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

Nazia Khan
Department of Biochemistry,
Shri Shivaji College of Arts, Commerce and Science, Akola (M.S.) India
nkhan247@gmail.com

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, ß-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 (Mortan *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 80º 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:

Inhibitory activity (%) = ($A_{control} - A_{sample}$) / $A_{control}$ X 100
(Hao *et al*. 2009)

RESULTS AND DISCUSSION

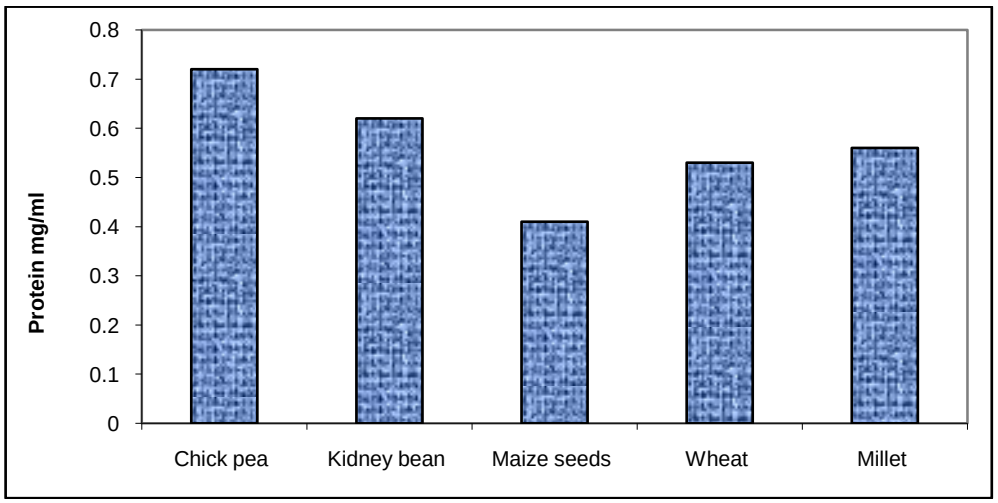
Protein extraction & determination

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 No.1: Amount of Protein in Seeds

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

Fig 1: Amount of protein in Seed



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.

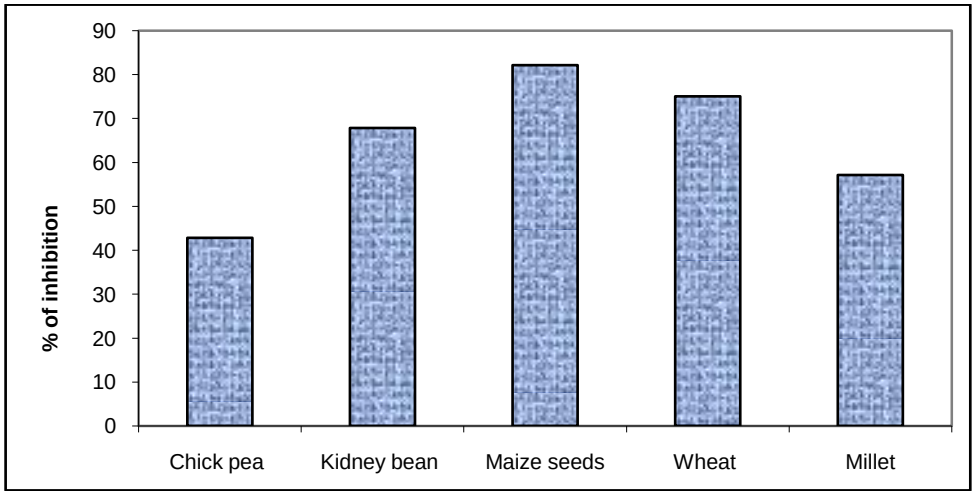
Effects of alpha amylase inhibitor on activities of *T. castaneum* alpha amylase *in vitro*

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

Sr. No.	Alpha amylase inhibitor source	% of inhibition
1.	Chick pea (*Cicer arietinum)*	**42.8**
2.	Kidney bean (*Phaseolus vulgaris*)	**67.8**
3.	Maize seeds (*Zea mays*)	**82.1**
4.	Wheat (*Triticum aestivum*)	**75**
5.	Millet (*Pennisetum typhoides*)	**57.1**

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



LITERATURE CITED

Chen MS, Feng G, Zen KC, Richardson M, Valdes-Rodrigues S, Reeck GR and Kramer KJ. 1992. Alpha amylase from three species of stored grain Coleopteran and their inhibition by wheat & corn proteinaceous inhibitor. *Insect. biochem. Mol. Biol.* **22:** 261-268.

Franco OL, Rigden DJ, Melo FR, Bloch Jr, C, Silva, CP and Grossi-de-Sa, MF. 2000. Activity of wheat alpha amylase inhibitors towards bruchid alpha amylases and structural explanation of observed specificities. *Eur. J. Biochem.* **267**: 1466-1473.

Figueira ELZ, Blanco-Labra A, Gerage AC, Ono EYS and Mendiola-Olaya E. 2003. New amylase inhibitor present in corn seeds active in vitro against amylase from *Fusarium Verticilloides*. *Plant Dis*.**87**:233-240.

Ishimoto M, Yamada T and Kaga A. 1999. Insecticidal activity of an alpha amylase like protein resembling a putative precursor of alpha amylase inhibitor in the common bean. *Phaseolus Vulgaris* L. *Biochemical et Biophysical Acta (BBA)* - Protein structure and molecular Enzymology. **1432** (1):104-112.

Ikeda K, Shida K and Kishida M. 1994. Alpha amylase inhibitor in Buckwheat seed. Faculty of Nutrition, Kobe Gakuin University, Nishi-ku, Kobe, Japan, Fagopyrum **14**: 3-6.

Kotkar HM, Sarate PJ, Tamhane VA, Gupta VS and Giri AP. 2009. Responses of midgut amylases of Helicoverpa Armigera to feeding on various host plants. *Journal of insect physiology*. **55** (8):663-670.

Leple JC, Bonade-Bottino M, Augustin S, Pilate G, Le TanDelplanque VD, Cornu AD, and Jonanin L. 1995. Toxicity of Chrysomela tremulae (Coleoptera: Chrysomelidae) of transgenic poplars expressing a Cystein proteinase inhibitor. *J of Mol.Breed.***1:** 319-328.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.***193:** 265-275.

Lee IS, Lee HS, Koo JC, Chun HJ, Lim CO, Mun JH, Song YH, and Cho MJ. 1999 Soyabean knutiz trypsin inhibitor (SKTI) confers resistance to the brown plant hopper in transgenic rice. *J. Mol. Breed.* **5**: 1-9.

Mortan RL, Schroeder HE, Bateman KS, Chrispeels MJ ,Armstrong E,and Higgins TJV. 2000. Bean alpha amylase inhibitor 1 in transgenic peas (*Pisum Sativam*) provides complete protection from pea weevil (*Bruchus pisorum*) under field conditions. *Proc. Natl. Acad. Sci.* USA 97; 3820-3825.

Richardson M. 1991. *Methods in Plant Biochemistry* (Rogers, L.J.Ed.), vol.5, pp.259-305, Academic Press, New York.

Sivakumar S, Mohan M, Franco OL, Thayumanavan B. 2006. Inhibition of insect pest alpha amylases by little and finger millet inhibitors. *Pesticide Biochemistry and physiology*. **85:** 155-160.

Shewry PR, Napier JA and Tatham AS. 1995. Effect of Soybean trypsin inhibitors on the development and development and midgut proteolytic activity of Tribolium Castaneum larvae. *Plant Cell.* 7:945-956.

Valencia-Jimenez, Arboleda JW, Lopez Avila A and Grossi-de-Sa MF. 2008. Digestive alpha amylase from Tecia solanivora larvae (Lapidoptera: Gelechiidae): response to pH, temperature and plant amylase inhibitors. *Bulletin of Entomological Research.* **98:** 575-579.

Xiaoyan Hao, Jiangui Li, Qiughua Shi, Jusong Zhang, Xiaoling He, and Hao Ma. 2009. Characterization of a Novel Legumin alpha amylase inhibitor from Chickpea (*Cicer arietinum* L.) seeds. *Biosci. Biotechnol. Biochem.* **73** (5): 1200-1202.