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Purification and characterization of a milk-clotting aspartic protease from *Withania coagulans* fruit

Mahmoud Salehi [a], Mahmoud Reza Aghamaali [a,*], Reza H. Sajedi [b], S. Mohsen Asghari [a], Eisa Jorjani [c]

[a] Department of Biology, Faculty of Science, University of Guilan, Rasht, Iran
[b] Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran
[c] Department of Biology, Faculty of Science and Engineering, Gonbad kavous University, Gonbad Kavous, Iran

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ABSTRACT

Withania coagulans fruit has traditionally been used as milk coagulant. The present study reports the purification and characterization of an aspartic protease from *W. coagulans* fruit. The enzyme was purified *via* fractional ammonium sulfate precipitation and cation exchange chromatography. SDS-PAGE analysis revealed the presence of a monomeric protein with molecular weight of 31 kDa. Proteolytic activity (PA) of the protease was evaluated using casein, and the milk-clotting activity (MCA) was analyzed by skim milk. The K_m and V_{max} values of the enzyme for casein were obtained to be 1.29 mg/ml and 0.035 μmol Tyr/min, respectively. Optimal temperature and pH were 65 °C and 5.5, respectively. After incubation of enzyme at 65 °C for 1 h, 73% of PA was remained which demonstrated high thermal stability of the enzyme. Mass spectrometry analysis of the purified protease and enzyme assays in the presence of protease inhibitors indicated that aspartic protease was the only responsible enzyme in milk coagulation. Furthermore, by investigating the effect of salts on enzyme activity, it was observed that both NaCl and $CaCl_2$ reduced enzyme activity. These characteristics of the protease suggest that the enzyme may be suitable for producing low salt content cheeses.

1. Introduction

Aspartic proteases (APs; EC 3.4.23.-), generally known as acid proteases, have been obtained from a variety of organisms including plants and animals. These enzymes have two aspartic acid residues at their active sites which can optimally be activated at acidic pH values and they are specifically inhibited by pepstatin A [1]. Amino acid sequences of aspartic proteases show high level of homology and three-dimensional structure of these enzymes have been preserved [2,3]. Plant aspartic proteases mostly present in seeds and they participate in storage protein degradation during ripening and seed germination [4]. Furthermore, they might have role in stress responses. Plant aspartic proteases are found in both heterodimeric and monomeric forms which present in vacuoles and extracellular spaces, respectively [5]. Aspartic proteases are helpful enzymes in food industry due to their high activity and stability in acidic conditions, for example aspartic proteases are utilized as milk coagulating enzymes in cheese production. Coagulation of milk is related to the hydrolysis of κ-casein by enzymes destabilizing casein micelles [6]. Chymosin (EC 3.4.23.4), the main component of calf rennet, is the best and most applicable milk clotting enzyme. However, The increasing price of calf rennet and growing demand for cheese have led to extensive investigations to find new rennet substitutes [7]. Substitutes with milk coagulating ability should present some of calf rennet specific properties like high ratio of milk clotting activity to PA and suitable thermal stability. It has been proven that microbial rennets obtained from genetically engineered bacteria are appropriate substitutes for calf rennet, but too much attention has been paid to natural plant rennets with favorable properties [8]. Traditionally, in some regions such as Mediterranean, West African and Southern European countries, plant extracts have been used as milk coagulating agent in cheese production. Some plants of the family Solanaceae such as *Solanum innacum*, *S. dubium* Fresen and *W. coagulans Dunal* have been utilized as sources of milk-clotting enzymes [9]. Shah et al. (2014) reviewed plant proteases as milk-clotting enzymes in cheese production [10]. Yegin and Dekker (2013) also reviewed the structural and biochemical properties of aspartic proteases from animal, plant and microbial origin [11]. However most plant coagulants have excessive PA which leads to poor cheese yield, bitter

* Corresponding author.
E-mail address: aghamaali@guilan.ac.ir (M.R. Aghamaali).

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taste and defects in cheese texture [12]. Therefore, many investigations are required to find efficient plant milk-clotting enzymes through which the various and high quality cheese can be produced [13]. *W. coagulans* (Solanacea) is a small shrub which grows widely in Pakistan, Afghanistan, India and Iran. The fruits of this plant have been widely employed for decades in manufacturing of traditional cheese from raw cow's milk. The milk coagulation property is related to the berry's pulp and husk, and it is assumed that the milk coagulation ability corresponds to existence of an enzyme in berries of the plant [14,15]. Fruit extracts of *W. coagulans* was suggested as a good substitute for animal rennet in manufacturing of cottage and Cheddar cheese, although Cheddar cheese produced using the mentioned extract give a sensible bitter taste, but by extending the ripening period the bitter taste could be decreased [16]. However, there are few studies about the biochemical characteristics of purified milk-clotting enzyme extracted from *W. coagulans*. Formerly, a monomeric 66 kDa aspartic protease with maximum milk clotting activity at pH 4.25 and 37 °C extracted from *W. coagulans* fruit is reported [17]. In a previous work, milk clotting properties of crude extract of *W. coagulans* fruits was investigated and some properties like the best extraction buffer solution, effect of temperature and salt concentration on milk coagulation time were determined and molecular weight of enzyme was estimated to be 25–35 kDa [18]. In the present study, the purification of aspartic protease enzyme from *W. coagulans* fruit is described and the properties of the purified enzyme for its application in cheese industry are characterized. It is shown that the activity in the purified preparation is associated with a monomeric aspartic protease with molecular weight of approximately 31 kDa.

2. Materials and methods

2.1. Plant material

W. coagulans (Eudicots, Asterids, Order Solanales, Family Solanaceae) has the vernacular name "paneer bad" in Iran. Sun dried fruits were purchased from a local grocery at Balochestan, east Iran.

2.2. Chemicals

Bovine serum albumin (BSA), E-64, EDTA, glycine, skim milk powder, pepstatin A, PMSF, β-mercaptoethanol and dithiothreitol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Casein was provided from Merck (Darmstadt, Germany). Coomassie Brilliant Blue R-250 and UNOsphere™ S cation exchange resin were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All the other chemicals were of analytical grade and used without further purification.

2.3. Enzyme extraction

Dried fruits of *W. coagulans* were grounded to a fine powder under liquid nitrogen in a mortar. The obtained powder was mixed with 50 mM Tris–HCl buffer (pH 8.0, containing 0.85% NaCl (w/v)), with ratio of 1 g per 5 ml, and the mixture was homogenized for 1 h at 4 °C. The homogenized mixture was centrifuged at 9000 rpm for 15 min at 4 °C, and the resultant supernatant was passed through filter paper to remove suspended particles. Crude extract was kept at 4 °C for further analysis and it was either used at the day of preparation. MCA, PA and the protein concentration were determined for the obtained enzyme extract.

2.4. Enzyme purification

2.4.1. Ammonium sulfate fractionation

The prepared solution was brought to 20% saturation (w/v) with a gradual addition of solid ammonium sulfate at 4 °C and then put on ice for 30 min. The resulting precipitate was removed by centrifugation at 9000 rpm for 20 min at 4 °C and re-suspended in 50 mM tris-HCl buffer, pH 8.0. This step was repeated twice to obtain 50% and 80% saturation, respectively. The protein solutions from each step were dialyzed against 50 mM Tris-HCl buffer (pH 8.0) three times for 6 h and the samples were centrifuged as previously described. Thereafter, MCA, PA as well as protein concentration was measured for each fraction. The fraction with good activity and quantity was used in the next step.

2.4.2. pH treatment

The enzyme was purified further and pH treatment was applied for it to be prepared for ion exchange chromatography. Briefly, the pH of the extract was adjusted to 4.5 by addition of 50 mM sodium acetate buffer. After incubation for 15 min at 4 °C, resultant mixture was centrifuged at above condition to eliminate insoluble proteins.

2.4.3. Cation exchange chromatography

The resultant extract was applied to UNOsphere™ S column (1.5 × 20 cm), formerly equilibrated with 20 mM sodium acetate buffer (pH 4.5). To remove unbound proteins, the column was washed with 40 ml of the same buffer, and then the bound proteins were eluted with isocratic steps at 0–0.2, 0.2–0.3, 0.3–0.4, 0.4–0.6 and 0.6–1.0 M of NaCl in 20 mM sodium acetate buffer (pH 4.5). The collected fractions were monitored at 280 nm and assayed for protease activity. Individual fractions under each peak which showed the highest level of protease activity were pooled and dialyzed with a 12-kDa cut-off dialysis membrane against 20 mM sodium acetate buffer (pH 4.5), twice at 3-h intervals, separately. These fractions were concentrated several times through a 3 kDa cut-off cellulose acetate membrane (Millipore, Molsheim, France). Aliquots were frozen at −20 °C, and specific activities of the final pools were determined. The purification process was carried out at 4 °C. The protein concentrations were measured by Bradford method [19] using bovine serum albumin as standard. All protein samples collected from the extraction and purification steps were analyzed by SDS-PAGE according to the procedure of Laemmli [20], under reducing conditions, with 12.5% acrylamide separating gel.

2.5. Mass spectrometry

Protein bands corresponding to the purified protease were cut out from the gel and they were digested in-gel by trypsin and the process was followed by post reduction and alkylation. The obtained peptides were analyzed by Matrix Assisted Laser Desorption Ionization Tandem Time-of-Flight mass spectrometry (Bruker ultraflex III MALDI-TOF/TOF). A mass spectrum was acquired between 800 and 5000 m/z and the ten strongest peaks, above a signal to noise threshold, were selected for MS/MS fragmentation. The produced tandem mass spectral data were searched against the NCBI non-redundant protein database using the Mascot search program (URL http://www.matrixscience.com) to generate peptide which were matched with associated expect values. The proteins were identified from peptide matches. The probability-based MOWSE score was calculated as 10 Log (P), where P illustrated the possibility of the observed match in a random occurrence. Protein scores more than 56 considered significant ($p < 0.05$).