ISOLATION AND CHARACTERIZATION OF α-AMYLASE DURING GERMINATION OF MAIZE GRAINS (*ZEA MAYS* L.)

Khin Sandar Linn[1], Myat Kyaw Thu[2], Thaw Thaw Win[3]

Abstract

α-Amylase (EC 3.2.1.1) break down long-chain carbohydrate, ultimately yielding maltotriose and maltose. In this research, α-amylase from germinating maize grains was extracted by using sodium chloride and acetate buffer pH 5.6 solution. Qualitative examination of α-amylase activity in the solution was carried out by using iodine staining method. Soluble starch was used as a substrate for α-amylase activity determination by measuring the absorbance of maltose using UV-visible spectrophotometer. α-Amylase activities during germinating of maize grains were studied by determining the daily enzyme activity. The maximum α-amylase activity was found on third day of growth. The protein content of enzyme solution was determined by Biuret method. The specific activity of enzyme solution was calculated to be 12.035 μmol min^{-1} mg^{-1}. The enzyme unit (EU) of crude α-amylase was found to be 165.01 EU per gram of maize grains. The optimum pH of α-amylase was found to be 5.6 in acetate buffer and optimum temperature was found to be 60 °C. The values of K_m and V_{max} treated statistically using the linear regression method were compared with various graphically methods (Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Wilkinson). The K_m and V_{max} values of α-amylase were found to be 0.192×10^{-2} g mL^{-1} and 1.978×10^{-3} M min^{-1}, respectively, from Lineweaver-Burk plot. The reaction order (n) for α-amylase was calculated to be 1.203 proving that the reaction order is first order. The activation energy (E_a) of α-amylase-catalyzed reaction was calculated to be 4.977 kcal mol^{-1}. In this research, cassava sample was used as a starch source for the preparation of maltose. The maltose content in the prepared sample was determined by using Dinitrosalicylic acid method.

Keywords : Germinating maize grains, α-amylase, soluble starch, maltose

Introduction

Maize (*Zea mays* L.) is a major staple food grain throughout the world, particularly in Africa, Latin America and Asia. Maize is a tropical plant (Fox and Manley, 2009) which requires warm temperatures. The maximum size of kernels is reputedly 2.5 cm. An ear commonly holds 600 kernels. The grains are about the size of peas, and adhere in regular rows around a white, pithy substance, which forms the ear. α-Amylase is a protein enzyme (EC 3.2.1.1) that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen, yielding glucose and maltose (Hussain *et al*., 2013).

Cereal α-amylases are known as enzyme originates from cereals grain such as wheat, barley, maize, rye etc. (Muralikrishna and Nirmala, 2005). It plays a dominant role in starch metabolism during grain development as well as germination. α-Amylases (endo-1,4-α-D-glucan glucohydrolase) are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units in the linear amylose chain (Anto *et al*., 2006).

Amylases are one of the main enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries (Souza and Magalhaes, 2010). α-Amylases can be obtained from plants, animals and

[1] Dr, Assistant Lecturer, Department of Chemistry, University of Yangon
[2] Dr, Professor, Department of Chemistry, University of Yangon
[3] MSc, Department of Chemistry, University of Yangon

microorganisms. The production of α-amylase is essential for conversion of starches into oligosaccharides. Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. Starch-converting enzymes are used in the production of maltodextrin, modified starches, glucose, maltose and fructose syrups.

Cassava starch is used as a starch source and enzymic hydrolysis of starch was carried out by using crude α-amylase from maize grain. The maltose content was measured by dinitrosalicylic acid method. The production of sugar syrups by enzymatic method is among the most advanced food technologies, characterized by higher yields, wide range of products, higher product quality and energy economy (Blanchard and Katz, 1995).

The aim of this research was to study the isolation and characterization of α-amylase during germination of maize grains.

Materials and Methods

Materials

Maize Grains samples and cassava starch were purchased from local shop, 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 α-Amylase from Maize Grains

Maize grains (Figure 1) were placed in wet sand and kept at room temperature. After day 1 of germination, maize grains (10 g) were taken and α-amylase was extracted with sodium chloride and acetate buffer pH 5.6 solution. The partially purified α-amylase was qualitatively examined by iodine staining method for its α-amylase activity. Soluble starch was used as a substrate for α-amylase activity determination by measuring the absorbance of maltose using UV-visible spectrophotometer. Similarly, the same procedure was carried out after day 2, 3, 4, 5, 6, 7, 8 and 9 germination. Protein content was determined by Biuret method using Bovine Serum Albumin (BSA) as standard at 550 nm. Specific activity was calculated by using enzyme activity and protein content.

(a) (b)

Figure 1 (a) Maize seeds (b) After third day germination

Characterization of α-Amylase from Maize Grains

α-Amylase properties of optimum pH, optimum temperature and effect of reaction time were determined by dinitrosalicylic acid method. The enzyme kinetic parameters of K_m, V_{max} and reaction order of α-amylase-catalyzed reaction were determined by dinitrosalicylic acid method (Mohamed *et al*., 2009).

Application of α-Amylase from Maize Grains

Cassava sample was used as a starch source for the preparation of maltose. The maltose content in the prepared sample was determined by using dinitrosalicylic acid method (Blanchard and Katz, 1995).

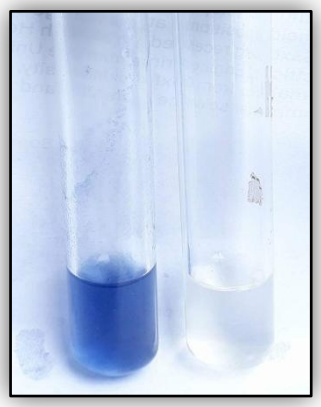
Results and Discussion

Extraction of α-Amylase from Maize Seed

In this work, crude α-amylase was extracted from germinated maize seed (Kigel *et al*., 1995). After day 1, germinated maize was taken and extracted with salt solution. Similarly, the same procedure was carried out after day 2, 3, 4, 5, 6, 7, 8 and 9 germination.

Qualitative Examination of α-Amylase by Using Iodine Staining Method

The iodine staining method is well suited for extensive kinetic analysis of purified enzyme (Normera, 1986). Thus, qualitative examination of α-amylase was done by using iodine staining method (Harper, 1977). In this work, a blank solution containing the mixture of starch and distilled water showed deep blue colour with iodine solution, whereas solution mixture containing the enzyme and starch solutions showed no colour with iodine solution (Figure 2). Therefore, α-amylase enzyme isolated from germinated maize hydrolyzes the starch by breaking down the α-glucosidic bond.



(a) **(b)**

Figure 2 Gelatinized starch solution treated with iodine solution
(a) without enzyme (control)
(b) with enzyme after 10 min at RT

Construction of Calibration Curve for Determination of Maltose Liberated from α-Amylase Action by DNS Method

Maltose can be used as a standard for estimating reducing sugar in unknown samples (Mohamed *et al*., 2009). Maltose reduces the pale yellow coloured alkaline 3,5-dinitrosalicylic acid (DNS) to the orange-red coloured, 3-amino 5-nitro salicylic acid. This intensity change in colour is measured using a UV-visible spectrophotometer as the absorbance at 540 nm wavelength. It was found that the nature of the plot of absorbance *vs.* concentration of maltose (Table 1 and Figure 3) was a straight line passing through the origin showing that Beer's Law was obeyed.

Table 1 Relationship between Absorbance and Concentration of Standard Maltose Solution

No.	Concentration of maltose (mM)	Absorbance at 540 nm
1	2.777	0.169
2	2.222	0.130
3	1.666	0.105
4	1.111	0.063
5	0.555	0.032
6	0.278	0.013

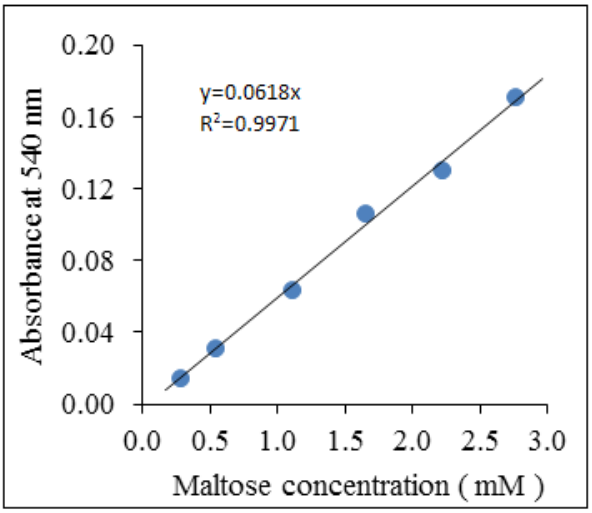


Figure 3 Plot of absorbance as a function of standard maltose concentration

Variation of α-Amylase Activity in Germinated Maize

During germination, a number of enzymes are synthesized and play a role in the complex process of maize production (Thoma, 1971). Especially enzyme degrading carbohydrate, α-amylase and proteins are formed. In this work, the maximum α-amylase activity was determined from the amount of maltose formed during the α-amylase-catalyzed reaction in the time scale of 1 to 9 days. The plot of α-amylase activity *vs.* day of germination (Table 2 and Figure 4) showed that the activity increased with the days and a maximum was reached at 3 days. Finally, the activity decreased with the increased in germination day. The maximum α-amylase activity was found to be 7.675 μmol min^{-1} mL^{-1} at day 3. α-Amylase was isolated from germinated maize by extraction with aqueous solution (acetate buffer).

Table 2 Relationship between Day of Germination and α-Amylase Activity

Germination (Day)	α-Amylase activity (μmol min^{-1} mL^{-1})
1	3.475
2	4.865
3	7.675
4	4.311
5	3.970
6	3.896
7	3.875
8	3.600
9	3.253

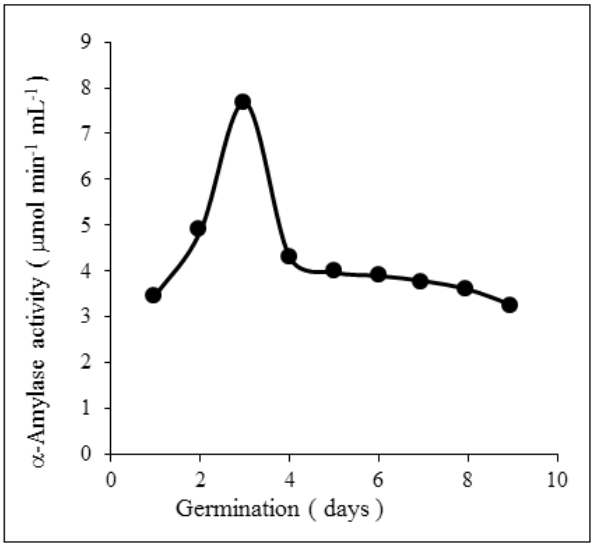


Figure 4 Variation of α-amylase activity with day of germination

Calibration Curve for Protein Determination by Biuret Method

Before determining the proteins content in sample solutions, it is necessary to construct a calibration curve by using standard protein concentration and their absorbance at 550 nm (Savary *et al.*, 1969). In this work, bovine serum albumin (BSA) was used as a standard protein. The different absorbance values were obtained for various standard protein solutions by using a UV-visible spectrometer. It was found that the nature of the plot of absorbance at 550 nm *vs.* concentration of protein (mg $mL^{-1)}$ (Table 3 and Figure 5), was a straight line passing through the origin showing that Beer's Law was obeyed.

Table 3 Relationship Between Absorbance and Concentration of Bovine Serum Albumin (BSA) Solutions

No.	Absorbance at 550 nm	Protein Concentration (mg mL^{-1})
1	0.006	0.024
2	0.014	0.048
3	0.022	0.072
4	0.030	0.098
5	0.035	0.120

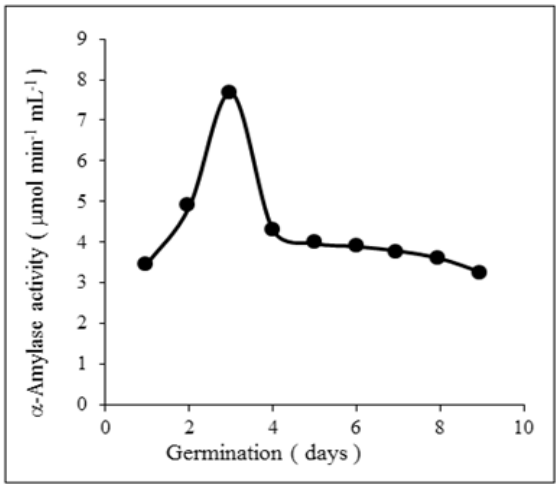


Figure 5 Calibration curve for standard protein solution

α-Amylase Activity, Protein Content and Specific Activity of the Enzyme Solution in α-Amylase by Biuret Method

α-Amylase activity was determined from the amount of maltose formed during the α-amylase-catalyzed reaction using starch as the substrate, according to the DNS method. The enzyme unit (EU) of crude α-amylase was found to be 160.01 EU per gram of maize grain. The protein content was determined by Biuret method. The protein content was observed to be 0.6377 mg mL^{-1}. Specific activity is the total activity divided by total protein. Specific activity was calculated by dividing the number of units/mL by the protein concentration in mg/mL to get μmol/min/mg. The specific activity was calculated to be 12.035 μmol min^{-1} mg^{-1}.

Optimum pH of α-Amylase Activity

At an optimum pH, an enzyme's activity is greatest. At pH above and below optimum pH, the activity of the enzyme is reduced and reaction rates are slower (Charles, 2007). In this work, different buffers of pH value 3.6 to 6.5 were used to determine the activity of the prepared α-amylase sample. The nature of the activity *vs.* pH curve of the enzyme (Table 4 and Figure 6) was obviously found to be unsymmetrical and the optimum pH was obtained at pH 5.6 with starch as substrate.

Table 4 Relationship between α-Amylase Activity and pH of Buffer Solution

Buffer	pH	α-Amylase activity (μmol min^{-1} mL^{-1})
Acetate	3.6	1.952
Acetate	4.0	3.941
Acetate	4.4	5.202
Acetate	4.8	5.796
Acetate	5.2	6.190
Acetate	5.6	7.479
Sodium Phosphate	6.0	3.556
Sodium Phosphate	6.5	4.554

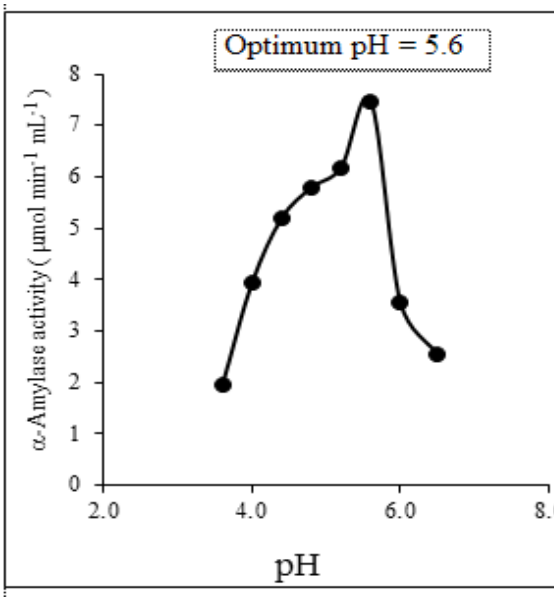


Figure 6 Plot of α-amylase as a function of pH solutions

Optimum Temperature of α-Amylase Activity

In this study, the effect of the temperature on the α-amylase activity was investigated in the temperature range from 40 to 90 ºC. The optimum temperature for α-amylase was found to be 60 ºC in acetate buffer pH 5.6 (Table 5 and Figure 7). The activation energy of α-amylase-catalyzed reaction was calculated by using Arrhenius equation (Atkins, 1994). Table 6 shows the relationship between temperature and velocity of α-amylase-catalyzed reaction. Figure 8 shows the graph for determination of activation energy and Arrhenius constant. By using the constant substrate concentration throughout the experiment, rate constant (K) in Arrhenius equation can

be substituted by velocity of the α-amylase-catalyzed reaction. The activation energy (E_a) was determined to be 4.977 kcal mol^{-1} from linear regression method.

Table 5 Relationship between α-Amylase Activity and Temperature of the Solution at pH 5.6

Temperature (°C)	α-Amylase Activity (μmol min^{-1} mL^{-1})	Velocity x 10^{-3} (M min^{-1})
40	5.434	1.208
45	6.004	1.334
50	6.803	1.512
55	8.452	1.878
60	9.117	2.206
70	6.899	1.533
80	5.619	1.249
90	5.243	1.165

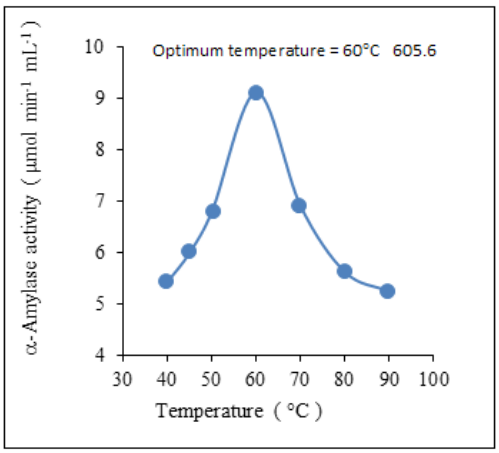


Figure 7 Plot of α-amylase activity as a function of temperature of the solutions at pH 5.6

Table 6 Relationship between Temperature and Velocity of theα-Amylase-catalyzed Reaction

Temperature (°C)	Temperature (K)	1/T (10^{-3} K^{-1})	Velocity × 10^3 (M min^{-1})	Log V
40	313	3.195	1.208	-2.9179
45	318	3.145	1.334	-2.8748
50	323	3.096	1.512	-2.8024
55	328	3.048	1.878	-2.7263
60	333	3.003	2.026	-2.6934

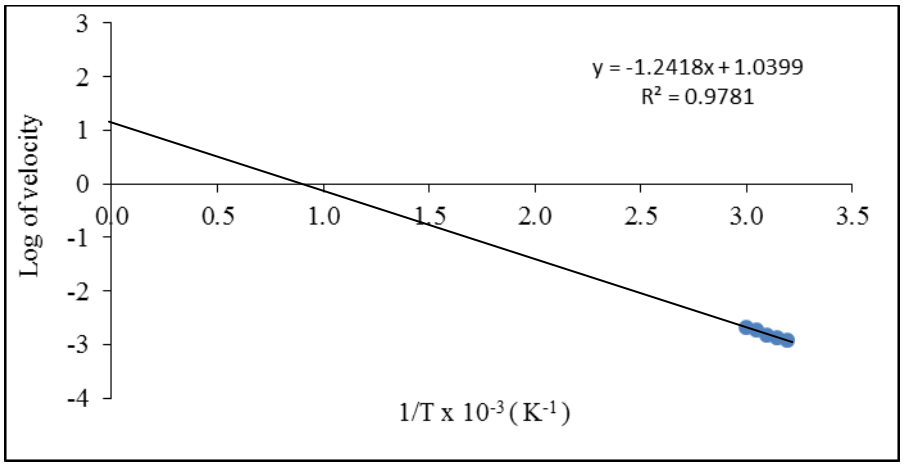


Figure 8 Plot of log of velocity as a function of 1/T for Germinated maize α-amylase

Effect of Reaction Time on α-Amylase-catalyzed Reaction

Among other conditions which control the rate of enzyme reaction, reaction time is an important factor in the determination of enzyme activity (Anderson, 1972).

In this work, the action of the α-amylase on soluble starch was studied in acetate buffer of pH 5.6. The amount of maltose liberated during the various reaction times of 3, 5, 10, 15, 20, 25, 30 and 35 min were determined by dinitrosalicylic acid method (Table 7). Figure 9 shows the plot of velocity of α-amylase reaction as a function of reaction time. At the beginning of the reaction (during 15 min), the reaction is very fast. Then, velocity decreased steadily. Therefore, in sequence studies, reaction time of 10 min was used for initial velocity measured in enzyme kinetic.

Table **7 Relationship between Reaction Time and Velocity of α-Amylase -catalyzed Reaction**

No	Reaction Time (min)	Concentration (mM)	Velocity (mM min^{-1})
1	3	12.012	4.004
2	5	12.159	2.432
3	10	16.711	1.671
4	15	16.793	1.119
5	20	17.484	0.874
6	25	17.763	0.711
7	30	17.993	0.599
8	35	18.272	0.522

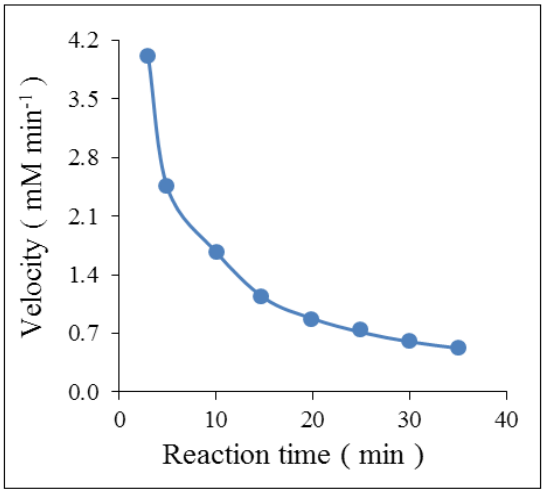


Figure 9 Plot of velocity of α-amylase-catalyzed reaction as a function of reaction time

Effect of Enzyme Concentration on α-Amylase-catalyzed Reaction

The activity of an enzyme is determined by the enzyme concentration (Walsh, 1968). As the enzyme concentration increases the rate of reaction increases linearly, because there are more enzyme molecules available to catalyze reaction. The validity of enzyme assay method was tested using different volume of enzyme. The enzyme activity was found to have a linear relationship with different volumes of enzyme solution ranging between 0.05 to 0.2 mL of enzyme (Table 8 and Figure 10).

Table 8 Relationship between α-Amylase Activity and Enzyme Concentration

No.	Enzyme solution (mL)	α-Amylase activity (μmol min^{-1} mL^{-1})
1	0.05	3.415
2	0.10	6.367
3	0.15	8.945
4	0.20	12.09

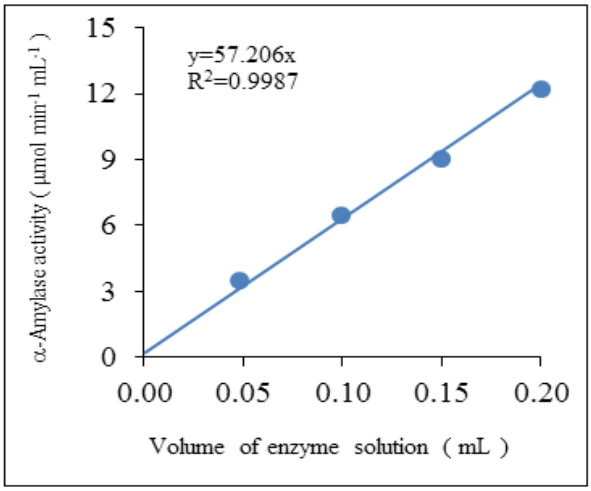


Figure 10 Plot of α-amylase activity as a function of volume of α-amylase solution of enzyme solution

Effect of Substrate Concentration on α-Amylase-catalyzed Reaction

In the present work, the velocities of enzyme reaction measured at different levels of starch concentration and their reciprocal values are shown in Table 9. The Michaelis-Menten plot of V *vs.* [S] is shown in Figure 11. If the concentration of starch was increased, the rate if reaction also increased until a point was reached where the enzyme was working as fast as it could; that was, it was transforming its maximum number of starch molecules each minute. At this point, the enzyme was said to be saturated, and further increases in the concentration of the starch would not increased the rate of reaction. The enzyme could worked no faster.

Most common transform is the Lineweaver-Burk plot which is also called double reciprocal plot (1/V vs 1/[S]) (Figure 12). From this Lineweaver-Burk plot K_m and V_{max} values were found to be 0.192×10^{-2} g mL^{-1} and 1.978×10^{-3} M min^{-1}, respectively. Figure 13 shows the Eadie-Hofstee plot of V vs V/[S]. K_m and V_{max} values obtained by this plot were 0.126×10^{-2} g mL^{-1} and 1.863×10^{-3} M min^{-1}, respectively. Hanes-Wilkinson plot, an alternative plot of [S]/V vs [S] based on Hanes equation gave a straight line as shown in Figure 14. From this plot, K_m and V_{max} values were calculated to be 0.114×10^{-2} g mL^{-1} and 1.843×10^{-3} M min^{-1}, respectively. Comparison of kinetic parameters of the α-amylase enzyme from different methods is shown in Table 10.

Table 9 Relationship between Substrate Concentration and Velocity of α-Amylase-

No.	$[S]\times10^{-2}$ (g mL^{-1})	$-[S]\times10^{-2}$ (g mL^{-1})	$1/[S]\times10^{2}$ (g^{-1} mL)	$V\times10^{-3}$ (M min^{-1})	$1/V\times10^{3}$ (M^{-1}min)	V/[S] (M min^{-1} g^{-1} mL)	$[S]/V\times10$ (gmL^{-1} M^{-1} min)
1	0.50	-0.50	2.000	1.219	0.820	2.438	0.410
2	0.75	-0.75	1.333	1.481	0.676	1.975	0.506
3	1.00	-1.00	1.000	1.676	0.597	1.676	0.597
4	1.25	-1.25	0.800	1.695	0.589	1.356	0.737
5	1.50	-1.50	0.667	1.719	0.582	1.146	0.873
6	1.75	-1.75	0.571	1.739	0.575	0.994	1.006

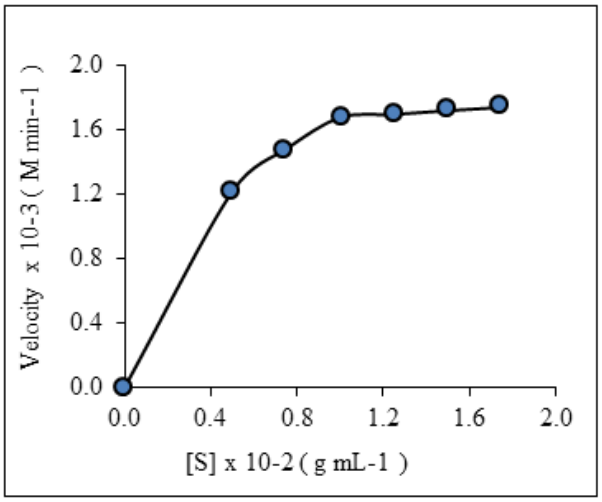


Figure 11 Michaelis-Menten plot used for graphic evaluation of V_{max} and K_m for crude α-amylase

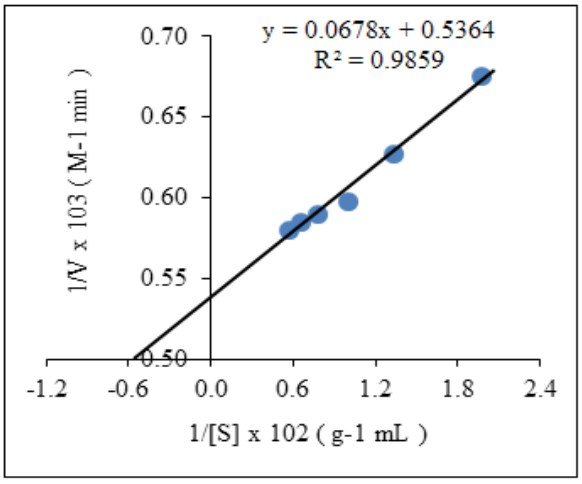


Figure 12 Lineweaver-Burk plot of 1/V *vs.* 1/[S] used for graphic evaluation V_{max} and K_m for crude α-Amylase

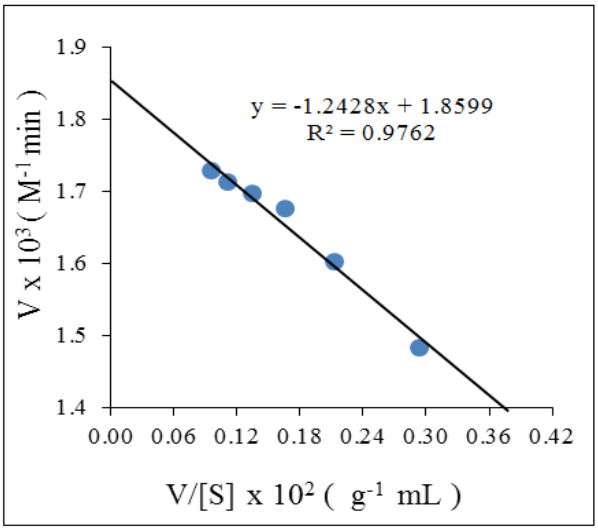


Figure 13 Eadie-Hofestee plot of V *vs.* V/[S] used for graphic evaluation of V_{max} and K_m for crude α-amylase

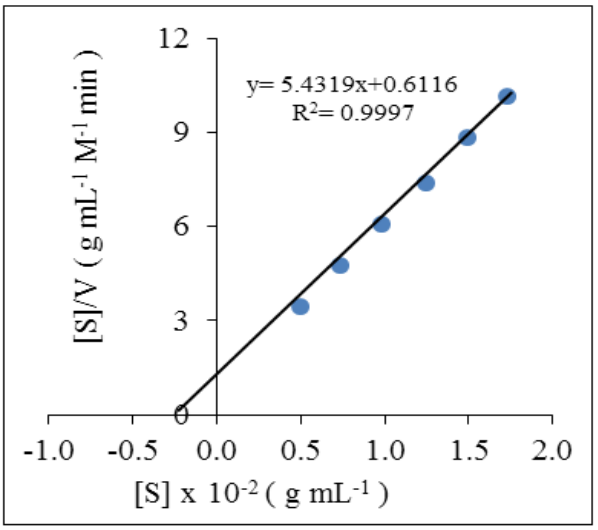


Figure 14 Hanes-Wilkinson plot of [S]/V *vs.* [S] used for graphic evaluation V_{max} and K_m for crude α-amylase

Table 10 Comparison of Kinetic Parameters of the α-Amylase Enzyme from Different Methods

No.	Methods	$V_{max} \times 10^{-3}$ (M min^{-1})	$K_m \times 10^{-2}$ (g mL^{-1})
1	Michaelis-Menten	1.739	0.285
2	Lineweaver-Burk	1.978	0.192
3	Eadie-Hofstee	1.863	0.126
4	Hanes-Wilkinson	1.843	0.114

Effect of

Reaction Order on α-Amylase-catalyzed Reaction

Reaction order refers to the number of molecules involved in forming a reaction complex that is component to proceed to product (s). A reaction characterized by the conversion of one molecules of A to one molecules of B with no influence from any other reactant or solvent is a first-order reaction (Martin, 1993). The plot of Log V/(V_{max}–V) *vs*. Log [S] will give a straight line from which reaction order (n) value can be computed from the slope (Tale 11 and Figure 15). The reaction order (n) for α-amylase was calculated to be 1.2033 proving that the reaction order is first order.

Table 11 Reaction Order for α-Amylase-catalyzed Reaction

No.	Log [S]	Log V/(V_{max}-V)
1	-2.602	0.037
2	-2.301	0.254
3	-2.125	0.688
4	-2.000	1.187
5	-1.903	1.275
6	-1.824	1.416
7	-1.757	1.576

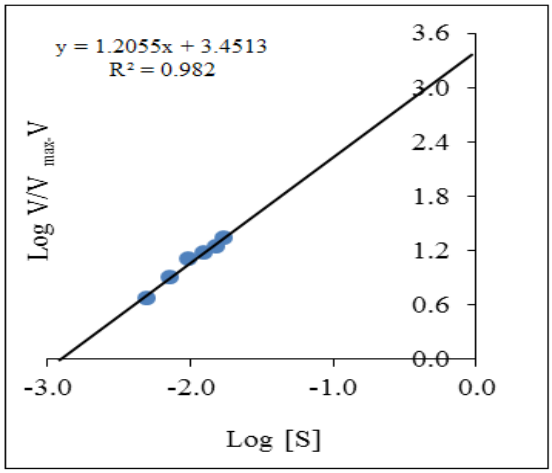
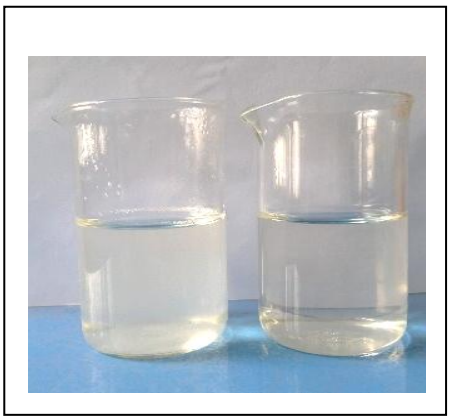


Figure 15 Plot of by V/V $_{max}$-V as a function of log [S] of α-amylase-catalyzed reaction

Conversion of Maltose from Cassava Starch using Maize Grain α-Amylase

Cassava starch is used as a starch source and enzymic hydrolysis of starch was carried out by using crude α-amylase from maize grain. Gelatinized starch solution converted to maltose after heating at 60 °C for 30 min. The maltose content was measured by dinitrosalicylic acid method. The percentage conversion to reducing sugars and maltose was computed with cassava sample having 3.983 %.



(a) (b)

Figure 16 (a) Gelatinized starch solution without enzyme (control)
(b) Gelatinized starch solution with α-amylase (converted to maltose)

Conclusion

α-Amylase from germinating maize was extracted by using sodium chloride and acetate buffer pH 5.6 solution. α-Amylase activities during germinating of maize grains were studied by determining the daily enzyme activity. α-Amylase activity in plant seeds increases rapidly after germinating the seeds. The maximum α-amylase activity was found at third day of growth. The enzyme unit (EU) of crude α-amylase was found to be 160.01 EU per gram of maize grain. The protein content was observed to be 0.6377 mg mL^{-1}. The specific activity of enzyme solution was calculated to be 12.035 μmol min^{-1} mg^{-1}. The optimum pH of α-amylase was found to be 5.6 in acetate buffer and optimum temperature was found to be 60 °C. The values of K_m and V_{max} treated statistically using the linear regression method were compared with various graphically methods (Michaelis-Menten, Lineweaver-Burk, Eadie-Hofstee and Hanes-Wilkinson). The K_m and V_{max} values of α-amylase were found to be 0.192×10^{-2} g mL^{-1} and 1.978×10^{-3} M min^{-1}, respectively, from Lineweaver-Burk plot. The reaction order (n) for α-amylase was calculated to be 1.203 proving that the reaction order is first order. The activation energy (E_a) of α-amylase-catalyzed reaction was calculated to be 4.977 kcal mol^{-1}. The maltose content was measured by dinitrosalicylic acid method. The percentage conversion to reducing sugars and maltose was computed with cassava sample having 3.983 %. α-Amylase is one of most advantageous because it is stable, inexpensive and widely used in the development of various applications.

Acknowledgements

The authors would like to thank the Myanmar Academy of Art and Science for allowing to present this paper and Professor and Head Dr Ni Ni Than, Department of Chemistry, University of Yangon, for her kind encouragement.

References

Anderson, A. K. (1972). *Essential of Purification Chemistry*. New York: John Wiley and Sons, Inc., pp.191-195

Anto, H., Trivedi, U. and Patel, K. (2006). "Alpha-amylase Production by *Bacillus cereus* MTCC 1305 using Solid-State Fermentation". *Food Technology and Biotechnology,* vol.44(2), pp. 241-245

Atkins, P. W. (1972). *Physical Chemistry*. New York: 5th Edition, Oxford University Press, pp.899-901

Blanchard, P. H. and Katz, F. R. (1995). *Starch Hydrolyzates. Food Polysaccharides and their Applications*. New York: 2nd Edition, Marcel Dekker, Inc., 99-121

Charles, H. H., Larry, C. B. and Norman, W. H. (2007). *A Laboratory for General, Organic, and Biochemistry*. New York: 5th Edition, Mc Graw Hill Co., pp.357-378

Fox, G. and Manley, M. (2009). "Hardness Methods for Testing Maize Kernels". *Journal of Agricultural and Food Chemistry*, vol.57**,** pp.5647-5657

Harper, H. A. (1977). *Review of Physiological Chemistry*. Tokyo: 16rd Edition, Maruzen Co. Ltd ; pp.124-143

Hussain, I., Siddique, F., Mahmood, M. S. and Ahmed, S. I. (2013). "A Review of the Microbiological Aspect of α-Amylase Production". *International Journal of Agriculture and Biology,* vol.15(5), pp.1029-1034

Jessop, W. J. E. (1961). *Pearsons Introduction to Biochemistry*. London: Heinemann Medical Book Ltd., pp.240-251

Kigel, J., Galili, G. and Dekker, G. M. (1995). *Seed Development and Germination*. New York: Marcel Dekker, Inc., pp.507-528

Martin, D. W., Mayer, P. A. and Rodwell, V.W. (1983). *Harpers Review of Biochemistry.* California: Lange Medical Pub., pp.77

Mohamed, S. A., Abdulrahman, L., Malki, A. and Kumosani, T. A. (2009). "Partial Purification and Characterization of Five á-Amylases from a Wheat Local Variety (Balady) During Germination". *Aust. J. Basic & Appl. Sci.*, vol.3(3), pp.1740-1748

Muralikrishna, G. and Nirmala, M. (2005). "Cereal α-Amylases An Overview". *Carbohydrate Polymers*, vol.60, pp.163-173

Nomera, N. (1986). *Inhibition and Activation of Barley Peptide Hydrolyses*. New York: McGraw-Hill Book Company, pp.291-310

Savary, J., Pu, P. H. and Sunderman, F. W. (1969). "A Biuret Method for Determination of Protein in Normal Urine". *Clinical Chemistry*, vol.14(12), pp.1160-1171

Souza, P. M. and Magalhaes, P. O. (2010). "Application of Microbial α-Amylase in Industry". *Braz . J. Microbiol.*, vol.41(4), pp.850–861

Thoma, J. A., Spradlin, J. E. and Dygert, S. (1971). *Plant and Animal Amylases in the Enzymes*. New York: Academic Press., pp.115-123

Walsh, E. O. F. (1968). An Introduction to Biochemistry. London: The English Press Ltd., pp.135-147