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The effects of temperature, pH and metal ions on alpha amylase activity of the brine shrimp *Artemia salina*

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Abstract. Nauplii of brine shrimp that hatched within 24 h were collected, homogenized, centrifuged and the supernatant was used as the crude enzyme preparation. In the reaction mixture for the amylase assay, various volumes of the enzyme preparation and the effect of reaction time on the initial velocity of amylase were determined. Also, the effects of pH and temperature on amylase activity and stability were investigated. Several metal ions were added to the amylase reaction to determine if they inhibited or enhanced the brine shrimp amylase activitiy. Results showed that homogenization with 20 times the buffer (w/v) was enough to produce linear relationship of the volume of the enzyme preparation and progress time with the initial activity of amylase. Maximal (and also optimal) temperature and pH were determined to be 40°C and pH 7.5, respectively. Thermal and pH stabilities ranged from 0°C to about 50°C and pH 6.0-7.5, respectively. The Michaelis-Menten constant (Km) for starch of the *Artemia* nauplii amylase was estimated using Lineweaver-Burk plot to be 0.11 µM maltose mg protein^{-1} (equivalent to 1.98 mg mL^{-1} glucose). Conclusion: The initial activity of the brine shrimp amylase could be measured using the enzyme volume and reaction time covered by the linear curves. *Artemia* amylase activity was optimal at 40°C and pH 7.5; it demonstrated high thermal and wide pH stability. Ca^{2+}, Fe^{2+}, Li^{+}, Mn^{2+}, Mg^{2+}, Na^{+} and Zn^{2+} inhibited amylase of the *Artemia* nauplii, retaining only 20% or less of the maximal activity. By the Km value, the physiological concentration of complex carbohydrates in the newly hatched *Artemia* nauplii was near the value equivalent to 0.11 µM maltose mg protein^{-1}.
Key Words: nauplii, thermal stability, pH stability, metal salts, amylase Km and Vmax.

Introduction. Knowledge of feeding strategies and adaptive digestive capacities of larval aquatic organisms under different food conditions and their effects on growth is of prime importance in aquaculture. Digestive enzyme activities of live prey and the mechanisms controlling them are thus deemed important in maximizing their enzymatic contribution to the larval absorption efficiencies for their corresponding substrates (Bayne 1976; Samain et al 1991; Ibarrola et al 1998, 2000), and this appears to be one of the principal factors explaining *in situ* growth variability (Huvet et al 2008).

α-Amylase (α-1,4 glucan-4-glucanohydrolase) is a key enzyme for carbohydrate digestion and is a limiting factor in absorption efficiency in bivalves (Moal et al 2000; Sellos et al 2003). In general, α-amylase can represent individual energy status, as observed in *Drosophila* when starch is the only carbohydrate source in the substrate (Powell & Andjelkovic 1983). α-Amylase catalyses the hydrolysis of internal (α-1,4) glucoside bonds in starch or related poly- and oligosaccharides. External factors affect the regulation processes of digestive enzymes such as the amylase in crustaceans (Guarna & Borowsky 1995; Le Moullac et al 1997). In *Artemia salina*, adaptation of amylase enzyme varies according to particular starch concentration in the diet (Samain et al 1980). This observation could probably extend to the existing high carbohydrate content of the brine shrimp right after hatching.

Brine shrimp (*Artemia salina*) has been a very convenient live food in the hatchery for a long time contributing its own digestive enzymes to aquatic larvae such as that of the mud crab *Scylla serrata* (Serrano 2013). *Artemia* nauplii contains 39% crude protein, 9.6% crude lipid, 24.4% ash and 6.4% glycogen (Ma et al 2001). In fish larvae, amylases are stimulated by glycolytic chains, glycogen, and starch (Peres et al 1998). A compiled proximate analysis data from 26 references revealed that the average values were 52.2% protein, 18.9% lipid, 14.8 carbohydrate and 9.7% ash (Leger et al 1987). The newly hatched instar I nauplii of *Artemia* contains 39% more energy and 34% less dry weight than older unfed instar stages (Vanhaecke et al 1983 from Leger et al 1987). The activity of amylase together with other digestive enzymes is high during larval development of *Artemia sinica* (Guangyu & Cheng 2002). Studies on the partial characterization of amylase from *Artemia* are rather scant and this information is important in the nutrition of larvae of aquatic organisms that are raised in the hatchery. *Artemia* nauplii contain considerably high carbohydrates at this stage. It is possible that the existing carbohydrates activate the activity of amylase in the nauplii that form an external source of the enzyme in the larvae of aquatic organisms. The ability of the dry dormant embryo of *Artemia* to survive for decades has been related to the presence in the cyst of high concentration of trehalose (Busa et al 1982). After resumption of development trehalose is used as the bulk source of energy and the bulk substrate of respiration (Clegg & Conte 1980). The aim of the present study is to characterize amylase in the newly hatched nauplii that could optimize its assays in the laboratory and may form a basis for successful larval culture in the hatchery.

Material and Method

Preparation of the tissue homogenate for enzyme assay. Nauplii of brine shrimp that hatched within 24 h were used in the assays. At this early stage of development, it has been demonstrated that the time of sampling does not matter since activity peaks of amylase occur from 1st to 10 days after hatching (Samain et al 1980). The same researchers have observed that these peaks are independent from diurnal and individual variations of amylase activity in *Artemia.*

About 100 *Artemia* nauplii were homogenized in 20 volumes of cold extraction solution (50 mM citrate phosphate buffer pH 7.0) at 1:20 ratio (wet tissue weight to volume) in an Ultra Turrax homogenizer. The homogenates were centrifuged at 4000 rpm for 15 min at 4^{o}C and the supernatant was used as enzyme preparation. Total soluble protein was measured following the procedure of Lowry et al (1951) with bovine serum albumin as standard. All enzyme assays were conducted within 4 h of homogenization and all samples for a single enzymatic assay were run in the same day. Blank controls, in which the reaction did not take place, were included in the routine assay.

Enzyme assays. All assays were carried out at 25^{o}C and values were taken as means of triplicate estimations ± the standard error of the mean (SEM). Each assay was conducted along with appropriate controls including non-enzymatic hydrolysis. α-Amylase activity was assayed as described by Bernfield (1955) implying that the increase in reducing power of a buffered starch solution was measured with 3,5 dinitrosalicylic acid (DNS) at 546 nm. The assay mixture consisted of 0.1 mL soluble starch solution, 0.5 mL of the enzyme preparation and 0.5 mL salt solution (homogenizing solution). The reaction was stopped by adding DNS solution and the mixture was heated for 5 min in boiling water, cooled in running tap water and the optical density read at 546 nm. Amylase activity was expressed in terms of µg maltose liberated from starch.

Reaction time and enzyme concentration. Progress curves were obtained from 0 to 60 min, and it was also found that the activity vs enzyme concentration curve was linear from 0.1 to 0.5 mL of the enzyme preparation.

Optimum pH and pH stability. The effect of pH on enzyme activity was determined in 50 mM citrate phosphate buffer (pH 5.9-8.0) at 25 °C. Enzyme pH stability was determined by incubating the enzyme preparation at different pH (pH 5.9 to 8.5) for 1 h at 0-4 °C, after which enzyme assays were performed.

Optimum temperature and thermal stability. The optimum temperature of the reaction was determined by conducting assays at different temperatures ranging from 25 to 40 °C. Thermal stability of the enzyme was determined by incubating the enzyme preparation at different temperatures ranging from 0-55 °C for 1 h, after which assay of the trypsin-like enzyme was done.

Estimation of Michaelis-Menten constant. The effect of substrate concentration on the brine shrimp amylase activity was investigated at a concentration range of $2.5x10^{-4}$ to $40x10^{-4}$ µmol min^{-1}, keeping the other components of the reaction mixture constant. The results were plotted by the double reciprocal plots (Lineweaver-Burk plots) to obtain the Michaelis-Menten constant (Km) of trypsin for its substrate.

Results. Amylase activity of *Artemia* nauplii exhibited linearity with enzyme concentration up to 0.5 mL (Figure 1). The linear part of the progress curves with time was until 40 min (Figure 2).

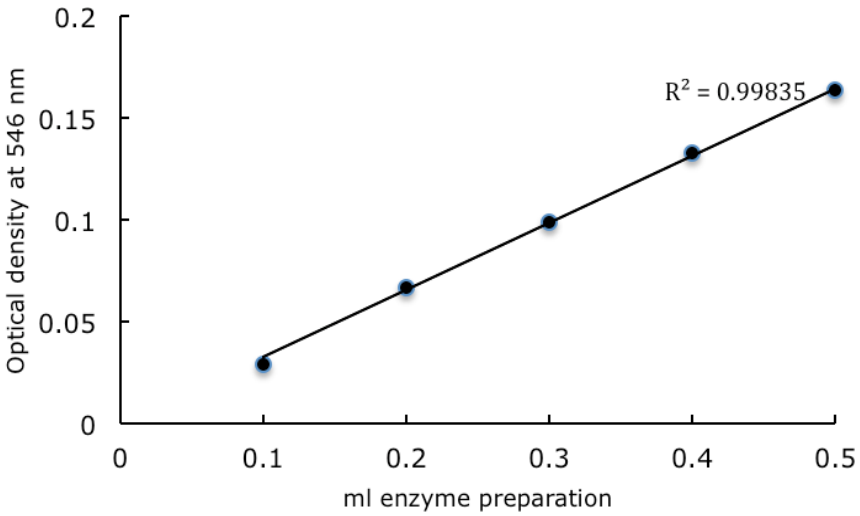

Figure 1. The effect of the volume of the enzyme preparation on amylase activity of the brine shrimp *Artemia*.

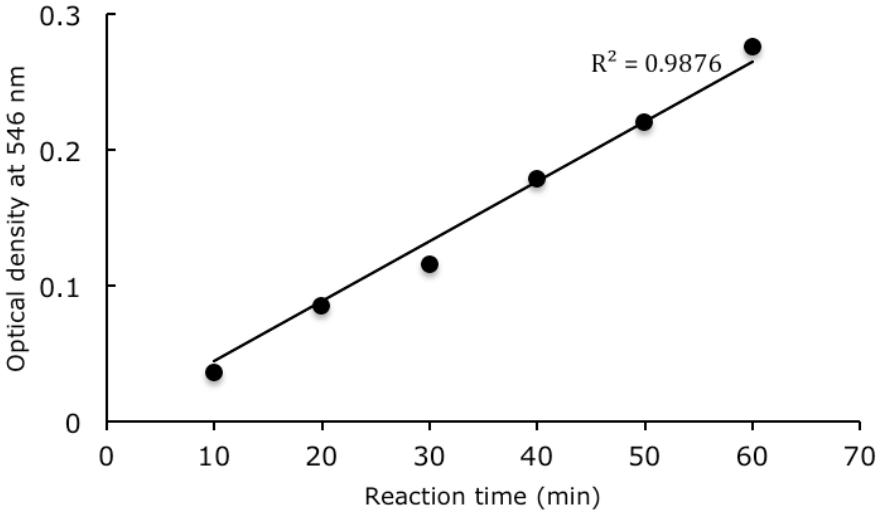

Figure 2. Progress curve of the activity of amylase from brine shrimp *Artemia*.

Amylase of *Artemia* nauplii showed slight activity changes with varying pH levels, exhibiting almost 80% or higher of the maximal activity even until pH 8.0. The optimum reaction pH was at pH 7.5 (Figure 3).

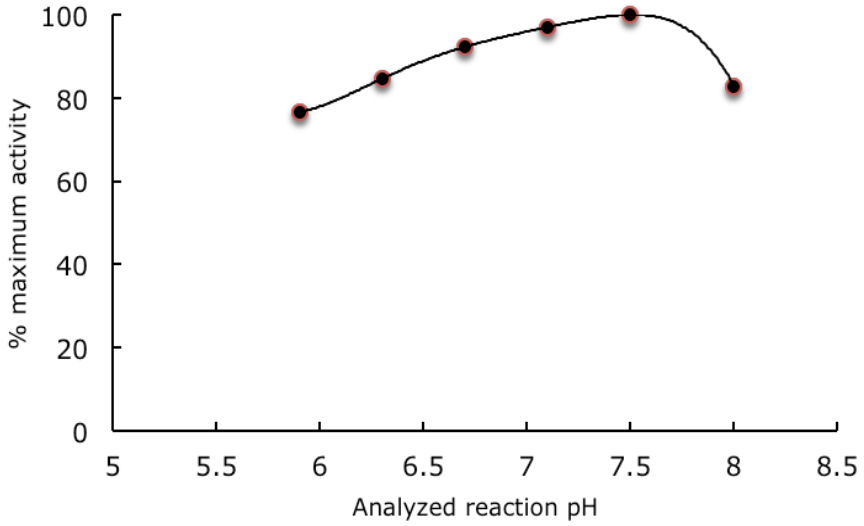

Figure 3. The effect of pH on amylase activity of the brine shrimp *Artemia.*

A nonlinear but proportional increase of amylase activity with temperature was observed in the brine shrimp amylase; optimal activity was observed at 40°C (Figure 4).

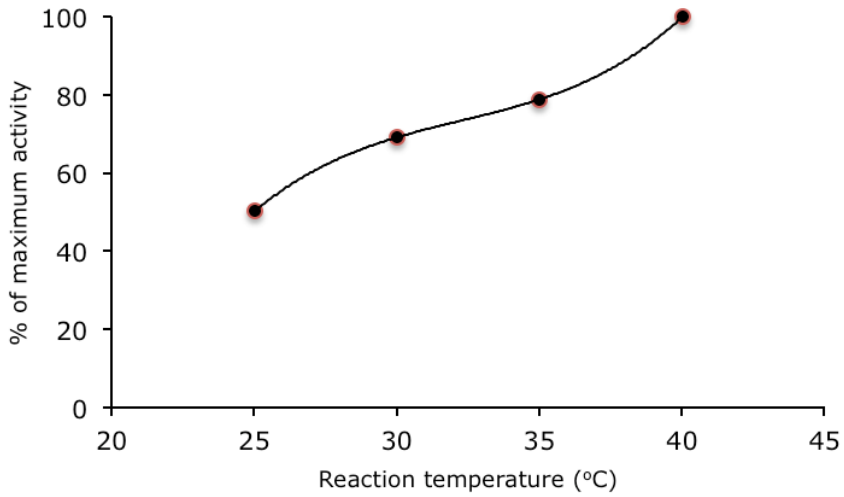

Figure 4. The effect of temperature (°C) on the amylase activity of brine shrimp *Artemia*.

The brine shrimp amylase showed slight activity changes when pH stability was investigated (Figure 5). It exhibited maximal values between pH 6.7-7.0. The enzyme was very thermostable even at 50°C, retaining 100 % of the initial activity (Figure 6).

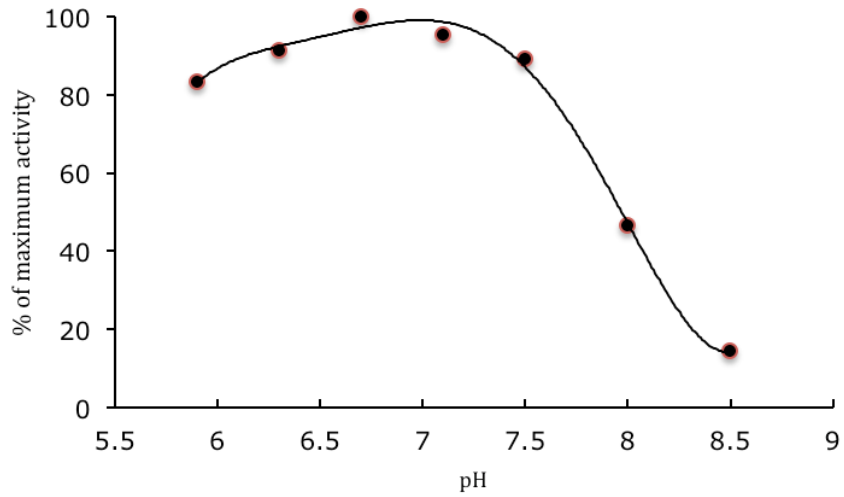

Figure 5. pH stability of amylase from *Artemia*.

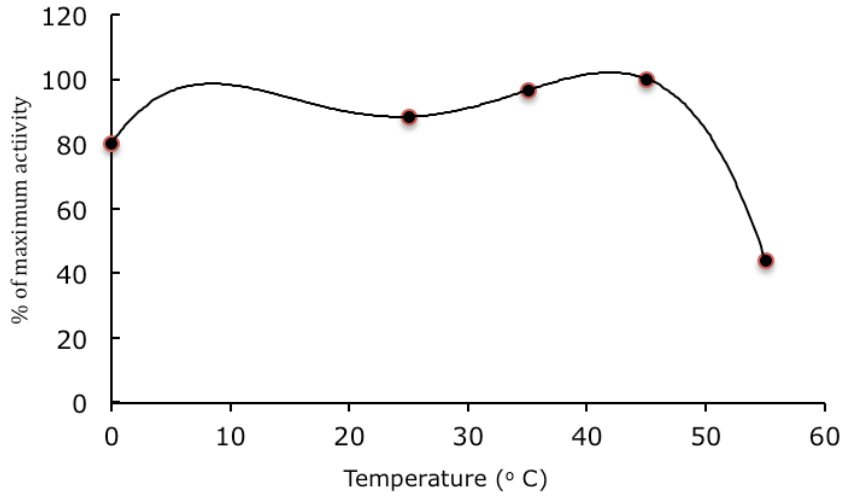

Figure 6. Thermal stability of amylase from the brine shrimp *Artemia*.

Most of the metals tested, namely Ca, Fe, Li, Mn, Na and Zn, markedly inhibited the brine shrimp amylase activity with less than 20% of the maximal activity (Table 1).

Table 1

The effects of metal ions on amylase activity in the brine shrimp

Metal ions	% of maximum activity
No metal ions	100.00
$CaCl_2$	9.38
$FeCl_3$	9.59
$LiCl_2$	13.86
$MnCl_2$	7.25
$MgSO_4$	10.23
NaCl	18.05
$ZnCL_2$	10.02

The determined Km for starch of the *Artemia* nauplii amylase was estimated to be 0.11 μM maltose mg protein^{-1} (Figure 7).

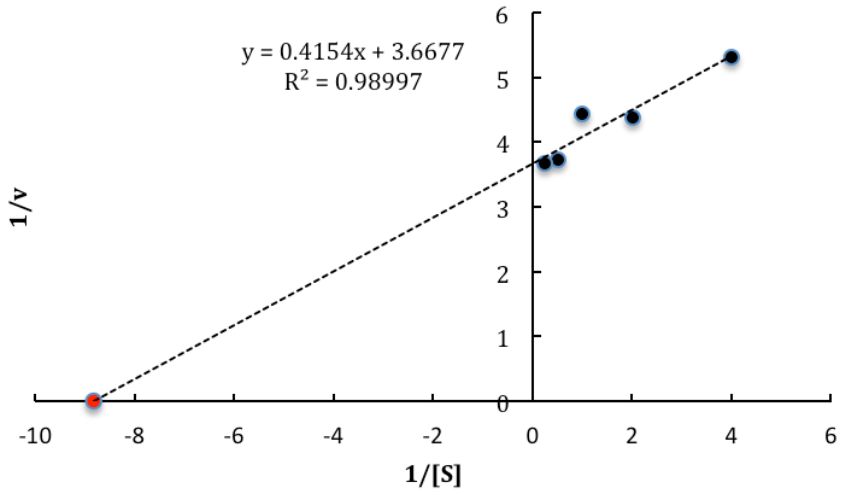

Figure 7. Lineweaver-Burk plot of the effect of substrate concentration on the initial velocity of the enzyme amylase from the brine shrimp *Artemia*.
[S] = substrate concentration in Molarity and v= initial velocity of the amylase in µM maltose mg protein^{-1}.

Discussion. The present study demonstrated in detail the procedural aspect of the enzyme preparation from the newly hatched *Artemia* nauplii. It was observed that the ideal dilution of the whole body tissue of the brine shrimp produced an almost ideal 45-degree angle of both the time of the reaction as well as the volume of the enzyme preparation against enzyme activity. This curve slope was ideal in the stopped-flow method of amylase assay specific to the whole brine shrimp. Also, the two curves were deemed important in every enzyme assay since they establish the condition for the measurement of activity, which should be based solely on initial velocity as a requirement of any enzyme kinetic measurement.

The enzyme exhibited very high activity in all the reaction pH tested, retaining almost 80% or higher of the maximum activity from pH 6-8; the optimal pH was observed at pH 7.5. The present study indicated that this enzyme preferred slightly alkaline pH for optimal activity which is in agreement with that reported for the amylase of the Antarctic krill *Euphasia superba*, which exhibited optimal activity at pH 7.2 (Van Wormhoudt et al. 1983). In contrast, the optimal pH observed for the calanoid copepod *Heliodiaptomus viduus* (Dutta et al 2006) and the optimal pH of a mixture of zooplankton and copepods were pH 6.0 (Mayzaud & Conover 1976; Mayzaud & Mayzaud 1981). Similarly, the *Acartia clausi* (a crustacean copepod) amylase exhibited optimum pH at 6.4 (Mayzaud 1985); while other copepod amylases exhibited optimal pH at 6.8 (Boucher & Samain 1974).

The narrow range of temperature in the present study resulted in more than 50% of the maximal activity between 25°C to 40°C; the activity exhibited continuous increase, albeit nonlinearly up to 40°C. This optimal temperature was in agreement with those observed in *Acartia clausi* at 40°C, retaining more than 50% of the activity between 30 and 50°C; the optimal activity was observed at 40°C (Mayzaud 1985). Those optimal temperatures reported for the activity of carbohydrase (endoglucanase) activity in rotifers in which more than 50% of the maximal activity was reported to be between 5 and 55°C, the optimal activity of which was at 37°C (Chang et al 1997). This is in contrast with those observed in *Heliodiaptomus viduus* with optimal activity observed at 30°C (Dutta et al 2006). The non-linear increase in the activity of amylase with temperature was an implied demonstration of adaptation to elevated temperature at the catalytic enzyme level without increasing the number of enzyme molecules (the volume of enzyme added was uniform in all reaction mixtures). This is presumed to be the interference of temperature on the enzyme-substrate (E-S) complex formation (Hochaka

& Somero 1973) as a way of increasing the efficiency of enzymatic reaction. Prolonged exposure at the temperature of 40°C, the temperature of maximal amylase activity in the present study, could inactivate the enzyme. This *in vitro* value did not necessarily define the natural physiological range of the brine shrimp and thus could be an artifact of the *in vitro* experiment (Traifalgar et al 1999).

Data on pH and thermal stability provides procedural techniques in the handling, preparation and temporary storage of the enzyme preparation. The stability of the crude preparation of the brine shrimp amylase (0-50°C and pH 6.0-7.5) was similar to that observed in the rotifer *Brachionus rotundiformis* (Traifalgar et al 1999).

In almost all enzymes, the presence of metallic cofactors like $Ba2^{+}$, $Ca2^{+}$, Co^{2+}, K^{+}, Ni^{2+}, Mn^{2+}, $Li^{+,}$ Cu^{2+}, Mg^{2+}, and Pb^{2+} is critical because they regulate enzyme activity. Their presence along with the food can increase or decrease amylase activity and thus the rate of digestion of carbohydrates, specifically starch. Examining the effects of metal ions may provide an indication of which amino acid residue can be found in the active or allosteric site of the enzyme by effecting a change in the conformation of these sites whether resulting in inhibition or activation. In fact, inhibition by metal ions provides more information on the active site than does activation. Similar with the results of the present study, Mg^{2+} and Mn^{2+} in *Euphasia superba*, *Acartia clausi* inactivated amylase activity. In *Heliodiaptomus viduus*, Mg^{2+} inactivated amylase but Mn^{2+} did not. Na^{+} exhibited no effect (Dutta et al 2006). Just like the observations of Dutta et al (2006), it was apparent that the actions of metallic ions on amylase activity varied from one species to another in the present study. Results from the rotifer amylase (Traifalgar et al 1999) were quite in contrast with that of the present study in which the rotifer amylase activity was enhanced or remained unaffected by the metal ions.

The Km value is an estimate of the affinity of the enzyme to its substrate and it also indicates the concentration of the substrate found normally in the body of the organism (i.e. its physiological concentration). The expression of amylase Km of other workers involving small preys was mg mL^{-1} glucose so that conversion was necessary for comparison purposes; the value estimated in the present study was 0.11 µM maltose to mg mL^{-1} glucose, the Km of the brine shrimp amylase for starch was 1.98 mg glucose mL^{-1}. This value was close to those found in unsorted neritic copepods (1.77 mg mL^{-1}) and *Heliodiaptomus viduus* (Dutta et al 2006) but less than that found in *Acartia calusi* (4.5 mg mL^{-1}). The low Km values of the brine shrimp amylase for starch in the present study, and those organisms whose amylase Km for starch exhibited similar values, demonstrated that their amylases possessed similar enzyme affinity to the starch substrate. The *V*max of the brine shrimp amylase under the Km value was estimated to be 93.3 µg glucose mg $protein^{-1}$ (converted from µg maltose mg $protein^{-1}$). Comparison of *V*max values with those reported by other workers was difficult because their expression of activity was µg glucose mL^{-1} min^{-1} while specific activity (µmol maltose mg $protein^{-1}$) was used in the present study.

Conclusion. The initial velocity of amylase from the brine shrimp *Artemia* nauplii could be measured using the linear curve of the volume of enzyme up to 0.5 mL and linear part of the reaction time until 40 min. The enzyme of the nauplii exhibited specific activities of 80% or more of the maximal activity even until 8.0, with optimal pH value at 7.5. The enzyme increased proportionately but nonlinearly with increasing temperature up to 40°C. The enzyme exhibited wide thermal and pH stabilities (from 0 to about 50°C and pH 6.0-7.5, respectively). All the metal ions inhibited the brine shrimp amylase retaining less than 20% of the maximal activity. The estimated Km for starch of the *Artemia* nauplii amylase was estimated to be 0.11 µM maltose mg $protein^{-1}$ (equivalent to 1.98 mg mL^{-1} glucose).

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