Trends in Life Sciences
An International Peer-Reviewed Journal
www.sciencejournal.in

INHIBITORY POTENTIAL OF SORGHUM α AMYLASE INHIBITOR ON THE DIGESTIVE ENZYME OF RED FLOUR BEETLE, *TRIBOLIUM CASTANEUM*

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ABSTRACT
The inhibitory potential of purified α amylase inhibitors of sorghum on the digestive enzyme of *Tribolium Castaneum*, a devastating insect pest of economic importance have been studied. Qualitative and quantitative differences in α amylase of *Tribolium Castaneum* upon feeding on natural and artificial diets were evident. Natural diets were legumes like Black gram, Pigeon Pea, Red lentil, Cowpea and Field bean. One hundred larvae were transferred to each of the above mentioned diets and also chickpea flour based artificial diet. Amylase activity and isoform patterns varied depending on host plant and/or artificial diet. Artificial diet- fed *Tribolium Castaneum* larvae had comparatively high amylase activity and several unique amylase isoforms might be due to its nutritionally balanced composition. On the other hand, total carbohydrates were the highest in chick pea and the lowest in red lentils while that of protein was the highest in black gram and the lowest in field bean. Correlation of amylase activities of *Tribolium Castaneum* with carbohydrate content of various diets suggested that *Tribolium Castaneum* regulates the levels of these digestive enzymes in response to composition of the diet. These adjustments in the digestive enzymes of *Tribolium Castaneum* may be to obtain better nourishment from the diet and avoid toxicity due to nutritional imbalance. An investigation of the differences in enzyme levels in response to macronutrient balance and imbalance highlight their importance in insect nutrition.

KEYWORDS: α amylase, Amylase inhibitors, Diet, Host plants, *Tribolium Castaneum*

INTRODUCTION

Pest insects affect food output directly by reducing the quality and quantity of the crop produced, or indirectly by serving as vectors of plant diseases. The red flour beetle, *Tribolium castaneum* (Herbst) is a serious pest of stored grains and products (Zettler, 1991). Conventional chemical based approaches to control this pest have failed due to development resistance against many insecticides (Kranthi *et al.*, 2002). Therefore there is an urgent need to develop safe, convenient, environmental and low-cost alternatives. Considerable efforts have been focused on plant derived materials for potentially useful products as bioinsecticides (Regnault Roger *et al.*, 2002). Development of resistance by pests and vectors against plant has been reported (R. Jbilou *et al.*, 2007). Bioinsecticides may be more rapidly degraded in the environment than synthetic compounds (Koul and Dhawiwal, 2001). Noticeable work has been done on interaction of insect proteinases with plant proteinases inhibitors with the objective of identifying potential inhibitors insect proteinases. (Giri et al., 1998; Giri *et al.*, 2003; Chougule *et al.*, 2005; Damle *et al.*, 2005; Srinivasan *et al.*, 2005; Tamhane *et al.*, 2007). The other important class of digestive enzymes from insects includes amylases. We have, therefore, focused our attention on *Tribolium castaneum* amylases and their inhibitors from plant sources as well. Amylases (α- 1, 4 glucan 4 glucanohydrolases; EC 3.2.1.1) catalyze hydrolysis of α-d-(1, 4) glucan linkage in starch components, glycogen and various other related carbohydrates to serve as an energy source (Franco et al 2000). Amylases are important digestive enzymes of many insects that feed exclusively on starchy seeds during larval and/or adult stages (Pereira *et al.*, 1999). In this regard, hymenopteran, dipteran and hemipteran amylase have been extensively investigated (Bandani *et al.*, 2009). Few reports on Lepidopteran amylases are available (De Sales et al 2008). Hitherto scarce information is available about amylases of *Tribolium castaneum* and their interaction with plant proteinaceous inhibitors. Nutritional regulation by an insect represents the integrated outcome of a highly complex set of interacting processes. Acquisition and allocation of different nutrient molecules required for survival and reproduction is central to these processes (Simpson and Raubenheimer, 1999). All these nutrient molecules are present in various ratios and concentrations within foods. They may also contain some harmful non-nutritive molecules. Of these, for availability of the major nutrients, regulation of enzymes gains priority.

In this present study we found (i) Biochemical properties of *Tribolium castaneum* (ii) Changes caused by feeding on various host plant tissues. Quantitative estimation of amylase activity was performed using enzyme assays while qualitative analysis was carried out using electrophoretic separation followed by in-gel visualization of amylase isoforms on polyacrylamide gels. As a path forward, amylase inhibitors from seeds of sorghum (*Sorghum bicolor* L.) were partially purified and their potential for inhibiting amylases of *Tribolium castaneum* larvae feeding on various host plants was established.

MATERIALS AND METHODS

Insect Culture

Cultures of the red flour beetle, *Tribolium castaneum* used for the study were established from an infested batch of rice purchased from local market in Akola. Cultures were maintained on crushed rice under ambient laboratory conditions (Rearing temperature was 29±1ºC and relative humidity 65±5% with 12 hours light). (Sadiya Kanvil *et.al*, 2006) and subsequent generations were used for feeding assays.

Feeding assay

Newly emerged larvae were allowed to feed on fresh legume pods Black gram (Vigna mungo L.) Pigeon Pea (*Cajanus cajan*) Red lentil (*Lens esculenta*) Cowpea (*Vigna catjang*) and Field bean (*Dolichos lablab*). One hundred neonates were transferred to each of the above mentioned diets and also chickpea flour based artificial diet. Diets were changed daily and never allowed to dry or be eaten completely. Insect cultures were maintained from first generation larvae to adult and second generation larvae were used for assay of amylase activity.

Estimation of total carbohydrates and protein

Total carbohydrate content in the diets used for feeding assays was estimated by phenol sulphuric acid method using glucose as a standard (Dubois et al., 1956). Soluble protein concentration in samples was determined by the Bradford method (Bradford, 1976). Bovine serum albumin was used as a standard.

Extraction and assay of *T. Castaneum* α amylase

The acetone defatted larvae of *T. Castaneum* were homogenized with 0.2M succinic acid buffer (1:5 wt/vol) pH 4.5. The extract was centrifuged at 10,000xg for 10 min at 4ºC. Supernatants were used as source of amylase. (Figueira *et al*, 2003) Endogenous amylase activity of all the diets was also estimated from proteins extracted using the procedure mentioned above. Amylase activity in crude homogenates and all host plant tissues was assayed by the dinitrosalicylic acid (DNSA) method (Bernfeld, 1955) using 0.25% soluble starch as substrate. Crude enzyme extract was incubated with 150 µl soluble starch prepared in sodium phosphate buffer (pH 6.9) containing 10 mM NaCl for 20 min at 37 °C.

The reaction was stopped by the addition of 500 µl DNSA and heating the tubes in a boiling water bath for 5 min. The absorbance was read at 540 nm after cooling on ice. A standard curve of absorbance against amount of maltose released was constructed to calculate amylase activity units. One amylase unit was defined as the amount of enzyme required to release 1 µM maltose/min at 37 °C under the given assay conditions.

Extraction and assays of sorghum seed amylase inhibitor

Whole seeds of sorghum were ground in a grinder to obtain a fine powder that was defatted with hexane, dried and ground again. It was stirred in six volumes of 0.1 M NaCl and 1% PVP for 2.5 h. Suspension was centrifuged at 12,000 × *g* and pH of the supernatant was adjusted to 7.0 with 1 M NaOH. Ammonium sulphate fractionation (40%) yielded a precipitate that was dissolved in and dialyzed against distilled water and centrifuged. An additional step of heating the partially purified extract at 60 °C for 10 min was performed. Supernatant containing partially purified inhibitors was used further for inhibitor assays (Giri and Kachole, 1996; Giri and Kachole, 1998).

Partially purified sorghum amylase inhibitor (6 µg) was pre-incubated with 20 U of amylase for 20 min and residual amylase activity was determined. Percent inhibition was calculated on the basis of residual amylase activity. The assays were performed in triplicate and repeated at least thrice.

In-gel visualization of amylase isoforms

The visualization of amylase activity present in crude homogenates of larval midguts and various diets was carried out using native PAGE (7%) with a stacking gel (4%) (Laemmli, 1970). The resolving gel buffer contained 1.5 M Tris–HCl (pH 8.8) while the stacking gel buffer contained 1.0 M Tris–HCl (pH 6.8). The electrode buffer comprised 25 mM Tris and 250 mM glycine (pH 8.3). The gels were run until the blue dye reached the end, approximately 3.5 h at a constant voltage of 200 V. For visualization of amylase activity, the gel was incubated in starch (1%) prepared in 0.02 M sodium phosphate buffer (pH 6.9) containing 10 mM NaCl for 30 min at 37 °C, briefly rinsed in water and amylolytic activity was stopped by transferring the gel to the staining solution (10 mM I_2 in 14 mM KI) for 5 min. Excess I_2 was washed off with water.

Light bands against a dark background indicated presence of active amylase isoforms. In-gel inhibition assays were performed by pre-incubating the gel for 30 min in partially purified sorghum inhibitor and then in starch (1%) for 30 min at 37 °C followed by staining solution to visualize the amylase activity bands.(Sivakumar *et.al*, 2006).

RESULTS

Dietary carbohydrate and protein content

Artificial and natural diets used for feeding assays of *T. Castaneum* were analyzed for total carbohydrate and soluble protein content (Figure 1).Total carbohydrate content was the highest in chick pea and the lowest in red lentil while that of protein was the highest in black gram and lowest in field bean.

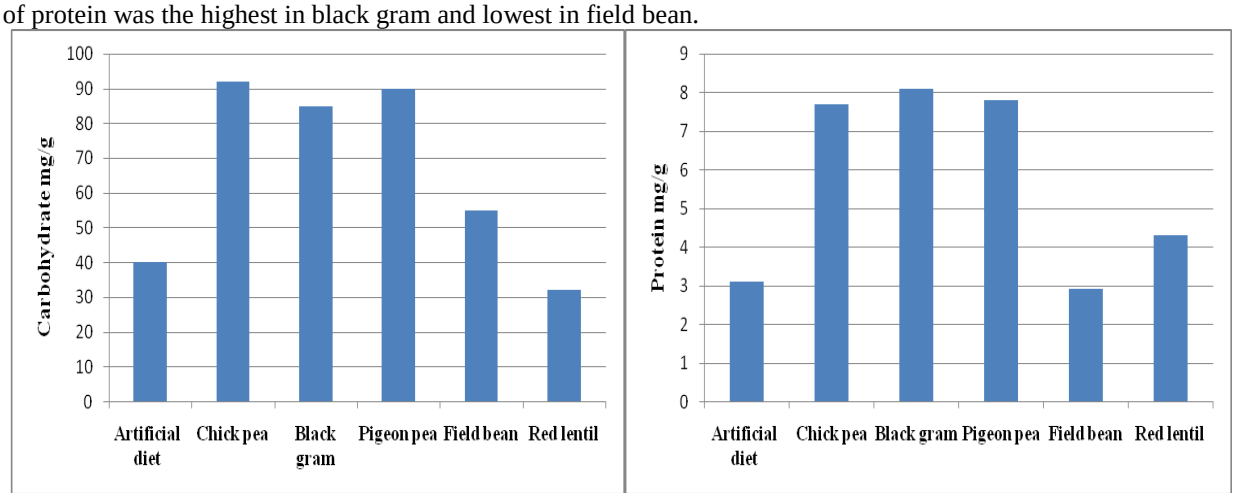
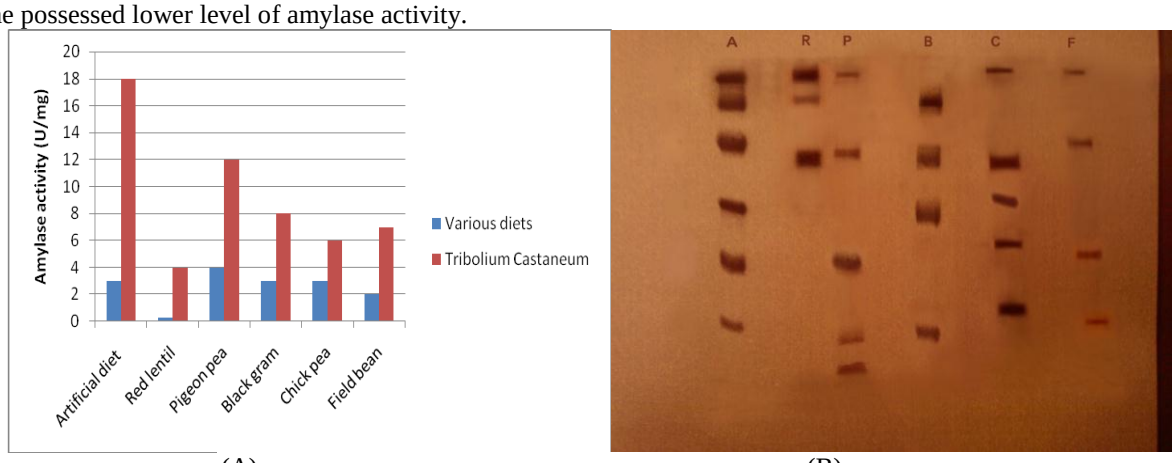


Figure1. Total carbohydrate and soluble protein content of the diet used for feeding assays. Carbohydrate and protein content of the diet used for feeding assays of *T. Castaneum* were estimated as mentioned in section 2.

α Amylase activity of *T. Castaneum* fed on various diets

Various isoforms of amylase obtained from larvae fed on artificial diets, red lentil, pigeon pea, black gram, chickpea, and field bean were visualized after resolution in native polyacrylamide gel (7%). Zymograms showed the presence of seven isoforms of amylase in various diets fed T. Castaneum gut extracts. The third amylase isoform was common in the gut extract of larvae fed on all diets. The seventh amylase isoform was unique for pigeon pea fed larvae while the first amylase isoform was absent in black gram fed larvae. Pigeon pea and chick pea fed T. Castaneum larvae showed five amylase isoforms while black gram and field bean(A2,A3,A4,A6) &(A1,A3,A5,A6) amylase isoforms respectively (figure 3 B). Quantitative estimation of amylase activity of gut tissue revealed significantly higher amylase activity in the gut extract of larvae fed on artificial diet than other natural diets. Amylase activity of larvae feeding on pigeon pea was particularly higher as compared to the other natural diets used in this study (fig.3 A).In contrast, field bean fed larvae possessed lower level of amylase activity.



(A) (B)

Figure 3. Tribolium Castaneum amylase activity profile. The larval gut extracts and diets used for feeding assays of T. *Castaneum* were assayed for amylase (A). Midgut extracts of *T. Castaneum* larvae fed on artificial & natural diets were separated on native polyacrylamide gels (7%), visualized for amylase (B). A= Artificial Diet, R= Red Lentil, P= Pigeon Pea, B= Black Gram, C= Chick Pea, F= Field Bean.

Inhibition of *Tribolium Castaneum* α amylases

The effect of partially purified Sorghum inhibitors on digestive amylases of *T. Castaneum* on various diets was determined by enzyme assay (figure 4). The results depicted that midgut amylase activity was efficiently inhibited by

sorghum amylase inhibitor. For inhibitory assays, various amounts of sorghum amylase inhibitor were titrated against known amylase activity units (20 U) from artificial diet-fed larvae to determine effective concentrations required for maximum inhibition. Assays revealed that amylases (20 U) from artificial diet-fed larvae could be inhibited up to 84% with partially purified amylase inhibitors from sorghum (6 µg). The gut amylase activity inhibition across the different hosts ranged from 90 to 96%.The highest inhibition was against gut amylase from the larvae fed on field bean was 96%. Amylases of pigeon pea fed larvae showed the least inhibition (93%) amongst the natural diets.

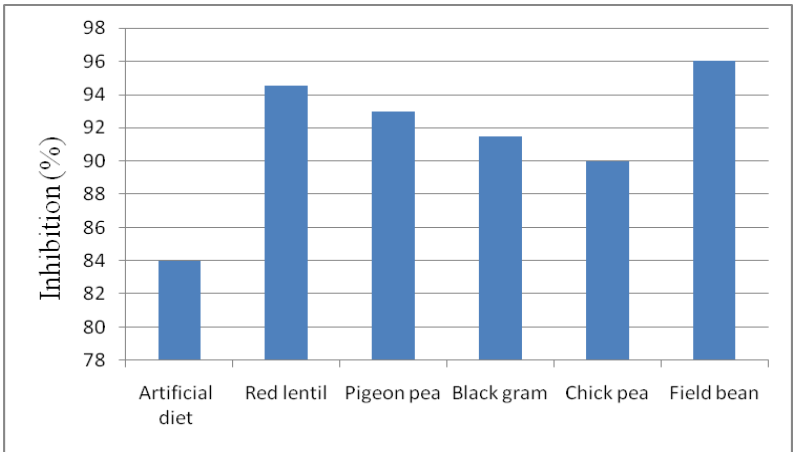


Figure 4. Inhibition of *Tribolium Castaneum* amylase activity by sorghum amylase inhibitors. Percent inhibition of amylase activity was assayed with sorghum inhibitor.

Discussion

In the present study, we attempted to characterize *T. Castaneum* digestive amylases in response to feeding on various host plants. In insects, density of diet consumed, temperature and pH are a few important factors that have a direct impact on activity of digestive enzymes responsible for providing energy and nutrition to the growing larvae (Sivakumar *et al.*, 2006). In our earlier study, we observed that *T. Castaneum* amylases exhibited increased activity at a highly alkaline pH and were active and stable in a broad temperature range 30–40 °C (Khan *et.al.*, 2010).

T. Castaneum being a devastating pest highly dependent on starchy food. We studied gut amylase activities of *T. Castaneum* feeding on legumes. Natural diet-fed *T. Castaneum* displayed three times lower gut amylase activities than those fed on artificial diet. Within the natural diets, pigeon pea fed *T. Castaneum* gut amylase activity was highest that of other natural diets fed larval gut amylases. High amylase activity in the artificial diet-fed larvae might be due to its nutritionally balanced composition. Artificial diets are usually complete foods designed for high insect performance and usually considered to be better than natural diets (Hari *et al.*, 2007). Such discrepancy in enzyme activities of artificial and natural diet-fed insects has been previously observed in labial salivary glucose oxidase of *Spodoptera exigua*. Artificial diet-reared *S. exigua* showed thirty times higher activity than was observed in insects reared on the legume, *Medicago truncatula* (Merkx-Jacques and Bede, 2005). In the present study, artificial diet-fed *T. Castaneum* displayed a unique pattern of amylase expression with six amylase isoforms. While seventh amylase isoform was unique for pigeon pea fed *T. Castaneum* larvae. All natural diets used in the present study showed the presence of two major common amylase isoforms (A3 and A6).

The soluble protein estimations of various diets indicated that black gram had the highest protein content (mg of soluble protein/g) and artificial diet had moderate (mg of soluble protein/g) protein content. On the other hand, total carbohydrates were the highest in chick pea, followed by pigeon pea and black gram. It has been reported that the efficiency of conversion of digested food into larval biomass depends on the activity of digestive enzymes (*Lazarevic et al.*, 2004). Red lentils fed T. Castaneum gut amylases were comparatively lower in concentration while surprisingly; higher amylase activities were present in protein and carbohydrate poor diets (Artificial diet). Considering these correlations between gut amylases with the diet carbohydrates, it seems that there exists an insect mechanism to precisely detect the diet contents and adjust the levels of these important digestive enzymes. The possible explanation for this could be that when carbohydrates are high even lower amounts of gut amylases seem to be sufficient for metabolism (Kotkar *et.al.*, 2009). In case of a control diet that has a particular balance of carbohydrate content, *T. Castaneum* gut amylase levels and their isoforms are the highest as compared to natural diets. While developing sustainable approaches for controlling coleopteran insects, it becomes essential to characterize digestive enzymes and their inhibitors from different sources. Studies indicate that specificities of insect amylases and plant amylase inhibitors

vary (Franco *et al.,* 2000). However, amylase inhibitors of different coleopteran insects have not been explored to that extent. In the present studies, sorghum amylase inhibitor was selected as it has: (i) three isoforms SIα-1, SIα-2 and SIα-3 that are reported to inhibit insect amylases and (ii) low activity against human salivary amylase (Bloch and Richardson, 1991). Our experiments indicated that partially purified sorghum inhibitors were active against most of the amylase activity from *T. Castaneum* fed on natural diets, whereas inhibition was partial in artificial diet-fed *T. Castaneum* gut amylase. *T. Castaneum* gut amylase inhibition was approximately 96% when the insects were reared on natural hosts and approximately 84% when insects were reared on artificial diet.

The correlation between *T. Castaneum* digestive enzymes and nutrient composition of the various diets reflects the adaptive nature of the polyphagous pest *T. Castaneum* gut amylase is regulated based on diet composition. A detailed biochemical and molecular analysis of gut amylase isoforms upon exposure of the insect to a particular diet will highlight their specific roles. It would be interesting to focus on amylase inhibitors from sorghum to devise a defence strategy against this highly devastating insect pest.

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