Journal of King Saud University – Science (2014) **26**, 53–58

King Saud University

Journal of King Saud University – Science

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ORIGINAL ARTICLE

Digestive α-amylase of *Bacterocera oleae* Gmelin (Diptera: Tephritidae): Biochemical characterization and effect of proteinaceous inhibitor

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Received 13 February 2013; accepted 4 May 2013

Available online 11 May 2013

KEYWORDS

Bacterocera oleae;
α-Amylase;
Characterization;
Plant origin inhibitor

Abstract Digestive α-amylase of *Bacterocera oleae* larvae was characterized and treated by an inhibitor to gain a better understanding of the degradation of nutritional molecules as a potential target for controlling the pest. Presence of α-amylase was confirmed in the gut of olive fruit fly through the use of a negative control in dinitrosalicylic acid procedure. An optimal pH of 5 was found for amylolytic activity in the gut. The enzyme had optimal activity in a broad range of temperatures 20–45 °C. Among used cations and specific inhibitors, Ca^{2+}, phenylmethylsulphonyl fluoride (PMSF) and ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA) had statistical differences on amylolytic activity indicating the presence of amino acid triad and Ca^{2+} in active site of the enzyme. A proteinaceous α-amylase inhibitor was extracted from *Polygonum persicaria*, a medicinal plant, that widely grows in North of Iran. IC_{50} value of PPAI was 0.062 mg/ml (i.e. 0.062 mg/ml of extracted inhibitor inhibited 50% of amylolytic activity in the gut of *B. oleae* larvae) and was temperature and pH dependent. The use of enzyme inhibitors from different plant sources may serve as an important pest control strategy via plant breeding programs. Identification of genes responsible for these inhibitor proteins could be a first step to provide a resistant variety of olive.

1. Introduction

Dietary carbohydrates are one of the most important macromolecules for insects and play a major role in physiological processes. To utilize carbohydrate sources in plants, herbivorous insects have to disrupt plant cell walls through the use of several enzymes including b-glucanases, xylanases, and pectinases (Terra and Ferreira, 2005). When carbohydrates reach the midgut lumen, hydrolysis is initiated by the action of amylases and glycosidases. α-Amylases play a central role in

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Peer review under responsibility of King Saud University.

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http://dx.doi.org/10.1016/j.jksus.2013.05.003

carbohydrate metabolism of microorganisms, plants, and animals. α-Amylases (1,4-α-D-glucan-4-glucanohydrolase; EC 3.2.1.1) are endoglycosidases which catalyze the hydrolysis of internal α-1,4-D-glucosidic linkages in starch and dextrins, thereby generating smaller dextrins and oligosaccharides with a C1-OH group in the α-anomeric configuration (Terra and Ferreira, 2005). Understanding the digestive enzyme function is essential especially when developing methods of insect control such as those involving the use of enzyme inhibitors in transgenic plants. Comparatively less is known about the inhibitors of alpha-amylase which might, on the other hand, be equally attractive candidates for conferring pest resistance to transgenic plants since many of them inhibit both proteinases and alpha-amylase (Fei et al., 2008).

Insect α-amylases are the calcium-dependent enzymes and may be activated by chloride at optimal pH (Terra and Ferreira, 1994). The best known insect α-amylase has been studies in *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and has been shown to consist of three domains in which central domain (domain A) is a (b/a) 8-barrel that comprises core of the molecule and includes catalytic amino acid residues. Domains B and C are almost opposite to each other, on each side of domain A (Strobl et al., 1998). Evolutionary, food sources play a critical role on the abundance and activity of insect α-amylases so that feeding on wool and plant tissues causes the lowest and the highest amylolytic activity, respectively (Zibaee et al., 2008).

Olive fruit fly, *Bacterocera oleae* Gmelin (Diptera: Tephritidae) is the most destructive pest of olive in Africa, Southern Europe, India, western Asia and California (Richard et al., 2003). Larvae feed inside the fruits and cause fruit loss before harvesting, decreasing oil quality and allowing for penetration of plant pathogens. Females lay eggs into the skin of the olive using their ovipositor (Richard et al., 2003). Larvae intensively feed inside the fruit, pupate and adult flies exit to disperse and mate.

Olive fruit fly has been introduced to the North of Iran since 2004 and causes severe damages almost 100% in some plant varieties (Mirrahimi et al., 2008). Control tactics of olive fruit fly rely on using pheromone and bite trap as well as chemical spraying. One of the promising controls of insect pests might be using enzyme inhibitors of plant origin. These inhibitors are known to be widespread in nature and comprise of one of the most abundant classes of proteins in the world because of their role in plant defense against insect herbivory (Laskowski and Kato, 1980). Comparatively less is known about the inhibitors of alpha-amylase which might, on the other hand, be equally attractive candidates for conferring pest resistance to transgenic plants since many of them inhibit both proteinases and alpha-amylase (Fei et al., 2008). Before designing any molecular and ecological experiments to introduce proteinaceous inhibitors, characterization of digestive enzymes like α-amylase is mandatory for understanding the digestion of nutritional molecules and determining the effects of xenobiotic proteins on the digestive system of insects. Hence, the objectives of the current study were to characterize digestive α-amylase of *B. oleae* and evaluate the effect of proteinaceous extract of *Polygonum persicaria* L (Polygonaceae) on the enzyme. In detail, (i) the presence of the enzyme was confirmed by using a negative control, (ii) optimal pH and temperature were determined, (iii) nature of enzyme's active site was determined by using cations and specific inhibitors and (iv) effective concentration of PPAI was determined on *B. oleae* α-amylase.

2. Material and methods

2.1. Insect rearing

The colony of *B. oleae* was established from field infested olives (variety Amigdalifolia) collected from Roudbar (North of Iran) in October, 2012. Larvae were reared on olive fruits (variety Amigdalifolia) at 25 ± 1 °C, 70% RH and 16L:8D in the laboratory. Third instar larvae were selected randomly for biochemical studies.

2.2. Sample preparation and enzyme assays

Third larval instars were separated from fruits and dissected under a stereomicroscope in saline solution (NaCl, 10 mM). Larval gut was removed and homogenized in pre-cooled homogenizer in distilled water (w/v). Homogenates were centrifuged at 13,000 rpm for 15 min at 4 °C. Supernatant was separated and kept at −20 °C for subsequent experiments as the enzyme source (Hosseinkhani and Nemat-Gorgani, 2003).

2.3. Determination of α-amylase activity

Activity of α-amylase was assayed according to a method described by Bernfeld (1955) using dinitrosalicilic acid (DNS) as the reagent and 1% (w/v) soluble starch as substrate with some modifications. Reaction mixture consisted of 50 µl Tris–HCl buffer[1] (20 mM, pH 7), 20 µl of starch and 10 µl of enzyme sample. The reaction was stopped after 30 min by the addition of 80 µl DNS[2] and heating in boiling water for 10 min prior to read absorbance at 545 nm. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg maltose in 30 min at 35 °C. The negative control contained all reaction mixtures with pre-boiled enzyme (for 15 min) to prove the enzyme presence in the samples.

2.4. Effect of pH and temperature on α-amylase activity

The effects of temperature and pH on α-amylase activity were evaluated by incubating enzyme reaction in different pH sets (3–12) for determination of optimal pH and different temperature ranges (20, 25, 30, 35, 40, 45, 50 and 60 °C). The procedure was continued as described above and absorbance was read at 545 nm.

2.5. Effect of different cations and inhibitors on α-amylase activity

Different concentrations of cations (0.5, 3 and 5 mM) and inhibitors (1, 3 and 5 mM) were assayed to find their possible effects on α-amylase activity in *B. oleae*. Ca^{2+}, Cu^{2+}, Fe^{2+}, K^{+}, Mg^{2+}, Mn^{+}, Na^{+} and Zn^{2+} were used as cations. Control consisted of substrate, buffer and enzyme. Inhibitors were

[1] Based on Henderson–Hasselbalch, molarity of buffer was calculated, stock solution was prepared and pH values were optimized by using HCl solution.

[2] To prepare 100 ml of DNS solution, 1 g of dinitrosalicilic acid was dissolved in 50 ml of distilled water. Then, 30 g of sodium potassium tartarate was dissolved followed by stirring. Finally, 20 ml of sodium hydroxide (2 N) was added and total volume was reached to 100 ml.

ethylenediamide tetraacetic acid (EDTA), ethylene glycol-bis (β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), triethylenetetramine hexaacetic acid (TTHA), diethyldithiocarbamate (DTC), phenanthrolien and phenylmethylsulphonyl fluoride (PMSF). These compounds were added to the assay mixture, then incubation was done at 30 °C for 30 min, and absorbance was recorded at 545 nm.

2.6. Extraction of α-amylase inhibitor from Polygonum persicaria (PPAI)

PPAI was extracted from stems as described by Baker (1989) and Melo et al. (1999). Powdered stems (10 g each) were mixed with a solution of 0.1 M NaCl, stirred for 2 h, and then centrifuged at 5000 rpm for 20 min. The pellet was discarded, and the supernatant was incubated at 70 °C for 20 min to inactivate major endogenous enzymes. Fractionation of the supernatant was done using 60% concentration of ammonium sulfate followed by centrifugation at 5000 rpm for 20 min at 4 °C. The 60% pellet containing the highest fraction of α-amylase inhibitors was dissolved in ice-cold sodium phosphate buffer (0.02 M, pH 7.1) and dialyzed overnight against the same buffer. This dialyzed solution was used as a source of amylase inhibitors in enzyme assays.

2.7. Inhibition of α-amylase by different concentrations of PPAI

To find possible inhibition of the digestive α-amylase, 50 µl of Tris–HCl buffer (20 mM, pH 7), 20 µl of 1% starch and each concentration of PPAI separately (0, 0.05, 0.1, 0.3, 0.5, 1 mg/ml) were incubated for 5 min. Then, 10 µl of the enzyme was added and the reaction continued as described earlier. Control contains buffer, starch 1% and each concentration of PPAI) separately[3]. Absorbance was recorded at 545 nm.

2.8. Effect of pH and temperature on α-amylase inhibition by PPAI

Effect of pH on α-amylase inhibition by PPAI was determined at different pH values using Tris–HCl buffer (20 mM) with pH set from 4–10. The amylase activity was assayed after incubation of the reaction mixture containing Tris–HCl buffer, starch 1%, PPAI (IC_{50} concentration) and enzyme. Absorbance was recorded at 545 nm. To find the effect of temperature on α-amylase inhibition by PPAI, reaction mixture containing Tris–HCl (20 mM pH, 9), starch 1%, PPAI (IC_{50} concentration) and enzyme was incubated at different temperature sets 20, 25, 30, 35, 40, 45, 50 and 60 °C. Absorbance was recorded at 545 nm.

2.9. Protein assay and statistical analysis

Protein concentrations were assayed according to the method described by Lowry et al. (1951). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test where significant differences were found at $P = 0.05$. IC_{50} values were calculated by POLO-PC software.

3. Results

Presence of α-amylase has been proved in larvae of *B. oleae* by using a negative control. Then, properties of the enzyme were determined under biochemical conditions. Present study demonstrated an optimal pH of 5 and a temperature range of 20–45 °C for amylolytic activity in the gut of *B. oleae* (Fig. 1). Regarding optimal temperature, a wide range of amylolytic optimal activity was observed in a temperature range from 20–45 °C (Fig. 1).

The present investigation revealed that Ca^{2+} at all concentrations and Mn^{+} (at 3 mM) significantly increased amylolytic activity in the midgut of *B. oleae* while other ions had no effect on α-amylase activity (Table 1). It was proved the presence of calcium in active site of the enzyme since chelating agent of calcium (EGTA) significantly decreased amylolytic activity (Table 2). Although, other chelating agents including DTC (for Cu^{2+}) and TTHA (for Mg^{2+}) have negative effects on enzymatic activity but their effect was not in accordance by ion assays (Table 2).

The present study employed α- amylase inhibitor extracted from the stems of *P. persicaria* (PPAI) with different concentrations I_{10}, I_{30} and I_{50} determined at 0.027, 0.044 and 0.062%, respectively (Table 3). Different concentrations of proteinaceous extract inhibited α-amylase of *B. oleae* to 100% (Fig. 2). IC_{50}[4] value of PPAI was calculated as 0.062 mg/ml. Although optimal pH of α-amylase was 5, PPAI produced the highest inhibition in the pH range of 8–10

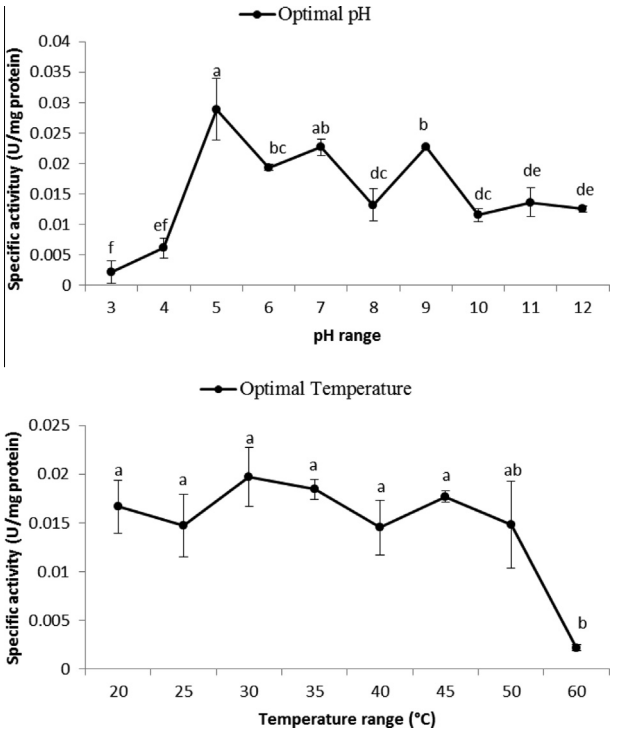

Figure 1 Optimal pH and temperature determination for digestive α-amylase of *B. oleae* larvae. Statistical differences have been shown by various letters (Tukey's test, $p \leqslant 0.05$).

[3] All defined concentrations of PPAI were used in reaction mixture to find their effects on amylolytic activity. Obtained data were inserted in POLO-PC software and IC values (Inhibitory concentrations) were calculated by line regression equation.

Table 1 Effect of mono- and divalent cations on α-amylase activity of *B. oleae*.

Compound	Concentration	Relative activity
Control	–	100
Ca	0.5	218.27*
	3	174.73*
	5	149.46*
Cu	0.5	74.70
	3	60.58
	5	0*
Fe	0.5	100.95
	3	88.72*
	5	75.29*
K	0.5	139.03
	3	131.62
	5	111.39
Mg	0.5	41.98*
	3	89.72
	5	39.27*
Mn	0.5	133.01
	3	237.10*
	5	139.62
Na	0.5	45.56*
	3	59.29*
	5	22.44*
Zn	0.5	57.99*
	3	60.86*
	5	64.34*

Control consisted of substrate, buffer and enzyme. ($F = 8.75$, $N = 30$ and $n = 3$).
* Indicate significant differences between values and the control (Tukey's test; $p = 0.05$).

Table 2 Effect of specific inhibitors on α-amylase activity of *B. oleae*.

Compound	Concentration	Relative activity
Control	–	100
DTC	1	10.52*
	3	44.95*
	5	6.35*
Phenanthroline	1	15.57*
	3	0*
	5	0*
EGTA	1	50.65*
	3	45.61*
	5	4.16*
EDTA	1	106.57
	3	114.25
	5	97.36
PMSF	1	83.11
	3	35.30*
	5	10.52*
TTHA	1	1.75*
	3	10.30*
	5	0*

Control consisted of substrate, buffer and enzyme. ($F = 3.21$, $N = 30$ and $n = 3$).
* Indicate significant differences between values and the control (Tukey's test; $p = 0.05$).

(Fig. 3). Also, the highest inhibition of the enzyme was shown at 20 °C, this inhibition was not statistically different from temperature ranges of 20–35 °C (Fig. 3).

4. Discussion

The present investigation clearly demonstrated the presence of a digestive α-amylase in the gut of *B. oleae* to digest polysaccharides like starch or glycogen. Similar findings have been reported on digestive α-amylase in *Musca domestica* L. (Diptera: Muscidae) (Espinoza-Fuentes and Terra, 1987), *Phlebotomus papatasi* (Scopoli) (Diptera: Psychodidae) (Jacobson and Schlein, 2001), *Lutzomyia longipalpis* L. (Diptera: Psychodidae) (Vale et al., 2012), *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Darvishzadeh et al., 2012).

Temperature and pH are the two key factors affecting biochemical reactions. Studies have shown an optimal pH of 6–7 in dipterans such as Bigham et al. (2010), 7–8 (Darvishzadeh et al., 2012), 8.5 (Vale et al., 2012) and even more (Vale et al., 2012) for α-amylase activity at 40–50 °C in the midgut of *L. longipalpis* larvae. Usually, it is believed that the optimal pH of enzymes reflects prevailing pH conditions in the midgut from which the enzyme has been extracted. Variation in the pH of midgut is related to different feeding habits and sources. Also, chemistry of olive fruits is changed during development and maturation of fruit and therefore the physiological state of fruits might contribute to the variation in pH from 5–7. Although studies related to optimal temperature of α-amylase in dipterans are lacking, it is well known that temperature will increase the rate of enzyme-catalyzing reactions by increasing kinetic energy and collision frequency of the reacting molecules (Mohammadi et al., 2010).

Cations (ions) are one of the important components of enzyme's site of activity. Terra and Ferreira (2005) believe that digestive α-amylases of insects are calcium-dependent enzymes and could be activated by chloride at optimal pH. In the current study, different concentrations of ions and some chelating agents were used to prove activation of α-amylase in *B. oleae* by calcium ion. Although some discrepancies were observed in case of Cu^{2+}. In fact, this could be attributed to structural similarity of Cu^{2+} with two others. Meanwhile, phenanthroline (Metalloproteinase inhibitor) and PMSF (Inhibitor serine, histidine and aspartic acid triad) had negative effect on α-amylase of *B. oleae*. Importance of amino acids and Ca^{2+} in active site of α-amylase has been elucidated in amylase structure of *T. molitor* (Strobl et al., 1998). α-Amylase of *T. molitor* had a central domain that comprises the core of the molecule and have the catalytic amino acid residues (Aspartate, Glutamate and Aspartate). Domains B and C have been placed on each side of domain A. The substrate-binding site is located in a long V-shaped cleft between domains A and B. A calcium ion is placed in domain B and is coordinated by Asparagine, Arginine and Aspartate. This ion is important for the structural integrity of the enzyme and seems also to be relevant due its

Table 3 Inhibiting parameters of PPAI on digestive α-amylase of *B. oleae*.

	IC_{10}	IC_{30}	IC_{50}	Slope ± SE	X^2	df
PPAI	0.027	0.044	0.062	3.6 ± 0.452	1.55	2

IC stands for inhibitor concentration.
$N = 30$.

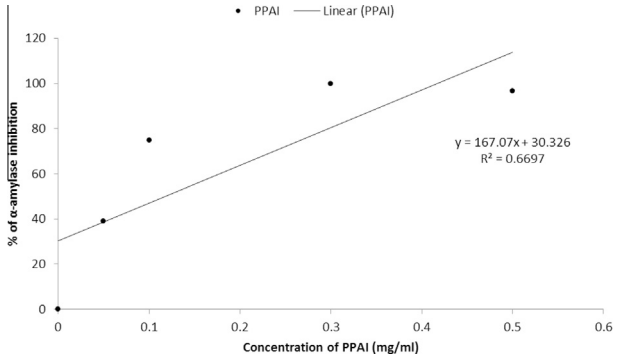

Figure 2 Inhibition of *B. oleae* α-amylase by different concentrations (mg/ml) of PPAI proteinaceous extract.

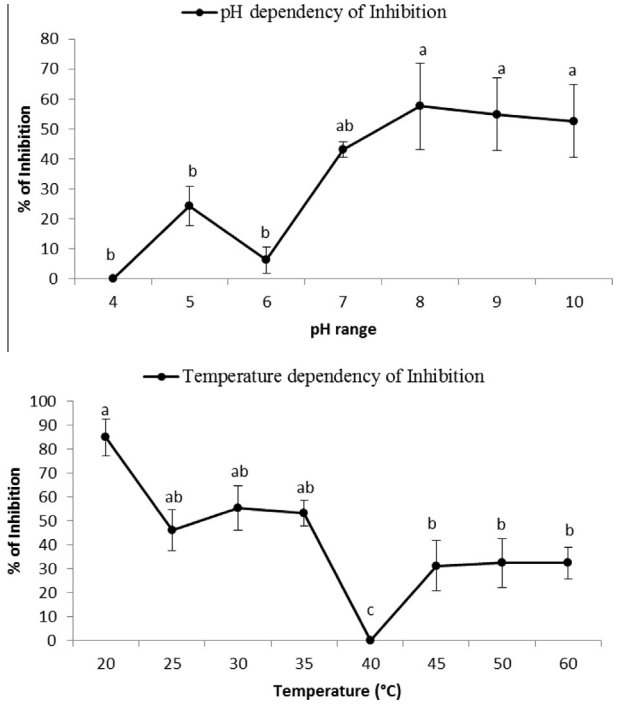

Figure 3 pH and temperature dependency of *B. oleae* α-amylase by PPAI. Statistical differences have been shown by various letters (Tukey's test, $p \leqslant 0.05$).

contact with Histidine (Strobl et al., 1998). Present results exhibit structural similarity of *B. oleae* α-amylase with found structure of insect α-amylase in involvement of amino acid triad (Inhibited by PMSF) and Ca^{2+} ion (Inhibited by EGTA).

Several classes of plant proteins have been discovered and characterized including lectins, ribosome-inactivating proteins (RIPs), protease inhibitors and α-amylase with demonstrated insecticidal effects against many insects (Franco et al., 2000). Inhibition of digestive α-amylase from larvae of *B. oleae* was achieved by using different concentrations of PPAI. Melo et al. (1999) reported inhibition of an α-amylase from *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) larvae by proteinaceous extract from cowpea seeds. Suzuki et al. (1994) extracted and purified two different α-amylase inhibitors from *Proteus vulgaris*. α-AI1 inhibits digestive α-amylases of *C. maculatus* and *Cryptocarya chinensis* and α-AI2 inhibits digestive α-amylase of *Zabrotes subfasciatus* Boheman (Coleoptera: Chrysomelliade) (Bonavides et al., 2007). Mehrabadi et al. (2010) determined dose-dependent inhibition of the α-amylase of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) by triticale extract (T-αAI).

Although, information on other dipterans is lacking, lepidopteran amylases demonstrated the highest inhibition at alkaline pHs that shows pH dependency of enzymatic inhibition. Effect of temperature on the activity of α-amylase does not support an earlier finding of Marshall and Lauda (1975). There was 10-fold increase in activity of the α-amylase inhibitor with an increase in temperature from 25 to 37 °C as reported by Marshall and Lauda (1975). This variance could be attributed to the nature of the inhibitor and the enzyme and the stability of the tertiary structure at the desired temperatures. Also, inhibition of the enzyme increased from 45 to 60 °C. At these temperatures, enzyme is degraded. So, this inhibition could be attributed to enzyme denaturation rather than inhibitory mechanism.

In the current study, activity of α-amylase was measured by dinitrosalisylic acid procedure and using negative control. Also, different concentrations of PPAI showed inhibitory effect on α-amylase of *B. oelae*. Our study has shown a strong inhibitory effect of PPAI on α-amylase activity of olive fruit fly, *B. oelae*. IC_{50} value of PPAI was 0.062 mg/ml and was temperature and pH dependent. The use of enzyme inhibitors from different plant sources may serve as an important pest control strategy via plant breeding programs. Hence, it is mandatory to determine biological activities of these compounds against digestive enzymes of insects under optimal conditions, prior to cloning the relevant gene to new varieties of plants for conferring pest resistance.

References

Baker, J.E., 1989. Interaction of partially-purified amylases from larval *Anagasta kuehniella* (Lepidoptera: Pyralidae) with amylase inhibitors from wheat. Comp. Biochem. Physiol. Part B 93, 239–245.

Bernfeld, P., 1955. Amylases, α and β. Methods Enzymol. 1, 149–158.

Bigham, M., Hosseininaveh, V., Nabavi, B., Talebi, K., Esmaeilzadeh, N.S., 2010. Effects of essential oil from *Teucrium polium* on some digestive enzyme activities of *Musca domestica*. Entomol. Res. 40, 37–45.

Bonavides, K.B., Pelegrini, P.B., Laumann, R.L., Grossi-de-Sá, M.F., Bloch Jr., C., Melo, J.A.T., Quirino, B.F., Noronha, E.F., Franco, O.L., 2007. Molecular identification of four different α-amylase inhibitors from Baru (*Dipteryx alata*) seeds with activity toward insect enzymes. J. Biochem. Mol. Biol. 40, 494–500.

Darvishzadeh, A., Hosseininaveh, V., Ghamari, M., 2012. Identification and biochemical characterisation of α-amylase in the alimen-

tary tract of Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae). Arch. Phytopathol. Plant Prot.. http://dx.doi.org/10.1080/03235408.2012.757858.

Espinoza-Fuentes, F.P., Terra, W.R., 1987. Physiological adaptation for digesting bacteria. Water fluxes and distribution of digestive enzymes in *Musca domestica* larval midgut. Insect Biochem. 17, 809–817.

Fei, Z.Z., Ting, T.N., Xu, Z.M., Ge, X.Z., Yan, H.M., 2008. The crystal water affect in the interaction between the *Tenebrio molitor* alpha-amylase and its inhibitor. Bioinorg. Chem. Appl.. http://dx.doi.org/10.1155/2008/469062.

Franco, O.L., Ridgen, D.J., Melo, F.R., Bloch Jr., C., Silva, C.P., Grossi-de-Sa, M.F., 2000. Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. Eur. J. Biochem. 267, 2166–2173.

Hosseinkhani, S., Nemat-Gorgani, M., 2003. Partial unfolding of carbonic anhydrase provides a method for its immobilization on hydrophobic adsorbents and protects it against irreversible thermoinactivation. Enzyme Microb. Technol. 33, 179–184.

Jacobson, R.L., Schlein, Y., 2001. *Phlebotomus papatasi* and Leishmania major parasites express α-amylase and α-glucosidase. Acta Trop. 78, 41–49.

Laskowski, J.R., Kato, M., 1980. Protein inhibitors of proteinases. Annu. Rev. 49, 593–626.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Marshall, J.J., Lauda, C.M., 1975. Purification and properties of phaseolamin, an inhibitor of α-amylase, from the kidney bean *Phaseolus vulgaris*. J. Biol. Chem. 250, 8030–8037.

Mehrabadi, M., Bandani, A.R., Saadati, F., 2010. Inhibition of Sunn pest, Eurygaster integriceps, α-amylases by α-amylase inhibitors (T-αAI) from Triticale. J. Insect Sci. 10, 179. Available online: insectscience.org/10.179.

Melo, F.R., Sales, M.P., Silva, L.S., Franco, O.L., Bloch Jr., C., Ary, M.B., 1999. α-Amylase inhibitors from cowpea seeds. Prot. Pept. Lett. 6, 387–392.

Mirrahimi, S., Khalaghani, J., Nouri, H., 2008. Effect of time, direction and site of sampling on olive infestation by olive fruit fly. Proceedings of 18th Iranian Plant Protection Congress, p. 413.

Mohammadi, D., Farshbaf Pour Abad, R., Rashidi, M.R., Mohammadi, S.A., 2010. Activity and some properties of *Helicoverpa armigera* Hubner and Spodoptera exigua Hubner (Lep.: Noctuidae) midgut protease. Munis Entomol. Zool. 5, 697–706.

Richard, R., Phillips, P.A., Stewart-Leslie, J., Sibbett, G.S., 2003. Olive fruit fly population in central and southern California 57. http://dx.doi.org/10.3733/ca.v057n04p122.

Strobl, S., Maskos, K., Wiegand, G., Huber, R., Gomis-Ruth, F.X., Glockshuber, R., 1998. A novel strategy for inhibition of a-amylases: yellow mealworm a-amylases in complex with the Ragi bifunctional inhibitor at 2.5A resolution. Structure 6, 911–921.

Suzuki, K., Ishimoto, M., Kitamura, K., 1994. CDNA sequence and deduced primary structure of an alpha-amylase inhibitor from a bruchid-resistant wild common bean. Biochem. Biophys. Acta 1206, 289–291.

Terra, W.R., Ferreira, C., 1994. Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. Part B 109, 1–62.

Terra, W.R., Ferreira, C., 2005. Biochemistry of digestion. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), . In: Comprehensive Molecular Insect Science, vol. 3. Elsevier, San Diego, California, USA, pp. 171–224.

Vale, V.F., Moreira, B.H., Moraes, C.S., Pereira, M.H., Genta, F.A., Gontijo, N.F., 2012. Carbohydrate digestion in *Lutzomyia longipalpis*' larvae (Diptera - Psychodidae). J. Insect Physiol. 58, 1314–1324.

Zibaee, A., Bandani, A.R., Kafil, M., Ramzi, S., 2008. Characterization of α-amylase in midgut and salivary glands of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), rice striped stem borer. J. Asia-Pacific Entomol. 11, 201–205.