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Purification and Characterization of β-amylase from Radish (*Raphanus sativus* L.) Root

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Abstract: β-amylase from mature radish root was isolated and purified to homogeneity for the study of enzyme characteristics. Purification steps involved acetone precipitation, DEAE-cellulose and Sephadex G-150 chromatography. The enzyme was purified 47.2 fold with 10.47 % yield, giving a final specific activity of 130 U/mg. Purity and homogeneity were judged by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Molecular mass of the purified enzyme was estimated by gel filtration chromatography and SDS-PAGE and it was found to be 61 kDa. Optimum pH of the purified enzyme was 6.0 and its activity was stable at pH ranged from 5.5 to 6.5. The enzyme showed maximum activity at 45°C and was found to be stable at temperature ranged from 0 to 45°C. K_m value of the enzyme for soluble starch was 1.12 mg/ml. Amylolytic activity of the enzyme for amylose and amylopectin was 95% and 80%, respectively. The enzyme had no effect on β-Limit dextrin, maltose, lactose and sucrose. Iodoacetic acid, N-Ethylmaleimide, urea, and Fe^{2+} reduced the activity of β-amylase moderately while Cu^{2+}, Pb^{2+}, Sn^{2+}, Hg^{2+} and p-hydroxymercuribenzoic acid (PCMB) almost completely ceased the enzyme activity. Amylolytic activity of purified enzyme was increased in the presence of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and EDTA.

Key words: β-amylase, *Raphanus sativus*, purification, characterization, radish, starch degradation.

INTRODUCTION

Amylolytic enzymes are widely distributed in plant tissues, e.g. in storage tissues such as seeds and tubers and in vegetative organs such as leaves. There exist two types of amylases in some species of plants, α-(E.C.3.2.1.1; 1-4-α-D-glucan glucohydrolase) and β-(E.C.3.2.1.2; 1-4-α-D-glucan maltohydrolase) amylases [1-2]. Beta-amylase (α-1, 4-glucan maltohyrolase, E.C.3, 2,1,2) is an exoamylase that attacks the non reducing ends of starches molecules, producing β-maltose and β-limit dextrin as products [3]. In starch-enriched tissues, β-amylase may play a role in the mobilization of starch during germination or sprouting tubers [1, 4-5]. Many reports have been demonstrated that β-amylase has a great commercial value in food and beverage industries. The enzyme is useful in structural studies of starch and glycogen. Marshal and Whelan [6] report on the removal of any contaminating β-glucosidase. The practical interest of β-amylase was concentrated on its capacity to produce maltose syrups from starch [7].

β-amylase has previously been purified and characterized from different types of plant sources and a few of microbial origin. In higher plants, the molecular characterization of β-amylase has been carried out on enzyme purified from the organs enriched in starch such as sweet potato tubers [8], leaves [9], bulbs [10], seeds of various cereal species such as barley [11], wheat [12], rice [2] and other higher plants such as soybean [13]. On the other hand, much less information is available on the purification and molecular characterization of β-amylase from root. The present study reports the purification of β-amylase from Radish (*Raphanus sativus* L.) root to an electrophoretically pure state along with its characterization.

MATERIALS AND METHODS

Chemicals: All chemicals were of analytical grade and purchased from Sigma Inc., USA. Soluble potato starch, amylopectin and amylose were also obtained from Sigma Co. p-Hydroxymercuricbenzoic acid (PCMB), N-Ethylmaleimide and Idoacetic acids were purchased from Fluka.

Plant Tissue: Mature, healthy *Raphanus sativus* (radish) roots were collected directly from the cultivation field during the winter season and these roots were used.

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Preparation of the Crude Extract: Fresh mature, healthy radish roots were washed thoroughly with distilled H_2O, cut into small pieces and ground with distilled H_2O and sand in a morter at 4°C. The suspension was then filtered through few layers of cheesecloth in a cold room. The filtrate was clarified further by centrifugation at 10,000 g for 15 minutes at 4°C. The supernatant was then dialyzed against distilled H_2O for 48h at 4°C and centrifuged at 10,000 g for 15 minutes at 4°C. The clear supernatant obtained was used as crude enzyme extract.

Purification of β-amylase: All enzymes purification steps were carried out at 4°C. The enzyme was purified to homogeneity following the four traditional successive steps:

Enzyme Precipitation: The crude extract was initially fractioned by 50% (v/v) chilled (-20°C) acetone saturation. After centrifugation at 10,000 g for 15 minutes, the precipitated pellets were collected and resuspended in two-pellet volume of cold buffer. The solution was dialyzed against 10 mM phosphate buffer of pH 7.0 for overnight. Undissolved particles were removed by centrifugation and the clear solution was used for further purification.

DEAE- cellulose chromatography: The enzyme preparation obtained from the previous step was passed over a DEAE-cellulose column (20.0 X 1.0 cm) previously equilibrated with 10 mM phosphate buffer of pH 7.0 at 4°C. The buffer was first passed through the column and then the enzyme was eluted with the same buffer containing a linear gradient of NaCl (0-500 mM) at a flow rate of 30 ml/h. Fraction with β-amylase activity was pooled and dialyzed against 10 mM phosphate buffer of pH 7.0 at 4°C and concentrated by Millipore.

Gel filtration chromatography: The dialyzed, concentrated enzyme preparation resulting from the anion exchange chromatography was applied to a column of Sephadex G-150 (150 X 3.0 cm) preequilibrated with 10 mM phosphate buffer of pH 7.0 at 4°C. The protein was eluted with the same buffer at a flow rate of 15 ml/h. Enzymatically active fraction from gel filtration step was pooled separately, concentrated by commercial sucrose and applied for molecular weight determination by gel filtration and electrophoresis.

Enzyme Assay: One milliliter of enzyme was added to 1 ml of 1% soluble starch containing 0.1 M phosphate buffer pH of 6.7 and the mixture was incubated at 37°C for 15 minute. The amount of reducing sugar produced was determined by Somogyi [14] and Nelson [15] methods. One unit of enzyme activity was defined as the amount; which catalyzed the formation of 1mmol of maltose under the assay conditions.

Protein concentration was determined by Lowry's phenol method[16], using crystalline BSA as the standard.

Determination of Molecular Weight by Gel Filtration Method: The molecular weight of the purified radish β-amylase was determined by gel filtration on Sephadex G-150 column (150 X 3.0) as described by Andrews[17]. β-galactosidase (MW-116 kDa), Phosphorylase (MW-92.5 kDa), Bovin serum albumin (MW-67 kDa), Carbonic anhydrase (MW-31 kDa), Trypsin inhibitor (MW-21.5 kDa) were use as standard. Standard proteins and purified protein were applied separately on to Sephadex G-150 column under identical conditions. The molecular weight of radish β-amylase was determined from the standard graph obtained by plotting the molecular weight of standard proteins against their elution volume.

Determination of Molecular Weight by SDS-polyacrylamide Gel Electrophoresis Method: The molecular weight of the purified radish β-amylase was determined by SDS-polyacrylamide gel electrophoresis according to the Laemmli[18]. The standard proteins were β-galactosidase (MW-116 kDa), Phosphorylase (MW-92.5 kDa), Bovin serum albumin (MW-67 kDa), Ovalbumin (MW-45 kDa), Trypsin inhibitor (MW-21.5 kDa). The PAGE was performed with 7% gels at 110 V and 30 mA. The protein bands on the gel was dyed by using of 0.25% Coomassie brilliant blue R-25 (CBB) solution containing 50 % methanol and 10 % acetic acid.

K_m Value of the Purified Radish Root β-amylase: K_m value of the radish root β-amylase was determined by the Lineweaver-Burk double reciprocal plot method. The initial velocity (V_O) was determined by measuring quantitatively the amount of the product (maltose) at various time intervals. The substrate concentration was varied between 0.66 and 2.0 mg/ml and the assay were performed at 45°C at pH 6.0.

Substrate Specificity: The substrate specificity of β-amylase was assayed in assay mixture containing different polysaccharides and disaccharides (1 mg/ml) at 45°C. At the time intervals aliquots were withdrawn to estimate the amount of released maltose.

Determination of Optimum pH and pH stability: The activity of amylase was measured at different pH values ranging from 3 to 9 (0.1M acctate buffer of pH 3.0-5.0, 0.1M citrate-phosphate buffer of pH 5.0, 0.1M phosphate buffer of pH 6.0-7.5, 0.1M tris-HCl buffer

of pH 8.0-9.0) at 37ºC. Starch solution (1 %) was made in above mentioned buffer of different pH values and was used as substrate for the amylase. Stability of the enzyme in buffer solution (0.1 M) of various pH values were examined by incubating the reaction mixture in the buffer for 30 minutes and then the remaining activity was assayed in the standard conditions (pH 6.0)

Determination of optimum temperature and thermostability: The activity of the amylase enzyme was measured at different temperature values (20-70ºC) using 0.1M-phosphate buffer, pH 6.0. The highest activity was expressed as 100%. To measure the thermostability, the enzyme was incubated at pH 6.0 at various temperatures for 10 minutes and the remaining activity was determined by the standard method.

Effect of Various Chemical Reagents and Metal Ions on the Activity of β-amylase: Effect of various compounds and metal ions on the activity on β-amylase was examined by incubating the enzyme solution at room temperature in the presence of the respective ion or compound for 5 minutes and at the end of incubation period, aliquots were withdrawn and assayed. In all of the above cases, the β-amylase activity is expressed as a percentage of the control enzyme activity (100%).

RESULTS AND DISCUSSION

The crude enzyme solution was extracted with distilled water from radish roots. The outline and results of the purification of β-amylase from radish roots are summarized in the Table 1.

Purification of Radish Root β-amylase: The crude enzyme solution was precipitated by the addition of acetone. Proteins are more easily denatured in organic solvents at temperatures above 10ºC [19]. So, special care was taken to work with chilled solvent. The mixture was allowed to stand for 2-3 hrs. The precipitate was collected by centrifugation at 15,000 g for 15 minutes at 4ºC. The pellet obtained was resuspended in two-pellet volume of cold buffer. The solution was dialyzed against 10 mM phosphate buffer of pH 7.0 for overnight. Undissolved particles were removed by centrifugation and the clear solution was used for further purification. The concentrated dialyzed enzyme solution from the previous step was passed over a column of DEAE-cellulose previously equilibrated with 10 mM phosphate buffer of pH 7.0 at 4ºC. The buffer was first passed through the column and then the enzyme was eluted with the same buffer containing a linear gradient of NaCl (0-500 mM). The elution profile is shown in the Figure 1. The proteins were eluted as one major peak (F-1) and two minor peaks (F-2 & F-3). Only the major fraction F-1 contained the β-amylase activity while other two fractions (F-2 & F-3) had no enzyme activity and were discarded. The enzymatically active fraction F-1 was pooled separately and the purity was checked by SDS-polyacrylamide gel electrophoresis. As shown in the Figure 2, F-1 fraction gave more than one bands indicating that it contained other proteins. The active fraction F-1 from the DEAE-cellulose step was pooled and concentrated by Millipore.

The concentrated solution was applied to a column of Sephadex G-150 (50 X1.0 cm) preequilibrated with 10mM phosphate buffer of pH 7.0 at 4ºC. The protein was eluted with the same buffer at a flow rate of 30 ml/h. The elution profile is shown in the figure 2. The fraction F-1 was separated into three peaks. One of which was major faction F-1c and other two fractions F-1a and F-1b were minor. Only the major fraction gave the enzyme activity and its purity was judged by SDS-polyacrylamide gel electrophoresis. This fraction contained pure β-amylase as it gave single band on polyacrylamide gel electrophoresis (Fig 3).

Characterization of the Enzyme: The purified radish root β-amylase shows many properties in common with other β-amylases. The molecular weight of radish root β-amylase, determined from the experiment with Sephadex G-150 gel filtration column was 62 kda. This was in good agreement with the molecular weight (61 kDa) determined by SDS-PAGE (Fig. 4). Hence, radish root β-amylase was a monomer. The present value (61 kDa) was very close to the molecular weights of β-amylase isolated from yam tuber (60 kDa) and ginseng (63 kDa) reported by Arai *et al.* [20] and Yamasaki *et al.* [21] respectively. Larger multimeric β-amylases have been reported from leaves of potato (111 kDa) [9] and potato tubers (122 kDa) [21].

As shown in the Figure 5, radish root β-amylase gave a characteristic bell shaped curve against different pH. The optimum pH of the purified enzyme was found in a range of pH from 5.5 to 6.5 with a maximum at pH 6.0. β-amylase from radish has a pH optimum for starch hydrolysis at 6.0, which is considerably lower than the optimum pH for β-amylase of potato, leaves[10]. Most β-amylases have an acidic pH optimum, ranging from pH 4.5 to 6.2 [12, 23-24]. However, the pH of amylase activity in extracts of sprouted potato tubers was reported by Ross and Davies [25] as 6.0. Hydrolysis of soluble starch by β-amylase isolated from pea epicotyls and leaves of *Arabidopis* had optima at pH 6.0 and 6.0 to 6.5, respectively [26-27], with which the present result was in good agreement.

Table 1: Summary of the purification of radish root β-amylase

Step	Total Protein (gm)	Total Activity (units)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude Extract	1625	4468.75	2.75	100	1
Acetone Precipitation & Dialysis	485.5	3997.5	8.2	89.18	2.9
DEAE-cellulose	59.4	2108.7	35	47.18	12.7
Sephadex G-150	3.6	468	130	10.47	47.2

Table 2: Effects of various substrates on radish root β-amylase

Substrate	Relative Activity (%)
Soluble starch	100
Alylose	95
Alylopectine	80
a-dextrin	68
Glycogen	30
b-Limit dextrin	0
Maltose	0
Lactose	0
Sucrose	0

Table 3: Effects of various metal ions on radish root β-amylase

Metal	Concentration (mM)	Residual Activity (%)
None	-	100
$CaCl_2$	0.1	175
$MgCl_2$	0.1	160
$ZnCl_2$	0.1	152
$FeCl_2$	1	33
$CuCl_2$	1	18
$PbCl_2$	1	17
$SnCl_2$	1	33
$HgCl_2$	1	13
$FeCl_3$	1	103

Table 4: Effects of various chemical reagents on radish β-amylase

Chemical Reagent	Concentration (mM)	Residual Activity (%)
None	0.1	100
EDTA	0.1	120
*PCMB	0.1	5
Idoacetic acid	0.1	87
N-Ethylmaleimide	0.1	97
Urea	0.1	95

*p-hydroxymercuribenzoic acid

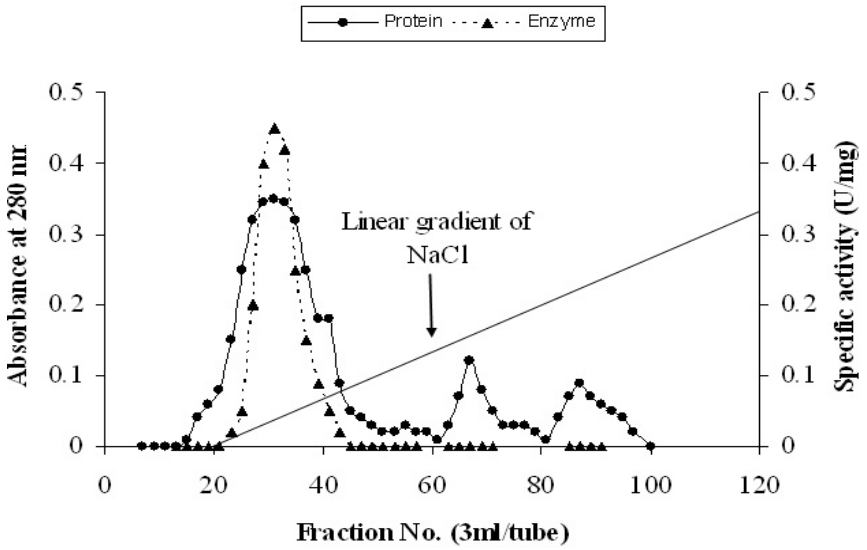

Fig. 1: Elution profile of radish root β-amylase on DEAE-cellulose column. The enzyme solution (485.5mg protein) obtained after acetone precipitation & dialysis was applied to the column previously equilibrated with 10 mM phosphate buffer pH 7.0 at 4^0C eluted with the same buffer containing a linear gradient of NaCl (0-500 mM).

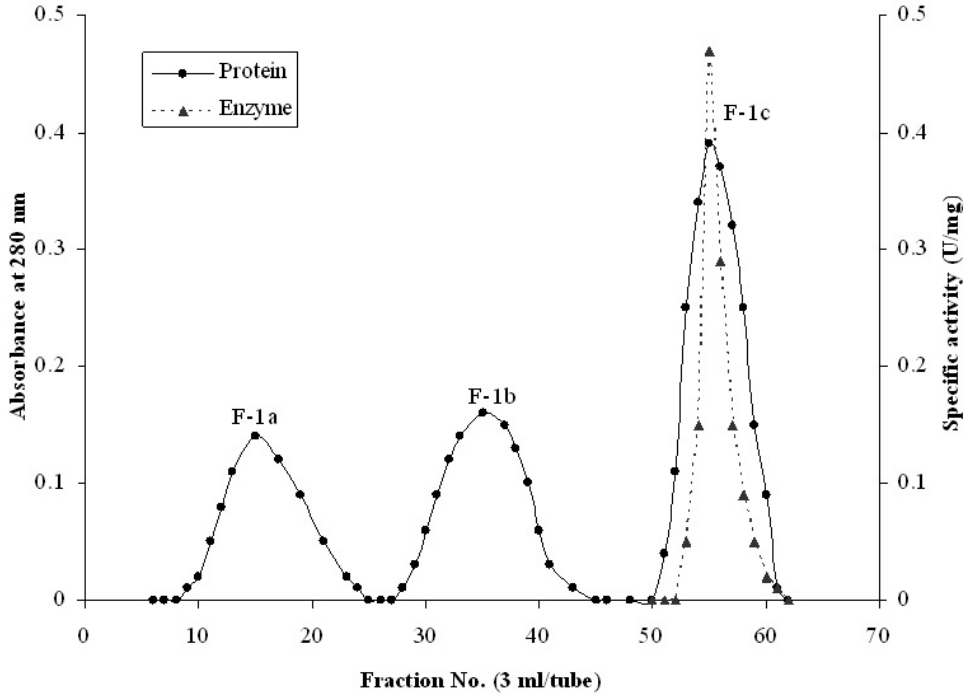

Fig. 2: Elution profile of F-1 fraction on Sephadex G-150 column. Protein (59.4 mg) was applied to column pre-equilibrated with 10 mM phosphate buffer pH 7.0 at 4^0C. The protein was eluted with the same buffer with same pH.

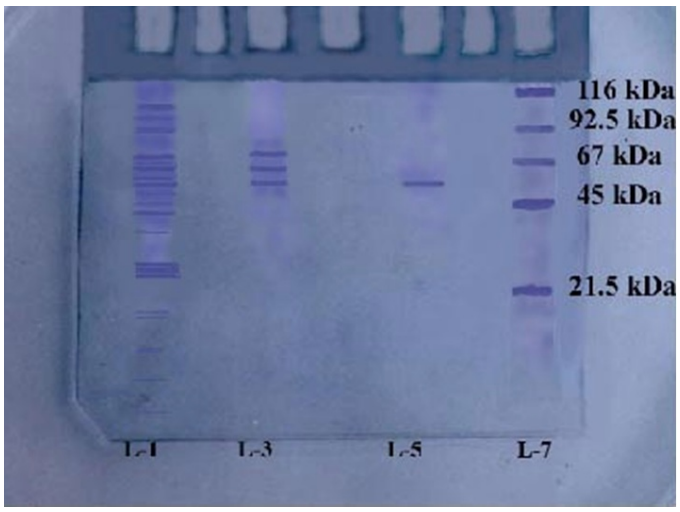

Fig. 3: Photographic representation of SDS-PAGE pattern of the purified enzyme.
L-1: Crude enzyme solution.
L-3: Fraction from DEAE-cellulose column.
L-5: Fraction from Sephadex G-150 column.
L-7: Marker protein solution.

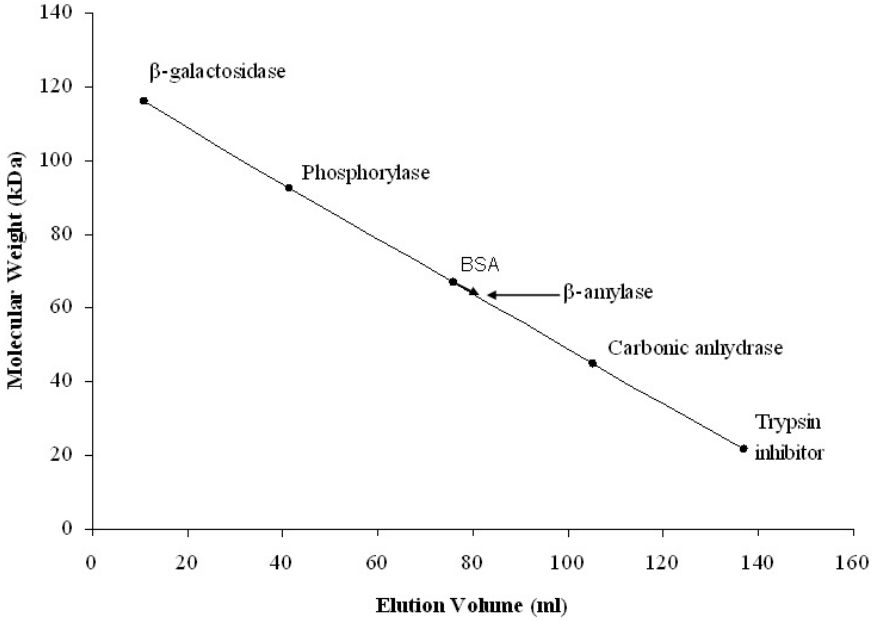

Fig. 4: Standard curve for the determination of molecular weight of radish root β-amylase by SDS-PAGE method.

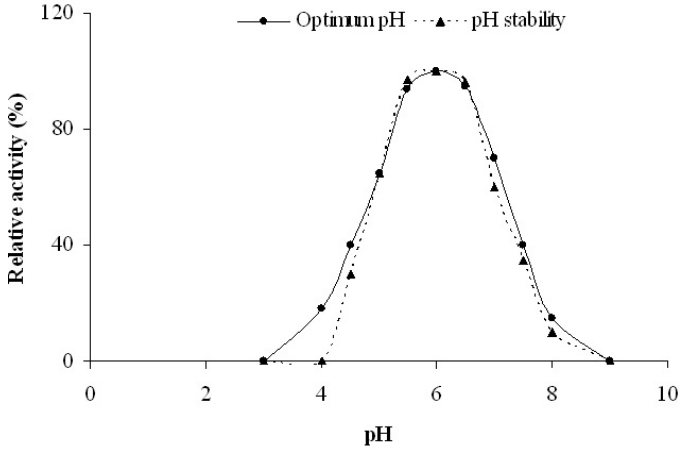

Fig. 5: Effect of pH on the activity of radish root β-amylase.

The activity of radish root β-amylase was found to be affected profoundly by temperature. The effect of temperature on the activities on the purified radish root β-amylase was in the range of 10-70°C. Optimum temperature and thermostsbility of the enzyme are shown in the Figure 6. The optimum temperature was at 45°C under the standard reaction conditions. The activity increased with increasing temperature to 30°-45°C, followed by a sharp decline to 70°C where complete inhibition in the activity was found. Shen *et al.* [28] reported the optimum temperature as 75°C for *Clostridium thermosulphurrogenes* β-amylase, Fan [22] reported as 55°C for potato tuber β-amylase, Dicko *et al.* [10] reported as 55°C for bulbs of Klattianus β-amylase, Which were considerably higher than our result, but was very close to that reported by Serafimova *et al.* [29] as 42°C, and was same as reported by Mori *et al.* [30] as 45°C. The enzyme was fully stable up to 45°C and the enzyme activity significantly decreased after 47°C.

The affinity of the enzyme for substrate was investigated using soluble starch as substrate. Under the standard assay conditions, the enzyme reaction was linear. The K_m value of the purified radish β-amylase obtained from the Lineweaver-Burk double reciprocal plot was 1.12 mg/ml (Fig 7). The K_m value of pea-epicotyl β-amylase was 1.67 mg/ml for soluble starch, which is relatively less high than our result. The k_m value of alfalfa (*Medicago sativa* L.) root β-amylase [31] was 5.9 mg/ml for soluble starch, which is higher than our result.

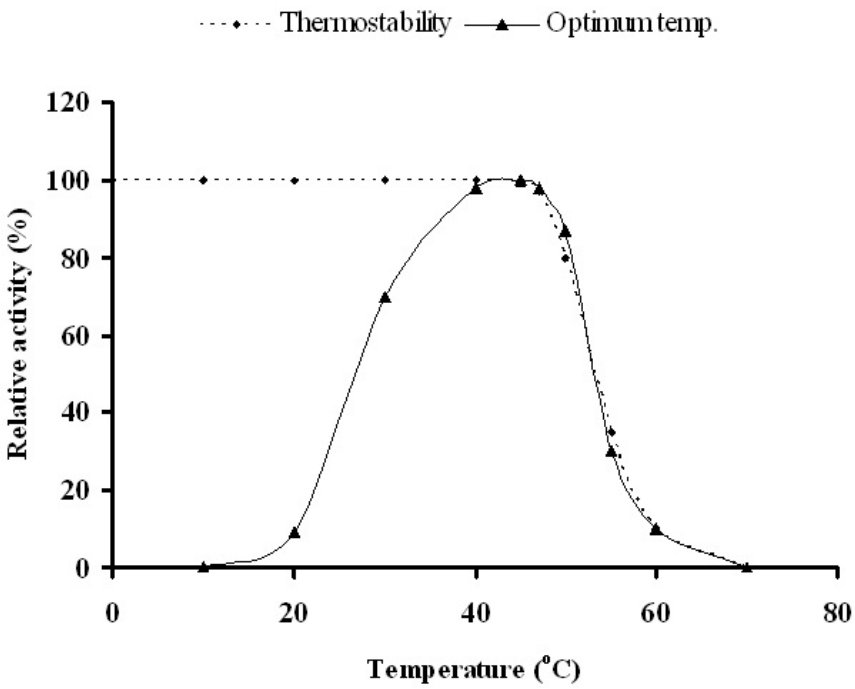

Fig. 6: Effect of temperature on the activity of radish root β-amylase

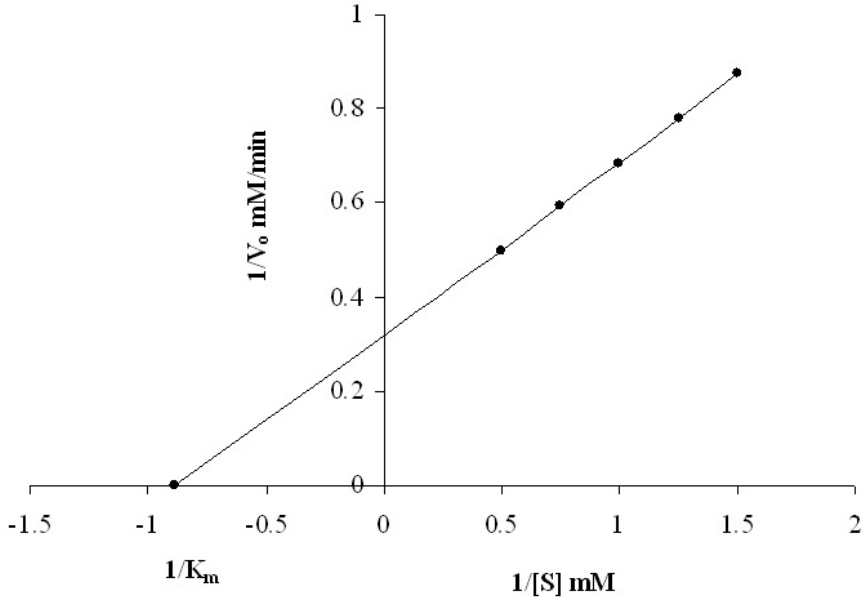

Fig. 7: Linewearver-Burk double reciprocal plot for the determination of K_m value of purified of purified radish root β-amylase.

The substrate specificity investigation showed that the only degradation product formed during soluble starch, amylose, amylopectin, a-dextrin, Glycogen is maltose. This confirms that the purified enzyme was a β-amylase. As given in table 2, the β-amylase gave about 100% activity when amylose and amylopectin were used as substrate but failed to hydrolyze maltose, β-limit dextrin, lactose and sucrose. These results coincide with those of other workers [1, 2, 24, 32-34].

The effects of various metallic salts and chemical reagents on the activity of β-amylase were shown in the table 3 and 4. Divalent cations like Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+} and EDTA increased the activity of the purified β-amylase. Iodoacetic acid, N-Ethylmaleimide,

urea, and Fe^{2+} reduced the activity of β-amylase moderately. The enzyme was almost completely inhibited by the presence of heavy metals such as Cu^{2+}, Pb^{2+}, Sn^{2+} Hg^{2+} and by SH-inhibitor such as PCMB, the activity being restored by thiol compounds such as cysteine. The result indicates that an SH group exits in the molecular structue, as in other plant β-amylase. Plant β-amylase has been reported to require free sulfhydrylgroups for activity and is inhibited by heavy metals as well as other other binding reagents [3, 12].

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