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ORIGINAL ARTICLE

Isolation and purification of α-amylase inhibitors and their *in vitro* and *in vivo* effects on *Tribolium castaneum* (Herbst) and *Callosobruchus maculatus* (F.)

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Abstract

Plant derived α-amylase inhibitors are proteinaceous molecules that regulate the enzyme activity in plants and also protect plants from insect attack. In the current study, 28 accessions of 19 plant species were screened for their α-amylase inhibitory activity. The durum wheat varieties, Beni Suef-1 and Beni Suef-5, showed strong α-amylase inhibitory activity and were subjected to further purification studies using ammonium sulfate fractionation and DEAE-Sephadex G-25 column. The isolated inhibitors were found to be stable at temperatures below 80°C with maximum activity obtained at 40–50°C. Also, they were stable in a wide pH range (2–12). The ion exchange products of purified α-amylase inhibitors from Beni Suef-1 and Beni Suef-5 varieties showed a molecular weight of 16 and 24 kDa, respectively. The purified α-amylase inhibitors were tested against *Tribolium castaneum* and *Callosobruchus maculatus* both *in vitro* and *in vivo.* There was linear inhibition of α-amylase activity with increasing inhibitor concentration until saturation was reached. Beni Suef-5 α-amylase inhibitor was more potent against α-amylase with lower IC_{50} values than Beni Suef-1 α-amylase inhibitor except in the case of *T. castaneum* larva. Kinetics analysis revealed that Beni Suef-1 and Beni Suef-5 α-amylase inhibitors are non-competitive types of inhibitors with high affinity toward α-amylase of *T. castaneum* and *C. maculatus.* Results of the *in vivo* studies demonstrated that α-amylase inhibitors isolated from durum wheat, Beni Suef-1 and Beni Suef-5 varieties, were very effective in inhibiting the development of *T. castaneum* and *C. maculatus* and could be used for future studies in developing insect resistant transgenic plants approaching α-amylase inhibitor genes.

Keywords: α-amylase, α-amylase inhibitors, durum wheat, *Callosobruchus maculatus, Tribolium castaneum*

Introduction

To regulate their enzyme activity, living organisms have created a large number of proteinaceous molecules that inhibit enzyme activities, known as enzyme inhibitors. In plants, in addition to their function as regulators of enzymes, inhibitors are believed to make plants less prefered and even lethal to insects (Sasikiran *et al.* 2002). Proteinaceous α-amylase inhibitors are a group of protenious inhibitors that bind to and inactivate α-amylase enzymes. In plants, α-amylase inhibitors are known to be found in seeds and tubers particularly in cereals and legumes where they act as regulators of endogenous enzymes and also as a defense mechanism against insects (Sasikiran *et al.* 2002). Plant derived α -amylase inhibitors have proven to be an effective biological mechanism for insect control (Mehrabadi *et al.* 2011; Neeta and Kamble 2016; Yadav *et al.* 2018).

The red flour beetle, *Tribolium castaneum,* and the cowpea weevil, *Callosobruchus maculatus,* are the most important pests responsible for severe losses of stored products, affecting their quantity and quality (Franco *et al.* 2000). Coleopteran insects, including *T. castaneum* and *C. maculatus,* rely mainly on α-amylase enzymes (Pereira *et al.* 1999). Plant derived α-amylase inhibitors were found to have *in vitro* and *in vivo* antimetabolic effects on *T. castaneum* (Chen *et al*. 1992; Tatun *et al.* 2014; Kasar *et al.* 2017) and *C. maculatus* (Farias *et al.* 2007; Wisessing *et al.* 2008; Gupta *et al.* 2014).

There is a growing interest in generating insect--resistant transgenic plants based on alpha amylase inhibitors (Franco *et al.* 2000), however the first step to develop such transgenic plants is finding new classes of α-amylase inhibitors and determining their specificity.

In the current study the biopotency of α-amylase inhibitors isolated from different plant accessions/ cultivars was evaluted against α-amylase from *T. castaneum* and *C. maculatus,* both *in vitro* and *in vivo.*

Materials and Methods

Collection of plant seeds

Seeds of 28 accessions of 19 plant species (Table 1) were obtained from the Faculty of Agriculture, Sohag University and Agricultural Research Center, Egypt.

Insect cultures

Tribolium castaneum (Coleoptera: Tenebrionidae) and *Callosobruchus maculatus* (Coleoptera: Chrysomelidea) populations were obtained from the cultures maintained at the Insect Physiology Laboratory, Faculty of Agriculture, Sohag University. Wheat grains and cowpea seeds were used to maintain *T. castaneum* and *C. maculatus*, respectively. The insects were kept in an incubator at 25 ± 2°C, 50–70% relative humidity (R.H.) and a photoperiod of 14 : 10/D : L h.

Extraction and purification of α-amylase inhibitors from seed powder (crude extract)

Crude extracts of selected seeds were obtained according to Baker (1987) with some modification. Finely ground seeds were defatted for 2 h with ice-cold acetone, filtered and then the powders were air-dried overnight. The defatted powders were extracted in 0.01 M sodium phosphate buffer (1 : 10 w/v), pH 7 containing 0.15 M NaCl. The buffer containing the powder was stirred for 2 h and kept for extraction with intermittent shaking at 4°C overnight, taking care that no foam was formed. The homogenate was then centrifuged at 10,000 rpm for 20 min at 4°C. The pellet was discarded and clear supernatant (crude extract) was collected and its α-amylase inhibitory potency against standard α-amylase enzymes was evaluated.

The crude extracts of durum wheat, Beni Suef-1 and Beni Suef-5 varieties, showed high inhibition activity against α-amylase for which they were subjected to ammonium sulfate fractionation at concentrations of 0–30, 30–60 and 60–90% under cold conditions. At each concentration, the α-amylase inhibitory activity and the protein content of each fraction were estimated. As the fraction F_{60-90} which corresponds to 60–90 saturation range showed the highest α-amylase inhibitory activity it was dialyzed and eluted by ion exchange chromatography in a column (50 × 1.2 cm, L × Dia) packed with DEAE-Sephadex G-25.

The selected ammonium sulfate fraction was dissolved in 15 ml of 20 mM Tris-HCl buffer, pH 8.0 and centrifuged. The clear supernatant was applied to the column and a flow rate of 15 ml · hr^{-1} was adjusted. Elution of unbound materials was carried out with 20 mM Tris-HCl buffer, pH 8 and then a gradient of (0–0.4) mM NaCl in 20 mM Tris-HCl, pH 8.0 was used to elute the bounded materials. The collected fractions (5 ml each) were tested for their α-amylase inhibition activities and the protein content was measured at 280 nm. Fractions which showed the highest α-amylase inhibition activities were separated.

Determination of α-amylase inhibition activity

The α-amylase inhibition activity was measured against standard α-amylase enzyme by determination of the reducing sugar (maltose equivalent) liberated under quantifying assay conditions. Different volumes of inhibitor crude extracts were pre-incubated with 25 µl of α-amylase (13 unit · mg^{-1}) at 37°C for 30 min before 400 µl of 1% starch solution dissolved in phosphate buffer 0.1 M (mol · l^{-1}), pH 6.9 containing 20 mM NaCl, 0.1 mM $CaCl_2$ was added. Themixture was then incubated for 30 min and the reaction was stopped by the addition of 250 µl dinitrosalycilic acid (DNSA) reagent (1 g DNSA, 30 gm sodium potassium tartarat and 20 ml of 2 N NaOH (w/v) dissolved in 100 ml distilled water and prepared fresh). Then the contents were heated in a boiling water bath for 10 min. The reaction mixture was cooled and diluted with 3 ml distilled water, and then the absorbance was recorded at 540 nm using UV/VIS-single beam spectrophotometer (Sigma). The control was set without inhibitor crude extract. All assays were repeated thrice. The reducing sugar released from starch was estimated as maltose equivalent from

Table 1. Alpha-amylase inhibitory specific activity and protein content of the tested germplasm accessions

No.	Accessions	AIU · g^{-1} seed	Protein content [mg · g^{-1} seed]	AIU · mg^{-1} protein
1	Barley (wild)	154.94	8.90	17.40
2	Castor oil plant (wild)	37.37	69.73	0.53
3	Chick pea	70.78	24.90	2.84
4	Cotton (Giza)	34.18	34.40	0.99
5	Cowpea IT81D1064	42.06	49.73	0.84
6	Cowpea IT93K2045-20	202.59	59.31	3.41
7	Drumstick tree	58.45	65.15	0.89
8	Durum wheat (Beni Suef-1)	12,467.07	28.73	433.94
9	Durum wheat (Beni Suef -5)	490.46	3.56	137.77
10	Faba bean (Masr 3)	36.13	18.24	1.98
11	Kidney bean (local)	84.52	37.42	2.25
12	Kidney bean (Hindi)	118.80	48.00	2.47
13	Kidney bean (Holand)	173.91	73.06	2.38
14	Kidney bean (Lima)	92.70	36.90	2.51
15	Kidney bean (yolosta)	38.595	89.98	0.42
16	Maize D333	86.49	22.65	3.81
17	Maize (wild)	150.27	6.06	24.77
18	Maize)Hi teck)	38.86	5.15	7.54
19	Maize D3444	41.69	22.06	1.88
20	Millet (wild)	38.26	11.56	3.30
21	Nalta jute	54.68	57.81	0.94
22	Parsley	50.96	58.65	0.86
23	Pea (Master B)	56.34	60.73	0.92
24	Pepper	39.02	37.32	1.04
25	Sorghum	34.64	6.90	5.02
26	Soybean (wild)	33.53	87.23	0.38
27	Sudan grass	72.29	237.32	0.30
28	Sweet corn (wild)	59.91	3.48	17.21

AIU = α-amylase inhibitory unit

the standard graph. One unit of α-amylase inhibitor was defined as the amount of -amylase inhibitor which inhibits 50% of α-amylase activity.

Protein determination

The protein content of inhibitor crude extracts of *T. castaneum* and *C. maculatus* was determined by Lowery's method (Lowery *et al.* 1951), using bovine serum albumin fraction V (Sigma) as the standard.

Characterization of α-amylase inhibitors

Thermal and pH stability

Thermal stability of the selected inhibitors was studied by incubating the inhibitors in a water bath at different temperatures, ranging from 30 to 100°C. After incubation, the inhibitor samples were cooled at 4°C for 10 min and centrifuged at 10,000 rpm for 10 min at 4°C. The α-amylase inhibitory activity of the treated inhibitors was determined as described above.

The effect of pH on the inhibitory activities of the selected α-amylase inhibitors was studied at different pH ranges (from 2–12) using the following buffers: a glycine-hydrochloric buffer for pH 2.0, a citrate-phosphate buffer for pH 3.0–5.0, a phosphate buffer for pH 6.0–8.0 and a glycine-NaOH buffer for pH 9.0–11.0 and potassium chloride-NaOH for pH 12.0 (Baker 1983). The purified α-amylase inhibitors were incubated in the buffer solution (pH 2–12) for 2 h at room temperature and the residual α-amylase inhibitory activities were measured as described earlier. All experiments were carried out in triplicate.

Determination of the molecular weight of α-amylase inhibitors protein

Molecular weight of α-amylase inhibitors was determined on discontinuous polyacrylamide SDS gel electrophoresis (SDS-PAGE) using a 4% stacking gel and a 10% resolving gel. Standard PUReGeNe genetix molecular weight protein markers employed β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35.5 kDa), restriction endonuclease Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa) were loaded to calculate the molecular weight of unknown proteins.

Preparation of larval gut homogenate

The method of Cohen (1993) was used for the extraction of α-amylase from *T. castaneum* and *C. maculatus*, with some modifications. The larvae and adults of both insect species were separately weighed and homogenized in 1 ml of cooled sodium acetate buffer, 20 mM, pH 7 containing (10 mM NaCl, and 20 mM $CaCl_2$) using a pre-cooled homogenizer. After centrifugation at 10,000 rpm at 4°C for 20 min, the supernatants were collected and transferred to a new tube and stored at –20°C for further use.

Inhibitory potential and kinetics of α-amylase inhibitors against α-amylase from *T. castaneum* and *C. maculatus*

To determine the IC_{50} (concentration of inhibitor required for 50% inhibition) values, different concentrations from each inhibitor (ammonium sulfate fraction) were used against α-amylase extracts of larvae and adults of both insects. Inhibitors were incubated with α-amylase crude extracts for 20 min at 37°C before addition of the substrate (10% starch) and the remaining α-amylase activity was measured as described above. Results were expressed as IC_{50}.

Lineweaver-Burk plots were used to determine the mechanism of inhibition against α-amylase of *T. castaneum* and *C. maculatus* (competitive or non-competitive) using different substrate concentrations (1%, 0.5% and 0.25% starch) in the absence and in the presence of an inhibitor. The inverse of enzyme activity was plotted versus the inverse of substrate concentration and the maximum velocity (V_{max}), Michaelis constant (K_m) and K_i were calculated.

In vivo effects of α-amylase inhibitors on larvae of *T. castaneum* and *C. maculatus*

Tribolium castaneum

Ammonium sulfate saturated fractions of the selected α-amylase inhibitors were incorporated with wheat flour at concentrations of 0.25, 0.5 and 0.1% (w/w) and kept in plastic Petri dishes. For each concentration, 10 newly hatched larvae of *T. castaneum* were introduced into the Petri dishes and kept under incubator conditions in the dark to maintain temperature and humidity. The bioassays were completely randomized with three replicates. The larvae were observed until 40 days after treatment and the mean larval weight, larval mortality, number of pupa and number of emerged adults were recorded at 2 day intervals.

Callosobruchus maculatus

To study the *in vivo* effect of α-amylase inhibitors on *C. maculatus* larvae, artificial seeds were prepared as described by Macedo *et al.* (1993). Ammonium sulfate saturated fractions of the selected α-amylase inhibitors were incorporated with chickpea flour at concentrations of 0.25, 0.5 and 0.1% (w/w) and the mixture was conditioned at 28°C and 70% R.H. for 3 days, and then used to completely fill gelatinous capsules (0.5 gm per capsules). The filled capsules were kept for 48 h in a growth chamber and 2–3 day old fertilized females were presented in each treatment for oviposition and incubated for 3 days at 27°C and 70% R.H. The excess eggs were removed from the artificial seeds and only 3 eggs/seed were left. Five replicates were performed for each concentration. Artificial seeds carrying the eggs were kept in the incubator under the same conditions and after 20 days, the artificial seeds were broken apart and the number and mean weight of any surviving larvae, pupa and/or adults found in each capsule were recorded.

Statistical analysis

Analysis of variance (ANOVA) was performed and the differences between treatments of the studied parameters (mean weight and mortality) were determined by least significant difference (LSD) at $p \leq 0.05\%$ level of significance.

Results

Alpha-amylase inhibitor activity

The α-amylase inhibitory activity of 28 accessions/cultivars belonging to 19 plant species was determined against standard α-amylase enzyme and presented in Table 1. All the accessions tested were found to have α-amylase inhibitory activity with wide inter-varietal variation. High α-amylase inhibitory activities of 433.94 and 137.77 AIU · mg^{-1} protein were recorded for durum wheat Beni Suef-1 and Beni Suef-5 varieties, respectively. Moderate α-amylase inhibitory activity was observed for maize (wild) (24.77 AIU · mg^{-1}

Table 2. Purification steps of α-amylase inhibitors isolated from durum wheat, Beni Suef-1 and Beni Suef-5 varieties

Inhibitor	Purification step	Total soluble protein [mg]	AIU	AIU · mg^{-1} protein	Recovery [%]	Fold of purification
Durum wheat (Beni Suef-1)	Crude extract	240.33	12,689	52.82	100	1.00
	$F_{60–90}$ $(NH_4)_2SO_4$ ppt	130.52	7,812	59.85	61.56	1.13
	DEAD Sephadex PI	24.32	4,409	181.29	34.73	3.43
	PII	28.62	5,912	206.57	46.59	3.91
Durum wheat (Beni Suef-5)	Crude extract	98.62	3,227	32.72	100	1.00
	$F_{60–90}$ $(NH_4)_2SO_4$ ppt	73.12	2,567	35.11	79.55	1.07
	DEAD Sephadex PI	22.57	1,380	61.14	42.76	1.86

AIU = α-amylase inhibitory unit; PI = first peak; PII = second peak

protein), barley (wild) (17.40 AIU · mg^{-1} protein) and sweet corn (wild) (17.21 AIU · mg^{-1} protein) while other accessions showed low α-amylase inhibitory activity. Based on these results, the durum wheat Beni Suef-1 and Beni Suef-5 varieties were selected for further purification steps.

Purification of α-amylase inhibitors

The crude extracts of durum wheat Beni Suef-1 and Beni Suef-5 were subjected to the ammonium sulfate precipitation step. The fraction $F_{60–90}$ which corresponds to 60–90% saturation range was found to be efficient for precipitating the α-amylase inhibitor in both varieties compared to other fractions for which it was then applied to DEAE-Sephadex G-25 column. In the ammonium sulfate precipitation step, 61.56% recovery and 1.13 fold of purification were obtained in the case of Beni Suef-1 variety while, 79.55% recovery and 1.07 fold of purification were obtained in the case of Beni Suef-5 variety (Table 2). The protein elution profile of Beni Suef-1 α-amylase inhibitor revealed that DEAE-Sephadex G-50 column yielded two peaks, PI and PII with 3.43 and 3.91 fold of purification, respectively, compared to the crude extract (Fig. 1). On the other hand, the DEAE-Sephadex G-50 column yielded only a single peak, PI with 1.86 fold of purification compared to the crude extract in the case of Beni Suef-5 variety (Fig. 2).

Thermal and pH stability

Figure 3 illustrates the thermal stability of α-amylase inhibitors isolated from durum wheat, Beni Suef-1 and Beni Suef-5 varieties. Beni Suef-1 α-amylase inhibitor was stable at temperatures below 80°C with maximum inhibition activity obtained at 40°C, however the inhibitor lost about 39% of its activity at 90°C and about 75% at 100°C. In the same way, Beni Suef-5 inhibitor was stable at temperatures below 80°C with maximum inhibition activity obtained at

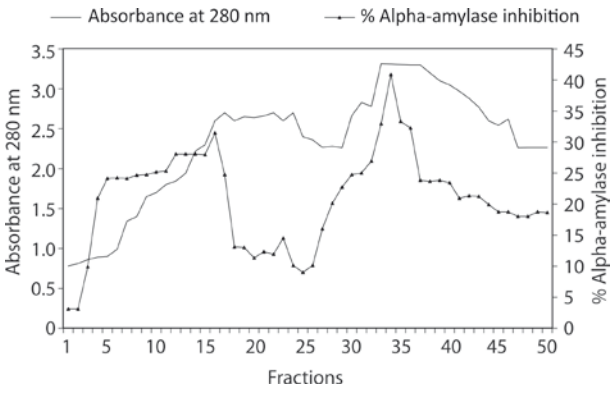

Fig. 1. Elution profile of DEAE-Sephadex G-50 of F60-90 from seeds of the durum wheat, Beni Suef-1 variety

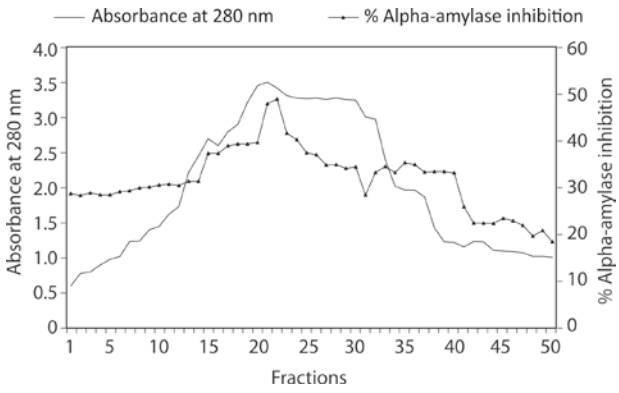

Fig. 2. Elution profile of DEAE-Sephadex G-50 of F60-90 from seeds of the durum wheat, Beni Suef-5 variety

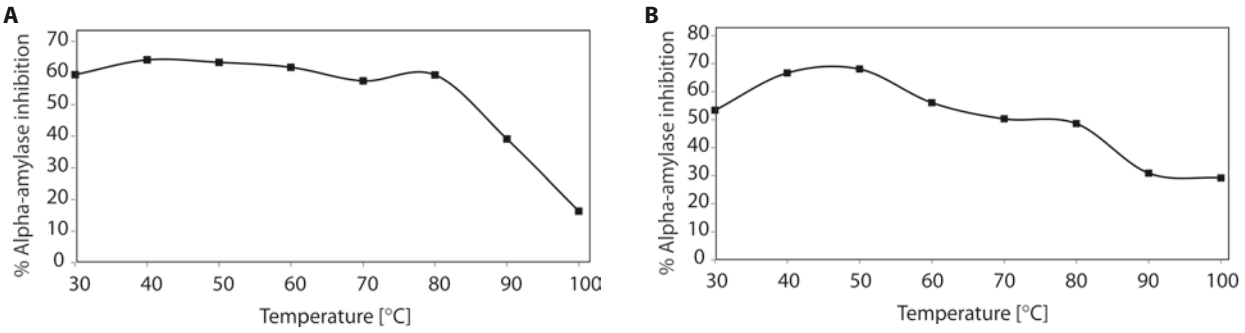

Fig. 3. Thermal stability profile of the Beni Suef-1 (A) and Beni Suef-5 (B) α-amylase enzyme activity

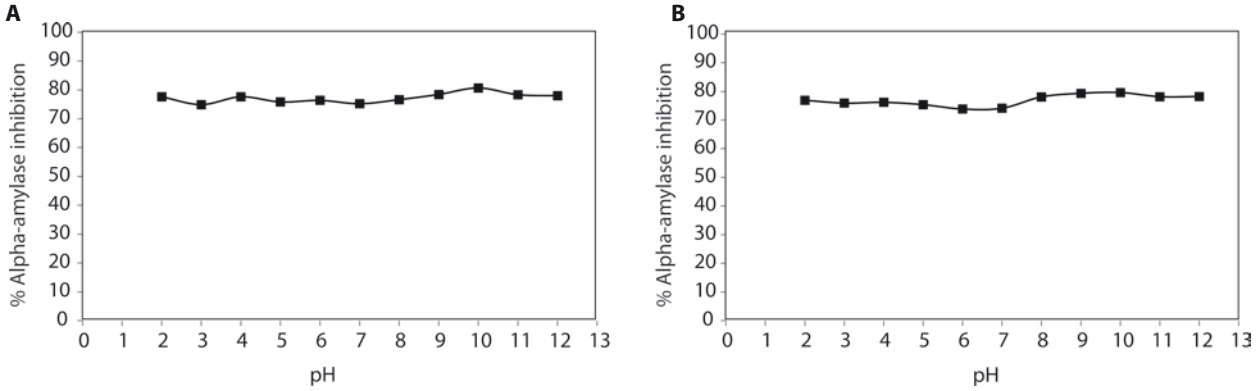

Fig. 4. pH stability profile of the Beni Suef-1 (A) and Beni Suef-5 (B) α-amylase inhibitors against α-amylase enzyme activity

50°C however the inhibitor lost about 55% of its activity at 90°C.

The stability of Beni Suef-1 and Beni Suef-5 α-amylase inhibitors at pH ranging from 2 to 12 is presented in Figure 4. Both inhibitors were almost stable at all the pH ranges without any significant changes in their inhibition activity.

Molecular weights of α-amylase inhibitors

The $F_{60-90}(NH_4)_2SO_4$ fractions and DEAE-Sephadex products of α-amylase inhibitor proteins isolated from durum wheat, Beni Suef-1 and Beni Suef-5 varieties, were resolved in 10% SDS-PAGE (Fig. 5). The ammonium sulfate fraction (F_{60-90}) of Beni Suef-1 variety resolved into five bands with molecular weights ranging from 45 to 16 kDa. The first peak (PI) of the DEAE-Sephadex was resolved in a two major protein bands of 16 and 24 kDa and a light band of 52 kDa could also be noticed, while PII was resolved into 2 protein bands of 24 and 16 kDa. In the case of Beni Suef-5 variety, the ammonium sulfate fraction (F_{60-90}) was resolved into seven protein bands with molecular weights ranging from 52 to 16 kDa. The DEAE--Sephadex product, PI was resolved into a single protein band of 16 kDa.

Inhibitory potential and kinetics of α-amylase inhibitors against α-amylase from *Tribolium castaneum* and *Callosobruchus maculatus*

Different concentrations of Beni Suef-1 and Beni Suef-5 α-amylase inhibitors were used to determine the IC_{50} values against α-amylase of larval and adult stages of *T. castaneum* and *C. maculatus*. Results revealed linear inhibition of α-amylase activity with increasing inhibitor concentrations until saturation was reached. Beni Suef-5 α-amylase inhibitor was more potent against α-amylase with lower IC_{50} values (Table 3) than Beni Suef-1 α-amylase inhibitor except in the case of *T. castaneum* larva. Beni Suef-1 and Beni Suef-5 α-amylase inhibitors were more potent against α-amylase of adult stages than α-amylase of larval stages in both insect species.

Kinetic analysis of α-amylase activity gave line reciprocal Michaelis-Menton (Lineweaver-Burk) plots, which enable the estimation of K_m and V_{max} values (Table 3). A decrease in V_{max} values with no change in K_m values was observed when α-amylase inhibitors were added to the reaction compared to the reaction in the absence of inhibitor which indicate that Beni Suef-1 and Beni Suef-5 α-amylase inhibitors are non-competitive types of inhibitors.

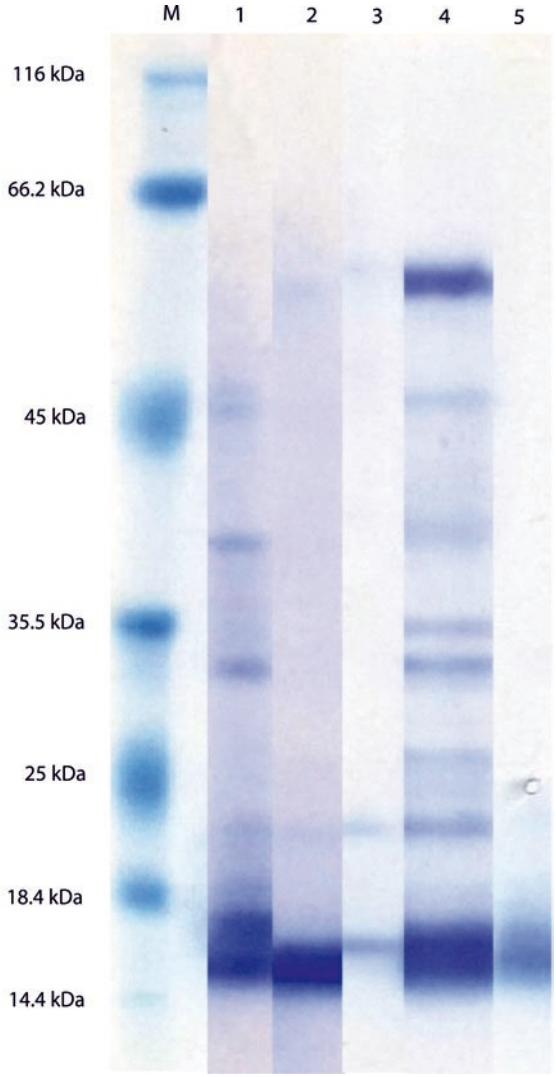

Fig. 5. SDS-PAGE analysis of durum wheat, Beni Suef-1 and Beni Suef-5 α-amylase inhibitors fractions, stained with coomassie blue. M = molecular weight marker; 1 = Beni Suef-1 $F_{60\text{-}90}$ fraction; 2 = Beni Suef-1 PI; 3 = Beni Suef-1 PII; 4 = Beni Suef-5 $F_{60\text{-}90}$ fraction; 5 = Beni Suef-5 PI

Beni Suef-1 and Beni Suef-5 α-amylase inhibitors have higher affinity (with lower K_i values) towards α-amylase of *T. castaneum* than that of *C. maculatus*. The affinity of Beni Suef-5 α-amylase inhibitor towards α-amylase of *C. maculatus* was higher than that of Beni Suef-1 α-amylase inhibitor. However, the affinity of both inhibitors towards α-amylase of *T. castaneum* was equal.

In vivo effects of α-amylase inhibitors on *Tribolium castaneum* and *Callosobruchus maculatus*

Tribolium castaneum

The antimetabolic effects of the α-amylase inhibitors, Beni Suef-1 and Beni Suef-5 have been assessed against *T. castaneum* by integrating the $F_{60-90}(NH_4)_2SO_4$ proteins into the artificial diet at different concentrations. The mean larval weight, larval mortality, number of pupa and number of emerged adults were recorded at intervals of 2 days up to 40 days after treatment (Table 4).

Larvae fed diets containing α-amylase inhibitors showed a noticeable reduction in the mean larval weight 2 days after treatment compared to the control larvae. Beni Suef-5 α-amylase inhibitor caused a higher reduction in the mean larval weigh than Beni Suef-1 α-amylase inhibitor at all concentrations. However, no significant differences were observed between the concentrations of the same inhibitor.

Low larval mortality, ranging from 10–20%, was observed 2 days after treatment in larvae with Beni Suef-1 α-amylase inhibitor while 50% mortality was observed in the larvae treated with Beni Suef-5 α-amylase inhibitor. At the end of the experiment (day 40) the larval mortality ranged from 40–50% in the case of Beni Suef-1 α-amylase inhibitor and 70–90% in the case of Beni Suef-5 α-amylase inhibitor. No mortality was recorded in the control larvae.

At the end of the experiment, 10 adults emerged in the control treatment compared to 4–6 adults and 1–3 adults in, Beni Suef-1 and Beni Suef-5 inhibitors, respectively.

Callosobruchus maculatus

The *in vivo* effect of Beni Suef-1 and Beni Suef-5 α-amylase inhibitors on *C. maculatus* was evaluated by incorporating the inhibitors into the diet using artificial seeds. Twenty days after treatment, the artificial seeds were broken and the number, mean weights of surviving and dead larva, pupa or/and adult found were recorded and presented in Table 5.

In the control treatment, only the larval stage was observed with no mortality. However, pupal and adult stages were observed in Beni Suef-1 and Beni Suef-5 α-amylase inhibitor treatments. In the Beni Suef-1 α-amylase inhibitor treatment all the larvae and adults found were dead except for three live larvae which were observed at the highest concentration of 1 : 1. The larval mortality was less in Beni Suef-5 α-amylase inhibitor treatment and only one live pupa and one live adult were recorded. There was a slight decrease in the number and mean weight of surviving larvae of Beni Suef-5 α-amylase inhibitor treatment compared to the control. The overall observations revealed that Beni Suef-1 α-amylase inhibitor was more potent against *C. maculatus* than Beni Suef-5 α-amylase inhibitor.

Discussion

Alpha-amylases are important digestive enzymes which act in the first phase of digestion of maltopolysaccharides (Da Lage 2018). The activity of α-amylase

Table 3. Kinetic analysis of α-amylase of larval and adult stages of *Tribolium castaneum* and *Callosobruchus maculatus* against α-amylase inhibitors

Insect species	Stage	α-amylase inhibitors	V_{max} [µmol maltose released · · min^{-1} · mg protein^{-1}]	K_m [mM]	K_i [µg]	IC_{50} [µg · ml^{-1}]
T. castaneum	larva	without inhibitor	0.2958	0.53		
		Beni Suef-1	0.1923	0.53	0.03	5.42
		Beni Suef-5	0.2000	0.53	0.03	12.35
	adult	without inhibitor	0.4338	0.66		
		Beni Suef-1	0.1786	0.66	0.04	2.66
		Beni Suef-5	0.2083	0.66	0.04	0.35
C. maculatus	larva	without inhibitor	0.4435	0.83		
		Beni Suef-1	0.4166	0.83	0.78	40.68
		Beni Suef-5	0.4232	0.83	0.12	2.29
	adult	without inhibitor	0.4103	0.67		
		Beni Suef-1	0.238	0.67	0.91	19.45
		Beni Suef-5	0.2325	0.67	0.26	2.11

V_{max} = maximum velocity; K_m = Michaelis constant; K_i = inhibitor constant; IC_{50} = half maximal inhibitory concentration

has been described as the most significant digestive enzyme in coleopteran species including *T. castaneum* (Sivakumar *et al*. 2006), and *C. maculatus* (Wisessing *et al.* 2008). Over the last decade, several studies have focused on naturally occurring α-amylase inhibitors. Alpha-amylase inhibitors are proteinaceous inhibitors that are found in seeds and tubers of many plant species especially cereals and legumes to regulate the endogenous enzymes and also act as a defense mechanism against insect attack (Sasikiran *et al*. 2002).

Transgenic plant approaches for α-amylase gene inhibitors offer an enticing alternative to chemical insecticides through the development of naturally resistant crops against target insect pests (Tyagi *et al*. 2014). Since the α-amylase inhibitors from different sources are highly diverse in terms of specificity against α-amylases of various insect species (Gavit *et al*. 2013), the search for suitable inhibitors and the study of their characteristics and their interaction with insect α-amylases is a very important step. In the current study the α-amylase inhibitory activity was screened in seeds of 28 accessions of 19 plant species. Two durum wheat varieties, Beni Suef-1 and Beni Suef-5, showed high inhibitory activity against standard α-amylase enzyme. Initially, ammonium sulfate was used to precipitate crude soluble protein extracts obtained from seeds of Beni Suef-1 and Beni Suef-5. Compared to other fractions, the $F_{60-90}(NH_4)_2SO_4$ proteins in both varieties showed strong inhibitory activity against α-amylase. $F_{80-100}(NH_4)_2SO_4$ was found to be suitable for the purification α-amylase inhibitor from *Amaranthus paniculatus* (Gavit *et al*. 2013) and F_{0-90} was suitable for *Sena alata* (Chandrashekharaiah 2018). The F_{60-90} protein was applied to ion exchange chromatography, DEAE-Sephadex G-50 column and the retained peak was assayed against α-amylase enzyme. Two peaks with high α-amylase inhibitory activity were observed in Beni Suef-1 variety and only one peak was observed in Beni Suef-5 variety. The purification procedure which followed resulted in recovery percentages ranging from 34.73 to 79.55% and purification ranged from 1.13 to 3.91 times. Puntambekar and Dake (2017) obtained a purification fold of 1.1–2 folds for α-amylase inhibitor from *Phaseolus vulgaris*. Working on *Vigna sublobata,* Kokiladevi *et al*. (2005) reported that the specific activity of a purified fraction using ammonium sulfate was 7.48 times that of the crude extract. On the other hand, high purification of 16 and 24.24 times were obtained in the purification of α-amylase inhibitor from *A. paniculatus* (Gavit *et al*. 2013) and *Sena alata* (Chandrashekharaiah 2018), respectively. As suggested by Prabhu and Pattabiraman (1980) and Babu and Subrhamnyam (2010), the low level of purification achieved in this study may be due to the high concentration of the inhibitor in the seed.

Temperature and pH are the major factors affecting enzyme inhibitory activity. The isolated inhibitors were found to be stable at temperatures below 80°C and maximum activity was obtained at 40–50°C and also the inhibitors were stable in a wide pH range (2–12). The high temperature and pH stability of α-amylase inhibitors were confirmed by other authors on α-amylase inhibitor isolated from different sources (Gavit *et al*. 2013; Puntambekar and Dake 2017; Chandrashekharaiah 2018). The high temperature tolerance and broad pH stability observed for Beni Suef-1 and Beni Suef-5 α-amylase inhibitors indicate their effectiveness in controlling a variety of phytophagous insects with different gut conditions.

Table 4. *In vivo* effects of α-amylase inhibitors on the mean larval weight, larval mortality, pupation and adult emergence of *Tribolium castaneum*

Treatment		Days																			
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
1		2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Control		0.0212	0	0	0	0.0212	0	0	0	0.0214	0	0	0	0.0214	0	0	0	0.0214	0	0	0
			12				14				16				18				20		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
		0.0032	0	8	0	0.0034	0	8	0	0.0035	0	4	4	0.0036	0	4	4	0.0076	0	0	8
			22				24				26				28				30		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
		–	0	2	8	–	0	2	8	–	0	2	8	–	0	2	8	0	0	0	10
			32				34				36				38				40		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
		–	0	0	10	–	0	0	10	–	0	0	10	–	0	0	10	–	0	0	10
Durum wheat (Beni Suef-1)			2				4				6				8				10		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	0.0144	20	0	0	0.0142	20	0	0	0.0157	30	0	0	0.0160	30	0	0	0.0156	30	0	0
	1 : 0.5	0.0150	0	0	0	0.0150	0	0	0	0.0160	0	0	0	0.0168	0	0	0	0.0160	0	0	0
	1 : 1	0.0152	10	0	0	0.0150	10	0	0	0.0147	20	0	0	0.0116	40	0	0	0.0115	40	0	0
			12				14				16				18				20		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	0.0136	40	1	0	0.0045	40	4	0	0.0024	40	5	0	0	40	4	2	0	40	1	5
	1 : 0.5	0.0160	10	0	0	0.0142	10	0	0	0.0127	10	2	0	0.0070	30	4	0	0.0016	30	6	1
	1 : 1	0.0089	40	2	0	0.0088	40	2	1	0.0036	40	2	2	0.0029	40	3	2	0	50	1	4
			22				24				26				28				30		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	–	40	1	5	–	50	0	5	–	50	0	5	–	50	0	5	–	50	0	5
	1 : 0.5	0.0016	30	6	0	–	40	1	5	–	40	1	5	–	40	0	6	–	40	0	6
	1 : 1	–	50	1	4	–	50	1	4	–	50	1	4	–	50	1	4	–	50	1	4
			32				34				36				38				40		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	–	50	0	5	–	50	0	5	–	50	0	5	–	50	0	5	–	50	0	5
	1 : 0.5	–	40	0	6	–	40	0	6	–	40	0	6	–	40	0	6	–	40	0	6
	1 : 1	–	50	1	4	–	50	1	4	–	50	1	4	–	50	1	3	–	50	1	4
Durum wheat (Beni Suef-1)			2				4				6				8				10		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	0.0094	50	0	0	0.0101	50	0	0	0.0084	60	0	0	0.0084	60	0	0	0.0083	60	0	0
	1 : 0.5	0.0089	50	0	0	0.0091	50	0	0	0.0030	80	0	0	0.0028	8	0	0	0.0025	90	0	0
	1 : 1	0.0070	50	0	0	0.0030	80	0	0	0.0030	80	0	0	0.0025	80	0	0	0.0018	90	0	0
			12				14				16				18				20		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	0.0061	70	1	0	0.0016	70	2	0	0.0017	70	1	1	0.0017	70	1	1	–	70	1	2
	1 : 0.5	0.0025	90	0	0	0.0024	90	0	0	–	90	1	0	–	90	1	0	–	90	0	1
	1 : 1	0.0017	90	0	0	0.0015	90	0	0	0.0012	90	0	0	0.0012	90	0	0	0.0012	90	0	0
			22				24				26				28				30		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	–	70	1	2	–	70	0	3	–	70	0	3	–	70	0	3	–	70	0	3
	1 : 0.5	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1
	1 : 1	0.0012	90	0	0	–	90	1	0	–	90	1	0	–	90	1	0	–	90	0	1
			32				34				36				38				40		
		W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A	W	M	P	A
	1 : 0.25	–	70	0	3	–	70	0	3	–	70	0	3	–	70	0	3	–	70	0	3
	1 : 0.5	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1
	1 : 1	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1	–	90	0	1

W = mean larval weight, M = larval mortality, P = number of pupa, A = number of adults; $LSD_{0.05}$ (W) = 0.0047
Data are mean of three replications

Table 5. *In vivo* effects of α-amylase inhibitors on the number, mean weight of surviving larvae and larval mortality of *Callosobruchus maculatus* using artificial seed method

Inhibitor		Larvae			Pupa			Adult		
		number	M.W. of survival	mortality [%]	number	M.W. of survival	mortality [%]	number	M.W. of survival	mortality [%]
Control		15	0.00280	0.00	–	–	–	–	–	–
Durum wheat (Beni Suef-1)	1 : 0.25	9	–	100.00	–	–	–	6	0	100
	1 : 0.5	10	–	100.00	1	0.0023	0	4	0	100
	1 : 1	12	0.00173	75.00	–	–	–	3	0	100
Durum wheat (Beni Suef-5)	1 : 0.25	15	0.00198	40.00	–	–	–	–	–	–
	1 : 0.5	14	0.00150	35.71	1	0.0015	0			–
	1 : 1	14	0.00200	64.28	–	–	–	1	0.0021	0
$LSD_{0.05}$			0.00049	6.66		0.0006				

M.W. – mean weight
Data are means of three replications

The ion exchange purified α-amylase inhibitors from Beni Suef-1 and Beni Suef-5 showed a molecular weight of 16 and 24 kDa. Gavit *et al.* (2013) reported α-amylase inhibitor with a molecular weight of 14.3 kDa from rice. Chandrashekharaiah (2018) reported α-amylase inhibitor 14 kDa from rice. Similarly, 15.488, 18.62 and 26.302 kDa α-amylase inhibitors from bean cultivars were reported by Gupta *et al.* (2014).

Kinetics analysis revealed that Beni Suef-1 and Beni Suef-5 α-amylase inhibitors are non-competitive types of α-amylase inhibitors with high affinity toward α-amylase of *T. castaneum* and *C. maculatus*. Abd El-latif (2014) reported that *in vitro* inhibition of digestive enzymes is not enough to determine the efficiency of enzyme inhibitors against insects due to the ability of insects to modify the composition of their digestive enzymes. Therefore, an inhibitor feeding test was conducted by integrating the $F_{60-90}(NH_4)_2SO_4$ proteins into the artificial diet of both insect species. The isolated α-amylase inhibitors strongly affected the life parameters of both insect species. Alpha-amylase inhibitors from wheat, barley and millet showed high potency against coleopteran insects (Farias *et al.* 2007). Plant purified α-amylase inhibitors were found to be effective against α-amylases from *C. chinensis* and *Helicoverpa armigera* (Gavit *et al.* 2013) and *C. chinensis* and *T. castaneum* (Gupta *et al.* 2014).

Conclusions

The findings of the present study show uniquely that α-amylase inhibitors isolated from durum wheat, Beni Suef-1 and Beni Suef-5 varieties, are very effective in inhibiting the development of *T. castaneum* and *C. maculatus* and could be used for future studies in developing insect resistant transgenic plants approaching α-amylase inhibitor genes.

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