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Three phase partitioning to concentrate milk clotting proteases from *Wrightia tinctoria* R. Br and its characterization

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ABSTRACT

Wrightia tinctoria stem proteases were partially purified for the first time through a non-chromatographic technique, three phase partitioning (TPP), to concentrate the milk clotting proteases. Various parameters like salt and solvent concentration that affect the partitioning of the protease were examined. Maximum recovery and purification fold of the protease activity were found in the interfacial phase (IP) with 60% ammonium sulphate and 1:1 crude enzyme to *t*-butanol. Optimum pH and temperature of the enzyme fraction were found to be 7.5 and 50 °C respectively. Inhibition studies revealed its serine nature. Non-denaturing PAGE, Zymography and 2D PAGE of IP revealed presence of three different caseinolytic proteases of molecular weights 95.62 kDa, 91.11 kDa and 83.23 kDa with p*I* 3.89, 5.45 and 5.43 respectively. Both aqueous and lyophilized form of IP were remarkably stable retaining complete activity at 4 °C for 3 weeks. Electrophoretic analysis of casein hydrolysate by IP at different incubation time indicated a time dependent substrate subunit specificity with hydrolysis of κ-casein commencing after 10 min followed by α and β caseins. This pattern was found similar to that by commercial vegetable coagulant, Enzeco®. Study details the effectiveness of TPP concentrated *W. tinctoria* proteases as a vegetable coagulant alternative in cheese making.

1. Introduction

Biotechnological advantages of proteases (EC 3.4.x.x) brand them as industrially important enzymes especially in food biotechnology. Cheese making is considered as an earliest known application of proteases. Coagulation of milk remains an elementary step in manufacturing cheese and is generally achieved by adding rennet (chymosin EC 3.4.23.4). Chymosin is the milk clotting enzyme obtained from young unweaned calf's stomach contents. Increased worldwide cheese demand along with many shortcomings associated with rennet including insufficient supply and consumer acceptability has led to the search for its alternatives from plant and microbe. Currently, around 90% of cheese produced is using genetically engineered chymosin/fermentation produced chymosin however with an escalated cost [1]. For these reasons, coagulants sourced from plants with aim of producing vegetarian cheese have become an emerging subject of interest in dairy technology.

Milk clotting proteases from plants have been extracted using different protocols such as extraction with aqueous, buffers (pH ranging from 3 to 8 and 0.05–0.2 molarity), sodium chloride solution either individually or in combination [2–8]. Traditionally vegetable coagulants from thistle of *Cynara cardunculus* is extracted in water [9]. Plant proteases are more advantageous owing to their comfort of accessibility and handling, enzyme extraction procedures and heightened consumer demand for alternate natural sources [10]. In addition, its use rises cheese acceptability by the lactovegetarian population and has an added advantage of enhancing their nutritional status. History of artisanal cheese preparation reveals usage of several plant extracts as milk coagulants even with their elevated caseinolytic activity [3, 4, 11]. Cardosins from *Cynara cardunculus* is one such example that have reached to the level of commercial cheese production for their characteristic flavour and texture [12]. An assortment of cheese manufactured from different milk sources using these plant proteases are available globally with Spain and Portugal contributing largely to different varieties [3, 9, 13].

Inhabitants of Kolli Hills in Southern and Eastern Ghats of India use extracts from *Wrightia tinctoria*, a deciduous tree of ethnomedical importance from Apocynaceae family, for local cheese preparations [14, 15]. However, no scientific reports on validation of their milk clotting potential are available till date to our knowledge. Wrightin, a latex protease from *W. tinctoria* has been purified using conventional chromatographic techniques and is established for its pharmaceutical applications [16]. Nevertheless, no reports investigating its milk clotting ability are available till date. In the present study, proteases from tender *W. tinctoria* stem were concentrated using three phase partitioning (TPP). TPP is a cost-effective bio separation method alternative to conventional chromatography based purification techniques that involves

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arduous and expensive steps along with scaling up difficulties for industrial production [17–19]. Principle behind this technique involves collective operations like isoionic, cosolvent, osmolytic and kosmotropic precipitation along with salting out of proteins [17]. In addition, TPP concentrates enzymes/proteins selectively in one phase and partitions other contaminants in another phase. This enhances recovery and purification along with ease of scaling down or up to semi- micro or litre levels respectively. Concentration of ammonium sulphate and ratio of crude enzyme (CE) to *t*-butanol has been experimentally proven to influence the degree of enzyme purification and activity recovery [20]. The current study describes for the first time the use of TPP for concentration of proteases from *W. tinctoria* stem crude extract for its milk clotting property using single step partitioning process. The effect of solvent concentration and salt saturation on recovery of protease activity has been studied. TPP derived protease fractions were characterized and assessed for their milk clotting potential through their ability to hydrolyze caseins. Stability of the enzyme fraction along with its lyophilized portion stored at 4 °C were also investigated for their enzyme activity and for their casein affinity for a period of 3 weeks.

2. Materials and methods

2.1. Plant source

Authenticated *W. tinctoria* R.Br. (Authentication no: 64) tender stem was collected from Sanjeevani Vatika, Department of Horticulture, Gandhi Krishi Vigyana Kendra (GKVK), Hebbal, Bangalore in the morning and processed the same day.

2.2. Chemicals

Skimmed milk powder, ammonium sulphate, *t*-butanol, trichloro acetic acid, casein, disodium hydrogen phosphate, citric acid, tricine, acrylamide, bis-acrylamide, calcium chloride, bovine serum albumin, rennin, dialysis membrane, trisodium citrate and tris base were procured from HiMedia laboratories Pvt. Ltd., Mumbai, India. FC reagent, sodium carbonate and tricine were purchased from Merck, India. S29534-Enzeco®, commercial plant coagulant was a kind gift from Enzyme development corporation, New York. All chemicals procured were of analytical grade.

2.3. Three phase partition (TPP)

Tender stem (5 g) of *W. tinctoria* was washed and homogenized in 25 ml of 0.05 M pH 5.5 sodium citrate buffer and filtered through muslin cloth. Filtrate was centrifuged for 20 min at 12138*g* at 4 °C. The supernatant was preserved as CE. CE was dialyzed overnight in cold against the same buffer. The dialyzed enzyme was subjected to TPP according to Gagaoua, with mild modifications [21]. Briefly, the dialyzed enzyme was saturated with 60% ammonium sulphate at room temperature (RT), 1:1 ratio of *t*-butanol was added, gently vortexed for few min and allowed to stand at RT for 1 h to enable three phase separations into upper butanol phase, middle interfacial phase (IP) and lower aqueous phase (AP). The upper phase was discarded using Pasteur pipette and IP and AP were carefully removed separately and IP was dissolved in minimum quantity of 0.05 M sodium citrate buffer (pH 5.5). The separated fractions were analyzed for their activity and protein content. The IP fraction was stored at −20 °C for further studies.

The effect of ammonium sulphate concentration (30, 40, 50, 60 and 70%) for TPP with constant 1:1 ratio of CE to *t*-butanol was explored. Effect of solvent, i.e., ratio between CE to *t*-butanol in range 1:0.5, 1:1, 1:1.5 and 1:2 also were investigated with constant 60% ammonium sulphate saturation. IP fraction with optimized parameters were used for further analysis.

2.4. Enzyme activity

Enzyme activity of the fractions and CE were assessed by their caseinolytic and milk clotting activity.

2.4.1. Caseinolytic activity (CA)

CA of the fractions and CE were analyzed using 1% (w/v) bovine casein dissolved in 0.05 M citrate phosphate buffer (pH 7.5) as substrate. Equal volumes (1 ml) of suitably diluted enzyme and substrate were incubated at RT for 1 h, followed by addition of 3 ml of 10% tri chloro acetic acid to stop the reaction and incubated for 1 h in ice. The mixture was centrifuged at 2688*g* at RT for 15 min. Amount of amino acid liberated was determined by measuring the absorbance at 280 nm using tyrosine as the standard spectrophotometrically (UV- Vis Spectrophotometer, Systronics double beam 2202, India). One unit of caseinolytic activity was defined as the amount of the enzyme that liberated 1 μg of tyrosine under the standard assay conditions [22].

$$\mathrm{CA}\left(\frac{\mathrm{U}}{\mathrm{ml}}\right) = \text{Tyrosine } (\mu\mathrm{g}) \times \left(\frac{\text{Dilution factor}}{\text{Volume of enzyme}}\right) \times \left(\frac{\text{Total volume}}{\text{Time of incubation}}\right)$$

2.4.2. Milk clotting activity (MCA)

MCA was evaluated for fractions along with CE in accordance to Arima, with slight modifications [23]. The substrate was dissolved in 0.01 M calcium chloride solution. 2 ml of substrate was incubated at 37 °C for 5 min followed by addition of 200 μl enzyme. MCA was calculated in Soxhlet units. The tubes were rotated at regular intervals and time taken for micellar formation was recorded.

$$\mathrm{MCA}\left(\frac{\mathrm{U}}{\mathrm{ml}}\right) = \frac{[2400 \times \text{volume of substrate (ml)}]}{\text{Time taken (s)}}$$

One milk-clotting unit was defined as the amount of enzyme that clots 10 ml of the substrate within 40 min (2400 s) at 37 °C.

2.5. Protein estimation

Lowry's method was used to estimate protein content [24]. Different volumes (100 μl–500 μl) of IP fraction was diluted to 1 ml with distilled water. 5 ml of alkaline copper solution was added, mixed thoroughly and allowed to stand at RT for 15 min. 0.5 ml of dilute (1:1) folin's reagent was added and absorbance at OD 660 nm was read after 30 min. BSA was used for calibration in the range of 40–200 μg/ml.

2.6. Milk clotting index (MCI)

MCI was calculated using the following formula [25].

$$\mathrm{MCI} = \mathrm{MCA} : \mathrm{CA}$$

2.7. Effect of pH and temperature on TPP - purified enzyme activity

Enzymes were incubated with 1% (w/v) casein dissolved in buffers of different pH [0.05 M sodium citrate (pH 4, 4.5, 5, 5.5), 0.05 M citrate phosphate (pH 6, 6.5, 7), 0.05 M Tris-HCl (pH 7.5, 8)] and at various temperature (30, 40, 50, 60, 70, 80 °C) following which their caseinolytic activity was evaluated as described earlier.

2.8. Inhibition studies

Effect of different protease inhibitors on the caseinolytic activity of TPP-purified enzyme was analyzed to identify the nature of protease.