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Purification and characterization of a novel milk-clotting metalloproteinase from *Paenibacillus* spp. BD3526

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ABSTRACT

In this study, a milk-clotting enzyme (MCE) isolated from *Paenibacillus* spp. BD3526 was purified and characterized. The MCE was purified 8.9-fold with a 10.11% recovery using ammonium sulfate precipitation and anion-exchange chromatography and the specific milk-clotting activity (MCA) reached 6791.73 SU/mg. The enzyme was characterized as a 35 kDa metalloproteinase, and the zymogen of which was encoded by a 1671 bp gene named zinc metalloproteinase precursor (*zmp*) with a predicted molecular weight of 59.6 kDa. The optimal temperature for MCA and proteolytic activity (PA) was 65 °C and 60 °C, respectively. The enzyme was stable over a pH range of 5.0–9.0 and at temperatures below 50 °C. The MCA was completely inactivated when the enzyme was heated at 60 °C for 30 min, and the PA was totally inactivated for 20 and 10 min when the enzyme was heated at 55 °C and 60 °C, respectively. The BD3526 enzyme was preferentially active towards κ-casein (*κ*-CN) and β-casein (β-CN), as determined by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), whereas the hydrolysis of α_s-casein (α_s-CN) was slow and comparable to that caused by chymosin and asparatic acid proteinase from *Rhizomucor miehei*. The cleavage site of the metalloproteinase in κ-CN was located at the Met_{106}–Ala_{107} bond, as determined by mass spectrometry analysis.

1. Introduction

The reported milk-clotting enzymes (MCEs) could be classified into four categories: aspartic [1,2], cysteine [3,4], serine [5–7] and metallo [8,9] proteinase; among all the types of proteinases, the ideal MCE is characterized by a high milk-clotting activity (MCA) to proteolytic activity (PA) ratio. Extracellular proteinases with MCA from microorganisms are one of the potential alternatives for calf rennet. Over the past few decades, more than one hundred fungi exerting MCE production abilities have been reported [10], and most of these MCEs are aspartic proteinases, with the exception of a metalloproteinase from *Termitomyces clypeatus* MTCC 5091 [8]. The specific cleavage of the Phe_{105}–Met_{106} and Ser_{104}–Phe_{105} peptide bonds of κ-CN by chymosin or commercial fungal-source MCEs induces milk curding [10]. Moreover, it has been reported that the κ-CN cleavage site for the metalloproteinase from *T. clypeatus* MTCC 5091 is the same as that for chymosin [8].

Apart from fungus-derived MCEs, more and more studies on bacterial sources of MCEs have been published in recent years. The MCEs secreted by *Bacillus subtilis* YB-3 [11], *Bacillus amyloliquefaciens* D4 [12], and *B. amyloliquefaciens* JNU002 [9] were purified and characterized as metalloproteinases, whereas those derived from *Bacillus sphaericus* NRC 24 [13] and *Bacillus licheniformis* USC13 [14] were identified as serine proteinases. Despite several reports on the production, purification, characterization, and application of various new bacterial MCEs [9,12,15,16], the protein sequences and milk-curding mechanisms of these MCEs have rarely been investigated.

A previous study investigated a novel *Paenibacillus* spp. strain, BD3526, with a high production of MCE in wheat bran broth. The maximum MCA reached 6469.72 ± 280.65 SU/mL, and the ratio of MCA to PA reached 8626.29 ± 374.20, which indicated that the enzyme of BD3526 could be potentially served as a microbial coagulant. The molecular weight of the enzyme (35 kDa) was determined through sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and gelatin zymography. In this work, the MCE produced by BD3526 was purified and characterized. The hydrolytic profiles of α_s, β, and κ-casein (CN) produced by the enzyme and the κ-CN cleavage site were also investigated.

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2. Materials and methods

2.1. Chemicals and reagents

The skim milk powder (Fonterra Ltd., Auckland, New Zealand) used for the skim milk agar preparation and milk-clotting activity (MCA) determination was composed of 0.8% (w/w) fat, 33.4% (w/w) protein, 54.1% (w/w) lactose, 7.9% (w/w) mineral, and 3.8% (w/w) moisture. Wheat bran, composed of 18.6% (w/w) protein, 6.2% (w/w) fat, 63.9% (w/w) carbohydrates, 3.38% (w/w) ash, and 7.89% (w/w) moisture, was purchased from a local market in Shanghai. Pepstain A, ethylene diamine tetraacetic acid (EDTA), and α_s-, β-, and κ-CN were purchased from Sigma–Aldrich, USA. The chemicals 2-iodoacetamide and phenylmethylsulfonyl fluoride (PMSF) were purchased from Meryer and Sinopharm Chemical Reagent Co., Ltd., China, respectively. Marzyme® 150 MG (*Rhizomucor miehei*, 662 SU/mg) and Chy-MAX® (recombinant chymosin, *Aspergillus niger* var. *awamori*, 310 SU/mg) were donated by DuPont Danisco (Vinay, France) and CHR. HANSEN A/S (Hørsholm, Denmark), respectively. All of the other chemicals used in the study were obtained from Sinopharm Chemical Reagent Co., Ltd., and were of analytical grade.

2.2. Maintenance of the culture and enzyme production

Paenibacillus spp. BD3526 was isolated from raw yak milk in Dangxiong country of Tibet and named *Paenibacillus bovis* sp. nov. The strain was previously deposited in the China General Microbiological Culture Collection Center (CGMCC No. 8333) and German Collection of Microorganisms and Cell Cultures (DSM No. 28815). The microorganism was maintained in a laboratory at −80 °C in lyophilized 10% (w/v) skim milk. Prior to use, the lyophilized powder was dissolved in 100 μL of sterilized water and streaked onto Tryptone Yeast Cystine (TYC) agar containing 15 g/L tryptone, 5 g/L yeast extract, 0.2 g/L l-cystine, 50 g/L sucrose, 20 g/L sodium acetate, 2 g/L sodium hydrogen carbonate, 2 g/L disodium hydrogen phosphate, 1 g/L sodium chloride, 0.1 g/L sodium sulfite, and 15 g/L agar at pH 7.2. The Petri dish was incubated at 30 °C for 48 h. The BD3526 colony that grew on the TYC agar was collected, inoculated in 20 mL of sterilized TYC broth in a 100 mL Erlenmeyer flask, and cultured on a rotary shaker at 30 °C at 180 r/min for 18 h. The culture broth was then used as inoculum (~5.0×10^8 CFU/mL) for the subsequent experiments.

Wheat bran broth (30 g/L) autoclaved at 121 °C for 20 min was used as the enzyme production medium. Enzyme production was carried out by flask fermentation with a 1.0% (v/v) inoculum size, followed by incubation on a rotatory shaker at 30 °C and 180 r/min for 72 h.

2.3. Enzyme preparation and purification

The fermentation broth was harvested by centrifugation at 14436 × *g* for 20 min at 4 °C. The supernatant was precipitated with ammonium sulfate at 20–80% saturation and centrifuged at 26916 × *g* for 15 min at 4 °C. The precipitate was suspended in 50 mL of 20 mM sodium phosphate buffer (Na_2HPO_4–KH_2PO_4, pH 7.0) and centrifuged at 26916 × *g* for 10 min at 4 °C to remove undissolvable materials. As the clear supernatant was a crude enzyme solution, it was dialyzed (14 kDa cutoff) against a 10 mM calcium chloride solution for at least 12 h to partially remove the residual ammonium sulfate. After the dialysis, the crude enzyme solution was lyophilized to powder for later purification.

The frozen crude enzyme powder was dissolved and subjected to DEAE Sepharose Fast Flow (GE Healthcare Bio-Sciences, Uppsala, Sweden) column (D 3.6 cm × 30 cm) that was previously equilibrated with 20 mM Tris–HCl buffer (pH 7.0). Elution was carried out with a linear gradient of NaCl from 0 to 0.75 M at a flow rate of 5 mL/min in 20 mM Tris–HCl buffer. The purified fractions showing enzyme activity were pooled, dialyzed, and lyophilized for further analyses.

For all the purification steps, the sample fractions were assayed for protein content and MCA. The protein concentrations were measured using the Lowry's method. The enzyme recovery and fold purification were calculated in terms of specific activity.

2.4. Identification of BD3526 MCE

The molecular weight of BD3526 MCE was determined as 35 kDa by sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE) and gelatin zymography in our previous study. The 35 kDa protein band obtained in SDS-PAGE was excised, subjected to in-gel digestion with trypsin. The resulting digests wedetermined by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (Applied Biosystems, Foster City, USA) were directly used for database search employing the MASCOT (Matrix Science, Inc., Boston, MA) peptide mass fingerprinting (PMF) search engine (http://www.matrixscience.com). Search parameters were set to 300 ppm and 0.9 Da error tolerance for MS and MS/MS spectra, respectively, with one missed trypsin cleavage allowance, and carbamidomethylation of cysteine and oxidation of methionine as fixed and variable amino acid modifications, respectively. The confidence threshold for protein identification was set to 95%.

Whole-genome sequencing of BD3526 (GenBank accession No. CP013023.1) was performed with a combined strategy of PacBio SMRT (single-molecule sequencing technology) and Illumina sequencing technology [17]. Gene prediction was performed in line with the predicted result of Glimmer 3.02. tRNA genes and rRNA genes were detected by tRNAScan, and RNAmmer, respectively. Gene function annotations were made through searching the NCBI non-redundant (NR), Kyoto Encyclopaedia of Genes and Genomes (KEGG) protein databases as well as the SEED protein databases. Clusters of orthologous group (COG) function classifications were performed using the NCBI Conserved Domains Database (CDD) [18].

2.5. Determination of the milk-clotting and proteolytic activity

The MCA was determined using the method described by Arima [19]. One Soxhlet unit (SU) of milk-clotting activity is defined as the amount of enzyme required to clot 1 mL of a substrate within 40 min at 35 °C. The MCA was calculated according to [20]: milk-clotting activity (SU) = $2400/T \times S/E$, where T is the time necessary for clot formation, S is the milk volume, and E is the enzyme volume.

The proteolytic activity (PA) was determined according to the casein digestion method described by [16]. One unit of PA was defined as the amount of enzyme that yielded a color equivalent to 1 μmol/mL of tyrosine per minute.

2.6. Effects of inhibitors, temperature, pH and calcium on enzyme activity

The effects of proteinase inhibitors were determined by incubating the BD3526 MCE with different concentrations of inhibitors solutions at 25 °C for 30 min. The type of group at the active site of the enzyme was determined with pepstain A, EDTA, PMSF, and 2-iodoacetamide inhibitor assays.

The MCA and PA at temperatures in the range of 35 °C–80 °C with 5 °C intervals were determined to investigate the optimal temperature of the BD3526 MCE. The thermostability of the MCE was measured by incubating the enzyme solutions in a water bath at 35 °C, 40 °C, 50, 55 °C, and 60 °C for 10, 20, 30, 40, and