KINETIC STUDIES OF α -AMYLASE FROM IMMATURE SEEDS OF *PHASEOLUS VULGARIS* L.

Latt Latt Chaw[1], Ni Ni Sein[2] , Thida Win[3]

Abstract

α-Amylases (1,4-α-D-glucan-glucanohydrolase EC 3.2.1.1) are found in human, several bacteria, yeast, fungi and seeds. This paper deals with the extraction of α-amylase from developing seeds of white kidney bean and studies on its kinetic properties. α-Amylase was extracted from immature white kidney bean (*Phaseolus vulgaris* L.) seeds by ammonium sulphate fractionation (20-60 %) and purified by gel filtration chromatography. The specific activity of purified α-amylase was 1.20 unit per mg of protein. The purity of enzyme was confirmed by non SDS-PAGE as a single band. The molecular weight of purified α-amylase was determined as 56.23 kDa. The optimum temperature and optimum pH for the α-amylase were 50˚C and 5.6, respectively. Kinetic parameters such as Michaelis-Menten constant K_m and maximum velocity V_{max} were 0.1267 $x10^{-2}$ g mL^{-1} and 1.64 $x10^{-5}$ M min^{-1} determined from a double reciprocal plot. K_m and V_{max}values determined by other plots were also found to be comparable.

Keywords : α-amylase, white kidney bean seed, *Phaseolus vulgaris* L.,ammonium sulphate fractionation, gel filtration chromatography

Introduction

α-Amylases (E.C.3.2.1.1.) (endo-1,4-α-D-glucanglucanohydrolase), are extracellular enzymes that randomly cleave the 1,4-α-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain (Irfan, 2012). This enzyme is employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Adu *et al*., 2005). α-Amylases are extensively used in food, textiles and pharma industries (Nerkar *et al*., 2011). α-Amylases are wide spread in nature, being found in animals, microorganisms and plants (Franco *et al.*, 2000). Commercially α-amylases are produced mostly from fungal sources, but they are also being extracted from different plant sources like barely, millets, wheat, sorghum, and maize. Immature white kidney bean seeds were chosen for the present study because of its wide distribution in Myanmar as a vegetable available throughout all the year.

The white kidney bean (*Phaseolus vulgaris* L.) is a warm season annual plant. The flowers vary in colour from white to purple. The seeds are rich in protein. White kidney beans are extensively cultivated in all parts of Myanmar.

The aim of the present study was to isolate α-amylase from immature seeds of white kidney bean and to study its kinetic properties.

Materials and Methods

Sample collection

Immature white kidney bean (*Phaseolus vulgaris* L.) seeds were collected from Sint Kaing Township, Mandalay Region.

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Extraction of α-Amylase from Immature White Kidney Bean Seeds

Immature white kidney bean seeds (200 g) were ground in a mortar to obtain a homogeneous mixture. It was then dissolved in 500 mL of 0.1 M acetate buffer (pH 5.6) and stirred for 30 min. The suspension was then centrifuged at 3000 rpm for 15 min to obtain crude enzyme extract. The crude α- amylase enzyme extract was first brought to 20 % saturation with solid ammonium sulphate (analar). After centrifugation for 15 min at 3000 rpm the supernatant was applied again with solid ammonium sulphate to achieve 60 % saturation. The resulting enzyme precipitate was collected by centrifugation at 3000 rpm for 15 min. The protein pellet dissolved in phosphate buffer (pH 5.6) and then it was then dialyzed against same buffer at 4 °C.

The partially purified α-amylase was dissolved in 0.1 M acetate buffer (pH 5.6). This solution was applied to a Sephadex G-100 gel filtration column (2.5×27 cm) previously equilibrated with the same buffer. The flow rate was adjusted to 12 mL/h by a mini pump and 1.5 mL fractions were collected per tube using a fraction collector. After collection, the protein content of each tube was checked by measuring the absorbance at 280 nm wavelength using a UV-visible spectrophotometer. Each tube was also measured from α-amylase enzyme activity by Nelson-Somogyi method (Nelson and Somogyi, 1973). The fractions that had the highest activity of α-amylase enzyme (fraction numbers 10-21, 34-38 and 41-47) were pooled and concentrated with acetone (1:9).

The protein content and α-amylase activity of enzyme solution in each purification step were analyzed by the Biuret method (Chykin, 1966) and Nelson-Somogyi method respectively.

The sample thus obtained was subjected to polyacrylamide gel electrophoresis using high molecular weight marker protein (calibration kit) from Pharmacia.

Determination of Optimum pH of α-Amylase-Catalysed Reaction

The effect of pH on activity of purified α-amylase, buffer solution from pH range of 1.0 to 10.7 were prepared by using hydrochloric acid-sodium chloride buffers (pH 1.6 and 2.2), acetate buffers (pH 3.6, 4.6, and 5.6), phosphate buffers (pH 6.2, 7.0, and 8.0) and sodium carbonate-bicarbonate buffers (pH 9.6 and 10.7). The catalytic activity of the enzymes in the above buffers was determined with starch as substrate and the assay was carried out for each buffer solution with an incubation period of 10 minutes.

A 0.1 mL of pH 1 hydrochloric acid-sodium chloride buffer solution was added into a test tube containing 0.1 mL of starch solution (2 %). Then, 0.1 mL of prepared enzyme solution was added and the contents were mixed well. After 10 min, the reaction was interrupted by adding 1 mL of alkaline copper reagent solution. The contents were then mixed thoroughly. The test tube was heated on a vigorously boiling water bath for 10 min. Next the test tube was cooled under running tap water for 1 min and 1 mL of arsenomolybdate colour reagent solution was added into the test tube. After shaking vigorouslythis solution was diluted to 10 mL with distilled water and mixed by inversion. The absorbance was measured at 750 nm. For blank solution, 0.1 mL of distilled water was used instead of 0.1 mL of prepared enzyme solution.

The whole of the above procedure was repeated with the other buffers solutions with different pH values.

Determination of Optimum Temperature of α-Amylase-Catalysed Reaction

For determination of optimum temperature for activity of the enzyme, the assay was carried out as the procedure mentioned above with the incubation temperature of 20 ^{o}C to 75 ^{o}C at pH 5.6 with an incubation period of 10 min.

Effect of Substrate Concentration on α-Amylase–Catalyzed Reaction

The enzymatic activity, was measured at different concentrations of starch (0.125 %, 0.25 %, 0.5 %, 1.0 %, 1.25 %, 1.50 %, 2.0 %, 2.5 % and 3.0 %). Then assay was carried out as mentioned earlier, with the incubation temperature of 50 ^{o}C at pH 5.6 with an incubation period of 10 min.

Results and Discussion

Purification of α-Amylase

In this study α - amylase was isolated from immature white kidney bean seeds by ammonium sulphate precipitation method followed by Sephadex G-100 gel filtration chromatography (Figure 1). α-Amylase activities, protein contents and specific activities of the enzyme solutions in each purification step are shown in

Table 1. The crude extract having specific activity of 0.10 was subjected to ammonium sulphate precipitation and resulted in specific activity of 1.20 μmol min^{-1} mL^{-1} mg^{-1} at the final purification step. So, 12.0 fold purification was achieved by purified α- amylase in this study.

Molecular Weight of α- Amylase

In this study protein from the pharmacia high molecular weight (HMW) calibration Kit: urease tetramer (480,000), urease dimer (240,000), albumin, bovine dimer (132,000) and albumin, chicken egg (45,000) were used for molecular weight determination. The homogeneity of the purified α-amylase was confirmed by a single band (Figure 2) using polyacrylamide gel electrophoresis. The molecular weight of a protein under investigation was determined by comparing its electrophoretic mobility with that of protein standards of known molecular weights (Table 2 and Figure 3). In the present study, R_f of α-amylase was found to be 0.75. Thus, the molecular weight was determined to be 56.23 kDa.

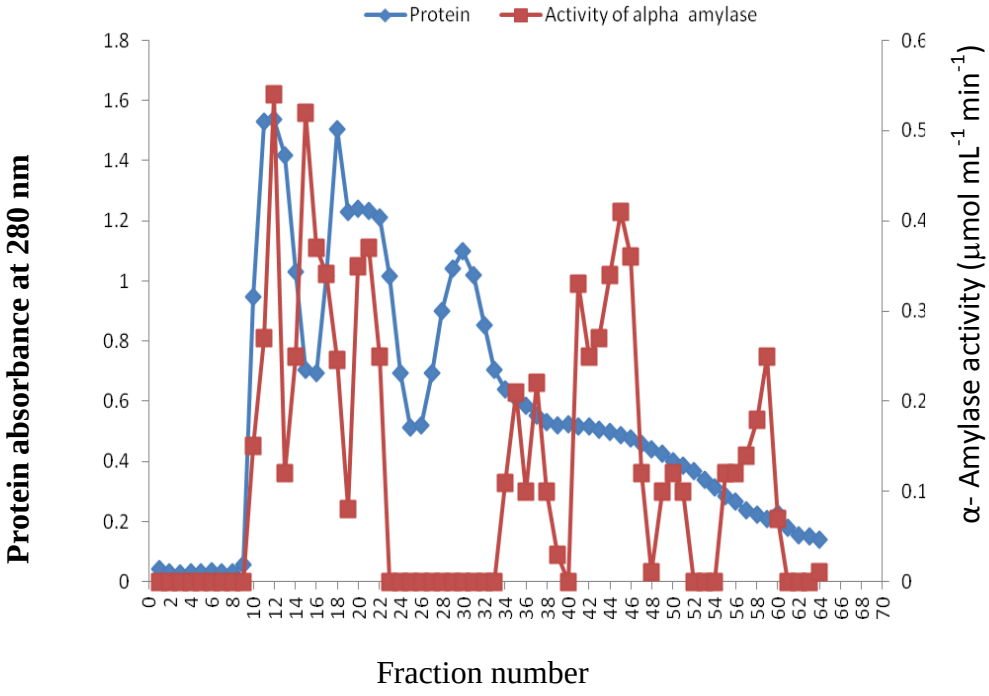

Figure 1 Chromatogram of α-amylase activity on Sephadex G-100

Table 1 Enzyme Activity and Specific Activity at Different Purification Steps

Purification steps	Total activity (µmol min^{-1})	Total protein (mg)	Specific activity (µmol min^{-1} mg^{-1})	Purification (fold)
Crude extract	781.2	7937.6	*0.10*	1
After 20 % ammonim sulphate precipitation	778.5	2412.0	0.32	3.2
After 60 % ammonium sulphate *precipitation*	149.5	243.3	0.61	6.1
Sephadex G-100	204.4	171	1.20	12.0

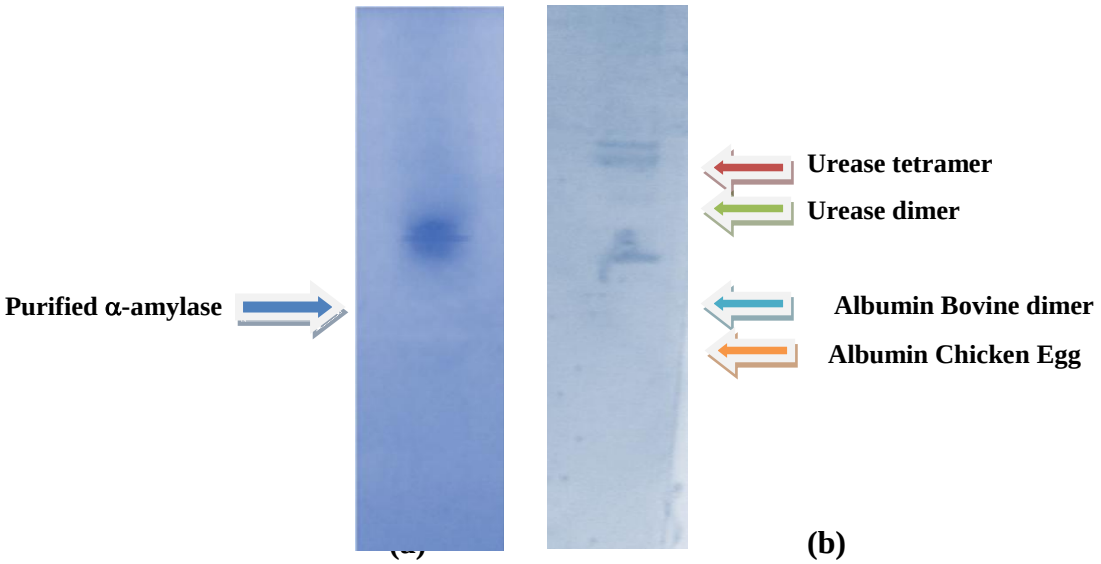

Figure 2 Photograph of polyacrylamide gel electrophoresis
Lane (a) Purified α-amylase
Lane (b) Standard marker proteins

Table 2 Relationship between Molecular Weight of Standard Protein Markers and Relative Mobility (R_f) Values

No.	Standard protein markers	Molecular weight (Dalton)	Log molecular weight	R_f
1	Urease tetramer	480,000	5.681	0.486
2	Urease dimer	240,000	5.380	0.527
3	Albumin, bovine dimer	132,000	5.121	0.625
4	Albumin, chicken egg	45,000	4.653	0.752

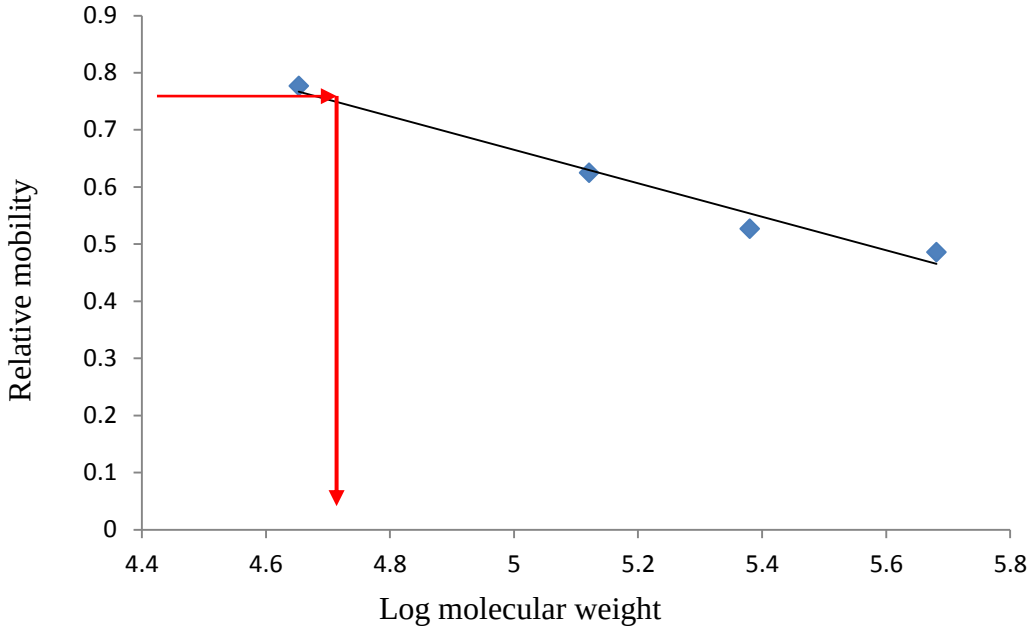

Figure 3 Log molecular weight of standard protein markers as a function of R_f values

Optimum pH of α-Amylase– Catalyzed Reaction

The effect of pH on the activity of immature white kidney bean α-amylase is shown in Figure 4. The enzyme activity increased steadily from pH 1 to 5.6 and then decreased with increasing pH. A decline in the enzyme activity was recorded on either side of pH 5.6. Enzyme showed the optimum activity at pH 5.6. It was reported that optimum pH of mango α- amylase was 5.5 (Yasin and Chaudhary, 1981). The optimum pH of α- amylase from *Phaseolus aconitifolius* was 7.0 (Chavan and Wadatkar, 2014). Khoo *et al*., (1994) reported that the α-amylase enzyme was found to have maximum activity at pH 6.0.

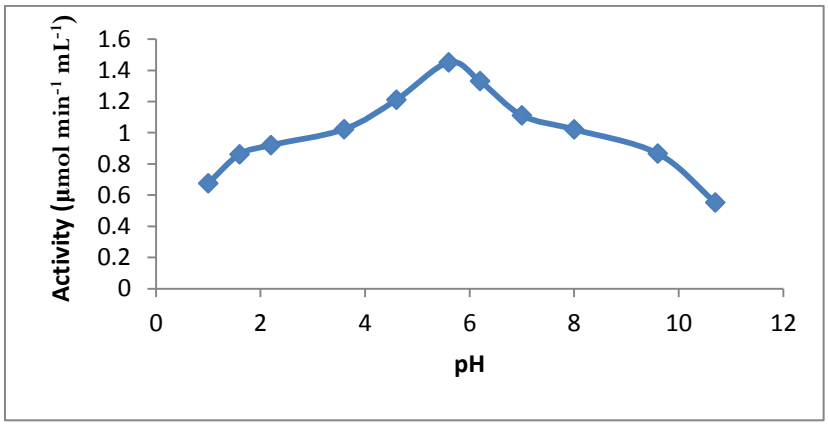

Figure 4 Plot of α–amylase activity as a function of pH of the solution

Optimum Temperature of α-Amylase– Catalyzed Reaction

Figure 5 shows the effect of temperature on the activity of immature white kidney bean α-amylase. Initially the activity increased with increase in temperature and then decreased after reaching a maximum at 50 ˚C. Mohamed *et al.*, (2009) reported that the optimum temperature for partially purified α-amylase from Wheat *Triticum aestivum* was 50 ˚C. Khoo *et al*., (1994)

reported that the optimum temperature for purified α-amylase was 55 ˚C. Plant amylases (Wheat alpha -1, Pearl millet alpha-1, and Safflower seeds) were found to have optimum temperature of 55 ^{o}C (Nerkar *et al*., 2011).

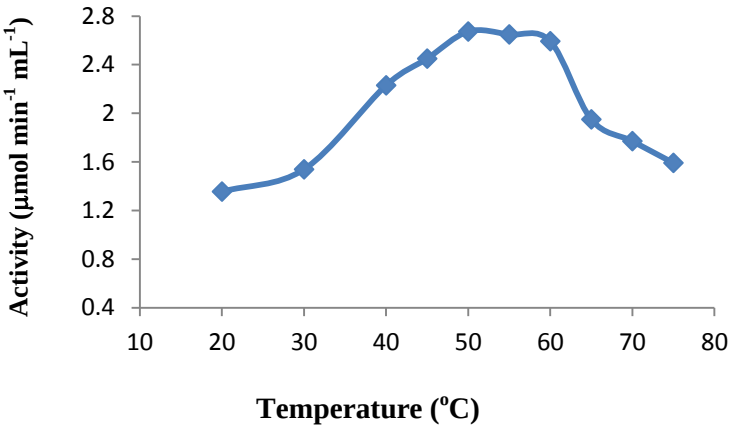

Figure 5 Plot of α–amylase activity as a function of temperature of the solution

Effect of Substrate Concentration

The effect of varying substrate on initial velocity of an enzyme catalyzed reaction is shown in Table 3 and Michaelis-Menten Plot (Figure 6). At relatively low starch concentration, initial velocity increased almost linearly with an increase in concentration of starch. In this region of the curve the reaction followed first order kinetics. At higher starch concentration, initial velocity increased by smaller and smaller extent in response to increase in concentration of starch. In this region of the curve, the reaction was mixed order type. Finally a point was reached beyond which there small was increased in the velocity with increased in the concentration of the starch. The reaction followed zero order kinetics in this region. At this stage, amylase was fully saturated with starch molecules. The immature white kidney bean α - amylase activity reached the maximum with an optimum substrate (starch) concentration of 2×10^{-2} g mL^{-1}. Kuiper *et al*. (1978) reported that the maximum activity of α-amylase enzyme was obtained at 1.67×10^{-2} g mL^{-1} of substrate (starch) concentrations.

Table 3 Relationship between Initial Starch Concentration and Velocity of α-Amylase Catalyzed Reaction

[S]×10^2 (g mL^{-1})	**-[S]×10^2 (g mL^{-1})**	**$\frac{1}{[S]}\times10^{-2}$ (g^{-1} mL)**	**v ×10^5 (M min^{-1})**	**$\frac{1}{v}\times10^{-5}$ (M^{-1}min)**	**$\frac{v}{[S]}\times10^{3}$ (M min^{-1} g^{-1} mL)**	**$\frac{[S]}{v}\times10^{-3}$ (g mL^{-1} M^{-1}min)**
0.1250	-0.1250	8.0000	0.8159	1.2256	6.5272	0.1523
0.2500	-0.2500	4.0000	1.0957	0.9127	4.3828	0.2282
0.5000	-0.5000	2.0000	1.2876	0.7766	2.5752	0.3883
1.0000	-1.0000	1.0000	1.4232	0.7026	1.4232	0.7026
1.2500	-1.2500	0.8000	1.4982	0.6675	1.1986	0.8343
1.5000	-1.5000	0.6667	1.5235	0.6564	1.0157	0.9846
2.0000	-2.0000	0.5000	1.5710	0.6365	0.7855	1.2731
2.5000	-2.5000	0.4000	1.5719	0.6362	0.6288	1.5904
3.0000	-3.0000	0.3333	1.5739	0.6354	0.5246	1.9061

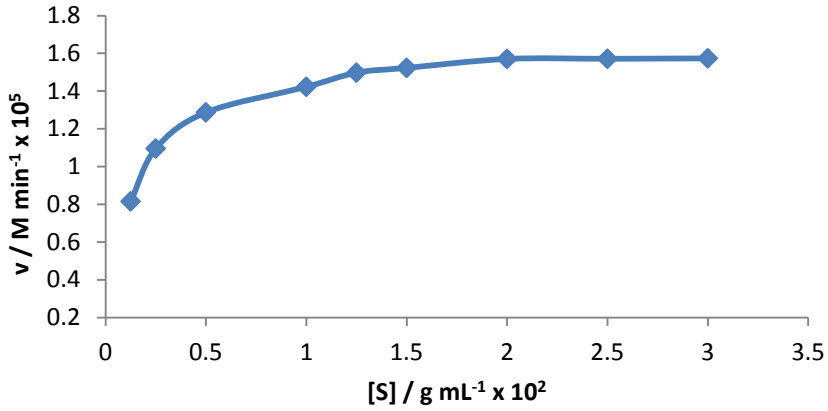

Figure 6 Michaelis-Menten plot used for graphic evaluation of V_{max} and K_m

Kinetic Parameters K_m and V_{max}

Each enzyme has a characteristic substrate concentration (K_m, the Michaelis-Menten constant) at which the reaction velocity is one-half maximal (Sawhney and Singh, 2000). Michaelis-Menten equation, $v = \frac{V_{max}[S]}{K_m} + [S]$ explains kinetics but, because it is nonlinear, is a little hard to deal with real practical data. K_m and V_{max} were found to be 0.1167×10^{-2} g $mL^{-1}$and 1.57×10^{-5} M min^{-1}, respectively, from Michaelis-Menten plot.

Most common transform is the Lineweaver-Burk plot which is also called double reciprocal plot ($\frac{1}{v}$ vs $\frac{1}{[S]}$ plot). The reciprocal transformation distorts the error in the measurements. As shown in Figure 7, the noisiest data are too heavily weighted when linear regression is used to determine the best straight line. From this Lineweaver-Burk Plot K_m and V_{max} values were found to be 0.1276×10^{-2} g mL^{-1} and 1.642×10^{-5} M min^{-1}, respectively.

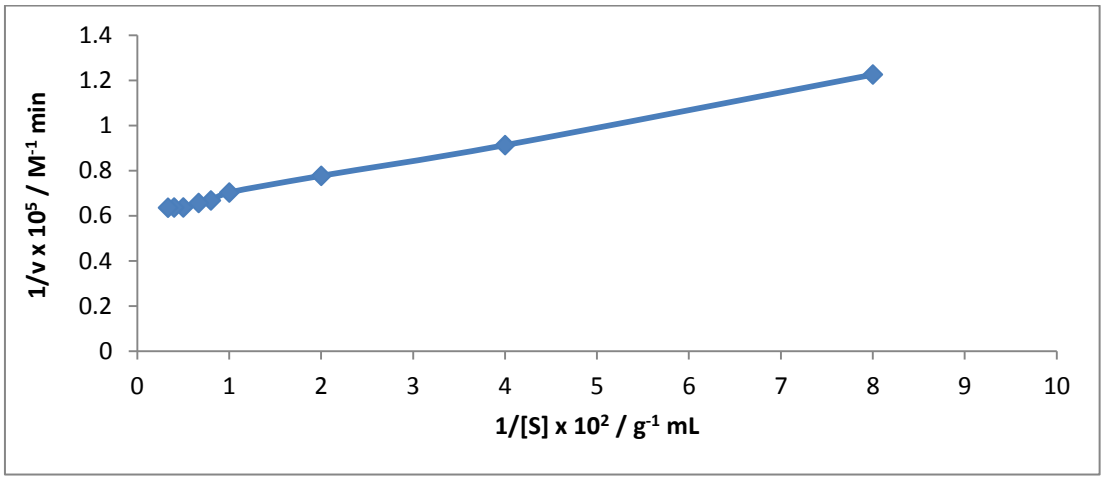

Figure7 Lineweaver-Burk plot of 1/v versus 1/[S] used for graphic evaluation of V_{max} and K_m

Figure 8 shows the Eadie-Hofstee plot of v vs $\frac{v}{[S]}$. This plot not only yields K_m and V_{max} in a very simple way but also magnifies departures from linearity which may not be apparent in a double reciprocal plot. K_m and V_{max} values obtained by this plot were 0.1276×10^{-2} g mL^{-1} and 1.643×10^{-5} M min^{-1}, respectively.

Hanes-Wilkinson plot, an alternative plot of $\frac{[S]}{v}$ vs [S] based on Hanes equation gave a straight line (Figure 9). From this plot, K_m and V_{max} values were calculated to be 0.1332×10^{-2} g mL^{-1} and 1.653×10^{-5} M min^{-1}.

Figure 10 is the direct linear plot or Eisenthal-Cornish Bowden plot. [S] values are plotted on the negative X-axis and observed v values on the Y-axis. This plot gave K_m and V_{max} values of 0.1300×10^{-2} g mL^{-1} and 1.640×10^{-5} M min^{-1}, respectively.

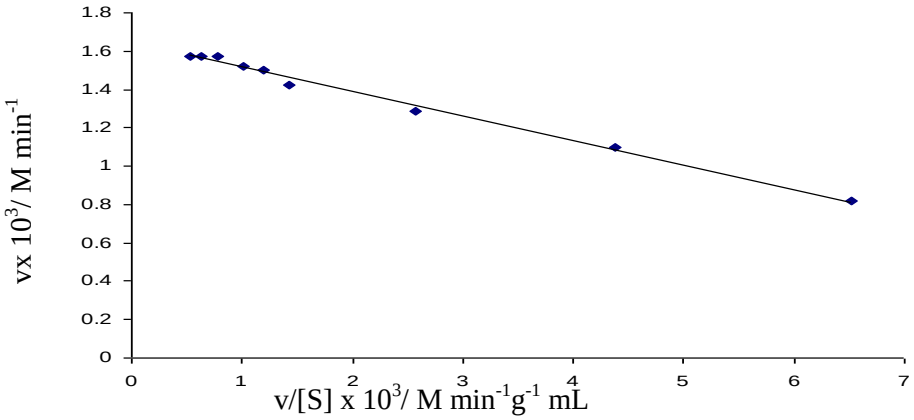

Figure 8 Eadie-Hofstee plot of v versus v/[S] used for graphic evaluation of V_{max} and K_m

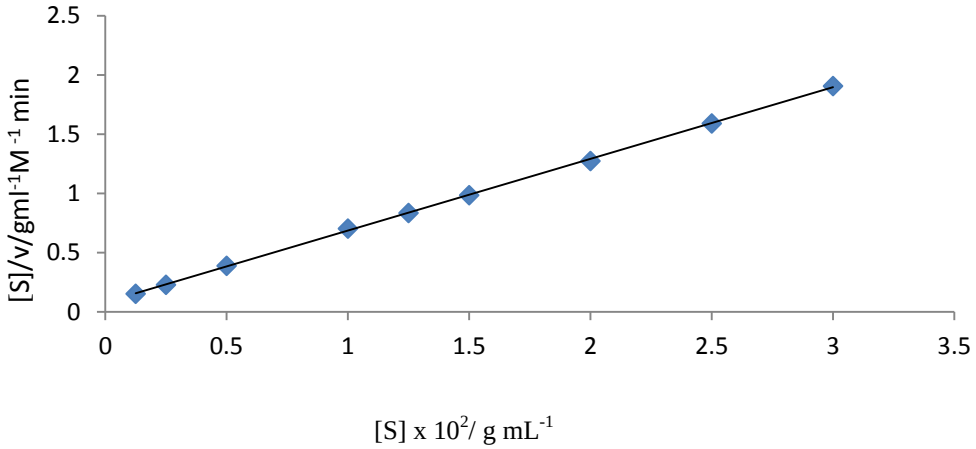

Figure 9 Hanes-Wilkinson plot of [S]/v versus [S] used for graphic evaluation of V_{max} and K_m

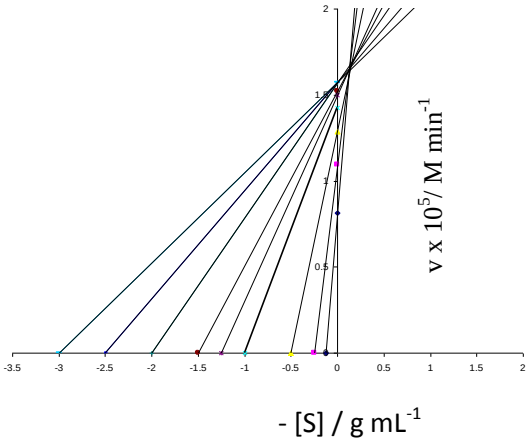

Figure 10 Eisenthal–Cornish Bowden plot of v versus –[S] used for graphic evaluation of V_{max} and K_m

K_m and V_{max} values obtained by different methods agreed with each other (Table 4). K_m value of α-amylase enzyme was reported as 2 mg mL^{-1} i.e., 0.2×10^{-2} g mL^{-1} (Kanwal *et al*., 2004). Moreover, K_m values of α - amylases from mango and *Carica papaya* were found to have same K_m value of 3.3 mg mL^{-1}, i.e., 0.33×10^{-2} g mL^{-1}(Annis, 1982).

Enzyme kinetics, K_m and V_{max} are significant coefficients in guiding scientific research and engineering design. The more firmly the enzyme binds to its substrate, the smaller will be the value of K_m. Results show good affinity of the enzyme for substrate i.e. starch. Moreover, K_m is independent of enzyme concentration and is a true characteristic of the enzyme under defined conditions of temperature, pH, etc. (Negi and Banerjee, 2009).

Table 4 Comparison of Different Methods for Reaction Kinetic Parameters of α amylase from White Kidney Bean Seeds

No.	Methods	$K_m\times10^{-2}$ (g mL^{-1})	$V_{max}\times10^{5}$ (M min^{-1})
1	Michaelis – Menten	0.1167	1.570
2	Lineweaver –Burk+	0.1276	1.642
3	Eadie – Hofstee+	0.1276	1.643
4	Hanes-Wilkinson+	0.1332	1.653
5	Eisenthal – Cornish Bowden	0.1300	1.640

+ Linear regression method

Determination of Reaction Order (n) for α- Amylase-catalyzed Reaction

The order of a chemical reaction with respect to the individual components is defined as power of the component concentration included into the rate equation. Depending on the substrate concentrations, the kinetics of an enzyme–catalyzed reaction may be described by the first–order rate equation.

The relationship between the rate of the reaction and substrate concentration has been formulated in most general terms as follows (Giese, 1973).

$$\text{Rate} = K[S]^n$$

Where, n = order of enzyme – catalyzed reaction

When the Michaelis–Menten equation is written in the form of a straight line, the Hill equation (Martin, 1993).

$\frac{\log v}{V_{max}-v} = n\log[S] - \log K_m$ is obtained. The equation states that, when [S] is low compared to K_m, the reaction velocity increases as the n^{th} power of [S].

In the present work, K_m and n values were determined from the plot of $\frac{\log v}{V_{max}-v}$ vs log [S] using the linear regression method (Table 4 and Figure 11). The reaction order (n) for α–amylase was calculated to be 1.0462 proving that the reaction order is first order.

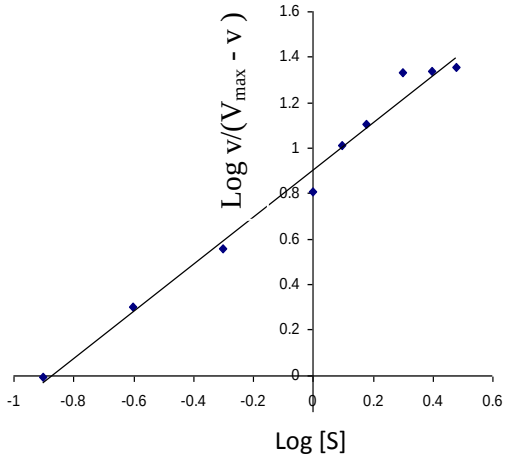

Figure 11 Plot of log $\frac{\mathbf{logv}}{\mathbf{V_{max} - v}}$ as a function of log [S] for α –amylase-catalyzed reaction

Conclusion

α-Amylase from immature white kidney bean seeds was isolated and purified by ammonium sulphate fractionation (20-60 %) followed by gel filtration chromatography and it was purified 12 fold compared to the crude extract. The molecular weight of purified α-amylase was determined as 56.23 kDa. The optimum pH of α-amylase was found to be 5.6 in acetate buffer and the optimum temperature of this enzyme was 50 ˚C. From the kinetic profile of the amylase enzyme the maximum velocity V_{max} and Michaelis-Menten constant K_m were observed to be 1.642×10^{-5} M min^{-1} and 0.1276×10^{-2} g mL^{-1} obtained by Lineweaver-Burk plot using linear regression method. For comparison purpose, K_m and V_{max} values were also evaluated from Eadie-Hofstee, Hanes-Wilkinson and Eisenthal-Cornish Bowden plots. The K_m values of α- amylase for starch by Eadie-Hofstee, Hane-Wilkinson and Eisenthal-Cornish Bowden plots were found to be comparable. The reaction order of the α- amylase catalyzed reaction was found to be 1.

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