In vitro Effects of proteinaceous alpha amylase inhibitors on red flour Beetle, *Tribolium castaneum*

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**ABSTRACT**  
This paper presents evidence for the occurrence of proteinaceous inhibitors isolated from Chick pea (*Cicer arietinum*), Kidney bean (*Phaseolus vulgaris*), Maize seeds (*Zea mays*), Wheat (*Triticum aestivum*) and Millet (*Pennisetum typhoides*). These seeds inhibitors exhibited inhibitory activity against alpha amylase from red flour beetle, *T. castaneum*. Out of five tested seed samples Wheat, Maize and Kidney bean inhibitors exhibited much inhibitory activity against α amylase from *T. castaneum*. It is of clinical significance that these seeds did not inhibit human saliva α amylase activity.  

**Keywords:** amylase, amylase inhibitor, *Tribolium castaneum*

**INTRODUCTION**  
In India, starchy foods are an important staple food, which is being destroyed by various storage pests every year on storage. The red flour beetle, *Tribolium castaneum* (Herbst) is one of the major starch dependent storage pests, which is responsible for severe stored grain losses (Chen et al. 1992). As these insects are totally dependent on α amylase for their survival, these enzymes are good target candidates for bio-insecticides by using alpha amylase inhibitors. Alpha amylase inhibitors are extensively found in many plant seeds and tubers, being particularly abundant in cereals and legumes. Leguminous plants have become a popular research source, due to the abundance of proteins and peptides involved in plant defense. Among these proteins are lectins, arcelins, chitinases, β-1, 3-glucanases, defensins, digestive enzyme inhibitors, naturally occur in many food plant and particularly abundant in cereals and legumes (Franco et al. 2002). The enzyme inhibitors impede digestion through their action on insect gut digestive alpha amylase and proteinases, which play a key role in the digestion of plant starch and proteins. The natural defenses of crop plants may be improved through the use of transgenic technology. Current research in the area focuses particularly on weevils as these insects are highly dependent on starch for their energy supply (Franco et al.2002). Several studies demonstrated the efficiency of proteinaceous inhibitors against digestive enzymes of important economic Coleopteran pests (Leple et al, 1995), especially when utilized in plants genetic engineered (Morton et al 2000, Lee et al 1999).

Many inhibitors of mammalian and insect alpha amylases have been isolated from different plant sources and subsequently characterized. (Xiaoyan Hao et al. 2009). As proposed by Richardson (Richardson et al. 1991), alpha amylase inhibitors may be conveniently classified by their tertiary structure into six different classes: lectin- like, knottin- like, cereal-type, kunitz-like, purothionin-like and thaumatin-like. Their specificity has been widely explored, with some capable of acting only against insect alpha amylases or against mammalian enzymes (Franco et al. 2000). A no. of inhibitors has received particular attention as attractive candidates for pest control. In this study, alpha amylase inhibitor from five different seeds, Chick pea (*Cicer arietinum*), Kidney bean (*Phaseolus vulgaris*), Maize seeds (*Zea mays*), Wheat (*Triticum aestivum*) and Millet (*Pennisetum typhoides*) was isolated and its inhibitory activities towards *T. castaneum* alpha amylases was measured.

**MATERIALS & METHODS**

1) Extraction of Proteinaceous alpha amylase inhibitors  
The seed flour extracted with 8 folds of volume (v/w) of 0.9% sodium chloride solution with stirring for 1 hr at room temperature. After extraction, the suspension was routinely heated at 80⁰ C for 10 min in order to inactivate endogenous amylase activity present. The heated suspension was then centrifuged at 3,000 rpm for 10 min. The supernatant obtained was assayed for amylase inhibitory activity (Ikeda et al. 1994).
2) **Estimation of Protein**
The protein concentration of crude alpha amylase inhibitor was determined by the method of Lowry et al. (1951) using Bovine serum albumin as standard.

3) **Extraction of alpha amylase from T. castaneum**
The acetone defatted larvae of *T. castaneum* were homogenized with buffer in a ratio (1: 5 w/v) to extract enzymes. The enzymes were extracted with succinic acid buffer 0.2M, pH 4.5. The suspensions were centrifuged at 10,000g for 10 minutes at 4° C. The supernatant were served as the source of α amylase (Figueira et al. 2003).

4) **Assay of alpha amylase & alpha amylase inhibitor activities**
Alpha amylase inhibitory activities were measured according to Ishimoto (Ishimoto et.al. 1999), with some modifications. 25 µl of *T. castaneum* alpha amylase preparation and 25 µl of the alpha amylase inhibitor extract were mixed and preincubated at 37 ºC for 30 min prior to the addition of 400 µl of substrate solution (1% soluble starch solution in 0.1 M phosphate buffer solution, pH 6.9,containing 20mM NaCl and 0.1 mM CaCl$_2$). After 10 min, the reaction was stopped by the addition of 250 µl dinitrosalicylic acid, followed by boiling for 10 min in a water bath. After the addition of 3 ml of distilled water, the solution was mixed and allowed to stand at room temperature for 15 min. and then $A_{546}$ was measured. Distilled water (25 µl) instead of alpha amylase inhibitor solution was used as the control. The alpha amylase inhibitory activity was calculated as follows:

\[
\text{Inhibitory activity (\%)} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

(Hao et al. 2009)

**RESULTS AND DISCUSSION**

**Protein extraction & determination**
Quantitative analysis of protein followed the method of Lowry et al. (1951) was conducted. The standard curve of BSA (Bovine Serum Albumin) was conducted. Amount of protein (mg) obtained from 5g of the seeds are shown in table 1.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Name of Seed</th>
<th>Amount of protein mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chick pea (<em>Cicer arietinum</em>)</td>
<td>0.72</td>
</tr>
<tr>
<td>2.</td>
<td>Kidney bean (<em>Phaseolus vulgaris</em>)</td>
<td>0.62</td>
</tr>
<tr>
<td>3.</td>
<td>Maize seeds (<em>Zea mays</em>)</td>
<td>0.41</td>
</tr>
<tr>
<td>4.</td>
<td>Wheat (<em>Triticum aestivum</em>)</td>
<td>0.53</td>
</tr>
<tr>
<td>5.</td>
<td>Millet (<em>Pennisetum typhoides</em>)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Fig 1: Amount of protein in Seed

![Graph showing the amount of protein in different seeds](http://jsrr.in)
Characteristics of alpha amylase
Profiles of amylase activity from *T. castaneum* were observed at various pH and temperatures. The amylase showed optimum pH for the hydrolysis of its substrate at pH 7.4. Amylase expressed the optimum temperature of 40 ºC.

Effects of alpha amylase inhibitor on activities of *T. castaneum* alpha amylase in vitro
Out of five tested seed samples Wheat, Maize and Kidney bean inhibitors exhibited much inhibitory activity against α amylase from *T. castaneum*. It is of clinical significance that these seeds did not inhibit human saliva α amylase activity. The presence of an α amylase inhibitor from Maize seeds with high inhibitory activity against *T. Castaneum* α amylase enzyme, indicating that this inhibitor probably could be used, through genetic engineering in the construction of transgenic plants with enhanced resistance towards stored grain beetle. In the present study, we observed that *T. Castaneum* amylases exhibited increased activity at a highly alkaline pH and were active and stable at 30-40 C. These results were concurrence with most lepidopteron reported so far. (Sivakumar *et al.* 2006; Valencia Jimenez *et al.* 2008; Kotkar *et al.* 2009).

Table No. 2: % of Inhibition of alpha amylase by different seeds

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Alpha amylase inhibitor source</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chick pea (<em>Cicer arietinum</em>)</td>
<td>42.8</td>
</tr>
<tr>
<td>2.</td>
<td>Kidney bean (<em>Phaseolus vulgaris</em>)</td>
<td>67.8</td>
</tr>
<tr>
<td>3.</td>
<td>Maize seeds (<em>Zea mays</em>)</td>
<td>82.1</td>
</tr>
<tr>
<td>4.</td>
<td>Wheat (<em>Triticum aestivum</em>)</td>
<td>75</td>
</tr>
<tr>
<td>5.</td>
<td>Millet (<em>Pennisetum typhoides</em>)</td>
<td>57.1</td>
</tr>
</tbody>
</table>

Fig. 2: Inhibition of alpha amylase activity of *T. castaneum* from various sources

LITERATURE CITED


