Study on the Activities of -Amylase from Soybeam

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Abstract

-Amylase enzyme, (EC 3.2.1.2) was extracted from soybean. Soybeans were obtained from Hou-Poun Township, Shan State. Firstly, removal of fat from soybean powder was done by using Soxhlet extractor. Isolation was done by using ammonium sulphate precipitation. -Amylase action on soluble starch was characterized by using iodine staining method. -Amylase hydrolyses soluble starch to maltose as a major product. The -amylase activity, mole of maltose liberated per min per ml, was determined by Nelson-Somogyi method. The protein content of the enzyme solution was also determined by Biuret method. Specific activity (enzyme activity per mg of protein) of enzyme solution in each purification step was also determined. The specific activity was found to be 117.112 mol min^{-1} mg^{-1} protein for final enzyme solution. It was found that the specific activity, the relative purity of enzyme was increased about eleven times from crude extract to final stages. The enzyme activity was found to be 6498.5584 EU per gram of soybean. The enzymic properties such as optimum pH of -amylase was found to be 4.6 in acetate buffer and optimum temperature was found to be 60°C. The valules 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 β-amylase were found to be 1.89×10^2 g mL^{-1} and 76.92×10^4 M min^{-1}, respectively, from Lineweaver-Burk plot. The reaction order (n) for -amylase was calculated to be 0.96 providing that the reaction order is first order. Therefore, this research work may assist the development of enzyme technology in Myanmar.

Keywords: Soybean, -amylase, soluble starch, maltose

Introduction

The soybean or soya is a species of legume native to East Asia, widely grown for its edible bean, which has numerous uses. It is so called golden bean or wonder bean for its colour and biological potential. The seeds have been used for centuries as an article of food in China, Japan, and Korea and constitute a valuable protein supplement to the rice diet. Soybean was used as a starch free diabetic food.

The soybean belongs to the family *L*eguminosae, sub family *papilionoideae* and the genus *Glycine* the cultivated form is *Glycine max (L) Merrill* . Soybean contains amylase, urease, lipoxidase, lipase, peroridase, protease, glucosidase, carboxylase, catalase, ascorbicasse, allantoinase, phytase and uricase. It is a good source of -amylase. The biological function of -amylase in soybean is obscure as the beans contain very little starch. However, the presence of amylase in soybeans has long to be recognized.

-Amylase, α-1, 4 glycan maltohydrolase (EC 3.2.1.2) are exoenzymes and attack alternate glycosidic bonds in normal starch chain, starting from the non reducing end, containing untail the entric chain is converted into maltose . -Amylase are found to be restricted in the higher plants. Amylase are one of the main enzymes used in industry. Such enzymes hydrolyze the starch molecules into polymer compossed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries (Yamamoto, 1988).

Materials and Methods

In this research, -amylase enzyme (EC 3.2.1.2) from soybean and purified by using successive ammonium sulphate precipitation. -Amylase action on soluble starch was characterized by using iodine staining method. The optimum pH, optimum temperature, effect of reaction at enzyme concentration and effect of substrate concentration at enzyme concentration were determined by using UV-visible spectrophotometric method.

Result and Discussion

Extraction of -Amylase from Soybean

The -Amylase enzyme was isolated from soybean sample obtained from Hou-Pon Township, Shan State and was partially purified by solid ammonium sulphate precipitation method.

In this research, the extraction was carried out in a soxhlet extractor using petroleum ether as solvent (the water bath temperature was 80°C) until the yellow colour of soybean fat disappeared in the glass extraction tube. The defatted soybean flour was suspended in 1L of 10 mM aceate buffer, pH 4.6. The suspension was stirred for 2:30 hr at room temperature and was stored in a refrigerator (Crude Extract). The pH of supernatant was adjusted to 5.2 by the addition of 0.1 M acetic acid (Acidic Extract). The precipitate was filtered and then centrifuged to remove starch and insoluble material. The enzyme was finally precipitated by addition of ammonium sulphate 80 % saturation. The precipitated formed was removed by centrifugation and discarded. Solid ammonium sulphate was slowly added to the crude extract under stiring to obtain soybean -amylase.

Qualitative Examination of Soybean -Amylase by Using Iodine Staining Method

The iodine staining method is well studied for extensive kinetic analysis of purified enzyme (Normer, 1986).

In this work, a blank solution containing the mixture of starch and distilled water show deep blue colour with iodine solution, whereas solution mixture containing the enzyme and starch solutions showed no colour with iodine solution (Fig). Therefore, -amylase enzyme isolate from soybean hydrolyzes the starch by breaking down the α-glucosidic bond.

- (b)

Figure 1. Qualitative examination of β-Amylase activity by using iodine staining method

- Sample Solution (b) Blank Solution

Construction of Calibration Curve for Determination of Maltose Liberated from β-Amylase Action by Nelson-Somogyi Method

For quantitative analysis of a compound by visible spectroscopy, it is firstly necessary to know the wavelength of maximum absorption (λ_{max}) (Varley, 1980). In the determination of λ_{max} of arsenomolybdate chromogenic compound, the standard maltose solution, reagents of Nelson and Somogyi (arsenomolybdate colour reagent and alkaline copper reagent) were used. The Somogyi reagent is used to interrupt the reaction of enzyme (β-Amylase) on starch and oxidative form of complex copper II include in an alkaline copper solution is also reduced by the sugar, specially, maltose. The wavelength of maximum absorption was found at 750 nm. 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.

No	Concntration of maltose (mM)	Absorbance at 750 nm
1.	1.85	1.32
2.	1.67	1.18
3.	1.3	0.92
4.	0.93	0.65
5.	0.74	0.52

Calibration Curve for Protein Determination by Biuret Method

To determine the concentration of a solution by measuring the amount of light it absorbs requires a quantitative relationship (Nomera, 1986). In this work, bovine serum albumin (BSA) was used as a standard protein. The different absorbance value were obtained for different protein concentration by using a UV-visible spectrophotometer. It was found that the nature of the plot of absorbances vs. concentration of protein at 550 nm (cf. Table) was a straight line passing through the origin showing that Beer's law was obeyed.

Table 2. Relationship between Absorbance and Concentration of Bovine Serum Albumin (BSA) Solution

No	Absorbance at 550 nm	Protein Concentration (mg mL^{-1})
1.	0.23	1
2.	0.46	2
3.	0.93	4
4.	0.39	6
5.	0.85	8

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

β-Amylase activity was determined from the amount of maltose formed during the β-amylase-catalyzed reaction using starch as the substrate, according to the Nelson-Somogyi method. The enzyme unit (EU) of crude β-amylase was found to be 6498.5584 EU per gram of soybean. The protein content was determined by Biuret method. The protein was observed to be 0.5198 mg mL^{-1}. The specific activity was calculated to be 117.112 mol min^{-1} mg^{-1}.

Table 3. Specific activities of -amylase sample in purification steps

No	Purification Steps	Activity (µmol min mL^{-1})	Protein content (mg mL^{-1})	Specific activity (µmol min^{-1} mg^{-1})	Degree of purification
1.	Crude extract	38.74	3.64	10.65	1.00
2.	Acidic extract	81.53	0.93	87.56	8.22
3.	discarded filtrate	8.34	0.50	16.76	1.57
4.	final enzyme	60.88	0.52	117.11	11.00

Optimum pH of β-Amylase Activity

Enzyme are very sensitive to change in pH and each enzymes functions best within a very limited range called its optimum pH. At pH above and below optimum pH the activity of the enzyme is reduced and reaction rates are slower (Wiseman, 1975). In this work, different buffers of pH value 1.62 to 9.6 were used to determine the activity of the prepared β-amylase sample. The nature of the activity *vs.* pH curve of the enzyme (Table 4 and Figure 3) was obviously found to be unsymmetrical and the optimum pH was obtained at pH 4.6 with starch as substrate.

No	Buffer	pH	β-Amylase activity (mol min^{-1} mL^{-1})
1.	HCL, NaCL	1.6	2.16
2.	HCL, NaCL	2.2	2.84
3.	Acetate	3.6	5.20
4.	Acetate	4.2	5.68
5.	Acetate	4.6	5.79
6.	Acetate	5.0	5.56
7.	Acetate	5.6	5.01
8.	phosphate	6.2	4.33
9.	phosphate	7.0	3.23
10.	Na_2CO_3, $NaHCO_3$	9.6	1.87

Optimum Temperature of β-Amylase Activity

In this study, the effect of temperature was increased from 30 to 85 ˚C while the substrate medium (2 % starch) was prepared and the optimum pH of 4.6 was fixed (Table 5 and Figure 4). It can be seen that the optimum temperature for β-amylase-catalyzed reaction was at 60 ˚C in accordance with the literature finding of 40 to 60 ˚C (Wiseman, 1975). It is obvious that the activity of β-amylase-catalyzed reaction was increased from 20 to 60 ˚C and then decreased from 60 to 85 ˚C.

No	Temperature (˚C)	β-Amylase Activity (mol min^{-1} mL^{-1})
1.	30	9.99
2.	35	10.25
3.	40	10.48
4.	45	10.69
5.	50	10.94
6.	55	11.09
7.	60	11.25
8.	65	10.88
9.	70	10.15
10.	75	9.74
11.	80	9.66
12.	85	9.48

Effect of Reaction Time on β-Amylase-Catalyzed Reaction

The reaction time is an important factor in determining the enzyme activity. The optimum temperature for enzyme activity increased inversely with the increase in reaction time (Anderson, 1972). In this work, the action of β-amylase on soluble starch was studied in acetate buffer of pH 4.6 at 37 ˚C. the amount of maltose liberated during the various reaction times of 2, 4, 8, 10, 15, 20, 25, 30, 35, 40, 45 and 50 were determined by Nelson-Somogyi method (Table 6 and Figure 5). Figure shows the plot of velocity of β-amylase reaction during 35 min, the reaction is very fast. Then, velocity decreased steadily. Therefore, sequence study, reaction time of 5 min was chosen for the enzyme kinetic experiment.

No	Reaction Time (min)	Velocity (mM min^{-1})
1.	2	10.21
2.	4	9.649
3.	8	8.009
4.	10	7.193
5.	15	4.963
6.	20	3.846
7.	25	3.085
8.	30	2.581
9.	35	1.941
10.	40	1.555
11.	45	1.297
12.	50	1.115

Effect of Enzyme Concentration on β-Amylase-Catalyzed Reaction

The activity of an enzyme is determined by the enzyme concentration (Ivan Woff, 1963). As the enzyme concentration increases the rate off reaction increases linearly, because there are more enzyme molecule available to the 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 volume of enzyme solution ranging between 0.1 to 0.4 mL of enzyme (Table 7 and Figure 6).

No	Enzyme Solution (mL)	β-Amylase Activity (mol min^{-1} mL^{-1})
1.	0.1	1.827
2.	0.2	2.994
3.	0.3	4.499
4.	0.4	6.099

Effect of Substrate Concentration on β-Amylase-Catalyzed Reaction

In the present work, the velocities of enzyme reaction measure at different levels of starch concentration and their reciprocal values are shown in Table 8. The Michaelis-Menten plot of V *vs*. [S] is shown in Figure 7. If the concentration of starch was increased, the of reaction also increased until point 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 starch would not increase the rate of reaction. The enzyme could not work faster.

Most common transform in the Linewaver-Bulk plot which is also called double reciprocal plot (1/V *vs.* 1/[S]) (Figure 8). From this Linewaver-Bulk plot K_m and V_{max} values were found to be 1.89×10^{2} g mL^{-1} and 1.69×10^{4} M min^{-1}, respectively. Figure 9 show the Eadie-Hofstee plot of V *vs*. V/[S]. K_m and V_{max} values were found to be 1.96×10^{2} g mL^{-1} and 1.92×10^{-4} M min^{-1}, respectively. Figure 10 show the Hanes-Wilkinson plot of [S]/V *vs*. [S]. K_m and V_{max} values were found to be 1.94×10^{-2} g mL^{-1} and 1.96×10^{-4} M min^{-1}, respectively. Comparison of kinetic parameters of the β-amylase enzyme from different method are shown in Table 9.

Table 8. Relationship between Substrate Concentration and Velocity of β-Amylase-Catalyzed reaction

No	[S]* 10^2 (g mL^{-1})	-[S] 10^2 (g mL^{-1})	1/[S]* 10^2 (g^{-1} mL)	V 10^4 (Mmin^{-1})	1/V 10^4 (M^{-1}min)	V/[S]* 10^2 (M$min^{-1}g^{-1}$ mL)	[S]/V 10^2 (g mL^{-1} M^{-1} min)
1	0.13	-0.13	8.00	4.19	23.81	33.60	2.98
2	0.25	-0.25	4.00	8.20	12.20	32.80	3.05
3	0.50	-0.50	2.00	14.46	6.92	28.92	3.46
4	1.00	-1.00	1.00	24.20	4.13	24.20	4.13
5	1.50	-1.50	0.67	30.90	3.24	20.60	4.86
6	2.00	-2.00	0.50	36.00	2.78	18.00	5.56
7	2.50	-2.50	0.40	40.10	2.49	16.04	6.23
8	3.00	-3.00	0.33	42.80	2.34	14.27	7.00

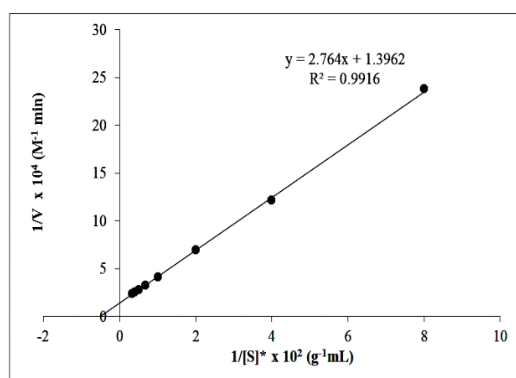

Table 9. Comparison of Kinetic Parameters of the β-Amylase Enzyme from Different Methods

No.	Method	$V_{max}\times10^{-3}$ (Mmin^{-1})	$K_m\times10^{-2}$ (g mL^{-1})
1.	Michaelis-Menten	1.49	1.54
2.	Lineweaver-Burk	1.69	1.89
3.	Eadie-Hofstee	1.92	1.96
4.	Hanes-Wilkinson	1.96	1.94

Determination of Reaction order (n) for β-Amylase-Catalyzed Reaction

In this work, K_m and n values were determined from the plot of log V/ V_{max} – V vs. log [S] for β-amylase sample using the linear regression method (Table 10 and Figure 11). The reaction order (n) for β-amylase was calculated to be 0.96 providing that the reaction is of the first order and this is in agreement with the literature.

Conclusion

β-Amylase samples were extracted from soybean obtained from Hou-Poun Township, Shan State; ammonium sulphate precipitation method was used. -Amylase action on soluble starch was characterized by using iodine staining method. The UV-visible spectrosphotometric determination was used to determine the amount of maltose that is formed by hydrolysis of β-amylase on starch, while Nelson-Somogyi method. The protein content of the enzyme solution was also determined by Biuret method. Specific activity (enzyme activity per mg of protein) of enzyme solution in each purification step was also determined. The specific activity was found to be 117.112 mol min^{-1} mg^{-1} protein for final enzyme solution. It was found that the specific activity, the relative purity of enzyme was increase about eleven times from crude extract to final stages. The enzyme activity was found to be 6498.5584 EU per gram of soybean. The enzymic properties such as optimum pH of -amylase was found to be 4.6 in acetate buffer and optimum temperature was found to be 60°C. The valules of K_m and V_{max} treated statistically using the linear regression method were compared with various graphically methods (Lineweaver-Burk, Eadie-Hofstee and Hanes-Wilkinson). The K_m and V_{max} values of β-amylase were found to be 1.89×10^2 g mL^{-1} and 76.92×10^4 M min^{-1}, respectively, from Lineweaver-Burk plot. The reaction order (n) for -amylase was calculated to be 0.96 providing that the reaction order is first order. Therefore, this research work may assist the development of enzyme technology in Myanmar.

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