increasing attention. This study detected and characterized hydrolytic and milk-clotting enzymes in these leaves. The proteins were examined by proteomics. A total of 3378 proteins were identified, mostly comprising enzymes of carbohydrate and protein metabolism, including 676 hydrolases, 548 oxidoreductases, glycoside hydrolases and proteinases. A serine/threonine-endopeptidase with a molecular mass of 56.146 kDa and an isoelectric point of 5.27 with milk-clotting activity was obtained from *M. oleifera* leaves by multistage ultrafiltration and anion exchange chromatography, and characterized by ESI mass spectrometry. The milk-clotting activity was inhibited over 95% by chymostatin, which confirmed its chymotrypsin-like serine protease nature. The protease had a milk-clotting activity/proteolytic activity (MCA/PA) activity ratio of 126.76, an optimal pH of 8.0, and an optimal temperature of 65 °C, indicating its potential use for cheese production and in food industry.

## 1. Introduction

In cheese manufacture, milk-clotting enzymes are the primary active agents of casein and whey hydrolysis. Due to various factors, such as rennet's limited availability and high cost, religious requirements, and diet habits in some countries, many researches have been focusing on discovering natural extracts from plants, such as papain from pineapple, ginger and artichoke flowers (Chazarra, Sidrach, López-Molina, & Rodríguez-López, 2007), ficin from *Calotropis gigantea* (Rajagopalan, Singh, & Bindhu, 2014) and *solanum dubium* seeds (Ahmed, Babiker, Mori, & Ahmed, 2011; Mori, Babiker, & Ahmed, 2010) and latex from *Ficus religiosa* (Kumari, Sharma, & Jagannadham, 2010). Growing in tropical and subtropical regions, *M. oleifera* represents a potential protein source (Rébufa, Pany, & Bombarda, 2018; Sánchez-Machado, Núñez-Gastélum, Reyes-Moreno, Ramírez-Wong, & López-Cervantes, 2010) and it is considered one of the most popular vegetables and animal feed in multiple nations, especially India, Pakistan, Philippines and multiple African nations.

Consumption of *Moringa oleifera Lam.* leaves as a new food source has been approved by the Chinese ministry of health. A recent study showed that due to the abundant nutrients and chemicals of its leaves (Oyeyinka & Oyeyinka, 2016), *M. oleifera* represents an important source of proteins in diet and animal feed (Teixeira, Carvalho, Neves, Silva, & Arantes-Pereira, 2014). *M. oleifera* leaves also contain flavonoids, phenolics, polyphenols, and polysaccharides. It was shown that the tree contains compounds with protease (Bijina et al., 2011), cytotoxic, antioxidant (Alhakmani, Kumar, & Khan, 2013; Cohen-Zinder et al., 2017), anti-arthritic, anti-inflammatory, fibrinogenolytic, wound healing and anticancer (Falowo et al., 2018; Gopalakrishnan, Doriya, & Kumar, 2016; Horwath & Benin, 2011; Khalafalla et al., 2010) activities. In some plants containing several proteins and growing in the tropical region, enzymes are secreted abundantly. These enzymes help resist larva attacks and digest proteins to accelerate plant growth (Oliveira et al., 2016). Indeed, multiple protein-degrading enzymes have been extracted and characterized from *M. oleifera* leaves, with some active proteins used for wastewater treatment, as well as lectins. However, these enzymes have not been comprehensively analyzed for their diversity. Crude extracts of *M. oleifera* seeds and flowers have undergone screening for milk-clotting activity with skim milk as substrate (Tajalsir et al., 2014). Meanwhile, skim milk is used for cheese production. Such activity was only obtained upon protein precipitation with 60% ammonium sulphate, suggesting low amounts of this protease in flowers (Nasr, Ahmed, & Hamid, 2016; Pontual et al., 2012). Recently, Satish, Sairam, Ahmed, and Urooj (2012) reported that the caseinolytic activity of *M. oleifera* leaf is significantly higher than the flower's ($p < 0.05$). However, milk-clotting enzymes from *M. oleifera* leaves have not been investigated in detail.

In the current study, we adopted an integrated GO application

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strategy in proteomic expression profile analysis, which has been widely used to annotate protein functions in several organisms. A previous pharmacologic study showed the efficacy of *L. rhinocerotis* TM02 sclerotium by proteome analysis, with subsequent isolation and identification of constituent (s) exerting anticancer effects (Yap, Fung, Ng, Tan, & Tan, 2015). Proteome assessment of scallop hepatopancreatic extract further explains the degradation of marine polysaccharides (Lyu et al., 2016). Multiple studies focusing on proteomics have provided insights into numerous functional protein components and diversity (Horie et al., 2008; Yan Zhang et al., 2014). Thus, great efforts should be made to develop more effective bioinformatics strategies in capturing enzymes from potential sources. In this study, we employed 2DLC–MS/MS shotgun proteomics to analyze protein composition and provide new insights into the milk-clotting activity of the protease isolated from *M. oleifera* leaves. Biochemical characterization was also carried out to estimate its potential in cheese-making industry.

## 2. Material and methods

### 2.1. Total protein extraction from M. oleifera leaves

In this study, we used the most widely cultivated *M. oleifera*, PKm2, collected in De Hong City, Yunnan Province, in July 2016. Total protein extraction from *M. oleifera* leaves was performed according to Shi, Wang, and Huang (2018). The BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China)was used to determine protein amounts, as directed by the manufacturer.

### 2.2. Protein digestion and high pH RPLC separation

Proteins were digested by routine methods. In brief, 200 μg protein was mixed with TCEP (10 mmol/L) and submitted to incubation at 37 °C for 60 min. Then, IAM was supplemented at 40 mM for a 40 min incubation away from light. Six volumes of chilled acetone were added, and reaction tubes were inverted three times and incubated at −20 °C until precipitate formation (4 h). After acetone removal by centrifugation (10,000 g, 4 °C, 20 min), the precipitate was solubilized with 200 μL (100 mmol/L) TEAB buffer. To every reaction, 5 μg of trypsin was added for incubation at 37 °C overnight. This was followed by drying under vacuum. Sample fractionation was carried out by high-pH reversed phase separation to enhance proteomic depth (Yin et al., 2015). After solubilization with loading buffer (ammonium hydroxide solution with 2% acetonitrile, pH 10), protein separation was performed on a high-pH reversed phase liquid chromatograph (RPLC, Acquity Ultra Performance LC; Waters, USA). The gradient elution was performed on high pH RPLC column (ACQUITY UPLC BEH C18 Column 1.7 μm, 2.1 mm × 150 mm, Waters, USA) at 200 μl/min with an elution gradient for 66 min, with ammonium hydroxide solution containing 80% and 2% acetonitrile (pH 10), respectively. Ten fractions were obtained for each sample.

### 2.3. Mass spectrometry

A Q Exactive mass spectrometer coupled with Easy-nLC 1200 was employed (electrospray voltage, 1.8 kV). A sample of 4 μl was used for nano LC-MS/MS. The protein sample was loaded on to a C18-reversed phase column (75 μm, 25 cm; Thermo, USA) in 0.1% formic acid in 2% acetonitrile, and resolved using a linear gradient of 0.1% formic acid in 80% acetonitrile at 300 nl/min. The data-dependent mode was employed for the mass spectrometer, switching automatically between MS and MS/MS. Full scan mass spectra (m/z 350–1300) were obtained at a mass resolution of 70 K; then, 20 successive high energy collisional dissociation (HCD) MS/MS scans (resolution, 17.5 K) were acquired. For MS/MS, normalized collision energy was 30. MS/MS data were analyzed with Protein Discoverer ™ Software 2.1 based on the Moringa database (19465 sequences) using the following criteria: tryptic digestion, ≤2 missed cleavages; fixed modification, cysteine carbamidomethylation; variable modifications, methionine oxidation and N-terminal acetylation. A false discovery rate (FDR) of 1% was adopted for validation.

### 2.4. Proteomic analysis of M. oleifera leaves

Gene ontology (GO) enrichment analysis of related proteins from *M. oleifera* leaves were carried out based on the Uniprot database and Blast2go (http://www.blast2go.com/b2ghome). Proteins were classified according to involvement in biological processes (BP), cellular components (CF) and molecular function (MF) (Conesa et al., 2005). GO terms enriched in a certain gene group were identified using EnrichPipeline[27]. Similar methods were employed to mapped the detected proteins for Kyoto Encyclopedia of Genes and Genomes (KEGG) and enrichment analysis (Kanehisa, Furumichi, Tanabe, Sato, & Morishima, 2017).

### 2.5. Purification and identification of a milk-clotting protease from M. oleifera leaves

*M. oleifera* leaves (80 g dry weight) was added to 400 ml NaCl (0.15 mol/L), homogenized and filtered. The obtained extract cleared by centrifugation was mixed with ammonium sulphate at 0–20%, 20–40%, 40–60% and 60–80%, respectively. The supernatant fractions with high milk-clotting activity were dialyzed (10 ml; 3.5 kDa cut off) against 2 L of distilled water (12 h) and 0.15 mol/L NaCl (2 h), respectively. Fractions with milk-clotting activity (MCA) were transferred into 10 KDa and 50 KDa Millipore ultrafiltration centrifuge tubes (Amicon Ultra-4, Ultracel-3 Membrane, Beckman Coulter Inc., Brea, CA), centrifuged at 4 °C (1300 × *g*, 40 min) and marked as C1, C2, C3 and C4 components. After dialysis, the samples were submitted to ammonium sulphate precipitation as above and loaded onto a Q-Sepharose fast flow column 1.8 × 80 cm) equilibrated with 20 mmol/L Tris buffer pH 8.0. Elution of bound proteins used gradients of 1 mol/L NaCl in TE buffer (0–30%, 30–70%, and 70–100%) at 1 mL per minute, collecting 3 mL fractions. The eluate was monitored at 280 nm with a UV detector to detect proteins. All active fractions in the MCA target peak were again transferred into 10 kDa Millipore ultrafiltration centrifuge tubes and centrifuged at 4 °C (1300 × *g*, 20 min), and the supernatants were stored at −20 °C. The extraction and purification processes were carried out at 4 °C to protect the enzyme. Protein concentration was measured by the Lowry method with BSA as the standard, and samples were assessed by 12% SDS-PAGE using a Mini Protein system (Bio-Rad, Watford, UK). The 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 Mascot 2.2 software (http://www.matrixscience.com/mascot_support_v2_2.html) to identify the main milk-clotting protein.

### 2.6. Assay of protease activity

Milk-clotting activity (MAC), according to Nouani et al. (2009), is the most crucial feature of enzymes employed in cheese manufacturing. The substrate was composed of 10% milk powder (low-heat skim milk powder) reconstituted with 5 mmol/L $CaCl_2$. It involved adding 0.2 mL of protease to 2 mL of substrate at 65 °C, and the time required for curd fragment generation was assessed. The MCA was defined in Soxhlet units (SU), i.e. the volume of milk coagulated/protease volume within 40 min. Proteolytic activity (PA) was detected with the modified method proposed by Mohanty et al. using the above substrate (Mohanty, Mukhopadhyay, Kaushik, Grover, & Batish, 2003) and 1.5% solution of bovine whole CN (pH 6.0 with 0.1 mol/L phosphate buffer). The preheated substrate (1 mL) was mixed with 100 μL of purified protease solution, followed by incubation (37 °C, 60 min) and addition

**Table 1**
GO term enrichment of *M. oleifera* leaves proteins.

| GO ID | GO Term | GO Class | Gene number | P value | Adjusted Pv |
|---|---|---|---|---|---|
| GO:0016787 | hydrolase activity | MF | 676 | 3.92E-23 | 8.36E-20 |
| GO:0016491 | oxidoreductase activity | MF | 548 | 9.15E-15 | 1.31E-14 |
| GO:0005198 | structural molecule activity | MF | 190 | 3.28E-14 | 6.68E-11 |
| GO:0016740 | transferase activity | MF | 524 | 8.41E-13 | 4.42E-10 |
| GO:0030234 | enzyme regulator activity | MF | 44 | 3.88E-08 | 2.10E-08 |
| GO:0016209 | antioxidant activity | MF | 53 | 8.22E-08 | 1.32E-07 |
| GO:0060089 | molecular transducer activity | MF | 20 | 6.79E-05 | 7.77E-04 |
| GO:0071704 | organic substance metabolic process | BP | 2382 | 3.30E-21 | 6.30E-20 |
| GO:0044237 | cellular metabolic process | BP | 2302 | 6.66E-14 | 3.64E-12 |
| GO:0042221 | response to stress | BP | 842 | 7.17E-12 | 2.19E-10 |
| GO:0009058 | biosynthetic process | BP | 1548 | 9.17E-09 | 1.12E-08 |
| GO:0044710 | single－organism metabolic process | BP | 1964 | 5.71E-05 | 2.22E-03 |
| GO:0044424 | intracellular part | CC | 2671 | 4.79E-15 | 8.68E-14 |
| GO:0005737 | cytoplasm | CC | 2494 | 7.82E-12 | 7.21E-09 |
| GO:0043231 | intracellular membrane－bounded organelle | CC | 2207 | 2.22E-06 | 3.24E-05 |
| GO:0005886 | plasma membrane | CC | 536 | 7.98E-04 | 1.98E-03 |

of 1 mL of 12% TCA. Optical density of soluble peptides was determined at 280 nm on a UV–visible spectrophotometer (Cintra 20, GBC Scientific Equipment Pty. Ltd., Dandenong, Victoria, Australia). A unit of PA was the quantity of protease required to increase the optical density by 0.001 in 1 min.

### 2.7. Inhibition assay

The serine protease inhibitor chymostatin, phenylmethanesulfonyl fluoride (PMSF) (8 mmol/L, 1 ml), the cysteine protease iodoacetamide, sodium tetrathionate, and the aspartic protease pepstatin A were supplemented to the milk-clotting protease solution (1 ml, 32 mg). The resulting sample was submitted to incubation (37 °C, 30 min) and assessed for proteolytic and milk-clotting activities. The following formula was adopted to assess inhibition: % inhibition = 100-[100 × (residual activity/activity in control without inhibitor)].

### 2.8. Determination of optimal temperature and pH

The optimum activity of the purified milk-clotting protease was determined at various pH values and temperatures. The reaction mixture containing 10% skim milk was incubated at a temperature range of 30–90 °C. Then, reactions were all performed at 65 °C until particle appearance.

Different pH values (2.0–12.0) were analyzed using the following buffers (0.1 mol/L): glycine-HCl (pH 2.0–3.5), citrate-phosphate (pH 3.5–6.0), sodium phosphate (pH 6.0–8.5), Tris-HCl (pH 8.5–10.0) and glycine-NaOH (pH 10.0–12.0). The maximum enzyme activity was 100% in the control group (without adjust pH), and relative enzyme activities were obtained for other groups.

### 2.9. Stability

The protease was treated with various pH and temperature conditions for 40 min. Then, residual proteolytic activity was determined (see above). The maximum enzyme activity was 100% in the control group, and relative enzyme activities were determined for the experimental groups.

### 2.10. Statistical analysis

The results were presented by triplicate in separate trials, the data were expressed as a mean of replicates ± standard deviation (SD) and subjected to an analysis of variance (ANOVA) using SPSS version 16.0 (SPSS Inc., Chicago, IL). Significant differences between treatment groups were analyzed by the Student's *t*-test (significance at $p < 0.05$).

## 3. Results and discussion

### 3.1. Protein analysis of M. oleifera leaves

*M. oleifera* leaf extract had 9.561 mg/mL protein. The shotgun proteomics approach revealed 8067 peptides corresponding to 3378 non-redundant proteins that were analyzed by 2DLC–MS/MS, and 522111 entries were matched in the Brassicales database. The unified dataset is one of the most comprehensive shotgun proteomics datasets ever reported for *M. oleifera* leaf proteins; 1261 non-informative proteins with predicted and/or uncharacterized properties would require a novel biological assembly.

### 3.2. Proteomic analysis of M. oleifera leaves

The 3348 proteins were subjected to molecular function categories of the GO database (2017_07). The results showed that the prominent categories involved molecular function and biological processes: hydrolytic activity, 676 proteins (GO: 0016787) (P value: 3.92E-23) and organic substance metabolism, 2382 proteins (GO: 0071704) (Table 1). There were 548 oxidoreductases, 524 transferases, 190 structural and molecularly active proteins, 53 antioxidant activity proteins, 44 enzyme regulatory proteins, and 20 proteins with molecular transduction activity. The extract contained a mixture of several hydrolytic enzymes, among which proteases as the key enzymes are responsible for the observed milk-clotting activity.

We obtained 305 maps using the *M. oleifera* proteins, many proteins of these pathways participate in energy, carbohydrate and protein metabolism (Table 2). KEGG suggested the above proteins were significantly enriched in pathways controlling glyoxylate and dicarboxylate metabolism (ko: 00630); protein processing in endoplasmic reticulum (ko: 04141); oxidative phosphorylation (ko: 00190); glycolysis/gluconeogenesis (ko: 00010); and the predictions for the alanine, aspartate and glutamate metabolism; cysteine and methionine metabolism; glycine, serine and threonine metabolism, which reflects leaf functions, providing valuable information about the functional and pharmacological research of *M. oleifera*.

### 3.3. Analysis of milk-clotting activities and protease purification

The hydrolytic feature of proteases is used for quality improvement of protein-rich foods. During screening of plant leaves for chemical components, a significant milk-clotting activity was detected in the 60% fraction of the plant. Upon loading the crude proteins for purification (after 60% ammonium sulphate followed by C2 and C3 ultrafiltration precipitation) on a Q-Sepharose fast flow column, C2 components were separated into four peaks (Q-I ~ Q-IV) (Fig. 1).

**Table 2**
KEGG enrichment of *M. oleifera* leaves proteins.

| Map ID | Map Title | Gene number | P value | Adjusted Pv |
|---|---|---|---|---|
| ko3435 | Energy metabolism | 439 | 1.50E-36 | 5.40E-33 |
| ko9074 | Carbohydrate metabolism | 476 | 7.40E-30 | 8.89E-29 |
| K15335 | Amino acid metabolism | 266 | 4.42E-28 | 2.21E-25 |
| ko00710 | Carbon fixation in photosynthetic organisms | 149 | 4.06E-20 | 5.18E-19 |
| ko00630 | Glyoxylate and dicarboxylate metabolism | 131 | 7.33E-17 | 2.22E-15 |
| ko04141 | Protein processing in endoplasmic reticulum | 84 | 5.06E-16 | 6.16E-15 |
| ko00010 | Glycolysis/Gluconeogenesis | 100 | 8.63E-11 | 3.34E-09 |
| ko00260 | Glycine, serine and threonine metabolism | 56 | 1.34E-10 | 2.24E-10 |
| ko00190 | Oxidative phosphorylation | 117 | 2.09E-05 | 4.04E-04 |
| ko00250 | Alanine, aspartate and glutamate metabolism | 56 | 6.39E-05 | 7.71E-04 |
| ko00270 | Cysteine and methionine metabolism | 66 | 5.30E-08 | 9.30E-06 |
| ko00220 | Arginine biosynthesis | 38 | 2.27E-04 | 8.00E-04 |
| ko00280 | Valine, leucine and isoleucine degradation | 36 | 8.86E-03 | 4.01E-03 |

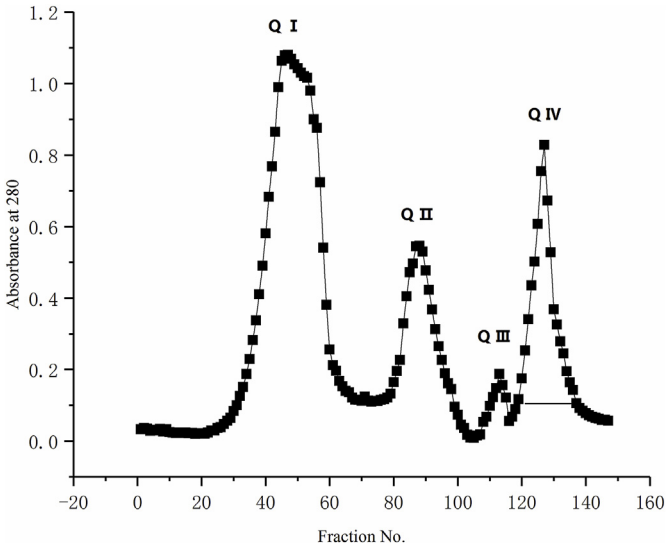


**Fig. 1.** Elution profile of curde enzyme after removal of anion exchange chromatography on Q-Sepharose fast flow column, pre-equilibrated with 20 mmol/L Tris buffer, pH 8.0. The bound proteins were eluted with a linear salt gradient of 0–1.0 mol/L NaCl. The fractions of the ascending limb of pool Q-IV (120–134) were pooled as indicated by the horizontal line.

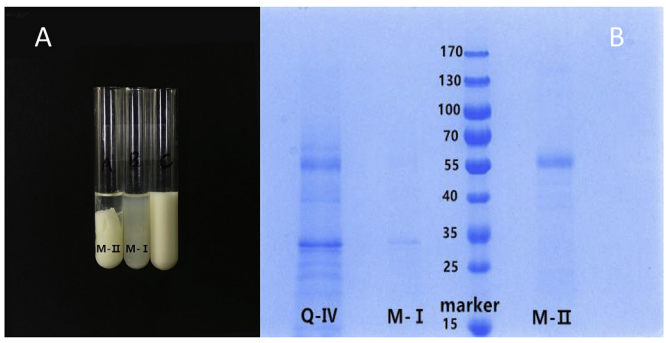


**Fig. 2.** (A) Milk-clotting activity of pure protease (lane M-II), pure protease (lane M-I) and negative control 1.0 mol/L NaCl; (B) Electrophoresis mapping of purified protease. Pool Q-IV, M-I and M-II showing marker, pure protease (non milk-clotting activity) after separation, pure protease (milk-clotting activity) after separation, respectively.

Among them, a single peak including eluting tubes 120 to 134 showed clotting activity, with higher activity than rennet. About Q-I peak, most miscellaneous proteins in the sample were in the flow-through, showing that this protein could not retained on Q- Sepharose column in a buffer not containing NaCl solution. Q-II peak ionic strength of the mobile phase increased to 0.2 mol/L NaCl, under which the Q-II peak appeared at 54 min. Decomposition of casein did not result in curds, and further increased ionic strength to 0.6 mol/L NaCl. A relatively uniform elution peak at 0.6 mol/L converted to the same NaCl buffer concentration as the negative control; strong curd dynamic test results indicated that Q-IV contained the target proteases. Further fractions from peak Q-IV were pooled and submitted to ultrafiltration with 10 KDa Millipore centrifuge tubes, and separation and purification of *M. oleifera* proteases by SDS-PAGE revealed the presence of 55 kDa and 35 kDa proteins (lanes M-II and M-I) (Fig. 2B). These findings indicated that the two proteases were completely separated and reached electrophoretic purity. The performances of these proteases were confirmed by milk-clotting experiments. The protein components of C3 underwent the above elution procedure to yield three elution peaks, and there was no clotting activity, indicating no or low concentration clotting component. Milk clotting did not occur when the 35 kDa protease was added at 0.84, 1.68 and 8.4 mg/mL to a mixture containing 10% skim milk. Reaction was initiated for 38 min at 42 mg/mL, and an extremely loose flocculent structure was obtained even at up to 84 mg/mL, representing the 35 kDa protease. The mixture was flocculent, indicating that the protease exhibited a low clotting activity and had no practical application value. When the 55 kDa protease was added at a 1.68 mg/mL, large clots were produced after 7 min. The clot surface was fine, smooth and elastic, with a satisfactory tissue state. With increasing protease ratio, milk-clotting was rapidly generated and reached stability at 6 min. The resulting clot was large, with an even structure and satisfactory tissue structure (Fig. 2A). These findings indicated that the purified protease with a molecular weight of 55 kDa was the main milk-clotting compound with a strong MCA. These purification steps are summarized in Table 3. The specific milk-clotting activities of the protease increased more than its PA during purification, and was 20-times higher than that of the filtrate, with an MCA/PA ratio of 126.76. This suggested the protease has a great potential to be developed as a

**Table 3**
Purification and enzymatic assays of extracts from *M. oleifera* leaves.

| Purification step | | | |
|---|---|---|---|
| Item | Filtrates | Crude enzyme | Purified enzyme |
| Volume (mL) | 400 | 20 | 10 |
| Milk-clotting activity (Soxhlet units/mL) | 13.33 | 174.60 | 253.27 |
| Total Soxhlet units | 5332 | 3492 | 2532.70 |
| Protein (mg/mL) | 39.9 | 24.50 | 5.40 |
| Total protein (mg) | 15960 | 490 | 54 |
| Specific activity (Soxhlet units/mg) | 0.33 | 7.13 | 46.90 |
| Relative purification fold | 1.00 | 21.61 | 142.12 |
| Proteolytic activity (U/mg) | 0.32 | 0.37 | 0.37 |
| Milk-clottingactivity:proteolytic activity (MCA/PA) | 1.03 | 19.27 | 126.76 |

milk-coagulating factor in the future. The commercial rennet possess highest clotting and lowest proteolytic activities, with an MCA/PA ratio of 4989.771. Although the MCA/PA ratio of the current milk-clotting protease (126.76) was reduced compared with that of the commercially available rennet, it was much elevated in comparison with those of proteins mentioned above. Huang, Chen, Luo, Guo, and Ren (2011) separated chymosin from ginger, whose highest MCA/PA ratio was 43.87; *Dregea sinensis* Hemsl contains a protease with an MCA/PA ratio of 27.31(Yali Zhang, Wang, Tao, & Huang, 2015). The higher enzymes with high MCA/PA ratios (such as fig, paw, pineapple and castor oil seed products) are indicated for cheese production, requiring short clotting time; in addition, biter tasty proteins are difficult to produce. Further studies to explore the mechanism underlying milk coagulation by serine/threonine-endopeptidases and to assess their use in cheese production are warranted.

### 3.4. Identification of the milk-clotting protease

Two protein bands with molecular weights of around 55 kDa and 35 kDa, respectively, were obtained by SDS-PAGE, and the 55 kDa protein band with curd activity was tested. The contrast UniProt protein database showed that the protein with the highest peak intensity was a serine/threonine-endopeptidase, with a relative molecular mass of 56146.35 Da and an isoelectric point of 5.27, exhibiting a hydrolytic activity (Fig. 3).

Historically, according to Tamer and Mavituna (1997) almost all enzymes employed to clot milk are aspartic proteases; however, cysteine and serine proteases have also been applied. Proteolytic activity assessment of *M. oleifera* leaves in presence of protease inhibitors (Table 4) revealed significant change after incubation with PMSF and chymostatin; meanwhile, marked reductions of milk-clotting activities were found in the PMSF and chymostatin groups ($p < 0.05$), reaching 28.63% and 36.55%, respectively. Compared with other protease inhibitors, the purified protease belonged to serine protease family. In addition, aspartic acid protease inhibitors have certain inhibitory effects on its enzyme activity. In the presence of pepstatin A, the hydrolytic activity on casein was not significantly changed, while the curd activity was reduced by 22.66%. Serine proteases represent one of the largest groups of proteolytic enzymes (Shah, Mir, & Paray, 2014; Tripathi, Tomar, & Jagannadham, 2011). The results showed that the most pronounced inhibition was obtained with PMSF, a well-known serine protease inhibitor, indicating the newly isolated protease is a serine protease. Inhibition of enzymes provides a preliminary characterization of their active sites and cofactors. Plant serine proteases maintain stability and activity under unfavorable temperatures and pH values, and even in presence of surfactants or oxidants. Therefore, they are very useful and cost effective for industrial purposes (Kumari, Sharma, & Jagannadham, 2012; Sharma, Kumari, & Jagannadham, 2012).

**Table 4**
Effects of protease inhibitors on the activity of purified enzyme. Different lowercase, uppercase letters indicate significant differences at $p < 0.05$.

| Protease class | Protease inhibitor Control | Residual protease Activity | |
|---|---|---|---|
| | | Milk-Clotting Activity (%) | Proteolytic Activity (%) |
| Control | | 100a | 100A |
| Aspartic protease | Pepstatin A | 77.34 ± 1.4b | 96.06 ± 0.8A |
| Cysteine protease | Sodium tetrathionate | 97.65 ± 3.3a | 95.43 ± 0.7A |
| | Iodoacetamide | 94.56 ± 2.7a | 98.1 ± 1.9A |
| Serine protease | PMSF | 28.63 ± 1.1c | 66.90 ± 1.1B |
| | Chymostatin | 36.55 ± 1.1c | 50 ± 0.2C |

### 3.5. Optimal pH and temperature, and stability of the purified milk-clotting protease

A certain amount of sample was drawn every 60 min to measure enzyme activity. The results showed that the enzyme in the 35–75 °C temperature range maintained for 60 min could keep 80% of its MCA, and exhibited the highest MCA ratio at 65 °C, demonstrating its potential use as an accelerating agent in the dairy industry (Fig. 4A). The enzyme exposed to pH values of 4.0–9.0 for 40 min still had more than 80% of its activity, and the optimal pH was 8.0. Increasing or decreasing the pH from this range reduced enzyme stability; at a pH value of 11.0, MCA was reduced to 20% (Fig. 4B). Regarding this notion, separation of the enzyme is important, since it could be used in alkaline environment. Most enzymes are not stable at high pH, e.g. from goats, sheep, and glutinous rice wine, which all have this shortcoming (Zhao, Wang, Zhang, Zhao, & Yang, 2015). They are weaker under alkaline than acid conditions, while the *M. oleifera* milk-clotting enzymes is consistently active. The similarly striking property of the milk-clotting enzyme from *Solanum dubium* seeds, a chymotrypsin-like serine protease with a pI value of 9.3, is its optimal activity at pH 11.0 (Mori, Morishima, Babiker, & Ahmed, 2009).

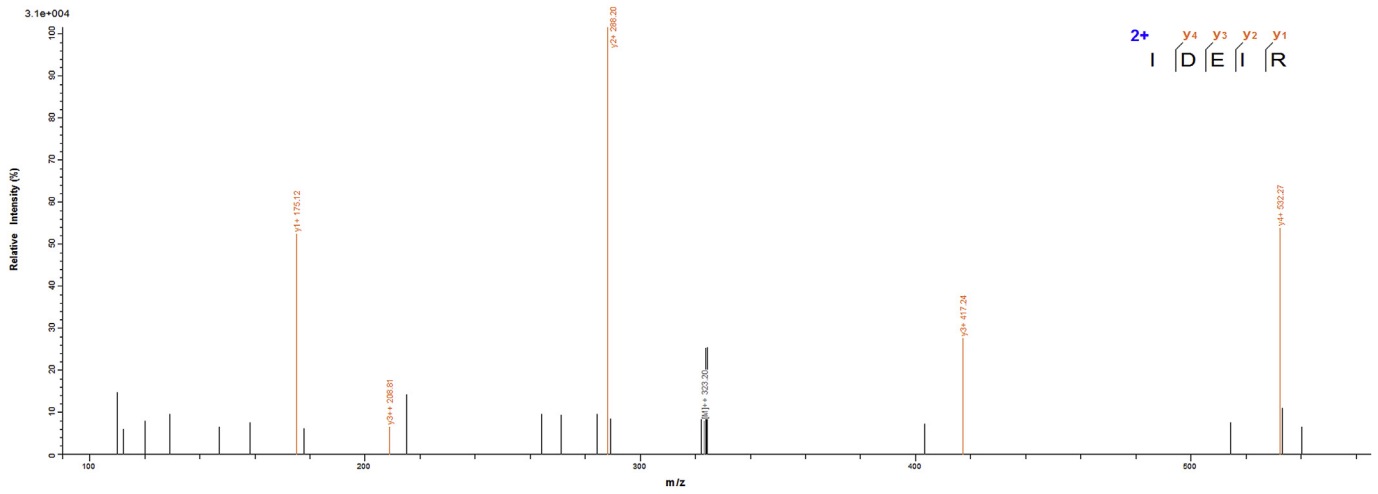


**Fig. 3.** Electro-spray ionization mass chromatogram of 56146.35 Da serine/threonine-endopeptidase. m/z = mass: charge ratio.

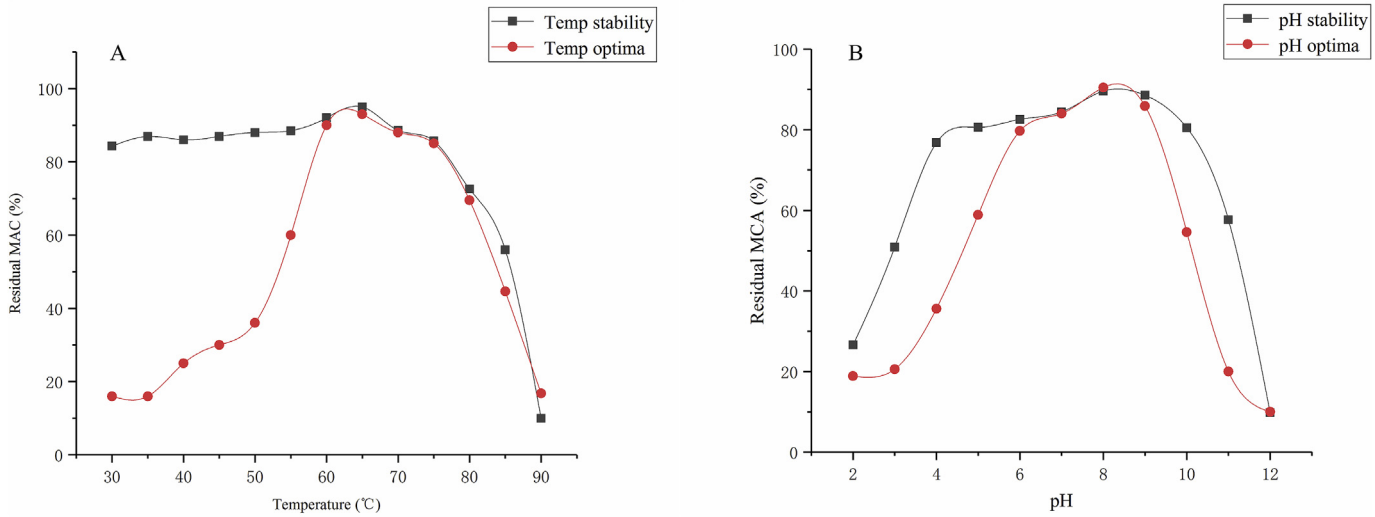


**Fig. 4.** Effect of temperature (A) and pH (B) on the milk-clotting activity (●) and stability (■) of serine/threonine-endopeptidase. The assay protocols are described in the material and method section.

## 4. Conclusion

*Moringa oleifera Lam.* leaves contain 3378 proteins, mostly including proteins with hydrolytic and oxidoreductase activities, which are associated with carbohydrate and protein metabolism. The separated and identified milk-clotting protease was a serine/threonine-endopeptidase of 56.146 kDa with an isoelectric point of 5.27. Its high specificity (MCA/PA ratio) combined with pH and temperature stability could help improve cheese production.

## Declaration of conflicting interests

The authors declared no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2019.04.035.

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