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Milk clotting and blood wash potential of acidic serine protease purified from Ocimum basilicum (sweet basil) seeds

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Research Article

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Posted Date: February 13th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8782507/v1

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Additional Declarations: No competing interests reported.

Milk clotting and blood wash potential of acidic serine protease purified from *Ocimum basilicum* (sweet basil) seeds

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Abstract

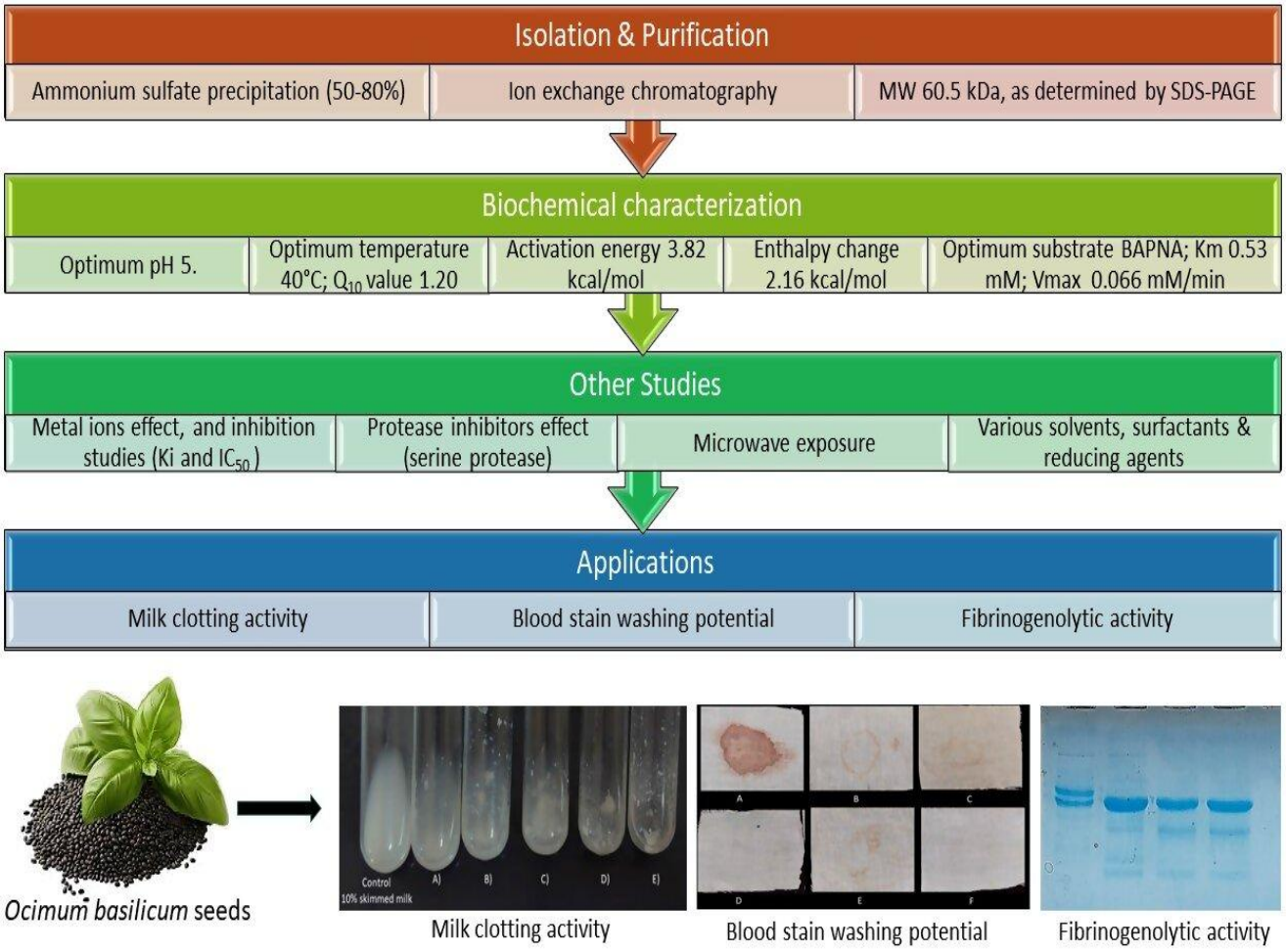
In this study we characterized a protease extracted from the mucilage and fat-free seeds of *Ocimum basilicum*. The purification process involved ammonium sulfate precipitation followed by ion exchange chromatography. The molecular weight of purified enzyme, as determined by SDS-PAGE was found to be 60.5 kDa. This protease was identified as a serine protease, as its activity was inhibited by phenylmethylsulfonyl fluoride (PMSF) and aprotinin. Cleavage of azo-casein indicates it to be endoprotease. The enzyme exhibited a maximum reaction velocity (Vmax) of 0.066 mM/min and a Michaelis constant (Km) of 0.53 mM using BAPNA as a substrate. The optimal pH for enzyme activity was found to be 5, and the optimal temperature was determined 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 demonstrated stability 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%. Among the metal ions investigated, Fe^{3+} was identified as an effective competitive inhibitor. Additionally, this protease showed significant effectiveness in removing blood stains when combined with sodium dodecyl sulfate (SDS) and was also 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, EGTA Ethylene glycol-bis(2-aminoethyl)tetraacetic acid,EDTA Ethylenediaminetetraacetic acid

Graphical Abstract



1 INTRODUCTION

Proteases are a diverse group of industrial biocatalysts classified into three main categories: proteinases, peptidases, and amidases. They are used across various industries and can be further divided into exopeptidases (which include amino and carboxy proteases) and endopeptidases (which include serine, aspartic, cysteine, and metalloproteases) [1].These enzymes are produced by animals, plants, and microorganisms, playing crucial roles in essential biological processes such as digestion, tissue healing, and inflammation. It has a crucial role in fibrinogenolytic activity, which is the process of breaking down fibrinogen, a key protein involved in blood clot formation [2]. The wide variety of protease sources and their unique characteristics make them suitable for many industrial applications, including in the food and dairy sectors, the leather industry, and pharmaceuticals [3, 4]. Proteases contribute to enhancing the taste, texture, and appearance of products. As demand for these enzymes continues to rise within various industries, the market for their production is expanding rapidly.

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 [5] . Their significance extends beyond plant biology, as they have potential applications in pharmacology and biotechnology. Additionally, these enzymes are used in feather processing, wastewater treatment, the photographic industry, the detergent industry, and biotransformation [6, 7]. Therefore, plant hydrolytic enzymes are crucial in therapeutic applications, making them promising targets for research into their unexplored medicinal and industrial value.

The basil plant (*Ocimum basilicum L.*), known as sweet basil or sabja, is an annual herbaceous member of the *Lamiaceae* family. It originates from the warmer regions of the Indo-Malayan area and is widely found in the tropical and hotter parts of the Indo-Pakistan subcontinent. This herb is rich in nutrients, providing between 11.4 to 22.5 grams of protein per 100 grams, as well as dietary fibre that ranges from 7.11 to 26.2 grams per 100 grams (both soluble and insoluble). Additionally, it contains various types of lipids [8].

The essential oil of *Ocimum basilicum* has demonstrated antimicrobial properties when tested against various bacterial strains [9]. For thousands of years, it has been used to treat digestive and nervous disorders and has been recognized as an antioxidant, anti-inflammatory agent, and antiviral. Additionally, it has shown effectiveness in treating ailments such as muscle cramps, insect-related issues, diabetes, and respiratory disorders [10, 11].

Ocimum basilicum (sweet basil) has recently attracted great attention because of its uses in ancient system for the treatment of various diseases [12]. It is used as a component in cosmetic products viz. perfumes, shampoos, lotions, oils and soaps [13, 14]. Its application in the treatment of various diseases, suggest, that the plant may also be a promising source of hydrolytic enzyme.

Recent advancements in biotechnology have made it easier and more affordable to isolate plant proteases. Additionally, there is a growing consumer preference for environmentally friendly products, which has increased the demand for plant-based enzymes, such as proteases, in detergents, food factories and more. The aim of this investigation was to characterize a protease derived from *Ocimum basilicum* (basil) seeds. This protease has been identified as a stable serine protease capable of withstanding challenging conditions, including the presence of various solvents and detergents, as well as maintaining stability across different pH levels and temperatures. Furthermore, this protease exhibits a broad spectrum of substrate specificity. The purified protease from *Ocimum basilicum* holds potential applications in both commercial and biotechnological fields.

2 MATERIALS AND METHODS

Ocimum basilicum seeds were purchased from the local market in Indore (India), GO VEGAN Company. N-αBenzoyl-DLarginine þ-nitroanilide (BAPNA)>99% pure, N-succinylPhe-p-nitroanilide, N-succinyl-Ala-Ala-Ala-p nitroanilide, Bovine serum albumin (BSA)>96% pure, Azocasien)>99% pure , gelatin 95% pure, Diethylaminoethyl (DEAE)-cellulose 52, PMSF, E-64 (L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine),Aprotinin,Pepstatin-A,Idoacetamide, Human fibrinogen were purchased from Sigma-Aldrich. Ammonium sulfate 98.5% extra pure, sodium dodecyl sulfate (SDS) 99% extra pure, $FeCl_3$, $BaCl_2$, $NiCl_2$, $ZnCl_2$,KCl, NaCl,$CaCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$ were (95–98% pure) purchased from SRL. Coomassie brilliant blue (CBB) 70% pure ,N, N,N, N-Tetramethylethylenediamine (TEMED) 99% pure, β-mercaptoethanol (β-ME) 99% pure, Dithiothretiol, bromophenol blue 99% pure, acrylamide 99.9% pure, N, N-methylene bis-acrylamide 99% pure,

silver nitrate pure, sodium thiosulphate 95.5 pure, citric acid extra pure, sodium carbonate, potassium citrate, citric acid, formaldehyde, phenol 99.7% pure, Dimethyl sulfoxide (DMSO) 99.9% pure, methanol, isopropanol, butanol, Triton X-100, Tween-80, Labolene and glacial acetic acid 99%pure were used of analytical grade.

2.1 Protease Purification from *Ocimum basilicum* Seeds

2.1.1 Mucilage and fat removal of *O. basilicum* seeds

O. basilicum seeds were soaked in water (1:40 (m/v)) for 2 hr at 4°C under stirring. Afterward, the mucilage was removed with a sieve, followed by cheesecloth filtration. The seed trace of the remaining mucilage was separated by centrifuge for 15 min at 10000rpm. Supernatant containing mucilage was discarded and mucilage free seeds obtained in pellet were kept for drying at 30˚C. Mucilage-free seeds were ground into powder using a mechanical grinder. Then, the resulting seed powder was defatted with petroleum ether (1:5 (m/v)) at room temperature, with four solvent changes and were stored at−20 °C [15].

2.1.2 Seed crude extract preparation

A crude extract was obtained by using mucilage and fat-free *O. basilicum* seed powder (10 g) in 0.05 M Tris–HCl buffer, pH 8.0 (1:10; m/v) under stirring for 5 hr at room temperature for proper mixing of homogenate. The homogenate was centrifuged at 10000 rpm for 45 min at 4˚C to remove all insoluble materials and the supernatant was collected.

2.1.3 Protease purification

Crude enzyme extract was precipitated by different concentrations of ammonium sulfate (0-30%, 30-50% and 50-80% saturation). A suitable quantity of ammonium sulfate salt was added to the supernatant and then centrifuged at 10000 rpm at 4˚C for 25 min. The precipitates were collected with a minimum quantity of 0.05 M Tris–HCl buffer, pH 8.0. further the fractions were dialyzed against the same buffer using a dialysis bag and were stored at -20°C.The supernatant obtained after 50-80% ammonium sulfate fractionation was found to have highest protease activity as measured using BAPNA as a substrate.

The dialysed 50-80% ammonium sulfate fraction was subjected to Ion exchange chromatography. Ion exchange chromatography is commonly used for protein purification, DEAE-Cellulose 52 (21 × 60 mm) column was used as the matrix in this study. The column was equilibrated with 0.05 M Tris–HCl buffer, pH 8.0. The dialyzed fraction was then loaded into the column. The bound protein was eluted batch wise with 0–0.5M NaCl at a flow rate of 0.7 mL/min. Fractions of 3 mL were collected and their absorbance at 280 nm recorded and protease activity was measured using BAPNA as a substrate.

2.2 Protein content determination

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.,[16] and absorbance at 280 nm was also recorded.

2.3 Determination of Proteolytic Activity

2.3.1 Azocaseinolytic activity

0.3% azocasein was used as a substrate and activity was performed with slight modification (Coêlho et al., 2016) [17]. 0.5 ml of protease (0.2mg) was incubated with 0.5 ml of 0.3% azocasein substrate for 2 hr at 37˚C. the reaction was stopped by using 10% TCA, incubated for 15 min at 4˚C and centrifuged at 10000 rpm for 10 min. 1M NaOH was added to neutralize the supernatant and was measured spectrophotometrically at 440 nm. In control, substrate was precipitated by 10% TCA before adding the enzyme. One unit of enzyme activity was defined as the amount of enzyme that increases the absorbance at 440 nm by 1 unit per minute of reaction in the given assay condition. The number of units of enzyme activity per mg of protein was described as specific activity of the enzyme.

2.3.2 Amidolytic activity

N-Succinyl-Ala-Ala-Ala-p-nitroanilide (Elastase substrate),N-α-Benzoyl-DL-arginine p-nitroanilide(BAPNA) (Trypsin substrate), N-Succinyl-L-phenylalanine-p-nitroanilide (Chymotrypsin substrate), were synthetic

substrates used to assay amidolytic activity of the purified protease measuring absorbance of þ nitroanilide released using spectrophotometer.

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. Similarly other two substrates were also made and used [18]. The amount of þ -nitroanilide release was measured by absorbance change at 410 nm (extinction coefficient=8.8 ml µmol-1 cm-1). The unit (U) of protease activity was defined as the amount of protease that yielded a 1µmol þ -nitroanilide per minute at 37 °C. Unit/ml = [△Abs× Reaction Volume (ml)]/ [EC× Time ×Protease volume (ml)] △Abs=increase in absorbance. EC=Extinction Coefficient.

2.4 Calculation of fold purification and yield

The purification fold was calculated to determine the number of purification times compared with the crude extract. Percentage yield was calculated to display the highest enzyme activity that can be achieved.

Fold purification = Specific activity of fraction / Specific activity of crude fraction

Yield (%) = Total enzyme activity of fraction x 100% /Total enzyme activity of crude fraction

2.5 Molecular weight determination using gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli's [19] for the analysis of protein profile of the eluted sample from the column chromatography. SDS-PAGE of the purified enzyme was done with 5% stacking gel and 10% resolving gel by using Mini prot (BioRad, CA). Molecular weight marker consisting of proteins of known molecular weight was used to estimate the molecular weight of protease. Sample to be run on SDS-PAGE was mixed with sample buffer comprising of 60mM Tris HCl (pH 6.8), 14.4 mM β-mercaptoethanol, 4% SDS, 25% glycerol (w/v), 0.01% Bromophenol blue dye. This mixture was boiled at 100 ˚C for 5 min. Electrophoresis was carried out at constant 60mA for 1.5 hrs .Finally the gel was stained with Silver staining method for visualization of bands [20].

2.6 Biochemical characterizations of protease

2.6.1 Determination of the Optimum pH and stability for the protease

Estimating of the optimum pH of the pure enzyme was done by replacing 0.05 M potassium citrate buffer pH 5 in protease assay 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. After adjusting the pH to the optimal level for activity, which is pH 5, the substrate was added. The reaction mixture was then kept at 40°C for another 2 hrs. The reaction was stopped according to the enzyme assay method. The relative activity at various pH levels was calculated by assigning a value of 100% to the maximum measured activity.

2.6.2 Determination of the Optimum temperature and stability for the protease

The optimum temperature for the purified enzyme was determined by incubating the reaction mixture of the protease enzyme assay at various temperatures, ranging from 4 to 80°C, for 2 hours. After incubation, the enzyme activity was assessed at each temperature to identify the optimal temperature for the protease enzyme. The enzyme was incubated at temperatures ranging from 4 to 80°C for 1 hr at a pH of 5 to assess its thermal stability. Following this, the temperature was adjusted to the optimal level of 40°C, and the substrate was added. The reaction was maintained for two hours, after which the enzyme assay was performed. The relative activity at different temperatures was calculated, with the maximum measured activity set at 100%.

2.6.3 Estimating the Temperature Coefficient (Q_{10} Value), Activation Energy (Ea), and Enthalpy (ΔH)

Activation Energy (Ea) was calculated by the Arrhenius equation: (Equation 1) [21]. In this equation, the temperature is denoted as T (in Kelvin, where K = °C + 273.15), the universal gas constant is R (0.008314 kJ/mol·K), the activation energy is represented by Ea (in kJ/mol), and the physical symbol for the rate constant, which indicates that all molecules involved have sufficient energy for the reaction, is A (when Ea = 0).

$$k = A.e^{\frac{-Ea}{RT}} \qquad (1)$$

To visualize the relationship, the log-normal growth rate plotted against 1/T provides a linearized version of this equation (2) [22]

$$\ln k = -\left(\frac{Ea}{R}\right)\left(\frac{1}{T}\right) \ln A \qquad (2)$$

Activation enthalpy was calculated from the equation,

$\Delta H = Ea - RT.$

The Q_{10} value is estimated via the following equation:

$$Q_{10} = e^{\left(\frac{Ea}{R}\right)\left(\frac{10}{T_2 T_1}\right)}$$

Where T1 is the initial temperature and T2 is the final temperature after a 10 °C rise from T1 [23].

2.6.4 Effect of substrate concentration on the reaction velocity

Using BAPNA as a substrate, we assessed the effect of increasing substrate concentration on the reaction velocity of the purified protease. The experiment was conducted for 2 hr at pH 5 and a temperature of 40°C. 0.2 mg of protease was used, with BAPNA concentrations ranging from 0.02 µM to 1 µM. The assay followed the methodology for amidolytic activity. For comparison, a blank was prepared using a range of substrate concentrations without any enzyme. The Michaelis constant (Km) and maximum velocity (Vmax) was determined using the Lineweaver-Burk plot.

2.6.5 Effect of microwave exposure on protease activity

The purified enzyme was kept 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 at pH 5 using BAPNA as a substrate. The relative activity at different time interval was calculated, with the control set 100% as maximum measured activity.

2.7 Effect of protease inhibitors on the protease activity

The effects of protease inhibitors on protease activity were studied using various types of protease inhibitors including serine protease inhibitors like PMSF (1-2mM) and Aprotinin (0.5-1 µM), cysteine protease inhibitors like Idoacetamide (1-2mM) and E-64 (L-trans-3-Carboxyoxiran-2-carbonyl-L-leucylagmatine) (50 µM), aspartic inhibitor like Pepstatin-A (10 & 50µM) and metalloprotease inhibitor like EDTA (1 & 5mM) and EGTA (1& 5mM). Before conducting the assay with the various protease inhibitors, the proteases were incubated for 45 min with the inhibitor. 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%.

2.8 Effect of different metal ions and determination of IC_{50} values and Ki constant

To determine the effects of metal ions on protease activity, same concentrations of 0.05mM of Na^{+}, K^{+},Mg^{2+}, Mn^{2+}, Ba^{2+}, Ca^{2+}, Ni^{+2}, Zn^{2+} and Fe^{3+}, were incubated with enzyme for 3 hr at room temperature. After incubation, protease assay was done using BAPNA as a substrate for 2 hr at 40°C. Activity of the enzyme was measured, and the activity in absence of metal ions was used as control (100% activity). The graph of activity (%) against metal ion concentration was used to determine the IC_{50} values. Three different concentrations of the

inhibitor were added to the reaction medium, along with five different fixed concentrations of the substrate (BAPNA), in order to calculate the Ki constant. Using the 1/[V] vs. 1/[BAPNA] values, Lineweaver-Burk graph were constructed. From these graph, the Ki constant and the type of inhibition were determined[24].

2.9 Effect of various solvents, surfactants and reducing and on protease activity

The stability of the protease was investigated in the presence of various surfactants (Triton X-100, SDS, Labolene [a laboratory cleaning detergent], and Tween-80) and organic solvents (Methanol, Isopropanol, Butanol, Ethanol, and DMSO). Reducing agents such as β-mercaptoethanol (β-ME) and dithiothreitol (DTT) were used at final concentrations ranging from 2 to 5 mM. The protease was incubated for 30 minutes with different concentrations of surfactants, reducing agents, or solvents. To initiate the reaction, the substrate was added at 40°C, and the incubation continued for 2 hr. The control activity, which contained no solvents, reducing agents, or surfactants, was defined as 100%. The activity was calculated as a percentage of the residual activity.

2.10 Milk clotting activity

Milk-clotting activity was measured using a slightly modified version of the method described by Arima, Ya, and Iwasaki (1970). 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. [25]

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.

2.11 Blood stain removal study

Clean cotton cloth pieces measuring 5 × 5 cm were stained with blood and allowed to dry. They were then incubated in a solution of 1% detergent containing 0.2 mg/ml of purified enzyme for 5 hr to assess stain removal efficacy. A control sample consisted of cotton cloth of the same size that had been stained with blood. Different conditions made for the study are as follows:

A) Cloth with blood stain

B) Blood stained cloth treated with tap water

C) Blood stained cloth treated with 0.05 mM potassium citrate buffer pH 5

D) Blood stained cloth treated with 0.2 mg/ml of purified enzyme

E) Blood stained cloth treated with 1% SDS

F) Blood stained cloth treated with 1% SDS and 0.2 mg/ml of purified enzyme

After 5 hrs, images were taken and compared with control [26].

2.12 Fibrinogenolytic activity

Human plasma fibrinogen was prepared in 20mM HEPES buffer with 145 mM NaCl and 5mM $CaCl_2$ pH 8 in concentration of 1 mg/ml. 20 µl of fibrinogen was incubated with 10 & 20µg of protease at 40˚C for 6 hr. Reaction volume was 40 µl in each vial. Fibrinogen stored at 4˚C and 37 ˚C was taken as a control to study self-cleavage of fibrinogen and was visualized using 15% SDS-PAGE.

2.13 statistical analysis

All the experiments done in triplicates. Mean and standard deviation were calculated using MS Excel. Graphs were plotted using MS Excel.

3 Results and Discussion

3.1 Isolation and purification of protease from *Ocimum basilicum* seeds

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 and *P. vulgaris* seeds [27] 28].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 or homogenate, 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. yielded two peaks of protease with 0.1 M NaCl and highest activity was present in first peak about 38.5 U/mg of lowest absorbance at 280 nm as shown in Figure 1.

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 (30-50%)	530.2	14.3	0.0082	3.28
Ion-exchange (DEAE-Cellulose 52) chromatography	7.5	0.20	0.0385	15.4

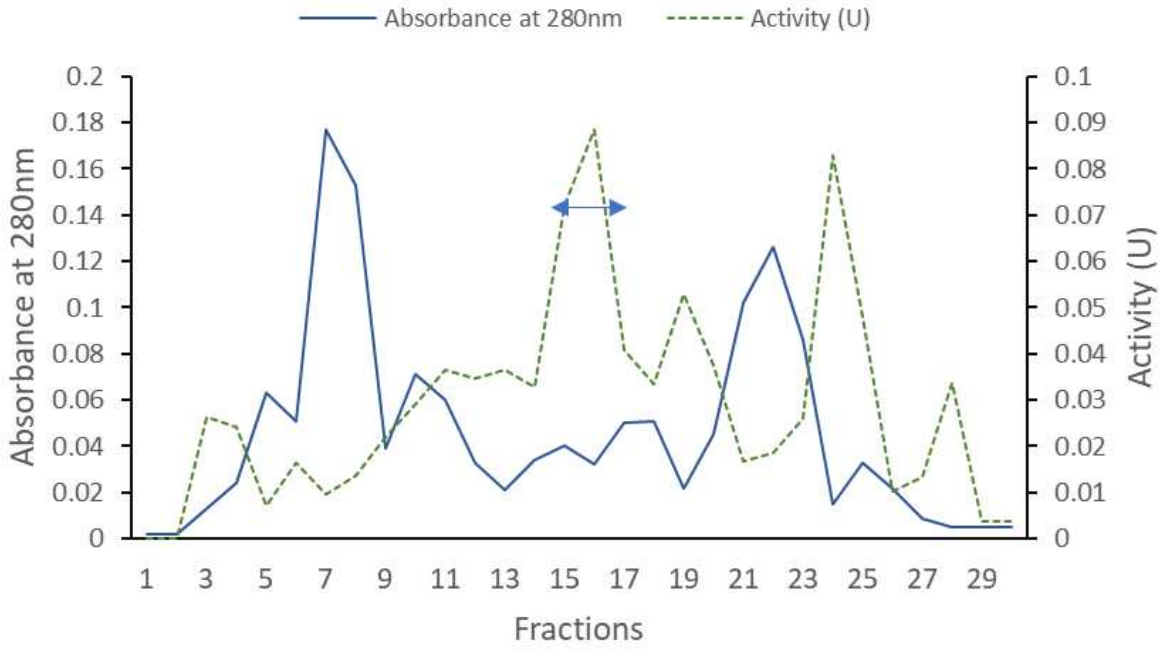


Figure 1 Chromatogram showing anion exchange chromatography using DEAE cellulose resins. The protein elution profile was obtained following a 50-80% ammonium sulfate fractionation step on the DEAE-cellulose column. Unbound proteins were removed by rinsing the column with equilibration buffer, which consisted of 0.05 M Tris-HCl at pH 8. The elution of the column was accomplished using 0.1 M NaCl. Fractions of 3 mL were collected, and each fraction was analyzed for protein content and proteolytic activity. Fractions 14 to 17, marked with an arrow, were pooled together.

3.2 Molecular weight determination by SDS-PAGE

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. The purified enzyme demonstrated a molecular weight of approximately 60.5 kDa under 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. Previously reported proteases from Coriandrum sativum and Xenorhabdus nematophila were found to have molecular weights of around 55 kDa and 60 kDa, respectively [29] [30].

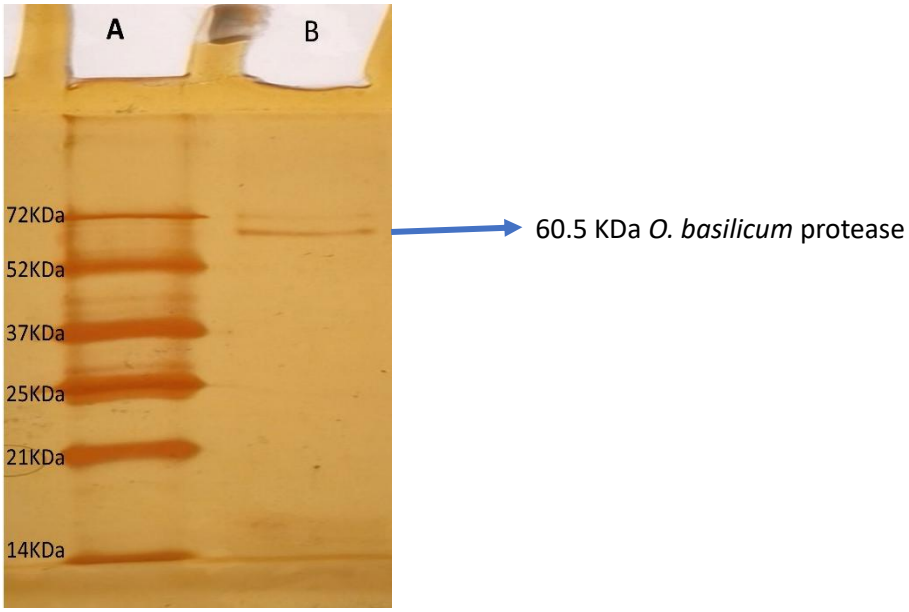


Figure 2 SDS–PAGE gel (10%) of purified O. basilicum protease; Lane a: Standard molecular-mass markers (kDa); Lane b: DEAE Cellulose column eluted active protease fraction in reducing condition with molecular weight 60.5KDa.

3.3 Determination of Proteolytic Activity and kinetic parameters

The protease from *O. basilicum* demonstrated significant activity in hydrolyzing substrates such as azocasein and bovine serum albumin (BSA). The highest activity was observed with 0.3% azocasein, compared to 1% BSA as a substrate. The Michaelis constants (Km) for azocasein and BSA were calculated to be 5.5 mM and 12.9 mM, respectively. Additionally, the maximum velocities (Vmax) were found to be 0.022 mM/min for azocasein and 0.056 mM/min for BSA, as determined using the Lineweaver-Burk plot. These results indicate that the protease has a greater affinity for azocasein compared to BSA.

However, among the synthetic substrates used in the study, e.g., N-α-Benzoyl-DL-arginine þ-nitroanilide (Trypsin substrate), N-Succinyl-L-phenylalanine-p-nitroanilide (Chymotrypsin substrate) and N-Succinyl-Ala-Ala-Ala-p-nitroanilide (Elastase substrate) *O. basilicum* protease cleaved only N-α-Benzoyl-DL-arginine þ-nitroanilide (Trypsin substrate) and did not cleave the substrates of chymotrypsin and elastase as shown in Figure 3.

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. 0.2 mg of protease was utilized, with substrate concentrations ranging from 0.02 mM to 1 mM, to construct a Lineweaver-Burk plot (Figure 4). The calculated values for Vmax and Km were 0.066 mM/min and 0.53 mM, respectively. These results indicate that the protease has a high affinity for BAPNA, making it the optimal substrate for this enzyme.

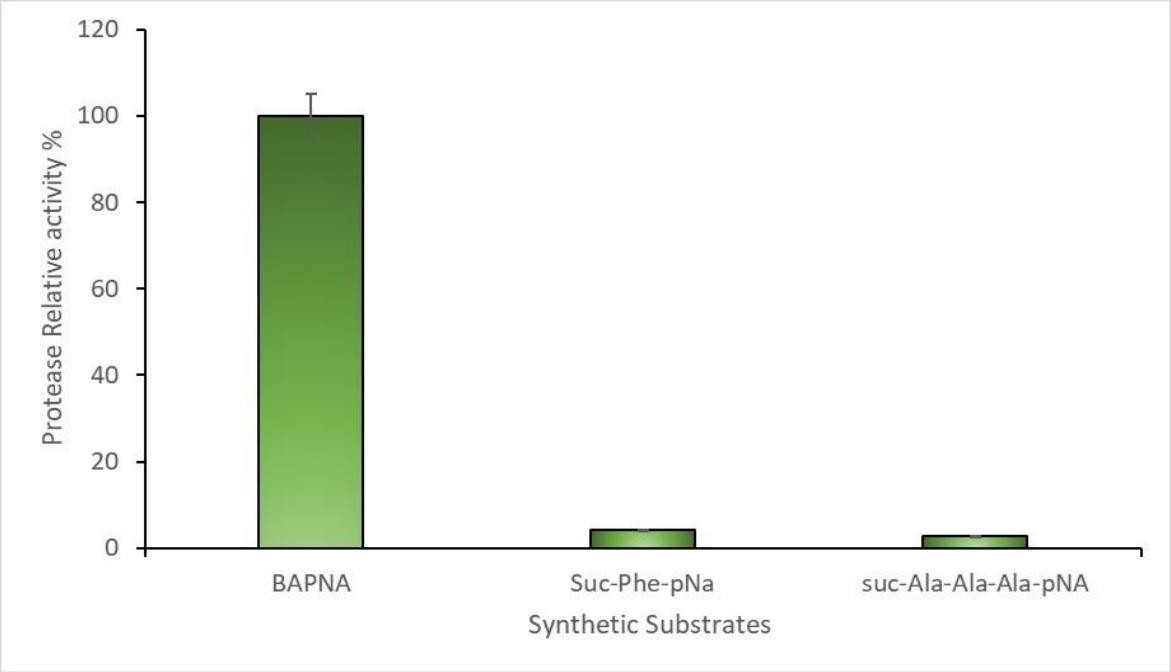


Figure 3 *O. basilicum protease activity against various types of synthetic substrates. Relative activity was assessed using a standardised quantity of isolated protease (0.2 mg) and the suitable substrates BAPNA (1 mM), N-succinyl phenylalanine-p-nitroanilide (1 mM), and N-succinyl-Ala-Ala-Ala-p-nitroanilide (1 mM). Data are means ±SD of triplicate measurements*

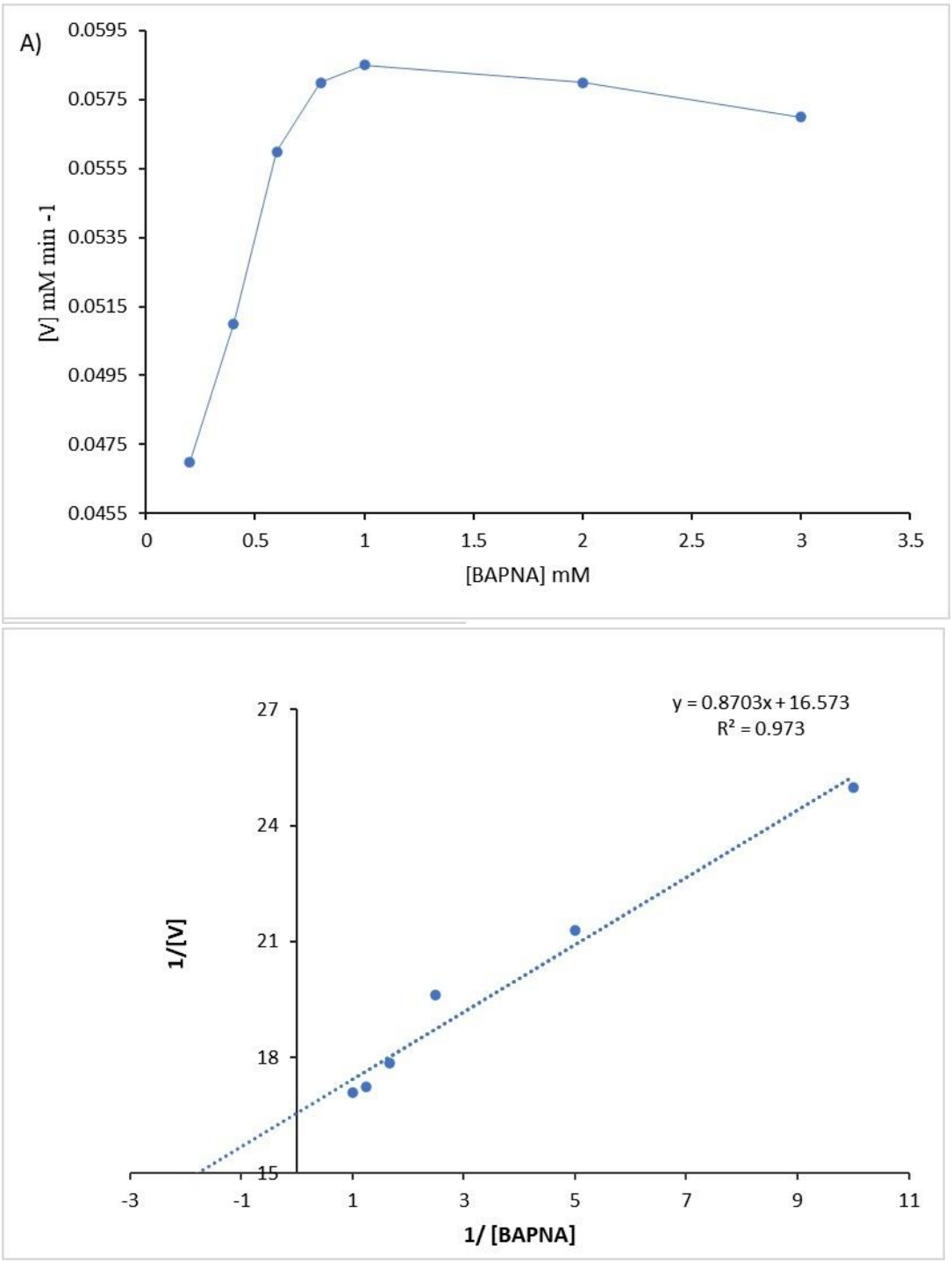


Figure 4 Effect of substrate concentration on reaction velocity of O. basilicum protease was studied using BAPNA as a substrate (A) Hyperbola curve; and (B) Lineweaver–Burk plot. Km values for the substrate was calculated from the Lineweaver–Burk plot.

3.4 Biochemical characterizations of protease

3.4.1 Determination of the Optimum pH and stability

The pH level significantly influences the activity of protease enzymes. As illustrated in the accompanying figure, the enzyme demonstrated its highest activity in cleaving BAPNA at a pH of 5, within a 0.05M potassium citrate buffer. The purified enzyme derived from *O. basilicum* exhibits slightly acidic properties, with an

optimum pH of 5 (Figure 5 A). Similarly, the optimum pH for the acidic protease enzyme from *Rhizopus oryzae* is reported to be 5.5 [31]. Research on the pure protease enzyme extracted from *Solanum tuberosum L.* revealed an optimum pH range between 4 and 5 [32]. In this study, the protease was incubated across a range of pH levels and assayed using BAPNA as a substrate at its optimum pH of 5. The enzyme demonstrated high stability within the pH range of 3 to 7 (Figure 5 B)

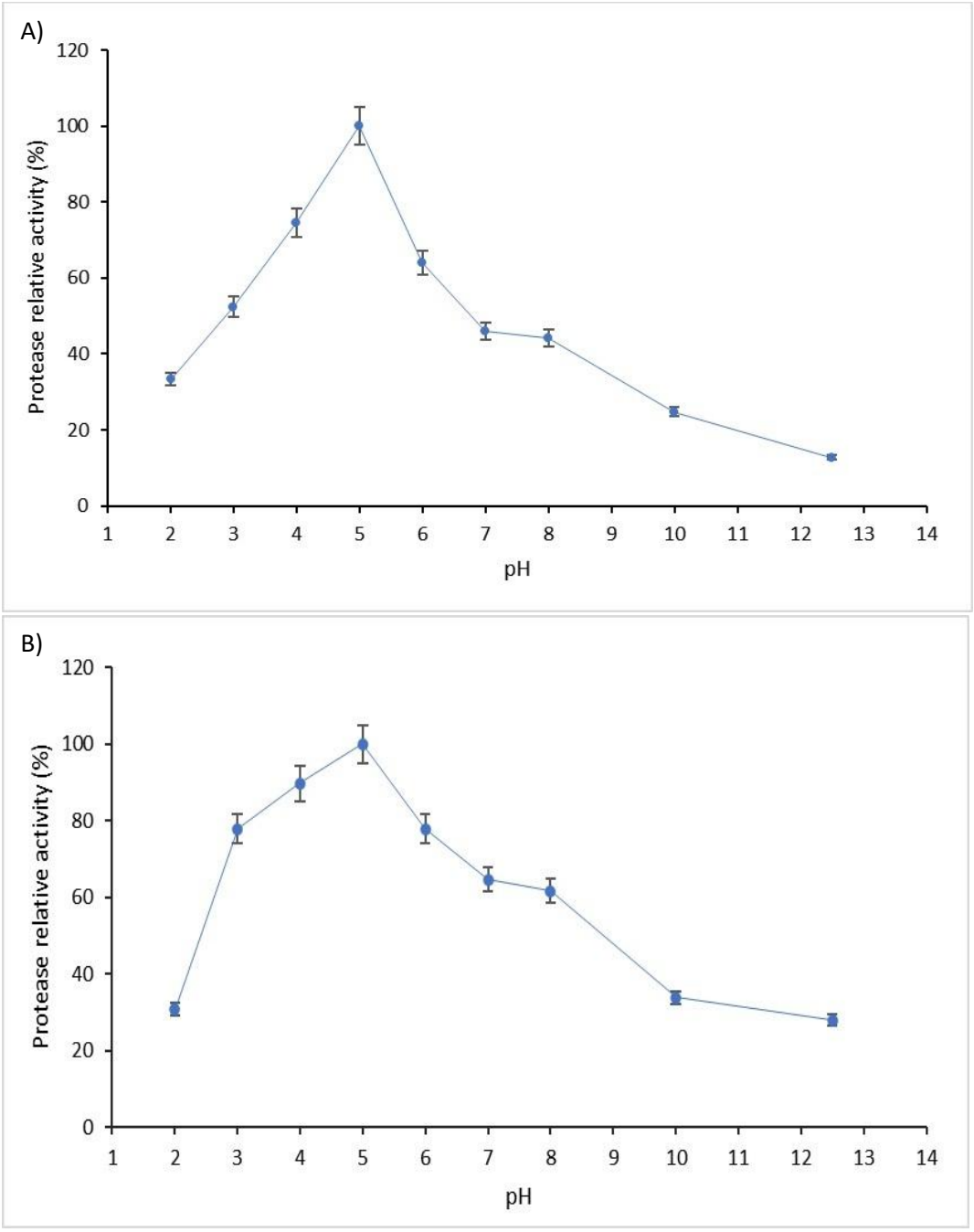


Figure 5 Effect of pH on protease activity and stability of O. basilicum protease A) Protease activity at different pH B) Effect of pH in Stability of O. basilicum protease.. Protease activity was performed at optimum pH after incubating protease at different pH for 1hr. Experiment was performed in triplicates and error bars above column represent Mean±SD

3.4.2 Determination of the Optimum temperature and stability

The obtained results (Figure 6 A) show the effect of different temperatures from 4 to 80°C on the purified protease activity of *O. basilicum* seeds. The enzyme activity was examined based on cleaving BAPNA as a substrate. This result shows isolated enzyme has an optimum temperature at 40°C. In some studies, the optimum temperature for enzyme activity has been reported to be 40°C in protease of *P. farinose* [33], *M. indicus* leaves [34] and *Phalaris minor* seeds [34].The enzyme was incubated at the temperatures from 4-80°C for 1 hr. The enzyme activity was stable at 4 to 50°C on the incubation time 2 hr. At 80 ◦C, the protease was denatured and no proteolityc activity was detected (Figure 6 B).

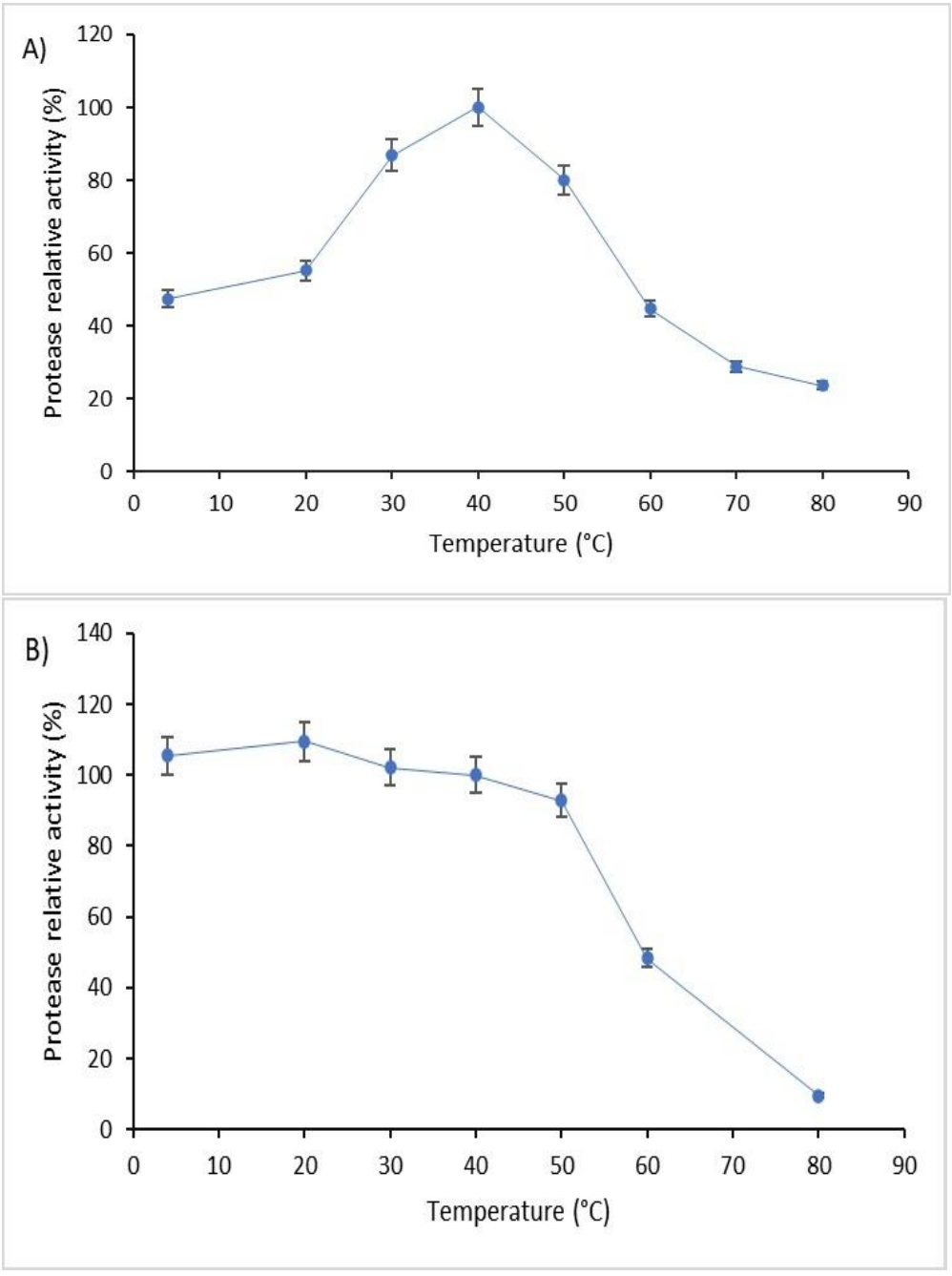


Figure 6 Effect of temperature on protease activity and stability of O. basilicum protease A) Protease activity at different temperature B) Effect of temperature in Stability of O. basilicum protease. Protease activity was performed at optimum temperature after incubating protease at different temperature for 1hr. Experiment was performed in triplicates and error bars above column represent Mean±SD

3.4.3 Determination of Activation Energy (Ea), Enthalpy (ΔH) and Temperature Coefficient (Q_{10} Value)

The highest protease activity was observed at 40 °C, with a noticeable decrease in activity as the temperature increased beyond this point. When plotting the logarithm of the reaction rate against the temperature (1/T), an inverted V-shaped graph was produced, as illustrated in the Figure 7.

From the slope of this plot, 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. These values of Ea, ΔH, and Q_{10} were comparable to those of carbonic anhydrase obtained from the kidneys of rainbow trout (*Oncorhynchus mykiss*) as reported by Söyüt and Beydemir in 2012 [35]. Additionally, many enzymes from various plant sources, including acid phosphatases, β-galactosidases, and both acid and alkaline proteases, have also exhibited similar thermostability.[36–38]

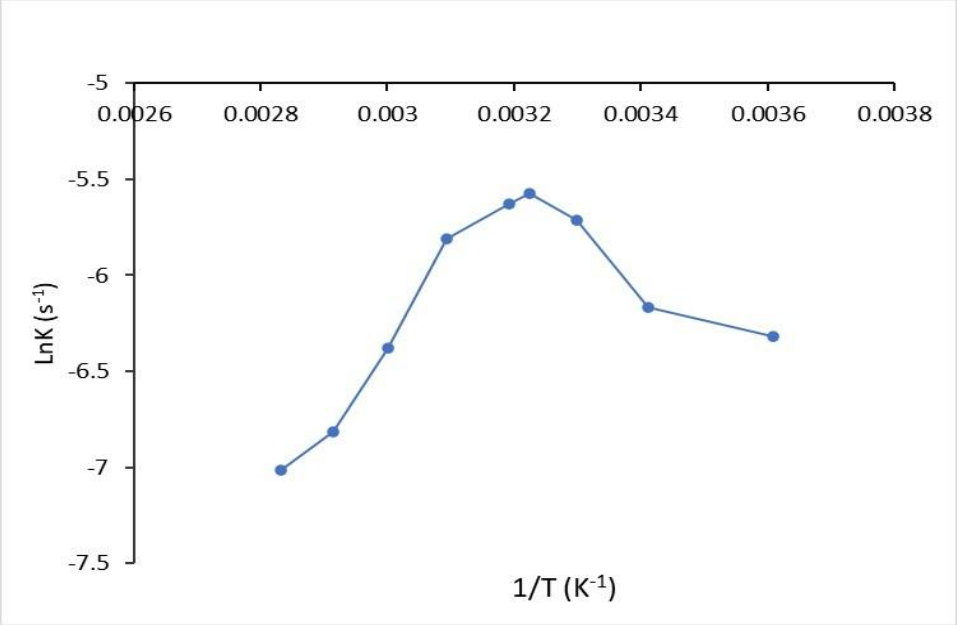


Figure 7 Arrhenius plot between ln (k)) and 1/T to calculate overall Activation Energy (Ea) and Enthalpy (ΔH) of purified O. basilicum protease.

3.4.4 Effect of microwave exposure on protease activity

Microwave (MW) irradiation affects enzyme activity through both thermal and nonthermal effects. 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. It was reported that Microwave treatment enhanced β-glucosidase enzyme activity, resulting in a 26% higher glucose concentration [39]. 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 (Figure 8). 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.

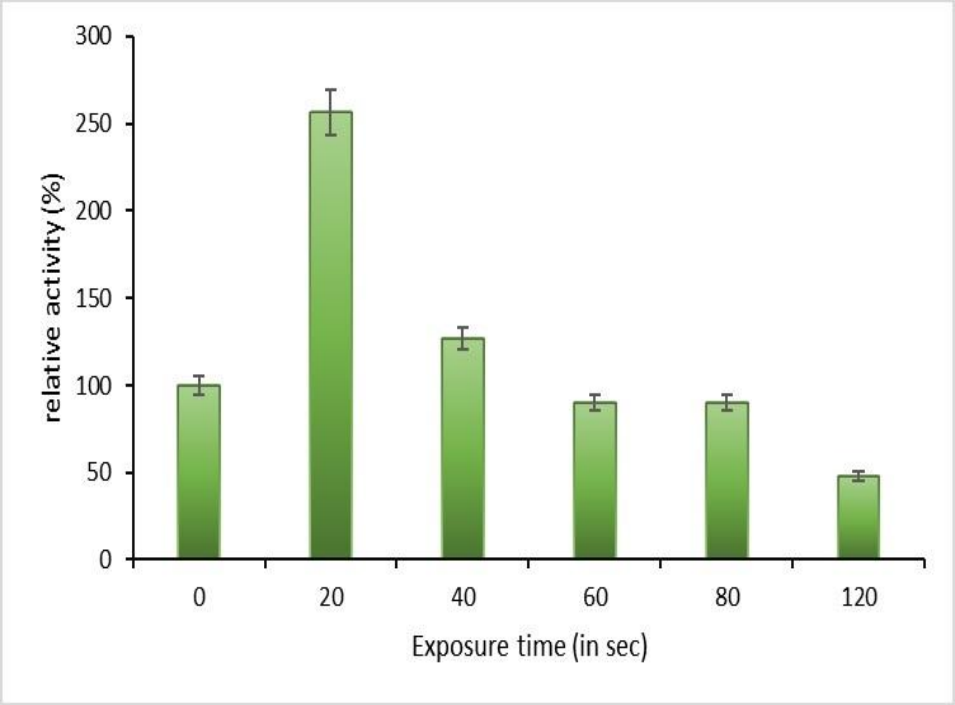


Figure 8 *Effect of Microwave exposure to purified O. basilicum protease at 900 Watt for different incubation time. Experiment was performed in triplicates and error bars above column represent Mean±SD*

3.5 Effect of protease inhibitors on the protease activity

Various specific protease inhibitors were used to identify the class of the isolated protease, as illustrated in the Table 2. The enzyme activity was found to be significantly reduced in the presence of PMSF and Aprotinin, while other inhibitors had a much smaller impact on its activity. Specifically, the enzyme was inhibited by 2 mM PMSF, resulting in a 92.8% inhibition, whereas Aprotinin exhibited a lesser effect, inhibiting the enzyme by up to 41% at a concentration of 1 µM. PMSF is known to sulfonate the essential serine residue at the enzyme's active site, leading to a decrease in enzyme activity [40]. Based on these results, the enzyme is classified as a serine protease, as it was inhibited exclusively by serine protease inhibitors.

Table 2 Protease inhibitors effect on purified protease of O. basilicum. Each value represents the mean of triplicates (Mean±Standard Deviation)

Protease Inhibitors	Concentration	Residual activity (%)±SD
No Inhibitor	-	100±0.003
Aprotinin	0.5µM	76.78±0.005
	1µM	58.90±0.007
PMSF	1mM	50.00±0.002
	2mM	7.14±0.001
E-64	50µM	103.75±0.030
IAA	1mM	87.50±0.012
Pepstatin A	10µM	82.14±0.013
	50µM	91.07±0.015
EGTA	1mM	66.07±0.011
	5mM	40.20±0.008
EDTA	1mM	100.92±0.005
	5mM	49.52±0.004

3.6 Effect of different metal ions and determination of IC50 values and Ki constant

To investigate the impact of various metal ions (at a concentration of 50 mM) on the activity of pure *O. basilicum* protease, we added salts for monovalent ions (K^+ and Na^+), divalent ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, and Ba^{2+}), and a trivalent ion (Fe^{3+}) to the reaction mixture. The experiment was conducted at pH 5 and 40°C (Figure 9 A). The protease activity without any added metal ions was considered as 100%. Na^+ ions decreased the protease activity when compared to K^+ ions as shown in result. Similarly effect was demonstrated by HIV-1 protease [41] .This occurs because sodium binds more strongly to acidic residues near the active site, which hinders the binding of substrates.In contrast, the addition of Ca^{2+} and Zn^{2+} significantly increased the activity to 140% and 113%, respectively The effect of 5 mM EDTA and 5 mM EGTA was studied on protease incubated with 5 mM Ca^{+2} ions. The results indicate a reversible effect, with activity decreasing by 52% and 60%, respectively. Similar results were reported in *Artocarpus* protease also [27]. , Mn2+ decrease the proteolytic activity by 40% of *O. basilicum* protease while other divalents have less effect on activity.It was reported earlier for human α3/4 fucosyltransferase III, that presence of Mn2+ alters the catalytic site of proteases, leading to decreased substrate affinity and activity, and this effect is not shown by other divalent cation [42].

Earlier it was studied that iron complexes reduce protease activity by oxidatively inactivating serine proteases, which generates ferryl (FeIVO) species. These ferryls oxidize sensitive amino acid side chains, notably cysteine,

tyrosine, and tryptophan, causing enzyme deactivation [43]. Similarly, the protease activity of *O. basilicum* was reduced by 92% in the presence of Fe^{3+} ions. The inhibition constant (Ki) was calculated to be 0.167 mM using three concentrations of Fe^{3+} (10, 30, and 50 mM). The type of inhibition was determined from a Lineweaver-Burk graph (Figure 9 C), revealing that Fe^{3+} acts as a competitive inhibitor for the protease. Earlier studies reported that iron acts as a competitive inhibitor for carbonic anhydrase obtained from the kidneys of Black Sea trout (*Salmo trutta Labrax Coruhensis*), with a Ki value of 0.7031 mM [44].

Moreover, the half maximal inhibitory concentration (IC_{50}) was measured to assess the effectiveness of an inhibitor in blocking a specific biochemical function. This value indicates the concentration of a heavy metal needed to achieve 50% of the maximum effect [45]. In this study, the IC50 value for iron ions of the purified protease was determined to be 5.8 mM. (Figure 9 B).

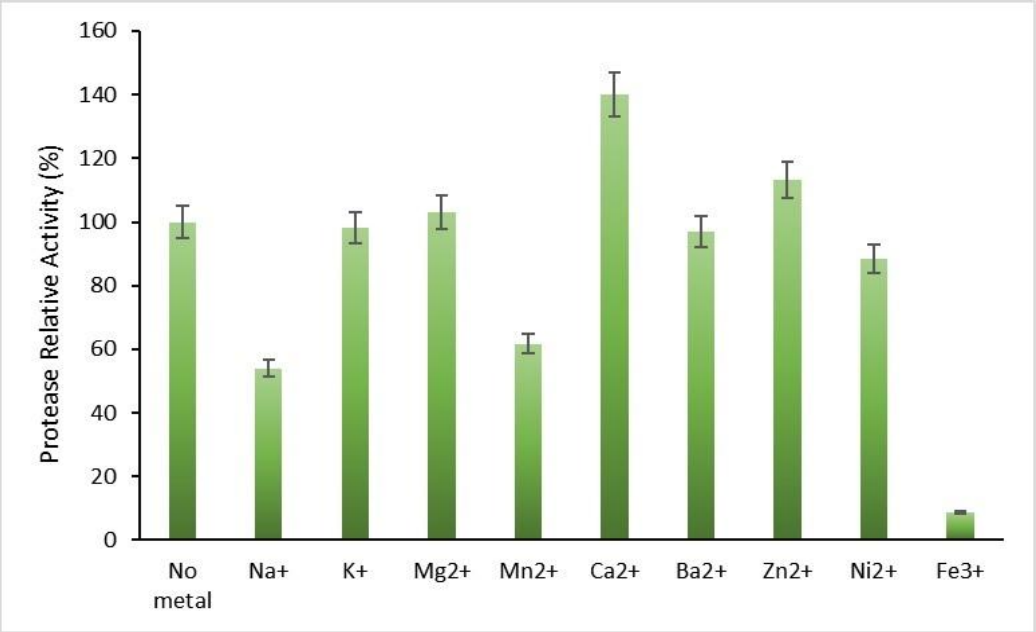


Figure 9 A ***Effect of different metal ions on O.basilicum protease activity. Experiment was performed in triplicates and error bars above column represent Mean±SD***

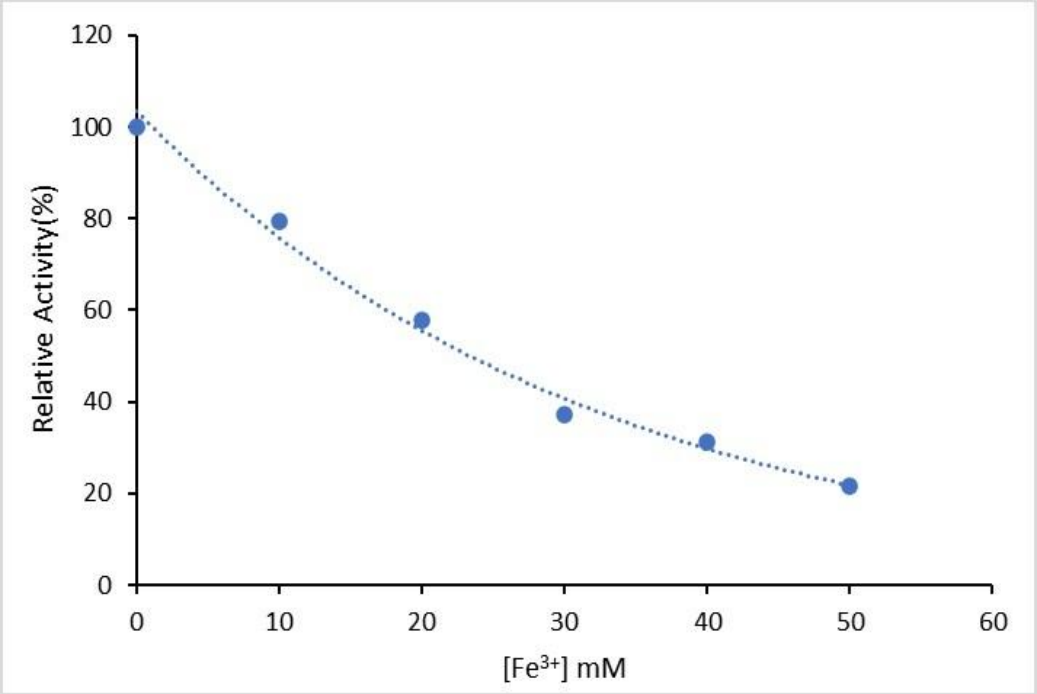


Figure 9 B Activity%-[Fe^{+3}] graph for determining IC_{50} value of Fe^{+3} ion

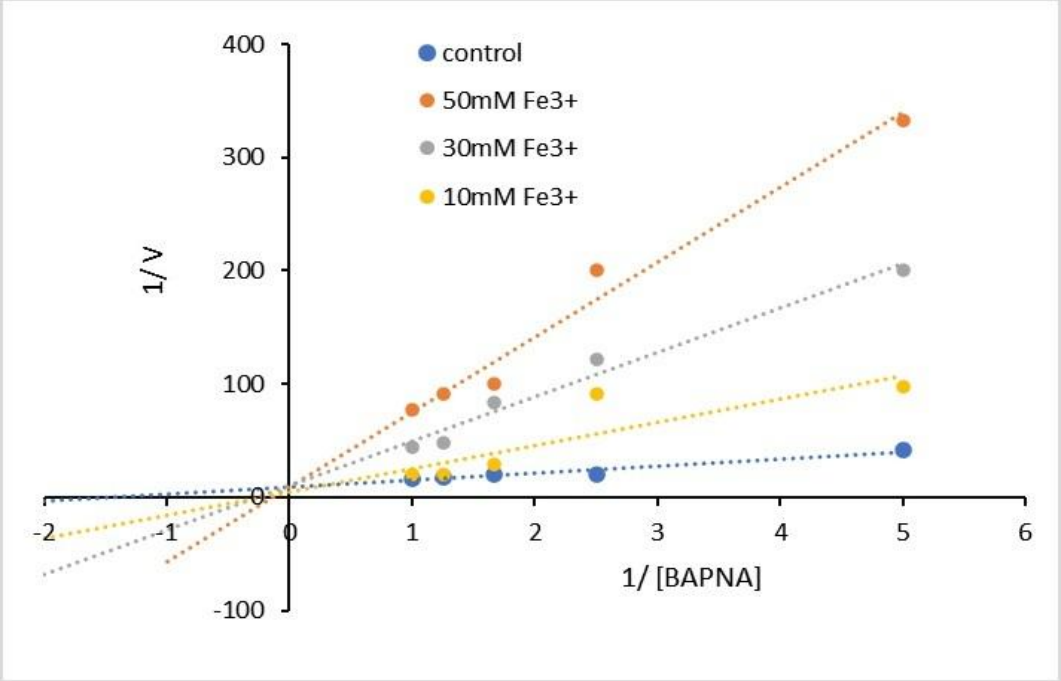


Figure 9 C Lineweaver-Burk graph with increasing substrate (BAPNA) concentrations and 3 different Fe^{+3} concentrations (10,30 and 50 mM) for determination of Ki.

3.7 Effect of various solvents, surfactants and reducing agents on protease activity

Plant proteases are enzymes whose structure and activity can be significantly affected by organic solvents [46]. These enzymes often undergo conformational changes in response to organic solvents, which can alter the

structure of the active site and lead to variations in enzyme activity. The polarity and hydrophobicity of the organic solvent are crucial factors that influence the stability and activity of the enzyme [47]. An investigation into the effect of different organic solvents on purified enzyme activity revealed that the presence of 10% ethanol resulted in an increase of more than 8% in proteolytic activity compared to the control, which contained no solvent. However, enzyme activity decreased to around 60% when 10% concentrations of methanol and butanol were used. In contrast, the enzyme experienced significant inhibition with DMSO and isopropanol, resulting in a 96% loss of activity at a concentration of 10%, compared to the activity observed at 30% and 50% concentrations (Table 3).

Table 3 Effect of different organic solvents, surfactant and reducing agent on purified O. basilicum protease. Each value represents the mean of triplicates (Mean±Standard Deviation)

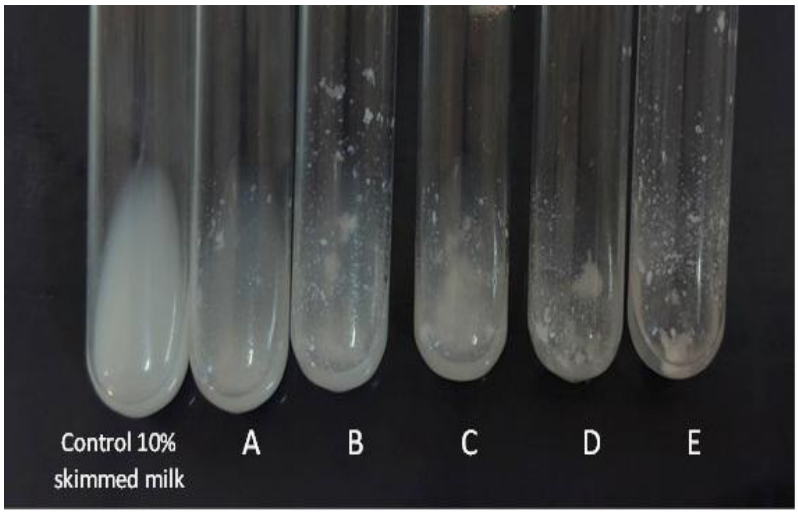
		Concentration	Relative activity (%)±SD
	Control	-	100±0.003
Organic Solvents	DMSO	10%	4.60±0.003
		30%	0.89±0.005
		50%	0.71±0.005
	Methanol	10%	35.71±0.002
		30%	33.03±0.003
		50%	5.35±0.002
	Ethanol	10%	108.92±0.001
		30%	105.35±0.001
		50%	101.78±0.002
	Isopropanol	10%	4.37±0.006
		30%	3.08±0.004
		50%	0.53±0.005
	Butanol	10%	37.58±0.002
		30%	31.25±0.001
		50%	9.82±0.002
Surfactants	Triton-X 100	0.2%	1.96±0.003
		0.5%	1.78±0.005
		1%	1.75±0.002
	Tween-80	0.2%	7.85±0.001
		0.5%	5.89±0.002
		1%	3.57±0.002
	Labolene	0.2%	0.53±0.006
		0.5%	0.51±0.005
		1%	0.50±0.005
	SDS	0.2%	84.82±0.002
		0.5%	53.39±0.001
		1%	32.50±0.001
Reducing Agents	β-ME	2mM	73.39±0.003
		5mM	57.14±0.002
	DTT	2mM	92.85±0.002
		5mM	85.17±0.001

Moreover, the isolated protease maintained over 84% activity at a 0.2% concentration of SDS. However, its enzymatic activity declined as the concentration of SDS increased. Other detergents, such as Triton X-100, Tween 80, and Labolene (a commercially available glassware cleaning detergent), completely inhibited protease activity compared to the control. The enzyme was found to be less stable in the presence of highly

concentrated detergents. Additionally, protease activity was examined using reducing agents like β-mercaptoethanol and dithiothreitol at concentrations of 2 mM and 5 mM. However, β-ME and DTT shows a 43% and 15% decrease in proteolytic activity at a 5 mM concentration, respectively.

3.8 Milk clotting activity

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 [48]. In experiments, 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 10. It was found that as the concentration of the enzyme increased, both the clotting time and the milk clotting units per milliliter (MCU/ml) decreased as mentioned in Table 4. Previous studies have reported that a serine-like protease from *Solanum dubium* seeds can coagulate fresh milk [49]. 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.



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

Table 4 Milk clotting activity of O. basilicum purified protease. MCU: Milk Clotting Unit.

Figure 10 Milk clotting activity of O. basilicum protease. 10% skimmed milk with potassium citrate buffer used as a control. A-E, milk with increasing protease concentration (0.03-0.14mg) as mentioned in Table 4.

3.9 Blood washing activity

Blood stains consist of RBC and serum proteins whom proteases break down into smaller, water-soluble fragments. This process enhances stain removal by allowing surfactants in detergents to work more effectively [50] . 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 11. 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. Additionally, proteases from microbial sources like *Aspergillus tamarii* and cysteine proteases from *Calotropis gigantea* latex have also proven effective in removing blood stains. [51] [52].

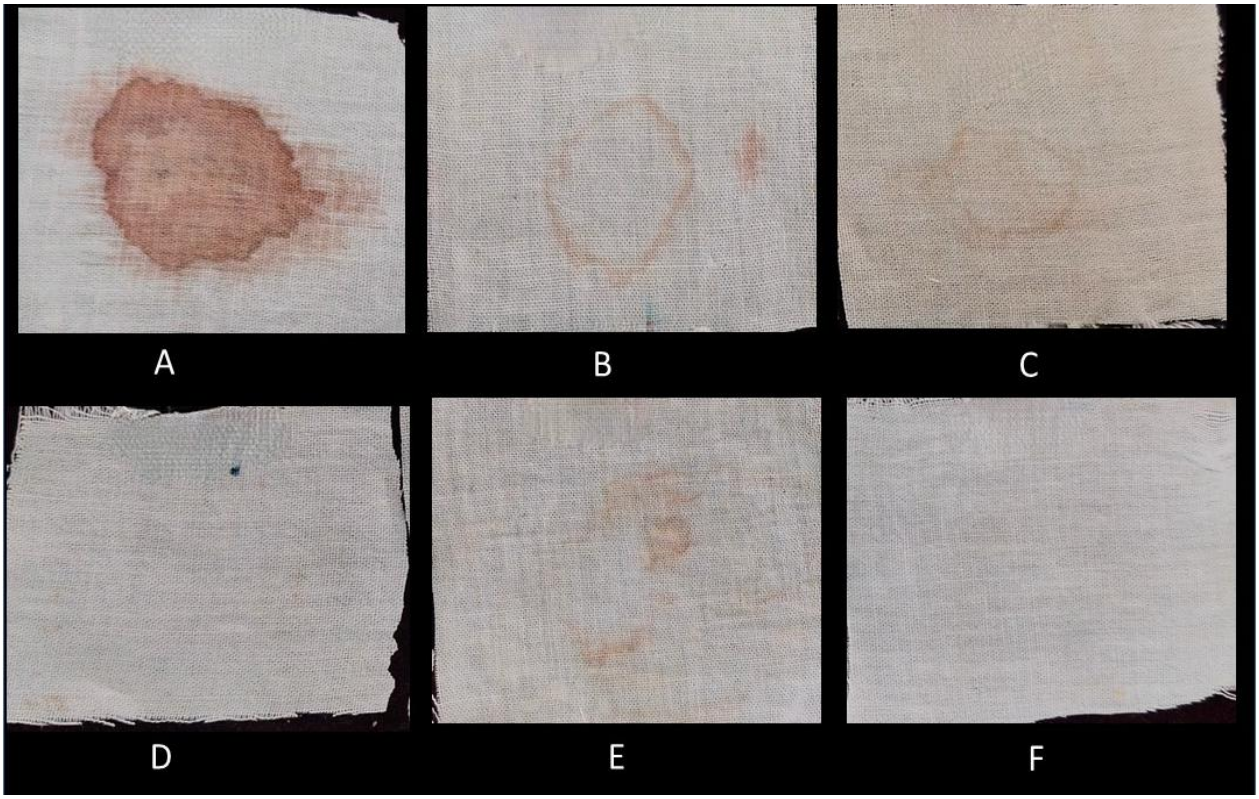


Figure 11 Blood stain washing activity of protease purified from O. basilicum (A) Unwashed blood-stained cloth (B) Tap water washing (C) Potassium citrate buffer pH 5 (D) Phosphate buffer+ 4ml of Protease (0.2mg/ml) (E) Potassium citrate buffer +0.1% SDS detergent (E) 4 ml of Protease (0.2mg/ml) +0.1% SDS detergent.

3.10 Fibrinogenolytic activity

Thrombin cleaves the alpha chain of fibrinogen, a key step in the coagulation cascade. This transformation converts fibrinogen into fibrin, facilitating the formation of a stable clot that prevents blood loss and aids in wound healing. The cleavage releases fibrinopeptide A and exposes binding sites, allowing fibrinogen to polymerize into insoluble fibrin strands [53].Human fibrinogen hydrolysis by protease from *O. basilicum* was visualized using 15% SDS-PAGE (Figure 12). Additionally, a previously reported cysteine protease from *Tabernaemontana divaricata* latex has demonstrated thrombin-like activity [51].*O. basilicum* protease was also tested for fibrinogenolytic activity. Fibrinogen incubated at -15℃ shows the distinct bands of α chain (Figure 12, lane A). Incubation at 37℃ shows autocleavage due to presence of some contaminating enzyme (Figure 12, lane B). α chain is remaining uncleaved but β chain is cleaved and disappear. A thicker γ chain band lower bands at C', C" and C'" are shown. When fibrinogen was cleaved with *O. basilicum* protease then α chain disappears complete (Figure 12, lane C) and C" band also disappeared. Also the intensity of γ band is decreasing indicating cleaveage of γ chain. This clearly indicated that *O. basilicum* protease is capable of cleaving fibrinogen α, β and γ chains. However, since γ- γ dimers are not formed it seems that the site of cleavage is different from that of thrombin [54].

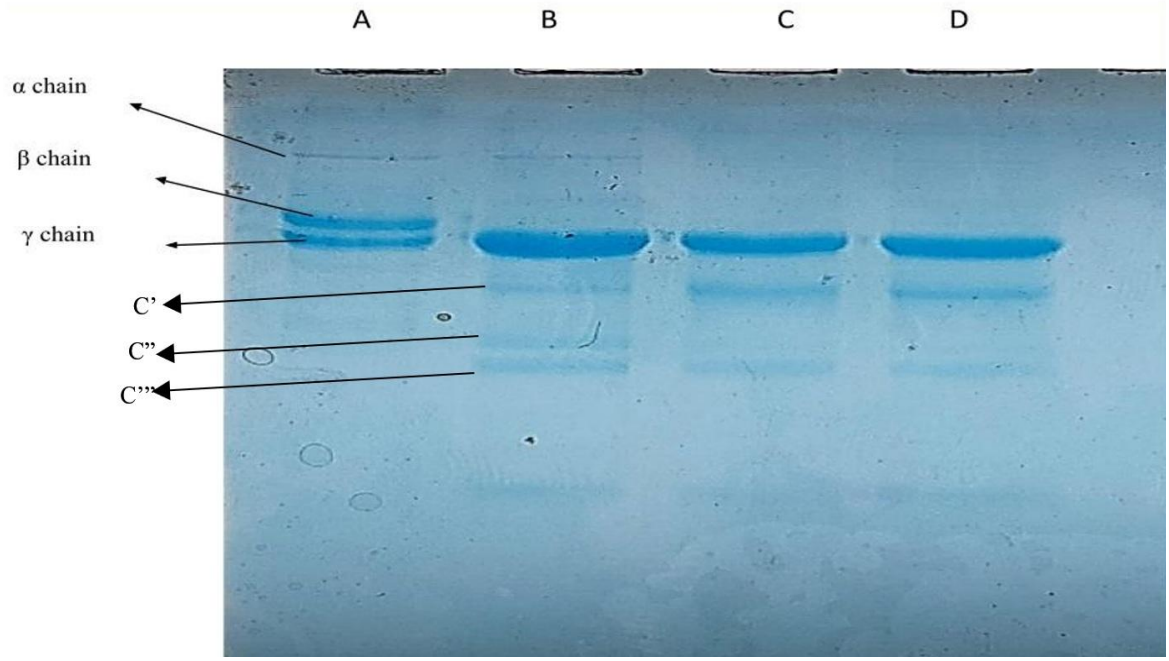


Figure 12 SDS-PAGE (15%) of fibrinogen cleavage by O. basilicum protease. Lane A and B: Control fibrinogen (20µg) incubated at -15°C and 37°C; Lane C and D: Fibrinogen (20µg) incubated with protease 20µg and 10µg respectively. 40 µl was loaded equally in each well.

4 Conclusion

In the recent study, the biochemical characterization of protease from the mucilage and fat-free seeds of *Ocimum basilicum* was conducted. The protease was purified through ammonium sulfate precipitation followed by ion exchange chromatography. The molecular weight of the purified protease was determined to be 60.5 kDa using SDS-PAGE.The protease exhibited narrow substrate specificity and was identified as an endopeptidase, with a Vmax of 0.066 mM/min and a Km of 0.53 mM, using BAPNA as a substrate The impact of pH and temperature on protease activity was tested, revealing an optimum pH of 5 and an optimum temperature of 40°C. The calculated activation energy (Ea), enthalpy change (ΔH), and Q_{10} values (between 30–40 °C) for the protease were 3.82 kcal/mol, 2.16 kcal/mol, and 1.2, respectively, as determined from the Arrhenius plot.The acidic protease demonstrated stability within a pH range of 3 to 7 and at temperatures between 4 to 50°C. The protease activity was found to increase by 56% and 26% when subjected to microwave exposure for durations of 20 seconds and 40 seconds, highlighting its efficiency in food processing. The purified protease was confirmed to be a serine protease, as its activity was inhibited by specific serine protease inhibitors such as PMSF and aprotinin.

Furthermore, the effect of metal ions was studied, revealing that Fe^{3+} exhibited the maximum inhibition as a competitive inhibitor. The influence of organic solvents and reducing agents was also examined. In this study, the protease, when combined with a detergent like SDS, proved effective in removing blood stains. 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 and milk clotting. The enzyme could serve as a potential ingredient in detergents due to its strong cleaning performance and may also find applications in the food industry for cheese production. Additionally, the *O. basilicum* protease cleaves fibrinogen albeit at sites different than thrombin.

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

Author Contributions: Anjali Vyas conducted all the experimental work and prepared the manuscript. Dr. Krishnan Hajela contributed to the concept and design of the experiments and was involved in revising the manuscript. Both authors participated in reviewing and approving the final version of the manuscript before submission.

Funding: The authors declare that this research received no specific grant from any funding agency.

Data Availability: not applicable.

Declarations:

Ethics and Consent to Participate: Not applicable.

Consent for Publication: All the authors have approved the manuscript and agree with its publication.

Competing Interests: No competing interests.

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