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The Effect of Temperature, pH, and Salt on Amylase in *Heliodiaptomus viduus* (Gurney) (Crustacea: Copepoda: Calanoida)

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Abstract: An interesting α-amylase has been obtained in large quantity (2400 U/g of body weight) with specific activity (20.22 U/g protein) from a freshwater zooplankton, *Heliodiaptomus viduus* (Gurney). Partially purified enzyme showed activity up to 70 ºC and demonstrated optimum activity at 30 ºC. The enzyme was active between pH 3.5 and 8.5, with maximum activity at pH 6.0. It retained its full activity at 30 ºC for 2 h, but became inactive at 60 ºC after 2 h, and at 70 ºC after 1 h. Enzyme activity was retained at 60% in 2 M NaCl after 24 h incubation, while full activity was found in 0.5 M NaCl for the same duration of incubation. Addition of metal ions like Fe^{2+}, Ba^{2+}, Co^{2+}, Ag^{2+}, and Mn^{2+} enhanced activity up to 130%-200% of the original activity, while K^{+} and Sn^{2+} caused a negligible increase in the activity. Addition of Hg^{2+} and Li^{2+} completely inhibited amylase activity, whereas Cu^{2+}, Mg^{2+}, and Pb^{2+} reduced activity to as little as 5% of original activity. Soluble starch, amylose, and amylopectin were completely digested by this amylase, whereas glycogen was hydrolyzed to a lesser extent. During hydrolysis of soluble starch, initially, maltose (G_2) and maltotetraose (G_4) were produced in similar magnitude, followed by a distinctly higher amount (> 80%) of maltose. Amylose was the most potential substrate with a K_m value of 1.82 mg/ml. The molecular mass was 50 kDa in the Native PAGE and no multiple forms were observed.

Key Words: *Heliodiaptomus viduus*, calanoid copepod, zooplankton, amylase

Introduction

Heliodiaptomus viduus is a commonly occurring calanoid copepod, which is found in large numbers in India throughout most of the year. It is a common natural source of food for fish, and thus is of great importance to fisheries and aquaculture. Although there are several works relating to amylase activity in calanoid copepods (Moal et al., 1981; Samain et al., 1981; Tande and Slagstad, 1982; Mayzaud et al., 1984; Mayzaud, 1985; Head and Harris, 1985; Landry and Hasset, 1985; Oosterhuis and Baars, 1985; Patel and Gouder, 1985; Samain et al., 1985; Mazaud et al., 1994), studies of the partial characterization of amylase from calanoid copepods are rather scant. Some of these are by Mayzaud (1980), Mayzaud and Mayzaud (1981), Mayzaud (1985), and Roche-Mayzaud and Mayzaud (1987). Investigation of amylase activity in freshwater calanoid copepods and its characterization at the species level has yet to be undertaken in India.

In this paper, amylase activity in *H. viduus* was investigated in relation to temperature, pH, NaCl concentration, and the effect of metal ions. In addition, quantity of hydrolyzed substrate, substrate affinity, and molecular mass of this enzyme were also determined.

Materials and Methods

Materials and chemicals

Dialysis membrane, 3,5-dinitrosalisylate reagent (DNS), sodium potassium tartrate, and potato starch, were purchased from Sigma Chemicals Company, USA. Whatman 1 mm and 3 mm chromatographic paper (Whatman Int. Ltd., England) and standard protein (Bio-Rad Laboratories, India Pvt. Ltd.) were used for analytical purposes. Other analytical grade chemicals were purchased from E. Merck Ltd (India).

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Collection, Isolation and Storage of *H. viduus*

Plankton samples were collected from a selected wetland in Midnapore (lat 20°33′N, long 87°11′E) with standard nylobolt plankton net (mesh size: 54 µ). These were then passed through a filter of fine net (Nylobolt PA 625; mesh size: 625 µm). Since this wetland had only 2 calanoid species, of which *H. viduus* was sufficiently large (~2.1 mm) (Pahari, 2001), it was quite convenient to identify and sort the species under a stereo zooming dissecting binocular microscope (Leica-MZ6). *H. viduus*, after sorting, were transferred to a buffer solution (20 mM, pH 6.0) and kept in glass vials at 4 °C for further processing. Prior to biochemical analysis, excess moisture was removed using filter paper.

Preparation of crude enzyme extract

The enzyme extract was prepared by homogenizing a known wet weight of *H. viduus* in phosphate buffer (20 mM, pH 6.0) and then centrifuging it at 10000 × g in a refrigerated centrifuge for 10 min at 4 ºC (Bakemann Avanti™ 30). The supernatant was then taken in a tube and stored at 4 ºC for further analysis.

Partial purification of enzyme

Supernatant (original activity: 240 µg ml^{-1} min^{-1}) was initially treated with 10% $(NH_4)_2SO_4$ and allowed to stand for 4 h, and was then isolated by centrifugation (10000 × g, 30 min at 4 °C). Finally, 60% $(NH_4)_2SO_4$ was added to the supernatant and it was allowed to stand overnight. The precipitate was recovered by centrifugation (15000 × g, 10 min at 4 °C), dissolved in 20 mM phosphate buffer (pH 6.0), and dialyzed overnight against the same buffer. The dialysate was used as the enzyme solution.

Assay α-amylase (1,4 α-glucanase) [EC 3.2.1.1]

Saccharolytic activity of α-amylase was measured with the DNS method (Bernfeld, 1955). The reaction mixture consisted 0.5 ml of 1% substrate solution dissolved in 20 mM phosphate buffer (pH 6.0), 0.4 ml of 20 mM phosphate buffer (pH 6.0), and 0.1 ml of enzyme solution. After incubation at 30 ºC for 5 min in a thermostable water bath, the reaction was stopped by the addition of 1 ml of DNS reagents. Samples were then placed in a boiling water bath for 15 min and subsequently cooled down to room temperature. Absorbance was measured using a spectrophotometer (Hitachi U2001) at 540 nm against a blank prepared using the identical method, except the enzyme solution was added to the mixture after the addition of the DNS solution. The OD values were then converted to micrograms of glucose equivalent using a standard graph obtained from the known concentration of glucose prepared with the same buffer solutions. A unit of enzyme activity was defined as the amount of enzyme that released 1 µg of reducing sugar as glucose standard per minute under the assay conditions specified.

Effect of temperature on amylase activity and stability

To determine the optimum temperature, amylase activity was measured at different temperatures for 5 min at pH 6.0. For thermal stability, the enzyme solution was kept at different temperatures for 60 min in phosphate buffer (20 mM, pH 6.0) and then immediately cooled in ice, and the residual activity was measured at the optimum temperature.

Effect of pH on amylase activity and stability

To determine the optimum pH, amylase activity was measured at different pH values for 5 min at 30 ºC. For pH stability, the enzyme solution at a desired pH, was kept at 4 ºC for 24 h, and then activity was measured at the optimum temperature.

Salt tolerance of amylase

The enzyme was incubated in 20 mM phosphate buffer (pH 6.0) containing various NaCl concentrations (0 to 5 M) for 2 and 24 h at 4 ºC, and activities were measured in the same way as described above.

Effect of metal ions on amylase activity

Various metal ions (Table 1) were added to the standard assay mixture and activity of the enzyme was measured in the same way as mentioned above.

Substrate specificity

To determine the substrate specificity, hydrolytic activity was assayed on the following substrates: starch,

amylose, amylopectin, and glycogen. Measurements of activity were carried out at various concentrations (0.1%-4.0% w/v) of substrate to establish the maximum activity under standard conditions.

Determination of the nature of enzyme action on substrate

The hydrolysis of soluble starch by the partially purified amylase was followed for 2 h under standard assay conditions. The reduction of the blue complex between starch and iodine was determined by the method described by Jensen and Olsen (cf. Jensen and Olsen, 1992). This reduction was compared with the production of maltose as previously described by Bernfeld (1955).

Paper chromatography

Determination of hydrolytic products

Hydrolytic products on different time scales were assayed by paper chromatography. The reaction mixture contained 2% starch solution instead of the 1% used in the standard method. Hydrolytic reaction after commencement was stopped by boiling the assay mixture for 5 min at the intervals of 5, 15, 30, 60, and 120 min. The hydrolyzed solution was spotted on the sheet of Whatman 1 mm chromatographic paper. The chromatographic solution contained a 4:1:5 n-butanol: acetic acid: water mixture. The chromatogram was stained with silver nitrate (Robyt and French, 1964) and dried after 5 min. Sugar areas were developed in alkaline methanol.

Quantitative assay of hydrolyzed products

Twenty-five microliters of the reaction mixture with 2% starch solution, incubated for 24 h, was spotted onto Whatman 3 mm chromatographic paper. Paper chromatography was carried out following the method described above.

For quantitative assay of hydrolyzed product from the enzyme, each spot was cut into several small pieces and shaken vigorously with 3 ml of elution mixture in a test tube. Small aliquots of these were centrifuged at 10,000 x g for 5 min. Supernatants were then processed to develop color with a phenol sulfuric acid method (Dubois et al., 1956). The OD was then measured at 490 nm using glucose as a standard.

Total protein measurement

Protein concentrations for determining specific activity were measured with the method described by Bradford (1976).

Native PAGE

Native polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) for α-amylase, excluding the presence of SDS. Gels were prepared at 10% and molecular mass was determined based on the comparative migration of the pre-stained standard marker proteins (Bio-Rad).

Activity staining was accomplished by the incorporation of a substrate (potato starch) into the acrylamide matrix of the resolving gel. When preparing gels, as described above, 0.5% soluble substrate was used in place of distilled water when the gels were cast. To observe the enzyme activity following electrophoresis, the spacers, along with the gel, were left between glass plates. The gel between the plates was then incubated for 30 min at 30 ºC in a buffer solution contained in a water bath to prevent any activation or inhibition. It was then stained with the appropriate staining solution as mentioned in the methods section.

Results

Heliodiaptomus viduus (Gurney) has a large amount (2400 U/g of body weight) of α-amylase with 20.22 U/mg protein-specific activity in crude enzyme extract.

The effect of temperature on partially purified enzyme activity was measured at pH 6.0 over a temperature range of 5-80 ºC (Figure 1). The optimum temperature of the enzyme was 30 ºC. Fifty percent of hydrolyzing activity occurred between 10-55 ºC. At 70 ºC, activity was 15% and at 80 ºC, activity was virtually undetectable.

The rates of thermal inactivation of the enzyme at pH 6.0 are shown in Figure 2. The enzyme retained 100% and 70% activity for 120 min when heated to 30 ºC and 40 ºC, respectively. At 50 ºC, 50% of its activity was retained for up to 85 min. The rate of thermal inactivation was faster at higher temperatures. At 60 and 70 ºC, the enzyme lost activity after heating for 120 and 60 min, respectively.

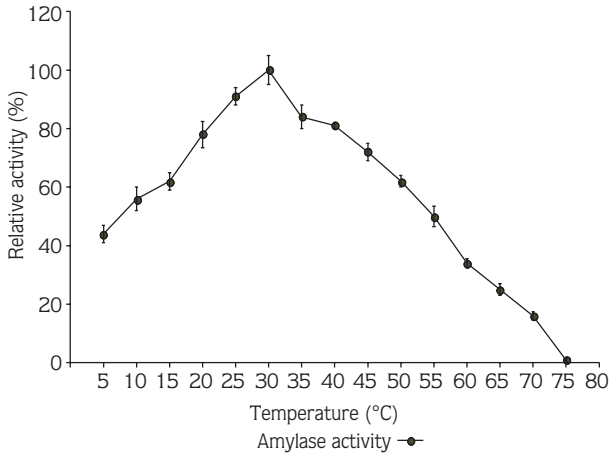

Figure 1. The effect of temperature (°C) on α-amylase.

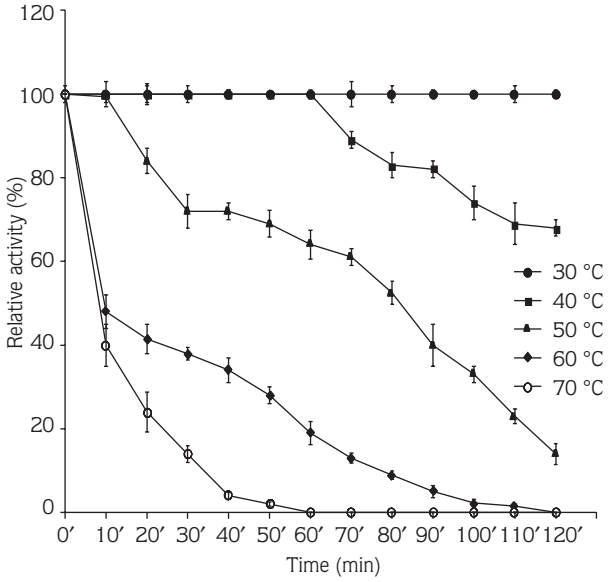

Figure 2. Thermostability of amylase.

The optimum pH and pH stability of the enzyme are shown in Figure 3. The optimum pH of the enzyme was 6.0. The amylase retained more than 50% of its original activity between pH 4.6 and 6.8. The enzyme was stable over a wide pH range. More than 50% residual activity was obtained between pH 4.7 and 9.0. The enzyme was not stable below pH 3.5 or above pH 10.0.

The amylase was stable in NaCl solution and retained 80% and 50% of original activity in 2 M and 5 M concentrations, respectively after 2 h of incubation at 4 ºC (Figure 4). Only 60% and < 10% of its original activity

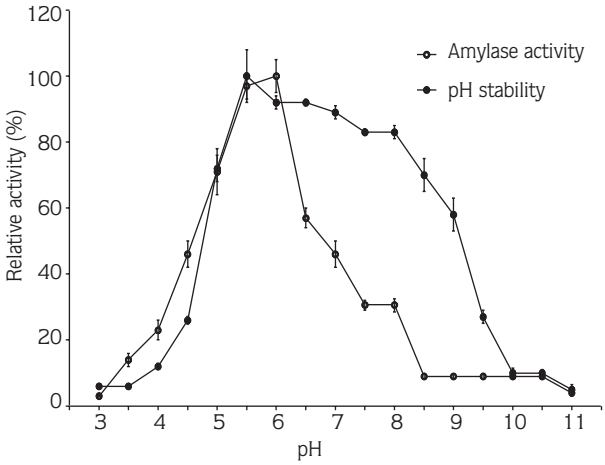

Figure 3. The effect of pH on amylase activity and pH stability.

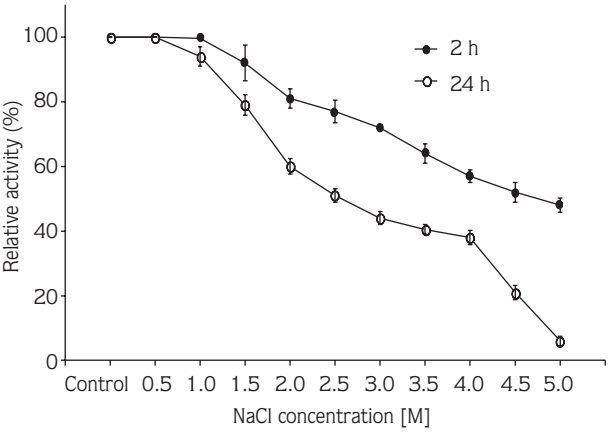

Figure 4. The effect of NaCl on amylase activity.

were retained in 2 M and 5 M NaCl, respectively after 24 h of incubation at the same temperature. Below 0.5 M NaCl concentration, the enzyme retained full activity for up to 24 h of incubation under the same conditions.

Metal ions like Hg^{2+} and Li^{2+} completely inhibited amylase activity, while Cu^{2+}, Mg^{2+}, and Pb^{2+} reduced activity to as little as 5% of original activity. In contrast to these, most of the metal ions enhanced activity. Fe^{2+}, Ba^{2+}, Co^{2+}, Ag^{2+}, and Mn^{2+} could enhance original activity by up to 130%-200%, while K^{+} and Sn^{2+} caused a negligible increase in activity (Table 1).

Affinities of the amylase for various substrates are given in Table 2. Partially purified enzyme showed no activity when tested against polysaccharides other than starch [D-Gluc α(1-4) Gluc]n, amylose [D-Gluc α(1-4)

Table 1. The effects of metal ions on amylase activity in *H. viduus*.

Metal ions (5 mM)	Relative Activity (%)	Induction (%)	Inhibition (%)
Control	100	-	-
NaCl	100	-	-
KCl	110	110	
$MgSO_4$	30		70
$CaCl_2$	130	130	
$FeCl_2$	148	148	
$BaCl_2$	159	159	
$CuSO_4$	5		95
$HgCl_3$	0		100
$CoCl_2$	198	198	
$Pb(NO_3)_2$	15		85
$LiCO_3$	0		100
$NiCl_2$	130	130	
$ZnSO_4$	100	-	-
$AgCl_3$	156	156	
$MnCl_2$	200	200	
$SnCl_2$	110	110	

Table 2. Influence of the type of substrate on the activity of partially purified amylase in *H. viduus* as % of the maximal activity.

	Amylose	*Starch*	Amylopectin	Glycogen	CM-Cellulose
Relative activity of α-amylase	100	97	48	12	0.0

Gluc]n, amylopectin [D-Gluc α(1-4) & α(1-6) Gluc]n, and glycogen [D-Gluc α(1-4) & α(1-6) Gluc]n. There was no hydrolytic activity found against CM-cellulose [D-Gluc β(1- 4) Gluc]$_n$. Therefore, the specificity appears to be restricted to α(1-4)-linked glucans. The reaction velocity followed Michaelis-Menten-type kinetics. The Lineweaver-Burke plot was used for the determination of K_m values for different substrates, which are given in Figures 5-8. The lowest K_m values, i.e. the highest substrate affinity, was for amylose (K_m = 1.82). This was followed by starch (K_m = 1.96), amylopectin (K_m = 2.86), and glycogen (K_m = 7.69), in relation to their corresponding V_{max} of 1420.45 µg ml^{-1} min^{-1}, 1162.79 µg ml^{-1} min^{-1}, 980.4 µg ml^{-1} min^{-1}, and 12.5 µg ml^{-1} min^{-1}, respectively.

The time course of hydrolysis of soluble starch by the partially purified amylase from *H. viduus* is shown in Figure 9. The iodine staining capacity steadily declined due to the release of reducing power. The extent of

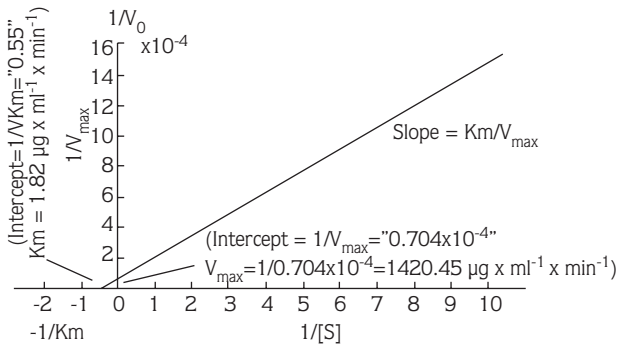

Figure 5. Lineweaver-Burke plot showing the Michaelis-Menten-type kinetics of the enzyme *amylase* on the amylose under the standard assay condition specified, indicating the K_m values under maximum velocity (V_{max}).

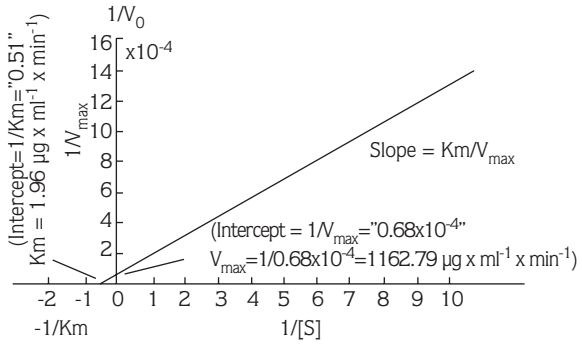

Figure 6. Lineweaver-Burke plot showing the Michaelis-Menten-type kinetics of the enzyme *amylase* on the starch under the standard assay condition specified, indicating the K_m values under maximum velocity (V_{max}).

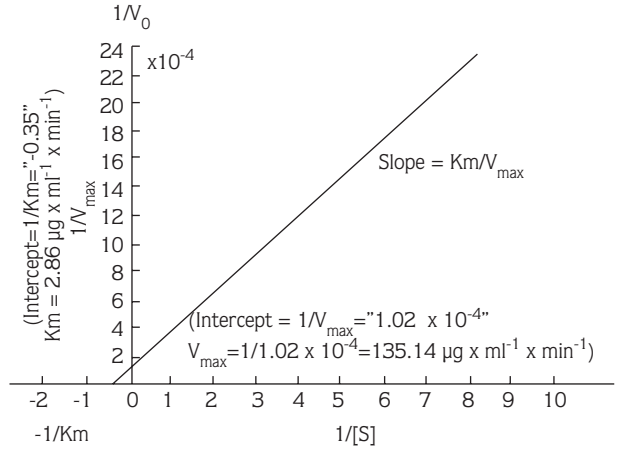

Figure 7. Lineweaver-Burke plot showing the Michaelis-Menten-type kinetics of the enzyme *amylase* on the amylopectin under the standard assay condition specified, indicating the K_m values under maximum velocity (V_{max}).

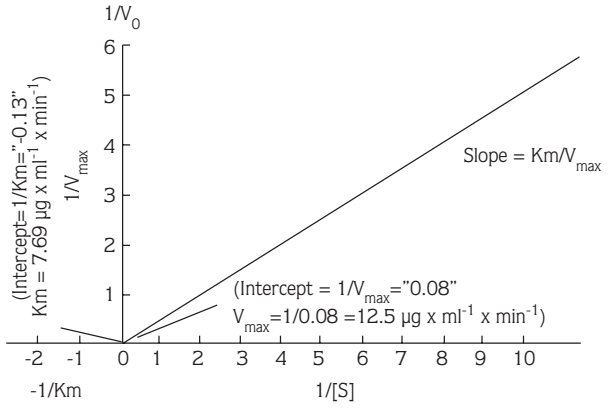

Figure 8. Lineweaver-Burke plot showing the Michaelis-Menten-type kinetics of the enzyme *amylase* on the glycogen under the standard assay condition specified, indicating the K_m values under maximum velocity (V_{max}).

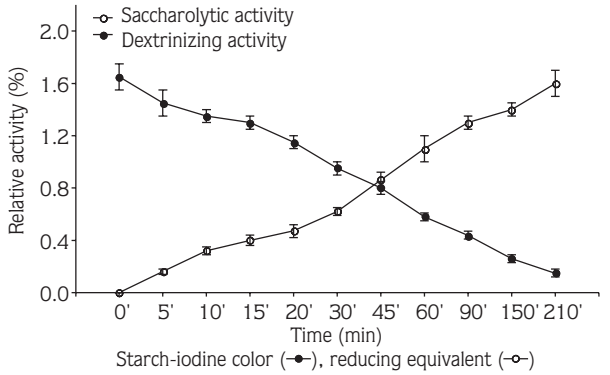

Figure 9. Time course hydrolysis of soluble starch by the partially purified α-amylase in *H. viduus* under standard assay conditions. Samples were withdrawn at different time courses (min) and processed for starch blue value and reducing sugar as described in the materials and methods section.

conversion of starch to maltose equivalents after 180 min of hydrolysis was about 90%, and this conversion was accompanied by a 10% decrease in iodine staining power.

Hydrolyzed products in various time courses (5 min to 2 h of incubation) of digestion are shown in Figure 10, exhibiting the degradation of substrate into products, mainly maltose (G_2) and maltotetraose (G_4). Glucose (G_1) and other malto-oligomers (maltotriose G_3, maltopentaose G_5, and maltohexose G_6) were absent during the course of hydrolysis of the substrate.

Eighty percent of the hydrolyzed product of this enzyme was found to be maltose (G_2) and maltotetraose (G_4) accounted for the remaining 20%.

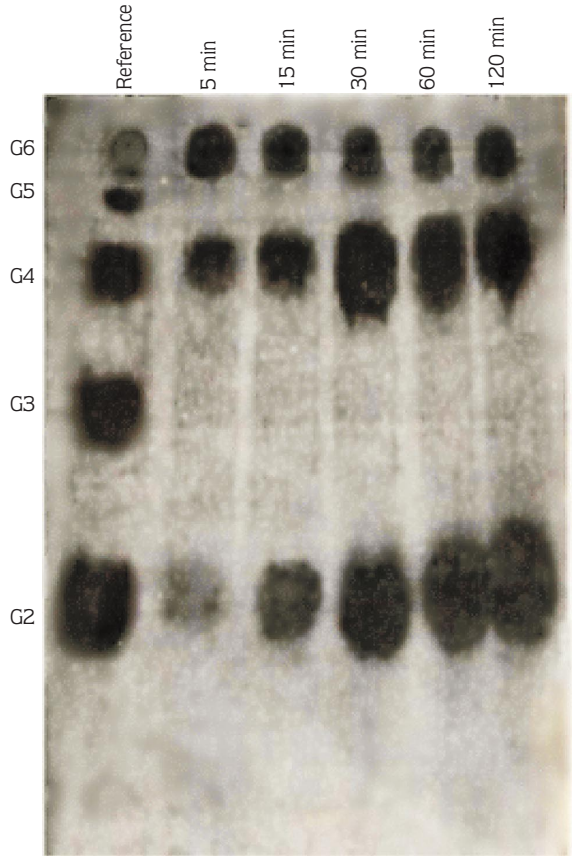

Figure 10. Hydrolyzed products in various time courses (5 min to 2 h) of digestion by amylase exhibited the degradation of substrate into products of mainly maltose (G_2) and maltotetraose (G_4).

The molecular mass was 50 kDa in the Native PAGE and no multiple forms were observed. The band showing the enzyme activity appeared as a clear area in the blue background for amylase (Figure 11).

Discussion

The present study is the first attempt toward the characterization of amylase in *H. viduus*. The presence of large amounts of this enzyme (2400 U/g of the body weight) in *H. viduus* is due to its herbivorous feeding habit. The broad temperature range of enzyme activity (> 50% of activity is between 10 and 55 ºC with the optimal at 30 ºC) is quite similar to amylase activity in *Acartia clausi*, which has > 50% of activity between 30 and 50 °C, with the optimal at 40 °C (Mayzaud, 1985), and carbohydrase (endoglucanase) activity in rotifers, where

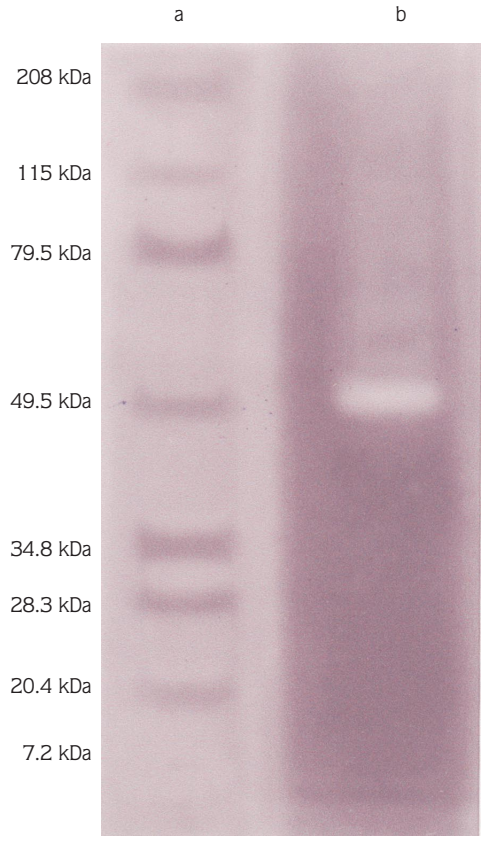

Figure 11. Native PAGE of partially purified α-amylase in *H. viduus*. Lane a: standard; Lane b: partially purified α-amylase. Molecular mass of the standard (pre-stained SDS-PAGE standard, broad range, Bio-Rad,) are: Myosin (208 kDa); β-Galactosidase (115 kDa); Bovine Serum Albumin (79.5 kDa); Ovalbumin (49.5 kDa); Carbonic anhydrase (34.8 kDa); Soybean trypsin inhibitor (28.3 kDa); Lysozyme (20.4 kDa); Aprotinin (7.2 kDa). Mobility is relative to the migration distance of Coomassie blue R-250 dye.

> 50% of activity has been reported between 5-55 ºC, with optimal activity at 37 ºC (Chang et al., 1997).

The enzyme also exhibited high activity in acidic or near neutral conditions. More than 50% of activity was seen from pH 4.6 to 6.8, with the optimal at pH 6.0. The present study indicates that this enzyme prefers slightly acidic pH for optimal activity. Similar preferred acidic conditions for activity of amylase have been reported by others. Mayzaud and Conover (1976) and Mayzaud and Mayzaud (1981) reported pH 6.0 as optimal for mixed total zooplankton and copepods. Boucher and Samain (1974) found pH 6.8 to be optimal for copepods and Mayzaud (1985) found pH 6.4 for *Acartia clausi* as optimal. Van Wormhoudt et al. (1983), however, reported an alkaline condition, 7.2 pH, as the optimum for α-amylase of the Antarctic krill, *Euphausia superba*.

Amylase in *H. viduus* exhibits maximum activity in the absence of NaCl, which is obviously an indication of conditions experienced in its freshwater habitat. Yet, this enzyme also exhibits its normal activity after incubation at 0.5 M NaCl concentration for 24 h, which suggests that saline environments may not adversely affect its digestive activity. In *Acartia clausi*, a marine copepod, amylase activity ranges between 10^{-4} and 0.25 M NaCl, with maximum activity occurring at 0.1 M NaCl (Mayzaud, 1985), whereas amylase in *H. viduus* showed normal activity at 0.5 M NaCl concentration (Figure 6).

In the enzyme action, metallic cofactors like Ba^{2+}, Ca^{2+}, Co^{2+}, K^{+}, Ni^{2+}, Ag^{3+}, Mn^{2+} Hg^{2+}, Li^{2+}, Cu^{2+}, Mg^{2+}, and Pb^{2+} are important because their presence or absence regulates enzyme activity. The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, and therefore the rate of digestion. As in the present study, Van Wormgoudt et al. (1983) and Mayzaud (1985) also reported inactivation of amylase caused by Mg^{2+} in *E. superba* and *Acartia clausi*, but in contrast to the present finding they reported inactivation caused by Mn^{2+}. Similarly, although Van Wormgoudt et al. (1983) and Mayzaud (1985) found enhancement as a result of Cu^{2+} and Na^{2+}, in the present study Cu^{2+} resulted in inactivation and Na^{2+} had no effect; however, as in the present study, Mayzaud (1983) also reported enhancement due to Co^{2+}. It appears that the actions of metallic ions on amylase vary from one species to the other.

Mayzaud and Mayzaud (1981) found the K_m value of amylase for unsorted neritic copepods to be 1.77, which is quite close to the K_m value for *H. viduus* (1.82 mg/ml), but it is less than the value obtained by Mayzaud (1985) for *Acartia* clausi (4.5 mg/ml).

This amylase has an interesting starch degradation property with reference to different time scales (Figure 12). During the first 30 min of hydrolysis, the yield of maltose (G_2) and maltotetraose (G_4) were equal; thereafter, the concentration of maltotetraose decreased and that of maltose increased. After 24 h of hydrolysis, we had 80% maltose and 20% maltotetraose. This enzyme is interesting in the sense that it can hydrolyze starch to produce large amounts of maltose (80%). Initially, the starch is broken down into G_2 and G_4, and

subsequently, G_4 is again hydrolyzed into 2 units of G_2, and as a result, G_2 gradually increases to a very high level. This property may be useful as an industrially valuable amylase, as is the case with maltose obtained from microorganisms like *Streptomyces hygroscopicus* (Hidaka and Adachi, 1980) and *Thermoactinomyces vulgaris* (Shimizu et al., 1978).

The molecular mass of this amylase was slightly higher (50 kDa) than that of the marine copepod Acartia clausi (44 kDa) (Mayzaud, 1985).

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