Isolation and Characterization of Protease Enzyme from Leaves of *Moringaoleifera*Lamk. (Dant-da-lun)

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Abstract

The aims of this research are to isolate protease enzyme and to study its enzymic properties and its application. Protease enzyme was isolated from leaves of *Moringa oleifera* Lamk. by using sodium chloride salt solution. Then successive ammonium sulphate precipitation method 20 % and 70 % was used. The leaves of *Moringa oleifera* Lamk. were collected from Aung San Quarter, Natmauk Township, Magway Region. Protease activity was determined by spectrophotometrically at 480 nm and casein was used as a substrate. The maximum wavelength, λ_{max} of tyrosine was found to be 480 nm by using spectrophotometer. The calibration curve of standard tyrosine was constructed. The protease activity was found to be 12.5 μmol min^{-1} mL^{-1} at final precipitation process. The protease enzyme had reaction time of 30 min. The optimum temperature of protease enzyme was found to be 45 ℃ at optimum pH 5.5. It was found that enzyme concentration increased protease activity also increased. The maximum velocity, V_{max} and Michaelis-Menton constant, K_m of protease enzyme were found to be 1.52 x 10^{-3} M min^{-1} and 0.85 x 10^{-2} g mL^{-1}, respectively. Protease enzyme can be used for depilating agent in leather processing and digestion of natural protein.

Keywords: protease, enzymic properties, precipitation method, tyrosine

Introduction

In this reserach, protease enzyme was isolated from leaves of *Moringa oleifera* L. and studied its enzymic properties. The leaves of *Moringa oleifera* L. were collected from Aung San Quarter, Natmauk Township, Magway Region. *Moringa oleifera* L. is one of the best medicinal plants in the tropical regions. It contains a mixture of several hydrolytic enzymes, in which protease are the key enzymes reported to show pharmacological activity (Bijina and Chandrasekaran, 2011).

Enzymes are complex protein molecules present in living cells where they act as catalysts in bringing about chemical changes in substance. Enzymes are produced by the cell of plants and animals. Most enzyme reaction rates are millions of times faster than those of comparable uncatalyzed reactions (Bairoch, 2000). Like other proteins, enzymes consist of amino acids linked together by peptide bonds. An enzyme molecule may contain one of these of paptide bonds. Six classes of enzymes are oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases.

Protease also known as proteinases or proteolytic enzymes, are a large group of enzymes. Protease belongs to the class of enzymes, hydrolases, which catalyses the reaction of hydrolysis of various bonds with the participitation of water molecule. By the Enzyme Commission rule, proteinase is EC 3.4.24.4. Where the number 3 indicates the hydrolases group, 3.4 indicates acting on peptide bonds, 3.4.24 indicates the peptidyl peptide bond of proteinase and the last number represents the second enzyme in its sub-sub-class (Arunachalam and Saritha, 2009).

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Casein is the name for a family of related phosphoproteins. These proteins are commonly found in mammalian milk. Casein has a wide variety of uses, from being a major component of cheese, to use food additive, to a binder for safety matches. As food sources, casein supplies amino acids, carbohydrates, and two inorganic elements, calcium and phosphorous (Das and Prasad, 2010). In this research, casein was used as the substrate in all the experimental studies of enzyme-catalyzed reactions. Tyrosine is a α-amino acid that is found in most proteins, is converted from the essential amino acid phenylalanine in the human body. Tyrosine is the product in this research.

Scientific Classification of *Moringa oleifera* L. (Dant-da-lun)

Botanical name	:	*Moringa oleifera* L.
Family	:	Moringaceae
Myanmar name	:	Dant-da-lun
Common name	:	moringa, drumstick tree
Part used	:	Leaves

Figure 1 The plant, leaves, flowers and fruits of *Moringa oleifera* L. (Dant-da-lun)

Materials and Methods

Sample Collection

Matured, fresh and green leaves of *Moringa oleifera* L. (Dant-da-lun) used for extraction of protease enzyme were collected from Aung San Quarter, Natmauk Township, Magway Region.

Determination of Wavelength of Maximum Absorption and Construction of Calibration Curve for Standard Tyrosine Solution

A standard tyrosine solution (1 mL) was added to a test tube containing (5 mL) of 0.5 M sodium carbonate solution and (1 mL) of 1 M sodium hydroxide solution. The mixture was shaken well. After 10 min, 0.5 mL of Folin-Ciocalteau (Phenol) reagent solution was added to mixture and blue colour appeared. After 30 min, this mixture was placed in the sample cell of UV-visible spectrophotometer. Trichloroacetic acid solution used as reagent blank was placed in the reference cell. The absorption sepctra were recorded from 420-600 nm and the wavelength of maximum absorption was found at 480 nm. The absorbance was plotted against the concentration of tyrosine to give the calibration curve.

Procedure for Extraction and Purification of Protease

Moringa leaves (4 g) were collected and crushed with 0.1 M sodium chloride solution. After homogenization, 20 mM phosphate buffer pH 5.5 was added. Then solid ammonium sulphate (25 g) and (45 g) was added to this mixture to obtain 20 % and 70 % saturation. Then it was stirred for 2 hours. After standing overnight, the precipitate protein containing protease enzyme was collected by centrifugation for 30 min at 1700 rpm. The crude enzyme precipitate was obtained (Mukhtar and Ikram, 2008).

Activity Assay of Protease Enzyme

A 0.75 mL of prepared enzyme solution was added to a test tube containing 1.25 mL of 1% casein solution and 0.5 mL of phosphate buffer solution. The mixture was shaken well and incubated at 30 ºC for 30 min. After incubation time, 1.25 mL of 5% trichloroacetic acid (TCA) was added. The mixture was centrifuged and 1 mL of supernatant was taken out. It was added to 5 mL of 0.5 M sodium carbonate solution and 1 mL of sodium hydroxide solution and kept for 10 min and 0.5 mL of Folin and Ciocalteau reagent was added. The test tubes were incubated for 30 min, the blue colour development. A blank solution was prepared by carrying out the procedure as described above expect that 0.75 mL of phosphate buffer was used instead of 0.75 mL of enzyme solution. The absorbance of both test and blank solutions were measured at 480 nm by using UV-Visible Spectrophotometer (Das and Prasad, 2010).

Determination of Reaction Time for Protease Enzyme

A 0.75 mL of purified enzyme solution was added to a test tube containing 1.25 mL of casein solution and 0.5 mL of pH (5.5) phosphate buffer solution. The mixture was shaken well and incubated at 30 ºC for 10 min. The remaining procedure was the same as those mentioned in activity assay. The reaction time of 20, 30, 40, 50, 60 min were carried out with the activity assay. The absorbance values were plotted against the respective reaction time.

Determination of Optimum pH for Protease Enzyme

The enzyme activities at pH (s) 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 were carried out the same as the above activity assay, respectively. The absorbance was measured and the optimum pH was obtained from protease activity vs. pH plot.

Determination of Optimum Temperature for Protease Enzyme

The activity assay was essentially the same except that the temperature as fixed variously at 30, 35, 40, 45, 50 and 55 ºC for each series of experimental runs, the casein solution (pH 5.5) were used. A blank solution was prepared by carrying out the procedure as described above except that 0.75 mL of phosphate buffer was used instead of 0.75 mL of enzyme solution. The absorbance was measured and the optimum temperature was obtained from protease activity vs. temperature plot.

Effect of Enzyme Concentration on Protease Activity

The different enzyme concentrations, viz, 0.1, 0.2, 0.3, 0.4 and 0.5 mL of purified enzyme solutions were prepared. Then the protease activity was measured. The absorbance values were plotted against the respective concentrations for each of the different enzyme solutions.

Effect of Substrate Concentration on Protease Activity

For each test tube, the reaction was initiated by adding the 0.75 mL of enzyme solution and 0.5 mL of phosphate buffer solution (pH 5.5) into test tube. The various percent concentration of casein solution 1, 2, 3, 4, 5 and 6%, respectively, were added to each test tube and then activity was measured at various concentrations.

Results and Discussion

Determination of Wavelength of Maximum Absorption and Construction of Calibration Curve for Standard Tyrosine Solution

In the present research, the absorption spectrum of tyrosine compound was recorded in the range from 420 to 600 nm. The wavelength of maximum absorption was found to be 480 nm shown in Figure 2. In UV-visible quantitative work, the visible spectra of standard tyrosine were scanned between 420 and 600 nm at different concentrations of 55.18, 27.16, 13.79, 6.89, 3.49 and 1.72 mM. The nature of the plot of absorbance vs. concentration of tyrosine was straight line passing through the origin showing that Beer's law was obeyed in Figure 3.

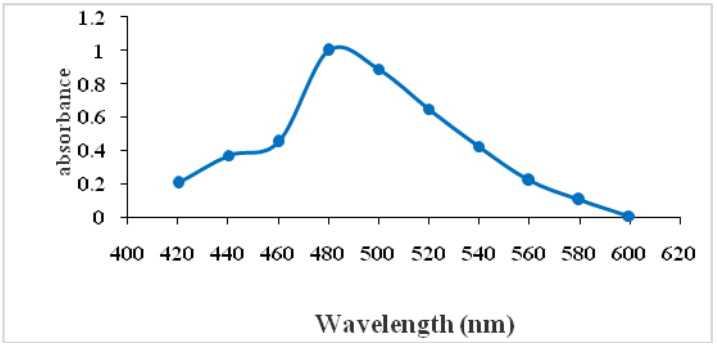

Figure 2 Wavelength of maximum absorption of standard tyrosine solution

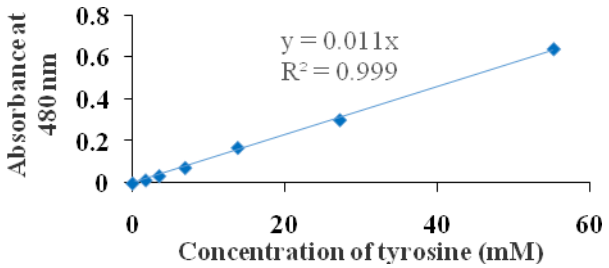

Figure 3 Plot of absorbance as a function of concentration for standard tyrosine

Protease Activity at Different Purification Steps

Extraction and purification are the first steps in the study of physicochemical, kinetic and biological properties of an enzyme. Purification involves a series of fractionations by which the enzyme protein is separated from the other proteins. In this research, the purification steps involve 20 % ammonium sulphate precipitation and 70 % ammonium sulphate precipitation methods. The activity of protease enzyme extract was found to be 12.5 μ mol $min^{-1}mL^{-1}$ shown in Table 1.

Table 1 Protease Enzyme Activity at Different Purification Steps

No	Main Steps Purification	Protease Activity (μ mol $min^{-1}mL^{-1}$)
1	Crude extract	12.5
2	After purification with 20 % ammonium sulphate saturation (filtrate discarde)	6.01
3	After purification with 70 % ammonium sulphate saturation (filtrate discarde)	1.18

Determination of Reaction Time for Protease

In this research, the amount of tyrosine liberated during various reaction times of 10, 20, 30, 40, 50 and 60 min were determined by UV-visible spectroscopic method in Figure 4. The rate of reaction decreases linearly with time up to about 40 min so that the reaction time of 30 min was chosen for the experiments.

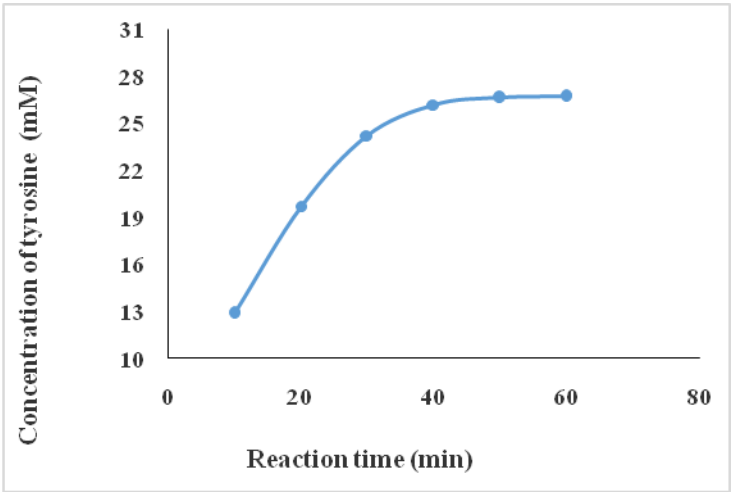

Figure 4 Plot of concentration of tyrosine liberated from protease-catalyzed reaction as a function of reaction time

Determination of Optimum pH for Protease

In this study, different potassium phosphate buffers of pH values ranging from 4.5 to 7.0 were used to determine the protease activity. The nature of the activity vs. pH curve of the enzyme in Figure 5 was found to be the optimum of pH 5.5. Before the optimum pH, the activities increased and after the optimum pH, the activities decreased due to the denaturing of enzyme.

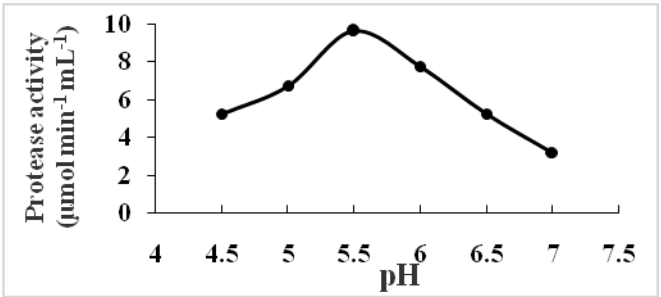

Figure 5 Plot of protease activity as a function of pH of the solution

Determination of Optimum Temperature for Protease

The optimum temperature for protease enzyme was found to be 45 ºC in phosphate buffer pH 5.5. The temperature range was from 30 to 55 ºC while the substrate medium and the optimum pH of 5.5 were fixed. It was obvious that the activity of protease was increased from 30 to 45 ºC and then decreased from 45 to 55 ºC. The results are shown in Figure 6.

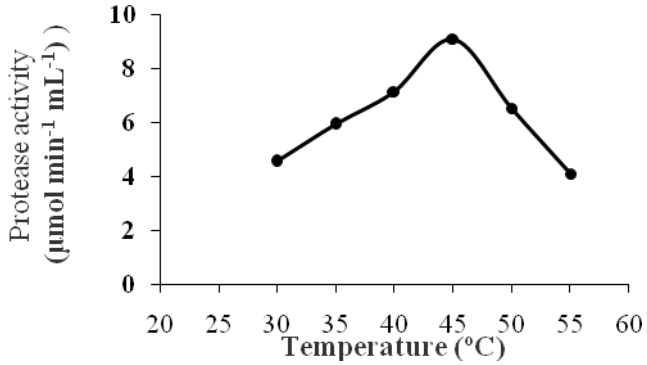

Figure 6 Plot of protease activity as a function of temperature of the solution

Effect of Enzyme Concentration on Protease Activity

The enzyme activity was found to have a linear relationship with different concentration ranging between 0.1 mL, 0.2 mL, 0.3 mL, 0.4 mL and 0.5 mL of enzyme volume. The results are described in Figure 7.

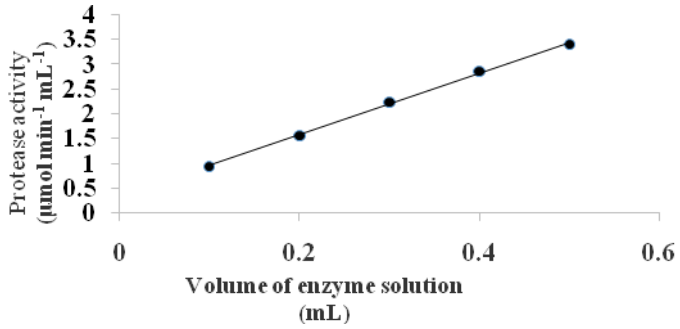

Figure 7 Plot of protease activity as a function of enzyme concentration

Effect of Substrate Concentration on Protease Activity

The effect of substrate concentration on protease activity was studied. The results are shown in Table 2. The important parameters K_m and V_{max} were calculated by the following Lineweaver-Burk equation.

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$$

From the above equation, the Michaelis – Menten constant, K_m was found to be 0.85 x 10^{-2} gmL^{-1} and maximum velocity, V_{max} was found to be 1.52 x 10^{-3} $Mmin^{-1}$, respectively.

Table 2 Relation between Casein Concentration on Velocity of Protease Catalyzed Reaction

*[S] (g mL^{-1})	1/[S] (10^2) (g^{-1}mL)	V (mM min^{-1})	1/V (mM^{-1} min)
0.01	1.00	1.06	0.94
0.02	0.50	1.38	0.72
0.03	0.33	1.48	0.67
0.04	0.25	1.51	0.66
0.05	0.20	1.55	0.65
0.06	0.16	1.59	0.62

*Initial casein concentration

Conclusion

In this study, the crude protease was isolated from green leaves of *Moringa oleifera* L. by using sodium chloride and ammonium sulphate precipitation methods. The protease enzyme (0.5 g) was extracted from (4 g) of fresh moringa leaves by using phosphate buffer. Protease activity was measured at 480 nm by using UV-Visible spectrophotometer. The standard calibration curve of tyrosine was straight line passing through the origin showing that Beer's law was obeyed. After 20 % and 70 % salt purification methods, protease activity was found to be 12.5 µ mol min^{-1} mL^{-1}. The reaction time of protease was found to be 30 min. The protease had optimum pH 5.5 and optimum temperature of 45 ºC. The kinetic profile of the protease enzyme showed that the maximum velocity (V_{max}) and Michaelis- Menten constant (K_m) were 1.52 x 10^{-3} M min^{-1} and 0.85 x 10^{-2} g mL^{-1} from the Lineweaver-Burk equation. According to literature, proteases are envisaged to have extensive applications in leather industry. Proteases have a wide range of applications such as detergent formulations, meat tenderization, cheese manufacture, protein hydrolysis, pharmaceutical industry and silk industry.

Acknowledgement

The authors are extremely grateful to Dr Khin Maung Oo, Rector, Dr Win Soe, Prorector and Dr Than Than Oo, Prorector, Magway University for their their permitting, understanding of this research work. The authors are also thankful to Dr Thidar Aung, Professor and Head, Department of Chemistry, Magway University, for her provision and suggestions of research facilities.

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