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ORIGINAL ARTICLE

Partial Purification of α-amylase Inhibitors in Wheat Kernels and the assessment of their Inhibitory activity upon α-amylase of *Aspergillus oryzae*

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ABSTRACT
Cereal and legume kernels have many α-amylase inhibitors that they have potential to inhibit the α-amylase activity from variant sources. The subject of this study was extraction of wheat seeds, partial purification of α-amylase inhibitors and the assessment of their inhibitory activity upon α-amylase of Aspergillus oryzae. In this regard, we precipitated extractions as three parts with different saturation percentages of ammonium sulfate. Then, we dialyzed, in order to remove ammonium sulfate and better check of their inhibitory activity upon A. oryzae α-amylase. With the assessment of the inhibitory activity of each sample on the basis of their absorbance in 540 nm and in comparison to the absorbance of negative control, we saw that only the fraction of 30% to 60% ($F_{30\text{-}60\%}$) of wheat extract had inhibitory activity upon defined α-amylase. Inhibitory percentage of this fraction was 95.77. In addition, for more characterization of these inhibitors, we conducted SDS-PAGE analyses. By assaying the activity of these inhibitors upon other α-amylase such as salivary and pancreatic α-amylases, this may enable us to use them as α-glucosidase inhibitors such as Acarbose and Miglitol for the treatment of type II diabetes. Also, the results might be applied in agriculture to produce transgenic plants that express α-amylase inhibitors against α-amylase of the pests.
Key words: *α-amylase inhibitors, partial purification, wheat kernel, Aspergillus oryzae*

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INTRODUCTION

Plants produce a myriad of molecules that are natural deterrents to pests and pathogens. These include carbohydrate molecules, polyanions, and several defense-related proteins such as chitinases, β-1,3-glucanases, lectins, α-thionins and β-thionins, defenses, and inhibitors of digestive enzymes [1]. This last group comprises α-amylase and proteinase inhibitors, which usually act by a competition mechanism, inhibiting the digestive enzyme activity that leads to insect death [2]. This property clearly shows a remarkable potential for the development of these proteins as biotechnological tools for application in agriculture for the control of pests and pathogens [3, 4].

α-amylases are a group of hydrolase enzymes which are found in microorganisms, plants and animals. These enzymes break α 1,4) glycoside bonds in polysaccharides such as starch and turn them into oligosaccharides [5].

α-amylases are inhibited by several families of proteinous and non-proteinous compounds [6]. Plant α-amylase inhibitors (α-AIs), particularly abundant in cereals and leguminosae, have been extensively studied, in part because they play a role in plant resistance to insect and microbial pests and also because they are major allergens involved in baker's asthma disease [4].

Recently, wheat α-amylase inhibitors are more considered because of their therapeutic effects on obesity and non dependent insulin diabetes [7]. In the last two decades, several α-amylase inhibitors have been described in numerous plant species [8, 9]. As proposed by Richardson [10], α-amylase inhibitors may be

conveniently classified by their tertiary structure into six different classes: lectin-like, knottin-like, cereal-type, Kunitz-like, γ-purothionin-like and thaumatin-like. Their specificity has been widely explored, with some capabilities of acting only against insect α-amylases or against mammalian enzymes [4, 8].
Oneda et al, purified 0.19AI from the blend of α-AIs of wheat kernel existing in crude preparation of them that they contain 0.19, 0.28, 0.36, 0.38, 0.53 and other α-AIs which was prepared according to the method reported previously by Choudhury et al. [7].
The purpose of the present study, is to provide a witness in vitro for showing the inhibitory potential of α-AIs exist in wheat kernel ,after the extraction and partial purification, upon the α-amylase of *A. oryzae* and indeed the inhibition of this enzyme could prevent dissociation of starch, therefore , whenever we could inhibit salivary and pancreatic α-amylase in vivo , this may enable us to use them as α-glucosidase inhibitors such as Acarbose and Miglitol for the treatment of type II diabetes.

MATERIALS AND METHODS

Partial purification of "*Triticum aestivum*" α-amyalse inhibitors from wheat seeds

Wheat seeds (*T aestivum*) were obtained from the agricultural research center of Sanandaj. Cotyledons were ground into flour and then extracted with 0.15M NaCl (1:5, w/v, meal to buffer ratio) with continuous stirring for 4 h at 4^0C. The material was then centrifuged at 10000g, 4^0C for 30 min [4]. The precipitate was discarded and supernatant was submitted to fractionation with ammonium sulfate. For this purpose, three fractions with different saturation percentages of ammonium sulfate were considered [($F_{0\text{-}30\%}$), ($F_{30\text{-}60\%}$) and ($F_{60\text{-}100\%}$)]. Following this step, for each of these fractions, dialysis was done. Materials acquired were stored in 4^0C, separately.

Determining the protein concentration of samples via the Bradford method

In this step, by means of Coomassie color reagent and bovine serum albumin (prepared in defined concentrations, as standard protein), the concentration of the protein samples was determined via Bradford method [11].

HPLC and relative estimation of molecular mass via SDS-PAGE

Following dialysis, the fractions obtained between 0% to 30% saturation ($F_{0\text{-}30\%}$), 30% to 60% ($F_{30\text{-}60\%}$) and 60% to 100% ($F_{60\text{-}100\%}$) were applied onto a HPLC column (Reprosi 100 C18) with a flow rate of 1.0mLmin^{-1}. TFA 0.1% was used as ion-pairing agent. Unfortunately, because the lack of collector, the materials in individual peaks could not be collected.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted as described by Laemmli [12] at room temperature, using protein ladder (10-200kDa) (PageRuler™ Unstained Protein Ladder #SM0661) and bromophenol blue as the tracking dye.

Preparation of enzymatic solution of *A. oryzae* and assessment of inhibitory activity

α-Amylase of *A. oryzae* was purchased from Fluka-Sigma-Aldrich company. This enzymatic powder contain 30 enzymatic units per each 1mg. For preparation of enzymatic solution, 1mg from enzymatic powder in 1ml distilled water solved.
In 3 tubes, the same amount of buffering starch was added as substrate and followed by adding the adequate volume from each of partial purified α-amylase inhibitors (i.e. in the first tube $F_{0\text{-}30\%}$, in the second one $F_{30\text{-}60\%}$ and in the third one $F_{60\text{-}100\%}$ were added), respectively. As a negative control, in 4th tube not added any inhibitors, distilled water was used instead. Resulting mixture for 5min in 37^0C was pre-incubated and then to each of 4 tubes, enzymatic solution added, all of them for 15min in 37^0C incubated. In the end of the period 1ml lugol solution for blocking the hydrolytic reaction of α-amylase added. The optical density of each sample in 540nm was measured and percentage of inhibitory activity was calculated by following formula [13, 14, 15]:

$$Inhibitory\ \% = \left(\left(OD_{negative\ control} - OD_{sample}\right)/OD_{control}\right) \times 100$$

RESULTS AND DISCUSSION

The partial purified inhibitor preparation from wheat seeds, as mentioned above, was obtained by aqueous solution extraction and ammonium sulfate precipitation [16] that was followed by dialysis. Each of the three fractions [($F_{0\text{-}30\%}$), ($F_{30\text{-}60\%}$), ($F_{60\text{-}100\%}$)] was applied onto HPLC column (Reprosi 100 C18) from each of them only one major peak was eluted (data for $F_{30\text{-}60\%}$ shown in Fig.1). The absorbance of the peaks monitored in 280 nm. Unfortunately, because of the lack of collector, the material in individual peaks could not be collected and for this reason, this step was done only for more characterization of fractions. When the fractions were tested against *A. oryzae* α-amylase, only the fraction ($F_{30\text{-}60\%}$) showed inhibitory activity about: 95.77% (±1.86) (data shown in Fig.3). As it was impossible to do almost complete purification via collection of each fraction, therefore, after doing SDS-PAGE, multiple bands for each fraction were acquired that we could compare with the former results in other studies to estimate

the relative molecular weight of possible inhibitors. The band with 14kDa of weight, could be compared with the previously-identified inhibitors WRP25, WRP26 and WRP27 [4] and the band with 28 kDa could be compared to 0.53 inhibitor. It should be noted that these are likely not to be quite true. According to the acquired results in this research, that show the existing of α-AI in $F_{30\text{-}60\%}$, therefore it could be said these results are compliance with the previous research.

In the previous research, after extraction, precipitation with ammonium sulfate has been done only in a certain fraction; for example, Charles Dayler et al. in 2005 [10], Wisessing et al. in 2010 [17], Franco et al. in 2000[4] and Weselake et al. in 1983 [18], performed precipitation by ammonium sulfate in the following fractions: $F_{0\text{-}85\%}$, $F_{0\text{-}80\%}$, $F_{20\text{-}40\%}$ and $F_{40\text{-}70\%}$, respectively.

In the current study, as noted before, there was no possibility to perform purification more; therefore, in the analysis of the inhibitor effects it should be considered that some contaminants are accompanied with them.

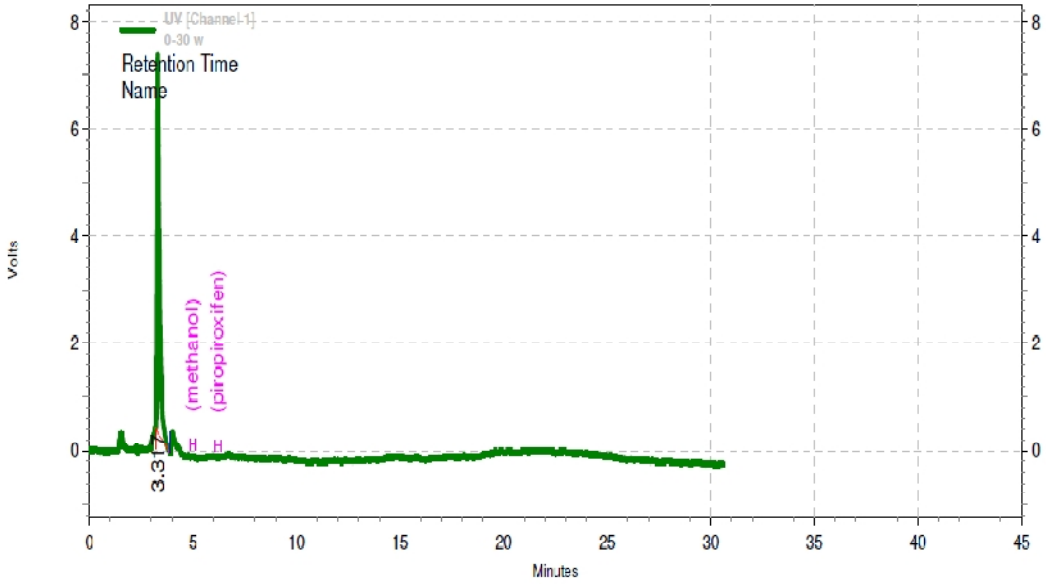

Fig.1. The major fraction obtained from HPLC column, (Reprosi 100 C18) with a flow rate of 1.0 $mLmin^{-1}$. TFA 0.1% was used as ion-pairing agent, and acetonitrile with 0-70% gradient was used up to 50^{th} min.

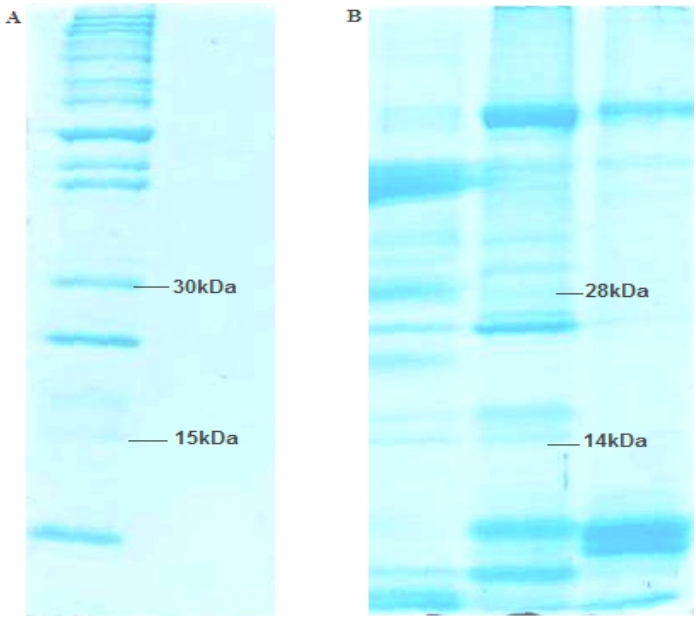

Fig.2. SDS-PAGE A: protein ladder; B: three fractions [($F_{0\text{-}30\%}$), ($F_{30\text{-}60\%}$) and ($F_{60\text{-}100\%}$)], stained with Coomassie-Blue.

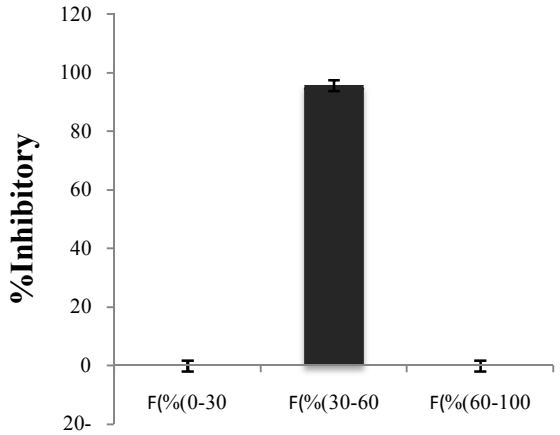

Fig.3. Inhibitory activities of three fractions [($F_{0\text{-}30\%}$), ($F_{30\text{-}60\%}$) and ($F_{60\text{-}100\%}$)] against α-amylase of *A. oryzae*.

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