Biochemical Characterisation of α-Amylase in Two Aphid Species, *Aphis fabae* Scopoli (Hemiptera: Aphididae) and *A. gossypii* Glover (Hemiptera: Aphididae)

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Abstract

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We identify and characterise α-amylases of the two aphid species, *A. fabae* and *A. gossypi*. To do this, α-amylases of the two insect species were extracted and their activities were determined using 1% soluble starch. Results showed that α-amylase, which hydrolyses starch, is present in both aphids. Also, it was shown that optimum pH and temperature for the α-amylases of both species is 7.0 and 40°C, respectively. Gel assays using zymogram analysis showed that in both aphid species more than one isoform (two isoforms) of α-amylases hydrolyszing carbohydrates are present.

Keywords: aphids; gel assay; starch

Aphids in general and especially two aphid species, *A. fabae* Scopoli (Hemiptera: Aphididae) and *A. gossypii* Glover (Homoptera: Aphididae), are the best known pests of agricultural ecosystems, causing serious damage to many agronomical and horticultural plants (Birch 1985; Gubran *et al*. 1992). Both of them are extremely cosmopolitan and polyphagous species infesting many plant species worldwide. They are able to multiply at immense rate thus producing dense population on their host plants under favourable environmental condition.

Aphids have specialised mouthparts (piercing and sucking stylets) for extracting plant sap from vascular tissue. Plant sap is usually rich in sugar and deficient in nitrogen (Miles 1987; Douglas 2006). To meet their requirements, they need to extract a lot of plant sap and excrete the remaining as honeydew. As a result heavy yield loss can occur especially when infestation is high. Damage to plants occurs as a result of direct feeding and excretion of its honeydew which is rich in hydrocarbons and free amino acids, resulting in pathogenic and saprophytic fungal growth (Klingauf 1987). Aphids make use of symbiotic bacteria such as *Buchnera aphidicola* exploiting them to synthesise amino acids lacking in their diet (Gunduz & Douglas 2009). There is an old view that aphids as plant sap feeders are not capable of digesting the ingested materials. This view has been questioned because of the existence of both biochemical and molecular evidence suggesting that this insect also uses hydrolytic enzyme to digest the ingested materials (Foissac *et al*. 2002; Pyati *et al*. 2011). In addition to the free amino acids, phloem sap contains polymers such as lectins, proteins, and peptides that should be digested in the insect's alimentary canal before absorption (Kehr 2006).

Regarding protein digestion, it is reported that aphids use proteinases, especially cysteine proteinase, in this process. Cysteine proteinase (Cathepsin L) is the major protease in the gut of *A. gossypii*, *Acyrthosiphon pisum,* and *Sitobion avenae* (Cristofoletti *et al*. 2003; Deraison *et al*. 2004; Pyati *et al*. 2011). In addition to Cathepsin L, Cathepsin B is reported to be present in the aphid gut (Rispe *et al*. 2007). However, regarding carbohydrate digestion in the

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gut no studies have been undertaken. If digestion of food in the aphid gut happens, then the aphids, like the other species of chewing insects, are prone to enzyme inhibitors. Using enzyme inhibitors to control insect pest has already been demonstrated to be an important system for the insect pest control since these inhibitors have detrimental effects on the insect growth and development by interfering in food digestion (Confalonieri *et al*. 1998). α-Amylase activity has been described from different species of several insect orders including Orthoptera, Coleoptera, Hymenoptera, Diptera, Lepidoptera, and Hemiptera (Baker & Woo 1985; Terra *et al*. 1988; Mendiola-Olaya *et al*. 2000; Zeng & Cohen 2000; Oliveira-Neto *et al*. 2003; Kazzazi *et al*. 2005; Safaei-Khorram *et al*. 2010; Darvishzadeh & Bandani 2012). α-Amylases are important enzymes involved in carbohydrate metabolism in insects, thus α-amylase inhibitors should be used in the control of agricultural pest. For example pea and azuki transgenic plants expressing α-amylase inhibitors from beans were completely resistant to *Bruchus pisorum* and *Callosobrouchus chinensis* which are two main pests of stored pulses (Svensson *et al*. 1986; Morton *et al*. 2000; Carlini & Grossi-de-Sá 2002; Franco *et al*. 2002). Also, bioassay results of a study using artificial diet showed that protease inhibitors in pea aphid (*A. pisum*), cotton aphid (*A. gossypii*), and peach potato aphid (*Myzus persicae*) (Rahbe *et al*. 2003; Ribeiro *et al*. 2006) can produce antimetabolic effects (detrimental effect on growth and development and reduced fecundity).

The understanding of biochemistry and physiology of digestion is essential when developing methods of insect pest control using enzyme inhibitors. Thus, the aim of the current study was to extract α-amylase from the digestive system of two aphid species, *A. fabae* Scopoli and *A. gossypii* Glover, and determine its characteristics using a specific substrate for the enzyme. The knowledge thus achieved should lead to better understanding of digestive physiology of the two aphid species which could be used to devise new management strategies for their control.

MATERIAL AND METHODS

Insects. Aphid populations were reared separately on their host in the greenhouse. Black bean aphids were reared on beans and for rearing of cotton aphid cucumber was used (temperature 25 ± 1°C and humidity 60 ± 10%). After assimilating the age of adult, the insects were collected for further testing.

Sample preparation and enzyme assays. Adult insects (50 individuals) were homogenised in a pre-cooled homogeniser (Sigma/Aldrich, Dorset, UK) (Teflon pestle with 0.1 mm clearance) in distilled water, then homogenates were put in the 1.5 ml centrifuge tubes and centrifuged at 15 000 *g* at 4°C for 15 minutes. Supernatant was separated and kept at –20°C for subsequent analysis as an enzyme source.

α-Amylase activity. α-Amylase activity was assayed by the dinitrosalicylic acid (DNS) procedure (Bernfeld 1955), using 1% soluble starch (Merck, Darmstad, Germany) solution as substrate as described by Bandani *et al*. (2009). A blank without substrate but with α-amylase extract and a control containing no α-amylase extract with substrate were run simultaneously with reaction mixture. All assays were performed in triplicates and with three time repetitions.

Effect of pH and temperature on the aphids' α-amylase activity. The effects of temperature and pH on α-amylase activity were examined using enzyme extract from the aphids as described by Kazzazi *et al*. (2005). Optimal pH was determined by using universal buffer (0.02M) (citrate, phosphate, and borate) with pH set at 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12. The effect of temperature on α-amylase activity was determined by pre-incubating of the reaction mixture at 10, 20, 25, 30, 35, 40, 50, and 60°C for 30 min followed by measurement of α-amylase activity as described previously.

Effect of activators and inhibitors on α-amylase activity. To test the effect of different ions on the enzyme activity, assays were performed in the presence of different concentrations of NaCl (5, 10, 20, and 40 mmol/l), KCl (5, 10, 20, and 40 mmol/l), $CaCl_2$ (5, 10, 20, and 40 mmol/l), and $MgCl_2$ (5, 10, 20, and 40 mmol/l) as described by Kazzazi *et al*. (2005). These compounds were added to the assay mixture, then incubation was done at 30°C for 30 min, and then absorbance was read like before. Also, suitable blanks were included in all enzyme assays.

Electrophoresis of α-amylase enzyme. The amylase present in crude homogenates after SDS polyacrylamide gel electrophoresis (PAGE) was visualised using

Table 1. Activity of α-amylase of two aphid species, *A. fabae* and *A. gossypii*

Aphid species (*n*)	Activity (U/ml) (mean ± SE)
A. faba	1.246 ± 0.0028
A. gossypii	1.225 ± 0.0045

n = 50 individuals

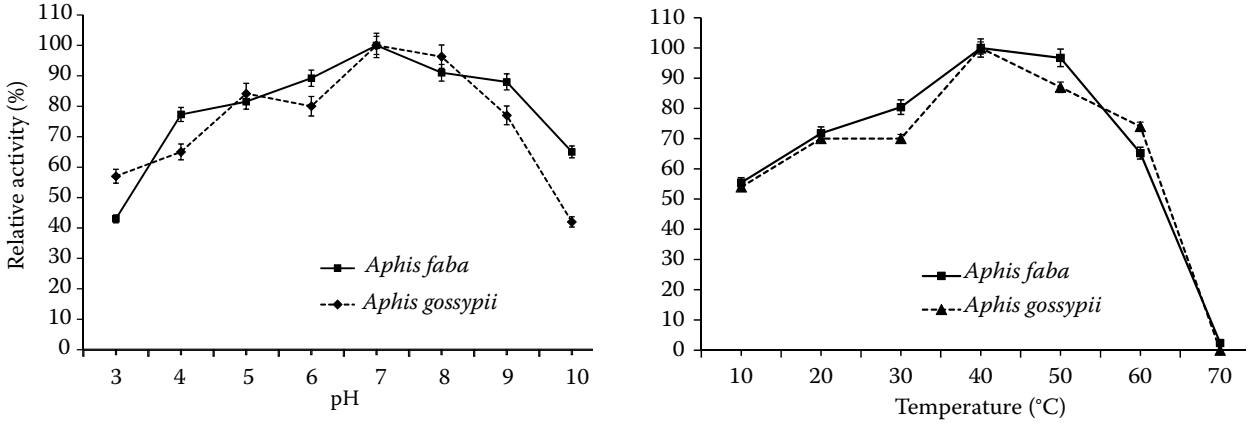

Figure 1. Effect of pH and temperature (°C) on the α-amylase activity of two aphid species, *A. fabae* and *A. gossypii*

the procedure described by Laemmli (1970) and Mehrabadi *et al.* (2010). Briefly, 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 100 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 shaking gently with 1% (v/v) Triton X-100 in phosphate buffer containing 2 mmol/l $CaCl_2$ and 10 mmol/l NaCl for 1.5 hours. Then the gel was placed in a solution of 1% starch for about an hour. Finally, the gel was rinsed with distilled water and treated with a solution of 1.3% I_2 and 3% KI to stop the reaction and stain the unreacted starch background. Zones of α-amylase activities appeared as a light band in a dark background (Mehrabadi *et al.* 2010).

Protein determination. Protein concentration was measured according to the method of Bradford (1976), using bovine serum albomin (Bio-rad, Hertfordshire, UK) as a standard.

RESULTS

α-Amylase activity. Studies showed that α-amylase enzyme is present in the two aphid species, *A. fabae* and *A. gossypii.* The activity of the enzyme was 1.25 U/ml for *A. fabae* and 1.23 U/ml for *A. gossypii*. As can be seen in Table 1, the activity of the amylase in *A. gossypii* is slightly higher than in *A. fabae*.

Effect of pH and temperature on α-amylase activity. The optimal pH for α-amylase activity in both aphid species, *A. fabae* and *A. gossypii*, has shown to be 7.0 (Figure 1). The enzyme activity in both species increased constantly from pH 3.0 to 7.0 and then decreased. Amylase was active over a broad range of temperatures from 10 up to 50°C. The optimal temperature for α-amylase activity was 40°C (Figure 1). However, after 50°C the amylase activity in both species was reduced, and steeply reached zero at 70°C.

Effect of activators and inhibitors on enzyme activity. The chemicals tested in the present study had different effects on the activity of α-amylase in the two aphid species, *A. fabae* and *A. gossypii* (Figure 2). Some chemicals such as $MgCl_2$ and NaCl did not significantly stimulate amylase activity in the species. However, high concentration of $CaCl_2$ caused a considerable reduction of their enzyme activity. KCl, on the other hand, prominently affected the α-amylase activity (Figure 2) increasing it almost linearly.

Zymogram analysis of α-amylase activity. Zymogram (Native page) analysis of α-amylase of the two aphid species, *A. fabae* and *A. gossypii*, by vertical slab gel electrophoresis proved the presence of α-amylase activity in both aphid species (Figure 3). Two different α-amylase bands with the same relative motilities were present in both species showing the importance of α-amylase in the insect's biology.

DISCUSSION

This is the first study bringing evidence that aphid species do possess carbohydrases, especially α-amylase enzyme for digestion of nutrient ingesting during feeding. It has already been reported that aphids use proteinases (endoprotease) in order to digest peptides and proteins ingested by the insect (Cristofoletti *et al.* 2003; Pyati *et al.* 2011). The phloem sap of the plants on which the aphids feed reportedly contains various molecules including proteins, small peptides, some enzymes, digestive enzyme inhibitors, and lec-

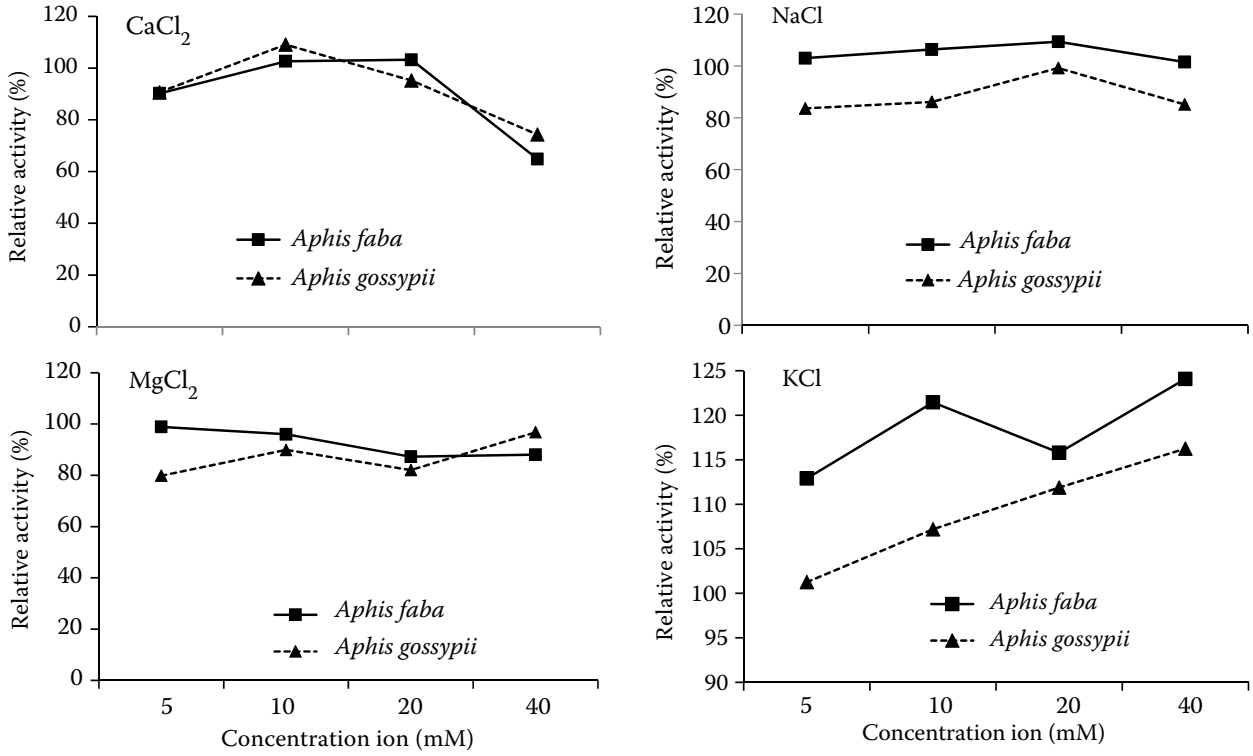

Figure 2. Effect of $CaCl_2$, NaCl, $MgCl_2$, and KCl on the activity of α-amylase of two aphid species, *A. fabae* and *A. gossypii*

tins (Kehr 2006). These molecules could be used by the aphids as potential nitrogen source. So, the old view that the aphids do not carry out hydrolysis of the ingested material inside the gut is no more valid (Pyati *et al.* 2011). Major protease that the aphids use to digest proteins in the gut seems to be derived from both groups of cysteine and serine proteinases (Cristofoletti *et al.* 2003; Rahbe *et al.* 2003; Pyati *et al.* 2011), although cysteine proteinases are likely to be more widespread (Terra & Ferreira 2005).

Bioassays showed that protease inhibitors incorporated in the artificial diet of the aphids produced anti-aphid effects. For example oryzacystatin I, which is a cysteine proteinase inhibitor obtained from rice, when added to artificial diet of the aphid species including *Myzus persicae, A. gossypii*, and *A. pisum*, produced antimetabolic effect in the aphids (Rahbe *et al.* 2003). This is in agreement with that the cathepsin-like proteinases are active in the gut of the aphids. Also serine protease inhibitors from pea seeds cause an anti-aphid effect on pea aphid (*A. pisum*) (Rahbe *et al.* 2003). Thus, this is in agreement with the reports that in aphids, in addition to cysteine proteinases, serine proteinases are present.

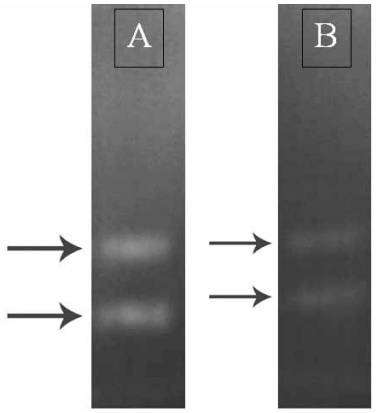

Figure 3. Native-PAGE gel electrophoresis of α-amylase of two aphid species, *A. fabae* (A) and *A. gossypii* (B)

In the current study proved that in the two aphid species, *A. gossypii* and *A. fabae*, α-amylases are present. α-Amylases activity was assayed in rose aphid (*Macrosiphum rosae*) by Darvishzadeh and Bandani (2012). Interestingly, α-amylases are present in two different isoforms (isoenzymes) showing importance of the amylase in the insect survival and development. Thus, as the demand for a higher carbohydrate digestion increased, the number of the carbohydrate digesting isoenzymes increased to meet it. Generally speaking, it has been hypothesised that the high content of starch or glycogen in the insect diet indicates the presence of the corresponding digestive enzymes in the alimentary canal of the insect (Applebaum *et al.* 1961; Bandani *et al.* 2001). The

presence of a number of isoenzymes in the alimentary canal of the insect is a strategy of how to suppress the inhibitory activity of the digestive enzyme inhibitor/s present in the diet in nature (Silva *et al.* 1999). It is interesting to note that optimum pH for the α-amylase activity was neutral (7.0) which is corresponding to optimum pH of proteinase in the aphid gut (Pyati *et al*. 2011).

Some compounds such as $MgCl_2$ and $CaCl_2$ had inhibitory effect on the aphids' α-amylase activity. The authors showed that NaCl stimulated the α-amylase activity in *Eurygaster integriceps*, however in the present study NaCl did not activate α-amylase of the two aphid species, the same as in some other insects, e.g. *Callosobruchus chinnesis* and *Bombyx mori* (Terra *et al*. 1996). KCl is the only compound that has stimulated α-amylase activity of both aphid species which is in agreement with data given by Kazzazi *et al*. (2005) who showed that α-amylase activity of the *Eurygaster integriceps* is stimulated by KCl. In conclusion, the presence of α-amylase activity as well as the existence of isoforms in the aphids' bodies indicate that this insect exploits α-amylase for the digestion of carbohydrates. In addition it was found out that α-amylases of these aphid species have almost the same characteristics as in the case of the other hemipterans (*Eurygaster integriceps*).

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