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Extraction, Partial Purification, Characteristics, and Antimicrobial Activity of Plant Protease From *Moringa Oleifera* Leaves

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Abstract

Introduction: Proteases are hydrolyzing enzymes and are considered to be one of the most important groups of enzymes for industry and are used in leather, pharmaceutical, and food industry along with detergents, and bioremediation processes. The main objectives of this study were (*i*) to extract, partially purify, and characterize the protease enzyme from Moringa (*Moringa oleifera*) leaves; and (*ii*) to investigate the effect of such an enzyme against some pathogenic bacteria.

Materials and Methods: This enzyme was extracted in a 0.1 M phosphate buffer pH 7. It was left for 24 hours in a refrigerator and was then filtered using filter paper Whatman no. 41. The aqueous filtrate was used to estimate the proteolytic activity.

Results: Purification by ammonium sulfate gave the best results at 50%-70% concentration which had the highest specific activity, highest purification fold with the percentage yield of 45.3%. Gel filtration by Sephadex G-100 gave the best specific activity and the best purification fold with the yield from fraction of 34%-43%. The protease enzyme has optimum pH 7 and optimum temperature 50°C. The enzyme was thermally stable at 40-70°C for 20-30 minutes. Some metal ions were activator on the enzyme like Mn^{2+} (highest), Ba^{2+}, Ca^{2+}, and Na^{+}. The efficacy of protease enzyme was improved when integration with antibiotic against certain bacterial including *Bacillus cereus* (S3), *Staphylococcus aureus, Salmonella typhimurium*, *Escherichia coli* O157:H7, and *Listeria monocytogenes*. Also, *E. coli* O157:H7, *L. monocytogenes*, and *Yersinia enterocolitica* did not show any growth at pH 10.

Conclusions: To conclude, it can be stated that protease enzyme can be considered as a promising agent, cheap, and safe source which is suitable for using in various industries.

Keywords: Antimicrobial, Casein, Characteristics, *Moringa oleifera*, Plants, Protease

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Introduction

Proteases are one of the three main groups of enzymes for industry which are used in the leather industry, detergents, medicinal, food industry, and bioremediation processes. Protease enzymes are very vital in digestion process as they hydrolyze the peptide bonds in the protein foods to release the amino acids that the body need.[1,2]

Proteases from plants had been used in several industries, such as detergent, food, leather, medicinal, and biotechnological application which is because of their high stability in excessive conditions, good solubility, substrate specificity, and activity over broad-ranging pH and temperature ranges. Proteases derived from plants, which are one of the main groups of proteolysis enzymes, are participatory in many regulatory operations in plants. Although they are the biggest class of proteases, the regulatory roles and functions of plant proteases are poorly known and not well understood, which is because of lacking identification. Most of the characterized and isolated plant proteases are broadly used in several procedures in the food industry. Therefore, the proteases are economical for use in industries.[3] Moreover, the search for new proteases from plant sources is continuing, the physiological roles of which the researchers hope to recognize to discover solutions that are usable and cost-effective in industries.[4]

Some enzymes present in our body act against bacteria in infection processes. The localized delivery of the enzyme along with antibiotics may provide better relief than antibiotics as a standalone treatment. However, proteolytic enzymes may reduce bio adhesive because of their mucolytic action.[5]

One of the best known medicinal plants is *Moringa oleifera* which is broadly spread in the tropical regions.[6] Moringa is usually mentioned as either Drumstick tree (describing the shape of its pods) or Horseradish tree (referring to the taste of its roots)[7] while less frequently referred to as 'The Tree of Life' or 'Miracle Tree' due to its economic importance and versatility.[8] It has a mixture of numerous hydrolytic enzymes,

in which proteases are the basic enzymes informed to show pharmacological action.[9,10]

The main objectives of this study were (*i*) to extract, partial purify, and characterize the protease enzyme from Moringa (*M. oleifera*) leaves; and (*ii*) to investigate the effect of such enzyme against some pathogenic bacteria.

Materials and Methods

Moringa leaves used in the present study were collected from the National Research Center, Dokki, Cairo, Egypt. Gel filtration medium (Sephadex G-100) was provided by Aldrich Chemical Company, Inc., USA. *Bacillus cereus* (ATCC133018), *Salmonella typhimurium* 9027, *Staphylococcus aureus* (ATCC 25175) obtained from the stock cultures of the Agricultural Research Center (ARC) in Giza. Also, *E. coli* O157: H7 (ATCC 6933) and *Listeria monocytogenes* V7 and *Yersinia enterocolitica* subsp. *enterocolitica* ATCC9610TM were obtained from Liofilchem S.r.l. Teramo, Italy.

Sample Preparation and Enzyme Extraction

Moringa leaves were collected and then were left to dry at room temperature. After dryness, they were ground with an electronic blinder. Protease enzyme was extracted by soaking 5 g of the dried sample in100 mL phosphate buffer pH 7 for 24 hours in a refrigerator and then filtered using filter paper Whatman no.41. Five grams of each sample of pumpkin seeds, sunflower seeds and, lupine seeds were soaked in 100 mL phosphate buffer pH 7 for 24 hours in a refrigerator. Then they were ground by mortar refrigerator and then filtered using filter paper Whatman no.41. The aqueous filtrates were used to estimate the proteolytic activity.[11]

Determination of Proteolytic Activity

Protease activity of supernatant was estimated by taking 1 mL of the substrate (1% casein dissolved in 0.1 M phosphate buffer pH 7) and incubated at 37°C for 15 minutes. One milliliter of the crude enzyme supernatant was added. The reaction mixture was incubated at 37°C for 20 minutes. The reaction was stopped by adding 2 mL of 0.4 M trichloroacetic acid (TCA) then it was filtered using filter paper Whatman no. 42. The mixture was further incubated at the same temperature for 20 minutes. For the blank, the substrate was precipitated with TCA before adding the enzyme solution and then treated as described above. One milliliter of the filtrate obtained after TCA precipitation was added to 5 mL of 0.4 M sodium carbonate solution and 1 mL of Folin's reagent and incubated at 37°C for 20 minutes for color development and reading absorbance at 750 nm. A standard curve was prepared using 0.1–1.0 mg/mL tyrosine (Sigma-Aldrich, St. Louis, MO) solutions. The proteolytic activity was expressed as units of μg tyrosine per milliliter.[12]

Determination of Protein Content

Protein content was estimated colorimetrically at 595 nm using Coomassie Brilliant Blue G-250 (CBB) and bovine serum albumin (BSA).[13] A known volume of protein sample was completed to a total volume of 1 mL by distilled H_2O. One milliliter of the dye solution was added to the sample and mixed. The blank was prepared by mixing 1 mL of distilled H_2O with 1 mL of the dye. The absorbance of the blue color formed was measured within 1 hour at 595 nm against blank. The standard curve covering the range of 1-50 mg/mL using BSA was carried out.

Partial Purification of Protease Enzyme

Precipitation by Ammonium Sulfate

Crude enzyme extract was precipitated by different concentrations of ammonium sulfate (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90% saturation).[14] A suitable quantity of solid ammonium sulfate was added to the supernatant and then centrifuged at 5000 rpm at 5°C for 20 minutes. The precipitates were collected with a minimum quantity of 0.1 M phosphate buffer pH 7. The supernatant fractions were dialyzed against the same buffer using a dialysis bag and were kept in the refrigerator for 6 days.

Gel Filtration by Sephadex G-100

The dialyzed supernatant fraction was purified by the gel filtration method[15] using Sephadex G-100 from (Aldrich Chemical Company, Inc., USA), column successively (2.5 × 40 cm) (Pharmacia, Uppsala, Sweden). The column was washed with 0.1 M phosphate buffer pH 7. The sample was applied to the column and eluted with 0.1 M phosphate buffer pH 7. Fractions were collected and assayed for enzyme activity and protein concentrate (mg mL^{-1}) at 750 and 280 nm.

Characterization of Protease Enzyme

Determination of the Optimum pH for the Enzyme

Estimating of the optimum pH of the pure enzyme was done by replacing 0.1 M phosphate buffer pH 7 in protease assay with the following buffer: 0.1 M acetate buffer (pH 4- 5), 0.1 M phosphate buffer (pH 6-7-8), and 0.1 M carbonate buffer (pH 9-10). The reaction was done using the proteolytic activity assay method.[16]

Determination of the Optimum Temperature for the Enzyme

The determination of the optimum temperature of the purified enzyme was performed. The reaction mixture of the protease enzyme assay was incubated at different temperatures ranging from 35 to 60°C for 20 minutes. The enzyme activity was then examined at each temperature to determine the protease enzyme's optimal temperature.[16]

Determination of the Thermal Stability for the Enzyme

Aliquots of enzyme extract were heat-treated for 20, 30, 60, and 90 minutes in water baths set at several temperatures of 40 to 90°C followed by fast cooling to 37°C and examined instantly for the remaining enzyme activity.[17]

Effect of Some Salts and Chelating Agents on the Purified Enzyme Activity

Effect of the presence of 5mM concentrations of $ZnSO_4.7H_2O$, $BaCl_2.2H_2O$, EDTA, $FeSO_4.7H_2O$, $CdCl_2$, $CuSO_4.5H_2O$, $MnSO_4.H_2O$, $CoCl_2.6H_2O$, $NiSO_4.2H_2O$, KCl, $CaCl_2.2H_2O$, NaCl and 1,10-penanthroline monohydrates effect on enzyme activity were studied. The enzyme was pre-incubated

with various salts and agents at 37°C for 30 minutes and the samples were placed in ice immediately. The activity was measured under standard assay conditions.

Antibacterial Effect of Protease Enzyme

The combination of protease enzyme from Moringa and the commercial antibiotic (FLUMOX) on pathogenic bacteria was evaluated by the well diffusion technique according to previous studies.[18,19] The nutrient agar was prepared in phosphate buffer pH7, acetate buffer pH4 and carbonate bicarbonate pH10.[20] The test was conducted against 6 common foodborne pathogenic bacteria: *B. cereus* (ATCC133018), *S. typhimurium* 9027, *S. aureus* (ATCC 25175), *E. coli* O157: H7 (ATCC 6933) and *L. monocytogenes* V7 and *Y. enterocolitica* subsp. *enterocolitica* ATCC9610TM. Each microorganism was cultured in brain heart infusion broth and was then spread onto the surface of nutrient agar plates. Three wells (8 mm in diameter) were made on the surface of nutrient agar. the first well was antibiotic (0.1 g/mL) as control. The enzyme with concentration (0.03 g/mL) and the antibiotic (0.1 g/mL) were mixed together by 1:1 v/v and that mixture was used to fill the second well. The enzyme with concentration (0.05 g/mL) and the antibiotic (0.1 g/mL) were mixed together by 1:1 v/v and that mixture was used to fill the third well. After incubation for 24 hours at 37°C, all plates were examined for the inhibition zone around the wells. The results were expressed as mm of the zone of inhibition.

Results and Discussion

Extraction of Protease Enzyme

The extraction of protease enzyme from leaves of *M. oleifera* plant, Pumpkin (*Cucurbita pepo*) seeds, Sunflower (*Helianthus annus*) seeds and, Lupine (*Lupinus polyphyllus*) are presented in Table 1. Extraction of leaves of *M. oleifera* plant with 0.1M phosphate buffer pH 7 gave the highest specific activity, total proteolytic activity and total protein content (0.435 U/mg, 5.058 U and 11.64 mg) respectively. This was followed by sunflower seeds which gave specific activity, total protease activity and total protein content (0.224 U/mg, 1.142U and 5.1 mg) respectively. Pumpkin seeds gave specific activity, total protease activity and total protein content (0.110 U/mg, 0.790 U and 7.2 mg) respectively, while lupine seeds gave specific activity, total protease activity and total protein content (0.218 U/mg, 1.76 U and 8.1 mg) respectively. Thus the Moringa leaves were chosen for further assays.

Partial Purification by Ammonium Sulfate and Gel Chromatography

Ammonium Sulfate Purification

The results indicated that 50%–60% and 60%–70% saturation gave the highest proteolytic activity, specific activity, total activity, and yield (%) (Table 2). The obtained results indicated that the degree of saturation of ammonium sulfate affected specific activity, proteolytic activity, total activity, and purification fold. The obtained results showed that 50%–70% saturation gave the highest specific activity, total activity, yield and rate purification (1.0786 U/mg, 2.8864 U, 45.347% and 4.393) (Table 3). Accordingly, the range of 50%-70% was selected for the potential purification of the proteolytic enzyme for leaves of *M. oleifera* plant. The highest specific activity of protein was achieved at 50% ammonium sulfate saturation.[21] The cotyledonary extract of germinating Indian bean (*Dolichos lablab* L. var *lignosus*) seeds was concentrated by ammonium sulfate fractionation and the 40%–60% saturated fraction with a specific activity, yield, and relative purification (1.06 Unit/mg, 89% and 2.5) respectively.[22] While another study found ammonium sulfate precipitation of alkaline protease from *B cereus* AG1, displayed the highest enzyme activity as well as specific activity at 80% saturation.

Table 1. Screening of Protease Activity in Certain Plant Species

Sample	Total Protease Activity (U)	Total Protein Content (mg)	Specific Activity (U mg^{-1} Protein)
Moringa leaves	5.058^{a}	11.64^{a}	0.435^{a}
Pumpkin seeds	0.790^{d}	7.2^{c}	0.110^{c}
Sunflower seeds	1.142^{c}	5.1^{d}	0.224^{b}
Lupine	1.76^{b}	8.1^{b}	0.218^{b}

Data are means of triplicate measurements. The means with the different capital (a, b, c…) superscript letters within the same column indicate significant ($P \leq 0.05$) differences between treatments.

Table 2. Ammonium Sulfate Precipitation of Protease From Moringa (*Moringa oleifera*) Leaves

Saturation of Ammonium Sulfate (%)	Total Protease Activity (U)	Total Protein (mg)	Specific activity (U mg^{-1} protein)	Yield (%)	Purification Fold
Crude enzyme homogenate	6.365	25.90	0.2455	100	1
0%-10%	0.0504	3.556	0.01416	0.79	0.06
10%-20%	0.0616	1.388	0.0444	0.97	0.18
20%-30%	0.0756	2.6704	0.0283	1.19	0.12
30%-40%	0.4556	3.6316	0.1254	7.16	0.51
40%-50%	0.9032	3.535	0.2555	14.19	1.04
50%-60%	1.550	1.686	0.9194	24.35	3.75
60%-70%	1.336	0.9932	1.3452	20.99	5.48
70%-80%	0.436	0.664	0.6566	6.849	2.67
80%-90%	0.592	0.444	1.333	9.301	5.43

Specific activity (activity/protein content); Total protease activity = activity ×volume of fraction; Total protein = protein content × volume of fraction; Yield = total activity of the fraction/total activity of crud enzyme × 100; purification fold = Specific activity of fraction / specific activity of the crude fraction.

Table 3. Purification of Protease From Moringa (*Moringa oleifera*) Leaves

Purification steps	Total Activity (U)	Total Protein (mg)	Specific Activity (U mg^{-1} Protein)	Yield (%)	Purification Fold
Crude enzyme	6.365	25.90	0.2455	100	1
Ammonium sulfate precipitation (50%-70%)	2.8864	2.676	1.0786	45.347	4.393
GF chromatography on Sephadex G-100	1.33	0.1180	11.271	20.89	4.591

Specific activity (activity / protein content); Total protease activity = activity ×volume of fraction; Total protein = protein content × volume of fraction; Yield = total activity of the fraction / total activity of crud enzyme × 100; purification fold = Specific activity of fraction / specific activity of the crude fraction.

The obtained purification fold was 1.49. The specific activity obtained after dialysis was 72.54 with 3.07 purification fold.[23] Cell-free clear solution (fermentation broth) from newly isolated *Bacillus* sp. BBXS-2 was used for ammonium sulfate precipitation (30%-100%) and showed protease activity at 75% saturation with 1.82-fold purification.[24] The highest enzyme activity, yield, and purification fold were found with a 40%-60% concentration of ammonium sulfate, i.e. 86% protease enzyme yield of 1.65 purification fold and 0.86 U/mg of protein-specific activity were found in kiwi fruit.[25]

Gel Filtration Using Sephadex G-100

The partially purified protease enzyme on a column of Sephadex G-100 showed 2 peaks with proteolytic activity (at fraction 34-43) & (at fraction 51) as shown in Table 3 and Figure 1. The results were obtained when the dialyzed enzyme was loaded on to Sephadex G-100 column equilibrated with 0.1 M phosphate buffer pH 7. Results for enzyme purification from Moringa leaves 1 revealed that fractions (34-43) gave purification fold, yield %, and specific activity (4.591, 20.89% and 11.271 U/mg protein) respectively. Another study has shown a single peak of the purified enzyme on Sephadex G-100.[21] The protease was eluted as a well-resolved single peak of caseinase activity identical with a single protein peak at NaCl concentration of 0.6M at fractions (19-23).[26] The specific activity increased from 62.00 to 86.51 U/mg and the purification fold was found to be 4.01.[27]

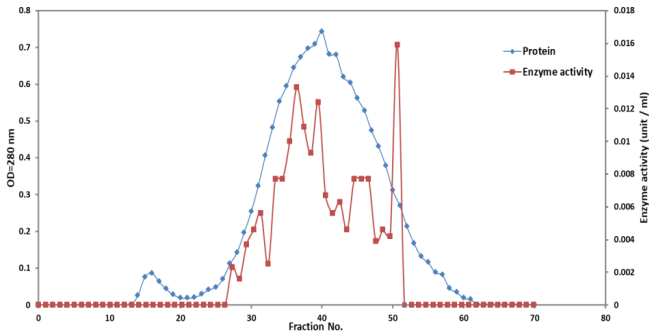

Figure 1. Chromatogram of Moringa (*Moringa oleifera*) Protease by Gel Filtration Chromatography on Sephadex G-100.

Characterization of Protease Enzyme

Determination of the Optimum pH

The pH always has a significant effect on the protease activity enzyme. The effect of several pH values on protease activity has been shown in Figure 2. The enzyme had the highest activity towards denatured casein at pH 7 and so the purified enzyme from Moringa leaves is neutral. Counter to the present study, another study informed that a maximum activity of protease enzyme was at pH 8. Hence, the enzyme was an alkaline protease.[10] The optimum pH of alkaline protease enzyme of *B. cereus* LS2B was pH 8.[28] Plumerin-R had an optimum pH at pH 7 with casein as substrate.[29] The research of the pure protease enzyme from *Bacillus* sp. TBRSN-1 resulted in that it was with an optimum pH of 7.[30]

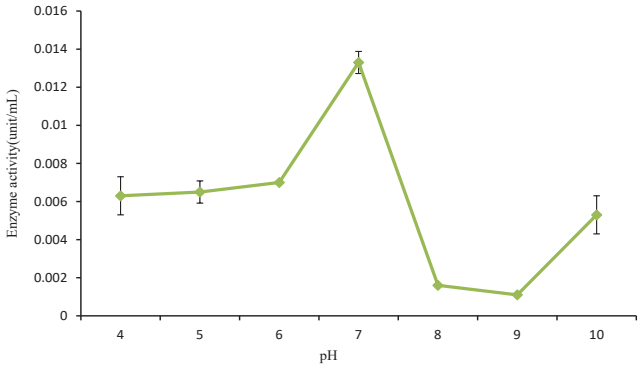

Figure 2. Effect of pH on the Purified Protease From Moringa (*Moringa oleifera*) Leaves.
Data are means ±SD of triplicate measurements.

Determination of the Optimum Temperature

The obtained results (Figure 3) show the effect of different temperatures from 35 to 60 ° C on the purified protease activity of Moringa (*M. oleifera*) leaves.

The enzyme activity was examined based on denaturing

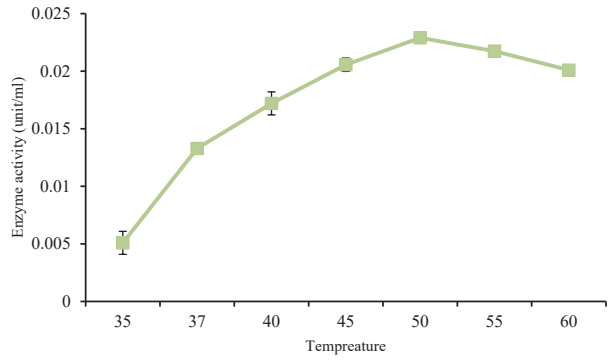

Figure 3. Effect of Different Temperatures on the Purified Protease From Moringa (*Moringa oleifera*) Leaves. Data are means ±SD of triplicate measurements.

casein as a substrate. Accordingly, the denaturation of casein was increased as reaction temperature from 35 to 50°C. The optimum activity was observed at 50°C while the activity slightly decreased at higher than 50°C. This result is in agreement with a study that has an optimum temperature at 50°C.[31] In some studies, the optimum temperature for enzyme activity has been reported to be 70°C, which differs from the findings of this study.[4,21] Also, in some reports, the maximum enzyme activity is at 37°C.[10]

Determination of the Thermal Stability for the Enzyme.

The enzyme was incubated at the temperatures from (40-90°C) for (20-90 minutes). The enzyme activity was stable at 40 to 70°C on the incubation times from 20-30 minutes. The enzyme activity decreased at 40°C and 70°C in incubation times from (30-60 minutes) then it was stable at (60-90 minutes). The enzyme activities at 50°C and 60°C decreased gradually in incubation times from (30-90 minutes). At temperatures 80°C and 90°C the enzyme activity had a sharp decrease at time of incubation from (20-30 minutes) then it was stable at (30-90 minutes) (Figure 4). Similarly, the partially purified protease from the two sources (*B. subtilis* AKAL7 and *Exiguobacterium indicum* AKAL11*)* was active within a broad range of temperatures. The protease was stable from 30 to 50°C. Yet, about 15%-25% of protease activity disappeared when the enzyme was treated at 55°C.These results indicated that the protease from both sources could be applied at a broad range of temperatures exceeding-room temperature.[32] It is a well-known fact that protein conformation changes at higher temperatures, and therefore, causes a reduction in the protease activity.[33,34] Thermal stability outlines showed that the enzyme kept total activity at 40°C while there was 11% decrease in activity at 50°C after 4 hours of incubation. When the enzyme was incubated at 70°C for 210 minutes, it lost its initial activity by half. Another study indicated protease from *Beauveria sp*. was stable at 30°C when incubated for 60 min with optimum temperature for enzyme activity at 50°C.[35] It was reported that a protease from *Fusarium* sp. was stable below 50°C, its optimum temperature for enzyme activity was 50°C. Enzymes derived from mesophilic microorganisms which were thermostable find usage in numerous commercial applications due to their low-cost preparations.[36] The cognizable thermostability of protease could be attributed to the presence of the carbohydrate moiety.[37,38]

Effect of Some Metal Ions and Chelating Agents

The effects of different metal ions on partially purified protease enzyme activity always exhibit differently. Some metal ions and other materials were added in concentration of 5mM as shown in Figure 5. Actually, Ca^{2+}, Ba^{2+}, Na^{+}, Mn^{2+} are activators, whereas Fe^{2+}, Cu^{2+}, EDTA are inhibitors of the partially purified protease enzyme activity. Also, Ni^{2+}, Co^{2+}, Cd^{2+}, K^{+}, Zn^{2+}, and O-Phenanthroline had completely inhibited the enzyme activity. A previous study [21] reported that Cu^{2+}, Ni^{2+}, Fe^{2+}, Ca^{2+} were activators, while Mn^{2+}, Ba^{2+}, Co^{2+} were inhibitors. The enzyme activity was inhibited by Fe^{2+} and Zn^{2+}, while it was raised in the presence of Ca^{2+} and Mg^{2+} and Cu^{2+}.[4,26] Some of the metal ions such as Ca^{2+}, Mg^{2+}, and Mn^{2+} raised and stabilized the protease enzyme activity which was potentially due to the activation by the metal ions. These cations also had been stated to raise the thermal stability of other *Bacillus* alkaline proteases. These results suggested that concerned metal ions apparently protected the enzyme against thermal denaturation and played an important role in maintaining the active confirmation of the enzyme at high temperatures. Other metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Al^{3+}, Na^{+}, Cd^{2+}, and EDTA did not show any noticeable effect on the enzyme activity.

Antimicrobial Test for Protease Enzyme

The protease enzyme from moringa leaves has no antimicrobial effect on the pathogenic bacteria. However, with the combination of the antibiotic, it improved the effect of its efficacy. From Table 4 and Figure 6, the antibiotic and the combination of the antibiotic with the enzyme affect all the tested strains including *B. cereus* (S3), *S. aureus*, *S. typhimurium*, *E. coli* O157: H7, *L. monocytogenes* except *Y. enterocolitica* which is resistant to them. Also, there is no growth of the *E. coli* O157: H7, *L. monocytogenes*, and *Y. enterocolitica* at pH 10. The combination of the antibiotic and the enzyme [FLUMOX (0.1 g/mL) + protease (0.03 g/ml)] had a higher effect compared to FLUMOX (0.1 g/mL) + protease

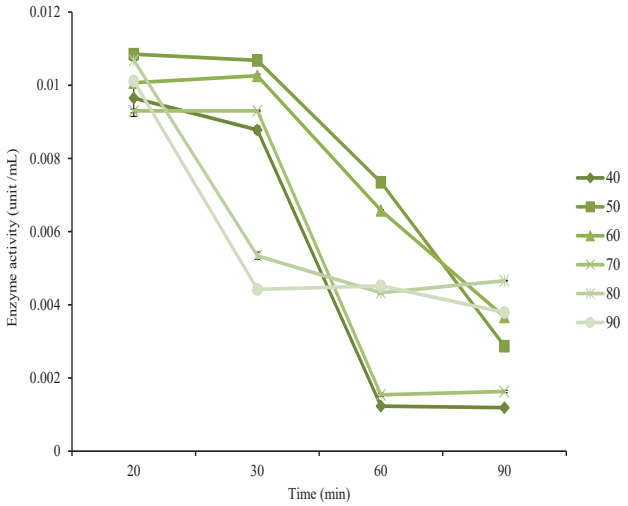

Figure 4. Thermal Stability of the Purified Protease From Moringa (*Moringa oleifera*) Leaves. Data are means ±SD of triplicate measurements.

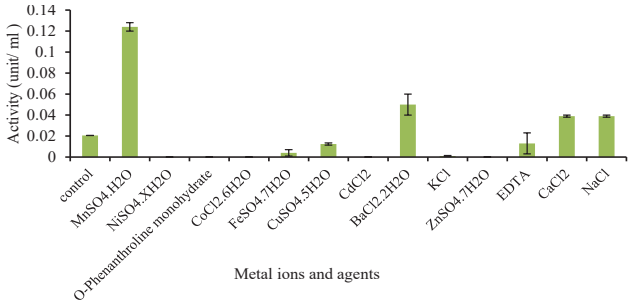

Figure 5. Influence of Some Metal Ions and Chelating Agents on the Purified Protease Enzyme.
Data are means ±SD of triplicate measurements

Table 4. Zone of Inhibition (in mm) of Pathogenic Microorganisms at 37°C in Alkaline, Neutral and Acidic Medium

Pathogenic strains	Inhibition Zone (mm)					
	pH 7 pH4*			pH 10		
	FLUMOX (0.1 g/mL)	FLUMOX (0.1 g/mL)+ protease (0.03 g/mL)	FLUMOX (0.1 g/mL)+ protease (0.05 g/mL)	FLUMOX (0.1 g/mL)	FLUMOX (0.1 g/mL)+ protease (0.03 g/mL)	FLUMOX (0.1 g/mL)+ protease (0.05 g/mL)
Bacillus cereus (S3)	35^{B}	37^{A}	25^{C}	38^{A}	37^{B}	37^{B}
Staphylococcus aureus	42^{A}	40^{AB}	39^{B}	44^{A}	41^{B}	40^{B}
Salmonella typhimurium	40^{A}	40^{A}	41^{A}	45^{A}	40^{B}	38^{C}
E. coli O157:H7	45^{A}	40^{C}	44^{B}	No growth	No growth	No growth
Listeria monocytogenes	45^{A}	41^{B}	40^{B}	No growth	No growth	No growth
Yersinia enterocolitica	R	R	R	No growth	No growth	No growth

*Not tested in pH4. The nutrient agar was not stable in the buffer, it does not solidify readily; R= resistant and normal growth. Data are means of triplicate measurements.

(0.05 g/mL) on all strains at pH 7 and at pH 10 except for *E. coli* O157: H7 and *S. typhimurium* at pH 7 which have the opposite effect.

Previous studies are in agreement with the results of the present study as they demonstrated that prescription of pineapple extract along with antibiotics increased the antibacterial effects of the drug and thus reduced the minimum inhibitory concentration of the antibiotic.[39] It showed antibacterial activity against *Streptococcus* mutant with a minimum inhibitory concentration of 2 mg/mL. Hence, lower doses of the antibiotic would be needed to treat the streptococcal infection. Also, this study demonstrated that pineapple extract containing bromelain enzyme complex is not effective in inhibiting bacterial growth by itself.

Agreeing with the results, another study indicated that the bromelain from the pineapple leaves and stem (in the case of gram-positive bacteria) did not show any antibacterial effect on *Enterococcus faecalis* and *Streptococcus mutans*, but enhanced the activity of the antibiotics ampicillin against *E. faecalis* and chlorohexidine against *S. mutans*.[40] The purified leaves and stem bromelain at 100 % concentration showed antibacterial activity against gram-negative *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*, but did not have any effect on *Serratia marcescens* at any concentration. The inference drawn from this study indicated that bromelain from the leaves and stem of *Ananas comosus* enhanced the activities of the antibiotics namely amoxicillin against gram-negative *A. actinomycetemcomitans* and *P. gingivalis* and ciprofloxacin against *S. marcescens*. Hence, it could be given to the patients suffering from periodontitis along with antibiotics to enhance its activity in the case of those resistant to antibiotics.

Contrary to these results, an inferred that the extracts possess significant growth inhibitory activity on test organisms like *S. aureus* and *Streptococcus pneumoniae* that are gram-positive and *E. coli* and *P. aeruginosa* that are gram-negative bacteria.[41] The efficacy of pineapple and pawpaw extracts against these bacteria may provide a scientific ground for the application of the fruits in the prevention and treatment of bacterial infections caused by the test bacteria. The usage of fruits, especially the ones tested in this research for therapeutic purposes, require continuous examination because the antimicrobial resistance pattern is continually evolving and *E. coli, S. aureus, Pseudomonas aeruginosa*, and *S. pneumonia* undergo progressive antimicrobial resistance continuously. Updated data and antimicrobial susceptibility profiles will continue to be essential to ensure the provision of safe and effective empiric therapy. Fruit-therapy has since been researched and has been observed that it has a reasonable therapeutic implication in combating diseases from which the above test bacteria have been isolated.

In the same context, the gram-positive bacteria (*B. subtilis* and *Streptococcus pyogenes*) are resistant to both crude bromelain extract (1.8 mg/mL) and standard bromelain (2 mg/mL) in neutral pH at an incubation temperature of 37°C. However, the gram-positive bacteria *(Corynebacterium* spp) is inhibited by crude bromelain extract (1.8 mg/mL) in neutral pH by showing an inhibition zone of 8.33 mm at a temperature of 37°C. The crude bromelain extract (1.8 mg/mL) completely inhibited *B. subtilis* and *Streptococcus*, while failed to inhibit the growth of *Corynebacterium* spp at pH value of 10.00 and temperature of 37°C. A combination of the crude bromelain and an antibiotic were most effective compared to either standard or crude bromelain.[20] The bromelain also showed antagonistic effects against *Alicyclobacillus acidoterrestris* strain.[42,43]

Similar to our results, bromelain had also shown antibacterial activity against Streptococcus mutant with a minimum inhibitory concentration of 2 mg/mL.[39] However, the bromelain enzyme extracted from leaves and stem of pineapple did not show antagonistic effect against *Enterococcus faecalis*.[44]

Conclusions

The present study summarizes that protease enzyme would be a promising agent, cheap, safe source, and suitable for use in various industries. Protease enzyme from *M. oleifera* had the highest specific activity among all tested samples. It has an optimum pH at 7 and an optimum temperature at 50°C. The enzyme was thermally stable at 40-70°C for 20-30 minutes. The Mn^{2+} ion highly improves the activity of the enzyme. Actually, it has been revealed that protease enzyme improves the efficiency of the antibiotic FLUMOX. The combination of the antibiotic and the enzyme FLUMOX (0.1 g/mL) + protease (0.03 g/mL) had a higher effect compared to FLUMOX (0.1 g/mL) + protease (0.05 g/mL) on all strains at pH 7 and at pH 10 except for *E. coli* O157:H7 and *S. typhimurium* at pH 7 which had an opposite effect.

Authors' Contributions

AMA-E: Methodology, resources, investigation, formal analysis, writing - original draft**.** DMS: Methodology, supervision, writing- review

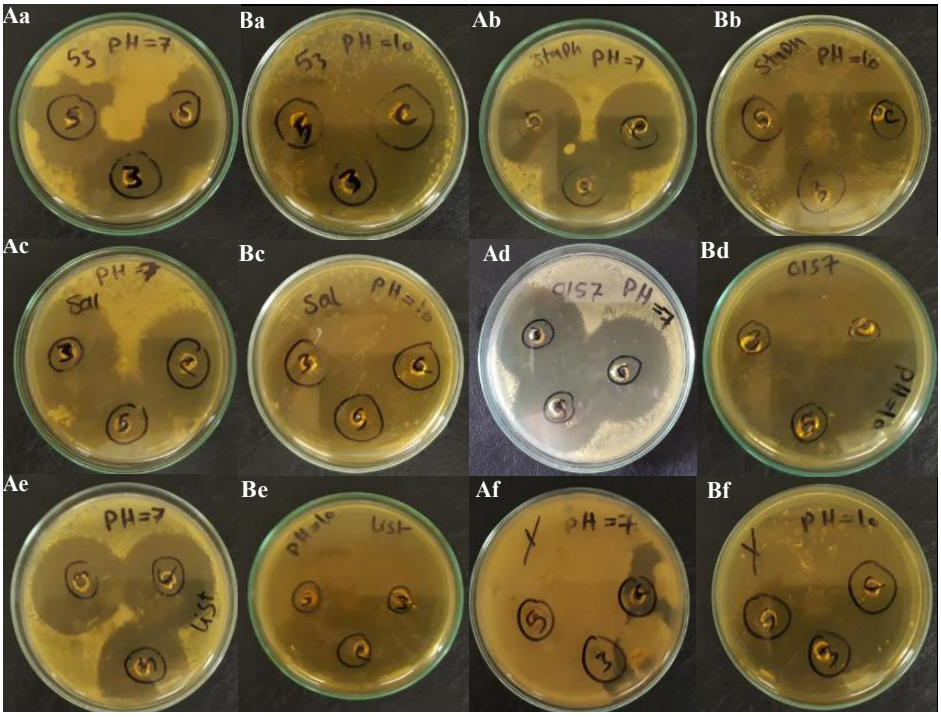

Figure 6. Zone of inhibition (in mm) of Microorganisms at 37°C in **(A)** neutral medium and **(B)** alkaline medium. **C**= antibiotic FLUMOX (0.1 g/mL); **3**= FLUMOX (0.1 g/mL) + protease (0.03 gm/ml); **5**= FLUMOX (0.1 g/mL) + protease (0.05 g/mL). **a:** *Bacillus cereus;* **b:** *Staphylococcus aureus***; c:** *Salmonella typhimurium***; d:** *E. coli* O157*: H7v* **; e:** *Listeria monocytogenes* **; f:** *Yersinia enterocolitica***.**

& editing. OAI: Methodology, visualization, data curation. NSA-R: Methodology, visualization, supervision, data curation, writing - review & editing. EMAE: Methodology, supervision, writing- review and editing.

Conflict of Interest Disclosures

The authors would like to declare that there are no conflicts of interest.

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