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Effects of the Two Extracted Agglutinins from *Rhizoctonia Solani* K ü hn (Cantharellales: Ceratobasidiaceae) on Digestive A -amylase of *Pieris Brassicae* L. (Lepidoptera: Pieridae)

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Abstract

Two galactose specific lectins, RSAI and RSAII, were purified from sclerotia of *Rhizoctonia solani* Kühn and their inhibitory effects were assessed on digestive α-amylase of *Pieris brassicae* Linnaeus. Results revealed molecular weight of 16 and 15.7 kDa and IC_{50} inhibitory concentration of 0.87 and 1.68 mg/ml for RSAI and RSAII, respectively. In native-PAGE experiment, both RSAs provoked disappearance of A_2 band of the enzyme and decreased sharpness of A_1 band. Determination of α-amylase kinetic parameters using Lineweaver-Burk analysis revealed that compared to the control, both lectins reduced V_{max} while K_m was increased only in the presence of RSAII. Therefore, kinetic parameters indicated non-competitive and mixed inhibitions for RSAI and RSAII, respectively. Moreover, RSAI and RSAII showed the highest inhibition within pH and temperature range optimal for enzyme activity in the control group, i.e., at pH values of 9 (RSAI) and 11 (RSAII) and temperature of 30°C. Incubation of RSAs at 50, 60 and 70°C showed high stability of RSAII while RSAI lost inhibitory potential at 70°C. Results of the current study clearly indicate negative *in vitro* effects of RSAs on digestive α-amylase of *P. brassicae*.

Keywords: *Pieris brassicae*; *Rhyzoctonia solani*; Lectin; Digestive α-amylase

Introduction

Dietary carbohydrates are energy sources for insect growth, locomotion, reproduction etc. In the digestive tract, carbohydrates are broken down into smaller molecules, monosaccharaides, and absorbed by midgut epithelial cells. Digestion of carbohydrates starts by the action of α-amylase (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) which catalyzes the endohydrolysis of α-D-(1-4)-glucan linkages in starch and glycogen to oligosaccharides [1]. Impairment of α-amylase activity, which may be caused by some food components, prevents sugar utilization in phytophagous insects. Plants produce a number of defense proteins that reduce digestive ability of phytophagous insects [2].

Lectins are ubiquitous carbohydrate-binding proteins that have been extracted from fungi, viruses, plants and animals [3]. These proteins are among the most important defense proteins in plants which are used against insects and pathogens [1]. These molecules can bind to monosaccharides or oligosaccharids of glycoproteins in midgut lumen or relevant receptors on the surface of midgut epithelial cells leading to enzyme inhibition or apoptosis [2-4]. Some lectins can bind to glycoproteins or chitin in the peritrophic matrix [5], and disturb circulation of digestive enzymes leading to their increased excretion [6]. Finaly, lectins may pass into the haemolymph through midgut epithelium and compromise insect immune system [7]. Several studies have reported harmful effects of lectins on insect larval weight, survival, feeding, metabolism, honeydew excretion, pupation, total development duration, adult emergence and fecundity [1,8-12]. Proteolytic degradation has been described as insect defense mechanisms against plant lectins [13]. However, it is expected that insects are not adapted to lectins from non-host plants and other organisms with which insects have not coevolved (e.g. plant fungi).

The large cabbage white butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), is a destructive pest of agricultural crops throughout Europe, North Africa and Asia causing massive damages to species from Brassicaceae and other related families of plants [14]. Larvae feed exclusively on leaves of host plants (Brassicaceae); piles of their feces make leaves rancid and allow fungal and bacterial pathogens to grow and penetrate plant tissues. Biological and chemical controls are the two main ways to alleviate damage of the pest. The third control procedure may be production of resistant host plants that pose the lowest risk to ecosystem. Because of many problems potentially caused by synthetic pesticides, the use of these chemicals for protection of agricultural plants is now limited and they are being replaced by resistant transgenic plants created through biotechnology [15]. Several studies have shown the potential of genes coding for lectins (the natural insecticidal proteins) to be used for plant transformation [16-20].

R. solani (Class: Basidiomycetes; Order: Polyporales) is a plant pathogenic fungus that has an asexual life cycle and exists primarily as vegetative mycelium and sclerotia. The sclerotia are important for the survival in soil under harsh conditions. RSA (*Rhizoctonia solani* agglutinin), initially purified by Vranken et al. [21], is a homodimeric protein composed of subunits, with high affinity to N-acetylgalactosamine (GalNAc) and more complex glycoproteins. In the current study, two lectin molecules have been extracted from a well-known fungus, *R. solani* Kühn and their *in vitro* inhibitory properties were examined against a digestive α-amylase of the large cabbage white

butterfly. In details, different concentrations of the purified lectins were prepared to find IC_{50} concentration. Then, the influence of pH and temperature on the α-amylase in addition to kinetic parameters were determined in the presence of the lectins at IC_{50} concentration and compared to the control group.

Materials and Methods

Insect rearing

Eggs of *P. brassicae* were collected from Canola fields and transferred to the laboratory at temperature of 25°C, 70% humidity and 16:8 light:dark photoperiod. Hatched larvae fed on cabbage leaves until molting into the fourth larval instar. Containers were cleaned every day, and fresh leaves were provided for the larvae.

Preparation of sepharose 4B-galactose column

Sepharose 4B-Galactose column was prepared based on a method described by Bulgakov et al. [22]. Briefly, after 20 ml of Sepharose 4B was suspended in 40 ml of 0.5 M Na_2CO_2 (pH 11.0), 2 ml divinylsulphone was added and the mixture was incubated for 70 min at room temperature with gentle shaking. Then, 500 mg of galactose in 50 ml 0.5 M Na_2CO_2 (pH 11.0) was added and incubation continued for additional 12 h. Prepared sorbent was washed by water, the unbound arm was blocked with ß-mercaptoethanol-containing buffer and finally it was packed into a 1.5 × 30 cm column. The sorbent was equilibrated with Tris-HCl (0.1 M) and used for the affinity purification of RSA.

Purification of RSA by affinity chromatography

Sclerotia of *R. solani* (provided from Herbarium of Department of Plant Protection, University of Guilan) were incubated in phosphate buffer (0.1 M, pH 7.1) for approximately 72 h at 4°C. Then, stems were grounded in the buffer to completely homogenize the tissue prior to additional incubation for 24 h. The mixture was filtrated by a layer of cheesecloth, and centrifuged at 5000 rpm for 20 min. Remaining debris was removed by passing the supernatant through filter paper (Whatmann No.4) [7]. Supernatant was precipitated by 0-60% ammonium sulfate and centrifuged at 5,000 rpm for 20 min. Debris was eluted in Tris-HCl buffer (0.1 M, pH 7) and dialyzed in the same buffer overnight [20]. Dialyzed samples were loaded into Sepharose 4B-galactose column equilibrated with Tris-HCl buffer (0.1 M, pH 7). The affinity column was washed with Tris-HCl buffer, and buffer containing 20 mM 1,3-diaminopropane (DAP) [11]. Fractions showing the highest protein content were pooled and used for forthcoming step. The lectin fractions obtained after the first affinity chromatography were loaded on DEAE-Cellulose fast flow equilibrated with DAP [11]. Finally, the lectin was eluted using Tris-HCl (0.1 M, pH 7.0) containing 0.5 M NaCl after DAP washing and freeze-dried. Purity of the samples and molecular weight were analyzed by SDS-PAGE stained with Coomassie Brilliant Blue [23].

Hemagglutinin assay

The experiment was performed in microtiter plates, according to Correia and Coelho [24]. The peaks from chromatography (50 µl) were two-fold serially diluted in 150 mM of NaCl before adding a 50 µl 2.5% (v/v) suspension of cow erythrocytes. The titer was expressed as the highest dilution exhibiting hemagglutination.

Preparation of midgut samples

Fourth instar larvae of *P. brassicae* were randomly selected and dissected under a stereomicroscope in ice-cold saline solution (10 mM). The midgut was separated from the larval body, rinsed in equal amount of ice-cold distilled water, placed in a pre-cooled homogenizer and was ground before centrifugation. Homogenates were transferred to 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were pooled (five midguts per sample) and stored at -20°C for subsequent analyses.

Assay of α-amylase

The method described by Bernfeld [25] was used to assay α-amylase activity. Briefly, reaction mixture contained 50 µl of universal buffer (Glycine, Succinate, 2-morpholinoethan sulfuric acid; 0.02 M, pH 9), 30 µl of soluble starch (1%) as substrate and 20 µl of the enzyme. After incubation for 30 min at 30°C, the reaction was stopped by adding 80 µl of DNSA (dinitrosalicylic acid) reagent and the color was developed by heating in boiling water for 10 min. Finally, the absorbance was read 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 30°C. Specific amylase activity was expressed in enzyme units per mg of proteins. Protein concentrations in midgut samples were determined according to the method described by Lowry et al. [26].

Inhibition of α-amylase by different concentrations of RSAs

Midgut homogenates of larvae in 50 µl of universal buffer (0.02 M, pH 9) were preincubated for 5 min with RSAs at different concentrations (0.1, 0.3, 0.5, 1, 1.7 and 2 mg/ml). After 5 min, 20 µl of starch (1%) was added and incubated for 30 min at 30°C. Then, 10 µl of the enzyme was added and reaction continued as described in previous section. Blanks contained all components except enzyme, i.e., universal buffer, 1% starch and each concentration of lectin. Half maximal inhibitory concentration (IC_{50}) was calculated for the two lectins.

Kinetic studies

Kinetic parameters for inhibited and control samples were determined with increasing concentrations of starch as a substrate (0.5-2.0%) in the presence of RSAs at IC_{50} concentration (0.87 mg/ml for RSAI and 1.68 mg/ml for RSAII) as well as in control samples without RSA. Lineweaver-Burk plot analysis was applied to estimate K_m and V_{max} values.

Effect of pH and temperature on α-amylase inhibition by RSAs

A range of universal buffers (0.02 M, 3-12) was prepared and inhibition of α-amylase was monitored at each pH set by incubating the midgut homogenates with RSAI and RSAII at IC_{50} concentrations (0.87 and 1.68 mg/ml, respectively). Control samples without inhibitor were run at the same pH values. Enzyme assays were performed as described earlier. The reaction mixtures containing universal buffer, starch, enzyme solution and IC_{50} concentration of each RSA (I or II), was incubated at temperature regimes of 15, 20, 25, 30, 35, 40, 45 and 50°C to measure effect of temperature on inhibition of RSAs on α-amylase of *P. brassicae*. Amylase activity in control samples without RSAs was also determined and percentage of inhibition was calculated based on the ratio of activities in inhibited and control samples.

Thermal stability of RSAs

To find stability of RSA molecules, three samples of RSAs (0.5 mg/ml, 500 µl in each) were incubated at 50, 60 and 70°C during different time intervals (10, 20, 30, 40 and 60 min). Inhibition studies were carried out with heated samples at each time interval as described in the section "Inhibition of α-amylase".

Inhibition in non-denaturing PAGE

Enzyme extracts were pre-incubated with of 0.87 mg/ml of RSAI and 1.68 mg/ml of RSAII for 30 min at 30°C, and then the remaining amylase activity was determined by SDS-polyacrylamide gel electrophoresis. SDS-PAGE was carried out using the procedures described by [18]. Concentrations of resolving and stacking gels were 10 and 4%, respectively. Electrophoresis was conducted at a voltage of 70 V until the blue dye reached the bottom of the slab gel [26]. Then, the gel was rinsed with distilled water and washed by shaking gently with 1% (v/v) Triton X-100. After that, gel was immersed in a solution of universal buffer (0.02 M pH 9) containing 1% starch, 10 mM NaCl and 2 mM $CaCl_2$ [27]. Finally, it was stained by solutions of 1.3% I2 and 3% KI to obtain white bands in dark backgrounds.

Statistical analysis

All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentized test.

Results and Discussion

Isolation and purification of RSAs

In our study, two lectin molecules, RSAI and RSAII, were purified from *R. solani* (Figure 1) and their lectin activity was confirmed by hemagglutinin assay. After affinity chromatography, two peaks were observed at protein concentrations of 32.87 and 26.08 mg/ml (Figure 1a) and molecular weight of 16 and 15.7 kDa (Figure 1b). Lectins are the carbohydrate-binding proteins composed of at least one carbohydrate binding domain for a specific mono- or oligosaccharide [11]. Fungal lectins are mainly isolated from fruiting bodies of higher fungi Ascomycota and Basiodiomycota [11,22], mycelia of lower fungi [28] and sclerotia of soil born plant pathogen *R. solani* [7,29,30]. Hamshou et al. [30] determined lectin concentrations in different strains of this fungus and reported the concentrations within a range from 0.058 to 7.5 mg/g of lectin in lyophilized mycelium or sclerotia.

IC_{50} determination of RSAs on digestive α-amylase of *P. brassicae*

Different concentrations of RSAs from 0.1-2 mg/ml were applied to find inhibitory effects of RSAs on digestive α-amylase of *P. brassicae*. The highest concentrations of RSAI and II inhibited more than 70% of amylolytic activity (Figures 2a and 2b). In gel electrophoresis, both RSAs completely removed A2 band of the enzyme and decreased sharpness of A1 (Figure 2c).

IC_{50} concentrations of these RSAs were calculated to be 0.87 and 1.68 mg/ml for RSAI and II, respectively (Table 1). Studies have reported six classes of α-amylase inhibitors including lectin-like, knottin-like, cereal-type, Kunitz-like, c-purothionin-like, and thaumatin-like which have structural diversities, different modes of inhibition and different specificity profiles against different α-amylases [1].

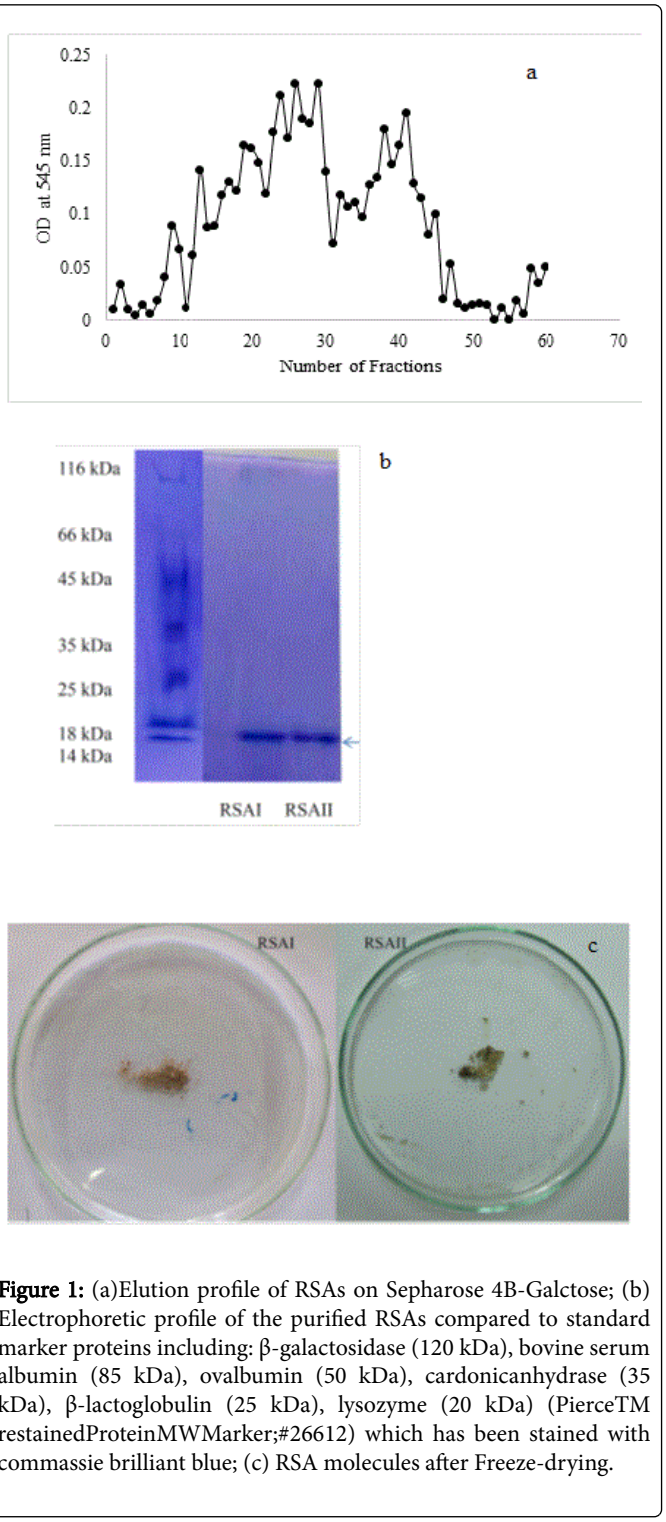

Figure 1: (a)Elution profile of RSAs on Sepharose 4B-Galctose; (b) Electrophoretic profile of the purified RSAs compared to standard marker proteins including: β-galactosidase (120 kDa), bovine serum albumin (85 kDa), ovalbumin (50 kDa), cardonicanhydrase (35 kDa), β-lactoglobulin (25 kDa), lysozyme (20 kDa) (PierceTM restainedProteinMWMarker;#26612) which has been stained with commassie brilliant blue; (c) RSA molecules after Freeze-drying.

In similar studies with plant lectins, inhibitory effects of a lectin extracted from *Citrullus colocynthis* L. on digestive α-amylase of *Ectomyelois ceratonia* Zeller (Lepidoptera: Pyralidae), a lectin from *Polygonum persicaria* L. on digestive α-amylase of *P. brassicae* and *Chilo suppressalis* Walker (Lepidoptera: Crambidae) found to be 1.5,

0.64 and 1.07 mg/ml, respectively [12,31]. Differential inhibitor efficiency of lectins point to differences in structure and specificity for sugars which determine their affinity properties and ability to reduce activityof target enzymes.

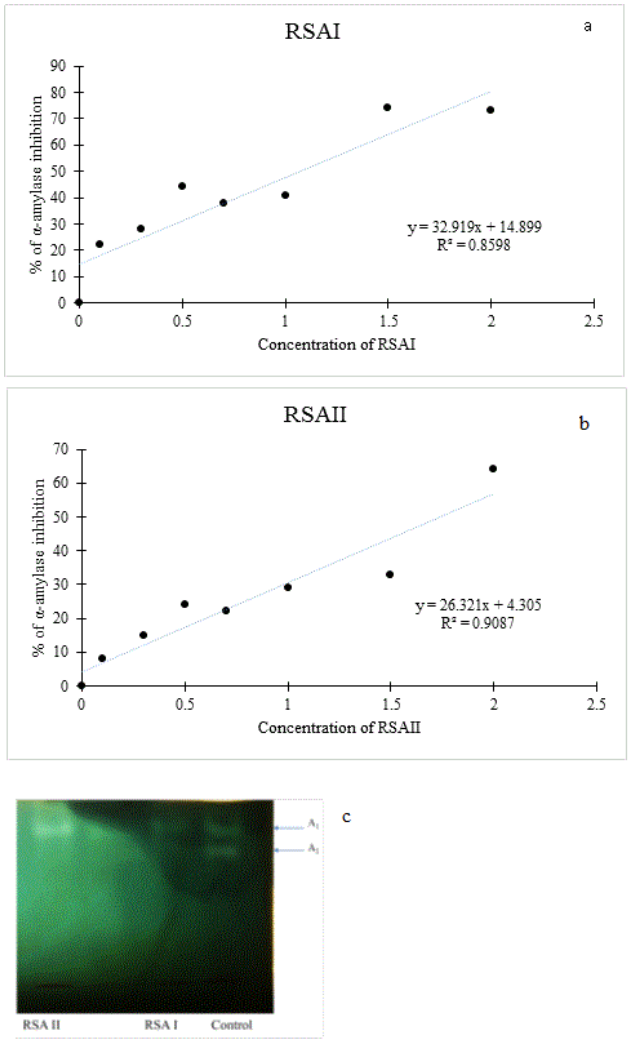

Figure 2: (a, b) Inhibition of digestive α-amylase from *P. brassicae* larvae by different concentrations of RSAI and II; (c) Inhibition of α-amylase zymogram by IC_{50} concentrations of RSAI and II which has been stained with I_2 and KI solution.

Treatment	IC_{10}	IC_{30}	IC_{50}	Slope ± SE	X_2	df
RSAI	0.13	0.40	0.87	1.559 ± 0.165	19.25	5
RSAII	0.31*	0.84*	1.68*	1.749 ± 0.19	12.15	5

Table 1: Inhibitory concentrations of RSAI and RSAII that inhibit 10, 30 and 50% of maximal activity of *P. brassicae* digestive α-amylase [*All values have been calculated by POLO-PC software].

Kinetic parametrs of α-amylase inhibition by RSAs

Lineweaver-Burk analysis of α-amylase inhibition by IC_{50} concentrations of RSAI and II revealed lower V_{max} of the enzyme in the presence of both RSAs (Figure 3). Changed (higher) value of K_m was observed only in the case of RSAII compared to the control (Table 2). These findings showed non-competitive inhibition of *P. brassicae* α-amylase for RSAI and mixed inhibition for RSAII. In non-competitive inhibition, inhibitor binds at sites different from the active site of the enzyme and causes a conformational change preventing enzymatic binding to substrates. In mixed inhibition, inhibitor binds to enzyme-substrate complex but prefers binding to the free enzyme. Ramzi and Sahragard [12] reported non-competitive inhibition of α-amylase from *E. ceratoniae* by lectin from *C. colocynthis*. Kinetic parameters of α-amylase from *C. suppressalis* inhibited by *P. persicaria* lectin demonstrated lower V_{max} and higher K_m values while non-competitive inhibition was observed on *P. brassicae* [31]. Different observed kinetic parameters may refer to different affinity of RSAs for α-amylase because of their different structural properties. So, crystallography of the extracted lectins would clearly explain the findings.

Treatments	V_{max} (U/mg protein)	K_m (%)	R^2
Control	0.33 ± 0.021a	0.70 ± 0.014b	0.93
RSAI	0.29 ± 0.070b	0.69 ± 0.033b	0.94
RSAII	0.29 ± 0.044b	0.94 ± 0.016a	0.92

Table 2: Kinetic parameters of digestive α-amylase activity (control) and amylase inhibition by RSAs [a, b-Statistical differences from the control are marked by various letters (Tukey test, $p \leq 0.05$)].

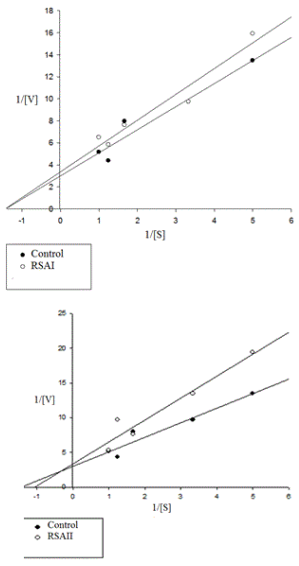

Figure 3: Double reciprocal Lineweaver-Burk plots indicating kinetic parameters of RSAs inhibition on digestive α-amylase of *P. brassicae*.

Effect of pH and temperature on α-amylase inhibition by RSAs

Biochemical reactions are significantly affected by pH and temperature of media since these factors may alter velocity and inhibition of enzymes. In the current study, the highest inhibition of α-amylase by RSAI and II were found at pH values of 9 and 11, respectively (Figure 4). In case of temperature, both lectins showed the highest inhibition at 25-35°C (Figure 5). In the control group, α-amylase of *P. brassicae* demonstrated the highest activity at pH values of 9-11 and temperature of 30°C (Figure 5). Temperature and pH may affect capability of enzyme to bind substrate and inhibitor. Majority of studies have reported the highest inhibition of insect α-amylases in the optimal pH and temperatures. Baker [32] found optimal pH of 7.5 for inhibition of *Anagasta kuehniella* (Zeller) α-amylase by an aqueous extract of wheat. Ramzi and Sahragard [12] found that the lectin extracted from *C. colocynthis* showed the highest inhibition of *E. ceratoniae* α-amylase at pH values of 8 and 9 as well as temperature of 35-40°C. The highest inhibition of *P. brassicae* α-amylase by *P. persicaria* lectin was found at pH values of 10 and 11 while the lectin on *C. suppressalis* showed the highest inhibition at pH 9 and temperature of 35°C [31,33].

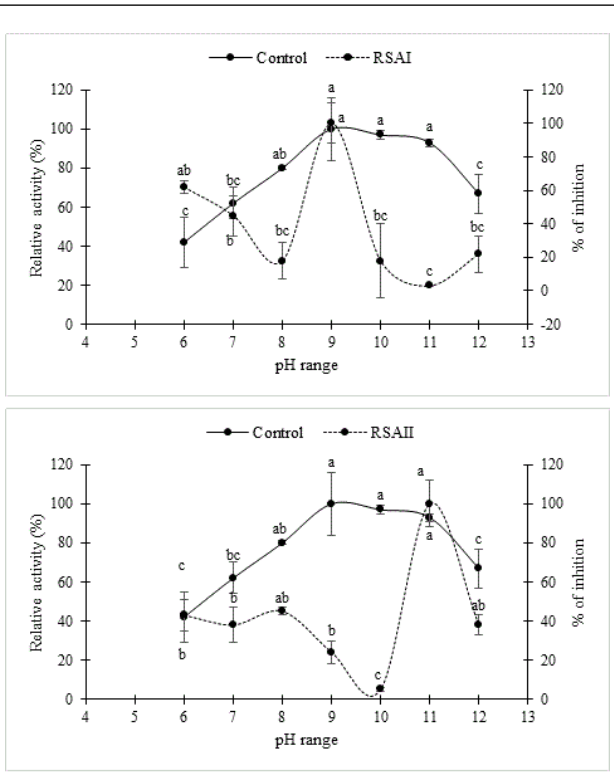

Figure 4: Effect of pH on digestive α-amylase activity from *P. brassicae* larvae in addition to its inhibition by RSAI and II. Statistical differences have been marked by various letters (Tukey test, $p \leq 0.05$).

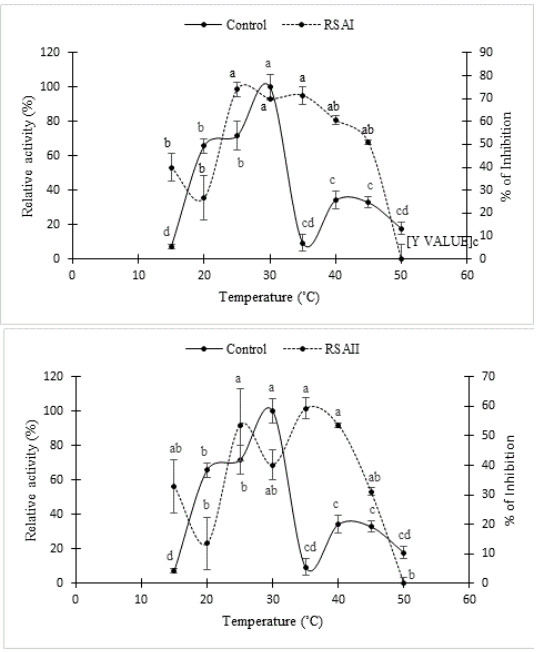

Figure 5: Effect of temperature on digestive α-amylase activity from *P. brassicae* larvae and its inhibition by RSAI and II. Statistical differences have been marked by various letters (Tukey test, $p \leq 0.05$).

Effect of heated RSAs on α-amylase inhibition

To find stability in high temperature regimes, the purified RSAs were kept for 10, 20, 30 and 60 min at 50, 60 and 70°C, and then their inhibition effects on *P. brassicae* α-amylase were evaluated. Results demonstrated that heated RSAs were stable in all temperatures regimes and all examined time intervals with the exception of RSAI at 70°C (Figure 6). Heated samples significantly decreased amylolytic activity versus control while RSAI incubated at 70°C showed no inhibition on the enzyme (Figure 6). Zibaee et al. [26] found that the lectin extracted from *P. persicaria* were stable at 50°C and it significantly decreased activity of α-amylase from *P. brassicae* while samples incubated at 60 and 70°C showed no inhibitory effects. Oneda et al. [34] observed that α-amylase inhibitor from wheat kernel were inactivated at 88°C for 30 min. Mehrabadi et al. [35] found thermal inactivation of T-αAI at 50-80°C.

Conclusions

Here, two lectin molecules have been isolated and purified from *R. solani* for the first time. *In vitro* studies revealed significant inhibition of digestive α-amylase from *P. brassicae* and its dependence on pH and temperature. Kinetic parameters point to non-competitive and mixed inhibtions of RSA I and II respectively. Also, the lectins were stable at high temperatures. The two purified lectin here had the almost same molecular weight which indicates their similar structural property with slight difference which needs to be clarified by crystallography. The difference might be referred to their functional groups leading to different biochemical reaction toward other molecules e.g. α-amylase.

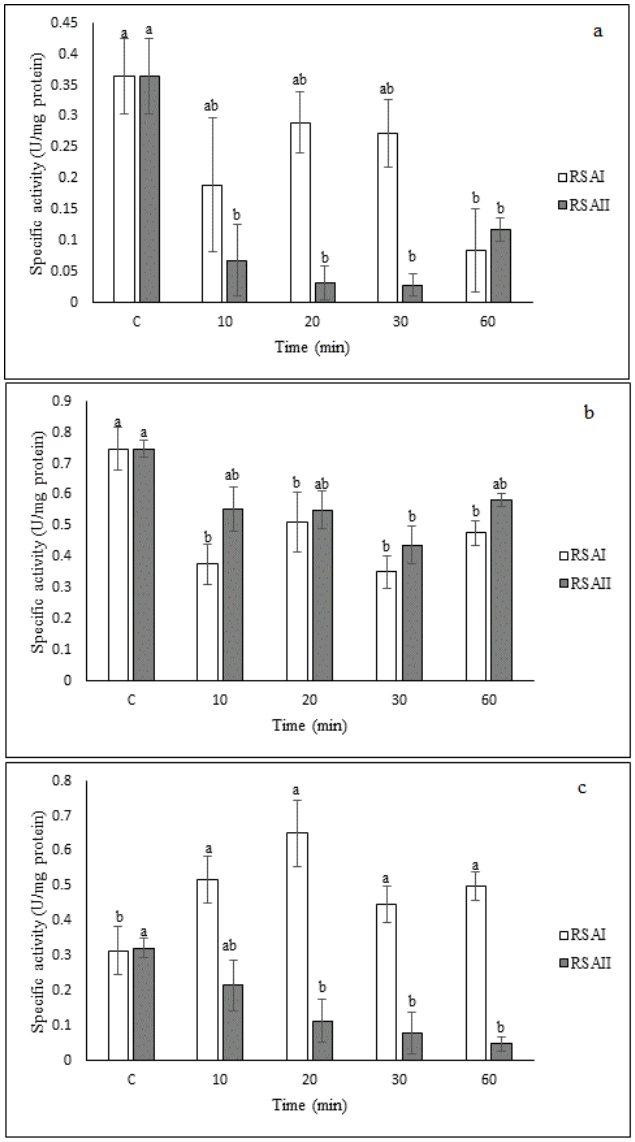

Figure 6: (a) Thermal stability of RSAs and their effects on digestive α-amylase of *P. brassicae* at 50°C; (b) Thermal stability of RSAs and their effects on digestive α-amylase of *P. brassicae* at 60°C; (C) Thermal stability of RSAs and their effects on digestive α-amylase of *P. brassicae* at 70°C [Statistical differences have been marked by various letters (Tukey test, $p \leq 0.05$)].

As a future work, the gene responsible to lectin synthesis in *R. solani* should be identified and transferred to host plant by molecular biology approaches. Expression of the given lectins in leaves of host plants which suppress population outbreaks of *P. brasicae* due to feeding deficiency.

Acknowledgement

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Authors' Contributions

The first author had 70 % contribution and others have a same share.

Conflict of Interest

There is no conflict of interest.

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