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Azadirachta indica derived compounds as inhibitors of digestive alpha-amylase in insect pests: Potential bio-pesticides in insect pest management

Amtul Jamil Sami

Institute of Biochemistry and Biotechnology, University of the Punjab, Lahore, Pakistan

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ABSTRACT

The study was undertaken to determine the role of Neem (Azadirachta indica) derived compounds as bio-safe pesticides, by studying the inhibition of a digestive enzyme, alpha amylase in insect pests. Three insects were selected for the study (1): Tribolium castaneum, (2): Aulacophora foveicollis and (3): Oxya chinensis. The three insects were tested for starch hydrolyzing activity in the salivary glands and gut. Two groups of compounds, Saponin and Azadirachtin were isolated from Neem plant and were used for the inhibition of amylase enzyme. Saponins and Azadirachtin were identified as enzyme inhibitors in T. castaneum while no alpha amylase enzyme inhibition was observed for O.chinensis, A.foveicollis. To determine the effect of Saponin and Azadirachtin on the expression of alpha amylase enzyme activity, T. castaneum were fed on diet mixed with Neem leaf powder, Azadirachtin and Saponins for 4 days. Proteins were then extracted and tested for total amylase activity. There was a decrease in the amylase activity. After 4 days mortality rate was 50%.

Keywords: *Azadirachta indica,* inhibitors, amylase, insect pests, *Tribolium castaneum, Aulacophora foveicollis and Oxya chinensis.*

INTRODUCTION

Starch is a highly organized mixture of two carbohydrate polymers, (1): amylose and (2): amylopectin. The two polymers are synthesized by plant enzymes and simultaneously packed into dense water-insoluble granules [1]. Starch is hydrolyzed by amylases present in animals, plants and microbes. Alpha amylases play a pivotal role in carbohydrate metabolism in microorganisms, plants and animals. α--1, 4-glucans-4-glucohydrolase comes under the family of endo-amylases which catalyzes the hydrolysis reaction of α-D-(1, 4) glucan linkages in carbohydrates (indicating starch, glycogen) [2]. This class of glycohydrolases is very important for the metabolism of carbohydrates in plant and animal kingdoms [3]. The enzymatic mode of action of alpha -amylase has not yet been completely understood. The reaction of α-amylase enzyme proceeds via double displacement mechanism. The catalytic residues of α-amylase are conserved among all enzymes [4]. Many insects such as *Tribolium castaneum, Aulacophora foveicollis* and *Oxya chinensis* feed on carbohydrates (starch and cellulose) during different stages of their lives. *T. castaneum* (Herbst) is one of the major starch dependent storage pests, which is responsible for the severe loss of stored grains [5]. As the insect is totally dependent on starch hydrolyzing enzymes for its survival, alpha amylase enzyme is a good candidate for the inhibition by plant derived molecules, for their defense. Enzyme inhibitors for Alpha amylase chitinase, ß-1, 3-glucanases, defending digestive enzymes are present in many plant seeds and tubers, especially in cereals and legumes. These inhibitors could be proteins and peptides, involved in plant defense including lectins, arcelins, etc. [6]. Plant derived glycohydrolase inhibitors have been isolated and characterized [7, 8]. Botanicals are insecticidal because they form complexes with digestive enzymes, as they are stable and dissociate slowly. Inactivation of digestive enzymes by inhibitors results in blocking of gut amylases including proteases, cellulases, etc., leading to poor nutrient utilization, growth retardation and death [9, 10].

A. indica (Neem) a local plant is a common bio pesticide [11-13]. It has been reported that *A. indica* contains bitter compounds with anti-feedant property that can interfere with hormonal processes in insects [12, 14]. Neem-based insecticides are not toxic to humans and many beneficial arthropods. Neem derived insecticides are being given a thought to replace synthetic insecticides in insect pest management programs [15] because they are environmental friendly.

There are a number of reports on inhibition of insect amylases by the extracts from different plants including Neem (16-19). The role of plant derived molecules has not been investigated, individually. Here, in this report, two Neem derived compounds (Azadirachtin and Saponin) were studied, for the inhibition of alpha amylase of three important insect pests (1): *T. castaneum, (2): A. foveicollis* and (3): *O. chinensis*. Results from this study could be useful in understanding the mechanism of inhibition of Azadirachtin and Saponins for insect pest alpha amylases.

MATERIALS AND METHODS

All the reagents used were of analytical grade. The experiments were repeated twice in triplicates.

Isolation of Neem derived compounds

For the extraction of Azadirachtin from Neem seed oil, the method used was as described by Dai *et al.,* 1999, 2001 [20, 21]. 100ml of oil was defated with 100 ml of diethyl ether; lower layer of the extract was extracted and washed with 200 ml of methanol. The extract was dried in the dark overnight. A yellow liquid was obtained which was dissolved in dichloromethane. The solution was tested for the presence of Azadirachtin, using vanillin reagent, based on the method, described by Dai *et al.,* 2001 [21]. A range of 10 to 100 µl of Azadirachtin was used for estimation. The amount of Azadirachtin was estimated by using a standard curve. An absorption maxima for vanillin Azadirachtin in complex was determined on spectrophotometer to confirm the presence of Azadirachtin [20, 21]. For the isolation of Saponin, Neem leaf powder was purchased from local shop in Anarkali Lahore. 6gm of Neem leaf powder was defatted with di-ethyl ether and Saponins were extracted in 100 ml of 50% methanol. The extract was filtered and methanol was evaporated. The aqueous extract was mixed with 100ml of n-butanol and after thorough shaking; top butanol layer containing saponins was separated by using a separating funnel. The extract was dried and white powder was dissolved in 5 ml of distilled water. The extract was tested for soap like activity.

Sample Collection and Enzyme Assay

The insects were collected from the local vegetation and food stores. The insects were weighed 2g and were homogenized in 40ml of 0.5M Tris-HCl buffer pH 8.5 and centrifuged at 10,000Xg for 10 minutes at 4°C. The supernatant was stored in 1ml aliquot in eppendorf tubes and was used as a source of enzymes. The substrate agar plates constituted of 2g agar, 1 g starch was dissolved in 100ml of buffer 0.1M (pH 4.0 citrate buffer / pH 8.0 Tris–HCl) and 20 ml of the dissolved agar was poured in a petri plate. After setting of the Agar, a hole was punched in the center of the plate. To check the presence of enzyme activity in the insects, 50 µl of the enzyme sample was placed in the center of the hole and plate was incubated at 50°C overnight. Next morning plates were stained with iodine reagent. The enzyme activity was identified by the appearance of clear zone (hydrolyzed starch) against blue area (un-hydrolyzed starch).

For inhibition studies, 50 µl of inhibitor (Azadirachtin and Saponin) was placed in the hole on the agar plate and incubated at 30°C for 20 minutes and then 50 µl of enzyme solution was added in the hole, as described above. After incubation overnight, plates were stained with the iodine reagent. Difference between the area of hydrolyzed and un-hydrolyzed starch for the test and control was compared. Effect of Neem derived compounds was also determined on human salivary amylase activity. Human saliva was collected after meal in a sterile vial and diluted 20 times with distilled water and used as a source of enzyme.

Bioassays for Inhibition of Amylase enzyme

To check the effect of Neem derived compounds on the level of expression of digestive enzymes, insect's larvae and adult insects were fed with a diet mixed with Neem leaf powder, Saponins and Azadirachtin for 4 days. The total soluble cellular proteins were extracted and tested for the level of expression of alpha amylase enzymes by the agar substrate plate method.

RESULTS AND DISCUSSION

Screening of Amylase activity in the insects

Starch is a major constituent of food grain among cellulose and xylose. A number of insect pests rely on cellulases and amylases for utilizing alpha and beta polymer of glucose, as a source of carbohydrates. Starch hydrolyzing enzymes are one of the major glycohydrolases produced by the insect. In the study three insect pests were used (1): *T. castaneum,* an insect in stored grains. (2): *A. foveicollis,* a pest of green vegetables and (3): *O. chinensis,* rice

grasshopper. All the insects were screened for alpha amylase activity. Results are shown in Figs 1-3. *O. chinensis* mainly lives on cellulose and starch produced by the rice plant. Starch hydrolyzing activity was checked at acidic pH (4.0) and alkaline pH (8.0) for the gut and salivary gland tissues. It was observed that amylase activity produced by *O.chinensis* was acidic, as in case of cellulose hydrolyzing enzyme activity [22]. Previously, it was reported that cellulose hydrolyzing enzyme present in *O. chinenesis* had an acidic pH optimum [22], for the salivary glands and gut enzymes. The amylase activity present in the salivary glands and the gut is active at acidic and alkaline pH values but shows maximum activity at acidic pH value (Fig. 1). Thus, the glycohydrolases of *O.chinensis* are acidic in nature.

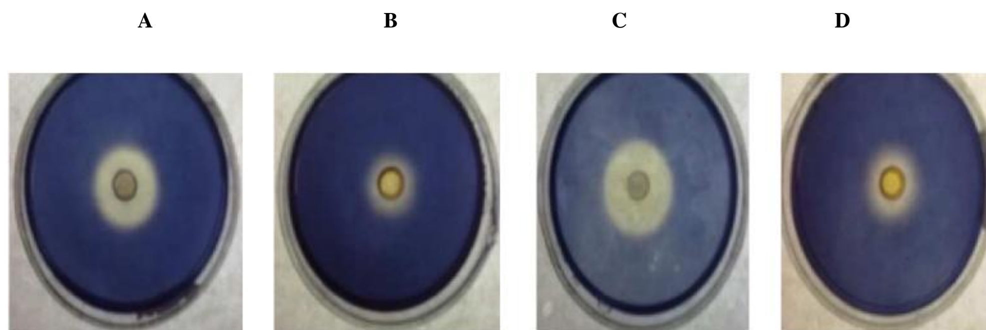

Fig 1: Screening of *Oxya chinensis*, for amylase activity in salivary gland and gut extracts on starch agar plates. A & B shows amylase enzyme activity from salivary gland extract (pH 4.0 and 8.0). C & D shows amylase activity from gut extract (pH 4.0 and 8.0).

Starch hydrolyzing activity of *A. foveicollis* was active at acidic as well as alkaline pH values. Results are shown in Fig.2 (A and B). Previously, we have reported that *A. foveicollis* produces alkaline cellulase hydrolyzing activity [23]. Slightly higher enzyme activity was observed at alkaline pH value (Fig 2). For *A. foveicollis* we have earlier reported that it produces alkaline cellulose hydrolyzing enzyme activity [23], thus it had an alkaline glycohydrolase enzyme system. Same is the case with *T. castaneum;* it had both alkaline cellulases and alkaline amylases [24, 25].

Fig 2: Screening of *A. foveicollis* total soluble proteins, for amylase activity in the crude body extract (A) pH 4.0 (B) pH 8.0, on agar starch plates.

Fig 3: Detection of alpha- amylase activity of *T. castaneum*, in the crude body total soluble proteins (A) pH 8.0 (B) pH 4.

Starch hydrolyzing activity present in the crude enzyme extract of *T. castaneum* was subjected to ion exchange chromatography on a CM Sepharose column (data not shown). A single band was appeared on zymogram after non-denaturing PAGE (Fig 4 a,b). This indicates that the enzyme was in its native form. In *Tribolium genome,* there is only one gene for alpha amylase enzyme activity. 261

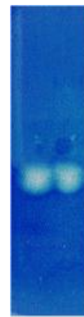

Fig 4: Zymogram of amylase of *T. castaneum shows* a single band for enzyme is visible on the zymogram after non-denaturing PAGE from the crude (a) and purified enzyme (b).

Inhibition of amylase by Neem derived inhibitors

Inhibition studies for amylase enzyme activity of *T.castenum, O.chinensis* and *A.foveicollis* were conducted on substrate-agar plates, as described in material and methods. It was observed that there was no inhibition of starch hydrolyzing activity of *A. foveicollis and O. chinensis* (Fig 5) while *T. castenum* alpha amylase was inhibited, at larval and insect stages (Fig 6).

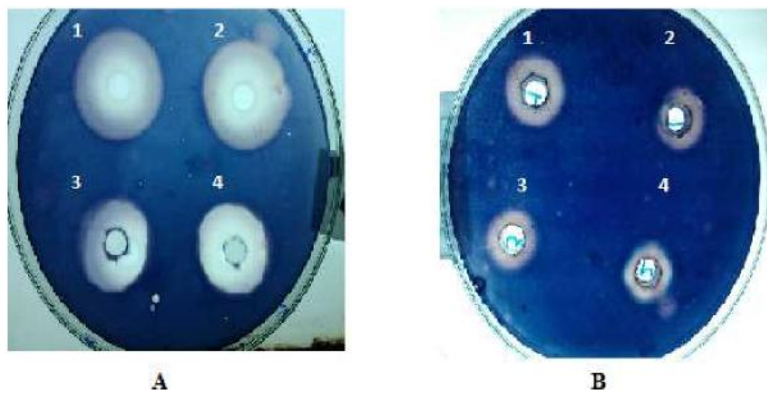

Fig 5: Alpha amylase of *A. foveicollis* (A) and *O. chinensis* (B). 1: control; 2: enzyme with crude Neem leaf extract 3; enzyme with Azdirachtin; 4: enzyme with Saponin. No inhibition is visible in any sample in each case.

The purified amylase activity of *T. castenum* larvae and insect was mixed with the inhibitor (1): Azadirachtin and (2): Saponins. Characterization of amylase inhibitors has been reported by a number of workers. The characterization of a novel Legumin alpha amylase inhibitor from Chickpea *(Cicer arietinum* L.) has been reported [8]. The inhibitory effect of Azadirachtin was also checked for human amylase. Results are shown in Fig. 6. It was observed that the Azadirachtin inhibition effect was around 20% of all samples except human amylase (Fig. 7) Human amylase was inhibited by saponins only . Results are shown in Fig. 6, 7.

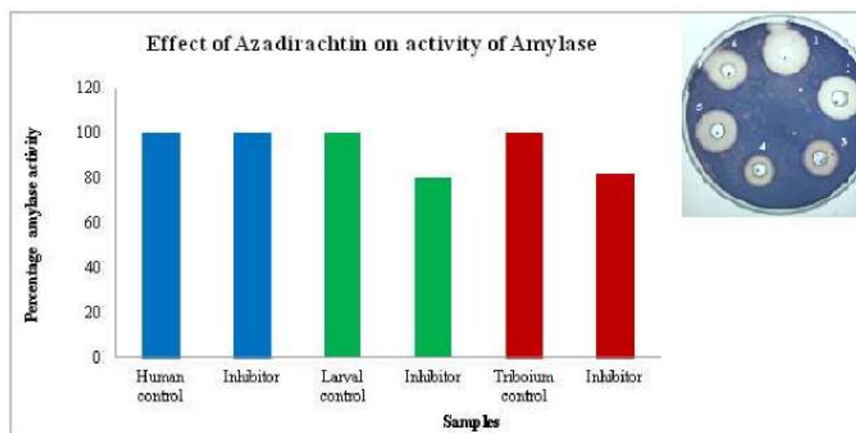

Fig 6: Percentage amylase activity in various samples in *T. casteneum with Azadirachtin* treated and untreated samples. Inseat showing an activity plate of amylase, 1: human saliva 2: human saliva with Azadirachtin; 3: *T. castaneum* amylase 4: *T. castaneum* amylase with Azadirachtin; 5: *T. castaneum* larval amylase; 6: *T. castenum* larval amylase with Azadirachtin.

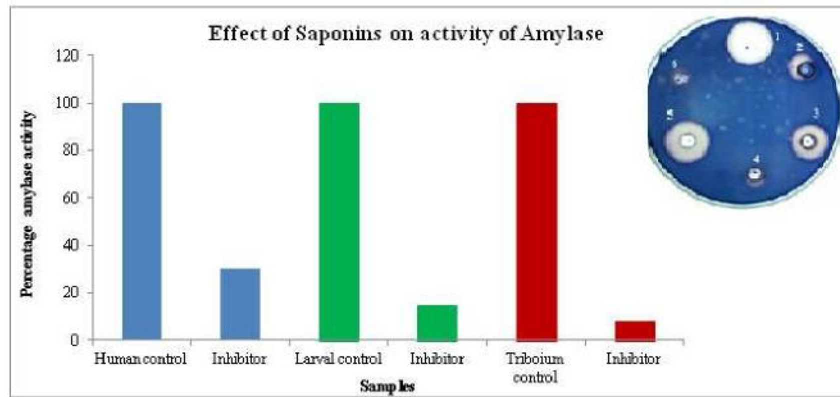

Fig 7: Percentage alpha amylase activity in saponins treated and un-treated samples for *T. castaneum*. Inseat showing an activity plate, 1: human saliva; 2: human saliva with Saponin; 3: *T. castaneum* amylase extract 4; *T. castaneum* amylase extract with Saponin; 5: *T. castenum* larval amylase extract; 6: *T. castenum* larval amylase extracts with Saponin.

Sivakumar *et al.* (2000) reported the inhibition of insect pest alpha amylases by millet plant derived inhibitors [26]. There is a notion that plant extracts are insecticidal because they form stable complexes with digestive enzymes which could inhibit the formation of substrate-enzyme complex. Thus leading to blocking of digestive enzymes resulted in poor nutrition, growth retardation and death. Further it has been reported previously that plant derived molecules mimic the substrate molecule as they unite with glucose molecule, for example, rutin [27].

Bioassays for the larvae and adults of *T. castaneum*

Impact of Neem derived compounds at the genetic level was studied by feeding the insects on the starch mixed with the Neem derived compounds. Insects were fed on starch mixed with Azadirachtin, Saponin and Neem leaf powder for four days. After four days it was noticed that there was 50% mortality. Thus, Neem leaf powder and its compounds acted as toxin for *T. castenum* at the larval and adult stages. The total soluble proteins were isolated from insects and larvae and tested for starch hydrolyzing activity. The effect of Neem derived compounds was determined by locating the enzyme activity on starch agar plates. The extracted proteins were loaded onto substrate agar plate and incubated overnight at 50°C. The substrate area hydrolyzed by each protein sample of treated insect was compared with the area hydrolyzed by the control insects (fed on a normal diet). It was recorded that the rate of expression of alpha amylase was 25% less for treated insects while Saponin inhibited 20%. Leaf powder was able to reduce only 10% expression of the enzyme protein, as compared to inhibition by Azadirachtin. Results are shown in Fig. 8. Thus, the role of Neem derived compounds may have some impact on the expression level. To have a complete picture, these results should be coupled with the isolation and quantification of mRNA for amylase enzyme. Neem derived Saponins stand a good merit as an inhibitor for amylase enzyme protein.

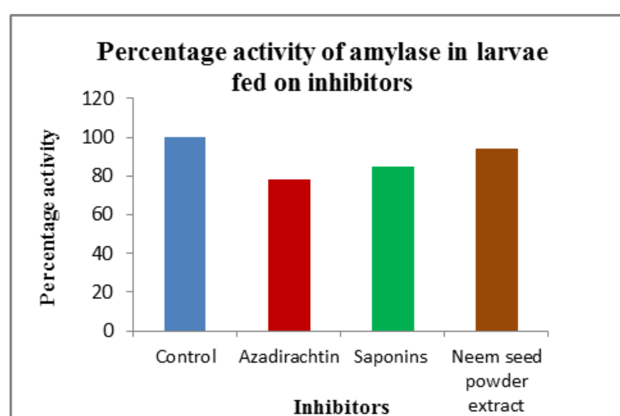

Fig. 8: Percentage inhibition of enzyme activity in *T. castaneum* larvae fed on Neem leaf extract, Saponins and Azadirachtin.

CONCLUSION

Azadirachta indica derived compounds (Saponins and Azadirachtin) act as inhibitors of digestive alpha-amylase of *T. castaneum*. .*Azadirachta indica* compounds have a potential to be used as bio-pesticides in insect pest management.

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