*Seed Res.* 54 (1): 19-23, June, 2026
doi: https://doi.org/10.56093/SR.v54i1.3

# Milk Clotting and Blood Washing Potential of Heat-stable Acidic Serine Protease Purified from *Ocimum basilicum* (Sweet basil) Seeds

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(Received November 2025; Revised January 2026; Accepted February 2026)

**ABSTRACT:** In this study for the first time we purified and characterized a serine protease of 60.5kDa purified from the mucilage and fat-free seeds of *Ocimum basilicum* by ammonium sulfate precipitation followed by ion exchange chromatography. Cleavage of azo-casein indicates it to be endoprotease. The enzyme exhibited a Vmax of 0.066 mM/min and a Km of 0.53 mM using BAPNA as a substrate. For enzyme activity the optimal pH was found to be 5, and the optimal temperature was found to be 40°C. The activation energy was measured at 3.82 kcal/mol, with an enthalpy change of 2.16 kcal/mol and a $Q_{10}$ value of 1.20. The protease was stable within a pH range of 3 to 7 and at temperatures from 4 to 50°C. Notably, exposure to microwaves increased its proteolytic activity by 56%. Additionally, this protease showed significant effectiveness in removing blood stains when combined with sodium dodecyl sulfate (SDS) and was able to clot milk. This research represents the first characterization of a protease from *Ocimum basilicum* seeds, highlighting its potential applications in the detergent industry and food production, particularly in cheese making.

**Keywords:** *Ocimum basilicum*, BAPNA, Plant protease, Blood stain wash, Milk clotting

**Abbreviations:** DMSO - Dimethyl sulfoxide, PAGE - Polyacrylamide gel electrophoresis, SDS - Sodium dodecyl sulphate, PMSF - Phenylmethylsulfonyl fluoride, BAPNA - Nα-Benzoyl-DL-arginine þ-nitroanilide, BSA - Bovine serum albumin, TCA - Tricarboxylic acid

Plant proteases are essential enzymes that play a critical role in various stages of a plant's life, including protein turnover and the recycling of amino acids for protein synthesis [1]. The basil plant (*Ocimum basilicum L.*), known as sweet basil or sabja, is an annual herbaceous member of the *Lamiaceae* family. *O. basilicum* seeds were purchased from the local market in Indore (Go Vegan Company India). *O. basilicum* mucilage was removed and deffated by the modified method described earlier [2]. A crude enzyme extract was prepared in a 0.05 M Tris–HCl buffer at pH 8.0 (1:10 m/v) and then precipitated with ammonium sulfate (50-80%) followed by ion exchange chromatography.

Using BSA as a standard, the concentration of the protease protein was determined at each step of purification, employing the method by Lowry et al., [3] and absorbance at 280 nm was also recorded. To determine the nature and molecular weight of purified protease, SDS-PAGE was performed according to the method of Laemmli's [4].

Protease activity was measured using BAPNA as a substrate. BAPNA stock (1 mM) was prepared by dissolving in minimum amount of DMSO. The reaction volume includes protease (20µg) in final volume of 0.5 mL of 0.05 M potassium citrate buffer pH 5 and 0.5 mL of 1 mM BAPNA to make the final reaction volume of 1 mL. Mixture was incubated for 2 hr at 37°C. The reaction was terminated by adding 0.3 mL of 30% acetic acid and finally absorbance was taken at 405 nm. Azocaseinolytic activity was determined using 0.3% azocasein and activity was performed according to method described earlier [5].

The effects of protease inhibitors on protease activity were studied using various types of protease inhibitors, the proteases were incubated for 45 min with the inhibitors. Following this, a substrate was added to initiate the reaction, which was then incubated for 2 hr at 40°C. The control activity, conducted without any inhibitors, was considered as 100%.

For the optimum pH determination, the enzyme protease activity was measured with the following buffer: 0.05 M glycin HCl buffer (pH 2), 0.05 M citrate buffer (pH 3-4-5), 0.05 M phosphate buffer (pH 6-7), 0.05 M carbonate buffer (pH 9-10) and 0.05 M glycin-NaOH (pH 12.5). the enzyme assay was performed for 2 hr at 37°C. The enzyme was incubated in different pH buffers for 1 hr at room temperature to perform a stability test.

For the optimum temperature determination, the enzyme protease activity was measured at various temperatures, ranging from 4 to 80°C, for 2 hr and stability was checked at optimum temperature. Effect of microwave exposure on protease activity was studied by incubating purified protease for 20, 40, 60,80 and120 seconds in microwave, operated at full power of 900 Watts. After incubation, the enzyme activity was assessed at 40°C for 2 hr.

For determining the activation energy (Ea ) and activation enthalpy (∆H), of the enzyme, protease enzyme activity was measured at different temperatures in the range 4 to 80°C [6]. Then, the Arrhenius plot was drawn logk-1/T and the Ea and ∆H were calculated from this graph. The temperature coefficient (Q10 value) for the increase in temperature from 30 to 40°C was estimated.

The Michaelis constant (Km) and maximum velocity (Vmax) was determined using the Lineweaver-Burk plot.The experiment was performed at optimum temperature and optimum pH in 1 ml reaction mixture as mentioned above, with BAPNA concentrations ranging from 0.02 µM to 1 µM. For comparison, a blank was prepared using a range of substrate concentrations without any enzyme.

A study on blood stain removal was conducted using clean cotton cloth squares measuring 5 × 5 cm. These squares were stained with blood and allowed to dry. They were then incubated in a 1% detergent solution containing 0.2 mg/ml of purified enzyme for 5 hours to evaluate the efficacy of stain removal. A control sample consisted of cotton cloth of the same size that had also been stained with blood. Various conditions were tested during the study to assess their effects on stain removal.

Milk-clotting activity was measured using a slightly modified version of the method described by Arima et al. (1970) [7]. The pH of the prepared substrate, which consisted of 10% skim milk (w/v) in 0.01 M $CaCl_2$, was adjusted to 6. After pre-incubating 2.0 ml of the substrate for 5 mins at 37°C, 0.03 to 0.14 mg of the enzyme was added. The formation of curd was then monitored at 37°C, with periodic manual rotation of the test tube. The termination point was recorded when distinct curd particles were visibly formed. The amount of enzyme required to clot 1 ml of the substrate within 40 min at 37°C is referred to as one milk-clotting unit.

Milk clotting activity (MCA) U/ml = (2400/ clotting time in sec) X dilution factor from the ratio of MCA to proteolytic activity (PA), the milk clotting index (MCI) was calculated.

Using the methodical process outlined above, a new protease was isolated and purified from *O. basilicum* seeds, resulting in a 15.4-fold increase in purity closer to those protease reported in *A. heterophyllus* seeds [8]. Table 1 summarizes the outcomes of the purification procedure. In the first stage, specific activity, total proteolytic activity, and total protein content were determined from the crude extract yielding values of 2.5 U/mg, 9.18 U, and 3672 mg, respectively. The precipitate produced from 50-80% ammonium sulfate demonstrated a specific activity of 8.2 U/mg. At this stage of the purification process, the enzyme achieved a yield of 14.3% and a 3.28-fold increase in purification. The precipitate was then subjected to anion exchanger DEAE-Cellulose52. Elution yielded two peaks of protease with 0.1 M NaCl and highest activity was present in first peak obtained at 280 nm as shown in Figure 1 A.

The initial peak of bound fraction was obtained through ion exchange chromatography and subsequently concentrated using a CentriconPlus-70 Centrifugal 100 kDa filter. The concentrated protease was then loaded onto a 10% SDS-PAGE gel alongside a protein marker as shown in Figure 2. The gel was stained using the silver staining method [9]. The purified enzyme demonstrated a molecular weight of approximately 60.5 kDa under

**Table 1.** Purification summary of *O. basilicum* seed protease

| Purification steps | Total protein (mg) | Yield (%) | Specific activity (Units/mg) | Fold purification |
|---|---|---|---|---|
| Crude extract | 3672 | 100 | 0.0025 | 1 |
| Ammonium sulfate precipitate (50-80%) | 530.2 | 14.3 | 0.0082 | 3.28 |
| Ion-exchange (DEAE-Cellulose 52) chromatography | 7.5 | 0.20 | 0.0385 | 15.4 |

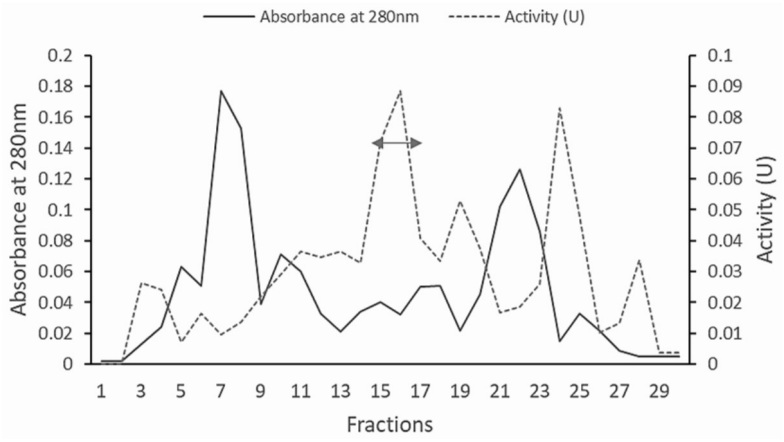



**Figure 1.** A) Chromatogram showing anion exchange chromatography using DEAE cellulose resins. The elution of the column was accomplished using 0.1 M NaCl. Fractions of 3 mL were collected, Fractions 14 to 17 marked with an arrow, were pooled together for further studies.B) SDS–PAGE gel (10%) of purified *O. basilicum* protease; Lane a: Standard molecular-mass markers (kDa); Lane b & c: DEAE cellulose column eluted active protease fraction in reducing condition with molecular weight 60.5KDa

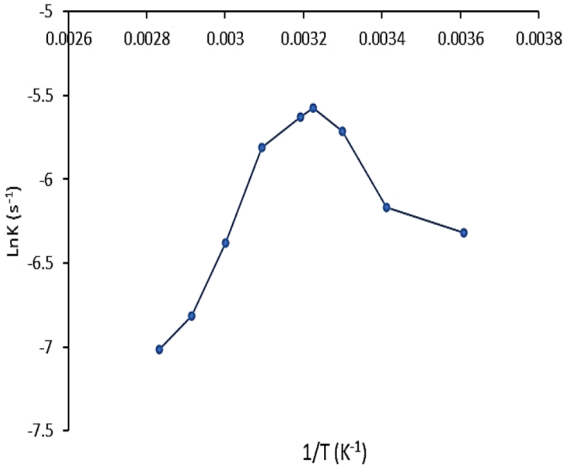



**Figure 2.** Arrhenius plot between ln (k)) and 1/T to calculate overall Activation Energy (Ea) and Enthalpy (ΔH) of purified O. basilicum protease

reducing conditions. The presence of a single band indicates the homogenous nature of the protein, which was estimated using known protein standards, as illustrated in the accompanying Figure 1 B.

The enzyme exhibited its highest activity in cleaving BAPNA at a pH of 5, in 0.05M potassium citrate buffer. The purified enzyme derived from *O. basilicum* demonstrates slightly acidic properties, with an optimum pH of 5. Additionally, the enzyme shows high stability within a pH range of 3 to 7. Furthermore, the isolated enzyme has an optimum temperature of 40°C, and its activity remains stable at temperatures ranging from 4°C to 50°C, over an incubation period of 2 hr, indicating its thermal stability. From the slope of Arrhenius plot (Figure 2), the calculated activation energy (Ea) of the protease was determined to be 3.82 kcal/mol, while the enthalpy change (ΔH) for the reaction was found to be 2.16 kcal/mol. The temperature coefficient ($Q_{10}$ value) for the increase in temperature from 30 to 40 °C was estimated to be 1.22.

The study examined the effect of increasing BAPNA substrate concentration on the rate of an enzyme-catalyzed reaction at a pH of 5 and a temperature of 40°C. The Michaelis-Menten equation was used to analyze how the enzyme's velocity varied with different substrate concentrations (Figure 3). The calculated values for Vmax and Km were 0.066 mM/min and 0.53 mM, respectively. These results indicate that the protease

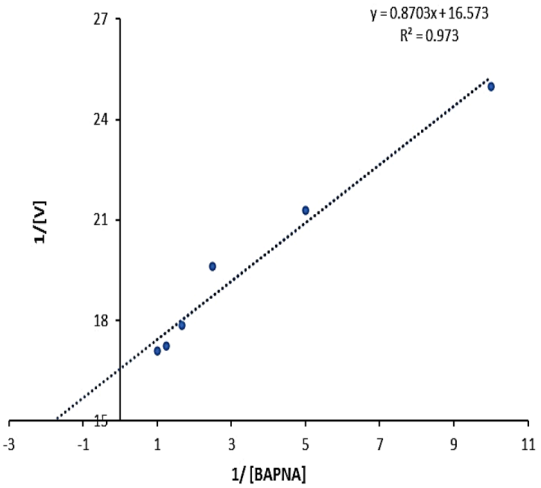



**Figure 3.** Lineweaver–Burk plot. Km values for the substrate was calculated from the Lineweaver–Burk plot using BAPNA as a substrate

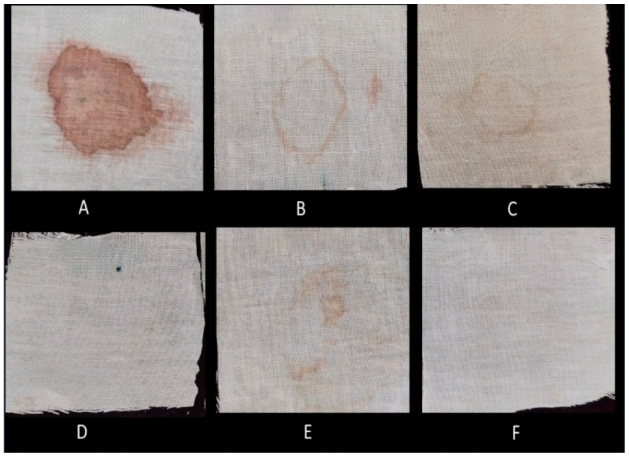



**Figure 4.** Blood stain washing activity of protease purified from *O. basilicum* (A) Unwashed blood-stained cloth (B) Tap water washing (C) Potassium citrate buffer pH 5 (D) Phosphate buffer+ 4ml of Protease (0.2mg/ml) (E) Potassium citrate buffer +0.1% SDS detergent (E) 4 ml of Protease (0.2mg/ml) +0.1% SDS detergent

has a high affinity for BAPNA, making it the optimal substrate for this enzyme. Also, the protease from *O. basilicum* found significant activity in hydrolyzing azocasein as a substrate.

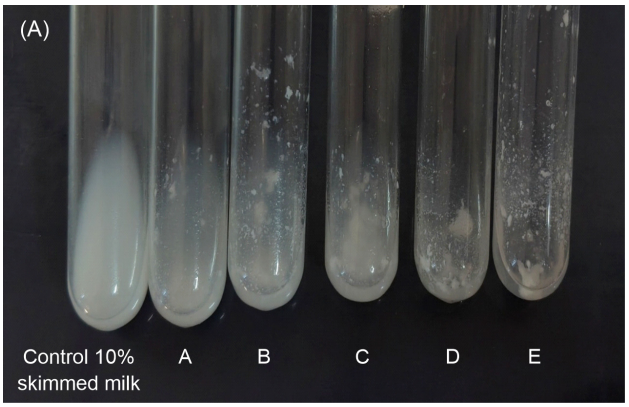
The enzyme activity was significantly reduced by PMSF and Aprotinin; PMSF inhibited activity by 92.8% at 2 mM, while Aprotinin inhibited it by 41% at 1 µM. PMSF sulfonates an essential serine residue at the active site, decreasing enzyme activity. These results classify the enzyme as a serine protease, as it was inhibited solely by serine protease inhibitors.

Enzymes are activated at optimal temperatures, but exceeding this temperature can lead to their inhibition. Understanding these mechanisms is essential for optimizing microwave applications in enzyme catalysis during food processing. When *O. basilicum* protease was subjected to microwave treatment at full power (900 Watts), an increase in enzyme activity was observed. Specifically, there was a 156% increase after 20 seconds and a 126% increase after 40 seconds of heating. The enzyme activity remained stable for up to 80 seconds of exposure but decreased by 50% after 120 seconds. The activity of the enzyme before incubation was regarded as 100% activity.

Our study indicates that *Ocimum basilicum* protease (0.2 mg/ml) can remove blood stains from fabric after 5 hours at room temperature. When combined with 1% SDS detergent, it significantly improves cleaning performance as shown in Figure 4. Experiments show that using a mixture of purified enzyme extract and detergent removes stains more effectively than either alone, indicating its potential as an alternative in the detergent industry.

This is the first study to characterize the protease isolated from the seeds of Ocimum basilicum and to report its properties for blood-stain removal. The enzyme could serve as a potential ingredient in detergents due to its strong cleaning performance.

The milk-clotting activity of plant protease enzymes is an important factor in their application within the dairy industry, particularly in cheese production. The primary stages of milk coagulation involve the hydrolysis of casein. In our experiments, the purified protease was able to coagulate skimmed milk, resulting in the formation of white curd. The effect of increasing protease concentrations (ranging from 0.03 to 0.14mg) on milk clotting activity was examined as shown in Figure 5 A. It was found that as the concentration of the enzyme increased, both the clotting time and the milk clotting units




(B)

| Protease concentration (mg) | Clotting time (sec) | MCU/ml |
|---|---|---|
| A) 0.03 | 180 | 952 |
| B) 0.05 | 175 | 489 |
| C) 0.08 | 145 | 389 |
| D) 0.11 | 130 | 326 |
| E) 0.14 | 120 | 281 |

**Figure 5.** Milk clotting activity of O. basilicum protease. (A) 10% skimmed milk with potassium citrate buffer used as a control. A-E, milk with increasing protease concentration (0.03-0.14mg). (B) Milk clotting activity of *O. basilicum* purified protease. MCU: Milk Clotting Unit

per milliliter (MCU/ml) decreased as mentioned in Figure 5 B. As research progresses, the role of plant proteases in milk clotting is likely to expand, presenting new opportunities for product development and addressing diverse consumer needs in the dairy industry.

## Acknowledgements

We thank School of Life Sciences, DAVV, Indore for providing us with all the necessary instruments and facilities.

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