PURIFICATION AND CHARACTERIZATION OF POLYPHENOL OXIDASE ENZYME FROM BANANA PEELS (*MUSA ACUMINATA* SIMMONDS)

Nyein Nyein Aye[1], Myat Kyaw Thu[2], Wai Lin Oo[3]

Abstract

An enzyme called polyphenol oxidase (1.10.3.1) is responsible for the browning events that occur when handling damage to the cells. Peels from bananas were gathered in the Thiri Mingalar market in the Yangon Region. Using ammonium sulphate precipitation (20-80 %), dialysis, and gel filtration chromatography on Sephadex G-100, the enzyme polyphenol oxidase (PPO) was extracted from banana peels. Using catechol as a substrate at 420 nm, the spectrophotometric technique was used to assess the polyphenol oxidase activity. The modified Lowry's method was used to determine the protein content using a standard of bovine serum albumin (BSA) at 550 nm. The purification of polyphenol oxidase over crude extract was 3.52 folds, and 0.18 % of the protein was obtained. pH 7.0 and 30 °C were found to be the optimum conditions. It was shown that after 5h of incubation at pH 7.0, polyphenol oxidase activity remained at approximately 68.6 %. A particular pH of 7.0 was used to study the heat sensitivity of polyphenol oxidase at various temperatures of 25, 30, and 35 °C for various incubation periods (0, 1, 3, and 5 h). At 30, 25, and 35 °C, polyphenol oxidase activity was shown to be relatively stable; after 5 h of incubation, it retained 67.8, 49.6, and 26.9 % of its original activity, respectively. The activation energy (E_a) of the polyphenol oxidase-catalyzed reaction was determined to be 2.404 kcal mol^{-1}.

Keywords: polyphenol oxidase enzyme, modified Lowry's method, Sephadex G-100, activation energy

Introduction

Banana is an important food source in the developing world and is one of the most important worldwide crops (Karakus and Pekyardimci 2009). Nowadays, banana commercialization is diversifying and expanding. But, during handling, storage, and processing, its tissue turns dark brown because of the action of polyphenol oxidase (PPO). The degree of browning in bananas after cutting was associated with polyphenol oxidase activity and the concentration of free phenolic substrates (Chaisakdanugull and Theerakulkait 2009).

Polyphenol oxidases are copper-containing oxidoreductase enzymes (Aziz *et al*., 2018). Polyphenol oxidases catalyze the hydroxylation of monophenols to o-diphenols and the oxidation of o-diphenols to o-quinones using molecular oxygen, leading to the formation of black or brown pigments (Aziz and AL-Sa'ady, 2016). In the food industry, polyphenol oxidases are very important enzymes due to their involvement in the enzymatic browning of edible plants. Enzymatic browning is often undesirable and is responsible for unpleasant qualities and losses in nutrient quality. PPO plays an important role as an efficient reagent for cleaning polyphenol-containing wastewater and has many applications in the fields of medicine, food processing, and wastewater treatment. Polyphenol oxidases are a group of enzymes found in almost all living organisms, including plants, animals, and microorganisms. The aim of this research was to study the extraction and characterization of polyphenol oxidase from banana peels (*Musa acuminata* Simmonds).

[1] Department of Chemistry, University of Yangon
[2] Department of Chemistry, University of Yangon
[3] Department of Chemistry, University of Yangon

Materials and Methods

Materials

The banana (*Musa acuminata* Simmonds**)** peel sample was purchased from a local shop in the Yangon Region. Bovine Serum Albumin (BSA) was purchased from Sigma Aldrich, England. All other chemicals used were of analytical reagent grade. In all investigations, the recommended standard methods and techniques involving both conventional and modern methods were provided.

Sample Preparation and Extraction of Polyphenol Oxidase from Banana Peels

Banana peels were washed with tap water, and 50 g of each banana peel was homogenized with 200 mL of 0.05 M potassium phosphate buffer (pH 7.0) containing 1% polyethylene glycol and 0.01 M ascorbic acid in a blender for 1 min. The homogenate solution was filtered through cheesecloth and the filtrate was centrifuged at 6000 rpm for 30 min. The proteins in the supernatant were collected. The enzyme solution was fractionated with solid ammonium sulphate, and a precipitate of 20 % saturation was collected by centrifugation at 6000 rpm, for 30 min. After standing for 2 h at 4 °C, the precipitate was removed by centrifugation for 30 min at 6000 rpm, and the supernatant was collected. Solid ammonium sulphate was then added to the supernatant to achieve 80% Saturation. After standing overnight, the precipitated protein containing polyphenol oxidase was collected by centrifugation for 30 min at 6000 rpm. The pellet was redissolved in (0.05 M potassium phosphate buffer, pH 7.0) and dialyzed against the same buffer in the dialysis tubing kit (m.w. cutoff 12000 – 14000 Da). At intervals of 4 h, dialysis buffer was changed twice. The dialyzed solution was kept at 4 °C after being dialyzed to remove ammonium sulphate.

Purification of Polyphenol Oxidase Enzyme by Using Gel Filtration Chromatography

Gel filtration was carried out using Sephadex G-100. Sephadex G-100 (4 g) was dissolved in 150 mL of 0.05 M potassium phosphate buffer (pH 7.0) and kept for 1 d. Then, Sephadex G-100 was loaded into the column and pre-equilibrated. The elution rate of this column was determined by using 0.05 M potassium phosphate buffer (pH 7.0) after some cotton wool was added to the column. The eluates were collected from the column by a fraction collector after the dialyzed enzyme solution was applied to the Sephadex G-100 column. The flow rate was adjusted to 1.5 mL/7min by the mini-pump, and 1.5 mL fractions were collected per tube. The elution process was continued until no absorbance was observed at 280 nm. The protein and PPO activities of each fraction were checked by measuring the absorbance at 280 nm wavelength using a UV-visible spectrophotometer. The fraction that had the highest polyphenol oxidase activity was pooled. The pooled polyphenol oxidase fraction was measured for protein content by the modified Lowry's method, and polyphenol oxidase activity was measured. Their purification degrees were determined by measuring specific activities.

Characterization of Polyphenol Oxidase from Banana Peels (*Musa acuminata* Simmonds)

Polyphenol oxidase properties of optimum pH, optimum temperature, pH stability, thermostability, reaction time, and enzyme concentration were measured by the spectrophotometric method (Tin Tin Myo, 2009).

Results and Discussion

Extraction of Polyphenol Oxidase from Banana Peels (*Musa acuminata* Simmonds)

In the present work, the polyphenol oxidase enzyme was isolated from banana peel samples obtained from Thiri Mingalar market, Yangon Region, and was partially purified by the solid ammonium sulphate precipitation method.

Purification of Polyphenol Oxidase Enzyme by Gel Filtration Chromatography

In this study, PPO was partially purified from *Musa acuminata* Simmonds Peels using ammonium sulphate precipitation and dialysis. The PPO activity of the precipitate at 20–80 % $(NH4)_2SO_4$ saturation was found to be the highest, and this saturation point was used for all the extraction processes. The PPO enzyme obtained by precipitating with solid ammonium sulphate was dialyzed. The molecules that were smaller than 12000 Dalton are out of the dialysis tube, and the PPO enzyme is located in the tube. The fractions showing PPO activity were obtained after gel filtration chromatography.

The protein contents and polyphenol oxidase enzyme activity in different stages of purification of polyphenol oxidase were investigated. The polyphenol oxidase activity was determined by measuring the initial rate of quinone formation, as indicated by an increase in the absorbance unit at 420 nm. The polyphenol oxidase activity is defined as the increase in absorbance at 420 nm per volume of enzyme solution per reaction time (min) per 0.001 absorbance unit using catechol as a substrate.

Figure 1 shows the chromatogram of polyphenol oxidase on Sephadex G-100 gel. The protein content of the eluate was checked spectrophotometrically at 280 nm and the enzyme activity was determined at 420 nm. The fractions with the highest activity were pooled. The relative purity of the enzyme was increased by about 3.52 folds over the crude extract. The yield percent was 0.18 %. The resultant enzyme protein was dissolved in phosphate buffer (pH 7.0) and UV-visible measurement was carried out. The degree of purity of each step is show in Table 1.

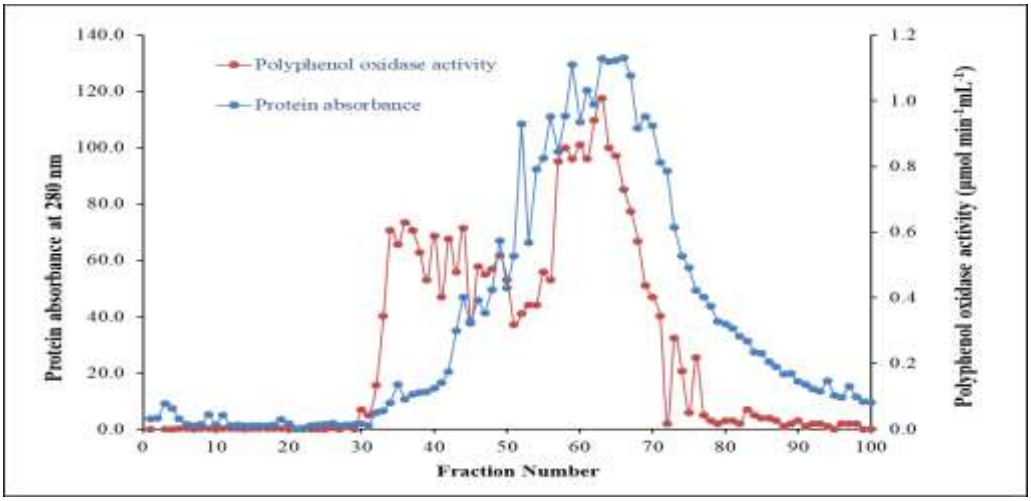

Figure 1. Chromatogram of polyphenol oxidase on sephadex G-100 gel

Table 1. Purification of Polyphenol Oxidase from Banana Peels

Fraction	Total Enzyme Activity(EU)	Total Protein Content (mg)	Specific Activity (EU/mg)	Protein Recovery (%)	Degree of Purity(fold)
Crude	611400	615.52	993.29	100	1
20 % $(NH_4)_2SO_4$ precipitation	659733	463.21	1424.24	75.25	1.43
80 % $(NH_4)_2SO_4$ precipitation	120400	49.87	2414.13	8.10	2.43
Dialysis	82983	28.80	2879.55	4.68	2.90
Sephadex G-100 gel separation	3927	1.12	3496.64	0.18	3.52

Optimum pH of Polyphenol Oxidase Activity

The enzyme is active at a limited range of pH. The activity of the enzyme is reduced and reaction rates are slower at pH levels above and below optimum pH (Hendrickson *et al*, 2007). In this work, phosphate buffer ranging from a value of 5.5 to 8.0 was used to determine the activity of the prepared polyphenol oxidase sample. The nature of the activity *vs* pH curve of the enzyme (Table 2 and Figure 2) was obviously found to be bell-shaped, and the optimum pH was obtained at pH 7.0 with catechol as substrate.

Table 2. Relationship between Polyphenol Oxidase Activity and pH of Potassium Phosphate Buffer Solution

No.	pH	Polyphenol Oxidase Activity (10^{-3}EU)
1	5.5	0.06
2	6.0	0.10
3	6.5	0.16
4	7.0	0.22
5	7.5	0.14
6	8.0	0.04

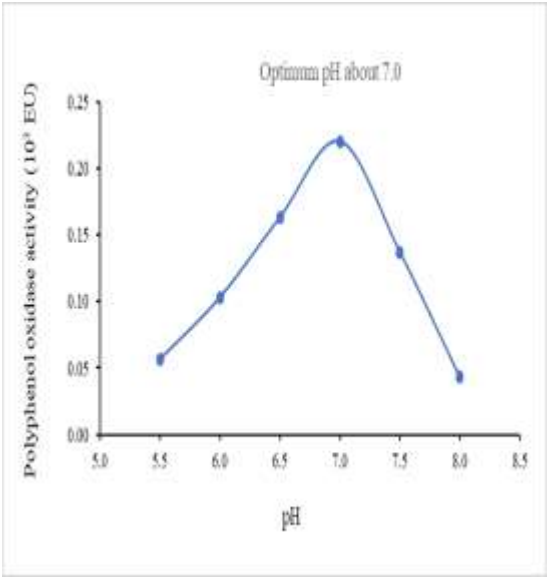

Figure 2. Plot of polyphenol oxidase activity as a function of pH of the solution

Optimum Temperature of Polyphenol Oxidase Activity

In common with other proteins, enzymes are sensitive to temperature changes (Campbell and Smith, 2000). In this study, the effect of temperature on polyphenol oxidase activity was investigated in the temperature range of 15°C to 50°C. The optimum temperature for polyphenol oxidase was found to be 30 °C in potassium phosphate buffer, pH 7.0 (Table 3 and Figure 3). It was found that the activity of polyphenol oxidase increased from 15°C to 30°C and then decreased from 30°C to 50°C.

The activation energy of the polyphenol oxidase catalyzed reaction was calculated by using the Arrhenius equation (Atkins, 1994). Table 4 shows the relationship between the temperature and velocity of the polyphenol oxidase-catalyzed reaction and the Arrhenius constant. By using the constant substrate concentration throughout the experiment, the rate constant (K) in the Arrhenius equation can be substituted by the velocity of the polyphenol oxidase-catalyzed reaction. The activation energy (E_a) was determined to be 2.404 kcal mol^{-1} from the linear regression method.

Table 3. Relationship between Activity of Polyphenol Oxidase-catalyzed Reaction and Temperature of the Solution at pH 7.0

No.	Temperature (°C)	Polyphenol oxidase Activity (10^{-3} EU)
1	15	0.27
2	20	0.30
3	25	0.33
4	30	0.36
5	35	0.31
6	40	0.29
7	50	0.12

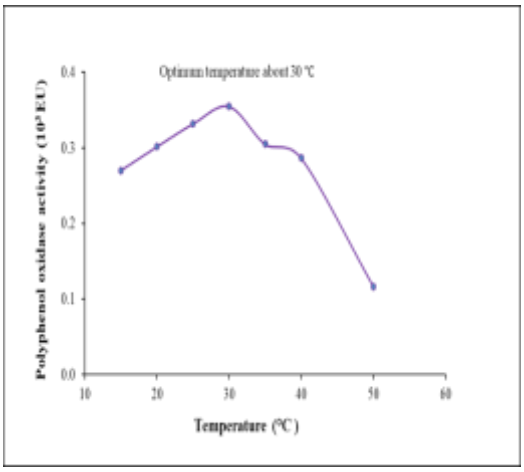

Figure 3. Plot of activity as a function of temperature of the solution at pH 7.0

Table 4. Relationship between Temperature and Velocity of Polyphenol Oxidase-catalyzed Reaction of the solution at pH 7.0

No.	Temperature (°C)	$1/T\times10^3$ (K^{-1})	Velocity (μmol min^{-1})	Log V
1	15	3.47	7.95	0.90
2	20	3.41	8.87	0.95
3	25	3.36	9.77	0.99
4	30	3.30	10.44	1.02

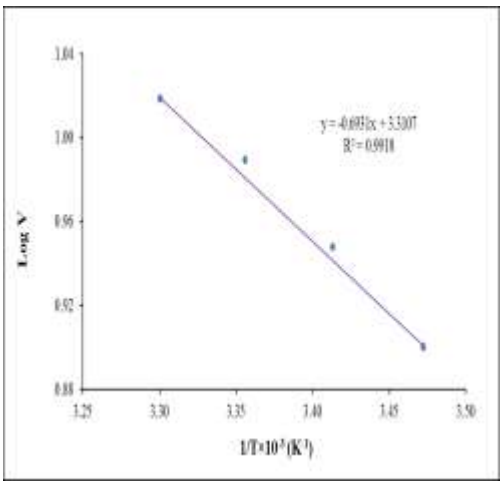

Figure 4. Plot of Log V as a function of 1/T for polyphenol oxidase activity

pH Stability of Polyphenol Oxidase activity at Different pH Values

pH profoundly affects the stability of enzymes (Sawhney and Singh, 2000). The pH stability is determined by pre-incubating the enzyme at various pH for a fixed time. The pH stability of polyphenol oxidase activity was studied by pre-incubating the enzyme at pH 6.5, 7.0, and 7.5 for 0, 1, 3, and 5 h (Table 5). From Figure 5, it can be seen clearly that the polyphenol oxidase activation at pH 7.0 was relatively stable, whereas at pH 6.5 and 7.5, activity decreased by about 60 % and 56 % of the original activity for 5 h of incubation.

Table 5. Relationship between Relative Activity of Polyphenol Oxidase Enzyme and Incubation Time at Different pH

pH	Incubation time (h)	Polyphenol oxidase activity (10^{-3} EU)	Relative activity (%)
6.5	0	0.27	100
	1	0.20	73.93
	3	0.13	48.87
	5	0.11	40.10
7.0	0	0.28	100
	1	0.24	87.92
	3	0.21	76.21
	5	0.19	68.60
7.5	0	0.23	100
	1	0.19	80.77
	3	0.15	62.82
	5	0.10	43.87

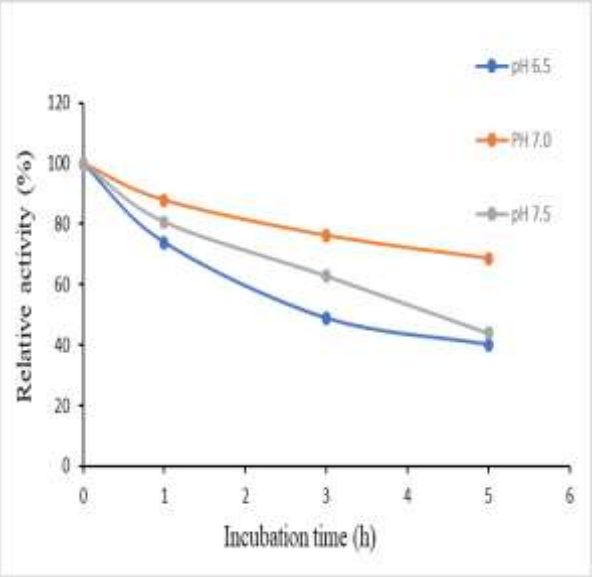

Figure 5. Plot of relative activity of polyphenol oxidase *vs* incubation time at different pH

Thermostability of Polyphenol Oxidase Activity at Different Temperatures

The maintenance of a defined functional state (chemical and structural properties that are required for activity) under extreme conditions refers to stability (Jaenicke and Bohm, 1998). The thermal stability usually increases with rising temperature and passes a maximum, followed by a decrease. The thermostability of polyphenol oxidase activity was studied using a particular pH of 7.0 at different temperatures of 25, 30, and 35 °C for various incubation times (0, 1, 3, and 5 h) (Table 6 and Figure 6). It was observed that the polyphenol oxidase activity was relatively stable at 30 °C until 5 h incubation. The polyphenol oxidase activities decreased by about 50 % at 25 °C for 5 h of incubation. It was interesting to find that the stability of polyphenol oxidase activity was relatively low at 35°C, as it decreased by about 73% of original activity for 5h of incubation.

Table 6. Relationship between Relative Activity of Polyphenol Oxidase Enzyme and Incubation Time at Different Temperatures

Temperature (°C)	Incubation time (h)	Polyphenol oxidase activity (10^{-3} EU)	Relative activity (%)
25	0	0.28	100
	1	0.24	84.72
	3	0.19	68.60
	5	0.14	49.64
30	0	0.33	100
	1	0.30	90.53
	3	0.26	77.64
	5	0.22	67.77
35	0	0.26	100
	1	0.18	69.36
	3	0.13	51.28
	5	0.07	26.92

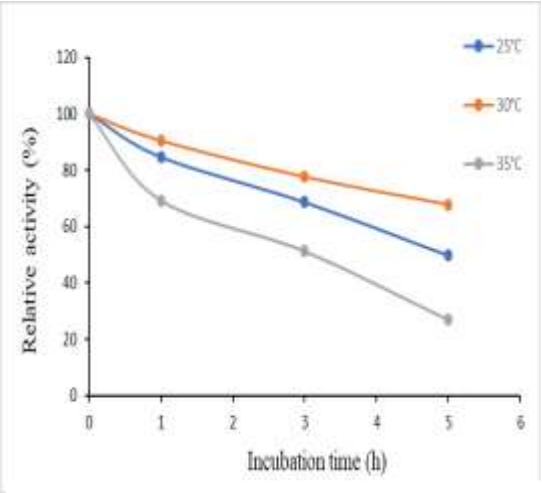

Figure 6. Plot of relative activity of polyphenol oxidase *vs* incubation time at different temperature

Reaction Time on Polyphenol Oxidase-catalyzed Reaction

Reaction time is also a major factor in enzyme activity. The significance of the reaction time is created by the different enzymes that record the starting time (Das and Prasad, 2010). In this work, the action of the polyphenol oxidase on catechol substrate was studied in phosphate buffer pH (7.0). The amount of orthoquinone product as a function of reaction times of 1, 3, 6, 9, 12, 15, 18, 21, and 24 min was determined at 420 nm by the UV-visible spectroscopic method (Table 7 and Figure 7). The figure shows the plot of the velocity of the polyphenol oxidase reaction as a function of reaction time. At the beginning of the reaction (during 5 min), the reaction is very fast. Then, velocity decreased steadily. Therefore, in sequence studies, the reaction time of 3 min was used for the initial velocity measured in enzyme kinetics.

Table 7. Relationship between Reaction Time and Different Velocity of Polyphenol Oxidase-catalyzed Reaction

No.	Reaction Time (min)	Velocity (10^5 M min^{-1})
1	1	1.27
2	3	0.75
3	6	0.51
4	9	0.39
5	12	0.33
6	15	0.28
7	18	0.25
8	21	0.22
9	24	0.19

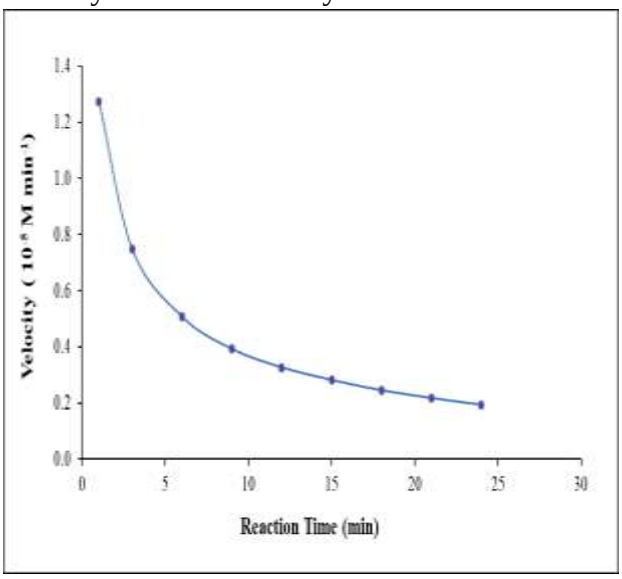

Figure 7. Plot of different velocity of polyphenol oxidase-catalyzed reaction as a function of reaction time

Conclusion

The ammonium sulphate precipitation method and gel filtration chromatography method (Sephadex G-100) were used to extract purified polyphenol oxidase from *Musa acuminata* Simmonds peels. Polyphenol oxidase activity was determined by a spectrophotometric method using catechol as a substrate at 420 nm. The specific activity and relative purity of the enzyme were increased by about 3.52 folds from the crude extract to the final purification step. The optimum pH of the polyphenol oxidase enzyme was found to be 7.0 in a potassium phosphate buffer and the optimum temperature was 30°C. At a pH value of 7.0, the polyphenol oxidase activity was relatively stable, whereas the polyphenol oxidase activity decreased by about 60 % and 56 % of original activity at 6.5 and 7.5, respectively, for 5 h incubation. At 25 and 35°C, polyphenol oxidase activities decreased by about 50 % and 73 % of its original activity, respectively, for 5 h of incubation. The activation energy (Ea) of the polyphenol oxidase-catalyzed reaction was found to be 2.404 kcal mol-1.

Acknowledgements

The authors would like to thank the Department of Higher Education, Ministry of Education, Yangon, Myanmar. We would like to express our sincere thanks to Dr Tin Maung Tun (Rector) and Dr Khin Chit Chit, Dr Cho Cho, and Dr Thidar Aye (Pro-Rectors), University of Yangon. Our warmest thanks are also extended to Professor Dr Ni Ni Than, Head of the Department of Chemistry, University of Yangon, for her kind encouragement, permission to submit this paper and many helpful suggestions.

References

Atkins, P. W. (1994). *Physical Chemistry*. New York: 5th Ed., Oxford University Press, pp. 899-910

Aziz, G. M., and A. J. R. AL-Sa'ady. (2016). "Extraction conditions of Polyphenol Oxidase from Banana Peel". *Baghdad Science Journal*, vol. 13 (3), pp. 251 - 263

Aziz, G. M., A. J. Al-Sa'ady, and D. S. Bedan. (2018). "Characterization and Immobilization of Purified Polyphenol Oxidase Extracted from Banana Peel". *Iraqi Journal of Biotechnology*, vol. 17 (2), pp. 13-22

Campbell, P. N., and A. D. Smith. (2000). *Biochemistry Illustrated*. London: 4th Ed., Churchill Livingstone Harcourt Publisher Ltd., ppt. 75-80

Chaisakdanugull, C, and C. Theerakulkait. (2009). "Partial Purification and characterization of Banana [Musa (AAA Group) 'Gros Michel']". *Internal Journal of Food Science and Technology*, vol. 44, pp. 840-846

Das, G. and M.P Prasad. (2010). "Isolation, Purification and Mass Production of Protease Enzyme from *Bacillus subtilis*". *International Research Journals of Microbiology*, vol. 1, pp. 026-031

Hendrickson, C.H., L.C. Byrd, and N.W Hunter. (2007) *A Laboratory Manual for General, Organic, and Biochemistry*, New York: 6th Ed., Ma Graw Hill Science/Engineering/Math

Jaenicke, R. and G. Bohm. (1998). "The Stability of Proteins in Extreme Environments", *Curr. Opin. Struct. Biol,* vol. 8 (6), pp. 738-748

Karakus, E. and S. Pekyardimci. (2009). "Purification and Biochemical Characterization of Polyphenol Oxidase from Alanya Banana (Musa caritalicevendishi)". *Asian Journal of Chemistry*, vol. 4, pp. 3138-3150

Sawhney, S. K., and R.Singh. (2000). Introductory Practical Biochemistry. New Delhi: Narosa Publishing House, pp. 88-96

Tin Tin Myo (2009). Isolation, Immobilization, and *Application of Polyphenol Oxidase from Avocado* (*Persea Americana Mill*.). Yangon: PhD Dissertation, Chemistry Department, Yangon University