Biomacromol. J., Vol. 4, No. 1, 35-45, July 2018.

Biomacromolecular Journal
www.bmmj.org

Novel Amylase in Coelomic Fluid and Body Extract from the Earthworm *Allolobophora Chlorotica*

P. Ramian[a], M. Arabi[a] and R. Hemmati[b,c,*]

[a]*Department of Zoology, Faculty of Basic Sciences, Shahrekord University, Shahrekord, Iran*
[b]*Department of Biology, Faculty of Basic Sciences, Shahrekord University, Shahrekord, Iran*
[c]*Biotechnology Research Institute, Shahrekord University, Shahrekord, Iran*

(Received 17 July 2018, Accepted 25 September 2018)

ABSTRACT

Amylases are a family of endoamylases that randomly hydrolyze starch. This study for the first time aimed to explore α-amylase enzyme activity and the existence of amylases in coelomic fluid and body extract from the earthworm *Allolobophora chlorotic.* In practice, the worms were homogenized in ice-cold water and centrifuged to obtain a supernatant form earthworm body. Moreover, to prepare coelomic fluid electric shock was used and subsequently the activities of amylase in coeloimc fluid and body extract were measured at different pHs and temperatures. Based on results, the optimal pH and temperature for amylase activity in the body extract and coelomic fluid were 8 and 50 °C, respectively. Additionally, thermostability and pH stability of the enzyme was measured at different temperatures and pHs. At the other extreme, results indicated that metal salts, including $CuSO_4$, $CaCl_2$, NaCl, $MgCl_2$, KCl have enhanced amylase activity and, $HgCl_2$ and EDTA have inhibited the enzyme form both sources. As a consequence, it can be concluded that novel amylases in coeloimc fluid and body extract from the earthworm *Allolobophora chlorotica* behave similarly and the amylase can be purified in large scale from the earthworms and exploited in various biotechnological applications.

Keywords: Earthworm, *Allolobophora chlorotica*, Amylase, Starch, Coelomic fluid

INTRODUCTION

Earthworms belong to the phylum Annelida and are characterized by their ability to hydrolysis of carbohydrates and digestion of leaf litters, roots, yeast, algae and fungi on soil [1]. The presence of earthworms can significantly affect soil fertility, microbial and enzymatic activities. The current study used the earthworm *Allolobophora chlorotica* which belongs to the class oligochaete and order Opisthopora. Colour of this species of earthworm varies from green to yellow or gray but primarily is represented by two color morphs: green and pink [2].

Earthworms contain abundant of enzymes useful for bioremediation of contaminated soils, in animal feeds for improving the digestion of proteins, lipids, and carbohydrates, and also in the saccharification of starch, detergents, animal and human therapeutics. Amylases are among the most important industrial enzymes and are of great significance for biotechnology. Such enzymes can hydrolyze the starch molecules into polymers composed of glucose units [3].

Currently, amylases represent 25-33% of the world enzyme market [4]. α-Amylases (E.C 3.2.1.1) are a family of endoamylases that randomly catalyze the hydrolysis of internal α,1-4 bonds present in starch [5]. These kinds of enzymes are found mostly in plants, animal tissues and microorganisms (7). α-Amylases are extensively employed in processed-food industry, the pulp and paper industry, texture and detergent formulations [3].

The amylase activity in the body of earthworm has been investigated by many researchers. In a study conducted by Kavian et al. on the earthworm *Lumbricus rubellus*, it was found that the water extract of the crude high-protein (68.80%)-containing dry powder of this worm contains amylase and three other enzymes [6]. In addition, the enzymatic activity of amylase and some other enzymes have

*Corresponding author. E-mail: roohullah.hemmati@sku.ac.ir

been identified in the gut of earthworms *Lampito mauritii* and *Eudrilus eugeniae* reared on various substrates [7].

Levels of various enzymes including amylase were investigated in the guts of the two earthworms *Eudrilus Eugenia* and *Eisenia foetida* [8]. In the previous study, the amylase activity was found to be 0.248 and in the latter 0.290 mg of maltos/min/mg of starch. In fact, amylase showed higher activity in the guts of both the earthworms [8]. In addition, Ueda *et al*. were also able to purify and characterize α-amylases (Amy I and Amy II) from *Eisenia foetida* [9].

Based on the literature, to the best of our knowledge, it appears that so far no work has been done on amylase from *Allolobophora chlorotica* and also no report has been published regarding the origin and presence of enzymes in the coelomic fluid of the earthworms. However, few studies have reported the possible applications of colomic enzymes. Thus, this study for the first time aimed to explore the activity of amylase (starch-hydrolyzing enzyme) in coelomic fluid and body extract from the earthworm *Allolobophora chlorotic* to receive more information associated with amylase from the mentioned earthworm. Here, an attempt has been made to compare the behaviors of amylase in the coelomic fluid and body extract at different pHs, temperatures and in the presence of different metal salts. These were accomplished with the aid of spectrometry measurements, examinations of thermal deactivation, measurements of optimal pH and temperature parameters using the DNS method [10].

MATERIALS AND METHODS

Collecting Earthworms and Preparation of Coelomic Fluid and Body Extract

The earthworm samples were collected from greenfield of Shahrekord University (Shahrekord, Iran) and then transferred to the laboratory. They were kept at room temperature for two days. After washing the samples 100 g of the earthworms were added to a beaker and to obtain coelomic fluid, and a 50 mM Tris buffer was poured into the samples. The samples were then shocked with electricity and their coelomic fluids were collected. Alternatively, to obtain body extract the samples without coelomic fluid were homogenized in the presence of buffer and the resultant mixture was centrifuged at 6000 rpm for 6 min. The prepared supernatant was used as a source of enzyme. All materials used in this study were purchased from Merck® representative in Iran.

Assay of the Amylase from Body Extract and Coelomic Fluid

Amylase activity was measured according to the method of Bernfeld [11]. Thus, 1mg/ml of body extract or coelomic fluid was added to 1% soluble starch in the presence of 50 mM Tris buffer, pH 8 and followed by incubation of the resulting mixture for 120 min at room temperature. Then, the reaction was stopped with adding 1 ml of DNS reagent and leaving the solution in a hot water bath at 60 °C for 5 min. The same procedure was done for the control samples except that they were treated with the enzyme after adding DNS reagent. Subsequently, the samples were centrifuged to remove a yellow-color supernatant (due to the amylase effect on the starch and production of reducing sugars, a colored compound was developed). The absorbance of each mixture was then measured at 540 nm by means of a spectrophotometer. A set of triplicate measurements was used. All experiments repeated in triplicates and the average values used to obtain the appropriate plots using Excel 2016. For each experiment, the value of absorbance at 540 nm was converted to concentration using maltose standard curve [12] and the activities of amylases wre reported in the terms of µmole/min.mg per assay volume. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the conversion of 1 micromole of maltose per minute.

Measurement of Concentration of Total Protein from Body Extract and Coelomic Fluid

The Bradford (1976) protein assay with bovine serum albumin (BSA) as the standard, was used to calculate the total protein concentration. The absorbance was measured at 590 nm by means of a spectrophotometer [13].

A Qualitative Test for the Existence of Amylase from Body Extract and Coelomic Fluid

To test the existence of amylase in the body of earthworm, a series of qualitative tests were performed in the presence of agar with starch as the substrate according

to the earlier report [14]. Thus, 1 mg ml^{-1} of the body extract and coelomic fluid as two forms of fresh and heat-denatured enzymes were separately poured into the plates and the effects of enzymes on the substrate were investigated.

The Effects of pH on the Enzyme Activity from Body Extract and Coelomic Fluid

To investigate the effects of pH on amylase activity, the following buffer systems were used: citric acid monohydrate, sodium hydrogen diphosphate, Tris, glycine-sodium hydroxide with a concentration of 50 mM across the pH range 4-11, as has been reported previously [14]. Each reaction mixture was separately prepared by the addition of one of the buffers to body extract or coelomic fluid (1mg/ml) and of starch (1%). The mixture was then incubated for 120 min at room temperature, and subsequently, DNS was added and the tubes were immediately placed in a boiling water bath at 60 C. Finally, after centrifugation at 6000 rpm for 6 min and removal of the supernatant, the absorbance was measured at 540 nm and then using a glucose standard curve and considering the total protein concentration and the time of assay, the absorbance values were reported in terms of specific activity (μmol min^{-1} mg^{-1}).

The Effect of Temperature on the Enzyme Activity from Body Extract and Coelomic Fluid

The amylase activity was tested by incubating the reaction mixture at a different temperature from 10-70 °C for 120 min (with a temperature interval of 10 °C) as has been reported previously [15]. For this purpose, each reaction mixture was separately prepared by the addition of one of the buffers to body extract or coelomic fluid (1 mg ml^{-1}) and of starch (1%). The mixture was then incubated for 120 min at room temperature, and subsequently, DNS was added and the tubes were immediately placed in a boiling water bath at 60 °C. Finally, after centrifugation at 6000 rpm for 6 min and removal of the supernatant, the absorbance of the supernatant was measured at 540 nm and then using a glucose standard curve and considering the total protein concentration and the time of assay, the absorbance values were reported in terms of specific activity (μmol min^{-1} mg^{-1}).

Assay of Thermal Deactivation of Amylase and Determination of Half-life Values

To run the aforementioned test, both for the body extract and coelomic fluid, thermal deactivation of the amylase was separately measured within the temperature ranges of 30, 40, 50, 60 and 70 °C, as follows: Practically, a 5-ml mixture containing each body extract or coelomic fluid was placed at the specific temperature for 120 min. For every 20-min interval, a volume of the incubated body extract or coelomic fluid was cooled on ice for 30 min and the activity of amylase was measured at room temperature and optimum pH. Finally, the absorbance of the solution was measured at 540 nm. A mixture of the substrate (starch) and DNS was used as a control and it was not heated but directly cooled on ice. Eventually, the half-life of the amylase was calculated for each of body extract and coelomic fluid at any given temperature as reported in the previous study [15]. For each sample, the enzyme activity was obtained using maltose standard curve and the absorbance values were converted to a specific activity (vide supra).

Determination of pH Stability of Amylase from Body Extract and Coelomic Fluid

The pH stability of the amylase was assessed under the following buffer systems: citric acid monohydrate pH 4; Tris pH 9 and glycine pH 10.6 with a concentration of 50 mM. Practically, the activity of a mixture containing 1 mg ml^{-1} each of body extract or coelomic fluid was measured in a certain pH level at room temperature for 120 min. In practice, for every 20-min interval, a volume of the body extract or coelomic which was being treated at different pHs, removed and assayed at the optimum pH (Tris pH 8). The slopes of pH stability plots were calculated for each of body extract and coelomic fluid at any given pH as reported previously [15] and the activities were measures as described above (vide supra).

The Effects of Different Ions and EDTA on Amylase Activity in Body Extract and Coelomic Fluid

The effects of the metal salts such as KCl, $MgCl_2$, $HgCl_2$, NaCl, $FeCl_3$, $CaCl_2$ and EDTA on the amylase activity were studied as follows: First, three concentrations (1, 5 and 10 mM) of each compound were prepared. Then,

the solution was blended with a reaction mixture containing 1 mg ml^{-1} of the enzyme, 1% starch substrate and incubated for 120 min at room temperature. This was followed by determination of amylase activities in the presence of each compound as described above (vide supra).

RESULTS

Qualitative Testing of Amylase in Coelomic Fluid and Body Extract

The outputs of the qualitative testing in the presence of starch on the agar plate demonstrated the existence of α-amylase in the coelomic fluid and body extract as shown in Fig. 1B. The presence of a transparent halo around fresh coelomic fluid and body extract on agar plates in comparison with Fig. 1A and the absence of any transparent halo around boiled coelomic fluid and body extract on agar plates are indicative of amylase activity.

The Effect of pH on Amylase Activity in Coelomic Fluid and Body Extract

Based on Fig. 2, (A) α-amylase in the body extract shows maximal activity at pH 8. In fact, α- amylase activity at pH 8 reaches a maximal value. (B) Similarly, α- amylase in the coelomic fluid indicate a high activity at pH 7 and 8, however, the activity at pH 8 reached a maximal point. As pH increases to more than 8, the enzyme activity decreases in both of body extract and coelomic fluid.

At higher and lower pHs, the activity of the enzyme reduces but it does not reach zero. The results suggest the existence of an alkaliphilic enzyme. The maximum activity of the enzyme was observed in the body extract and coelomic fluid and then in the neutral and acidic medium.

Determination of pH Stability of Amylase in Coelomic Fluid and Body Extract

According to the results of amylase stability in both the body extract and coelomic fluid, shown in Fig. 3 and Table 1, the slope of the line is lowest at pH 8 than those for pHs 4 and 11. It is suggesting a higher stability of the enzyme at pH 8. For the both body extract and coelomic fluid, at more alkaline and acidic pH, the activity of the enzyme reduces as time passes to reach 120 min.

The Effect of Temperature on Amylase Activity in Coelomic Fluid and Body Extract

Figure 4 shows the activity of the amylase in the temperature range of 10-70 °C. Regarding the Fig. 4, it is shown that amylases in the body extract and coelomic fluid with higher activity within the temperature range of 40- 50 °C and the maximum activity of the enzymes are observed at a temperature of 50 °C. Additionally, at two temperatures, 10 and 70 C, the activity of the enzymes has reached to less than half of the maximal value.

Determination of Thermal Deactivation and Half-life of Amylase

The results of thermal inactivation of the amylases in the body extract and coelomic fluid in the temperature range 30-70 °C are shown in Fig. 5. The slopes of the lines (k_d) and half-life of amylases from body extract and coelomic fluid are depicted in Tables 2 and 3, respectively. Considering the results shown in Fig. 5 and Tables 2 and 3, enzymes are more stable at lower temperatures than the higher ones. Clearly, as temperature increases from 60 to 70 °C, the activity of the enzyme abruptly declines. Moreover, the slope of the line (k_d) together with the resultant half-life values suggest that the enzymes are more stable at lower temperatures but as time passes, it apparently loses its stability at higher temperatures.

The Effects of Metal Salts and EDTA on the Enzyme Activity

The effects of metal salts on amylases exist in the body extract and colelomic fluid are shown in Figs. 6A and B. The results in Fig. 6A showed that $CaCl_2$ has a stimulatory effect on the amylase activity from body extract and maximal effect was found at 10 mM. A stimulatory effect was observed for $FeCl_3$ up to 5 mM, however, at higher concentrations, this effect is reversed. Also, $MgCl_2$ has a stimulatory effect on the enzyme activity but this effect is reduced as salt concentration increases and reaches 10 mM. A stimulatory effect on the enzyme activity was found for KCl which was reversed at higher concentrations. In contrast to other salts, $HgCl_2$ has an inhibitory effect on the enzyme activity; this effect increases as the concentration of salt rises. Furthermore, EDTA has a slight inhibitory effect on the enzyme activity and maximal effect is found at 5

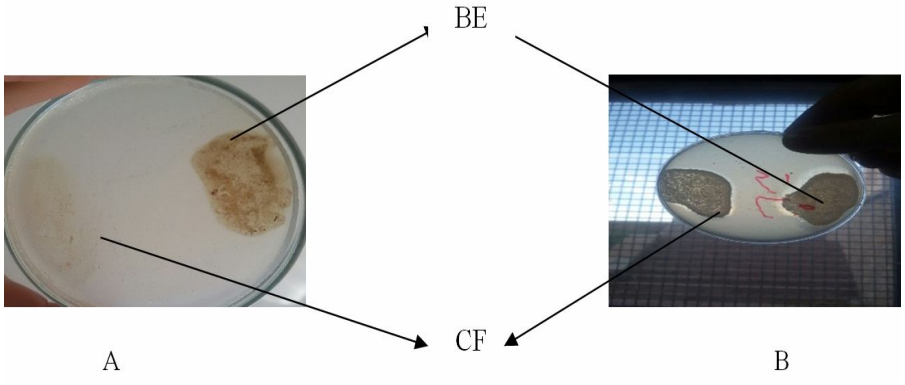

Fig. 1. The qualitative testing of α-amylase in heat-treated (A) coelomic fluid (CF) and body extract (BE) and (B) fresh samples on starch-containing aga plate (BE:body extract and CF: coelomic fluid). Note the formation of a transparent halo around the fresh extract of BE and CF and the absence of the halo around heat-treated samples on the starch-containing agar plates. Fresh and boiled body extract and coelomic fluid incubated on starch-containing agar plates for 24 h at room temperature.

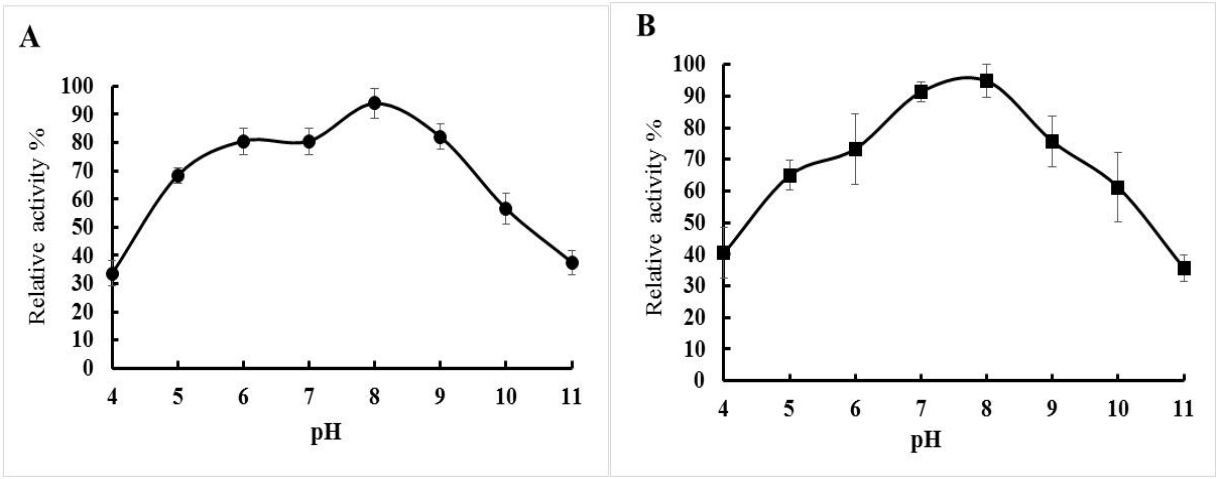

Fig. 2. The effect of various pH levels on the amylase activity (A) in the body extract and (B) in the coelomic fluid. Following the addition of 1 mg ml^{-1} of total protein to a mixture containing 50 mM of each used buffer in the presence of starch 1% at room temperatures, the mixture was incubated for 120 min. DNS was then added to the mixture and heated at 60 °C, centrifuged at 6000 rpm for 6 min and the absorbance of upper phase read at 540 nm. Consequently, absorbance values were converted to concentration using a glucose standard curve to draw the plots and find optimal pH level. All experiments repeated in triplicate.

mM. CuSo4 with a concentration of 5 mM decreases the enzyme activity; it has no effect at 1 mM but a stimulatory effect is observed at 10 mM.

In the case of coelomic fluid, considering Fig. 6B the effects of metal salts on amylase activity revealed that $CaCl_2$ has a powerful stimulatory effect on the amylase activity and this effect increases as the salt concentration rise. A slightly stimulatory effect is observed for $FeCl_3$, however, at higher concentrations, this effect declines. Also, $MgCl_2$ has a stimulatory effect on the enzyme activity but

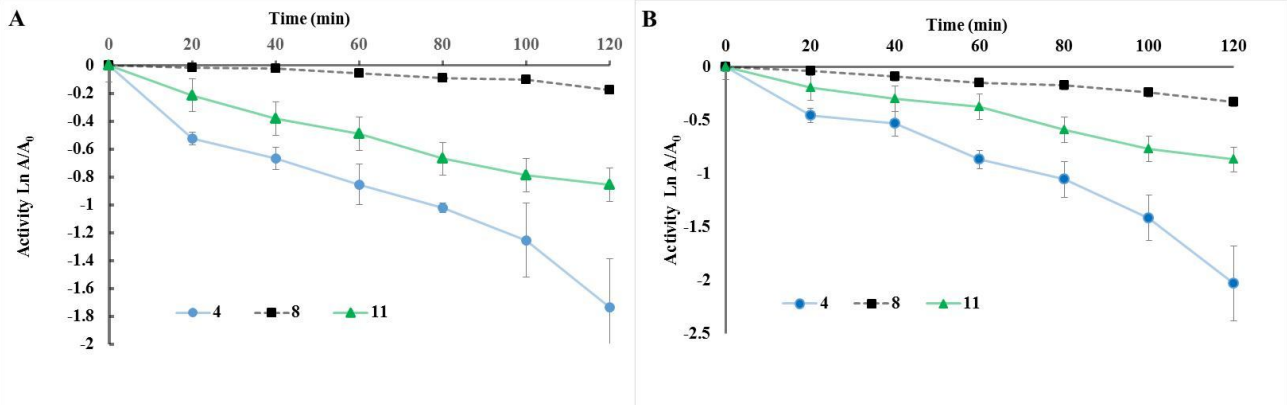

Fig. 3. The pH stability of the α-amylase at pH 4, 8 and 11, (A) in the body extract and (B) in the coelomic fluid. (A: The enzyme activity at any time; A_0: The enzyme activity at time zero). The activity of a mixture containing 1 mg ml^{-1} each of body extract or coelomic fluid was measured at each pH level for 120 min at room temperature in the presence of 50 mM of different buffers. In practice, for every 20-min interval, a volume of the body extract or coelomic which was being treated at specific pH, removed and assayed at the optimum pH (Tris pH 8). For this, DNS was then added to the mixture of assay and heated at 60 °C, centrifuged at 6000 rpm for 6 min and the absorbance of upper phase read at 540 nm. Consequently, absorbance values were converted to concentration using a glucose standard curve. All experiments repeated in triplicate. Note the relatively constant slope of stability plots at pH 8.

Table 1. The Slope of the Lines of pH Stability Plots from Fig. 3

Source	pH	Slope (min^{-1})
Body extract	4	-0.0125
Body extract	8	-0.0014
Body extract	11	-0.0071
Coelomic fluid	4	- 0.0152
Coelomic fluid	8	-0.0025
Coelomic fluid	11	-0.0072

this effect is reduced as salt concentration increases and reaches 10 mM. Oppositely, $HgCl_2$ has an inhibitory effect on the enzyme activity; this effect increases as the concentration of salt rises. In any concentration, KCl has a stimulatory but EDTA an inhibitory effect on the enzyme activity. $CuSO_4$ with a concentration of 1 mM has a stimulatory effect on the enzyme activity but this effect reduces as the concentration of salt rises.

DISCUSSIONS

Based on the quantitative and qualitative results obtained from the current study it appears that both body extract and coelomic fluid of the earthworm *Allolobophora chlorotica* contain amylase. Many studies have reported the various enzymes in the earthworm [7,8] however; no attempt has been made to investigate amylase in coelomic

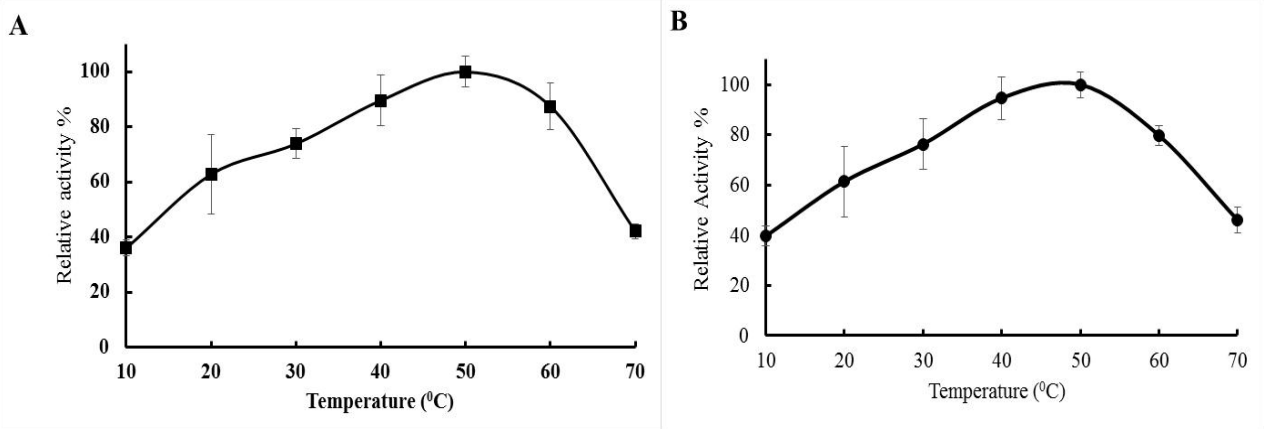

Fig. 4. The effects of various temperatures on the amylase activity (A) in the body extract and (B) in the coelomic fluid. Following the addition of 1 mg ml^{-1} of total protein to a mixture containing 50 mM of Tris buffer in the presence of starch 1% at room temperature, pH 8, the mixture was incubated for 120 min. DNS was then added to the mixture and heated at 60 °C, centrifuged at 6000 rpm for 6 min and the absorbance of upper phase read at 540 nm. Consequently, absorbance values were converted to concentration using a glucose standard curve to draw the plots and find optimal temperature. All experiments repeated in triplicate.

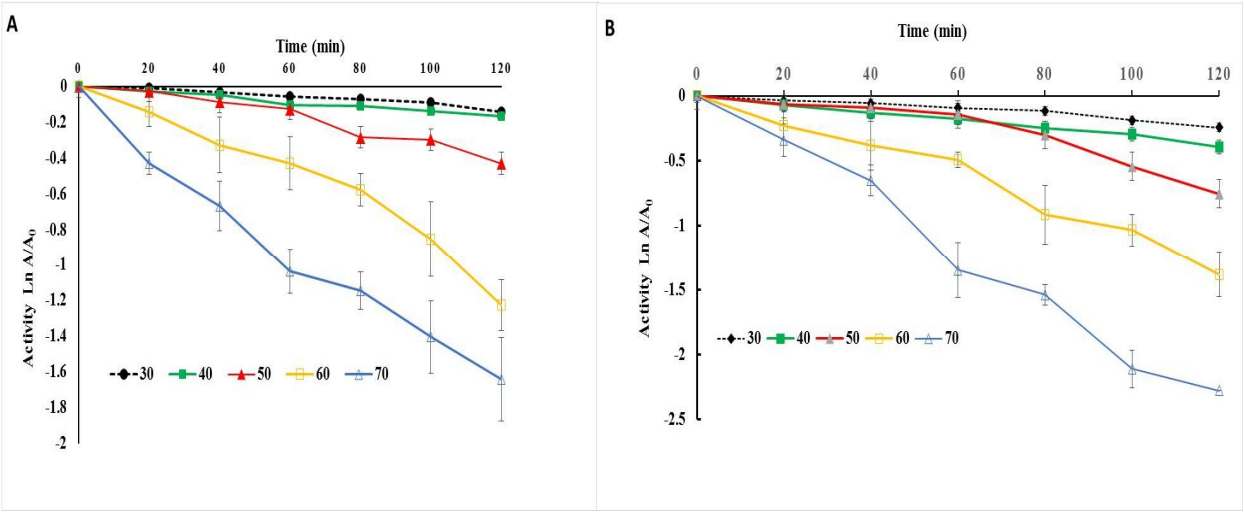

Fig. 5. The thermal deactivation of the amylase, (A) in the body extract and (B) in the coelomic fluid. (A: The enzyme activity at any time; A_0: The enzyme activity at time zero). The activity of a mixture containing 1 mg ml^{-1} each of body extract or coelomic fluid was measured at each temperature for 120 min at 25 °C. Practically, for every 20-min interval, a volume of the body extract or coelomic fluid which was being treated at specific temperature, removed and assayed in Tris buffer pH 8. For this, DNS was then added to the mixture of assay and heated at 60 °C, centrifuged at 6000 rpm for 6 min and the absorbance of upper phase read at 540 nm. Consequently, bsorbance values were converted to concentration using a glucose standard curve. All experiments repeated in triplicate.

Table 2. The Slope of the Lines from Thermal in Activation Plots and the Resultant Half-Life Values Obtained for Amylase in the Body Extract (the Slope of the Plot: k_d; the Half-life: $T_{1/2}$)

Source	Temperature (°C)	k_d (min^{-1})	$T_{1/2}$ (min)
Body extract	30	-0.0011	630
Body extract	40	-0.0014	495
Body extract	50	-0.0036	192.5
Body extract	60	-0.0095	72.9
Body extract	70	-0.0131	52.9

Table 3. The Slope of the Lines from Thermal in Activation Plots and the Resultant Half-life Values Obtained for Amylase in the Coelomic Fluid (the Slope of the Plot: k_d; the Half-life: $T_{1/2}$)

Source	Temperature (°C)	k_d (min^{-1})	$T_{1/2}$ (min)
Coelomic fluid	30	-0.0021	346.5
Coelomic fluid	40	-0.0031	223.5
Coelomic fluid	50	-0.0062	113.6
Coelomic fluid	60	- 0.0112	61.8
Coelomic fluid	70	-0.0201	34.4

fluid of the earthworm. Thereby, this study for the first time has indicated the amylase activity in coelomic fluid compared to that of body extract from the earthworm *Allolobophora chlorotic* under various parameters such as temperature and thermal stability, optimum pH, different metal ions and EDTA.

Here, the effects of various temperatures on the amylase activity of the earthworm in the current study showed that the optimum temperatures for these enzymes both in body extract and coelomic fluid are 50 °C (Fig. 4) and the enzymes is moderately thermostable. According to the results obtained from Tables 3 and 4, at temperatures 30, 40 and 50 °C, the amylase in the body extract and coelomic fluid is more stable with a longer half-life. In addition, the enzyme in coelomic fluid shows slightly less thermal stability than those of the body extract, and at higher temperatures, the enzyme activities from both sources abruptly decline (Fig. 5). In this research, more enzyme activity at higher temperatures could be seen. Thermostability of this novel amylase has a genetic basis and can be attributed to amino-acid sequence, the enzyme structure and the presence of osmolytes in the body extract and coelomic fluid.

Similarly, a previous report has found that stability and activity of amylase will increase in the presence of calcium ions and a calcium-sodium-calcium metal triad. Additionally, the research has claimed that the calcium bond causes the A and B regions of the enzyme to be linked together and, consequently, increases the thermostability of the enzyme [16].

In our study, the maximal enzyme activities for both body extract and coelomic fluid were observed at pH 8 when an alkaline buffer solution was used however, the activity of the enzyme showed a decreasing trend in extreme alkaline or acidic conditions (Figs. 2 and 3).

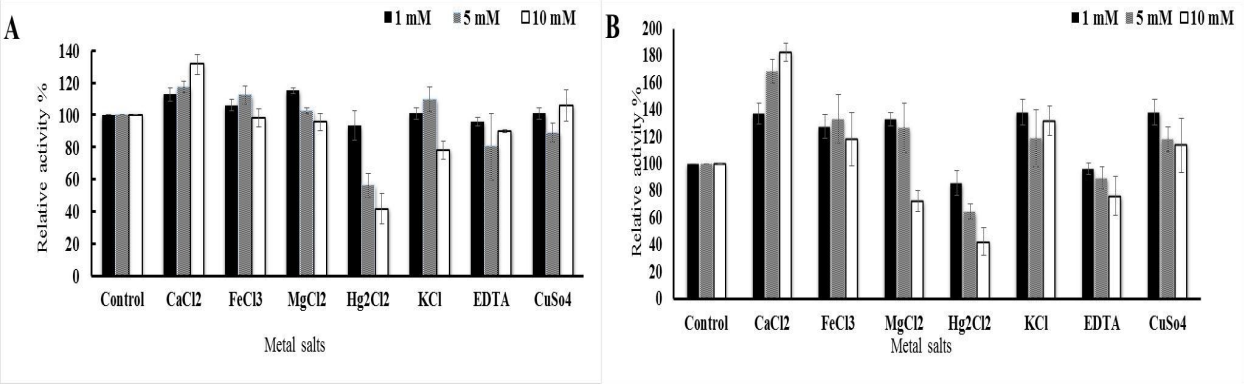

Fig. 6. The effects of different salts and EDTA in various concentrations on amylase activity (A) in the body extract and (B) in the coelomic fluid. Following the addition of 1 mg ml^{-1} of total protein to a mixture containing 50 mM Triss buffer in the presence of starch 1% at room temperature, the mixture was incubated for 120 min. DNS was then added to the mixture, heated at 60 °C , centrifuged at 6000 rpm for 5 min and the absorbance of upper phase read at 540 nm. Absorbance values were converted to concentration using a glucose standard curve and the plots were drown. All experiments were repeated in triplicate.

The effects of monovalent, divalent and trivalent metal salts and EDTA with three concentrations of 1, 5 and 10 mM on the amylase activity of body extract and coelomic fluid from the earthworm in the current study were not consistent because the components of body extact and coelomic fluid may interfere in the experiments (Fig. 6). Based on a previous research, the activity of an enzyme is affected by many compounds such as metal ions (known as stimulatory or inhibitory factors) mediate the interaction between substrate and enzyme [17]. Furthermore, it has been demonstrated that amylase is a calcium-dependent enzyme so that in the presence of appropriate calcium concentration, enzyme activity for both sources can be increased. In fact, most amylases contain at least one calcium ion and the enzyme has a high affinity for calcium ions, stronger than those of other ions. Considering the earlier report, the number of calcium ions bound to amylase varies from one to ten [18].

The inhibition of the amylase in this research at higher concentrations of Mg^{2+} ions could be due to competition between the exogenous cations and the protein associated cation which has led to decrease in amylase activity as investigated in the previous study [19]. Interestingly, the results of the later study showed that Hg_2Cl_2 has an inhibitory effect on amylase activity in line with our findings. Regarding the inhibition by Hg_2Cl_2, it can be explained that oxidation of at least a sulfhydryl group at the active site of the enzyme may lose the catalytic activity or vary the conformation of amylase due to unusual disulfide bonds form on amylase which may lead to enzyme aggregation [20].

The previous report has indicated that EDTA is a ligand wrapped around the calcium ions. The effect of EDTA on the calcium ion of α-amylase varies and it could have potent inhibitory effect to no effect at all on the enzyme activity [19].

Purification and characterization of amylases have been reported from different kinds of animals. For example, Zółtowska has purified amylase from the muscle and intestine of the parasitic helminth of pigs *Ascaris suum* which has shown maximum activity at pH 7.4, and the enzyme from muscle at pH 8.2 and the optimum temperature range was 40-50 °C [21]. Another study demonstrated that the optimum activity for amylase in *Trogoderma granarium* (Dermecidae; Coleoptera) was at alkaline pH (broad optimum in the range 8-11) [22].

Raw-starch-digesting and cold-adapted α-amylases (Amy I and Amy II) from the earthworm *Eisenia foetida* were characterized and purified by Ueda *et al*. [9]. Both enzymes were most active at pH 5.5 and 50 °C and stable at

pH 7.0-9.0 and 50-60 °C. The two enzymes i.e. Amy I and II exhibited activities at 10 C. The enzymes while hydrolyzed raw starch into glucose, maltose, and maltotorios as end products, were inhibited by metal ions Cu^{2+}, Fe^{2+} and Hg^{2+} However, EDTA had no effect on the enzyme activity.

Rafiei *et al.* were able to purify and characterize α-amylase in Moroccan locust, *Dociostaurus maroccanus* Thunberg (Orthoptera: Acrididae). The optimum activity was found at pH 6.0 and 45 °C. Hg^{2+} and EDTA decreased α-amylase activity, whereas K^{+}, Fe^{2+}, Mg^{2+}, Ca^{2+} increased enzyme activity [23].

Based on a previous report, the α-amylase obtained from the antarctic psychrophile *Alteromonas haloplanctis* A23 is a marked heat-labile enzyme [24], however, the α-amylase in the current study was active not only at lower temperatures but also exhibited some activity at 70 °C.

While amylases can be obtained from fungi and bacteria as *Aspegillus* sp.[25], *Rhizoporus* sp. [26] and *Bacillus* sp. [27] with limited activities at low temperatures, the α-amylase of the earthworm in the current study, however, is still active at lower temperatures.

CONCLUSIONS

Due to the similarity of the activities obtaind for coelomic fluid and body extract of the earthworm and nearly equal half-life values at higher temperatures, a potential explanation for this finding could be that amylase in the coelomic fluid originates from that of body extract which has been reported to be produced in the intestine [8]. As amylase is a digestive enzyme and not being used for defensive purposes therefore, it can be concluded that amylase in coelomic fluid is probably essential for early digestion. After swallowing, the processes of digestion and absorption continue through the gut. Of course, further investigation is needed to test this hypothesis. On the other hand, when purifying the enzyme from a low-cost source in large scale, the novel α-amylase can be exploited in various biotechnological industries.

CONFLICT OF INTEREST STATEMENT

The authors state that they have no conflict of interest.

REFERENCES

[1] P. Prat, M. Charrier, S. Deleporte, Y. Frenot, Pedobiologia 46 (2002) 417.
[2] C.N Lowe, K.R. Butt, Biol. Fertil. Soils, 43 (2007) 495.
[3] P.M.D. Souza, Brazilian J. Microbiol. 41 (2010) 850.
[4] M. Sahnoun, S. Bejar, A. Sayari, M.A. Triki, M. Kriaa, R. Kammoun, Process Biochem. 47 (2012) 18.
[5] Z. Konsula, M. Liakopoulou-Kyriakides, Process Biochem. 39 (2004) 1745.
[6] M. Kavian, S. Ghatnekar, P. Kulkarni, Bioresour Technol. 69 (1999) 161.
[7] K. Parthasarathi, L. Ranganathan, Trop. Ecol. 41 (2000) 251.
[8] M.L. Prabha, I.A. Jayaraaj, R. Jeyaraaj, S. Rao, Indian J. Biotechnol. 6 (2007) 567.
[9] M. Ueda, T. Asano, M. Nakazawa, K. Miyatake, K. Inouye, Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 150 (2008) 125.
[10] D. Mehta, Int. J. Biol Macromol. 79 (2015) 570.
[11] G. Gubern, F. Canalias, F. Gella, Clin. Chem. 41 (1995) 435.
[12] B. Dojnov, N. Božić, V. Nenadović, J. Ivanović, Z. Vujčić, Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 149 (2008) 153.
[13] S.C. Akazawa, H. Tokuyama, S. Sato, T. Watanabe, Y. Shida, W. Ogasawara, J. Biosci. Bioeng. 125 (2018) 155.
[14] H.R. Johnsen, K. Krause, Int. J. Mol. Sci. 15 (2014) 830.
[15] A. Homaei, J. Mol. Catal., B Enzym. 118 (2015) 16.
[16] M. Machius, N. Declerck, R. Huber, G. Wiegand, Structure. 6 (1998) 281.
[17] W. Li, X. Zhou, P. Lu, Biotechnol. Adv. 23 (2005) 271.
[18] N. Rana, A. Walia, A .Gaur, Natl. Acad. Sci. Lett. 36 (2013) 9.
[19] A. Aygan, B. Arikan, H. Korkmaz, S. Dincer, O. Colak, Braz. J. Microbiol. 39 (2008) 547.
[20] Y. Ohmiya, T. Takeda, S. Nakamura, F. Sakai, T. Hayashi, Plant Cell Physiol. 36 (1995) 607.
[21] K. Zółtowska, Acta Biochim. Pol. 48 (2001) 763.
[22] V. Hosseininaveh, A. Bandani, P. Azmayeshfard, S.

Hosseinkhani, M. Kazzazi, J. Stored. Prod. Res. 43 (2007) 515.

[23] B. Rafiei, M. Ghadamyari, S. Imani, V. Hosseininaveh, A. Ahadiyat, Toxin Rev. 35 (2016) 90.

[24] G. Feller, F. Payan, F. Theys, M. Qian, R. Haser, C. Gerday, FEBS J. 222 (1994) 441.

[25] J.I. Abe, K. Nakajima, H. Nagano, S. Hizukuri, K. Obata, Carbohydr. Res. 175 (1988) 85.

[26] T. Takahashi, K. Kato, Y. Ikegami, M. Irie, J. Biochem. 98 (1985) 663.

[27] L.M. Hamilton, C.T. Kelly, W.M. Fogarty, Biotechnol. Lett. 21 (1999) 111.