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The Inhibitory Activity of Triticale, Rye and Black Nightshade Seed Proteinaceous Extracts against Potato Tuberworm Digestive α-Amylase and Protease

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ABSTRACT

Potato tuberworm, *Phthorimaea operculella* Zeller (Lepidoptera; Gelechiidae) is a worldwide pest of solanaceous crops. Larvae feed inside galleries in foliage, stems and tubers making chemical control unsuccessful, so other control methods should be applied. In recent years many plants have received genes that encode toxic proteins as a strategy to resist insect pests. In this study, optimal pH and temperature of digestive α-amylase and protease activities of potato tuberworm and the effect of triticale (X *Triticosecale wittmack* cv. Sanabad), rye (*Secale cereale* L. cv. Danko) and black nightshade (*Solanum nigrum* L.) seed proteinaceous extracts against enzymes activities were evaluated using starch 1% and azocasein 2% as a substrate, respectively. The optimum pH of *α*-amylase and protease activities was found to be highly alkaline. Enzymes inhibition assays showed that amylase activity was significantly affected by extracts from triticale and rye by pH (P= 0.05; maximum effect at pH 9) and influencing of protease activity by extracts mentioned above did not vary by pHs 8-11 and 9-11, respectively. Extracts from black nightshade seed had no effect on enzymes activity. Inhibition manner of various concentrations; 1.5, 0.75, 0.375, 0.187 and 0.093 (mg protein ml^{-1}) of extracts were dose-dependent. Maximum inhibitory effect occurred at the highest concentration and the minimum was at the lowest concentration. In polyacrylamide gel assay, both enzymes, without inhibitors showed two isozymes, which at highest concentration of extracts, both bands disappeared or their intensity decreased. So, these proteins can be introduced to be encoded in producing resistant potato crops against potato tuberworm.

Keywords: Cereals, Digestive enzymes, Potato tuberworm.

INTRODUCTION

The potato tuberworm, *Phthorimaea operculella* (Zeller), is a worldwide pest of solanaceous crops especially devastating to potato. Although potato tuberworm is primarily a pest of potato, it can also be found in other solanaceous plants (Rondon, 2010). It is one of the most important pests of potato in many temperate and tropical regions of the world. The pest is native to South America. In Iran it was first reported in potato fields in Karaj. The larvae mine leaves, stems, and petioles cause irregular galleries, and excavate tunnels through tubers. Foliar damage to the potato crop usually does not result in significant yield losses but infested tubers especially in non-refrigerated systems may have reduced marketability. Several approaches are available for the development of an integrated pest management system for potato tuberworm. Also, since the larvae feed inside the tubers, pesticide application in order to control this insect is not successful because

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the insects are not exposed to the insecticides (Rondon, 2010). Since insect pests rely on digestive enzymes such as α-amylase and proteases for their feeding and moreover large scale pesticides application causes deleterious effects to human environment and also the occurrence of resistance in the insect pests against the pesticides, enzyme inhibitors encoded in transgenic plants could be an alternative strategy to control phytophagous insect-pests (Gatehouse *et al*., 1998; Franco *et al*., 2002). Cereals and legumes seeds are the rich sources of digestive enzyme inhibitors (Franco *et al.*, 2002). Therefore, it is advisable to characterize digestive enzymes as well as to do *in vitro* and *in vivo* bioassay with plant proteinaceous inhibitors in order to achieve a control strategy based on digestive enzyme inhibitors (Harrison and Bonning, 2010). So, the goal of the current study was to investigate the effect of seed proteinaceous extracts from triticale, rye and black nightshade on the amylolytic and proteolytic activities of the potato tuberworm.

MATERIAL AND METHODS

Insect Rearing

A population of potato tuberworm was obtained from the insect physiology laboratory (University of Mohaghegh Ardabili) and maintained on potato tubers (*S. tuberosum* L. cv. Agria) in plastic containers in the incubator set at 30±1 °C and 55% RH (Relative Humidity).

Enzyme Preparation

Insect enzyme extraction was done based on procedures described by Mehrabadi *et al.* (2012). The fifth instar larvae of potato tuberworm was used for enzyme extraction, because the most feeding occurs in this instar. The larvae were randomly selected, cold-immobilized on ice for 10 minutes and carefully dissected in distilled water under stereomicroscope (Nicon WD®). Guts were separated and grounded in 1.5 ml of phosphate buffer at pH 7 and homogenized with homogenizer (Ultra turax T8®). The 1.5 ml homogenates from preparations were centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatants were transferred to a new tube and stored at -20 °C for further use as an enzyme source.

Optimal pH of α-Amylase and Protease Activity Determination

The optimal pH of α-amylase and protease activities were determined using different pH values; 8, 9, 10, 11 and 12 of universal buffer (Hosseinkhani and Nemat-Gorgani, 2003) containing Glycin (0.02M), 2-morpholinoethansulphonic acid (0.02 M) and succinate (0.02 M). To determine the optimal pH of *α*-amylase activity, 10 µl of enzyme extract (1.5 mg protein ml^{-1}) was dissolved in 65 µl universal buffer at distinct pH, then 25 µl of starch solution 1% was added to the reaction as a substrate and the mixture was incubated at 40 °C for about 30 minutes. Then the reaction was stopped by adding 100 µl DNS (DiNitroSalicilic acid) according to Bernfeld, 1955 and heated in boiling water for 10 minutes. Then absorbance of the reaction mixture was read at 540 nm by using ELISA reader. To determine the optimal pH of protease activity, 10 µl enzyme extract was incubated with 50 µl azocasein 2% as a substrate in 40 µl of distinct pH at 45 °C for about 60 minutes. According to Saadati *et al.* (2011), the reaction was stopped by adding 100 µl TCA (TriChloroAcetic acid) and kept in the refrigerator at 4 °C for about 30 minutes, followed by centrifugation at 13,000 rpm for about 20 minutes to precipitate non-hydrolyzed substrate. Finally the absorbance of reaction mixtures was measured at 405 nm.

Optimum Temperature of *α*-Amylase and Protease Activity Determination

The effect of temperature on *α*-amylase and protease activities was determined by

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incubating the reaction mixtures including enzyme extract, different pH values of universal buffer and the specific substrate for each enzyme (starch for α-amylase and azocasein for protease) at various temperatures; 30, 35, 40, 45 and 50°C for 30 and 60 minutes, respectively. After stopping the reactions by adding DNS and TCA to α-amylase and protease mixtures, the amylolytic and proteolytic activities were measured as described before.

Seed Protein Extraction Procedure

Seeds of rye (*Secale cereale* L. cv. Danko) and triticale (X *Triticosecale wittmack* cv. Sanabad) were supplied by Seed and Plant Improvement Institute, Karaj, Iran. Black nightshade (*S. nigrum* L.) seed was obtained from Agriculture and Natural Resources Research Center of East Azarbaijan, Khosroshahr, Iran. According to Baker (1987) and Melo *et al*. (1999), seeds were milled completely, and then 30 grams of grinded seeds from each plant was mixed separately with 100 ml of 0.1M NaCl and stirred for 90 minutes, then the mixture was centrifuged at 8,000 rpm for about 30 minutes at 4°C. The pellet was discarded and proteins were concentrated using a saturation of 70% ammonium sulfate followed by centrifuging the mixture at the same condition. The pellet was dissolved in ice-cold Tris-HCl buffer (0.02 M and pH 7.0) and dialyzed against the same buffer for about 20 hours. Then this dialyzed solution was transferred to 1.5 ml tubes and placed at 70°C for about 15 minutes in order to inactivate the enzymes within the seeds. Finally, the 1.5 ml tubes were centrifuged at the same condition and the supernatants were transferred to other tubes and frozen at -20°C as an inhibitor source in enzyme inhibition assays.

The Effect of pH on Inhibitory Activity of Seed Extracts

In-vitro assay of the effect of pH on inhibitory activity of seed extracts including triticale, rye and black nightshade on α-amylase and protease activities was assayed. At 55µl of given pH value of universal buffer (8, 9, 10, 11 and 12), 10µl enzyme extract was pre-incubated with 10µl of each seed extract solution at 40°C for 15 minutes for amylase inhibition assay and 60 minutes at 45°C for inhibition assay of protease. Then starch 1% solution as α-amylase substrate and azocasein 2% as protease substrate were added to each enzyme mixture. Appropriate blanks were included in the experiments, too. The inhibition percentage of α-amylase and protease (% I) was calculated according to Mehrabadi *et al*. (2011):

$\%I_{\alpha\text{-amylase}}$ = [(ΔA540 Control-ΔA540 Experiment)/ΔA540 Control]×100

$\%I_{protease}$ = [(ΔA405 Control-ΔA405 Experiment)/ΔA405 Control]×100

The Effect of Different Concentrations of Seed Extracts on Enzymes Activity

The effect of seed proteinaceous extracts on α-amylase and protease activities was determined as described by Mehrabadi *et al.* (2010). Various concentrations including 1.5, 0.75, 0.375, 0.187 and 0.093 mg ml^{-1} protein of seed extracts were prepared by diluting the most dense extract (1.5 mg ml^{-1} protein). Then 10 µl of enzyme extract at defined pH (the optimum pH of each enzyme activity) was pre-incubated with each of the above-mentioned contemplations. Then specific substrate of both enzymes was added to the mixtures and the continuation of assay was done as described before.

All assays were performed with three replicates and 20 samples.

Semi-denaturing Native-PAGE

Electrophoretic detection of amylolytic and proteolytic activity was done basically according to the procedures described by Laemmli (1970) and Walker *et al.* (1998). Amylolytic activity was detected using 10%

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(w/v) polyacrylamide gel co-polymerized with 0.5% starch according to Mehrabadi and Bandani (2010) and 4% for stacking gel with 10% SDS. Electrophoresis was conducted at a voltage of 70V at 4°C until the blue dye reached the bottom of the gel. Then, the gel was rinsed with distilled water and washed by 1% (v/v) Triton X-100 buffer for about 30 minutes followed by incubation in Tris-base buffer (pH 9.0) containing 2 mM $CaCl_2$ and 10 mM NaCl for about 2 hours. Finally, the gel was treated with a solution of 1.3% I_2 and 3% *KI* to stop the reaction and stain the un-reacted starch background. Proteolytic activity was detected using 10% (w/v) polyacrylamide gel co-polymerized with 1% gelatin. Electrophoresis was conducted at a voltage of 70V at 4°C until the blue dye reached the bottom of the gel. Then, the gel was rinsed with distilled water and washed by 2.5% (v/v) Triton X-100 buffer for about 60 minutes followed by incubation in Tris-base buffer (pH 11) for about 24 hours. Finally, the gel was treated with staining buffer as described by Hosseininaveh *et al.* (2007) containing 50% (v/v) methanol, 10% (v/v) acetic acid and 0.25% (w/v) Coomassie blue R-250 to stain the un-reacted gelatin background for about 24h and was finally treated with destain buffer containing 10% (v/v) methanol and 5% (v/v) acetic acid for about 4 hours.

Zones of *α*-amylase and protease activities appeared at light bands against a dark background.

Protein Determination

Protein concentration of enzymes extracted of insect gut and proteinaceous extract of seeds was measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, Munchen, Germany) as a standard.

Material Supply

Azocasein, Bovine Serum Albumin (BSA), succinic acid disodium salt, and Ammonium PerSulfate for electrophoresis (APS) were supplied by Sigma (St Louis, MO, USA). Tris, phosphate buffer solution (pH: 7.0), 2-hydroxy-3,5-DiNitroSalicylic acid (DNS), potassium sodium tartrate tetrahydrate, starch soluble, TriChloroAcetic acid (TCA), sodium hydroxide, ammonium sulfate, acrylamide, N,N′-methylene diacrylamide, dodecyl Sulfate Dodium Salt (SDS), 2-MorpholinoEthaneSulfonic acid (MES), sodium chloride, calcium chloride, phosphoric acid, glycerol, potassium iodide, iodine, coomassie brillant blue G 250, bromophenol blue, and N,N,N′,N′-Tetramethyl ethylenediamine (Temed) were purchased from Merck (Darmstadt, Germany). Methanol was from Arman Sina (Tehran, IRI); glysin from Scharlau (Barcelona, Spain) and Triton X-100 from Applichem (GmbH in Darmstadt, Germany). Spectrophotometric measurements were made using ELISA reader, BioTek® (Winooski, VT), ELx800.

Statistical Analysis

Data was analyzed using MSTAT-C software by Single factor ANOVA followed by mean comparison with Tukey's Honestly Significant Difference test (HSD).

RESULTS

Optimum pH of *α*-Amylase and Protease Activity

Some features of α-amylase and protease enzymes in the potato tuberworm gut were determined in introductory experiments. We found that the optimal pH of *α*-amylase (Figure 1-a) and protease (Figure 1-b) was in the alkaline range with a peak at about pH 9 and 11, respectively. The α-amylase and protease activity level in optimal pH was 0.024319 and 0.13 U min^{-1} mg^{-1} protein, respectively.

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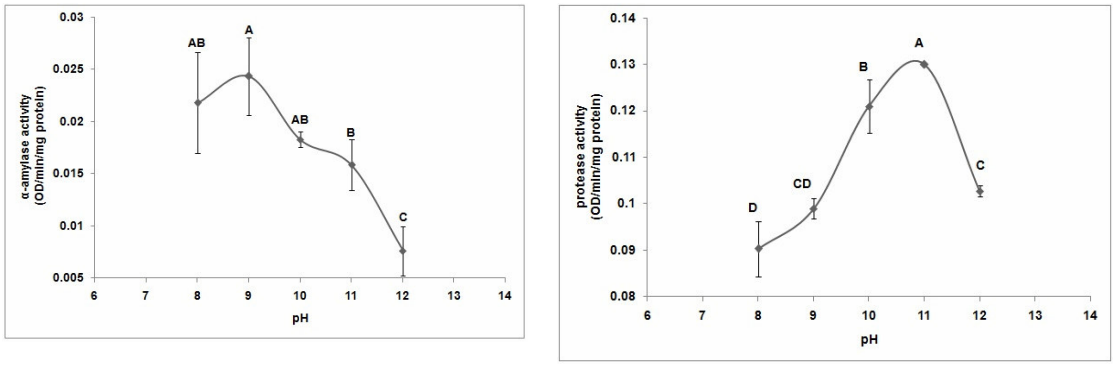

(a) (b)

Figure 1. The effect of pH on α-amylase (a) and protease (b) of potato tuberworm. Means followed by the same letters are not significantly different by Tukey's test (P< 0.05).

Optimal Temperature of *α*-Amylase and Protease Activities

In addition, several temperatures including 30, 35, 40, 45 and 50°C were tested to find out the optimum temperature of α-amylase and protease activities. The highest activity of α-amylase was detected at 40°C and the peak of protease activity was found at 45°C, (Figure 2). Enzymes activity level at optimal temperature was 0.015522 and 0.018392 U min^{-1} mg^{-1} protein, respectively.

The Effect of pH on the Inhibitory Activity of Seed Proteinaceous Extracts

Inhibitory activity of seed extracts on α-amylase and protease activities was evaluated in different pH values to study the importance of pH factor in insect midgut. The results signified that the extract of black nightshade could not affect the α-amylase activity (data not shown), so the inhibition assays were carried out with two other plants. The effect of extracts from triticale and rye on α-amylase differed at several pH

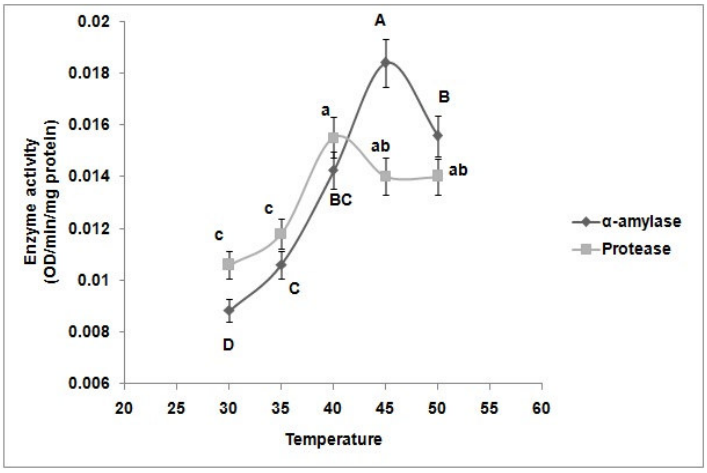

Figure 2. The effect of temperature on potato tuberworm *α*-amylase and protease. Means followed by the same letters are not significantly different by Tukey's test (P< 0.05).

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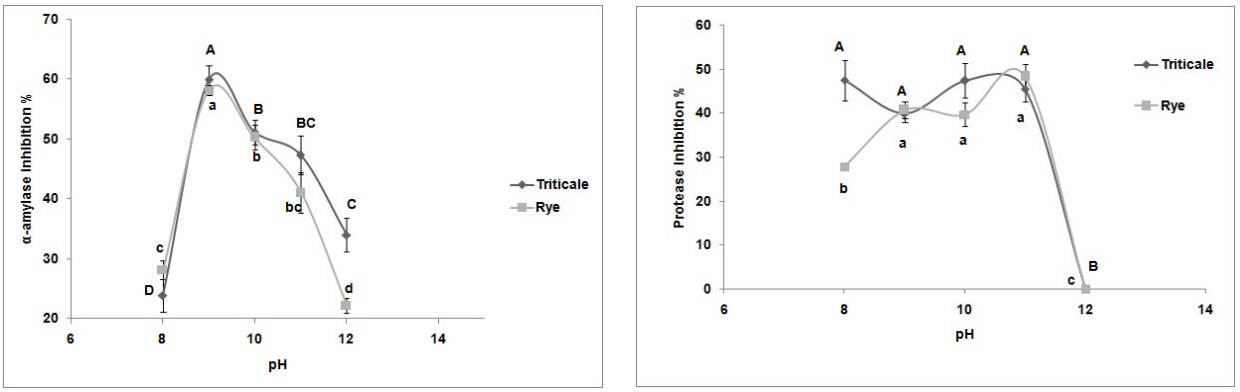
values, so that, the highest inhibition percentage of α-amylase by both extracts was observed at pH 9 (Figure 3-a). Whereas, protease activity by triticale and rye extracts inhibited significantly at 8-11 and 9-11 pH values, respectively (Figure 3-b).

The Effect of Different Concentrations of Seed Extracts on the α-Amylase and Protease Activity

The introductory assay was carried out to estimate the protein concentration of seed extracts in order to start the enzyme inhibition assays. Both extracts showed dose dependent style of inhibition. Various concentrations; 1.5, 0.75, 0.375, 0.187 and 0.093 (mg protein ml^{-1}) of the extract from triticale inhibited α-amylase activity by 61.98, 50.41, 38.81, 25.89 and 19.91% and the extract from rye decreased the enzyme activity by 55.42, 43.34, 32.42, 23 and 20.62% (Figure 4-a). Also protease activity was reduced by the extract from triticale by 55.80, 50.51, 32.01, 23.59 and 11.61% and by the extract from rye by 41.82, 29.68, 20.35, 15.69 and 8.83% (Figure 4-b).

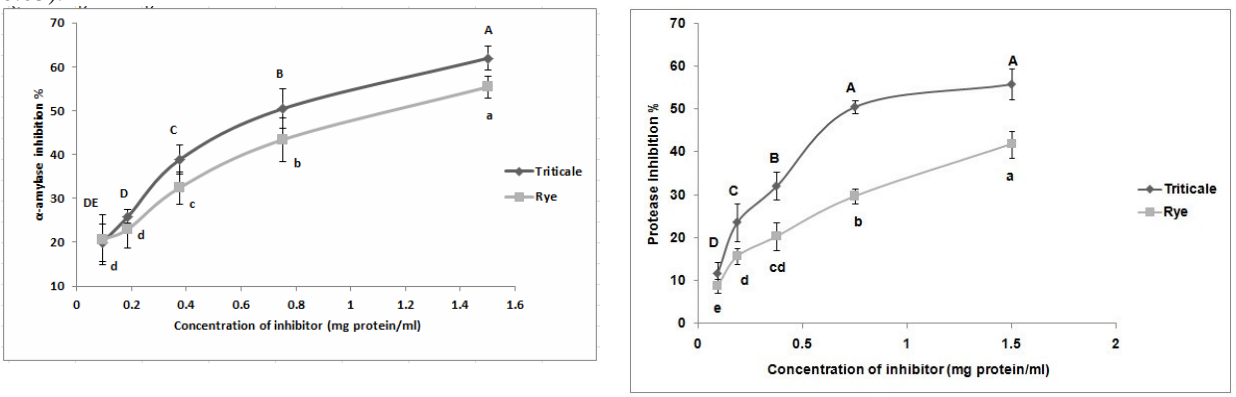
In-gel Assay of the Effects of Seed Proteinaceous Extracts on the *α*-Amylase and Protease Activity

Assays in gel, showed that there were two major isozymes of α-amylase and protease enzymes in the larval gut. When different



(a) (b)

Figure 3. The effect of pH on inhibition of potato tuberworm *α*-amylase (a) and protease (b) by extracts from triticale and rye. Means followed by the same letters are not significantly different by Tukey's test (P< 0.05).



(a) (b)

Figure 4. Inhibition of potato tuberworm *α*-amylase (a) and protease (b) by different concentrations of triticale and rye. Means followed by the same letters are not significantly different by Tukey's test (P< 0.05).

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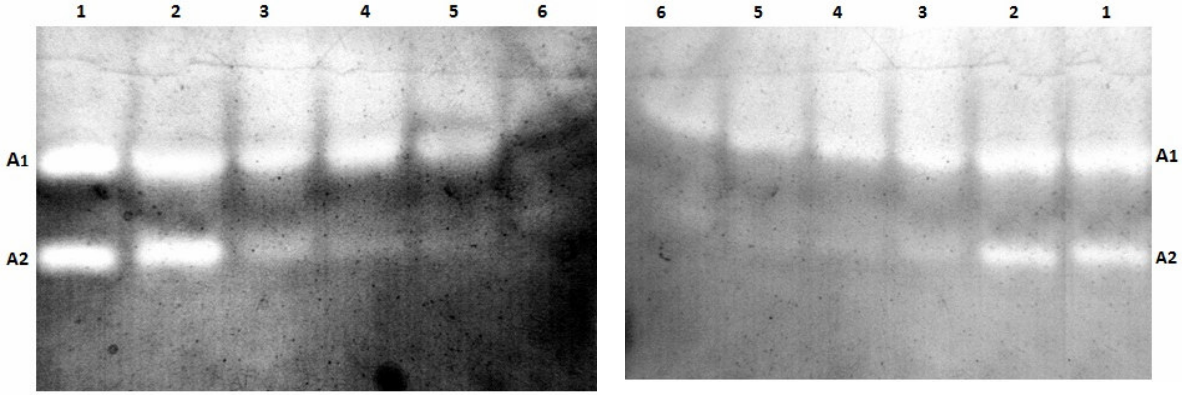
concentrations of seed extracts were used, the intensity of bands differed in both cases. At the lowest concentration of seed extracts (0.093 mg ml^{-1} protein) both bands were faint. At the highest concentration of extract from triticale both bands of α-amylase (Figure 5-a) and one of the two bands of protease (Figure 6-a) disappeared. Whereas, the highest concentration of rye extract only decreased the intensity of bands of both enzymes (Figures 5-b and 6-b).

DISCUSSION

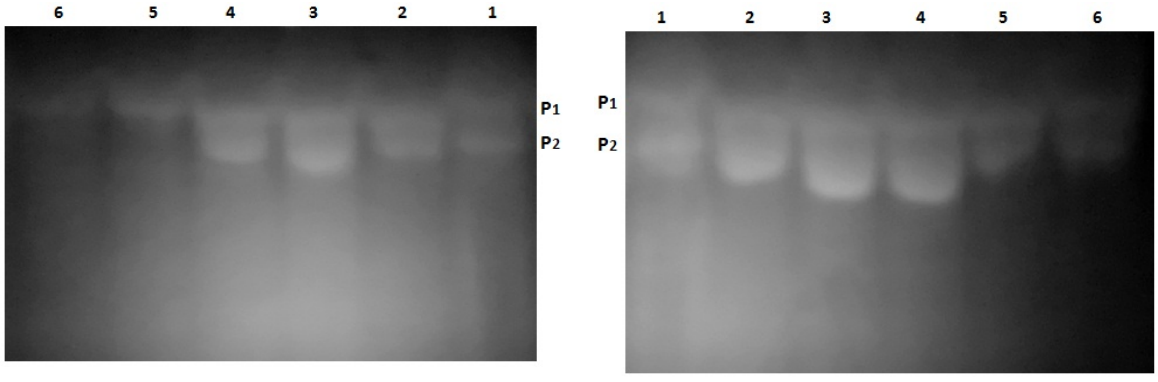
The potato crop is one of the most important food crops, along with rice, wheat and maize all over the world (Ross, 1986; Douches *et al*., 2004). Unfortunately, severe losses may occur in storage, especially in developing countries where low income farmers cannot afford refrigerated storages (Rondon, 2010).

In this study, for the first time, digestive α-amylase and protease enzymes of potato tuberworm were characterized and also the effect of extracts from two plants of cereals and black nightshade which is the wild host of the pest was tested on enzymes activities. Many of Lepidopteran insects live on a polysaccharide-rich diet and require digestive α-amylase to break down and



(a) (b)

Figure 5. In gel inhibition assay of the effect of different concentrations of plant extracts on the α-amylase of potato tuberworm using 0.5% starch as substrate. Lane numbers are as follow: (1) Enzyme extract with no inhibitor, (2) 0.093, (3) 0.187, (4) 0.375, (5) 0.75, (6) 1.5 mg ml^{-1} protein of extract from triticale (a) and rye (b). A1: Alpha-amylase first isozyme, A2: Alpha-amylase second isozyme.



(a) **(b)**

Figure 6. In gel inhibition assay of the effect of different concentrations of plant extracts on the protease of potato tuberworm using 1% gelatin.

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utilize the starch in their food sources. These amylases play a very important role in starch digestion and in insect survival (Borzoui *et al*., 2013).

Plant seeds are known to contain a variety of enzyme inhibitors that are thought to be involved in defense mechanisms against herbivores. Among these proteins, the α-amylase and proteinase inhibitors are found in several legume and cereal seeds. There are many examples where enzyme inhibitors, especially those isolated from cereals including wheat (*Triticum aestivum* L.), barley (*Hordeum vulgareum* L.), sorghum (*Sorghum bicolor* L.), rye (*S. cereale* L.) and rice (*Oryza sativa* L.) seeds inhibit amylase and protease from insect guts (Franco *et al.,* 2002; Valencia- Jiménez *et al*., 2008). Therefore in the current study, two plant seeds from cereals have been tested for inhibitory effects on two digestive enzymes of potato tuberworm larvae.

The acidity of the contents of digestive tract is the main factor affecting the digestive enzymes (Terra and Ferreira, 1994). It has been reported that high levels of acidity in the phytophagous lepidopteran gut is dealing with high levels of tannins in their foods. At low acidity these materials join the insect enzymes which leads to reduced digestion performance. The digestive tract of Lepidopteran insects such as Mediterranean flour-moth *Anagasta kuehniella* Zeller, *Plodia interpunctella* Zeller and many other Lepidoptera is extremely alkaline (Baker, 1989; Sivakumar *et al*., 2006; Amorim *et al*., 2008; Pytelková *et al*., 2009). In the present study, the results of pH assays were in consent with previous assays. As we observed, potato tuberworm α-amylase and protease exhibited increased activity at alkaline pHs 9 and 11, respectively. Also, it was found that pH factor affected the inhibition of α-amylase and protease activity of potato tuberworm by seed extracts. An inhibitor which reduces enzyme activity at the optimum pH of enzyme activity can be used in producing transgenic plants against insect pests. The highest inhibition of α-amylase and protease activity by extracts from triticale and rye was observed at the optimum pH of the enzymes activity. There are many other reports which have confirmed that inhibition manner of digestive α-amylase and protease by seed proteinaceous extracts is pH dependent, e.g., the inhibitory effect of wheat seed extract on digestive α-amylase of *Plutella xylustella* L. (Borzoei *et al.*, 2013), inhibition of proteolytic and amylolytic activity of *Tenebrio molitor* L. by plant proteinaceous seed extracts (Dastranj *et al.*, 2013).

The effect of seed proteinaceous extracts was also dose dependent, as a gradual increase in the amount of the enzyme inhibition was observed along with a gradual increase in the amount of the seed extract concentrations. Mehrabadi *et al.* (2010) found that the effect of triticale seed extract on the α-amylase activity of the Sunn pest (*Eurygaster integriceps* Puton) was dose dependent. They found that the lowest concentration of triticale seed extract (0.25 mg) inhibited about 10% of enzyme activity, while the highest dose of seed extract (1.5 mg) caused 80% inhibition of enzyme activity.

The role and mechanism of action for most of these inhibitors are being studied in detail and their respective genes have been isolated. These genes have been used for the construction of transgenic plants to be incorporated in integrated pest management programs (Lawrence and Koundal, 2002).

In conclusion, since the insects such as potato tuberworm are greatly dependent on α-amylases for their survival and digestion, along with the starch as the main and the most necessary material in their food, amylase could be a good target for insect control through α-amylase inhibitors (Franco *et al*., 2002; Svensson *et al*., 2003; Sivakumar *et al*., 2006). Results showed that, potato tuberworm α-amylases are more sensitive to be inhibited by tested proteinaceous extract from triticale and rye seeds in comparison with protease. Finally, it should be said that triticale and rye seed

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extracts can be used to produce resistant potato crops against potato tuberworm.

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فعالیت مهارکنندگی عصاره های پروتئینی بذر تریتیکاله، چاودار و تاجریزی سیاه روی آنزیم آلفا-آمیلاز و پروتئاز گوارشی بید سیب زمینی

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چکیده

بید سیب زمینی آفت بسیار مخرب گیاهان بادنجانیان در سراسر دنیا می باشد. تغذیه لاروها درون دالان های ساقه، برگ و غده، باعث عدم موفقیت کنترل شیمیایی می گردد، بنابراین باید به دنبال کاربرد سایر روش های کنترلی بود. در سال های اخیر گیاهان متعددی ژن های کد کننده پروتئین های سمی را در راستای مقاومت به آفات دریافت نموده‌اند. در این مطالعه، اسیدیته و دمای بهینه فعالیت آنزیم های آلفا-آمیلاز و پروتئاز گوارشی بید سیب زمینی و اثر مهاری عصاره پروتئینی بذر تریتیکاله: رقم دانکو، چاودار: رقم سناباد و تاجریزی سیاه بر فعالیت این آنزیم ها به ترتیب با استفاده از زیرنهشت نشاسته ۱٪ و آزوکازئین ۲٪، ارزیابی گردید. هر دو آنزیم، در رنج قلیایی بیش ترین فعالیت را داشتند. در سنجش مهار کنندگی، آلفا-آمیلاز توسط عصاره های تریتیکاله و چاودار در اسیدیته ۹ بیش از سایر اسیدیته‌ها مهار شد و تاثیر پذیری پروتئاز در اسیدیته های ۸-۱۱ و ۹-۱۱ اختلاف چندانی نشان نداد. عصاره تاجریزی سیاه فعالیت مهارکنندگی نشان نداد. لذا ادامه آزمایشات با دو گیاه تریتیکاله و چاودار صورت پذیرفت. میزان مهارکنندگی غلظت‌های ۱/۵، ۰/۷۵، ۰/۳۷۵، ۰/۱۸۷ و ۰/۰۹۳ (میلی گرم پروتئین بر میلی لیتر) عصاره ها وابسته به دز بود. بیشینه مهارکنندگی در بالاترین غلظت و کمترین میزان در پایین‌ترین غلظت مشاهده گردید. در ژل پلی آکریل آمید، دو ایزوزایم برای هر دو آنزیم تشخیص داده شد که در بالاترین غلظت عصاره ها، هر دو ایزوزایم حذف گردیده یا از شدت آن ها کاسته شد. این داده ها نشان دادند که پروتئین های موجود در بذر تریتیکاله و چاودار می توانند در تولید گیاه سیب زمینی تراریخت مقاوم به بید سیب زمینی معرفی گردند.

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