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Evaluation of amylase activity in fruit fly, *Drosophila melanogaster* and the inhibitory effect of common bean, *Phaseolus vulgaris* extract

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Abstract
Fruitfly (*Drosophila melanogaster)*, a dipteran species has been known to consume or ruin vast quantities of carbohydrate rich plant products. To study the starch digesting activity of amylase and the inhibitory effect of common bean (*Phaseolus vulgaris)* at different temperatures this experiment was undertaken. Amylase activity from homogenates was measured by Colorimetric method of Caraway (1959). At 37 ˚C amylase activity was found to be 101.73 mg/dl and at 42 ˚C it was 136.38 mg/dl indicating that amylase in drosophila works better at higher temperature. The comparative inhibitory activity of *Phaseolus vulgaris* at 37 ˚C and 42 ˚C was 85.14 mg/dl and 105.65 mg/dl respectively showing a potential trend of effectiveness of the inhibitor at lower temperature. From the experiment it can be said that in tropical as well as in subtropical areas the consumption of starch rich plant products by drosophila will be high as it acts better at higher temperature.

Keywords: Amylase, Climate change, Diptera, Global, *Phaseolus vulgaris*

1. Introduction
α-amylase (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1), which hydrolyze starch and other polysaccharides is a key enzyme to digest polysaccharide in many organisms.[1] This enzyme constitutes a family of endo-amylases catalyzing the hydrolysis of α-D-(1,4)-glucan linkages in starch components, glycogen and other carbohydrates. The presence of several, sometimes divergent amylase molecules enables organisms to digest a broad range of substrates, in a broad range of environmental conditions [2]. These enzymes are found in plants, animals and microorganisms where they play important roles in carbohydrate metabolism [3].
Amylase is a very important enzyme used by insects to hydrolyze carbohydrate. α-amylases have been found in several insects, especially those feeding on starchy food during larval stage, depend on their α-amylase for survival [4, 5].
Plant α-amylase inhibitors are found in several plants including common bean *Phaseolus vulgaris*, play a key role in natural defence against those insects which feed on starchy food. These inhibitors, which are particularly abundant in legumes and cereals represent a potent tool in engineering crop plants due to their role as defence factors against insect pests and pathogens [5, 6, 7]. Search on starch digestion as a target for control of starch-dependent insects was stimulated in recent years after results showed that α-amylase inhibitors from *Phascolus vulgaris* L. seeds are detrimental to the development of cow pea weevil *Callosobruchus maculates* (F.) (Coleoptera Bruchidae) and Azuki bean weevil *Callosobruchus chinensis* (L.). Pea and azuki transgenic plants expressesing α-amylase inhibitors from common beans were completely resistant to *Bruchus pisorum (L.) C. chinensis* weevils [8, 9]. Weevils such as *Sitophilus* sp. have such high levels of α-amylase that they are able to overcome the inhibitors in their diet [10].
Drosophila melanogaster is a fruit fly, a little insect about 3mm long, of the kind that accumulates around spoiled fruit. It is also one of the most valuable of organisms in biological research, particularly in genetics and developmental biology. *Drosophila* has been used as a model organism for research for almost a century, and today several thousand scientists are working on many different aspects of the fruit fly. Its importance for human health was recognized by the award of the Nobel prize in medicine/physiology to Ed Lewis, Christiane Nusslein-Volhard and Eric Wieschaus in 1995. *Drosophila* has been known to over winter in

storage facilities, where it can consume or ruin vast quantities of food. The fruit fly also lays its eggs on unripened fruit and is considered a pest in many areas [11, 12].
As many insect species depend on the effectiveness of their amylases for survival, fruit fly is also no exception. Therefore considering the potential of this insect for the benefit of human being as well as to study the carbohydrate digesting capacity of amylase this experiment was undertaken.

2. Materials and Methods

2.1 Sample collection: The insects were cultured in glass vial by keeping cut fruit (banana) at 25 ˚C and only 3 days old adult insects were taken as the test insect for the whole duration of the experiment from June to September of 2014.

2.2 Sample preparation: Enzyme samples from *D. melanogaster* were prepared by the method of Cohen (1993) with slight modifications. Adult insects were randomly selected, killed and weighed in the electrical weight balance. The insects were then ground in a homogenizer in 1 ml of ice-cold saline buffer (0.006 M NaCl) giving a final strength of 10 mg per ml of homogenate.
The homogenate was centrifuged at 10,000 rpm for 5 minutes. The required supernatant was used and for subsequent analysis it was stored at -20 °C.

2.3 Extraction of α-amylase Inhibitor: Amylase Inhibitor from seeds of common bean was extracted [13, 14]. Ground seeds (10 g) were mixed with a solution of 0.1*M* NaCl and stirred for two hours, followed by centrifugation at 10,000 g for 30 min. The pellet was discarded, and the supernatant was incubated at 70˚ C for 20 min to inactivate major endogenous enzymes. Fractionation of the supernatant was done using different concentrations of ammonium sulfate (20, 40, 60, and 80%) followed by centrifugation at 10,000 g for 20 min at 4 ˚C. The 60% pellet containing the highest fraction of amylase inhibitors was dissolved in ice cold sodium phosphate buffer (0.02 *M* and pH 7.0) and dialyzed overnight against the same buffer. This dialyzed solution was used as a source of amylase inhibitors in enzyme assays.

2.4 Determination of amylase activity in sample homogenate:
Amylase enzyme activity was measured by Colorimetric method [15].
In 3 test tubes labelled as “Blank”, “Control” and “Unknown” 0.5 ml of amylase substrate was taken. In the blank test tube instead of sample homogenate 0.01 ml of phosphate benzoate buffer, in the control 0.01 ml of sample homogenate (Boiled for 10 minutes) and in the unknown 0.01 ml of sample homogenate was added. Then all the tubes were placed in 37 ºC heating bath for 3-5 minutes. After an interval of 3 minutes 0.010 ml (10 µl) of sample homogenate was added to each ‘Test’ tube and 0.010 ml (10 µl) of phosphate benzoate buffer solution to each ‘Blank’ tube with the help of micropipette. After mixing gently the tubes were immediately returned to incubator (37 °C) for exactly 7½ minutes.

1) The tubes were then removed from incubator and 4.0 ml of distilled water was added immediately to each test tube followed by addition of 0.5 ml amylase color reagent. Solution in the tube was gently mixed by inversion.
2) Absorbance of each tube was measured at 570 nm against zero set within 30 minutes of adding the color reagent.

2.5 Determination of amylase activity by inducing inhibitor
The same procedure was performed as for amylase assay, only the inhibitor (10 µl) was added in all the ‘Test’ tubes just after adding the sample homogenate in it. The amylase activity was determined by measuring absorbance at 570 nm.
Both the experiments of amylase activity i.e. with and without inhibitor, were performed at two different temperatures- 37 °C and 40 °C.

2.6 Procedure notes
One amylase unit is defined by Caraway as the amount of enzyme that will hydrolyse 10 mg of starch in 30 minutes to a state at which no color is produced by the addition of iodine. Therefore, in 7½ minutes with a 0.01 ml sample, 0.25 mg of starch is required per reaction mixture.

2.7 Stability of endpoint reaction
The final color produced in the reaction is stable for about 30 minutes.

2.8 Calibration

$$\frac{1000\text{ml}}{2000\text{ml}} \text{X} \frac{0.5\text{ml}}{10\text{mg}} \text{X} \frac{30\text{min}}{7½\text{min}} \text{X} \frac{100}{0.01\text{ml}} = 1000\ \text{U/dl}$$

Calculation of the unknown is based on the fractional decrease in starch multiplied by 1000 U/dl, the activity present if all starch is digested. Since the reaction becomes nonlinear when about one half substrate is used, linearity is limited to 500 U/dl.
The whole procedure was performed for four months and the results of all the study parameters so obtained during the period of investigation were statistically analyzed. The mean, the standard deviation of mean, the standard error of mean for each set of data were calculated using MS Excel.

3. Results & Discussion

Contrary to a wide distribution of glycosidases in plants and microbes, the occurrence and distribution of glycosidase enzyme in insects are yet to be explored quantitatively. Studies of these enzyme is still in an incipient stage in insects. But, few reports demonstrated the occurrence of several glucosidase enzymes in different insects [16, 17].
In the present study from the array of glycosidases present in the insect***s*** salivary gland and different parts of digestive tract, the salivary amylase was selected as the model glycosidases representing the α glycosidase series. *Drosophila* was selected as the insect under study which may be exploited through their glycoside degrading capacity. The normal food habit of the representative insects selected in the present study was mainly starchy material.

On interpretation of the presented information it emerged out that the polysaccharide mainly starch, consumption capacity of drosophila under the present study, primarily depends upon amylase. Amylase extracted from drosophila had acted very efficiently on starch. At 37 ˚C amylase activity was 101.73 mg/dl and at 42 ˚C it was 136.38 mg/dl. (Fig-1). This indicates that amylase in drosophila works better at higher temperature.
The amylase activity at 37 °C was 42.72% and at 42 °C was 57.72%. Increase in 5 °C temperature has increased the amylase activity to about 14.55% (Fig-2). So with every 1 ˚C increase in temperature it was found that there was a proportionate increase of 2.91% amylase activity.

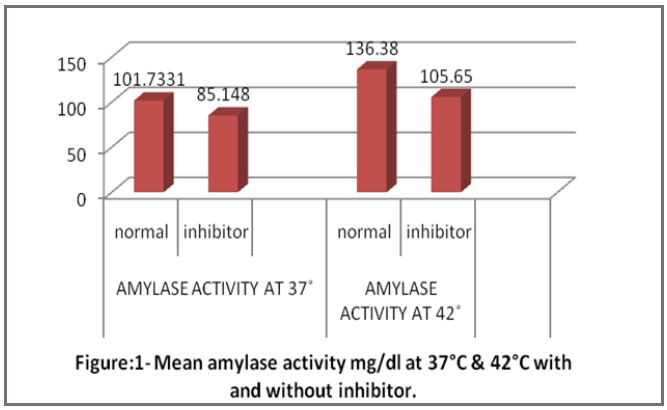

Figure:1- Mean amylase activity mg/dl at 37°C & 42°C with and without inhibitor.

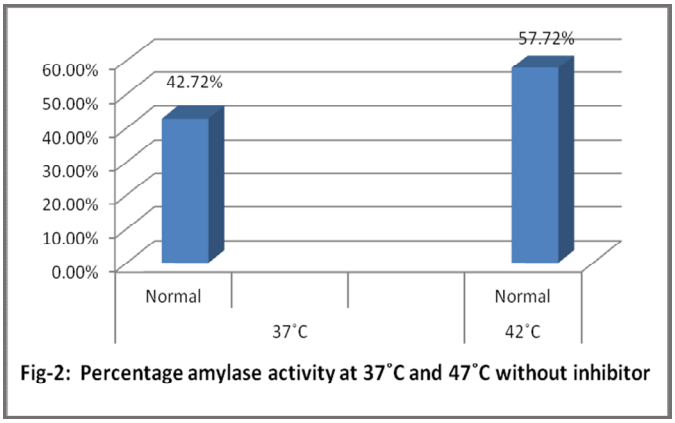

Fig-2: Percentage amylase activity at 37˚C and 47˚C without inhibitor

On the contrary the bean extract which was used as inhibitor of amylase activity had reduced the activity of amylase to a greater extent. At 37 ˚C the amylase activity without inhibitor was 54.43% and with inhibitor was 45.56%. So, about 10% amylase activity was reduced by this inhibitor (Fig-3).

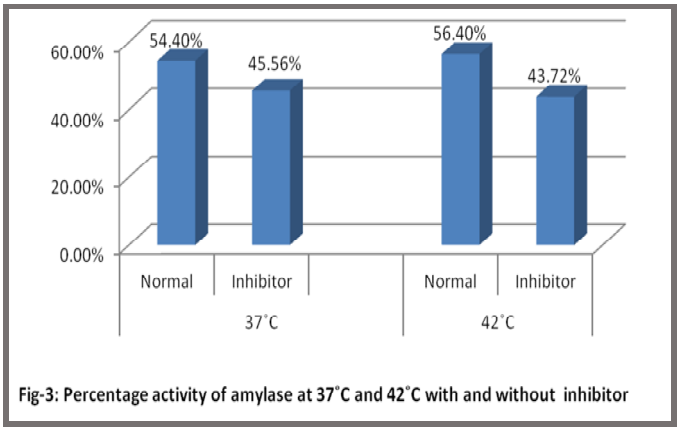

Fig-3: Percentage activity of amylase at 37˚C and 42˚C with and without inhibitor

From the present study it was evident that at 42 ˚C the normal activity of amylase was 136.38 mg/dl and with inhibitor was 105.65 mg/dl. The normal percentage was 56.4% and with inhibitor was 43.72% respectively (Fig-1 & 3). The specific inhibitor had reduced the amylase activity to about 12.68% which is a significant extent ($p<0.05$).

The comparative inhibitory activity of *Phaseolus vulgaris* at 37 ˚C and 42 ˚C was 85.14 mg/dl and 105.65 mg/dl respectively. Here also a potential trend of effectiveness of the inhibitor is seen at lower temperature than the higher temperature. With every 1 ^{0}C temperature rise there was a decrease of 2.18% in inhibitory activity of *Phaseolus vulgaris* (Fig-4).

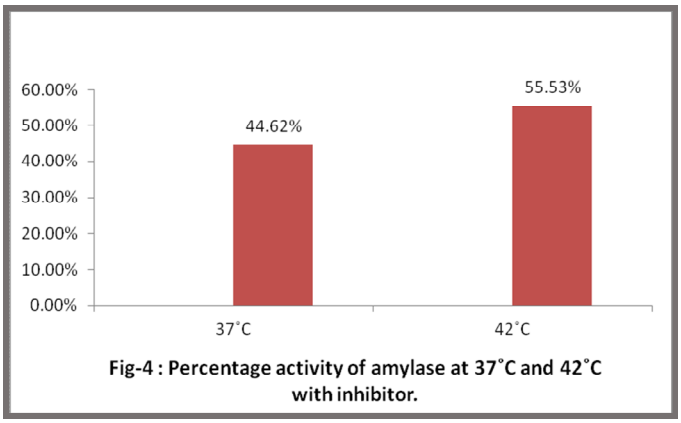

Fig-4 : Percentage activity of amylase at 37˚C and 42˚C with inhibitor.

Plants secondary metabolites can act as protective agents in plants against insects either by acting as repellents or causing direct toxicity. Many different types of secondary metabolites including alkaloids have been demonstrated to confer resistance to different plant species against insects [8, 9].

From the experimental findings it can be predicted that Drosophila amylase has effective activity on starch at higher temperature. So in tropical as well as in subtropical areas the consumption of starch rich plant materials will be high as it acts better at higher temperature. Visualising the present scenario of global temperature increase due to greenhouse gases, the drosophila amylase will automatically have an advantageous state to cause maximum damage to starch rich material. On the contrary the effect of common bean to inhibit the amylase activity will also decrease inversely as the temperature increases.

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