

IDENTIFICATION AND CHARACTERIZATION OF ALPHA-AMYLASE IN CITRUS SWALLOWTAIL, *PAPILIO DEMOLEUS* (LINNAEUS, 1758) (LEPIDOPTERA: PAPILIONIDAE)

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ABSTRACT: *Papilio demoleus* (Linnaeus, 1758) (Lepidoptera: Papilionidae), commonly known as the lime or citrus swallowtail, has a successful dispersal and becoming a major pest of citrus plants in wide area of the world including the Middle East. The insect has been reported to feed on every citrus cultivars and varieties thus it has potential to become a major pest in area where it has been reported. Host plant resistance and natural plant products offer a potentially rational method for the insect pest control since they are safe to the environment as well as human being and non target organisms. Therefore, new control methods such as digestive enzyme inhibitors which interfere with function of the insect digestive enzymes are needed because these compounds affect insect growth and development but not non-target organisms. The aim of the current study was to investigate and characterize α -amylase activity of the larval stage of the lime swallowtail. To do this, the insect larvae were collected and transferred to the lab where their enzymes have been extracted. For enzyme assay soluble starch (1%) has been used. The study showed that α -amylase is present in the insect gut and is used for carbohydrate digestion. The optimum temperature and pH for the activity of α -amylase was 50°C and 8.0, respectively. Native page electrophoresis showed that only one amylase band is present in the insect gut. Understanding of the digestive physiology and α -amylase activity of Citrus Swallowtail is important when new management strategies for this economically important pest are devised.

KEY WORDS: α -amylase, lime swallowtail, characterization, optimum temperature and pH.

Papilio demoleus L., commonly known as the lime or citrus swallowtail, has a successful dispersal and becoming a major pest of citrus plants throughout Asia including Iraq (Eastwood et al., 2006) and the Middle East (Farid, 1987; Badawi, 1981). *P. demoleus* feeds on the foliage of citrus trees and is regarded as a citrus pest, particularly in southern and Southeast Asia (Margabandhu, 1933; Ebeling, 1959; Wyniger, 1962). Citrus swallowtail (*Papilio demoleus*) produces 5 generation per year in warm temperature. However, the number of generation is greatly influenced by temperature since nine generation per year has been recorded for the insect near the equator (Chen et al., 2004). Under ideal experimental conditions in India, a generation time of over 30 days has been reported for it (Pathak & Rizvi, 2003).

The insect has been reported to feed on every citrus cultivars and varieties thus it has potential to become a major pest in area where it has been reported (Larsen, 1984). Chemical pesticides are used to control this insect pest in some area. However, problems associated with pesticide use in integrated pest management program limited their use. Thus, the public concern regarding residual toxicity of pesticides remaining in the agricultural products, the occurrence of pesticide resistance, and environmental impact of pesticides have driven the modern world to call for more environmentally-benign alternatives to

control insect pest (Hagstrum & Subramanyam, 1996). Recently, a great deal of research has been carried out on new methods especially plant derived materials (Regnault-Roger et al., 2002).

Host plant resistance and natural plant products offer a potentially rational method for the insect pest control since they are safe to the environment as well as human being and non target organisms (Andow, 2008). Therefore, new control methods such as digestive enzyme inhibitors which interfere with function of the insect digestive enzymes are needed because these compounds affect insect growth and development but not non-target organisms.

Different types of alpha-amylase inhibitors are found in microorganisms, plants and animals. There are six different alpha-amylase inhibitors including lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin like (Mehrabadi et al., 2011). These inhibitors have a good potential to be used in integrated pest management program (IPM) in order to control insect pest. They show diverse structural differences thus causing different mode of actions and diverse specificity against target enzymes. Different alpha-amylase inhibitors from different plant species have different molecular structures, leading to different modes of inhibition and different specificity profiles against a wide range of insect alpha-amylases.

Among insects, α -amylase has been purified or characterized in a number of species including those in the orders Diptera (Terra et al., 1977), Lepidoptera (Kanekatsu, 1978; Kusano & Tanabe, 1986; Baker, 1989), Hemiptera (Hori, 1972), Orthoptera (Moore & Davis, 1985), and particularly those in granivorous Coleoptera (Applebaum, 1964; Podoler & Applebaum, 1971; Buonocore et al., 1976; Baker & Woo, 1984; Chen et al., 1992). However, to date no information is available concerning the presence and properties of such enzymes in the lime swallowtail. In these studies, and in nearly all studies of α -amylase in insects, the enzyme activity is characterized by using a soluble starch substrate. Hot-water soluble starch is an extremely sensitive substrate for α -amylase. An understanding of how digestive enzymes act on their substrate in insects is essential to develop new methods of insect control. Determination of digestive enzyme reactions to different inhibitors is a winning procedure to develop transgenic plants programs to control phytophagous insects (Bandani et al., 2001).

MATERIALS AND METHODS

Insect

Last instar larvae were collected from their hosts from Mazandaran province, Iran, during October and November 2011. The insects were transferred to the Laboratory of the Insect Physiology and kept at $27\pm 1^\circ\text{C}$ and 16L:8D photoperiod on the host plants.

Sample preparation and enzyme assays

Sample preparation was done as described by Allahyari et al. (2010) with slight modifications. Briefly, larvae were placed on ice (about 5 min) for immobilization and dissected under binocular. Larval gut was divided into three section including foregut, midgut, and hindgut and each section was removed separately and homogenized in pre-cooled homogenizer in distilled water. Samples then were put in the 1.5 ml centrifuge tubes and centrifuged at 15000 rpm for 15 min at 4°C . Supernatant was separated and kept at -20°C for further analysis as an enzyme source.

α -amylase assay

α -Amylase activity was assayed using the dinitrosalicylic acid (DNS) procedure (Bernfeld, 1955), using 1 % soluble starch (Merck, Darmstadt, Germany) as the substrate. Ten microliter of the enzyme in addition to 10 microliter substrate (soluble starch) and 80 microliter universal buffer (0.02 M) containing succinate, glycine and 2-morpholinoethanesulfonic acid (pH 7) were incubated in 30 min at 35 °C. After addition of DNS and heating in the boiling water for 10 min, the reaction was stopped. Then, absorbance was read at 540 nm. In the blank, instead of enzyme, distilled water was used. All assays were repeated at least three times.

Effect of PH and temperature on enzyme activity

The effects of temperature and pH on α -amylase activity were examined. Optimal pH was determined using universal buffer with pH set at 3, 4, 5, 6, 7, 8, 9, 10 and 11. The effect of temperature on α -amylase activity was determined by incubating the reaction mixture at 20, 30, 35, 40, 45, 50, 60 and 70 °C for 30 min followed by measurement of residual activity.

Electrophoresis

The amylase present in crude homogenates was visualized using SDS–polyacrylamide gel electrophoresis (PAGE) by procedure described by Laemmli (1970) and Campos et al. (1989), with minor modification. SDS–PAGE was performed in a 10 % (w/v) separating gel and a 5 % stacking gel, both with 0.05 % SDS. The electrode buffer was prepared based on the method of Laemmli (1970), but SDS was not used. The sample buffer contained 25 % stacking buffer (0.5 mol/ L Tris–HCl [pH 6.8]), 20 % glycerol, 2 % SDS, 0.005 % (w/v) bromophenol blue, but no mercaptoethanol, and it was not heated. Electrophoresis was conducted at room temperature at 120 V until the blue dye reached the bottom of the slab gel. To prepare gels for α -amylase assay, the gel was rinsed with water and washed by shaking gently with 1 % (v/v) Triton X-100 in phosphate buffer [pH 7] containing 2 mmol CaCl₂ and 10 mmol NaCl for 1.5 h. Staining the gel with 0.05% KI and 0.05% I₂ solution visualized α -amylase activity as light bands in dark background.

Protein determination

Protein determined based on the method of Bradford (1976) and bovine serum albumin (Bio-Rad, München, Germany), used as a standard for measuring the concentration of protein.

RESULTS

α -Amylase activity

This study showed that α -amylase activity was present in three different gut sections of the lime swallowtail. Results showed that the amount of enzyme activity in three gut sections were different. The enzyme activity in the foregut, midgut and hindgut were 1.19, 1.81 and 0.89 U/min, respectively (Table 1). As can be seen in Table 1 the greatest amount of the enzyme activity (1.81 U/min) was present in the midgut section of the larvae and the least amount of the enzyme activity (0.89 U/min) was present in the hindgut section of the larval gut.

Effect of PH and temperature on enzyme activity

The α -amylases activity in Citrus Swallowtail showed an optimal pH of 8 (Fig. 1). The enzyme activity increased from pH 3 to 8, and then decreased with increasing

pH (Fig. 1). Amylase was active over a broad range of temperatures. The optimal temperature for α -amylase activity was 50 °C (Fig. 2).

Electrophoresis

Analysis of enzyme homogenates by zymogram analysis showed that only one band of alpha amylase was present in the larval gut (Fig. 3).

DISCUSSION

Alpha-Amylases play an essential role in carbohydrate metabolism in insects (Franco et al., 2002). The present study revealed the presence of alpha-amylase activity in the gut of *P. demoleus*. The presence of one alpha-amylase isoforms in gut extracts of *P. demoleus* represents a strong evidence of the importance of digestive nature of the enzyme in this pest.

Citrus Swallowtail alpha-amylase activity pH was determined to be around 8. Our results show that the digestive alpha-amylase in *P. demoleus* has alkaline optimal pH, which is consistent with the optimal pH that has been reported for other lepidopteran species. (Valenci a-Jimenez et al., 2008).

High pH gut has been reported for the Lepidoptera thus optimal pH for the α -amylase activity in these insects has been shown to be high (> 7.0). There are reports that pH values of 9.0 for *Chilo suppressalis* (Lepidoptera: Crambidae), 9.2 for *A. mylitta*, 12.0 for *Acherontia atropos* (Lepidoptera: Sphingidae), 10.8 for *Lasiocampa quercus* (Lepidoptera: Lasiocampidae), 11.3 for *Manduca sexta* (Lepidoptera: Sphingidae) and 10.8 *Lichnoptera felina* (Lepidoptera: Noctuidae) (Dow, 1984; Zibaee et al., 2009). It has been reported that high values of gut pH in insects is adaptation to feed on plant materials rich in tannins (Chapman, 1998), which at lower pH values bind to proteins thus decreasing the digestion efficiency (Dow, 1986).

Acidic pH optima for alpha-amylases have been reported in other insect-pests, such as coleopteran insects. Generally, optimal pH is corresponding to the pH prevailing in the gut from which the enzyme has been extracted (Bandani et al., 2001; Baker, 1983; Terra et al., 1996). Optimal pH values for amylases in larvae of several coleopterans were 4 to 5.8 (Baker, 1983) and in *Lygus spp.* (Heteroptera), the optimal pH value was 6.5 (Zeng & Cohen, 2000). The optimal temperature for Citrus Swallowtail alpha-amylase activity was 50°C, the enzyme was active over a broad temperature range from 20 to 60°C. Analysis of enzyme homogenates by electrophoresis indicated a single band in polyacrylamide gel. Characterisation of insect digestive enzymes greatly facilitates the understanding of the mechanisms involved in selectivity and helps to design new and more specific strategies for insect control.

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LITERATURE CITED

Allahyari, M., Bandani, A. R. & Habibi-Rezaei, M. 2010. Subcellular fractionation of midgut cells of the sunn pest *Eurygaster integriceps* (Hemiptera: Scutelleridae): Enzyme markers of microvillar and perimicrovillar membranes. *Journal of Insect Physiology*, 56: 710-717.

Andow, D. A. 2008. The risk of resistance evolution in insects to transgenic insecticidal crops. *Collection of Biosafety Reviews, International Centre for Genetic Engineering and Biotechnology*, 4: 142-199.

- Applebaum, S. W.** 1964. The action pattern and physiological role of *Tenebrio* larval amylase. *J. Insect. Physiol.*, 10: 897-906.
- Badawi, A.** 1981. Studies on some aspects of the biology and ecology of the citrus butterfly *Papilio demoleus* L. in Saudi Arabia (Papilionidae: Lepidoptera). *Zeitschrift fuer Angewandte Entomologie*, 91 (3): 286-292.
- Baker, J. E. & Woo, S. W.** 1985. Purification, partial characterization and postembryonic levels of amylase from *Sitophilus oryzae* and *Sitophilus granarius*. *Arch. Insect Biochem. Physiol.*, 2: 415-428.
- Baker, J. E.** 1983. Properties of amylases from midguts of larvae of *Sitophilus zeamais* and *Sitophilus granarius*. *Insect Biochem.*, 13: 421-428.
- Baker, J. E.** 1989. Interaction of partially-purified amylase from larval *Anagasta kuehniella* (Lepidoptera: Pyralidae) with amylase inhibitors from wheat. *Comp. Biochem. Physiol.*, 93B: 239-246.
- Bandani, A. R., Amiri, B., Butt, T. M. & Gordon-Weeks, R.** 2001. Effects of efrapeptin and destruxin, metabolites of entomogenous fungi, on the hydrolytic activity of a vacuolar type ATPase identified on the brush border membrane vesicles of *Galleria mellonella* midgut and on plant membrane bound hydrolytic enzymes, *Biochim. Biophys. Acta.*, 1510: 367-377.
- Bernfeld, P.** 1955. Amylases, α and β . *Method Enzym.*, 1: 149-158.
- Bradford, M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.
- Buonocore, V., Poerio, E., Silano, V. & Tomasi, M.** 1976. Physical and catalytic properties of α -amylase from *Tenebrio molitor* L. larvae. *Biochem. J.*, 153: 621-625.
- Campos, F. A., Xavier-Filho, J., Silva, C. P & Ary, M. B.** 1989. Resolution and partial characterization of proteinases and α -amylases from midgut of larvae of the bruchid beetle *Callosobruchus maculatus* (F). *Comparative Biochemistry and Physiology Part B*, 92: 51-57.
- Chapman, R. F.** 1998. *The Insects: Structure and Function*, 4th edition. Cambridge: Cambridge University Press.
- Chen, M. S., Feng, G., Zeng, K. C., et al.** 1992. α -Amylase from three species of stored grain coleopteran and their inhibition by wheat and corn proteinaceous inhibitors. *Insect Biochem. Mol. Biol.*, 22: 261-268.
- Chen, X. Y., Wen, R. Z. & Chen, H. D.** 2004. Observation on the bionomics of *Papilio demoleus* in Guangzhou. *Entomological Knowledge*, 41 (2): 169-171.
- Dow, J. A.** 1984. Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.*, 24: 633-635.
- Dow, J. A.** 1986. Insect midgut function. *Adv. Insect Physiol.*, 19: 187-328.
- Eastwood, R., Boyce, S. L. & Farrell, B. D.** 2006. The provenance of Old World swallowtail butterflies, *Papilio demoleus* (Lepidoptera: Papilionidae), recently discovered in the New World. *Ann. Entomol. Soc. Am.*, 99 (1): 164-168.
- Ebeling, W.** 1959. *Subtropical fruit pests*. Division of Agricultural Science, University of California, Berkeley. 436 pp.
- Farid, A.** 1987. Some bio-ecological features of citrus butterfly in south-east Iran. *Entomologie et Phytopathologie Appliquees*, 54 (1-2): 129-137.
- Franco, O. L., Rigden, D. J., Melo, F. R. & Grossi-de-Sa, M. F.** 2002. Plant alpha amylase inhibitors and their interaction with insect alpha amylases structure, function and potential for crop protection. *Eur. J. Biochem.*, 269: 397-412.
- Hagstrum, D. W. & Subramanyam, B.** 1996. *Integrated Management of Insects in stored products*. Marcel Dekker, Inc, New York, 426 pp.
- Hori, K.** 1972. Comparative study of a property of salivary amylase among various heteropterous insects. *Comp. Biochem. Physiol.*, 42B: 501-508.

- Kanekatsu, R.** 1978. Studies on further properties for an alkaline amylase in the digestive juice of the silkworm, *Bombyx mori*. J. Fac. Text. Sci. Technol., 76: 1-21.
- Kusano, T. & Tanabe, S.** 1986. Enzymatic properties of the midgut amylase activity and its changes during development in the cabbage armyworm, *Mamestra brassicae* L. Kontyu (Tokyo), 54: 12-24.
- Lammi, U. K.** 1970. Cleavage of structural proteins during the assembly of bacteriophage T4. Nature, 227: 680-685.
- Larsen, T. B.** 1984. The zoogeographical composition and distribution of the Arabian butterflies (Lepidoptera; Rhopalocera). Journal of Biogeography, 11: 119-158.
- Margabandhu, V.** 1933. Insect pests of oranges in the northern Circars. J. Madras Agric. 21: 6068.
- Mehrabadi, M., Bandani, A. R., Saadati, F., & Mahmudvand, M.** 2011. α -Amylase activity of stored products insects and its inhibition by medicinal plant extracts. Journal of Agricultural Science and Technology, 13: 1173-1182.
- Moore, K. C. & Davis G. R. F.** 1985. Physical and catalytic properties of amylase from the alimentary canal of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.). Archs Int. Physiol. Biochim., 93: 171-174.
- Pathak, M. & Rizvi, Q.** 2003. Effect of different temperatures and hostplants on the developmental behavior of lemon butterfly, *Papilio demoleus*. Indian J. Entomol., 65 (4): 496-499.
- Podoler, H. & Applebaum, S. W.** 1971. The α -amylase of the beetle *Callosobruchus chinensis* - properties. Biochemical Journal, 121: 321-325.
- Regnault-Roger, C., Philogène, B. J. & Vincent, C.** 2002. Biopesticides of Plant Origin. Tec & Doc Eds. Paris, 337 pp.
- Terra, W. R., Ferreira, C. & De-Bianchi, A. G.** 1977. Action pattern, kinetical properties and electrophoretal studies of an alpha-amylase present in midgut homogenates from *Rhynchosciara americana* (Diptera) larvae. Comp. Biochem. Physiol., 56: 201-209.
- Terra, W. R., Ferreira, C., Jordão, B. P. & Dillon, R. J.** 1996. Digestive enzymes. In: Lehane MJ, Billingsley PF (eds), Biology of the Insect Midgut, Chapman and Hall, London, pp. 153-193.
- Valencia-Jimenez, A., Arboleda, J. W., Lopez-Avila, A. & Grossi-de-Sa M. F.** 2008. Digestive α -amylases from *Tecia solaniivora* larvae (Lepidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. Bull Entomol Research., 98: 575-579.
- Wyniger, R.** 1962. Pests of crops in warm climates and their control. Verlag fur Recht und Gesellschaft, Basel, Switzerland. Verlag fur Recht und Gesellschaft, 555 p.
- Zeng, F. & Cohen, A. C.** 2000. Partial characterization of α -amylase in the salivary glands of *lygus Hesperus* and *L. lineolaris*. Comparative Biochemistry and Physiology, 126: 9-16.
- Zibae, A., Bandani, A. R. & Ramzi, S.** 2009. Characterization of α and β -glucosidases in midgut and salivary glands of *Chilo suppressalis* Walker (Lepidoptera: Pyralidae), rice striped stem borer. Comptes Rendus Biologies, 332: 633-641.

Table 1. α -Amylase activity of the the *Papilio demoleus*.

Organ	Total activity (U/min)	Total protein (mg)	Specific activity (U/mg protein)
Foregut	1.19 \pm 0.14	2.41	0.49 \pm 0.05
midgut	1.81 \pm 0.18	2.77	0.65 \pm 0.06
Hindgut	0.89 \pm 0.09	1.39	0.64 \pm 0.06

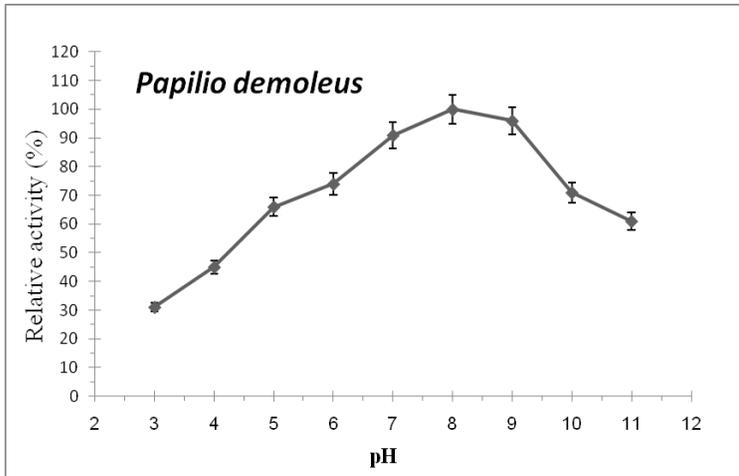


Figure 1. The effect of pH on the activity of the α -amylase activity of the *Papilio demoleus*.

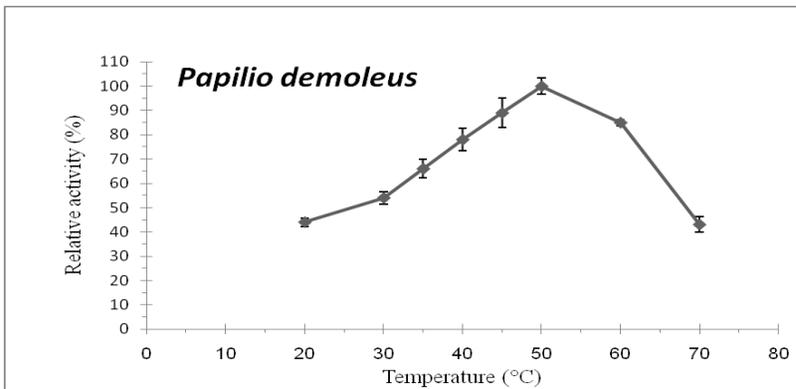


Figure 2. The effect of temperature on the α -amylase activity of the *Papilio demoleus*.

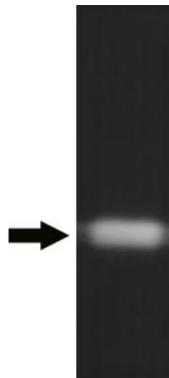


Figure 3. Native gel electrophoresis of α -amylase activity of the *Papilio demoleus*.