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Original Research Article

Induction of new forms of amylases indicates adaptation in *Tribolium castaneum* towards wheat α-amylase inhibitors

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Abstract

α-Amylase inhibitors are extensively found in many plant seeds, tubers and abundant in cereals and legumes. These molecules play key role in plant defense towards pests such as *Tribolium castaneum* which is almost a cosmopolitan pest. In this study, we describe the isolation of α-amylase inhibitors from five seed samples Wheat (*Triticum aestivum*), Soyabean (*Glycine max*), Chana (*Cicer arietinum*), Tur (*Cajanas cajan*), Masur (*Lens culinaris)* and the isolation of α-amylase from *Tribolium castaneum* and its characterization and their interaction under different physicochemical conditions.The α-amylase inhibitors from five seed samples and amylase from *T. castaneum* were extracted with phosphate buffer and the inhibition of α-Amylase activity of *T. castaneum* were measured in % using five seed sample extracts. Furthermore, the patterns of expression of inhibitor fed larvae with control were resolved on Native PAGE and it is found different from control. Highest inhibitory activities were observed for Chana 66.01% and lowest for Soyabean 35.89%. As feeding *T. castaneum* on wheat α-AI, triggers the expression of newer form of α-Amylase, it indicates further investigation to find such a inhibitor which is capable of inhibiting all the isoforms of α-Amylases.
Key Words: α-amylase, α-amylase inhibitors, *Tribolium castaneum, Cicer arietinum.*

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INTRODUCTION

α-Amylase inhibitors are proteinaceous enzyme inhibitors and found in many plant seeds such as legumes, cereals and plant as secondary chemicals and play a key role in plant defense toward pests and pathogens, which cause severe damages to field crops and stored grains. The α-amylase inhibitors inhibits the activity of α-amylases which is essential for the carbohydrate metabolism (Ryan, 1990 and S. Sivakumar *et.al.*2006*)*. In most cases the mechanism of inhibition by amylase inhibitors occurs through the direct blockage of the active center at several subsites of enzyme. Inhibitors exhibiting "dual" activity against mammalian and insect α-amylases establish contacts of the same type in alternative ways (Francois payan, 2004). These inhibitors show different specificities against amylases from different sources. α- amylase (1-4-glucan-4-glucano hydrolases EC 3.2.1.1) constitute a family of endoamylases that catalyzes the hydrolysis of -1,4 linked sugar polymers, such as starch and glycogen into oligosaccharides. The enzyme plays a key role in carbohydrate metabolism of microorganisms, plants and animals. Moreover, several insects, one of those is *T. castaneum* that feed on starchy food during larval and adult stages depends on their amylases for their survival (O. L Franco *et.al.,* 2002). The *T. castaneum* life cycle takes from 40-90 days and the adult can live for three years, is a cosmopolitan pest attacking and causing serious damage to stored commodities such as cereals, flour, meal, cracker, beans, spices, dried pet food, dried flowers, nuts, seeds, chocolates, and even dried museum specimens(Via 1999, Wetson and Rattlingourd2000). Conventional chemical based approaches to control this

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pest have failed due to development of resistance by this pest against many insecticides (Kranthi *et.al.,* 2002). There is an urgent need to develop safe, convenient, environmental, low cost alternatives and considerable efforts have been focused on plant derived materials for potentially useful products as bioinsecticides (Regnault Roger *et.al,* 2002). Plant α-amylases inhibitors show great potentials tools to engineer resistance of crop plants against pests. Here we report the isolation of amylases inhibitors and their inhibitory effect on *T. castaneum* amylases.

MATERIAL AND METHODS

Collection of Seed samples: The five seed samples Wheat (*Triticum aestivum*), Soyabean (*Glycine max*), Chana (*Cicer arietinum*), Tur (*Cajanas cajan*) and Masur (*Lens culinaris)* were collected from local market.

Extraction of amylase inhibitors from seed samples: For extraction of α-amylase inhibitors, with some modification Guzman- Partidas (2007) method was used. Each of the five seed samples, were grinded to obtain fine powder, defatted with acetone. The solvent was removed by filtration and the seed powders were air-dried. These fine powders were overnight extracted with distilled water at 4^0C. The seed extract were overnight kept at 4^0C in 1 M ammonium sulphate 40-60%, and then centrifuged at 15000g for 10 minutes, the supernatant served as source of amylase inhibitors.

Extraction of amylase from *T. Castaneum:* The acetone defatted larvae of *T. castaneum* were homogenized with 0.2 M phosphate buffer having pH 6.9 in a ratio (1:6w/v) to extract enzymes. The suspensions were centrifuged at 10,000g for 10 minutes at 4^oC. The supernatant was used as the source of amylase. The protein concentrations of the larvae were measured for determining specific activity with the method described by Bradford (1976).

Effect of Temperature and pH on enzyme activity: The effect of temperature on activity of *T. castaneum* amylase was examined. It was determined by incubating the reaction mixture at 10, 20, 30, 40, 50, 60, 70, 80^0C for 20 minutes followed by measurement of activity. The Amylase activity was also measured at different pH (ranging from 4-10) using sodium phosphate buffer.

Inhibition assay for *T. castaneum* α –amylase: The inhibition of amylase activity of *T. castaneum* was measured in % using five seed extracts. In each tube 200 ul of seed extracts 1 ml of insect amylase in phosphate buffer was added, incubated at 37^0C for 15 minutes, 0.5ml of 1% buffered starch was added to each tube incubated at 37^0C for 15 minutes. The reaction was stopped by adding 3'5-DNSA (1.5ml), boiled for 10-15 minutes cooled; absorbance at 540 nm was recorded (Bernfeld, 1955). The larvae were feed on diet containing α-AIs, and were investigated for amylase activity and expression pattern of Amylase. Native polyacrylamide gel electrophoresis was carried out according to the method of Laemmli (1970) for amylase control and the amylase from inhibitor feed larvae, gels were prepared at 10%. After electrophoresis, gel was incubated with phosphate buffer at pH 6.9 for 20 minutes, then immersed in 1% starch dissolved in phosphate buffer pH 6.9 for 30 minutes following 2 minutes washing with buffer and then distilled water. Gel was stained with 10 mM iodine in 4 mM potassium iodide for 5 minutes. The excess Iodine was washed off with cold distilled water, amylases were visible against blue background (S. Sivakumar *et.al*., 2006).

RESULT AND DISCUSSION

In the present study, α-Amylase inhibitors from five seed samples were isolated. α- amylase inhibitors are extensively found in many plant seeds and tubers and abundant in cereals and legumes. These molecules play a key role in plant defence toward pest and pathogens. These inhibitors shows different specificities against α-amylases from different sources, inhibitors with a wide specificity spectrum are strongly favoured for insect control (S.Sivakumar*et.al*.2006). The number of α-amylase inhibitors isolated and identified so far is extremely large occurs in microorganisms, higher plants and animals. Proteinaceous α-amylase inhibitors have different polypeptide scaffolds and are grouped by their tertiary structures into six classes lectin like, knottin like, cereal type, kunitz like, ϒ-purothionin-like and thaumatin-like (Francoise, 2004). The α-Amylase from *T. castaneum* was extracted with phosphate buffer 0.2 M, pH 6.9.Several insects, one of those is *Tribolium castaneum* that feed on starchy food during larval and adult stages, depends on their α-Amylases for carbohydrate metabolism, releasing mixture of oligosaccharides for energy production. Due to their importance, different forms of α-Amylases can be found in a unique insect species, to guarantee the digestion process efficiently. (J.E. Baker *et.al.,* 1983). In insects, only α-amylases have been found to hydrolyze long α-1,4 glucan chains, such as starch or glycogen. (Terra *et.al.*, 1996).Amylase activity has been described in several insect orders, including Coleoptera, Hymenoptera, Diptera, Lepidoptera and Hemiptera. (Baker and Woo, 1985, Zeng and Cohen, 2000). The amount of protein in insect extract was estimated and found to be 1.72mg/ml. The effect of temperature on the activity of *T. castaneum* α-amylase was examined showing that these amylases are thermolabile. The highest amylase activity of *T. castaneum* was observed at 40^0C.

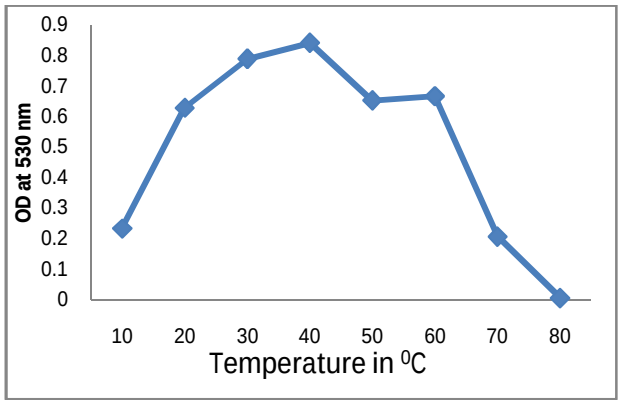

Figure 1: Effect of temperature on α-amylase of *T. Castaneum*

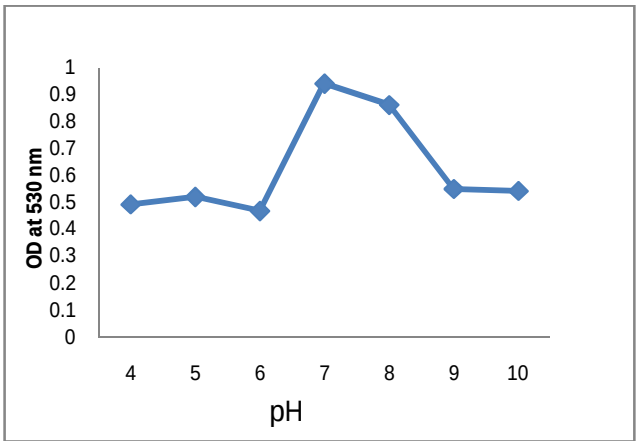

Figure 2: Effect of pH on α-amylase of *T. castaneum*

The effect of pH on the activity of *T. castaneum* α-amylase was also examined showing highest activity at pH 7. These results of temperature and pH were concurrence with most lepidopteron reported so far. (Sivakumar *et al.* 2006; Valencia Jimenez *et. al.*, 2008)

The isolated α-amylase inhibitors were checked for their inhibitory effect on *T. castaneum* α-amylase with respect to physico-chemical properties. All the five seed samples showed the presence of amylase inhibitors. The highest inhibitory activity was shown by Chana (66.01 %) and the lowest inhibitory activity by Soyabean (35.89 %).

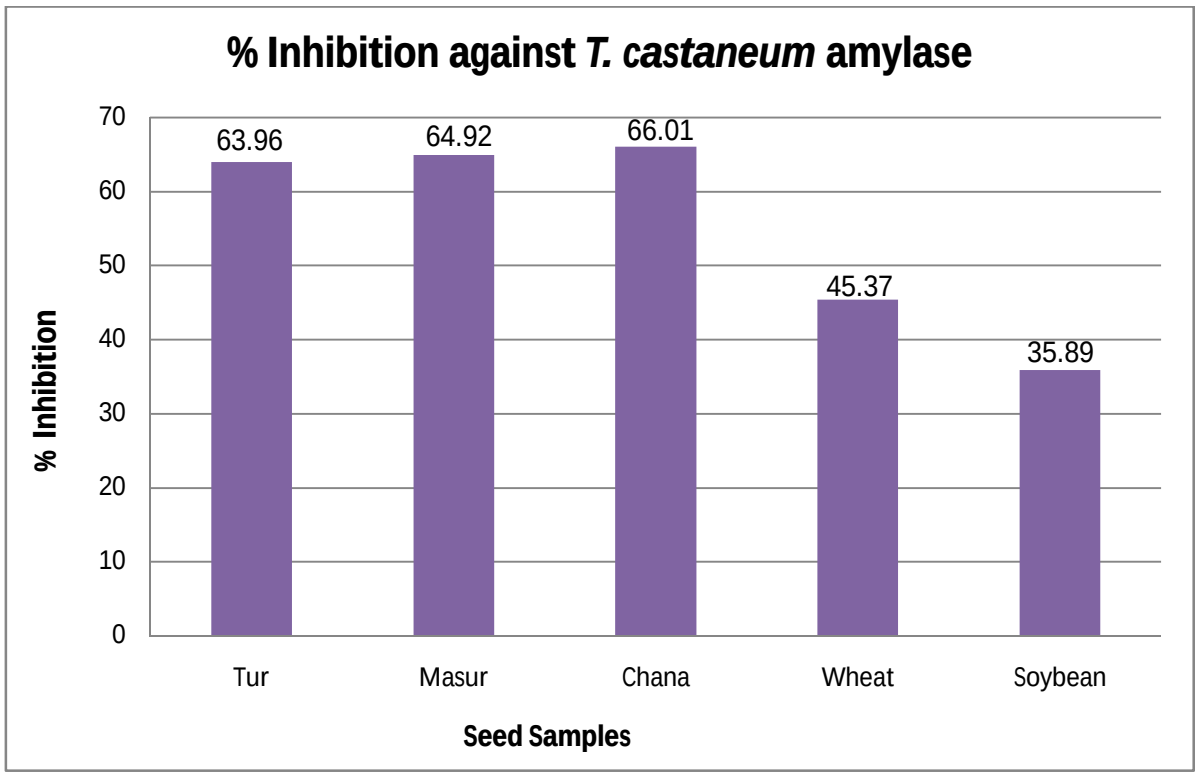

Figure 3: % Inhibition against *T. castaneum* amylase

The differential inhibitor potential of the four amylase isoinhibitors of wheat flour to the amylases of rice weevil *Sitophilus oryzae* and red flour beetle, *Tribolium castaneum* (Herbst) was studied (Feng *et.al.*, 1996). The zymogram analysis shows that the pattern of expression obtained under the influence of wheat inhibitor fed larvae was different from normal this is due to expression of other isoforms, when one isoform of amylase is inhibited

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by wheat α-AIs then other isoforms are expressed, this indicates its adaptation.

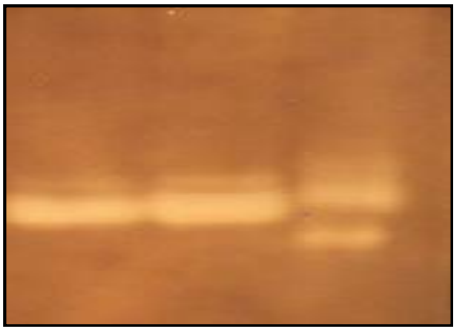

Figure 4: Native PAGE analysis of amylase. (Lane1 and 2 are control; 3 is Inhibitor fed larvae)

Since plant proteinaceous inhibitors of proteases and amylases play protective role against the attack of insects and as they are bioinsecticides, this study provide important information that could contribute to pest management programs.

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