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PARTIAL PURIFICATION OF CYSTEINE PROTEASE FROM *NIGELLA SATIVA* L SEEDS

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ABSTRACT: Plant proteases are playing the major role in the dairy industry; studies on related enzymes are still needed due to their application in the cheese making process. Here we report the purification and characterization of the cysteine protease from *Nigella sativa L* (Ranunculaceae). The purified protease was characterized as a cysteine protease by inhibitor study it was confirmed through Zymogram assay. The maximum activity of the enzyme was observed at pH 6.5 and temperature optimum at 50°C. The enzyme activity was stimulated by the Fe^{2+} ions and enzyme activity inhibited by Mg^{2+}, Mn^{2+}, $Ca2^{+}$, Cu^{2+} and Ni^{2+} ions. The enzyme was stable in NP-40 compare to Triton X-100 and SDS. The purified protease has the ability to coagulate milk, based on the results that the purified protease may have the potential application in the dairy industry.

KEYWORDS: Cysteine Protease, Enzyme extraction, Protein purification, *Nigella sativa*, milk clotting activity.

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1. INTRODUCTION

Proteolysis is the prime bio-catalytic property ascribed to a class of enzymes called proteases. Proteolysis is the hydrolysis of the peptide bond by attacking the carbonyl group of the peptide. In humans, nearly 990 known protease genes were reported. In addition, about 1605 known protease inhibitor genes have been reported in human [1]. Based on the amino acid present at the active site of the protease and the mechanism of the peptide bond cleavage, proteases can be classified into

six groups: cysteine (Cys), serine (Ser), threonine (Thr), glutamic acid (Glu), aspartate (Asp) proteases and matrix metalloproteases [2] [3] [4]. Ser, Cys and Thr proteases act directly as nucleophiles that attack an amide carbonyl C, whereas Asp, Glu and metalloproteases activate a water molecule that then acts as a nucleophile. The enzymes are also classified into exopeptidases and endopeptidases by the position of the peptide bond in a protein they cleave [5]. Plant proteases are involved in plant physiology and development process. They play a major role in processes such as protein turnover, degradation of mis-folded proteins, senescence and the ubiquitin/proteasome pathway [6]. It is also responsible for the post-translational modification of proteins by limited proteolysis at highly specific sites. They are involved in diverse cellular processes, including defense mechanisms, photo-inhibition in the chloroplast and photomorphogenesis in the developing seedling and programmed cell death. Proteases are implicated in all aspects of the plant life cycle ranging from mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs [7]. The study reveals that the protease inhibition activity in the naturally abundant of succinic acid both trypsin and papain. Succinic acid may be a good anti-viral agent in the future with fewer side effects [8]. *Nigella sativa L* (Ranunculaceae) has been used as a traditional medicine for many years. It is used to treat many diseases. *Nigella sativa* (*N. sativa*) L is an annual flowering plant, which is grown and used as an herbal medicine all over the world and is native to South and Southwest Asia [9]. It is an annual grassy plant with green to blue colored flowers and black trigonal seeds. The seeds are the main source of the active compounds of the plants [10]. This plant is known all over the world by different common (folkloric) names, such as Habbat al-barakah in Arabic, Siah-Daneh in Persian, and black cumin or black seed in English. Based on historical records, this plant was known as far back as 1400 years ago and its seeds were extensively used for flavor [11]. In Unani traditional medicine, *N. sativa* is considered as an herbal medicine for a number of diseases. *N. sativa*, as an herbal medicine with a rich historical background, has been traditionally used in the treatment of several diseases, such as infertility, fever, cough, bronchitis, asthma, chronic headache, migraine, dizziness, chest congestion, paralysis, hemiplegia, back pain, dysmenorrhea, obesity, diabetes, infection and inflammation, rheumatism, hypertension, and gastrointestinal disorders such as flatulence, dyspepsia, diarrhea, and dysentery [12] [13] [14]. Initially, the chemical components of *N.sativa* seeds were reported to contain mainly oils, proteins, carbohydrates, ashes, fibers and moisturizers [15]. The plant contains pharmacologically active components such as nigellicine, nigellidine, thymoquinone (TQ), dithymoquinone, thymol, and carvacrol [16].

2. MATERIALS AND METHODS

Extraction of *Nigella sativa* seed protein:

The *Nigella sativa* plant seeds were purchased from traditional medicine shop and washed with distilled water and air dried. Seeds were ground and taken to a fine powder using laboratory mixer. The fine powder was defatted and depigmented with 1: 3 ratios of ice cold acetone and hexane, filtered and air dried at room temperature. The fine defatted powder was stirred with five volumes of 20 mM sodium phosphate buffer, pH 6.5 overnight in a shaker incubator at 20°C. The solution was centrifuged at 10,000 rpm and the supernatant solution was collected and stored at -20°C for further analysis.

Protease and protease inhibitor assay

Casein agar plate method

The supernatant solution was used for protease assay using casein agar plate method [17]. The agar was prepared along with 1 % (w/v) casein autoclaved and poured onto petri dishes. The plates were allowed to solidify, after which the wells were punched on the agar according to the number of samples. Samples were heated at 70°C for 15 minutes. Samples were loaded and marked on the corresponding wells. These plates were incubated overnight at 37°C.

Ammonium sulfate precipitation

The crude protein solution was fractionated by ammonium sulfate precipitation [18] at different concentrations (0-60% and 60-90%). The ammonium sulfate was added slowly from 0-60% (w/v) saturation to the crude extract while keeping on ice with gentle stirring. After complete dissolution of ammonium sulfate, the mixture was kept at 4°C to allow overnight precipitation. Precipitated protein was collected by centrifugation at 10,000 rpm for 30 minutes at 4°C.

Dialysis

The precipitate obtained after prolonged precipitation was dissolved in 20 mM Sodium Phosphate, pH 6.5 and dialyzed against the same buffer using 10 kDa cut off membrane at 4°C overnight and further for 4 hours against fresh buffer.

Proteolytic assay (UV method)

The enzyme activity was measured using UV spectroscopy with slight modifications of the method described by Beynon and Bond, 1989 [19]. A volume of 100 µl of sample was added to an equal volume of 1 % casein (w/v) and incubated at 37°C. After 30 minutes, the reaction was stopped by adding 200 µl of 10 % ice-cold TCA and incubated for another 30 minutes. This is followed by centrifugation at 10,000 rpm for 30 minutes at 4°C. The clear supernatant in which the lytic fragments of casein were expected was analyzed for its absorption at UV-280 nm. As described, the proteolytic activity for the ammonium sulfate fractions, crude was analyzed by UV method with positive (Papain) and negative (Buffer) controls.

Purification by ion exchange chromatography

Ion exchange chromatography was carried out using weak anionic DEAE FF 1ml (GE) column for the active fraction (0-60%). The column was pre-equilibrated with 20 Column volumes (CV) of each buffer, Buffer A [20 mM Sodium Phosphate pH 6.5] and Buffer B [20 mM Sodium Phosphate pH 6.5, 1 M NaCl] at the flow rate of 1ml/min. The fractionated samples were clarified (12,000 rpm 30 minutes at 4°C) before loading onto the column. Unbound fractions were washed with 20 CV buffer A and bound proteins were collected in 40 CV by buffer B at the flow rate 1ml/min using a linear salt gradient. All the fractions were analyzed for their enzymatic activity and the purity of each fraction was checked by 12% SDS-PAGE with 20-200 kDa protein markers.

Characterization of the purified enzyme from *N. sativa* seeds

Protease inhibition analysis

The purified protein was analyzed for its proteolytic activity by radial diffusion method [17]. For which iodoacetic acid (IAA) [Cysteine protease inhibitor] and phenylmethylsulfonyl fluoride (PMSF) [Serine protease inhibitor] were used to characterize the nature of the protease. IAA was dissolved in water while PMSF in 10% ethanol. The purified protease was incubated with 2 mM IAA and 2 mM PMSF in different vials and residual proteolytic activity was measured using the radial diffusion method at 37°C overnight.

Effect of metal ions

Protease activity was measured by Anson, 1938 [20], Beynon & Bond, 1989 method [19]. The reaction mixture containing 40µl of the purified enzyme, an equal volume of 1% casein solution, pH 6.5 and 5mM concentration of metal ions (Ca^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+} and Ni^{2+}) were incubated at 37°C for 30 minutes, the reaction was stopped by adding 80µl of 10% trichloroacetic acid and the mixture was incubated at room temperature for 30 minutes. After incubation, the reaction mixture was centrifuged at 10,000 rpm for 15 minutes and the absorbance of the supernatant was read at 280 nm. A control assay of the enzyme activity was done without inhibitors and resulting activity was considered as 100 %. The effect of each agent was determined by measuring the enzyme activity using the casein digestion method.

Application of the protease as a detergent additive

Stability of the protease was examined in the presence of different surfactants (SDS, Triton X-100 and NP 40) three different concentrations 50mM, 100mm and 150mM respectively. The enzyme was incubated for 30 min at 37°C with those detergents and the residual protease activity was measured as described earlier.

Effect of pH and temperature on proteolytic activity

To determine the effect of pH on the enzyme, samples were buffer exchanged with different buffers of varying pH (20 mM Sodium acetate, pH 4.5 & 5.5); 20 mM Sodium phosphate 6.5 & 7.5; 20 mM Tris pH (8.5 & 9.5) by dialyzing for 12 hours at 4°C. The enzyme activity was

analyzed as described earlier. The effect of temperature of the enzyme activity was determined by incubating the enzyme at different temperatures 20°C, 40°C, 60°C and 80°C for 30 minutes. The caseinolytic activity of the enzyme was observed as a zone of digestion.

Zymogram

Zymography was carried out at 4°C using 1% casein as substrate, copolymerized with the polyacrylamide gel. After running SDS-PAGE at 50 mV, the gel was washed twice with 50 ml of re-naturation buffer (2.5 % Triton X-100) for 2 hours to remove the SDS. Followed by this, the gel was incubated overnight at 37°C in 50 mM Tris buffer, pH 6.5 containing 50 mM NaCl and 10 mM $CaCl_2$. The gel was stained with Coomassie Brilliant Blue G-250. The appearance of the clear, colorless zone upon blue background depicted the proteolytic activity of the enzyme [21].

Milk–clotting activity

Milk-clotting activity was measured by diluting [22] 1 ml of skimmed milk in 9 ml solution containing 10 mM $CaCl_2$ and 10 mM $MnSO_4$ and the pH was adjusted to 6.5. Then, a volume of 200 µl of the sample was mixed with the same solution, incubated at 37°C for 10 minutes and the clotting activity was observed.

3. RESULTS AND DISCUSSION

The seeds of *N. sativa* were collected and extracted using 20mM sodium phosphate buffer (pH 6.5). The crude protein extract fractionated using ammonium sulfate and fractions were used for further analysis. The dialyzed fractions of 0-60% and 60-90% were analyzed for their proteolytic activity using casein-agar plate method. The results showed proteolytic activity in the crude (Figure 1. Plate 1) and 0-60% ammonium sulfate fraction (Figure 1. Plate 2). The 0-60% fraction showed better activity when compared to the crude or the other fractions. When the enzyme activity of the crude and fractions were analyzed by UV method, it was evident that the activity of the 0-60% fraction corresponds to the activity of papain (Figure 2). Based on the result, the same fraction was used for further purification by ion exchange chromatography. The fractions were analyzed for their enzymatic activity and the purity of each fraction was checked by 12% SDS-PAGE. The bound fraction is in its purified form and shows protease activity. (Figure 3&4). The molecular weight of the purified protein was calculated as 20kDa approximately.

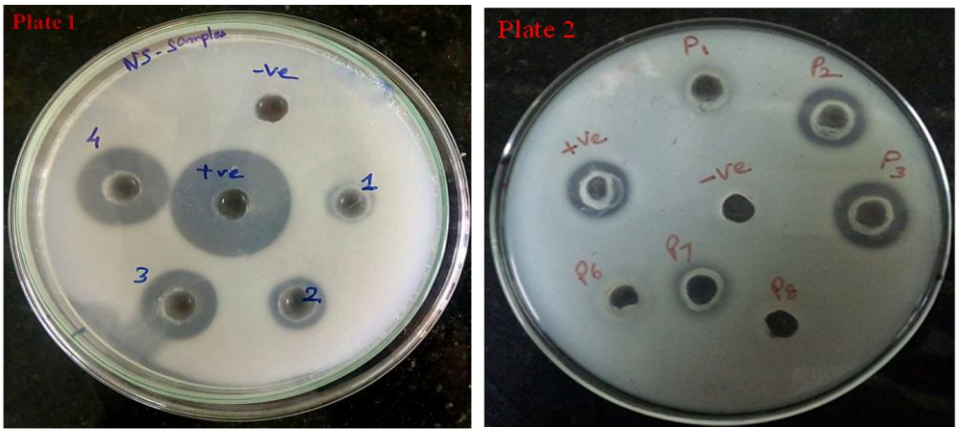


Figure 1: Protease activity plates. **Plate. I.** 1. Pre heated crude sample 40µl, 2. Pre heated crude sample 60µl, 3. Crude sample 40µl, 4. Crude sample 60µl, +Ve. Papain, -Ve. Tris buffer. **Plate II.** P1. 0-60% fraction 20µl, P2.0-60% fraction 40µl, P3. 0-60% fraction 60µl, +Ve. Papain, -Ve. Tris buffer, P6. 60-90% fraction 20µl, P7. 60-90% fraction 40µl, P8. 60-90% fraction 60µl.

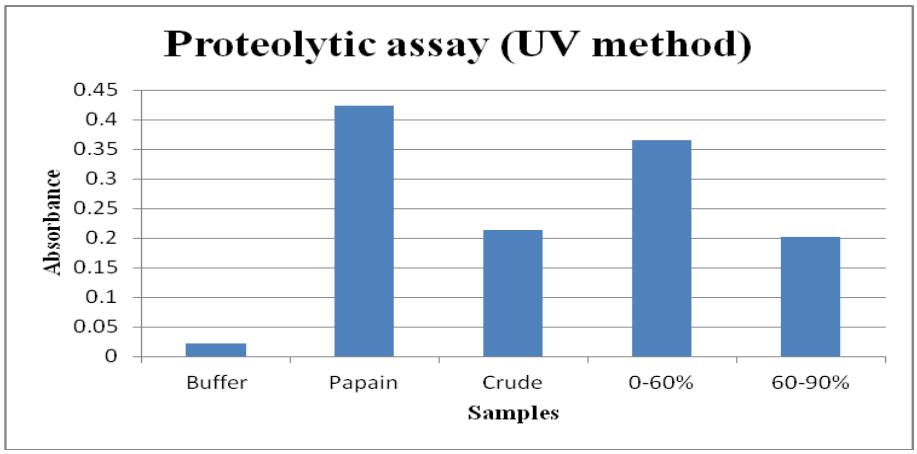


Figure 2: Protease activity of the crude sample and fractions compared with Papain by UV method

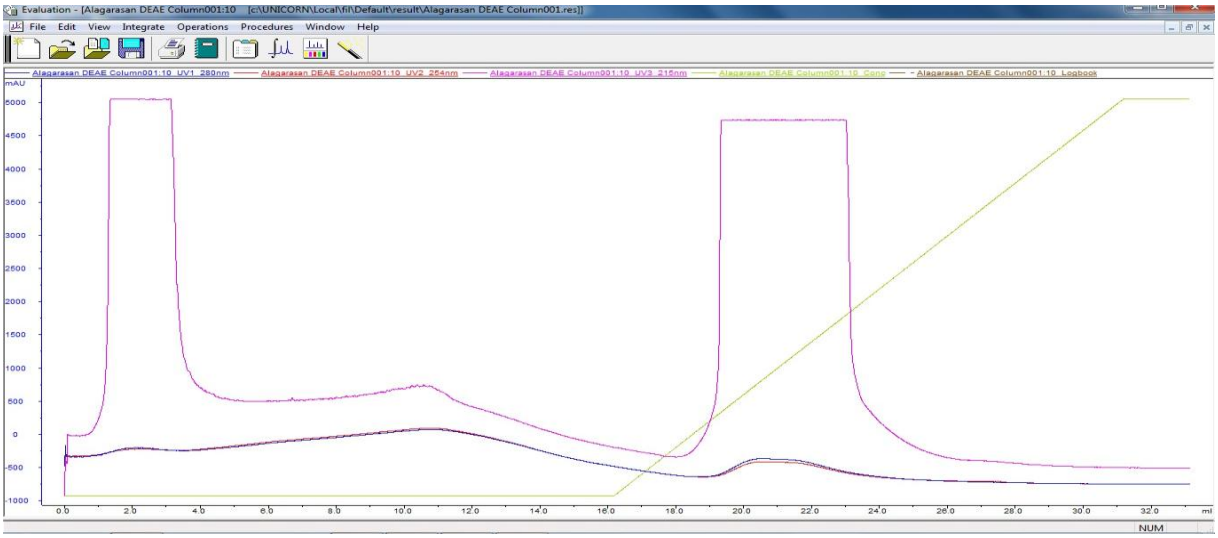


Figure 3: Ion Exchange Chromatography of the 0-60% fraction in DEAE Sepharose column

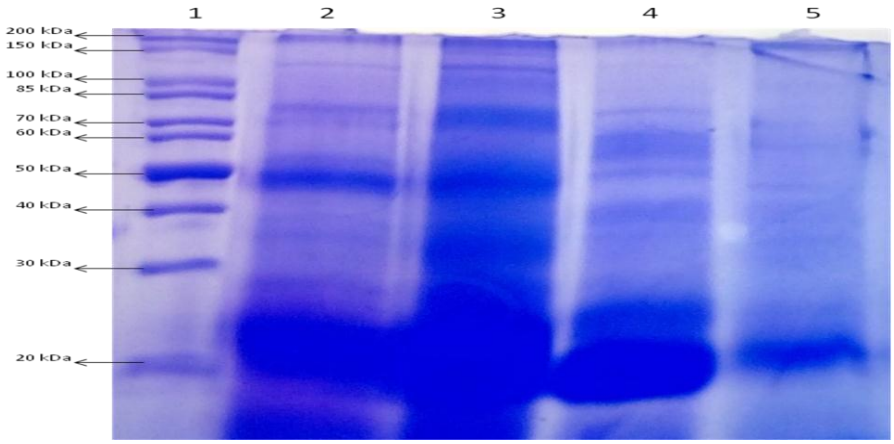


Figure 4: 12% SDS PAGE. Lane 1. 20 – 200kDa marker, lane 2. Crude sample, lane 3. 0-60% fraction, lane 4. 60-90% fraction, lane 5. Purified protease.

Characterization of the purified enzyme from *N. Sativa* seeds

Protease inhibition analysis

The purified protease showed the effect against IAA as a cysteine alkylating agent. The proteolytic activity of the purified protease was completely arrested by IAA and this indicated that this protease belongs to the family of cysteine proteases (Figure 5. Plate1). A clear zone of digestion in the presence of PMSF confirmed that the purified protease is not a serine protease (Figure 5. Plate 2). Similar inhibition studies are reported in the cysteine proteases. Such as ginger proteases P1 and P2 as a rennin replacement [23], highly stable ervatamin [24]

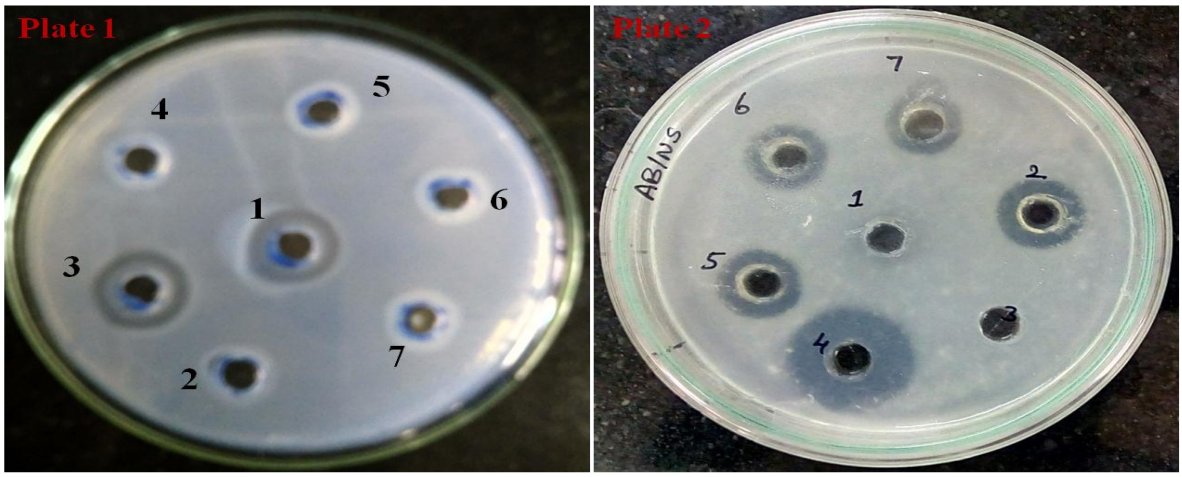


Figure 5: Characterization of the purified protease. Plate 1. 1. +ve 5µg Papain, 2. –ve Tris Buffer, 3. 40 µl of purified protease, 4. 40 µl of purified protease + 10 µg IAA, 5. 40 µl of purified protease + 20 µg IAA, 6. 40 µl of purified protease + 40 µg IAA, 7. Papain + IAA. Plate 2. 1. Buffer (Negative Control), 2. 40 µl of purified protease, 3. Trypsin + IAA, 4. +ve 5µg Trypsin, 5. 40 µl of purified protease + 10 µg PMSF, 6. 40 µl of purified protease + 20 µg PMSF, 7. 40 µl of purified protease + 40 µg PMSF.

Effect of metal ions

The effect of metal ion activity of the purified enzyme was measured in the presence of metal ions at the concentration of 5mM. Based on the results, activities of enzymes were stimulated in the presence of Fe^{2+} (Relative enzyme activity 157%) ions (Figure 6). It may because of the allosteric regulation. The strong inhibitory effect was observed in the presence of Mg^{2+}, Mn^{2+}, $Ca2^{+}$, Cu^{2+} and Ni^{2+}. It may be the conformational changes in the enzyme. The study reported purified ginger protease strongly inhibited by the metal ions, such as Hg^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+} [25].

Detergent stability

The effect of detergent stability on the activity of the purified enzyme was measured in the presence of different detergents and at different concentrations (50mM, 100mM and 150mM). Based on the results activities of enzymes were stable in the presence of NP-40 in all the concentrations. (Figure 7). The study was reported cysteine protease of *Euphorbia nivulia* latex excellent stability and compatibility on different detergents [26].

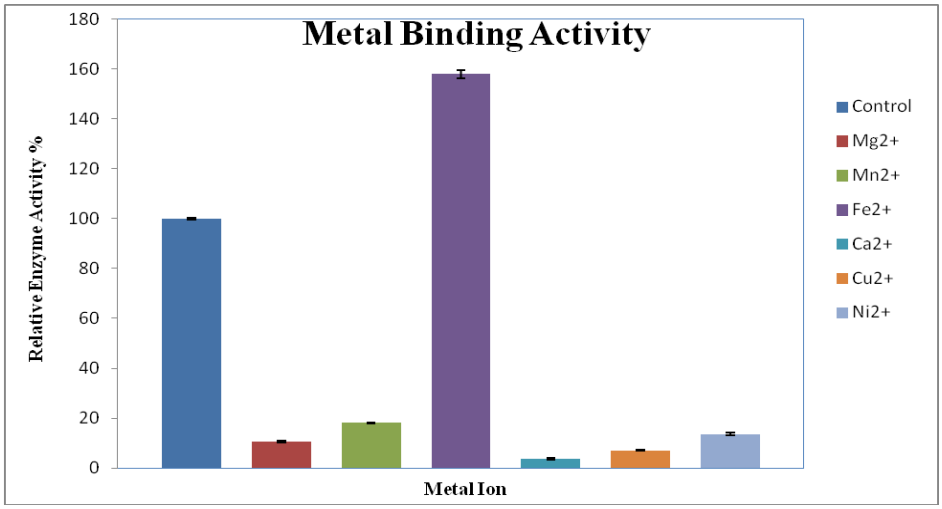


Figure 6: Effect of metal ions on enzyme activity. The results are presented on graph. Bars represent means ± standard deviations for three replicates.

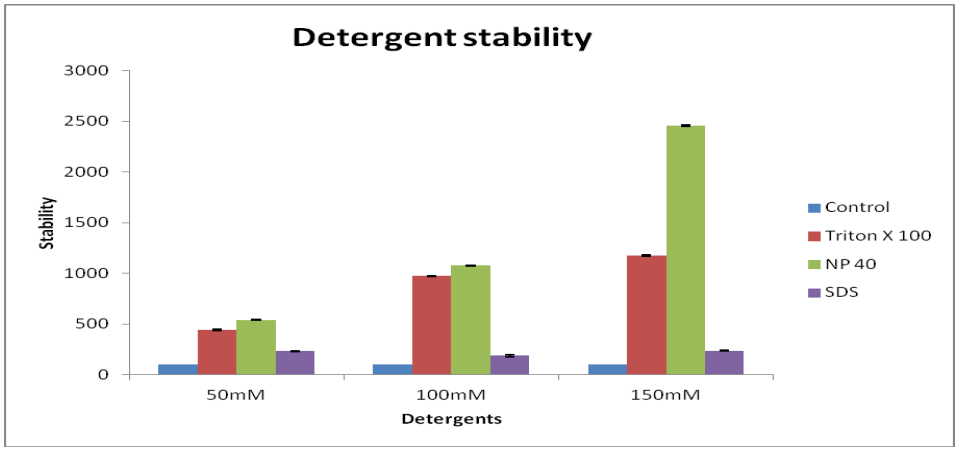
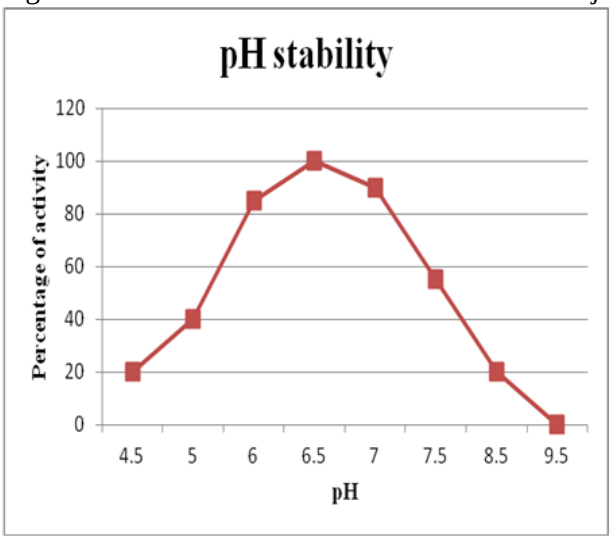


Figure 7: Effect of detergents on enzyme activity. The results are presented on graph. Bars represent means ± standard deviations for three replicates.

Effect of pH and Temperature on the activity of enzyme

The protease activity was tested against the pH ranging from acidic to basic (4.5 – 9.5) for the purified enzyme using casein as a substrate. The optimum activity of the enzyme was observed at pH 6.2 (Figure 8). Enzyme activity dipped down with increasing pH. All commercially available rennet's shows more in relative milk coagulation activity in pH drops from pH 7 towards pH 4-5 [27]. The caseinolytic activity of the protein was carried out at the different temperature from 20°C to 80°C using casein agar plate method. The enzyme activity sharply increased up to 60°C, above which the activity decreased. The optimum activity of the enzyme was observed at 50°C (Figure 9). The purified *N. sativa* protease enzyme has an excellent scope in dairy industry for this high thermal stability. Protease require for different applications for specific pH and temperature optimum for best activity of protease enzyme. The cheese-making industry requires an acidic pH optimum protease for their use [28]. The same cysteine protease enzyme reported in ginger has the optimum pH 5.5 and temperature stability of the ginger protease enzyme stable up to 55°C [29].



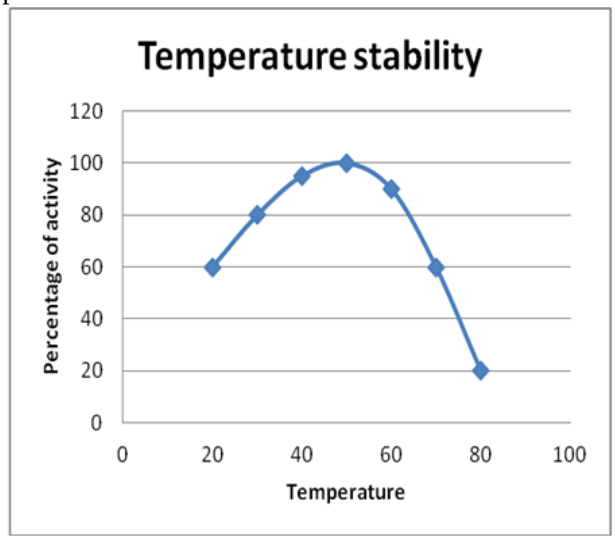


Figure 8. pH stability of purified protease **Figure 9**. Temperature stability of purified protease

Zymography

The property of proteolysis in the purified protein was also confirmed by zymography using casein as a substrate. The casein zymographic results showed a clear zone of digestion on the gel (Figure 10), this result showed the proteolytic activity of the purified protein from the *N.sativa* seeds. The study revealed that detection and characterization of proteases and protease inhibitors using zymography analysis [30].

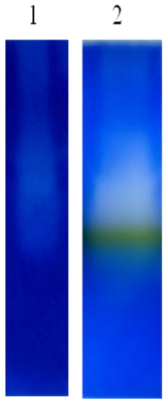


Figure 10: Caseinolytic activity on zymography. 1. 0-60% fraction, 2. Purified protease.

Milk clotting activity

The purified enzyme also exhibited the property of milk coagulation (Figure 11). Thus, the protease from *N. sativa* could benefit the dairy industry as milk coagulant, substitute or in addition to calf rennet, and for the enhancement of cheese ripening process in order to save time and cost of storage for maturation of cheese. Milk coagulants are essential for cheese making and plant proteases have been isolated from different plant sources and reported milk clotting activity [31]. *Albizia julibrissin* tree seeds have been reported that milk clotting activity without any bitterness in cheese after three months of ripening [32].

Figure 11: Milk clotting activity of the purified protease. 1. Crude sample, 2. 0-60% fraction, 3. Purified protease, 4. Positive control, 5. Control.

4. CONCLUSION

N. sativa was used globally for various ailments in the traditional knowledge. This study has been aimed to purifying and characterizing the functions of bioactive proteins from the seeds. The crude extract was fractionated by ammonium sulfate and the protease of approximately 20 kDa has been purified by ion exchange chromatography. The purified protease was characterized as cysteine protease by inhibitor study. The purified cysteine protease has metal and detergent stability, pH stability at 6.5 and optimum thermal stability is 50°C. Apart from the proteolytic activity, it also has the ability to coagulate milk. From this study, we conclude that the cysteine protease enzyme from the *Nigella sativa* seeds may be used in the cheese making industry.

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CONFLICT OF INTEREST

There is no conflict of interest.

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