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## Extraction, Purification, and Inhibitory Effect of Alpha-Amylase Inhibitor from Wheat (*Triticum aestivum* Var. *Zarrin*)

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**Abstract:** Plant alpha-amylase inhibitors show great potential as tools to engineer resistance of crop plants against pests. They are also drug-design targets for treatment of diabetes and digestion disorders. These inhibitors also known as sensitizing agents in human. The numerous form of alpha-amylase inhibitors was reported. In this study alpha-amylase inhibitor was extracted from Iranian wheat cultivar (*Triticum aestivum* v *zarrin*), precipitated and purified by anion exchange fast protein liquid chromatography. Electrophoresis of purified protein showed 0.66 relative mobility. Total hydrolytic activity of human salivary and bacillus subtilis alpha-amylase were inhibited 97.07% and 89.97% respectively by collected purified alpha-amylase inhibitor.

**Key words:** Alpha-amylase inhibitor, wheat, ion exchange chromatography, human salivary

### Introduction

The relative inefficacy of alpha-amylase inhibitors in affecting human digestion of starch has been highlighted by recent scientific and public controversy over the commercial sales of so-called starch-blockers or slimming pills (Richardson, 1991). Alpha-amylase and its inhibitors is drug-design targets for the development of compounds for treatment of diabetes Obesity and Hyperlipaemia (Octavio and Rigden, 2000). Studies of the structures of the numerous enzyme inhibitors found in cereal grains have led to the recognition of a super family of homologous proteins which includes inhibitors of alpha-amylase, proteinase and bifunctional inhibitors active against two or more classes of enzymes (Alam and Gourinath, 2001; Octavio and Rigden, 2002; Richardson, 1991).

The first alpha-amylase inhibitor determined was that of the monomeric 13 kD known as 0.31 form, from wheat (Kashlan and Richardson, 1981). Other dimeric 0.19, 0.23, 0.28, 0.53 form of wheat inhibitors of exogenous alpha-amylase were later shown (Oneda *et al.*, 2004; Kondo and Ida, 1995; Roy and Gupta, 2000; Richardson, 1991; Octavio and Rigden, 2002).

The favored hypotheses about physiological roles of the enzyme inhibitors in seeds is that they act as storage or reserve proteins, as regulators of endogenous enzyme or as defensive agents against the attacks of animal predators and insect or microbial pests. It seems likely that in certain species these proteins may fulfill a combination of these functions (Octavio and Rigden, 2002; Octavio and Rigden, 2000; Richardson, 1991). Also plant alpha-amylase inhibitors show great potential as tools to engineer resistance of crop plant against pests (Octavio and Rigden, 2002).

Nutritional and metabolic effects of enzyme inhibitors certainly some of inhibitors found in cereal and legume

seeds can inactivate the salivary and pancreatic enzymes of humans (*pick* and *wober*) and their susceptibility to inactivation in the stomach appears to be rather variable (Singhand Bblundel, 2001). Many are destroyed by cooking but some retain inhibitory activity even after baking (Richardson, 1991).

The amylase inhibitors present in seeds currently used as food present few nutritional problems for healthy people but may have some toxicological significance in the diets of infants who have a lower production of pancreatic alpha-amylase than adults and for patients with impaired peptic or gastric function (Brieteneder and Radauer, 2004; Richardson, 1991; Shewry *et al.*, 2001). Also one inhibitor of insect alpha-amylase isolated from barley flour is the major allergen associated with baker's asthma disease (Barber *et al.*, 1989). The inhibition is strictly competitive and in the 1:1 complexes all of the activities of the enzyme are completely abolished.

Crystallographic, nuclear magnetic resonance (NMR) and mechanistic studies all indicate that the inhibitors act as highly specific substrates for the enzyme. They inhibit at a unique peptide bond called the reactive site peptide bond.

The reaction mechanisms involved in the inhibition of alpha-amylase by plant protein inhibitors are not clearly understood (Silan, 1986). But there are suggestion that reducing sugars which are covalently bound to the inhibitor polypeptide chain may play a major role in the mechanism or that the inhibitor may induce conformational changes in the enzyme molecule.

Recently demonstrated that when the barley bifunctional inhibitor binds to the endogenous alpha-amylase it affects a specific tryptophan residue of the enzyme which is essential for productive enzyme-substrate binding (Richardson, 1991).

In this study albumin like proteins were extracted from wheat (*Triticum aestivum* var. *zarrin*) seeds. Alpha-

amylase inhibitor precipitated with ammonium sulfate and purified by exchange chromatography applied on AKTA FPLC. The collected purified protein detected by SDS-PAGE. Reduction in human salivary and bacillus subtilis alpha-amylase activity was measured by total hydrolytic activity assay.

Isolation and detection of alpha-amylase inhibitor from Iranian wheat cultivar (*zarrin*) and study of its effect on starch digestion in human salivary contrast to other inhibitors in cereal grains which were reported are the aim of this investigation work.

## Materials and Methods

**Extraction of alpha-amylase inhibitor:** *Zarrin* (*Triticum aestivum* var. *zarrin*) kernels were obtained from urmia agriculture research institute. Five complete kernel (0.2113g) ground in 2ml water (1:10 w: v ratio) (Kondo and Ida, 1995). Extraction was carried out at 20°C for 1h. The supernatants are separated by centrifugation (20min at 10,000g) (Doonan, 1996) and reached to the 2.5ml volume whit buffer A (Tris-HCl20mM) (Amersham, 1998).

**Fractional precipitation with Ammonium sulfate:** 0.485 g solid ammonium sulfate was added to protein solution at two steps to take the concentration from 35% to 50% F (35-50). The precipitated protein recovered by centrifugation in each steps (5000g at 30min). The protein pellet was suspended in 2ml of buffer A (Doonan, 1996; Amersham, 1998).

**Dialysis:** The protein suspension was transferred to visking dialysis tubing and dialyzed against two changes of buffer against 200ml of buffer A and allowing 8-10 h for equilibration after adjustment the pH (pH.8 as the buffer A). Dialysis was repeated against the distilled water 200ml for 2h. The dialyzed sample was centrifuged at 5000g for 15min (Doonan, 1996; Amersham, 1998).

**Fast protein liquid chromatography (FPLC) method:** A first purification step was achieved by ion-exchange chromatography. The crude extract after extraction, precipitation and dialysis was applied on *Hiprep TM 16/10 DEAE FF* prepaked column using *AKTA FPLC TM* (Amersham Biosciences Pharmacia, Uppsala Sweden) system. The column equilibrated with 100 ml start buffer (A) (low ionic strength, 20mM Tris - HCl pH 0.8) at 0.5 ml /min flow rate, equilibration is complete when the PH of elute are the same as buffer A (PH.8). The sample 75  $\mu$ L was loaded and then injected 0.30ml of buffer A was passed through the column 60 min and then the elution buffer B (20mm Tris – HCL + 1m NaCl PH.8) high ionic strength was applied elution was collected (Doonan, 1996; Amersham, 1998). All buffers and samples must be passed through a 0.22 $\mu$ m filter (GSWP, Millipore crop Bed ford, mA) or centrifuge at 10000g for 15 minutes

before use in *AKTA FPLC* system.

**Gel electrophoresis:** SDS-PAGE was performed by method of Laemmli (1970) Separated protein collected from Fractions of FPLC was Visualized by staining whit coomassie brilliant blue. The running and stacking gel concentration was 15% and 3% of poly acryl Amid respectively. 20  $\mu$ l of laemmli solution added to 20  $\mu$ l of sample and 35 $\mu$ l of mixture was injected to the running gel (Hames and Rickwood, 1990).

**Assay of  $\alpha$ -amylase inhibitor activity:** Total hydrolytic activity assay used to determinate the reduction in human salivary and bacillus subtilis  $\alpha$ -amylase activity when the  $\alpha$ -amylase inhibitor which purified and collected from FPLC fraction added to reaction mixture. 1ml of samples was homogenized in 6 ml ice-cold grinding medium which consists of 50mM 2-(N-morpholino) ethan-sulphonic acid (Mes), brought to pH 6.0 with NaOH, 5mM CaCl<sub>2</sub>, and 5%(v/v) glycerol. The homogenate was filtered through several layers of Mira cloth and filtrate was centrifuged (15 min at 40000 g). 3.5-Di nitro salicylic acid used as a alkaline colour reagent 1 ml of the incubation mixture (3ml soluble starch 2% and 3 ml extracted sample) after 30 min incubation in 30°C was added to an equal volume of alkaline colour reagent mixed thoroughly and heated for 5 min in boiling water bath. Samples (with there replication) including:

- 1) Alpha-amylase from aus Bacillus subtilis (MerckArt. 1329) without inhibitor as a blank
- 2) Alpha-amylase from aus Bacillus subtilis (MerckArt. 1329) mixed with same volume (1:1) of alpha-amylase inhibitor purified and collected by FPLC from *zarrin* wheat grains
- 3) Diluted (1:3 with distilled water) human salivary without inhibitor as a blank
- 4) Diluted human salivary mixed with same volume (1:1) of alpha-amylase inhibitor purified and collected by *FPLC* from *zarrin* wheat grains, samples then cooled to room temperature and stored for at last 30min. absorbance at 546nm was measured using (S 2100 Diode Array spectrophotometer Biowave Biochrom WPA) against a reference and blank. Complete method is described in plant biochemistry method (Richardson, 1991).

## Results and Discussion

Analytical anion exchange chromatography of protein fractions precipitated with ammonium sulfate from wheat seeds by *AKTA FPLC* yielded one peak shown in Fig. 1. This peak was monitored at 280nm. Peak was resolved at 100% buffer B (Tris-HCl+1M NaCl pH. 8) gradient flow and eluted at 80 min. SDS-PAGE analysis of the fraction is shown in Fig. 2. Relative mobility is

Table 1: Inhibitory activities of  $\alpha$ -amylase inhibitor from zarrin wheat grain collected from FPLC fraction determinate based on maltose concentration standard cure with total hydrolytic activity determination assay against human salivary and *Bacillus subtilis*  $\alpha$ -amylase

Samples	Optical density at 546 nm Mean (3 Replication) $\pm$ SD	Maltose content ( $\mu$ M) Mean (3 Replication) $\pm$ SD
Standard $\alpha$ -amylase from bacillus subtilis 1 mg/1 ml Blank Without inhibitor	0.808 $\pm$ 0.006	27.43 $\pm$ 0.006
Standard $\alpha$ -amylase from bacillus subtilis 1 mg/1 ml Plus Inhibitor (1 : 1) ml	0.580 $\pm$ 0.016	2.75 $\pm$ 0.016
Human salivary Without inhibitor 1 ml	0.991 $\pm$ 0.004	129.86 $\pm$ 0.004
Human salivary Plus Inhibitor (1 :1)ml	0.823 $\pm$ 0.016	3.80 $\pm$ 0.016

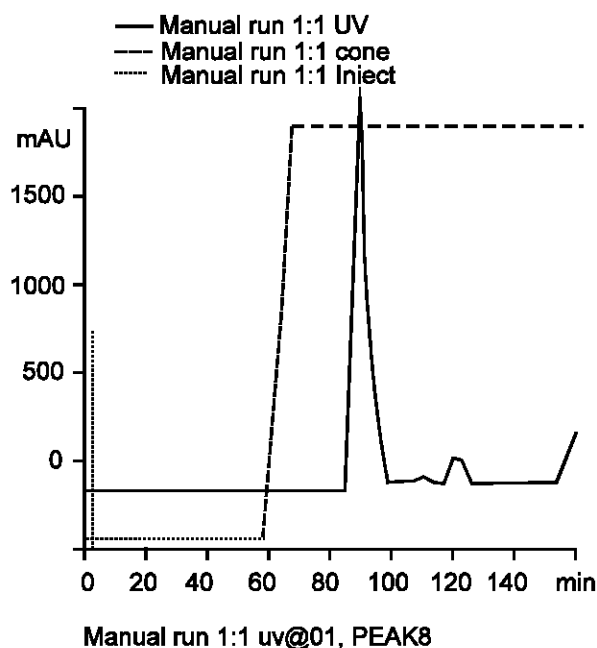


Fig. 1: Analysis of wheat alpha-amylase inhibitor Fraction F(35-50) by anion exchange fast protein liquid chromatography using DEAE FF prepaked column on AKTa FPLC TM .protein was eluted at 100% buffer B (20mM Tris - HCl+1M NaCl PH.8) at minute 80.at.0.5 ml/min flow rate. This peak was monitored based on mille absorbance at 280nm

around 0.66 RM. Two bands were observed and demonstrated that the inhibitor is dimeric. This result was in very close agreement to that reported by Maeda *et al.*, 1983; Richardson, 1991. The protein was bonded to DEAE column at pH 8.0. This shown that the net surface charge of the protein is anionic, negatively charged and the isoelectric point of the protein is below pH. 8.

DEAE is a weak ion exchangers it is possible for a bulky anion like a negatively charged protein to exchange with chloride ions. This process can later be reversed by washing with chloride ions in the form of NaCl Solution

(buffer B). Such washing removes weakly bound protein, first followed by more strongly bound proteins with greater net negative charge. These specificities are correlated to amino acid sequences of the protein.

Alpha-amylase inhibitor of the cereal family is composed of 120-160 Amino-acid residues forming five disulfide bonds. these inhibitors are also known as sensitizing agents in human upon repeated exposure, causing allergy, dermatitis and baker's asthma associated with cereal flour.

Other alpha-Amylase inhibitors such as 0.19, 0.28, WRP25, 0.53, WRP26, WRP27, and RBI were reported by researchers, that named according to their gel electrophoresis mobility relative to bromophenol blue. The protein bond in Fig. 2 show relative mobility around 0.66 RM which similar to that reported by Maeda *et al.*, 1983; Franco (2000) (Amersham, 1998; Octavio and Rigden, 2002; Payan, 2004; Pueyo *et al.*, 2000; Richardson, 1991; Kondo and Ida, 1995).

The enzyme inhibitors impede digestion through their action on digestive  $\alpha$ -amylase and proteinases which play a key role in the digestion of plant starch and portions. These proteins inhibit  $\alpha$ -amylase from birds, bacilli, insects and mammals. Inhibition of human salivary  $\alpha$ -amylase is characterized by  $K_i = 0.2$ , nm. A variety of assays are available for  $\alpha$ -amylase inhibitor activity determination (Alam and Gourinath, 2001; Berk *et al.*, 1981; Goff and Kull, 1995; Ramasubbu and Ragunath, 2004; Richardson, 1991; Strumeyer and Fisher, 1983). The method used here is based on monitoring the reducing groups which have been formed by hydrolytic cleavages of inter sugar bands. In principle this assay allows the determination of not only the total hydrolytic activity but also that of distinct hydrolases such as exo - endo - amylases and disbranching enzyme. Varying amounts of maltose used for calibration ,therefore total inhibition of hydrolytic activity affects on maltose content and we will enable to compare activity of wheat  $\alpha$ -amylase different  $\alpha$ -amylase based on this character.

Although a diagnostic method based on the different inhibition levels of salivary and pancreatic isozymes by wheat amylase inhibitor has been developed (Strumeyer

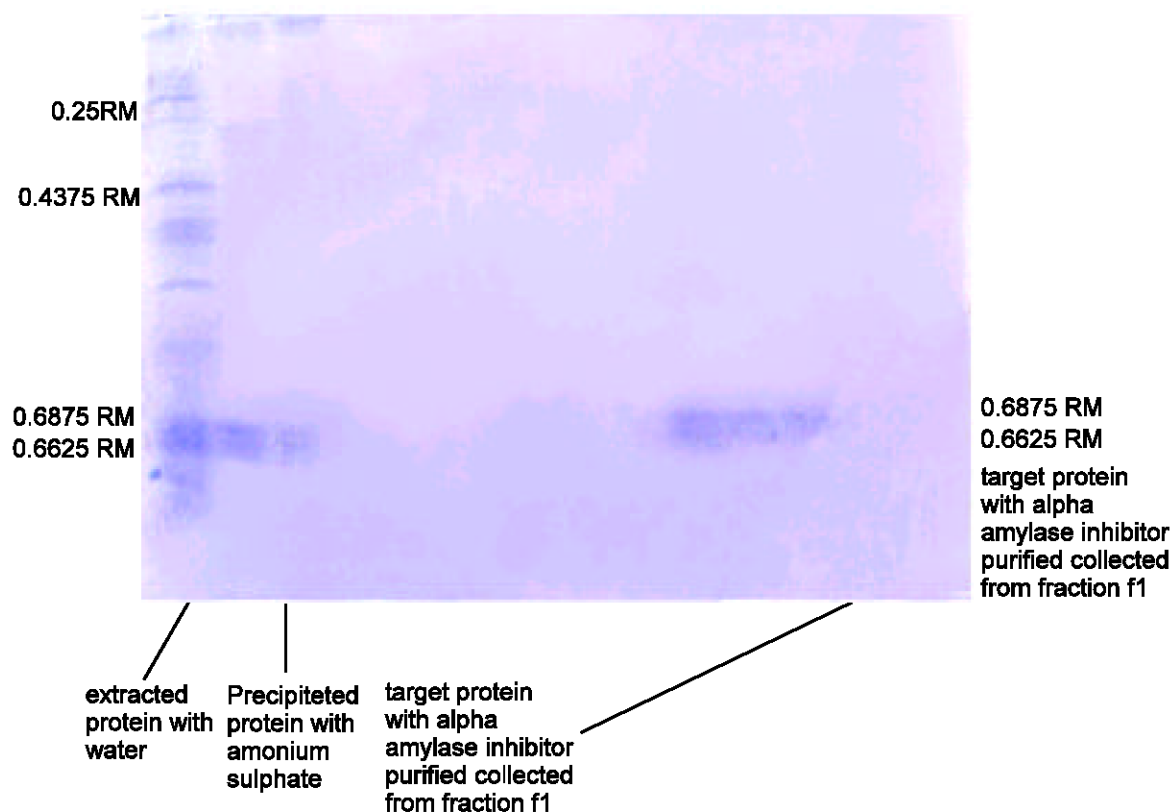


Fig. 2: Gel electrophoresis of wheat alpha-amylase inhibitor collected from FPLC Fraction using SDS-PAGE method. Mobility of protein bond relative to bromo phenol blue shown as RM.1) Albumin like protein extracted with water. 2) precipitated protein with ammonium sulfate F (35-50). 3,4,5,6) Target protein collected from FPLC fraction shown 0.66 RM consider as dimmer protein .result of inhibitory effect (Table 1) show it is alpha-amylase inhibitor.

and Fisher, 1983; Ramasubbu and Ragunath, 2004). The inhibitory activities of  $\alpha$ -amylase inhibitor isolated from zarrin wheat grains against human Salivary and bacillus subtilis  $\alpha$ -amylase showed in Table 1. previous results have also shown abroad inhibition specificity with this protein active against enzymes from rice weevil (*Sitophilus oryzae*), *red flour beetle* (*Tribolium castaneum*), *yellow meal worm*, and human saliva (Octavio and Rigden, 2002). Our result showed strong inhibition (97.07%) of human salivary compare (89.97%) inhibition of bacillus subtilis by inhibitor (Fig. 3). This strong inhibition present few nutritional problems for healthy people because their inactivation in stomach and disruption by cooking. This result confirmed that inhibition is strictly competitive and in 1:1 ratio of enzyme/inhibitor. Major of the enzyme activities (not all as reported) abolished. This potential suggesting that these inhibitors could be used as tools of engineered resistance of crop plants against insect and microbial pests. High inhibitory activities of these inhibitors against human salivary that our result confirmed suggest its

potential in prevention and therapy of obesity and diabetes. Living organisms use enzyme inhibitors as a major tool to regulate glycolytic activities of alpha-amylase. In most cases the mechanism of inhibition occurs through the direct blockage of the active center at several sub sites of the enzyme (Payan, 2004). The clinical usefulness of these inhibitors for determining the ratio pancreatic to salivary isoamylase activity in serum was evaluated and implies as inhibitor test in clinical laboratories (Berk *et al.*, 1981; Suehiro and Otsuki, 1984; Oneda *et al.*, 2004) This study combine the determination of inhibitory specificities with some structural explanation such relative molecular weight that will improve essential in the better understanding of alpha-amylase inhibitor interactions. The most promising candidate from this work is 0.66 protein which strongly inhibits hydrolytic activity of both human salivary and bacillus subtilis alpha-amylase. In Iran and all of the world wheat use as a major food source. it is important to investigate possible nutritional, allergenic and toxicological

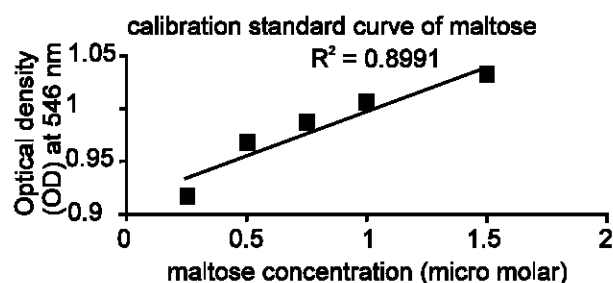


Fig. 3: Determination of reducing groups of maltose by the dinitrosalicylic acid method. Varying amounts of maltose (0-1.5  $\mu$ mol) processed as described in method. Standard curve equation used for determination of maltose content in samples.

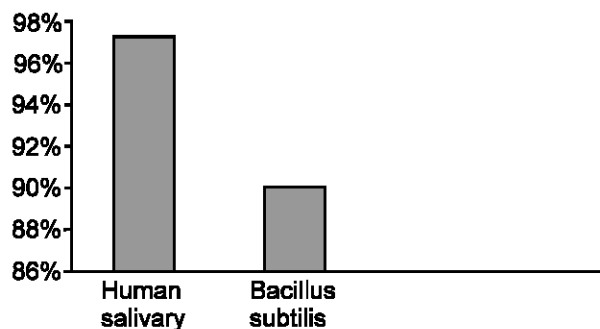


Fig. 4: Inhibition of human salivary and bacillus subtilis hydrolytic activity by wheat alpha-amylase inhibitor collected from FPLC fraction calculated based on result of Table 1.

problems that may be caused by these inhibitors. Extracted and purified inhibitor may be used as a drug-design target for treatment of diabetes by improving their diet. It was observed by our results that specificities of the inhibitor extracted from Iranian cultivar wheat (zarrin) are not exactly the same as other wheat and cereal inhibitors which are reported in papers. Therefore, it is important to suggest a wide and deep investigation of enzyme inhibitors that exist in Iranian cereals.

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