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Purification of a Moderately Thermal Stable Amylase from Earthworm *Allolobophora Choloretica* for Starch Processing

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ABSTRACT

Amylase catalyzes the hydrolysis of starch, glycogen and gives rise to certain products such as maltopentose, maltotetrose, maltotriose, maltose, and glucose. In the present study, earthworm *Allolobophora choloretica*, from Lumbricidae family, was used an animal model system. First, the earthworm cell extracts were precipitated using a gradient of saturated ammonium sulfate and multi-step dialysis, and then α-amylase was purified by Amicon® Ultra filter. The purified enzyme was analyzed on SDS-PAGE and a single 37 kDa band observed on the gel. Subsequently, the optimum pH and optimum temperature of the purified α-amylase were estimated to be 7 and 53 °C, respectively. Based on our results, at extreme acidic and alkaline pH conditions, the enzyme showed higher pH stability at pH 9 than 4. Moreover, the values of $\Delta H_D^{\mp}$, $\Delta S_D^{\mp}$ and $\Delta G_D^{\mp}$ were 63.75 kcal.mol^{-1}, 0.113 cal.mol^{-1}K^{-1} and 26.66 kcal.mol^{-1}, respectively. In conclusion, the purified moderately thermophilic amylase from earthworm *Allolobophora choloretica* can be exploited in different industries.

Keywords: Amylase, *Allolobophora choloretica*, Thermal stability, Purification

INTRODUCTION

Earthworms are scientifically classified as animals belonging to the order Oligochaeta, class Chaetopoda, phylum Annelida. More than 4400 species of earthworms are found worldwide. They have been existed on the earth since 660 million years ago. These beneficial animals feed on soil organic matters. Earthworm's digestive system spreads throughout their body and they have skin respiration. The movements of coelomic fluid of the earthworms act like an open circulatory system [1]. An earthworm is a rich source of enzymes which are used for the improvement of the soil biological characteristics and also in animal feeding for the improvement of the digestion of proteins, lipids and carbohydrates [2], in animal foods for improving digestion of proteins, lipids and carbohydrates. These enzymes can be used in detergents and the saccharification of starch [3].

It has also been reported that while earthworms use organic matter as their nutrient source, the microorganisms ingested along with these nutrients produce the enzymes that make the nutrients available for the worm's use [4]. These earthworm's intestinal microbiota are different from those present in the surrounding soils. Earthworms are able to change the composition of soil microbial communities as they pass through their digestive systems. Ingested microbial populations play a key role in earthworm nutrition by helping in the breakdown of organic matter, particularly the components that the earthworms cannot utilize in their natural state [5]. Earthworms have the ability to hydrolyze carbohydrates and therefore act on the leaves and roots of plants, yeasts, algae and fungi in the soil [6].

Enzymes are non-toxic, biodegradable and efficient or selective biocatalysts with a high level of safety, low energy consumption and environmentally friendly productions [7]. They are used in various industries, including detergent, paper, textile, food and feed industries. The history of the

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industrial enzyme production dates back to 1894, when Dr. Jhokichi Takamine began producing digestive enzymes from *Aspergillus oryzae* using wheat bran [8]. Amylase is a member of Glycoside hydrolases (GH). In GH reactions water acts as an acceptor of a transferred group. If the group acceptor in the reaction of GH is a hydroxyl or phosphate group, then the GH are called transglycosylases and phosphorylases respectively [9]. GH are able to cleave the linkages from an end or middle of the starch chains [10].

Different GH families have been described in Carbohydrate-Active enZymes (CAZy) Database (http://www.cazy.org). The families are related in terms of amino acid sequence and three-dimensional structure. In the Database, glycoside hydrolase have been classified into the 171 GH families. In addition, amylolytic enzymes have been classified into the GH13, GH15 and GH31 families (http://www.cazy.org). GH13 is the most prominent family among GH families. GH13 family is also divided into 43 subfamilies from GH13_1 to GH13_43. GH13 family share three protein domains A, B and C. Domain A is composed of a common three- dimensional structure status of (β/α) 8 barrel, domain B is a variable-length loop located within domain A strands. Finally, domain C is a Greek key motif at the C-terminal end of the hydrolases [11,12]. Amylases have been widely used for decades in different food and detergent industries that catalyzes the hydrolysis of starch to molecules such as maltopentose, maltotetrose, maltotriose, maltose and glucose [13,14]. β-Amylase is an exoamylase and releases maltose from the non-reducing ends of the starch. However, α-amylase is an endoamylase and randomly hydrolyzes internal bonds of starch [15]. Although other amylolytic enzymes are involved in the degradation of starch in animals, the presence of α -amylase is essential in this process [16]. It has been reported that enzymes in earthworm's intestines degrade organic materials for easier absorption of their constituents (Devi and Prakash, 2017). For a long time, researchers thought that invertebrates lack polysaccharide hydrolytic enzymes; however, nowadays, we know that earthworms and also insects are expressing these enzymes. Digestive enzymes of oligochaetes are responsible for degradation of the foods (S *et al.*, 2016). Earthworms decompose soil organic matters such as carbohydrates by cellulase and α-amylase (Akazawa *et al.*, 2020). Actually, earthworms are bioreactors that produce important hydrolytic enzymes such as α-amylase to convert macromolecules in the soil into smaller molecules. Earthworm's intestine protects enzymes against temperature and other inactivators and thus stabilizes the enzymes (Prabha *et al.*, 2007). According to a previous research by Ramian *et al.*, it was demonstrated that in addition to amylase from body extracts, the enzyme exists in the coelomic fluid of the earthworm *Allolobophora choloretica,* and shows characteristics similar to those of intestinal amylase. The previous study also showed that the origin of coelomic and intestinal amylases are identical for the two enzymes according to the results of their activities, optimum pH, pH stability and the effects of different metal ions on the activities of these enzymes [3]. In 1993, Zhang and colleagues showed that amylase is produced in mid- and foregut of earthworm *Pontoscolex corethrurus*. They also claimed that the earthworm itself produces amylase in the intestine to degrade the starch in the environment (Zhang *et al*., 1993).

Meanwhile, Akazawa *et al.* in 2020 and Ueda *et al*. in 2008 purified and characterized α-amylases from earthworm *Eisenia fetida* and shown that the earthworm produces intestinal amylase [13,14].

As discussed above, up to now few studies have been conducted on the enzymes of earthworm particularly amylases [6,13,14], while they are producing amylases which may be resistant against extreme pH and higher temperatures.

Therefore, we hypothesized that purified novel amylase from earthworm could exhibit remarkable properties for potential application in different industries. We know that the earthworms can be provided from earthworm farm as a beneficial source to purify amylase. In the current study, this is for the first time that we tried to extract, purify and characterize amylase from earthworm *Allolobophora choloretica* for possible industrial applications.

MATERIALS AND METHODS

Reagents and Compounds

Casein purchased from Sigma Aldrich (St. Louis, MO, USA), the protein marker was purchased from Sinaclon company (www.sinaclon.ir, Iran), and the other chemicals

were obtained from Merck (Darmstadt, Germany).

Sampling and Protein Extraction and Dialysis

Earthworms *Allolobophora choloretica* as an animal model system were collected from the campus of Shahrekord University, Shahrekord, Iran, and transferred to the animal physiology laboratory. The studied earthworms were collected from the natural soils of Shahrekord University campus. Mass of earthworms with dark regions on their skins were separated from others, washed and viewed under three-dimensional stereoscope. Then, earthworms were identified according to the current identification keys. Then 10 g of the earthworms were washed with chilled phosphate buffer to remove surface and gut residues and then poured into a porcelain mortar and crushed and powdered by drying with liquid nitrogen. The samples were placed in phosphate buffer and the resulting suspension was sonicated at 4 °C for 10 min with a 1 min time intervals. The resulting solution was centrifuged twice for 10 min (12000 × g) at 4 °C, the supernatant was collected and the precipitate discarded.

Extraction was carried out using gradient ammonium sulfate precipitation. We hypothesized that lower hydrophilic proteins are precipitated with lower concentration and highly hydrophilic proteins are precipitated at the higher concentrations of ammonium sulphate. Thereby, the experiment started with 45% ammonium sulfate and pellet was precipitated and the supernatant was removed and assayed after dialysis. Then, the sample was precipitated with 52% ammonium sulphate and then supernatant was removed, dialyzed and assayed. The steps were repeated with 62 and 75% ammonium sulphate and in the end, the precipitated proteins in the presence of 75% ammonium sulfate and dialyzed.

In more detail, first earthworm cell extracts were brought to 45, 52, 62 and 75% saturation with solid ammonium sulphate at 4 °C. The mixture was left overnight at 4 °C. Following precipitation with 45, 52 and 62% ammonium sulphate, the supernatant was removed and dialyzed and the pellet was discarded. Finally, pellet of 75% saturation sulphate was centrifuged at 12,000 × g for 20 min at 4 °C and dissolved in 50mM phosphate buffer (pH 7.0).

The final enzyme solution (precipitated with 70% ammonium sulfate) was placed in a dialysis membrane with a cut-off point of 14 kDa at 4 °C. The dialysis membrane was immersed in 25 mM potassium phosphate buffer as a dialysis buffer while it was stirring and kept at about 4 °C for 24 h [17].The buffer was changed at 2, 4, 8 and 12 h after starting dialysis.

Purification of 37 kDa Protein and SDS-PAGE Analysis

The dialyzed supernatant was analyzed on SDS-PAGE in which two protein bands were observed. Then using a 50 kDa (cut off) Amicon centrifugal filter, the two bands were separated. Finally the 37 kDa amylase was concentrated with a 10 kDa (cut off) Amicon centrifugal filter and again the single band was visualized on SDS-PAGE. The molecular weight was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli [18]. Protein bands were detected by staining with Coomassie Brilliant Blue R-250.

Enzyme Assay, Determination of pH and Temperature Profiles

The amylase activity was measured *via* the determination of the amount of reduced sugar released during incubation with starch. 3 nM amylase and 1% starch dissolved in 50 mM potassium phosphate buffer were incubated at pH 7.0 for 30 min at 25 °C. The enzymatic reaction was stopped by adding DNS. The amount of reduced sugar was determined by measuring the absorbance at 540 nm using a biochrome 110 PRO spectrophotometer. The unit of enzymatic activity is defined as the amount of enzyme produced by one micromole of reduced sugar per minute at 25 °C and pH 7.0 in a potassium phosphate buffer with 1% starch as substrate [3,19].

To determine optimum pH , in the temperature range of 0 to 75 °C, in the presence of 50 mM potassium phosphate buffer and 1% starch at pH 7.0, the curve of enzymatic activity was drawn against different temperatures [20]. The pH profile of amylase activity was plotted by measuring the enzyme activity at room temperature and in a 50 mM mixed buffer (sodium acetate, potassium phosphate, tris and glycine) in the range of 5-11 in the presence of 1% starch, and the optimal pH was determined accordingly [3,20].

Evaluation of Thermal and pH Stability

To determine the thermal inactivation constants (k_d), amylase was incubated at 5,37, 45 and 55 °C in the presence of 50 mM potassium phosphate buffer at pH in the absence of 1% starch for 0, 15, 30, 45, 60, 75 min; then, the equal volume of the solution was withdrawn at 15-min time intervals and cooled on ice for 30 min, and then the residual activities were assayed 50 mM potassium phosphate buffer and 1% starch at pH 7 at room temperature. Finally, k_d constants were determined by drawing the graph of lnA/A0 versus time. The inactivation activation energy of thermal denaturation ($E^{\mp}_{a(D)}$) was also calculated by plotting $\ln k_d$ against 1/T using the Arrhenius plot [20]. Finally, other thermodynamic parameters were calculated as follows:

$$\Delta G^{\#}_D = -RT\ln(k_d * h/k_B * T)$$

$$\Delta H^{\#}_D = E_{a(D)} - RT$$

$$\Delta S^{\#}_D = (\Delta H^{\#}_D - \Delta G^{\#}_D)/T$$

$$t_{1/2} = \ln 2/k_d$$

where h = 6.63×10^{-34} Js, $k_B = 1.38 \times 10^{-23}$ J K^{-1}, T = temperature in Kelvin and R = 8.314 J K^{-1} mol^{-1} .

To evaluate the pH stability of the purified amylase at extreme pH values, 0.11 nM enzyme was incubated in a 50 mM buffers (citrate buffre pH 4, potassium phosphate buffer pH values 6,7 and8 , Tris buffer pH 9) at the 55 ºC for 75 min and at a 15-min time interval an aliquot of the solutions were transferred to a new microtube and assayed in the presence of 50 mM potassium phosphate pH containing 1% starch at room temperature .After that, the plot of activities (lnA/A0) against time was drawn and the slopes to evaluate the stability of the enzyme against extreme pH conditions [20].

In order to calculate the thermal deactivation constant (k_d) the purified amylase was incubated at different temperatures and after 0, 15, 30, 45, 60, 75 min, an aliquot of the incubated enzyme was removed and incubated on ice for 30 min. and then, the enzymatic activities were measured. Temperature stability plot was drawn according to Fig. 4 and the slopes of the plots were considered as k_d and using the Arrhenius plot ($\ln k_d$ against 1/T) the value of $E^{\mp}_{a(D)}$ was calculated.

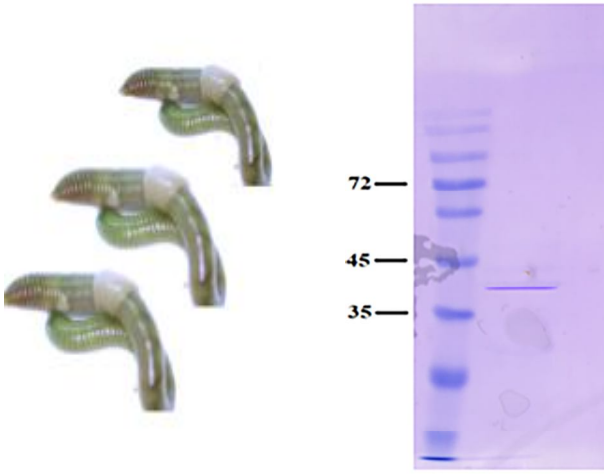


Fig. 1. Analysis of purified amylase on the SDS-PAGE. Cell extracts were precipitated with a gradient of ammonium sulfate and after centrifugation precipitated proteins were dialyzed for 24 h by 50 mM potassium phosphate dialysis buffer with pH 7 at 4 °C and then the final step of amylase purification was carried out by Amicon 50 kDa filter.

RESULTS

Extraction, Purification and SDS-PAGE Analysis of α-Amylase

The cell extract from the body of *Allolobophora choloretica* earthworm was precipitated with a gradient of ammonium sulfate, and after each precipitation, dialysis was performed. The final sample was separated on SDS-PAGE and 60 and 37 kDa protein bands were observed. Then two protein bands were isolated by a 50 kDa Amicon filter and the 37 kDa protein was concentrated using a 10 kDa Amicon filter. SDS-PAGE analysis revealed a single 37 kDa band with starch-degrading activity and considered as pure α-amylase (Fig. 1). The purified single band amylase was compared with other amylases from invertebrates (Table 1). According to Table 1 the molecular weight of the purified amylase from *Allolobophora choloretica* is close to those of the insects.

Table 1. Characteristics of Amylases Purified from other Invertebrates

Species	Enzyme	Optimum temperature (ºC)	Optimum pH	Molecular weight (kDa)	Ref.
Allolobophora sp	Alpha-amylase	53	7	37	This study
Eisenia fetid Walki	Alpha-amylase	45	5.5	64	[13]
Eisenia fetida	Alpha-amylase	50	5.5	60	[14]
Ascaric summ	Intestine alpha-amylase	50	7.4	74, 83	[21]
	Muscle alpha-amylase	40	8.2	59	
Anddrallus spindens	Salivary alpha-amylase	35-40	9	26	[22]
	Middle gut alpha-amylase	45	6-8	21.3	[23]
Maize weevil	Alpha-amylase	40-35	7-5	53.7	[24]
Callosobruchus maculatus	Alpha-amylase	60-50	5	50	[25]
Pieris brassicae L.	Alpha-amylase	35	8	88	[26]
Eurygaster integriceps	Alpha-amylase	30-40	6.5	49-72	[27]

Enzyme Activity Assay and Substrate Specificity

The 37 kDa protein was assayed and showed amylolytic activity. To assay 37 kDa amylase, we used an equal concentration of heat-inactivated amylase as control and fresh amylase and the reaction mixture containing heat-inactivated enzyme remained unchanged and no product was measured by DNS assay method. Moreover, in another experiment we added 5 drops of 10% (w/v) iodine to the reactions containing heat-inactivated amylase and the reactions containing fresh amylase under assay condition. After 30 min of incubation the blue color of the tube containing fresh amylase was disappeared but the blue color of the heat-inactivated tube remain unchanged. Altogether, these two experiments demonstrated that the understudy 37 kDa purified protein is an amylase. To test substrate specificity we used starch, cellobiose and chitin. The enzyme cannot hydrolyze cellobiose and chitin while it degrades starch and produces products which can be measured using DNS test.

Determination of Temperature and pH Profile

The effect of temperature on purified amylase was investigated in the temperature range of 5 to 70 °C. According to Fig. 2, the enzyme exhibited its maximum activity at 53 °C. Moreover, according to Fig. 3, the optimal pH of the novel amylase was at 7.

Thermal and pH Stability

As summarized in Table 2, according to the thermal stability of the enzyme, different thermodynamic parameters have been calculated. To calculate the value of ($E_{a(D)}^{\mp}$) the equations given in subsection 2.5 were used. Subsequently, equations given in subsection 2.5 were used. Subsequently,

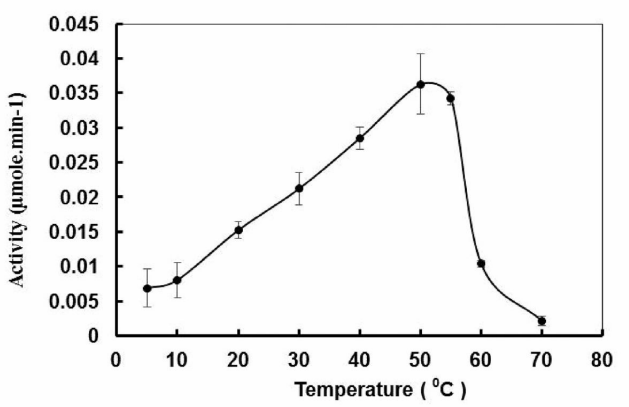


Fig. 2. The curve of temperature profile of α-amylase. The experiments were performed at different temperatures by the addition of 0.11 nM purified amylase to a 50 mM potassium phosphate buffer containing 1% starch at pH 7. The activities were measured by DNS method and the end product was read at 540 nm.

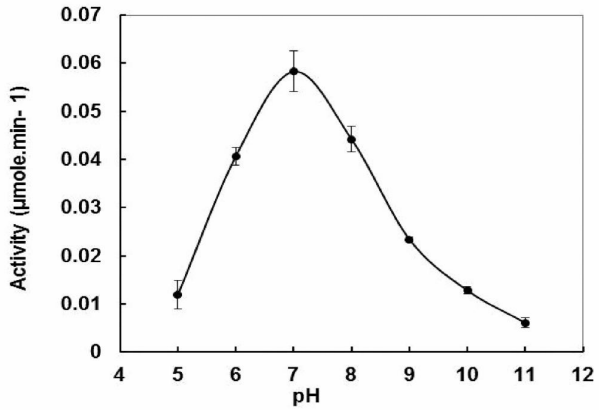
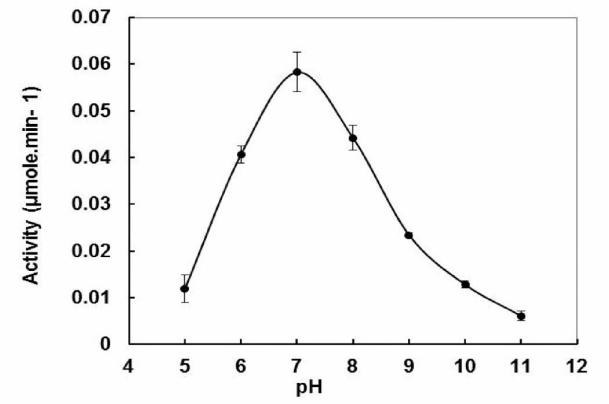


Fig. 3. The curve of pH profile of amylase purified from *Allolobophora choloretica*. The experiments were initiated by the addition of 0.11 nM purified amylaseto each buffer (sodium citrate buffer 50 mM pH 5, potassium phosphate buffer 50 mM pH 7 and 6, Tris buffer 50 mM pH 8 and 9, 50 mM glycine buffer pH 10 and 11) containing 1% starch and then the mixtures were incubated at room temperature. Finally, the activities were measured by DNS method and the end product was read at 540 nm.



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the values of $\Delta S_D^{\mp}$, $\Delta H_D^{\mp}$ and $\Delta G_D^{\mp}$ were calculated at different temperatures. According to Fig. 5, the slope of the Arrhenius plot gives the value of $E_{(D)}^{\mp}$ is 32. 4 kJ mol^{-1} and the values of $\Delta S_D^{\mp}$, $\Delta H_D^{\mp}$ and $\Delta G_D^{\mp}$ are 113.070 cal mol^{-1} K^{-1}, 63.749 kcal mol^{-1} and 26.662 kcal mol^{-1}, respectively.

Moreover, to evaluate the pH stability of the 37 kDa α-amylase, the enzyme activity was measured at pH values of 4, 7 and 9 in the time range of 0 to 75 min. The results were depicted in Fig. 6 and the calculated slopes of the plots showed that the steeper the slope, the enzyme is more sensitive to pH conditions. Thus, from Fig. 6, we can see that the stability of amylase at extreme pH 9 is higher than that of pH 4. However, the lowest slope value of 0.005 is related to optimum pH 7 and thereby, considering the slopes, it was found that the amylase purified from the earthworm *Allolobophora choloretica* at pH 7 showed the highest stability.

Table 2. Calculated Energy and Entropy Parameters of Purified Amylase

Temperature (K)	k_d	ln(k_d)	$t_{(1/2)}$ (min)	$\Delta H_D^{\mp}$ (kcal mol^{-1})	$\Delta G_D^{\mp}$ (kcal mol$^{-1)}$	$\Delta S_D^{\mp} \times 1000$ (cal mol^{-1} K^{-1})
278	0.0007	-7.2644	990	63.849	22.507	148.712
310	0.0060	-5.1160	115.5	63.785	23.841	128.825
318	0.0124	-4.3900	55.9	63.769	25.830	119.306
328	0.0724	-2.6255	9.6	63.749	26.662	113.070

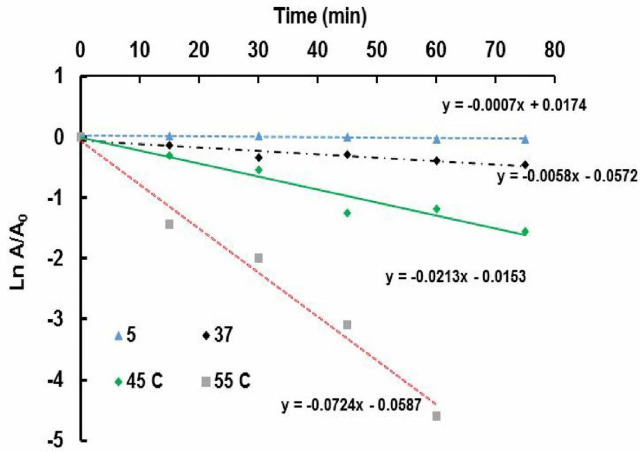

Fig. 4. Evaluation of the effects of different temperatures on the thermal inactivation of purified amylase. First, 0.11 nM of purified amylase was added to a solution containing 50 mM potassium phosphate buffer pH 7 and incubated at different temperatures and then at 15-min time interval aliquots of solutions were removed and incubated on ice for 30 min. Finally, the cooled fractions of the enzyme were assayed at temperature in the presence of a 50 mM potassium phosphate buffer containing 1% starch at pH 7 and the activities were measured by DNS method. The slopes of the plots give k_d (A_0 is activity at zero time and A is activity at any time).

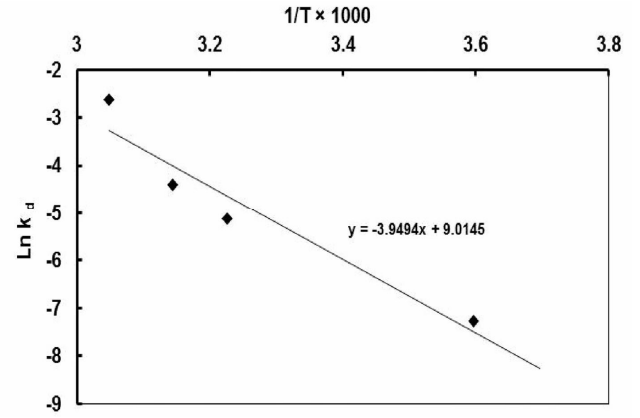

Fig. 5. Arrhenius plot of the purified amylase. Arrhenius plot was drawn using the slopes obtained from Fig. 4. From the solpes of the Arrhenius plot, activation energy of thermal inactivation $E^{\mp}_{a(D)}$ was estimated for the purified amylase.

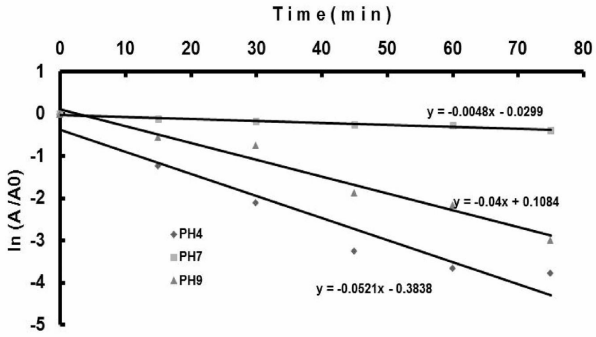

Fig. 6. Evaluation of pH stability of amylase at pHs 4, 7 and 9. In practice 0.11 nM of purified amylase was added to a 50 mM buffer containing each sodium citrate buffer pH 4, potassium phosphate buffer pH 7, tris buffer pH 9 and equal volumes of the solutions were removed at 15-min time intervals and assayed at optimal pH 7 in potassium phosphate buffer pH 7 containing 1% starch at room temperature and the activities were measured using DNS method (A_0 is enzyme activity at time zero and A is enzyme activity at any time).

DISCUSSION

Here, we extracted and purified active α-amylase from earthworm with a gradient ammonium sulfate saturation and the final step of purification using an Amicon filter. Based on our results, the 37 kDa purified protein exhibited amylolytic activity while according to Table 1 the previous studies have reported a 60 kDa amylase for earthworms. Although, in our study we partially purified a 60 kDa protein and assayed in the presence of starch, the 60 kDa amylase from *Allolobophora chlorotica* showed a lower activity than that of the 37 kDa. For this reason, we selected 37 kDa amylase for further studies.

The optimum temperature of amylase activity in the present study is close to the temperature range of the amylase from *Eisenia fetida* listed in Table 1, although the molecular weights are different. The optimum pH of the 37 kDa α-amylase is 7 while those of *Eisenia fetida* earthworm α-amylase isoenzymes were 5.5, probably due to the habitat conditions of the studied species. At optimal pH, charges on the protein surface and enzyme active site are

formed and well distributed so the active site binds to the substrates with high affinity conveniently.

According to the Table 1, among invertebrates the most of characterized α-amylases are from insects. In addition, based on the table, the purified amylase from earthworm *Allolobophora chlorotica* in terms of molecular weight is similar to those of insects such as *Morimus funereu* and *Anddrallus spindens*. As shown in Table 1, the molecular weights of amylases from invertebrates are ranged from 21.3 to 83 kDa. Considering the lower molecular weight of the purified amylase in the current study, it can be predicted that the purified amylase from earthworm *Allolobophora chlorotica* might has a structure more similar to those of amylases from insects.

Based on the results presented in Table 1, the new amylase purified from *Allolobophora chlorotica* is a thermophilic enzyme. However the $\Delta H_D^{\mp}$ values decrease with rising temperature the values of $\Delta S_D^{\mp}$ decrease which indicate the decrease in the structural disorder. Moreover, decreases in the values of $\Delta S_D^{\mp}$ compensate the decreases in $\Delta H_D^{\mp}$ values and result in the thermal stability of amylase at higher temperatures. In addition, with an increase in temperature, the values of $\Delta G_D^{\mp}$ increase which in turn indicate enzyme rigidity as well as an increase in thermal stability. According to Table 2, the half-lives ($t_{1/2}$) of the enzyme at lower temperatures are increased significantly compared with the higher temperatures. The slopes of thermal inactivation plots (k_d) have been used to calculate thermodynamic parameters as well as half-lives of the enzyme at the different temperatures.

Furthermore, according to the pH stability the enzyme is less stable at pH 4 than pH values of 7 and 9, due to formation of unwanted charges at the surface and in the active site of the enzyme. As we know at acidic pH, a large number of negative charges are neutralized while a large number of positive charges are formed on and the structure of the amylase enzyme is disturbed. The results of the current study are different from those of the previous research conducted by Ramian *et al.* [3]. For example the optimum pH of amylase in the latter study on the unpurified amylase in body extract and coelomic fluid of the *Allolobophora choloretica* was 8. In contrast, the purified amylase in the current research showed an optimum activity at pH 7. Moreover, the unpurified enzyme in the previous research showed a considerable stability at pH 11 while the purified enzyme is inactivated rapidly at this pH. Finally, the unpurified amylase in the form of cell extract at previous research showed wider pH and temperature profiles. Altogether, the characteristics of unpurified amylase from *Allolobophora choloretica* reported by *Ramian et al.* [3] are the average of activities of amylase isoenzymes and also the averages of the stability of the isoenzymes in the earthworm cell extract. In contrast, the results of the current study only indicate the properties of the purified 37 kDa amylase from the earthworm *Allolobophora choloretica*.

CONCLUSIONS

The purified novel α-amylase showed amylolytic activity while the molecular weight of the novel purified α-amylase from earthworm *Allolobophora choloretica* was calculated as 37 kDa. It exhibits optimum activity at 53 °C and pH 7.0. The amylase was shown to be a moderately thermophilic enzyme and relatively stable against alkaline pH condition. Although it has been demonstrated that earthworm's intestine contains α-amylase, the origin and the type of 37 kDa α-amylase remain to be answered. Since the cultivation of earthworms as source of beneficial enzymes is simple, economic and straightforward, the large amounts of this α-amylase could be extracted and purified. The enzyme can be used in industries, including detergent, paper, textile, food and feed industries.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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