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SOME PROPERTIES OF ENDOGENOUS α-AMYLASE INHIBITOR FROM WHEAT GRAIN

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ABSTRACT

The protein with endogenous α-amylase inhibitor activity was extracted and purified from wheat (Triticum aestivum) grains through 70% ammonium sulphate fractionation, ion-exchange chromatography on DEAE-Sephacel and gel-chromatography on Toyapearl HW-50. The molecular weight and isoelectric point of protein were estimated about 21 kD and 7.0 respectively. The inhibitor repressed of high pI wheat α-amylase isozymes, but had no effect on amylases of microbial and animal origin. The inhibitor also exhibited activity towards serine protease subtilisin. The inhibitor was the most active at pH 7.8 to pH 8.0 and was stable up to 90° C for 10 minutes. The protein is localized in the peripheral parts of the seed, and in the starchy endosperm.

Keywords: α-amylase, isoenzymes, subtilisin, inhibitor, wheat

INTRODUCTION

Recently scientists have been intensively studying proteinaceous inhibitors of proteases and amylases of plants and seeds due to their great theoretical and practical importance. Legume grains are particularly rich in these inhibitors. Legume inhibitors are widely used in scientific research, medicine and agriculture **(Mosolov *et al.*, 2005)**. Cereal seeds also contain a variety of amylase inhibitors of protein nature. Most of the known inhibitors are active against exogenous α-amylase of insects and mammals **(Silano *et al.*, 1987; Svenson *et al.*, 2004; Islamov *et al.*, 2007)**. Protein inhibitors against endogenous α-amylase were studied comparatively less. The first group of inhibitors is usually attributed to the components of the defense system of plants **(Gorjanovich *et al.*, 2009)**. The physiological role of inhibitors of the second group, apparently, is to regulate the activity of endogenous enzyme during seed maturation and germination **(Taufel *et al.*, 1997)**.

One of the well-studied member of this group is a bifunctional α-amylase/subtilisin inhibitor first discovered in barley grains (BASI) **(Weselake *et al.*, 1983; Mundy *et al.*, 1983)**. The molecular weight and pI (isoelectric point) of the protein are about 20 kD and 6.9 respectively. The inhibitor is able to suppress the activity of both α-amylase II of barley and serine protease of microorganisms. In grain the inhibitor is synthesized at the period of maturity, and the level of its activity is controlled by the ABA hormone **(Robertson *et al.*, 1989)**. Similar BASI inhibitors were later found in the seeds of wheat, rye, triticale and rice **(Weselake *et al.*, 1985; Zawistowska *et al.*, 2007)**. The functioning and regulation of these inhibitors have been studied intensively at present. On the basis of the study of the protein structure this family has been referred to Kunitz-type inhibitor. In the mechanism of enzyme-inhibitor interaction the important role is given to hydrated forms of calcium **(Svenson *et al.*, 2004)**.

Cereal α-amylase is highly polymorphic and is represented by two main groups: α-amylase I and α-amylase II. The pI of these groups were around 4.5 and 5.8 respectively. Isozymes of the two groups differ in the degree of affinity to calcium cations, sensitivity to pH and temperature stability **(Fursov *et al.*, 1986; Muralikrishna *et al.*, 2005)**. In the hydrolysis of wheat starch a high importance is given to the α-amylase II, which carries out the primary attack of granules. An increased activity of α-amylase, due to grain damage resulted by pre-harvest sprouting (PHS), significantly reduces the quality of flour and bread **(Kruger *et al.*, 1994)**. From this perspective, the study of inhibitors as natural regulators of grain α-amylase represents a high scientific significance **(Abdul-Hussain *et al.*, 1989; Kanzaki *et al.*, 1993)**.

In our study we have obtained a highly purified endogenous α-amylase inhibitor from the grain of soft spring wheat and investigated some of its physical and chemical properties.

MATERIAL AND METHODS

Inhibitor Purification

α-Amylase inhibitor from wheat grain was purified according to the procedure described for barley inhibitor **(Weselake *et al.*, 1983)** with some modifications. Wheat grains (*Triticum aestivum L.* cv Kazakhstanskaya 10) were ground in the mill LM-120 (Perten Instruments). 200 mL of 0.02 M Na-acetic buffer pH 5.2 with 1 mM $CaCl_2$ were added to 50 g of whole meal. The mixture was stirred for 2 hours at 4 °C and centrifuged at 8000g for 20 minutes. The protein of supernatant was precipitated by $(NH_4)_2SO_4$ in the range of saturation from 40 to 70% and dialyzed against 5 mM Tris-buffer pH 8.0 with 1 mM $CaCl_2$. The insoluble precipitate was removed by centrifugation, and the supernatant was applied to a DEAE-Sephacryl (Pharmacia) column (1.2 cm × 8 cm) equilibrated with buffer for dialysis. Elution was performed in a step gradient of NaCl from 0.07 to 0.11 M.

Enzyme Purification

The total α-amylase from germinated wheat grain was purified by the method of glycogen precipitation **(Gilmanov *et al.*, 1981)**. Separation of the groups of α-Amy 1 and α-Amy 2 was performed on CM-Sepharose (Pharmacia) column (1.2 cm × 6 cm) equilibrated with 0.02 M Na-acetic buffer pH 5.2. Elution of the enzyme fractions was carried by the step gradient – first by starting buffer and then by 0.08 and 0.2 M buffer. For a clearer separation of two α-amylase groups the stage of elution by 0.12 M buffer was additionally introduced. Native electrophoresis confirmed the presence of isozymes α-Amy 2 in the fraction of 0.08 M buffer, and isozymes α-Amy 1 – in the fraction of 0.2 M buffer. The isolated groups of α-amylase were concentrated on Amicon cell and stored at 4°C for further use.

Inhibitor Assay

The assay of the inhibitory activity was carried out at pH 8.0 (50 mM Tris-buffer) and pH 5.2 (50 mM Na-acetic buffer) supplemented with 1 mM $CaCl_2$. A protease activity was assayed after incubation of various concentrations of inhibitor with 10 μg subtilisin at 30 °C for 30 min. One unit of activity was

defined as the amount of inhibitor, which after the precipitation of proteins by trichloroacetic acid on the filtrate gives a reduction of the absorbance comparing to the control on 0.01 unit in min. at 280 nm **(Shulgin *et al.*, 1985)**.

Heat Stability Studies

To study the temperature stability of the inhibitor and the inhibitor-enzyme complex the samples were preincubated at various temperatures (65 to 90^{0}C) for different time frameworks, whereupon the activity was measured as it was described earlier.

Electrophoresis and Isoelectrofocusing

SDS-electrophoresis of proteins during inhibitor purification was performed in 10% PAG plates using the Laemmly method **(Laemmly *et al.*, 1970)**. Native electrophoresis of α-amylase and the inhibitor was carried out in 7.5% PAG according to the method **(Gilmanov *et al.*, 1981)**. Isoelectrofocusing of the inhibitor was carried out in 1 mm plate 5% PAG in the pH range from 3 to 10 (Ampholyte, Sigma) at a voltage of 600 V.

RESULTS AND DISCUSSION

In previous studies it was shown that barley grain contains protein factor, which is able to convert the malt α-amylase II into α-amylase III **(Weselake *et al.*, 1983)**. The protein factor was obtained and purified to more than 100 fold. In subsequent studies it was found that this factor can inhibit the activity of high pI α-amylase of cereal grains such as barley, rye, oats and wheat. Similar inhibitors have been discovered also in other species of cereals, including wheat **(Weselake *et al.*, 1985)**.

In order to obtain and study the properties of wheat inhibitor, we employed the scheme for barley inhibitor purification using some modifications. In our study, instead of linear, we used a narrow step gradient of salt (70 to 110 mM NaCl). Furthermore, at the final stage of purification we used gel-filtration on a firmer sorbent Toyapearl HW-50. SDS-electrophoresis of the wheat inhibitor during its purification is presented on Fig.1. The molecular weight of the inhibitor corresponded to 21 kD. The isoelectric point of the protein was estimated at 7.0 (Fig.2). The electrophoregrams illustrated that the highest purity corresponded to the inhibitor obtained from the bran, which we subsequently used in further work. We have also analyzed the effect of the inhibitor on two groups of isoforms of wheat α-amylase – Amy1 and Amy2 (Fig.3). It was found that the inhibitor is highly specific and is able to suppress the activity of isozymes only with high pI (α-Amy1). The obtained inhibitor showed no activity against a number of α-amylase of microbial and animal origins.

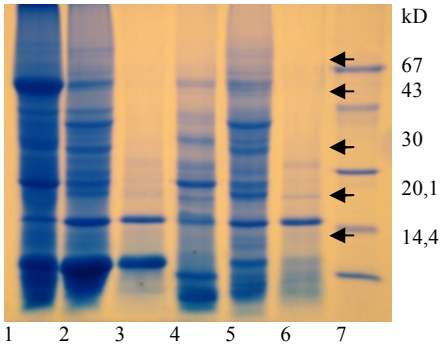

Figure 1 SDS-electrophoresis of wheat α-amylase inhibitor during purification; 1-3 – whole grain proteins after the precipitation by ammonium sulfate, ion-exchange chromatography and gel-filtration respectively; 4-6 – bran protein after precipitation by ammonium sulfate, ion-exchange chromatography and gel-filtration respectively; 7 – MW markers.

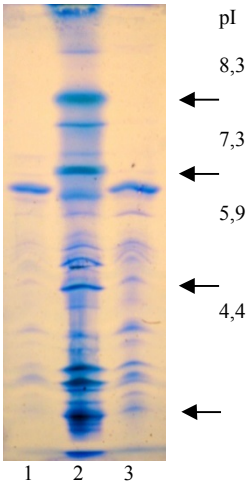

Figure 2 Isoelectrofocusing of wheat α-amylase inhibitor; 1 and 3 – inhibitors obtained from bran and whole grains respectively; 2 – pI markers.

These findings point to the close similarity of properties and specificity between the obtained protein and the barley inhibitor BASI. Therefore, we have tested the inhibitory effect of the obtained protein on subtilisin. The results shown on Fig. 4, indicate the ability of the inhibitor to effectively suppress the activity of the enzyme. Thus, the inhibitor obtained from wheat is bifunctional and belongs to the family of ASIs.

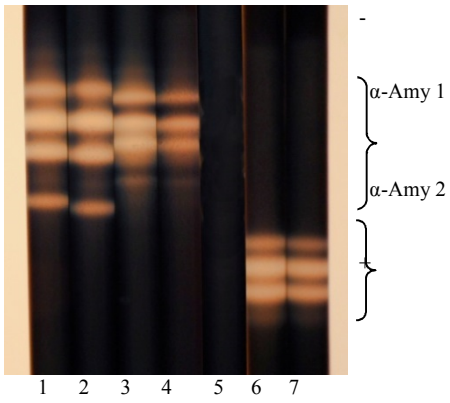

Figure 3 Effect of the inhibitor on wheat α-amylase isozymes. 1 – 5 correspond to inhibitor concentrations of 0, 10, 20, 30 and 50 µg/ml respectively; 6 and 7 correspond to inhibitor concentrations of 0 and 50 µg/ml, enzyme concentration – 30 µg/ml.

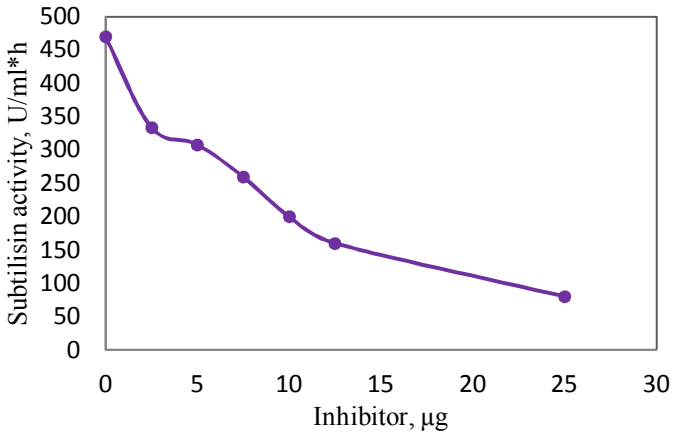

Figure 4 Effect of inhibitor on Subtilisin activity

Later on, we have investigated the effect of pH and high temperature on the activity of the obtained inhibitor and the stability of the inhibitor-enzyme complex (with α-Amy1 group). It was found that the inhibitor is most active in slightly alkaline media (pH range from 7.8 to 8.0), whereas at pH 5.2 its activity dropped to almost zero, especially in the condition of heat treatment. However, at pH 8.0 the inhibitor showed a very high thermostability and did not lose its activity even at a temperature of 90^o C for 10 min. (Table 1). This fact is particularly noteworthy, since it was previously reported on relative

thermolability of BASI **(Weselake et al., 1983)**. On the other hand, there is an evidence of high temperature stability of other inhibitory proteins with similar properties, such as Kunitz trypsin inhibitor, the 0.19 α-amylase inhibitor from wheat **(Roychandhuri *et al.*, 2003; Azarkan *et al.*, 2006; Oneda *et al.*, 2004)**.

Table 1 Effect of high temperature on inhibitor activity

Sample	Inhibitory activity, units	Loss of inhibitory activity, %
Control	3990	0
70 °C, 10 min	3810	4.5
80 °C, 10 min	3950	1.1
90 °C, 10 min	3980	0.3

As the inhibitor itself, its complex with the enzyme was also quite thermostable and has not decayed completely at 65 °C for 15 min. (Table 2). The high stability of the complex apparently is due to very close pI values of the inhibitor and α-Amy1 isoforms, as well as to the presence of calcium in the enzyme molecule, which enhances a mutual affinity of both components of the complex. Unlike the barley inhibitor **(Weselake *et al.*, 1983)**, the wheat inhibitor did not change significantly the mobility of α-amylase isozymes during native electrophoresis (Fig.5).

Table 2 Temperature stability of inhibitor-enzyme complex

Sample	Amylase activity, units	Complex decomposition percentage, %
Control	3460	0
65 °C, 5 min	3510	1.4
65 °C, 10 min	4340	20.3
65 °C, 15 min	5570	37.9

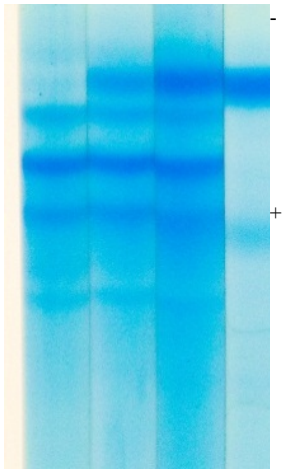

Figure 5 Native electrophoresis of inhibitor-α-Amy 1 isoenzymes complex
1-3 – inhibitor-enzyme complex (inhibitor concentrations correspond to 10, 20 and30 µg/ml respectively, the enzyme concentration is 20 µg/ml); 4 – inhibitor, 20 µg/ml; 5 - α-Amy 1 isoenzymes 20 µg/ml.

We have investigated the localization and distribution of inhibitory activity in the various particles of wheat seed. For the analysis of the inhibitory activitywe used whole grains, bran (fraction 1 with shells and aleurone), bran (fraction 2 with the embryo, scutellum and subaleurone endosperm), as well as the endosperm flour. From the data illustrated in Table 3, the inhibitor is present ubiquitously, but its specific activity is highest in the endosperm. For comparison, we have also measured the inhibitory activity in barley grain, which showed that inhibitor content is higher in barley than in wheat in more than two times. But despite this, the wheat is also a good source of cereal α-amylase inhibitor.

Table 3 Inhibitor content in various seed parts

Source	Inhibitory activity / mg protein
Barley grain	7010
Wheat grain	2680
Wheat bran (fraction 1)	1980
Wheat bran (fraction 2)	2330
Wheat endosperm flour	2700

CONCLUSION

Through the fractionation of wheat grain proteins by ammonium sulphate, followed by column ion-exchange chromatography and gel-filtration, we have obtained a highly purified inhibitor of endogenous α-amylase. The molecular weight and isoelectric point of protein were about 21 kD and 7.0 respectively. The inhibitor showed high temperature stability and maintained its activity even at 90°C for 10 min. The protein is localized both in the peripheral parts of the seed (embryo and aleurone) and in the starchy endosperm. The inhibitor demonstrated a high specificity and was active against wheat α-amylase Amy1 and protease subtilisin, which indicates that the inhibitor belongs to ASI family.

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