

## Some properties of $\alpha$ -amylase in the digestive system and head glands of *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae)

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**Abstract:** Biochemical characteristics of  $\alpha$ -amylase in the digestive system and head glands of *Cryptolaemus montrouzieri*, a key predator of citrus mealybug, *Planococcus citri* (Pseudococcidae), were studied. The major isoform of  $\alpha$ -amylase with the same molecular weight was detected in both gut and head glands loaded on polyacrylamide-starch gel electrophoresis. Moreover, a minor band with much lower intensity was observed in zymogram analysis of gut. Results showed that the specific activity of  $\alpha$ -amylase from head glands ( $0.89 \pm 0.02$   $\mu\text{mol}/\text{min}/\text{mg}$  protein) was significantly more than that of digestive system ( $0.76 \pm 0.01$   $\mu\text{mol}/\text{min}/\text{mg}$  protein) in common condition (temperature equal to  $25 \pm 1$  °C). The optimal pH and temperature for  $\alpha$ -amylases activity were determined to be nearly 4 and 50°C in digestive system and 6 and 60 °C in head glands, respectively. EDTA (Ethylenediamine tetra acetic acid),  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Ca}^{2+}$  inhibited the enzyme activity but  $\text{Ba}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Hg}^+$  and  $\text{K}^+$  enhanced enzyme activity in digestive system. EDTA and all tested metal ions except  $\text{Ba}^{2+}$  inhibited the enzyme activity of head glands. Detectable levels of  $\alpha$ -amylase activity in the insect reflect adaptation of the coccinellid for using starch granules or sugars (honeydew; sugary excreta of homopterans; and nectar) as a source of food in addition to predatory habits.

**Keywords:**  $\alpha$ -amylase, digestive enzyme, *Cryptolaemus montrouzieri*

### Introduction

The coccinellid, *Cryptolaemus montrouzieri* Mulsant, a key predator of citrus mealybug, *Planococcus citri* (Risso) (Pseudococcidae), is native to Australia and has been used in many control programs against a number of mealybug species around the world (Obrycki and Kring, 1998). This insect was introduced from Australia to north Iran, as mass reared and released in tea plantations and citrus orchards for control of *P. citri*. One factor

limiting the adoption of predator coccinellids in augmentative biological control is cost-effective mass production. The use of factitious foods may lead to lower production costs by reducing space and manpower requirements for mass rearing of the predator and its prey and by enhancing mechanization of rearing procedures. For this propose, study of digestive enzymes is so imperative to find and apply new mass rearing technologies.

$\alpha$ -amylase ( $\alpha$ -1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) is one of the key enzymes involved in digestion and carbohydrate metabolism in insects that is synthesized and secreted by midgut epithelial cells, along with other digestive enzymes (Terra *et al.*, 1996). As many insect species depend on the effectiveness of their amylases for survival,

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characterization studies of insect amylases are not only of interest for comparative investigations, but they can also contribute to clarify the compatibility of some natural diets with insect development.

The ladybug *C. montrouzieri* can be mass produced in the laboratory using mealybugs (grown on pumpkin or buds of potato) as food, but the predator can to some extent be reared with a freeze-dried artificial diet consisting of beef liver (5 g), hen's egg yolk (5 g), sucrose (1 g), honey (1 g), yeast (1 g), milk powder (0.5 g), brewer's yeast (0.5 g), groundnut oil (0.3 g), multivitamin (0.04 g), vitamin E (0.04 g), niphagine (0.004 g) and water (16 ml), showing this species would produce amylase, which would allow it to use the artificial diet (Venkatesan *et al.*, 2001).

The purpose of the present study is to identify and characterize the  $\alpha$ -amylase activity from digestive system and head glands of *C. montrouzieri* adult in order to gain a better understanding of their digestive physiology. Although some research has been done on amylase activity of coccinellids (*Coccinella septempunctata*) (Kharsun and Vinokurov, 1976), based on our knowledge, this is the first study on amylase activity in *C. montrouzieri*. Findings of this study will hopefully lead to better realization of mass rearing strategies for the predator.

## Materials and Methods

### Chemicals

3, 5-Dinitrosalicylic acid (DNS), Triton X-100 and EDTA (Ethylenediamine tetra acetic acid) were obtained from Sigma (St. Louis, MO, USA). All other chemicals (reagent grade) were obtained from Merck (Merck, Darmstadt, Germany).

### Insect rearing

A colony of *C. montrouzieri* was established from 200 adults obtained from Nashtarood insectarium (Mazandaran, Iran). Predators were reared on *P. citri* infested squash and potato in the cages (25 × 25 × 30cm) under laboratory conditions (25 ± 1 °C, 60 % ± 10 RH, and a photoperiod of 16: 8 h L:

D). Adult insects were randomly selected for measuring of enzyme activity.

### Sample preparation

Adults were immobilized on ice and dissected under a stereo microscope in ice-cold saline buffer (6 mole/l NaCl). Whole of the alimentary canal and head capsule were separately removed and digestive system content was eliminated. The digestive system and head glands of ten individuals were used as one sample. Each sample was replicated 3 times. Samples were homogenized in cold double-distilled water using a hand-held glass homogenizer and centrifuged at 13000 × g for 15 min at 4 °C. Supernatants were collected and stored at -20 °C for subsequent analyses.

### Determination of $\alpha$ -amylase activity and protein concentration

$\alpha$ -amylase activity was determined by the 3, 5-dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1 % soluble starch as substrate. Ten  $\mu$ l of the gut or head glands extract was mixed with 40  $\mu$ l of the buffer which consisted of 20 mM acetic-citrate buffer (pH adjusted to 4 for digestive system and to 6 for head glands extract) and 50  $\mu$ l of 1 % (w/v) starch and incubated at room temperature (25±1 °C) for optimal time periods as tested before (15 min for gut  $\alpha$ -amylase and 30 min for head glands  $\alpha$ -amylases). The reaction was stopped by adding 100  $\mu$ l dinitrosalicylic acid reagent and heated in boiling water for 10 min prior to reading absorbance at 545 nm with a Microplate Reader Model Stat Fax® 3200 (Awareness Technology Inc.). One unit of  $\alpha$ -amylase is defined as the amount of the enzyme that liberates 1.0  $\mu$ mole of reducing sugar/min with maltose as a standard (Sharifi *et al.*, 2011). Protein concentration was determined by the Bradford (1976) method, using bovine serum albumin as standard.

Normality of distribution was tested by Kolmogorov-Smirnov test (a nonparametric test for testing goodness of fit of data). The data of  $\alpha$ -amylase activity were evaluated statistically using independent samples t-test and a commercial statistical program (SPSS 16.0).

### Polyacrylamide gel electrophoresis and zymogram analysis

Non-denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8 %) was carried out as described by Davis (1964) and electrophoresis was performed with 100 V at 4 °C. After the run, the gel was transferred to a 2.5 % (w/v) aqueous solution of Triton X-100 for 30 min at room temperature with gentle agitation in order to permit renaturation of the enzymes. Then, the gel was rinsed with deionized water and washed for 45 minutes at 4 °C in 25 mM of acetic-citrate buffer (pH adjusted to 4 for digestive system and to 6 for head glands extract). The washed gel was incubated in fresh acetic-citrate buffer containing 1 % (w/v) soluble starch, at 30 °C for 60 min. After briefly rinsing the gel in deionized water, amylolytic activity was stopped by transferring the gel to the staining solution, Lugol's [1.3 % (w/v) I<sub>2</sub>, 3 % (w/v) KI] at an ambient temperature. After coloration, light bands against the dark background indicated the presence of active  $\alpha$ -amylases (Asadi *et al.*, 2010).

### Effect of pH and temperature on enzyme activity

The pH profiles of the  $\alpha$ -amylases were determined at room temperature ( $25 \pm 1$  °C) in a universal buffer containing phosphate, glycine and acetate (25 mM of each) adjusted to various pHs (pH 2 to 11) by adding HCl or NaOH for acidic and basic pH values, respectively (Asadi *et al.*, 2010). Before determining activity, the reaction mixtures were incubated at different pHs at room temperature for 5 min. The activities of the enzymes were determined by incubating the reaction mixture at different temperatures ranging from 20 to 70 °C for 5 minutes in 20 mM acetic-citrate buffer, pH adjusted at 4 for digestive system and at pH 6 for head glands extract. Enzyme activity was measured by the standard assay method mentioned above.

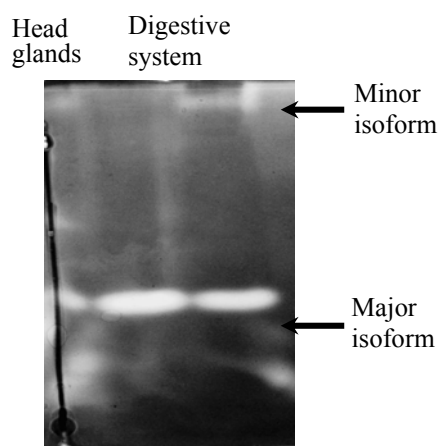
### Effect of metal ions and EDTA on $\alpha$ -amylase activity

The effects of various metal ions and EDTA (ethylene diamine tetraacetic acid) on  $\alpha$ -amylase activity were investigated at 20 mM concentration. All the metal ions were added as chloride salts. The enzyme was pre-incubated with metal ion or EDTA and 20 mM acetic-citrate buffer (pH adjusted to 4 for digestive system and to 6 for head glands extract) for 30 min at room temperature; then the same procedure for the amylase assay was performed, and amylase activity was determined by measuring absorbance at 545 nm.

## Results

### Zymogram analysis and amylase activity

The crude extracts of *C. montrouzieri* adult were analyzed by native PAGE. After  $\alpha$ -amylase activity staining, the major isoform of  $\alpha$ -amylase was clearly detected in both tissues with the same molecular weight (Fig. 1). Zymographic analysis of  $\alpha$ -amylase activities (Fig. 1) revealed that digestive system has at least two distinct  $\alpha$ -amylase bands: one closely associated with high intensity and a minor band with much lower intensity. In head glands, the minor band was not observed but the major one had a higher intensity compared to that from gut.



**Figure 1** Zymogram of  $\alpha$ -amylases from *Cryptolaemus montrouzieri* adult.

The data of  $\alpha$ -amylases activity at room temperature condition ( $25 \pm 1$  °C) (optimal temperature for development and rearing) revealed that  $\alpha$ -amylase is present in the digestive system and head glands of *C. montrouzieri* adult. The specific activity of  $\alpha$ -amylase from head glands ( $0.89 \pm 0.02$   $\mu\text{mol}/\text{min}/\text{mg}$  protein) was significantly more than that of digestive system ( $0.76 \pm 0.01$   $\mu\text{mol}/\text{min}/\text{mg}$  protein) ( $t = -0.61$ ,  $df = 4$ ,  $p < 0.01$ ) at room temperature, that agree with results of zymogram analysis.

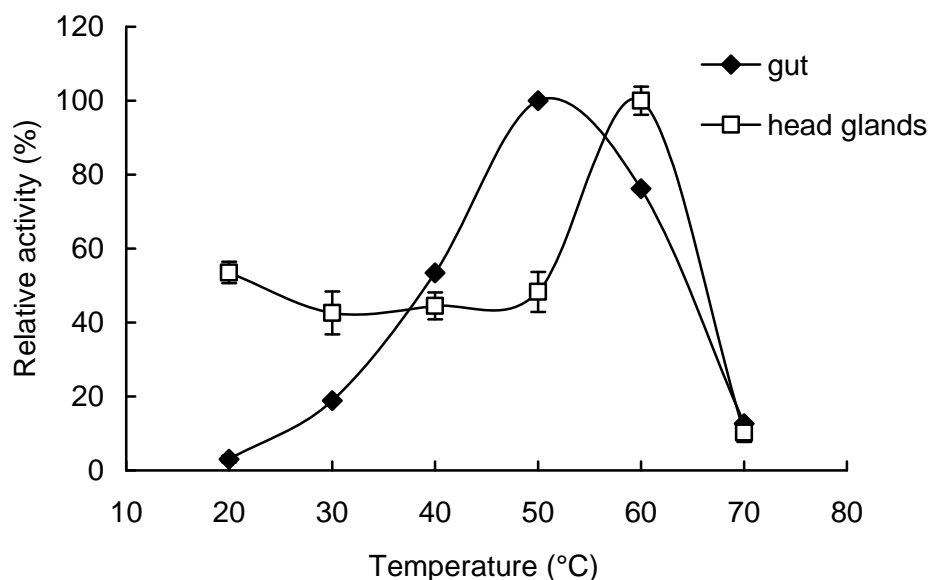
### Effect of temperature and pH on $\alpha$ -amylase activity

The  $\alpha$ -amylase activity was determined at different temperatures ranging from 20 to 70 °C (Fig. 2). The optimal temperatures for digestive system and head glands  $\alpha$ -amylases of the adult were 50 and 60 °C, respectively. A broad temperature activity profile was also observed for head glands  $\alpha$ -amylase compared with digestive system  $\alpha$ -amylase. A sharp decrease in activity was observed over 60 °C for those two  $\alpha$ -amylases.

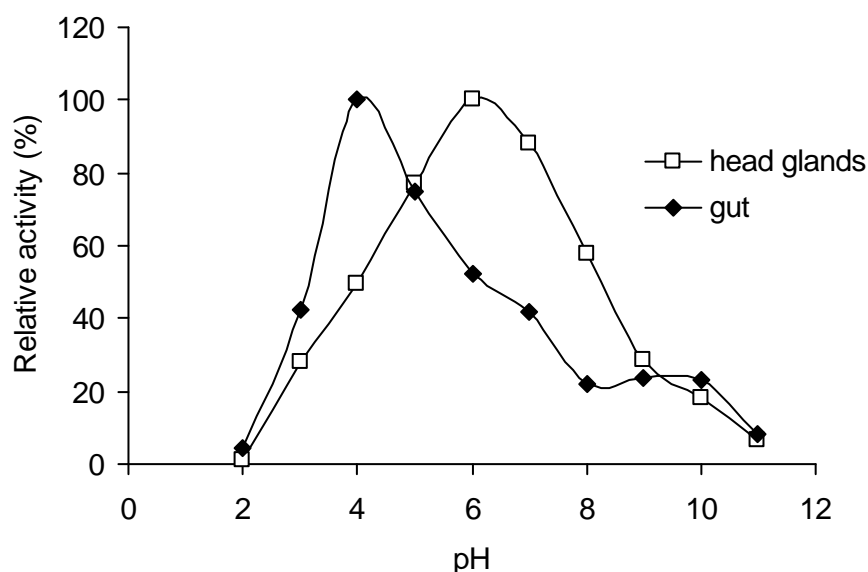
The influence of pH on the activity of digestive system and head glands  $\alpha$ -amylases is shown in Fig. 3. The optimal pH for digestive system and head glands  $\alpha$ -amylases were 4 and 6, respectively. The enzyme activity was retained over 50 % of its maximal activity in the pH range of 4 to 7.

### Effect of metal ions and EDTA on $\alpha$ -amylase activity

The  $\alpha$ -amylase activity was measured at optimal pH for each of gut and head glands in the presence of various metal ions and EDTA (20 mM). As shown in Table 1, the addition of  $\text{Ba}^{2+}$  increased enzyme activity in both  $\alpha$ -amylases, whereas the addition of EDTA,  $\text{Na}^+$ ,  $\text{Hg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  and  $\text{Fe}^{2+}$  decreased their activity. Furthermore,  $\text{K}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Hg}^+$  and  $\text{Mn}^{2+}$  increased  $\alpha$ -amylases activity of digestive system but decreased  $\alpha$ -amylases activity of head glands.



**Figure 2** Effect of temperature on the activity of  $\alpha$ -amylases from gut and head glands of *Cryptolaemus montrouzieri* adult.



**Figure 3** Effect of pH on the activity of gut and head glands  $\alpha$ -amylases of *Cryptolaemus montrouzieri* adult.

**Table 1** Effect of various metal ions (20 mM) and EDTA (20 mM) on  $\alpha$ -amylase activity in *Cryptolaemus montrouzieri*. All the metal ions were added as chloride salts.

Metal ions	Relative $\alpha$ -amylase activity (%)	
	Gut	Head glands
<b>Control</b>	<b>100</b>	<b>100</b>
Na <sup>+</sup>	60.92 ± 1.35	51.43 ± 3.56
K <sup>+</sup>	122.2 ± 1.51	60.26 ± 1.38
Mg <sup>2+</sup>	10.39 ± 1.52	66.54 ± 3.76
Co <sup>2+</sup>	75.80 ± 2.67	59.12 ± 3.36
Ba <sup>2+</sup>	203.6 ± 3.54	170.2 ± 1.42
Zn <sup>2+</sup>	178.8 ± 0.94	94.38 ± 4.09
Ca <sup>2+</sup>	83.34 ± 2.72	89.80 ± 4.62
Fe <sup>2+</sup>	84.50 ± 3.04	61.25 ± 2.55
Hg <sup>+</sup>	169.4 ± 1.07	34.26 ± 2.86
Hg <sup>2+</sup>	98.55 ± 2.31	32.54 ± 1.49
Mn <sup>2+</sup>	101.1 ± 2.76	43.56 ± 3.05
EDTA	19.92 ± 1.39	33.64 ± 1.69

## Discussion

The ability of insects to use plant or animal materials for food is indicated by the presence of specific digestive enzymes. Evidence of feeding adaptations was suggested by the salivary and midgut digestive enzymes in many insect orders (Torres and Boyd, 2009;

Asadi *et al.*, 2010; Sharifi *et al.*, 2011). Associated with each of the gnathal segments (mandibular, maxillary and labial) may be a pair of glands although they are not usually all present together. The most commonly occurring head glands are the labial glands which are present in all the major orders of insects. In most insects they function as salivary glands (Chapman, 1998). Glands in the head capsule of coccinellid beetles with a discussion on some aspects of gnathal glands were studied by Pradha (1939).

The specific activity of  $\alpha$ -amylase from head glands was significantly more than that of digestive system at room temperature condition that correspond with results of zymogram analysis. Two amylase activity bands and one amylase activity band were detected with polyacrylamide-starch gel electrophoresis of gut and head glands, respectively. However, as depicted in the figure 1, the light band intensity concerning to  $\alpha$ -amylase activity in head glands was more than in gut that correspond with results of  $\alpha$ -amylase activity. The presence of detectable amylase in the head glands and digestive system of *C. montrouzieri*, indicated

that the coccinellid could ingest starch granules or disaccharides from various sources and digest them in the gut. This fundamental enzyme is found in some zoophagous (Kharsun and Vinokurov, 1976; Torres and Boyd, 2009), zoophytophagous (Zeng and Cohen 2000a; Boyd *et al.*, 2002) and phytophagous (Bandani *et al.*, 2009; Sivakumar *et al.*, 2006) insects.

The ability to use carbohydrate sources for food might enable *C. montrouzieri* to survive in the absence of prey for short periods of time (Venkatesan *et al.*, 2001). The digestion of glycogen, obtained from arthropod prey, might also explain the presence of these enzymes in the gut. The presence of amylase indicates the ability of *C. montrouzieri* adults to use starch, either by direct ingestion (artificial diet) or by ingestion of plant material from the digestive system of their prey. Also pollen, honeydew (excreta of homopterans) and nectar constitute a significant, if not essential, food item for most coccinellids, such as *C. montrouzieri* (Heidari and Copland, 1993; Cottrell and Yeargan, 1998; Lundgren, 2009).

Results from this study showed that  $\alpha$ -amylase activity of *C. montrouzieri* was retained over 50 % of its maximal activity in the pH range of 4 to 7. The optimal pH for digestive system and head glands  $\alpha$ -amylases were 4 and 6, respectively. Moreover, the digestive amylases of several insects of coleoptera were very active at pH values ranging from slightly acidic to neutral. The acidic gut of beetles is supposed to permit maximum amylase activity. The optimal pHs of  $\alpha$ -amylase were found at 5 for *Hypothenemus hampei* (Coleoptera: Scolytidae) (Valencia-Jime'nez *et al.*, 2000) and at 6 for larger grain borer, *Prostephanus truncatus* (Coleoptera: Bostrichidae) (Mendiola-Olaya *et al.*, 2000).

The enzyme activity gradually declined at temperatures beyond 50 °C (Fig. 2). This condition was different to those found in other insects, such as  $\alpha$ -amylases from *Zabrotes subfasciatus* (Coleoptera: Bruchidae), *Tribolium castaneum* (Coleoptera: Tenebrionidae),

*Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) which showed higher activities at 37 °C (Sivakumar *et al.*, 2006; Safaei Khorram *et al.*, 2010). Optimal temperature range was determined to be 30 – 40 °C in *Eurygaster integriceps* (Hemiptera: Scutelleridae) (Bandani *et al.*, 2009).

As  $\alpha$ -amylases are essential enzymes for insect growth and development, inhibitors of  $\alpha$ -amylase may have detrimental effects on the insect's life cycle when present in the diet. It is well known that some animal amylases are activated or inhibited by certain ions and inhibitory effect of some mineral compounds on the digestive enzymes may offer a disadvantageous condition for digestion of food (Payan, 2004). Zeng and Cohen (2000b) showed that EDTA and SDS reduced  $\alpha$ -amylase activity of *Lygus* spp. This could be due to enzyme denaturation of  $\text{Ca}^{2+}$  omission from enzyme structure. Similarly, in our study EDTA had an inhibitory effect on  $\alpha$ -amylase activity. Rate of activity was reduced in gut and head glands by 80 and 66 %, respectively. Magnesium chloride decreased the salivary  $\alpha$ -amylase activity of *E. integriceps* (Sa'adati Bezdi *et al.*, 2008). Similarly, magnesium chloride decreased the head and gut  $\alpha$ -amylase activity of *C. montrouzieri*. Also calcium chloride decreased the head and gut  $\alpha$ -amylase activity of *C. montrouzieri*. However, in some insects it has been reported that  $\alpha$ -amylases are metalloproteins that require calcium for maximum activity. Midgut  $\alpha$ -amylase of *T. molitor* was slightly activated by  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  (Applebaum *et al.*, 1961). Zeng and Cohen (2000b) found NaCl was one of the activators of the salivary amylase of a zoophytophagous heteropterans, *Lygus hesperus* Knight. In contrast, sodium chloride decreased the head and gut  $\alpha$ -amylase activity of *C. montrouzieri*.

In various predacious insects, sugars can increase survival in the absence of prey. Sucrose greatly prolonged adult survival under no-prey conditions in *Stethorus japonicus* (Coleoptera: Coccinellidae), the predator of spider mites (Kishimoto and Adachi, 2010). In conclusion,

according to the results of this study (availability of  $\alpha$ -amylase activity), the addition of non-prey foods (Extrafloral nectar, honeydew, honey, simple carbohydrates) to the diets of *C. montrouzieri*, might aid in their biological control efficacy by fueling immigration into crop systems, increasing survival during periods of low prey availability and diapause, and increasing their reproductive ability (Heidari and Copland, 1993; Cottrell and Yeargan, 1998; Venkatesan et al., 2001; Lundgren, 2009).

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## برخی خواص آنزیم آلفا آمیلاز در لوله گوارش و غدد کپسول سر کفشدوزک شکارگر *Cryptolaemus montrouzieri* (Coleoptera: Coccinellidae)

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**چکیده:** خواص بیوشیمیایی آنزیم آلفا آمیلاز در لوله گوارش و غدد کپسول سر کفشدوزک *Cryptolaemus montrouzieri* شکارگر مهم شپشک مرکبات (*Planococcus citri* Pseudococcidae) بررسی شد. پس از انجام الکتروفورز روی ژل پلی‌آکریل آمید-نشاسته، یک باند اصلی مربوط به آلفا آمیلاز با وزن مولکولی مشابه در لوله گوارش و غدد کپسول سر کفشدوزک مشاهده گردید. به‌علاوه یک باند ضعیف‌تر در ژل مربوط به لوله گوارش دیده شد. در شرایط متعارفی (دمای محیط  $1 \pm 25^\circ\text{C}$ ) فعالیت مخصوص آنزیم آلفا آمیلاز در غدد کپسول سر  $(0.02 \pm 0.089)$  میکرومول بر دقیقه در میلی‌گرم پروتئین) به‌صورت معنی‌داری بیش از فعالیت آنزیم در لوله گوارش  $(0.01 \pm 0.076)$  میکرومول بر دقیقه در میلی‌گرم پروتئین) بود. دما و pH بهینه برای فعالیت آنزیم آلفا آمیلاز در غدد کپسول سر به‌ترتیب  $60^\circ\text{C}$  و  $6$  و در لوله گوارش  $50^\circ\text{C}$  و  $4$  تعیین گردید. اتیلن دی آمین تتراسیتیک اسید و یونهای منیزیم، سدیم، کبالت، آهن و کلسیم باعث مهار فعالیت آنزیم و یونهای باریم، روی، جیوه و پتاسیم باعث افزایش فعالیت آنزیم آلفا آمیلاز در لوله گوارش حشره شدند. اتیلن دی آمین تتراسیتیک اسید و همه یونهای فلزی بررسی شده به‌جز یون باریم باعث مهار فعالیت آنزیم در غدد کپسول سر شدند. وجود مقادیر قابل‌بازیابی از آنزیم آلفا آمیلاز در این کفشدوزک بیانگر توانایی این حشره در استفاده از منابع قندی مانند نوش یا شهد گیاهی و یا عسلک شپشکهای میزبان به‌عنوان یک منبع غذایی می‌باشد.

**واژگان کلیدی:** آلفا آمیلاز، آنزیم هضم‌کننده، *Cryptolaemus montrouzieri*