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Research Article

Characterization of digestive α-amylase in the midgut of willow leaf beetle *Plagiodera versicolora* (Coleoptera: Chrysomelidae)

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Abstract: Willow leaf beetle, *Plagiodera versicolora* is an important pest of willow trees that feeds on the leaves both as larvae and as adults. α-Amylases (EC 3.2.1.1) are the major insect digestive enzymes that catalyze the endohydrolysis of long α-1,4-glucan chains such as starch and glycogen. In the current study, α-amylase activity was studied in the midgut of larvae and adults of *P. versicolora.* Amylase activity in the midgut of larvae and adults was 0.6807 and 0.1162 µmol/min/mg protein, respectively. The optimal pH for α-amylase activity of larvae and adults was 4 and 8, respectively and the optimal temperature for both was 35 °C. The enzyme activity of larvae was inhibited by the addition of Na^{2+}, K^{+} and Zn^{2+}. K^{+} (at 5 mmol) had the most positive effect on α-amylase activity in adults. EGTA had significant influence on decreasing the enzyme activity in larvae. EDTA had the most effect on increasing the activity of the enzyme in adults. Plant amylase inhibitors play important role against insect pests. Therefore, the characterization of digestive enzymes and the examination of inhibitors on the enzyme activity could be useful in tackling insect pests.

Keywords: *Plagiodera versicolora*, midgut, α- amylase

Introduction

The imported willow leaf beetle, *Plagiodera versicolora* Laicharting (Coleoptera: Chrysomelidae) is one of the most important pests on several species of willow, poplar, birch and hazelnut around the world (Toros, 1996; Aslan, 2001). It has a bivoltine life cycle (Ishihara *et al*., 1999), two or three generations may occur in a year depending on moisture and temperature (Aslan 2001). *P. versicolora* overwinters as adults (Hood, 1940). Females lay clusters of 2 to 48 eggs, with an average clutch containing 15-19 eggs (Wade, 1994; Crowe, 1995). Larvae pass throught three instars in a period of 8 to 20 days (Schnaider, 1957). Both larvae and adults feed on leaves of the host trees. Adults prefer young leaves, make holes in the leaves but the larvae intensively feed on older ones (Çanakçıoğlu and Mol, 1998, 2000). Damage of the beetle has been detected on different willow species namely musk willow (*Salix aegyptica* L.), white willow (*Salix alba* L.) and weeping willow (*Salix babylonica* L.) in Guilan Province, northern Iran although that is recorded on *Populus* spp. (general distribution), *Rosa* spp. (Kohgiluyeh and Kermanshah Provinces) and *Salix* spp. (Northern and central Provinces) in Iran as a leaf borer (Abaii, 2000).

Digestive amylase is one of the key enzymes involved in carbohydrate digestion and metabolism (Daone *et al*., 1975; Buonocore *et al*., 1976). The enzyme converts starch to maltose, which is then hydrolyzed to glucose by an α-glucosidase. In

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insects, only α-amylases (α-1, 4-glucan-4-glucanohydrolases, EC 3.2.1.1) hydrolyse long α-1,4-glucan chains, such as starch or glycogen (Terra *et al*., 1996). There is a variety of natural compounds like plant protein inhibitors that affect amylase activity (Franco *et al*., 2002). Many organisms depend on the effectiveness of amylases for their survival since they utilize a poly-saccharide-rich diet (Marchaiah and Vakil, 1984).

The study of insect digestive enzymes is important because the gut is the major interface between the insect and its environment. Therefore, an understanding of digestive enzyme function is essential when developing methods of insect control, such as enzyme inhibitors and transgenic plants, which are considered as the safe approaches (Bandani *et al*., 2001; Goshal *et al*., 2001; Maqbool *et al*., 2001). Inhibitors of insect α-amylase have been shown to be an effective means for control of insect pests. Considering the importance of carbohydrate digestion as a target for *P. versicolora* control, it is clear that the hydrolyzing enzymes of carbohydrates need more attention. Although there is good information on proteolytic activity of *P. versicolora* as reported by Zibaee and Hajizadeh (2013), no studies have been made to characterize α-amylase activity in the midgut of the *P. versicolora*. Therefore the aim of the current study was to identify and characterize digestive α-amylase activity in the *P. versicolora* midgut in order to gain a better understanding of the digestive physiology of this insect. This knowledge may lead to new management strategies for pest control.

Materials and Methods

Insect rearing

Adults of *P. versicolora* were collected in summer 2013 from the infested leaves of *Salix aegyptica* L. (Salicaceae) in Rasht, Guilan province, Iran. They were reared in transparent plastic jars (6 × 8 cm) lids of which had a hole covered with muslin cloth for aeration. A piece of wet cotton was put in each container to provide moisture to leaves of *S. aegyptica*. Rearing media were maintained at 22 ± 2 °C, 70 ± 10% RH, and a photoperiod of 16:8 (L: D) hours. Rearing was continued for at least one generation to have a uniform group of adults and third instar larvae to initiate the biochemical experiments (Zibaee and Hajizadeh, 2013),

Insect dissection and preparation of midgut samples

Larvae and adults were immobilized by placing them on ice after which they were rinsed in water. The larvae and adults were dissected under a dissecting microscope in distilled water and the midgut removed. The midguts then were transferred to 1.5 ml centrifuge tubes and centrifuged at 13000 rpm for 20 min at 4 °C. The supernatants were pooled and stored at −20 °C for subsequent analyses (Zibaee and Hajizadeh, 2013).

α-Amylase assay

The α-amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck, Darmstadt, Germany) as substrate. Ten microliters of the enzyme were incubated for 30 min at 35 °C with 50 µl universal buffer (20 mM, pH 7.0) and 30 µl soluble starch. The reaction was stopped by addition of 100 µl DNS (dinitrosalicylic acid) and heated in boiling water for 10 min. DNS is a color reagent which reacts with the reducing groups released from starch by α-amylase action. Absorbance was then measured at 545 nm. A standard curve of α-amylase absorbance against the amount of maltose released was constructed to facilitate the calculation of the amount of maltose released during the α-amylase assays. Serial dilutions of maltose in the universal buffer (pH 7) were made to produce the following ranges of concentrations: 0.125, 0.25, 0.5, 1 and 2 mg mL^{-1}. The reaction mixture containing 50 µL of soluble maltose, 270 µL distilled water and 50 µL of DNS was heated in boiling water for 10 min, and then the absorbance was read at above-mentioned wave length. One unit of α-amylase activity is defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C. A blank was run containing all reaction components except for enzyme.

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Effect of pH and temperature on α-amylase activity

Optimal pH was determined using universal buffer with pH set at 3-12. The effects of different temperatures on the enzyme activity were determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45 and 50 °C for 30 min. The enzyme assay was done as described in the "α-amylase assay" section (Zibaee and Hajizadeh, 2013).

Effect of ions on α-amylase activity

The effects of various ions on the enzyme activity were measured by adding three concentrations (0.5, 3 and 5 mmol) of chloride salts of Na^+, K^+, Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} to the assay mixture, then the activity was measured after 30 min. A control was also measured (no compounds added).

Effects of inhibitors on α-amylase activity

To test the effect of different inhibitors on the α-amylase activity, assays were performed in the presence of different concentrations (2, 4, 6, 8, 10 mmol) of triethylenetetramine hexaacetic acid (TTHA), ethylene glycol-bis (β-aminoethylether) N, N, N′, N′-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA) and diethyldithiocarbamate (DTC). The purified enzyme (10 µl) was incubated with different concentrations of each inhibitor for 30 min prior to the addition of substrate. All the inhibitors were solubilized in 50 µl sodium phosphate buffer (pH 7.0) (Zibaee and Hajizadeh, 2013).

Protein determination

Protein concentration was determined by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

Statistical analysis

Data were compared by one-way analysis of variance (ANOVA) followed by Tukey's student test when significant differences were found at P = 0.05 (SAS, 1997). Differences between data of the effect of ions and inhibitors on the enzyme activity were compared by the LSD (least significant difference) test after ANOVA. The comparison of α- amylase activity in the midgut of larvae and adults was done by *t*- student test ($P < 0.05$).

Results

Midgut α-amylase activity

Amylase activity was detected in the midgut of adults and larvae of *P. versicolora*. Amylase activity in the midgut of adults was 0.1162 µmol/min/mg protein while in the midgut of larvae was 0.6807 µmol/min/mg protein (Fig. 1).

Effect of pH and temperature on enzyme activity

The α-amylase activity in the midgut of both larvae and adults was increased gradually from 20 to 35 °C and after that decreased to 50 °C. The optimal temperature for both was found to be 35 °C (Fig. 2). Optimal pH for the enzyme activity of larvae was 4. After that, the enzyme activity in the midgut of larvae dropped rapidly. The optimal pH value for the adults was 8. An acceptable increasing of the enzyme activity was also observed at pH 10 (Fig. 3).

Effect of ions and inhibitors on amylase activity

The compounds used in this study had different effects on the activity of α-amylase in the midgut of larvae and adults. The most significant positive effect on the enzyme activity in the midgut of adults was observed at 5 mmol concentration of K^+. However, Mn^{2+} (at 0.5 mmol) and Ca^{2+} (at 5 mmol) significantly increased the enzyme activity in adults. Meanwhile, the enzyme activity was significantly inhibited by 5 mmol concentration of Zn^{2+}. Our results also revealed that Na^{2+}, K^+ and Zn^{2+} (at all the mentioned concentrations) had significant negative effect on the α–amylase activity in the midgut of larvae (Table 1).

EDTA showed the most significant effect on increasing the α-amylaes activity in adults. However, EGTA (at 2, 4 and 6 mmol) had significant positive effect on the enzymatic activity in adults. DTC (at all of the concentrations) and EDTA (at 8 mmol) significantly increased the enzyme activity in larvae but EGTA (at all the concentrations) decreased it (Table 2).

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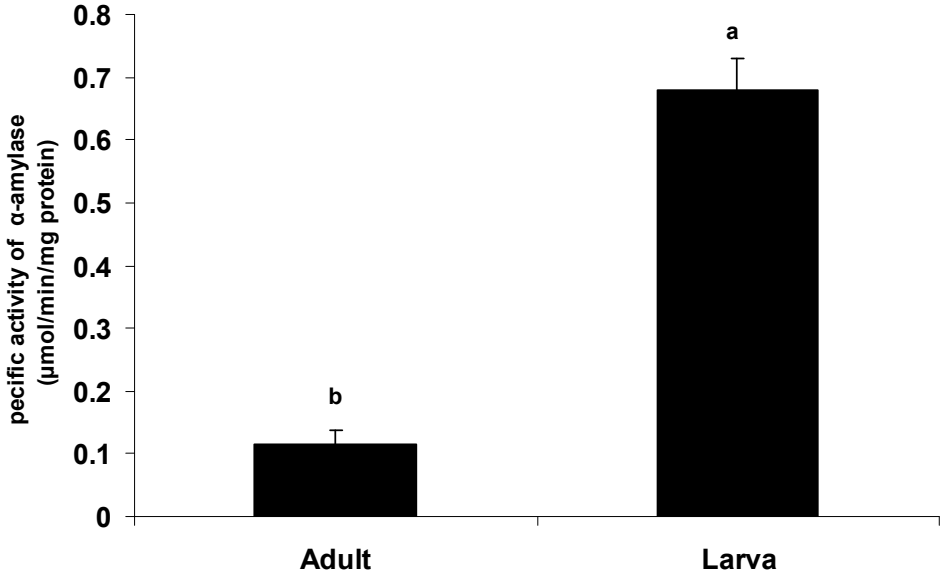

Figure 1 Activity level of α-amylase in the midgut of *P. versicolora* larvae and adults. Different letters indicate that the activity of the enzyme in larvae and adults is significantly different from each other by *t*- student test ($P < 0.05$).

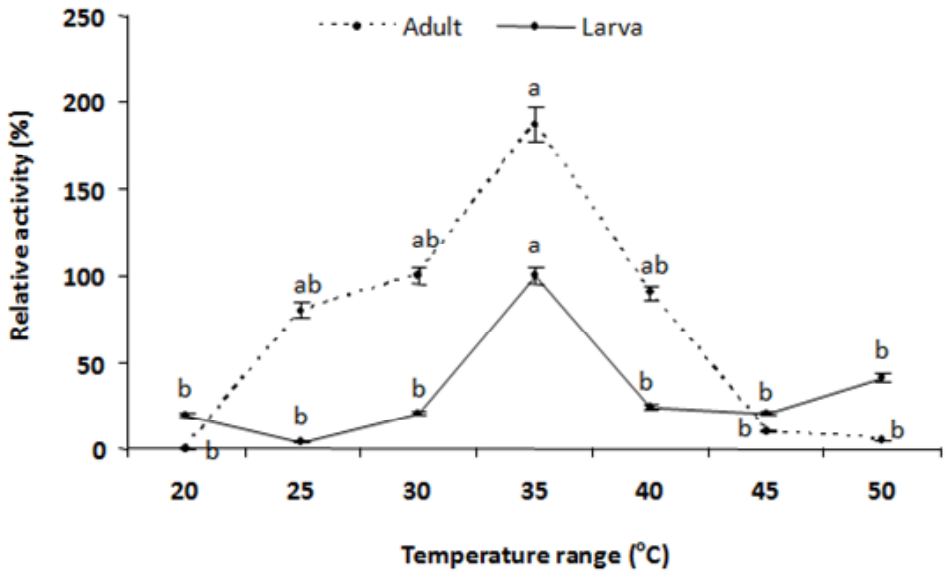

Figure 2 Effects of temperature on α-amylase activity from midgut extracts of *P. versicolora* larvae and adults. Means followed by the same letters in each line are not significantly different from each other by Tukey's test ($P < 0.05$).

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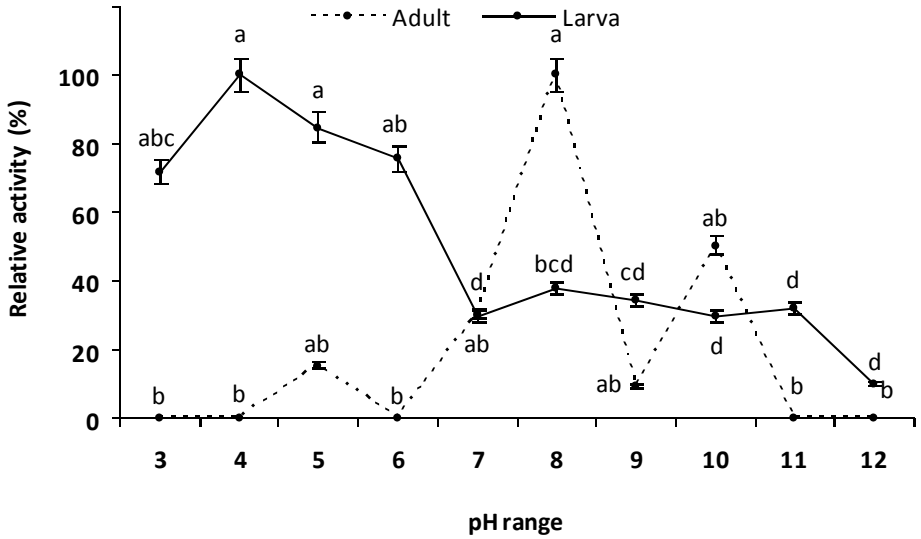

Figure 3 Effects of pH on α-amylase activity from midgut extracts of *P. versicolora* larvae and adults
Means followed by the same letters in each line are not significantly different from each other by Tukey's test (P < 0.05).

Table 1 Effects of ions on α-amylase activity from midgut extracts of *P. versicolora* larvae and adults.

Compound	Concentration of ions (mmol)	Relative activity of α-amylase in adult (%)	Relative activity of α-amylase in larvae (%)
Mn	Control	100b	100a
	0.5	172.0a	48.9bc
	3	5.4c	39.4c
	5	134.4ab	91.5ab
Mg	Control	100a	100a
	0.5	33.3ab	6.4b
	3	59.1ab	94.0a
	5	5.4b	68.1a
Ca	Control	100b	100a
	0.5	65.6b	48.2b
	3	58.6b	89.7ab
	5	171.0a	76.4ab
Na	Control	100a	100a
	0.5	29.0b	31.9b
	3	44.1ab	13.1b
	5	29.0b	26.1b
K	Control	100b	100a
	0.5	66.7b	19.5b
	3	88.2b	41.1b
	5	336.6a	58.7b
Zn	Control	100a	100a
	0.5	54.8ab	0b
	3	112.9a	8.9b
	5	0b	1.1b

Means followed by the same letters in each column are not significantly different (LSD test; $P < 0.05$).

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Table 2 Effects of inhibitors on α-amylase activity from midgut extracts of *P. versicolora* larvae and adults.

Compound	Concentration of inhibitors (mmol)	Relative activity of α-amylase in adult (%)	Relative activity of α-amylase in larvae (%)
TTHA	Control	100c	100ab
	2	61.5e	61.7c
	4	123.1b	66.7bc
	6	87.9cd	110a
	8	147.3a	22.5d
	10	68.1de	110.8a
EDTA	Control	100c	100b
	2	241.5c	36.9c
	4	339.0b	54.5c
	6	280.5b	40.2c
	8	304.9b	180.3a
	10	575.6a	38.9c
DTC	Control	100ab	100d
	2	31.0b	320.0b
	4	144.0a	241.7c
	6	92.9ab	235.0c
	8	8.3b	310.0b
	10	57.1ab	450.0a
EGTA	Control	100c	100a
	2	754.1a	59.8ab
	4	380.4b	6.8c
	6	272.6bc	33.3bc
	8	98.0c	21.7bc
	10	102.1bc	5.2d

Means followed by the same letters in each column are not significantly different (LSD test; $P < 0.05$).

Discussion

α-amylase activity in other insects has been observed to be within a range from 0.0673 to 1.2 U/mg protein (Gutierrez *et al*., 1990; Abraham *et al*., 1992; Ferreira *et al*., 1999; Mendiola-Olaya *et al*., 2000). In this study, α-amylase activity showed highest activity at 35 °C with a high activity at about 25 to 40 °C, which can be attributed to the environment in which the insects live and feed. This value was higher than that of the α-amylase activity in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) (25 °C; Barbosa Pereira *et al*., 1999). However, this value was lower than that of the α-amylase activity in *Rhyzoptertha dominica* (Coleoptera: Bostrichidae) (40 °C; Priya *et al*., 2010). Mendiola-Olaya *et al.* (2000) reported that the optimum temperature of midgut α-amylase in *Prostephanus truncates* Horn. (Coleoptera: Bostrichidae) is 40 °C and at higher temperatures the enzyme activity drops quickly. In general, coleopteran insects demonstrated a definite increase in α-amylase activity between 30 and 40 °C (Shivkumar *et al*., 2006).

Optimal pH values for amylases in the larvae of several coleopterans were 4-5.8 (Baker, 1983) and for *P. versicolora* adult and larva was recorded at 8 and 4, respectively. Our results are similar to those reported for many coleopteran larval amylase which are known to be active in

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the neutral to slightly acidic pH range (Applebaum and Konijn, 1965; Podoler and Applebaum, 1971; Buonocore *et al.,* 1976). Optimum pH generally corresponds to the pH prevailing in the midguts from which the enzymes are isolated. These results showed that α-amylase is active in a broad range of pH. Amylases in the insects are generally most active in neutral to slightly acidic conditions (Baker, 1983; Terra *et al*., 1996). D'Amico *et al*. (2000) showed that α-amylase has the most activity in pHs which are next to 7. Mehrabadi and Bandani (2009) reported that salivary α-amylase of *Eurygaster maura* (Hemiptera: Scutelleridae) had the highest activity at pH 6-7.

Insect amylases are calcium-dependent enzymes and are activated by chloride with displacement of the pH optimum. Activation also occurs with anions other than chloride, such as bromide and nitrate, and it seems to depend upon the ionic size (Terra and Ferreira, 1994). In the present study some compounds were used as activators and inhibitors which made different effects on α-amylase activity. This study showed that Na^+ decreased activity of the enzyme and it was similar to results reported for α-amylase activity of *Chilo supperssalis* (Lepidoptera: Pyralidae) when NaCl was used (Zibaee *et al*., 2008). Khorram *et al*. (2010) showed that digestive amylases from some insect species e. g. *Leptinotarsa decemlineata* Say. (Coleoptera: Chrysomelidae) are inhibited by K^+ and Mg^{2+} which is precisely consistent with the current study in *P. versicolora*.

Because of important biochemical role of α-amylase in insect growth and development, when the action of this enzyme is inhibited, insect nutrition is impaired, its growth and development retarded and eventually death occurrs due to starvation (Oliveira-Neto *et al*., 2003). However, it has been reported that α-amylases are metalloproteins that require calcium for maximum activity. Calcium also affords stability for the amylases from a variety of sources, including insects, to both pH and temperature extremes (Baker and Woo, 1985).

Reducing the use of chemical pesticides is the initial goal to produce insect-resistance transgenic plants which could reduce the cost to the farmer and the consumer as well as reducing the insecticide load in the environment (Da Silva *et al*., 2004). Our personal view is that the purification and characterization of more insect α-amylases will considerably help us to come to an understanding about the importance of designing new strategies for the pest control.

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تعیین ویژگی‌های آلفا-آمیلاز گوارشی در روده‌ی میانی سوسک برگخوار بید *Plagiodera versicolora* (Coleoptera: Chrysomelidae)

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چکیده: سوسک برگخوار بید *Plagiodera versicolora* آفت مهم درختان بید است که در هر دو مرحله لاروی و حشره کامل از برگ‌های این درختان تغذیه می‌کنند. آلفا-آمیلازها (EC 3.2.1.1) از مهم‌ترین آنزیم‌های گوارشی حشرات هستند که بر روی زنجیره بلند آلفا ۱ و ۴ گلوکان نظیر نشاسته و یا گلیکوژن عمل می‌نمایند. در پژوهش حاضر، فعالیت آلفا-آمیلاز در معده میانی لارو و حشرات کامل *P. versicolora* مورد بررسی قرار گرفت. فعالیت آلفا-آمیلاز در معده میانی لارو و حشرات کامل به‌ترتیب ۰/۶۸۰۷ و ۰/۱۱۶۲ میکرومول بر دقیقه بر میلی‌گرم پروتئین به‌دست آمد. pH بهینه برای فعالیت آلفا-آمیلاز لارو و حشرات کامل به‌ترتیب ۴ و ۸ به‌دست آمد و دمای بهینه فعالیت آلفاآمیلاز در لارو و حشرات کامل، ۳۵ درجه سلسیوس به‌دست آمد. یون‌های Na^{2+}، K^{+} و Zn^{2+} از فعالیت این آنزیم در لاروها جلوگیری کردند. یون K^{+} (غلظت ۵ میلی‌مولار) بیشترین تأثیر مثبت را بر فعالیت آلفا-آمیلاز در افراد بالغ داشت. EGTA تأثیر معنی‌داری در کاهش فعالیت آنزیم در لاروها داشت. EDTA بیشترین تأثیر را بر افزایش فعایت آنزیم در افراد بالغ داشت. در کنترل آفات گیاهی، مهارکننده‌های گیاهی آمیلاز حائز اهمیت هستند. از این‌رو، بررسی آنزیم‌های گوارشی و تأثیر مهارکننده‌ها بر روی فعالیت آنزیم می‌تواند در کنترل حشرات آفت مفید باشد.

واژگان کلیدی: *Plagiodera versicolora*، معده میانی، آلفا-آمیلاز

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