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Purification of *Moringa oleifera* Leaves Protease by Three-Phase Partitioning and Investigation of Its Potential Antibacterial Activity

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Abstract

One of plant-based products for dental care is plant-based proteolytic enzymes which are principally proteases. In order not to damage the protein and bioactive content, an efficient method should be employed for their purifications. As such, three-phase partitioning (TPP) was used to purify protease from moringa (*Moringa oleifera*). TPP is an emerging, promising, non-chromatographic and economical technology which is simple, quick, efficient and often one-step process for the separation and purification of bioactive molecules from natural sources. It involves the addition of salt (ammonium sulphate) to the crude extract followed by the addition of an organic solvent (butanol). The protein appears as an interfacial precipitate between upper organic solvent and lower aqueous phases. The various conditions such as ammonium sulphate, ratio of crude extract to t-butanol and pH which are required for attaining efficient purification of the protease fractions were optimized. Under optimized conditions, it was seen that, 35% of ammonium sulphate saturation with 1:0.75 ratio of crude extract to t-butanol at pH 7 gave 4.94-fold purification with 96.20% activity yield of protease in the middle phase of the TPP system. The purified enzyme from *Moringa oleifera* has no antimicrobial effect on the pathogenic bacteria tested. However, this purified enzyme, can

be considered as a promising agent, cheap, and safe source which is suitable for using in various industries.

Keywords

Three-Phase Partitioning, *Moringa oleifera*, Protease, Protein Purification, Antimicrobial

1. Introduction

We are witnessing dramatic progress in the production of new enzyme-catalyst over recent years with the present high demand for better use of renewable resources and the burden on industry to work within an environment-friendly [1]. Proteolytic enzymes, often called proteases or peptidase or proteinase, remain one of the major groups of industrial enzymes and occupy 60% of the total global enzyme sale. They have a wide range of application in various industries to make a change in product taste, texture, and appearance and in waste recovery. Besides this, they have extensive applications in food industry, laundry detergents, leather treatment, bioremediation processes, and pharmaceutical industry [2]. The applications of plant based proteolytic enzymes in dental oral care have been tested as well [3]. Belonging to Moringaceae family, *Moringa oleifera* is a fast growing, perennial, angiosperm tree that may grow as high as 7 to 15 m and reach a diameter of 20 to 40 cm at chest height. It is generally regarded as a vegetable, high protein type, a source of cooking oil in the developing world and a medicinal plant called as "miracle trees", "man's best friend", "medicine chest". Indigenous in South Asia, recently, it has garnered medical and socioeconomic attention in the tropics and subtropics such as in Western, Eastern, and Southern Africa; tropical Asia; Latin America; the Caribbean; and the Pacific Islands where it is now being widely cultivated and has naturalized [4] [5]. Taking into account the importance of *Moringa oleifera* which is medicinally valuable with overlapping uses in treating myriads of ailments and diseases, it is necessary to conduct a research on how to use its leaves as a protein source and natural antimicrobial. As such, in order not to damage the protein and bioactive content, an efficient method should be employed. Several conventional purification methods are used for recovering and purifying proteins, enzymes, enzymes inhibitors, oils and carbohydrates. These conventional techniques such as ammonium sulphate precipitation followed by size-exclusion and ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, electrophoresis or some combination of these methods are considerable cost, are time consuming, burdensome providing low yields and not suitable for large scale production [6]. As such, the development of new separation and purification techniques which can reduce the processing time, can be more effective, can have lower energy input, can be non-toxic and can possess environmentally friendly characteris-

tics has been a matter of great interest. One of the recent techniques which have been attractive to the scientists is the use of Three-Phase Partitioning (TPP) [7]. Three phase partitioning is an emerging, promising, non-chromatographic and economical technology which is simple, quick, efficient and often one-step process for the separation and purification of bioactive molecules from natural sources [8]. The principle of this technique consists in mixing the aqueous solution containing proteins with solid salt such as ammonium sulphate and a water-miscible aliphatic alcohol usually t-butanol in order to obtain three phases. This three-phase system formed comprises upper, middle and lower phases. The upper organic phase (t-butanol) which is containing nonpolar and hydrophobic compounds (pigments, lipids etc.) is separated from the lower aqueous phase that contains polar compounds such as proteins, saccharides and other polar compounds by an interfacial protein precipitate. The partitioning process is affected by the hydrophobicity, molecular weight, charge, isoelectric point of a protein and also temperature and content of the separation medium (salt type and concentration, organic solvent type and concentration, pH and protein amount) and also by the physical conditions of the phase system proteins show different partitioning behaviour in TPP systems. It has been used to purify a number of proteins and enzymes with high recovery and purity levels [9] [10] [11]. Therefore, the purpose of this work is to extract, purify protease enzyme from *Moringa oleifera* leaves and to investigate its antibacterial activity.

2. Material and Methods

2.1. Chemical Material

Casein, bovine serum albumin (BSA), Ammonium sulphate, tert-butanol were obtained from Sigma-Aldrich Chemical Company (St. Louis, USA). All the chemical and reagents were of analytical grade.

2.2. Plant Material and Microorganisms

The fresh mature *Moringa oleifera* leaves were collected from Abomey-Calavi district in Benin. These leave were authenticated under vouchers numbers YH 739 HNB by Benin national herbarium, University of Abomey-Calavi, Benin. The reference microorganisms used include bacteria and a yeast: *Microcus luteus* ATCC 10240; *Staphylococcus aureus* ATCC 29213; S*treptococcus pneumoniae* ATCC 49619, *Proteus mirabilus* A24974; *Pseudomonas aeruginosa* ATCC 27853; *Escherichia coli* ATCC 25922, *Candida albicans* MHMR. These strains are from the collection of Laboratory of Biology and Molecular Typing in Microbiology.

2.3. Methods

2.3.1. Protein Determination

The total protein content of the solutions at different stages of protein purification was determined by Bradford method [12]. In this assay series of BSA standard solutions (0.02 - 0.25 mg/ml) were used to prepare the standard curve.

Bradford assay was performed by adding 2 ml of Bradford reagent to 0.1 ml of each standard solutions or unknown protein solution and mixed by using vortex mixer. The blank was prepared by mixing 2 ml of distilled H_2O with 0.1 ml of the dye. All tubes are kept at room temperature in dark conditions for 10 minutes and the absorbance was read at 595 nm. The protein content was calculated from the bovine serum albumin (BSA) standard curve.

2.3.2. Protease Activity Assay

The enzymatic activity of protease was determined as described by [13] with slight modifications. In this assay, tyrosine (500 - 25 µM) was used as a standard and casein (0.65%) as substrate. Briefly, 2.5 ml of casein is added to 0.5 ml protein solution or distilled water for the Blank (3 ml of standard solution at different concentration). After mixing by vortex, they were incubated in a water bath at 37˚C for 30 minutes. Then, 2.5 ml of 10% trichloroacetic acid is added to all test tubes to stop the reaction. After mixing the samples were centrifuged at 9000 rpm for 2 minutes at 4˚C. To 0.5 ml of the supernatant, 2.5 ml of 0.5 M sodium carbonate and 0.5 ml of three fold diluted Folin-Ciocalteu reagent were added. After mixing, the samples were incubated at 37˚C in dark condition during 30 min for colour development. The quantity of liberated tyrosine was determined spectrophotometrically at 660 nm. One unit (1 U) of protease activity was described as the amount of enzyme required to liberate 1 µg/min tyrosine from casein per min under standard analysis conditions. The information given for protease activity assays are mean values of triplicate assays in which the standard deviations were always smaller than 10%.

2.3.3. Preparation of Crude Protease Extract

The crude extract of protease was obtained as described by [14] with slight modifications. So, after dryness at room temperature, the *Moringa oleifera* leaves were ground with an electronic blinder. Protease enzyme was extracted by soaking 10 g of the dried sample in 200 ml phosphate buffer (50 mM, pH 7.5) for 24 hours for 4˚C. The homogenate was filtered using filter paper Whatman no.1. Then, the filtrate was centrifuged at 9000 rpm for 15 min at 4˚C. The supernatant represented the protease extract and used for three phase partitioning. The protein concentration and specific activity of the enzyme were determined as 1.94 mg/ml and 0.95 U/mg, respectively.

2.3.4. Three Phase Partitioning of Protease

TPP experiments were carried out as described by [15] with slight modifications. Thus, the crude extract of protease (2 ml containing 3.68 U and 3.88 mg protein) was saturated at room temperature with ammonium sulphate to the desired level at room temperature. The mixture was vortexed to dissolve the salt. Then, the t-butanol was added. After vortexing gently, the mixture was allowed to stand for 1 hour at room temperature. Afterwards, the mixture was centrifuged at 4500 rpm for 10 min at +4˚C to facilitate the separation of the three phases. Then, the upper t-butanol phase was removed by a Pasteur pipette. The lower aqueous phase

and the interfacial phase were separated carefully. The interfacial precipitate was dissolved in phosphate buffer (50 mM, pH 7.5). Each of phases was analysed for enzyme activity and protein content. The experimental conditions at which the highest enzyme activity observed were selected for the further experiments.

1) Effect of Ammonium Sulphate Saturation

The effect of salt concentrations (20%, 25%, 30%, 35%, 40%, 45% and 50%) (w/v) on the crude enzyme extract for the TPP at the constant crude extract: t-butanol ratio (1.0:1.0) was investigated.

2) Effect of t-butanol

TPP experiments were performed by applying various t-butanol ratios (crude extract: t-butanol; 1.0:0.5, 1.0:0.75, 1.0:1.0, 1.0:1.25, 1.0:1.5, and 1.0:75) with a constant ammonium sulphate saturation at 35% at room temperature. The experimental conditions at which the highest enzyme activity observed were selected for the further experiments.

3) Effect of pH

After, the t-butanol and ammonium sulphate, effects with different pH values of medium study were tested. Crude extract was saturated with 35% ammonium sulphate and pH was adjusted to 3, 4, 5, 6, 7, 8, 9 and 10, then 1.0:0.75 t-butanol was added and the best pH value on the partitioning behaviour of proteases was investigated.

2.4. Antibacterial Effect of Protease Enzyme

The evaluation of the antimicrobial effect of the extracts on the strains was performed according to the disc diffusion method described by [16] with slight modification.

2.4.1. Preparation of Bacterial Suspension

For each strain, an 18 h pre-culture was prepared by inoculating to 1 mL of Muller Hinton broth one young colonies of each strain obtained from a 24-hour culture on Muller Hinton agar. The broths were incubated for 18 h at 37˚C. The inocula were prepared by diluting the 18 h pre-cultures in saline solution to achieve a turbidity of 0.5 McFarland (*i.e.* 10^8 CFU/mL for bacteria) and 10^7 CFU/mL for *C. albicans*. Each bacterial inoculum was used within 15 min to 30 min after the preparation.

2.4.2. Sensitivity Test

Under aseptic and sterile conditions (equipment and environment), 1 ml of the bacterial culture reduced to 10^6 CFU/ml (fungal culture 10^7 CFU/mL) with distilled water was used to inoculate a petri dish containing Mueller-Hinton agar approximately 3 mm thick. In each petri dish, 5 sterile blotting paper discs (6 mm diameter) were placed under aseptic conditions. The discs were aseptically impregnated with 30 µL of purified protease enzymes using a micropipette. The petri dishes were left for 15 minutes at room temperature for pre-diffusion. Incubations were ideally carried out within 15min of disc deposition, but not ex-

ceeding 30 min at 37˚C for 24 hours. After the incubation period, the plates were examined for any zones of inhibition. Each experiment was performed in triplicate.

3. Results and Discussion

Currently, there is a wide range of herbal products that are considerable importance in food and pharmaceutical industries including dental care. Among these herbal products, plant-based proteolytic enzymes, which are mainly proteases have received special attention due to their properties [17]. In order to purify the protease enzyme from *Moringa oleifera* leaves for its potential activity as antibacterial agent, three phase partitioning (TPP), a novel strategy is used. TPP is proved to be an excellent procedure for enzyme extraction and purification [18] [19]. In TPP system, the upper organic phase contains non-polar compounds like pigments, lipids, enzyme inhibitors etc. The polar compounds such as saccharides are generally partitioned in the lower phase. The middle phase is composed of precipitated proteins and enzymes [20]. In the present study, the *Moringa oleifera* protease is dominantly partitioned in the middle phase of the system. In order to obtain suitable phase system for efficient TPP, various process parameters including the amount of $(NH_4)_2SO_4$ for the precipitation, crude extract to t-butanol ratio and also pH were optimized. The starting protein concentration (containing 1.84 units/ml of protease activity) was 1.94 mg/ml.

3.1. Effect of Ammonium Sulphate Saturation on *Moringa oleifera* Protease Partitioning

One of the most common salts used in TPP is ammonium sulphate as it is responsible for protein-protein interaction and precipitation by salting-out mechanism [7]. As such, in order to determine the best ammonium sulphate saturation, salt concentration was studied by varying it from 20% to 50% (w/v) while keeping other experimental conditions constant (crude extract to t-butanol ratio 1:1; v/v, constant temperature and pH) and the results are shown in **Figure 1**. As can been seen from this figure, the concentration of ammonium sulphate is important for the TPP process. The purification fold and activity recovery are extremely improved up to 3.74 and 88.35 respectively when the ammonium sulphate is increased to 35% (w/v). Similar results were obtained by [21] [22]. At lower concentrations of salt (20% w/v), the recovery and fold purification are less (30.07% and 1.92 respectively). Also, further increase in salt concentration up to 50%, decreases the recovery percentage from 88.35% to 41.25%. Several researchers have noticed that an increase in salt saturation generally lead to a decrease in purity [23] [24]. This might be attributed to the irreversible denaturation of protein because of excessive dehydration. For this reason, it was decided to continue the enzyme isolation in the middle phase with 35% ammonium sulphate concentration.

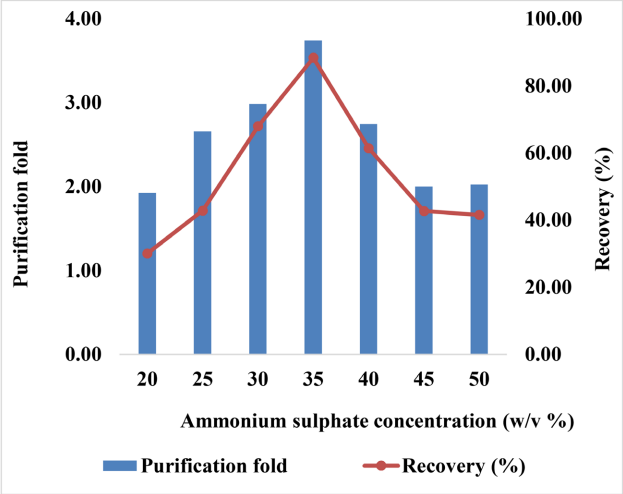

Figure 1. Effect of varying saturations of ammonium sulphate on the degree of purification and activity recovery of Moringa oleifera leaves proteases. The crude extract (2 mL containing 3.68 U) was brought to different levels of saturation ammonium sulphate (20%, 25%, 30%, 35%, 40% 45% and 50%) and t-butanol was added in the ratio of 1:1 (v/v) with respect to the volumes of the aqueous extract. The mixture was kept at room temperature for 1 h for three-phase partitioning. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content.

3.2. Effect of Crude Extract to T-Butanol Ratio on *Moringa oleifera* Protease Partitioning

The particularity of TPP is the use of tert-butanol as it exhibits a higher boiling point (84˚C) and is much less flammable compared with hexane, acetone, methanol, and ethanol [25]. It is a C4 non-ionic kosmotrope that is very soluble and miscible in water, but after the addition of solid salt, becomes hydrated and acts as a differentiating solvent. Furthermore, due to its size and branched structure, it does not cause denaturation of the partitioned enzyme as it is unable to permeate inside the folded three dimensional structure of protein due to its larger molecular size. So, it can combine with ammonium sulphate to separate organic impurities, such as enzyme proteins and pigments from a crude extract [26] [27]. Thus, after the selection of $(NH_4)_2SO_4$ saturation, the effect of crude extract to t-butanol ratio for plant-protease partitioning in the TPP system was investigated. The crude extract of protease: t-butanol ratio is changed (1.0:0.5; 1.0:0.75; 1.0:1.0; 1.0:1.25; 1.0:1.50; 1.0:1.75) by maintaining the salt concentration of 35% (w/v). As shown in **Figure 2**, the highest extraction yield of protease (88.47%) was obtained by using 1.0:0.75 crude extract to t-butanol ratio. A previous study for the three-phase partitioning of ficain from Ficus carica latex is in agreement with our results that 1.0:0.75 (v/v) ratio is sufficient to get best partitioning results (purification fold 6.4% and 167% of activity recovery) [28]. However, when t-butanol to crude extract ratio is less than 1.0:0.75, the extraction yield of protease is less. The reason is because the less amount of t-butanol is not adequate for separation as it does not create effective synergies with ammonium sulphate according to Yang. Contrary to our results, the ratios of 1.0:0.5 [29] 1.0:1.0 and

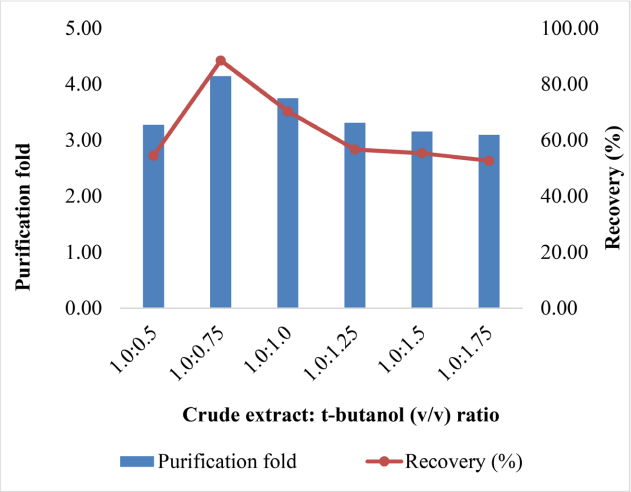

Figure 2. Optimization of crude extract to t-butanol ratio for the recovery of *Moringa oleifera* proteases. Various amounts of t-butanol were added to crude extract (2 mL containing 3.68 U) and saturated with 35% ammonium sulphate in the following volumetric ratios 1.0:0.5; 1.0:0.75; 1.0:1.0; 1.0:1.25; 1.0:1.50; 1.0:1.75. The mixture was kept at room temperature for 1 h for three-phase partitioning. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content.

1.0:1.25 [15] were also selected by researchers since they gave the highest recoveries and fold purification. From these findings, the ratio of crude extract to t-butanol of 1.0:0.75 was selected with $(NH_4)_2SO_4$ at 35% (w/v) for investigating the effect of pH value on the TPP system.

3.3. Effect of pH on *Moringa oleifera* Protease Partitioning

Another important step in protein purification with TPP is to determine the effect of ambient pH on separation [24]. Protein concentration by salting out depends on the sulphate concentration and pH-dependent net charge of the proteins. Electrostatic forces and binding of sulphate anions to cationic protein molecules, which promote macromolecular contraction and conformational shrinkage, are the main causes of the strong sulphate pH dependency in salting out. Proteins tend to precipitate most readily at their pI (isoelectric point). Below the pI, proteins are positively charged and can be quantitatively precipitated out by TPP [20] [25]. On the other hand, negatively charged proteins are more soluble and not easily precipitated [7] [30]. A pH range between 3.0 and 10.0 is selected to search the impact of pH on the purification of *Moringa oleifera* protease and the results are shown in **Figure 3**. The protease was partitioned to the interphase with giving 4.94-fold purification and 96.08% activity recovery of the enzyme at pH 7.0. When the pH of the medium increased from 3.0 to 7.0 the activity recovery (%) of protease gradually increased in the middle phase and then declined by increasing the pH. The similar observations are also noticed for the three-phase partitioning of zingibain. The zingibain was selectively partitioned into the interphase at pH 7.0 with increased yield (215%) [15]. Counter to the present study, the study of [31] on extraction, purification, and activity of pro-

tease from the leaves of *Moringa oleifera* informed that a maximum activity of protease enzyme was at pH 8. The possible reasons for the findings of this study are contrary to those of [31] is the method used. In fact, they used a conventional purification scheme including ammonium sulfate precipitation followed by chromatography, whereas we used TPP in our study.

3.4. Overall Purification of *Moringa oleifera* Protease

The overall purification profile of protease from *Moringa oleifera* leaves by TPP is summarized in Table 1. As understood from the obtained results, it can be said that protease has tendency to concentrate in the interfacial phase of the TPP. The optimum process parameters are 40% (w/v) of ammonium sulphate saturation, 1.0:1.0 (v/v) protease: t-butanol and pH 7.0. Under this optimized

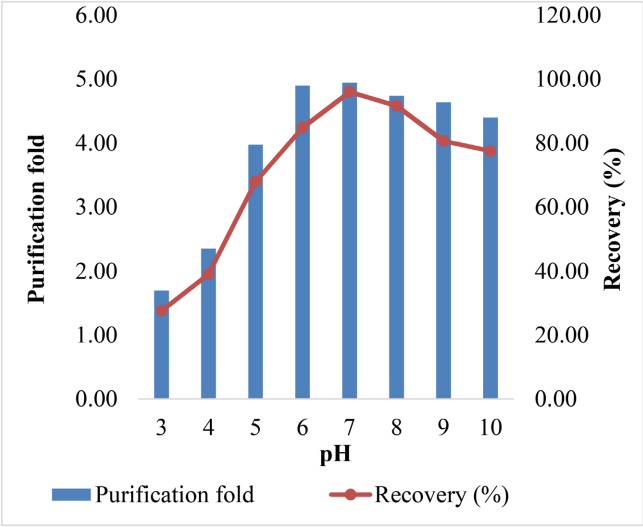

Figure 3. Influence of pH on the degree of purification and activity recovery of Moringa oleifera proteases. The ammonium sulphate (35%, w/v) was added to the crude extract of protease (2 mL containing 3.68 U). The pH of the medium was adjusted to different pH values (3 - 10). This was followed by addition of t-butanol in a ratio of 1:0.75 (crude extract to t-butanol). The mixture was kept at room temperature for 1 h for three-phase partitioning. The interfacial precipitates formed were collected and then estimated for enzyme activity and protein content.

Table 1. Overall purification of proteases from *Moringa oleifera* by three-phase partitioning[a].

Purification step	Total activity (U)	Total protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	3.68	3.88	0.95	1	100
Interfacial phase of TPP	3.54	0.754	4.69	4.94	96.20
TPP aqueous phase	0.002	0.26	0.01	0.10	0.06

[a]The ammonium sulfate (35%, w/v) was added to the extract of Moringa oleifera protease (2 containing 3.68 U), after then pH was adjusted to pH 7. Afterwards, t-butanol was added to the enzyme extract to the ratio of 1:0.75 (v/v) (crude extract: t-butanol). Three phases were spied on clearly. The upper phase was decanted and then the lower aqueous phase and interfacial precipitate were tested for enzyme activity and protein amount. Each experiment was carried out in triplicate and the difference in the readings was less than ±10%.

condition, the protease is concentrated and partitioned with the highest activity recovery and purification fold of 96.20% and 4.94, respectively. There are several studies that related to the purification of many proteases from several sources (plants animal, microbial) with TPP. Under optimized conditions these proteases were extracted, concentrated and purified by using TPP with various activity recovery and purification fold degrees [15] [26] [32].

3.5. Antibacterial Test of Protease Enzyme

The protease enzyme from *Moringa oleifera* leaves has no antimicrobial effect on the pathogenic bacteria tested namely *Microcus luteus* ATCC 10240; *Staphylococcus aureus* ATCC 29213; *Streptococcus pneumoniae* ATCC 49619, *Proteus mirabilus* A24974; *Pseudomonas aeruginosa* ATCC 27853; *Escherichia coli* ATCC 25922, *Candida albicans* MHMR. Similar results were obtained by [33] through extraction, purification of bromelain from pineapple and determination of its effect on bacteria causing periodontitis. Also the findings of [34] and [14] indicated that the plant proteases alone did not show any antibacterial effect on pathogenic bacteria.

4. Conclusion

TPP employed for the extraction and purification of protease from moringa (*Moringa oleifera*) has shown potential to be an attractive process as the primary purification step. The enzyme was efficiently partitioned in the middle phase of TPP with 35% (w/w) ammonium sulfate saturation, 1:0.75 (v/v) ratio of crude extract: t-butanol at pH 7. TPP is a relatively recent but fast developing, simple, cost-effective, quick and also a non-chromatographic separation technique which is economical for protein purification. Thus, this technique has been widely used for the recovery and purification of various enzymes from newer sources that have many advantages in comparison to traditional separation and purification techniques like fast, simple, scale-applicable and economic. In current study, it has been revealed that *Moringa oleifera* protease enzyme has no antimicrobial activity on the strains tested. However, there is a need further studies to provide a scientific ground for the application of the *Moringa oleifera* leaves protease in the prevention and treatment of bacterial infections.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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