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Properties and Specific Functional Features of Wheat Grain α-Amylase/Subtilisin Inhibitor

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Abstract—A protein bifunctional inhibitor of endogenous α-amylase and subtilisin has been isolated from wheat grain and purified. The inhibitor specifically inactivates α-amylase isozymes with high isoelectric point values (group α-AMY1) and has almost no effect on the α-AMY2 isozymes with low isoelectric point values. This enzyme does not belong to glycoproteins and has a molecular weight of 21 kDa and an isoelectric point of 7.2. The protein displays a relatively high thermostability and pH optimum of 8.0; its inhibitory activity requires the presence of Ca^{2+} cations. The inhibition of excess α-amylase in wheat grain with a low falling number by the purified protein is studied.

Keywords: Triticum aestivum L., bifunctional inhibitor, α-amylase, isozymes, subtilisin, falling number

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INTRODUCTION

Cereal grains contain manifold amylase inhibitors of a protein nature. Most of them are active towards exogenous α-amylases (1,4-glucan-4-glucohydrolases, EC 3.2.1.1) of bacteria, insects, and mammals. There are a few protein inhibitors known to act on their own (endogenous) α-amylases, but they are rather poorly studied [1–4]. The group of endogenous α-amylase inhibitors is regarded as a component of the plant defense system (immunity). The physiological role of endogenous α-amylase inhibitors most likely consists of regulation of the endogenous enzyme activity during grain ripening and germination, though there is still no direct evidence for this assumption. Many representatives of both groups are ascribed to the PR protein family, which is involved in pathogenesis [5, 6].

The best-known protein inhibitor of grain α-amylases is the bifunctional α-amylase/subtilisin inhibitor (**BFI**), which was first discovered in barley grain and designated BASI (barley amylase/subtilisin inhibitor) [7, 8]. This inhibitor is able to suppress the activities of barley endogenous α-amylase and *Bacillus* sp. serine proteinase. BASI-like inhibitors were later discovered in the grains of other cereal species, such as wheat, rye, rice, and triticale [9–12]. So far, BASI has been the center of attention because of its important role in regulation of the α-amylase content in malting barley, which influences malt quality. This is an issue of considerable applied interest. As was also demonstrated, BASI is involved in plant protection against microbial and fungal pathogens, as well as abiotic stresses. The protein structure, regulation of its activity, its functioning in grain, and genetic polymorphism have been studied in detail [13–16]. A BFI is also present in wheat grain and is referred to as WASI (wheat amylase/subtilisin inhibitor). The data on this protein are limited. However, this protein inhibitor, along with protective functions, can play a certain role in the control of flour quality and its bread-backing characteristics by inhibiting undesirable excess α-amylase activity in the ripening grain and during grain storage.

The α-amylase of germinating wheat grain displays a high polymorphism. This enzyme is represented by two major groups, namely, α-AMY1 with basic pI values (6.3–7.5) and α-AMY2 with acid pI values (4.9–6.0). The isozymes of these groups are encoded by different gene subfamilies and somewhat differ in their structure, properties, regulation, and functions in the kernel [17, 18]. α-AMY1 ("germination" α-amylase) plays an important role in starch hydrolysis, namely, in the initial attack of starch grains. This enzyme activity drastically increases as a result of grain damage caused by preharvest sprouting (PHS) or the presence of specific α-amylase form, late maturity α-amylase (LMA). This considerably decreases the quality of flour and bread [19, 20]. Thus, the study of BFIs as natural regulators of grain α-amylase is an important problem.

The goal of this work was to isolate a highly purified BFI of α-amylase/subtilisin from wheat grain and examine some of its biochemical properties and specific functional features.

MATERIALS AND METHODS

BFI purification. Dormant wheat (*Triticum aestivum* L. cv. Kazakhstanskaya 10) grain was ground in an LM-120 (Perten Instruments, Sweden) laboratory mill. A weighed flour sample (50 g) was supplemented with 200 mL of 20 mM acetate buffer (pH 5.2) containing 1 mM $CaCl_2$. The mixture was stirred for 2 h at +4°C and centrifuged at 8000× *g* for 20 min. Protein was precipitated from supernatant with ammonium sulfate (40 to 70% saturation) and dialyzed against 10 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$. The precipitate was removed by centrifugation, and the supernatant was loaded onto a column (1.2 × 8 cm) filled with DEAE Sepharose (Sigma, United States) equilibrated with the buffer for dialysis. The protein was eluted with a stepwise NaCl gradient (0.07 to 0.11 M). The fractions containing inhibitory activity were concentrated on an UM-10 filter in an Amicon cell (Millipore, United States) and assayed by affinity chromatography on immobilized α-amylase.

The purified wheat α-amylase was immobilized on Sepharose 4B activated with CNBr (Amersham Biosciences, Sweden) according to the conventional protocol for binding of the protein ligand [21]. Before loading the protein, the sorbent was equilibrated with 0.05 M phosphate buffer (pH 7.6) and, after loading, was washed with the starting buffer and then with the same buffer supplemented with 1 M NaCl and 8 M urea. The bound protein inhibitor was eluted with 75 mM acetic acid. The fractions that contained the inhibitor were immediately neutralized with 1 M NaOH to pH 7.0–7.2 to prevent inactivation of the inhibitor and then concentrated and stored at 4°C.

Purification and separation of α-amylase. The total α-amylase from germinating wheat grain was purified by glycogen (USB, United States) precipitation in the presence of 40% ethanol [22]. The isozyme groups α-AMY1 and α-AMY2 were separated on a column (1.2 × 6 cm) with CM-Sepharose (Sigma, United States) equilibrated with 20 mM acetate buffer (pH 5.0). The proteins bound to the ion exchanger were eluted with stepwise gradients of buffers: first, the starting buffer and then twice with 0.08 and 0.2 M acetate buffer (pH 5.0). The fractions containing α-amylase were concentrated and stored at 4°C.

Determination of BFI activity. The antiamylase activity of the inhibitor was determined in 50 mM acetate Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and 50 mM acetate buffer (pH 5.2) containing 1 mM $CaCl_2$. The degree of inhibition was assessed according to the decrease in α-amylase activity. The mixture of inhibitor and enzyme (20 μL each) was supplemented with the corresponding to buffer to a volume of 1 mL, incubated for 15 min at 30°C, supplemented with 1 mL of β-dextrin (1 mg/mL), and additionally incubated for 15 min. A mixture without inhibitor was used as a control. The reaction was stopped by adding 4 mL of iodine solution containing 0.005% J_2 and 0.05% KJ to determine the optical density at 540 nm. The change in optical density of the control by 0.01 during 1 h in the presence of inhibitor in a volume of 1 mL was taken as the unit of inhibitor activity [7].

The antiprotease activity of inhibitor was determined according to the degree of inhibition of subtilisin A (Sigma, United States) activity in 0.1 M Tris–HCl buffer (pH 7.8) during hydrolysis of 1% casein used as a substrate. The protease activity was determined after the incubation of 10 μg of subtilisin and different quantities of inhibitor for 30 min at 30°C. The proteins were than precipitated with trichloroacetic acid to measure the optical density of the filtrate at 280 nm. The amount of inhibitor that decreased optical density of the filtrate versus control by 0.01 over 1 min was taken as the activity unit [23].

Assessment of BFI thermostability. To estimate thermostability, the protein inhibitor was preincubated for different time periods at temperatures of 65 to 90°C to measure the activity as described above.

Protein electrophoresis and isoelectric focusing. The proteins were separated by electrophoresis in the presence of sodium dodecyl sulfate (**SDS**) in 10% polyacrylamide gel (**PAG**) plates according to Laemmli [24]. The electrophoresis under native conditions was conducted in 7.5% PAG columns as described in [22]. Isoelectric focusing (**IEF**) was performed in 6% PAG plates with Servalyt 3-10 (Serva, Germany) in a Multiphor II (LKB, Sweden) device at a voltage of 600 V.

Detection of glycoproteins in gel. A Pierce Glycoprotein Staining Kit (Thermo Scientific, United States) was used according to the manufacturer's protocol to stain glycoproteins in PAG after SDS electrophoresis.

Determination of falling number (FN). The FN of the grist (ground grain) produced from commercial grain was determined with a Falling Number 1700 (Perten Instruments, Sweden) device according to International Standard (**ISO**) 3093-2009 [25].

The experiments and measurements of enzyme activities were performed in three replicates. The data are shown as arithmetic means and their standard deviations.

RESULTS AND DISCUSSION

A three-stage purification comprised of protein precipitation with ammonium sulfate (30–70% saturation) from water extract, ion exchange chromatography, and gel chromatography was used to isolate and study the properties of wheat grain BFI. In this process, the fractions with antiamylase activity were collected. The degree of purity of the target protein at each stage was controlled by SDS electrophoresis. It is evident from the electrophoretic pattern (Fig. 1) that the protein has a molecular weight of 21 kDa. According to IEF data, the isoelectric point (pI) of the pro-

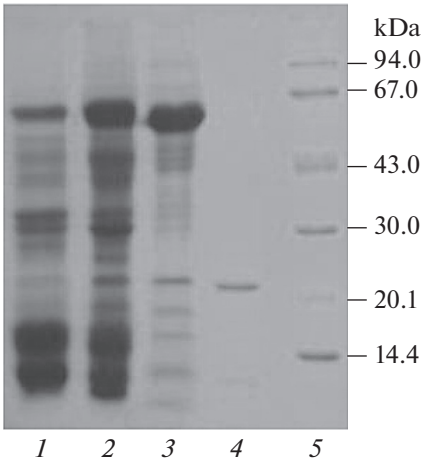

Fig. 1. SDS gel electrophoresis of (*1*) the proteins from wheat grain extract; (*2*) precipitated with $(NH_4)_2SO_4$ (30–70% saturation); (*3*) after chromatography on DEAE-Sephacel; (*4*) after affinity chromatography on α-amylase–Sepharose; and (*5*) the molecular weight markers phosphorylase b (94.0 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

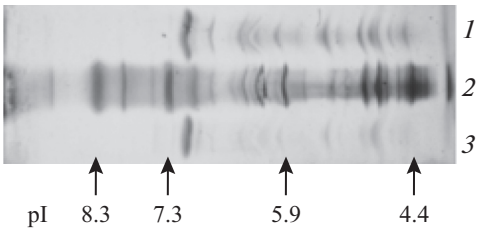

Fig. 2. Isoelectric focusing in PAG of (*1*) the proteins of wheat grain extract after heating for 15 min at 80°C; (*2*) purified BFI; and (*3*) the isoelectric point markers whale myoglobin (8.3), horse myoglobin (7.3), conalbumin (5.9), and ferritin (4.4).

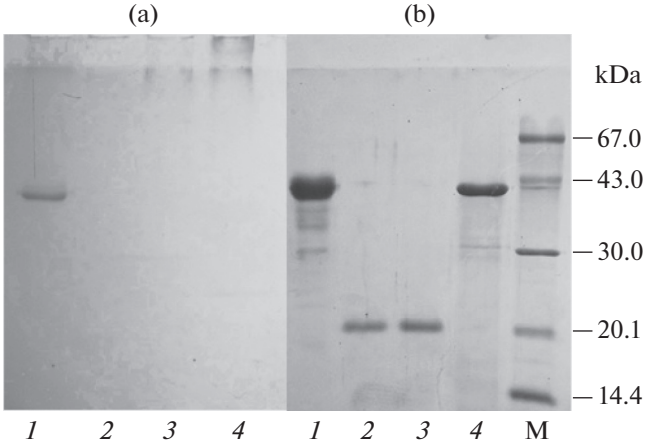

Fig. 3. Staining of (a) glycoproteins and (b) proteins in PAG after SDS electrophoresis: (*1*) horseradish peroxidase (positive control); (*2*) soybean trypsin inhibitor (negative control); (*3*) purified wheat BFI; (*4*) wheat α-amylase; and (M) molecular weight markers.

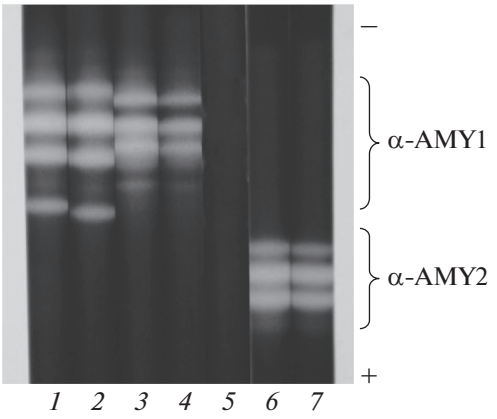

Fig. 4. The effects of (*1–5*) 0, 10, 20, 30, and 50 μg/mL purified BFI on AMY1 and (*6* and *7*) 0 and 50 μg/mL of this BFI on 30 μg/mL AMY2 from germination of wheat grain after 30-min preincubation of enzymes with inhibitor.

tein was in the neutral pH range and amounted to 7.2 (Fig. 2).

An important protein characteristic is the presence or absence of carbohydrate moiety. The presence of this moiety in a molecule confers the protein with some properties, including an increased stability towards proteolysis and high temperature, and promotes its directed transport. Thus, it was of interest to clarify whether the isolated inhibitor was a glycoprotein. SDS electrophoresis according to Laemmli with subsequent staining of gels for glycoproteins and total protein was performed for this purpose. Glycoproteins were stained with a special reagent kit (see Materials and Methods), and proteins were stained with Coomassie brilliant blue. As is evident from the electrophoretic pattern (Fig. 3), BFI, as well as the related soybean trypsin inhibitor, had no glycoprotein component. Note that the α-amylase of the wheat grain was also negative in the reaction for carbohydrates, although it is known that several cereal enzymes carry a carbohydrate moiety in their molecules [26].

The effect of the purified inhibitor on two major groups of wheat α-amylase isozymes (AMY1 and AMY2) was studied. The endogenous α-amylase isozymes were separated by ion exchange chromatography. Native gel electrophoresis demonstrated that α-AMY2 isozymes were eluted from the ion exchanger with 0.08 M acetate buffer and α-AMY1 isozymes, with 0.2 M buffer. The inhibitor appeared to be highly specific and able to inactivate isozyme activities with high pI values (α-AMY1) but was inefficient towards α-AMY2 group (Fig. 4).

The inhibitor was inactive towards several bacterial and mammalian α-amylases but inactivated the subtilisin A from *Bacillus licheniformis*. The data on activity inhibition of this bacterial proteinase are shown in Fig. 5.

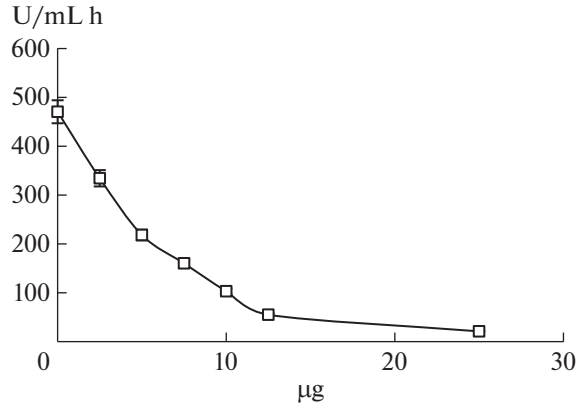

Fig. 5. Effect of purified wheat BFI on subtilisin A.

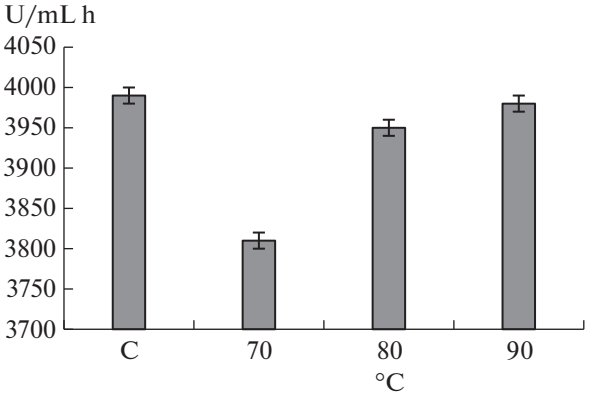

Fig. 6. Effect of high temperature (°C) on the activity of purified wheat BFI towards subtilisin A (*C,* control).

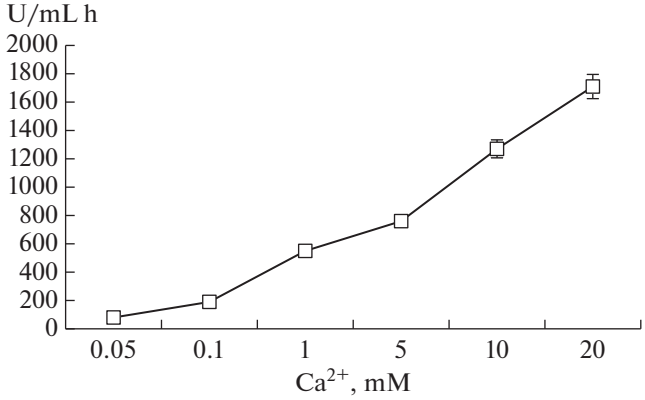

Fig. 7. Effect of concentration of calcium ions on the thermostability of inhibitor.

The results suggest a close similarity in the physicochemical properties and strict selectivity of the effect on wheat α-amylase of the isolated protein inhibitor and BFI of α-amylase/subtilisin from barley (BASI) [27].

The effects of ambient pH and high temperature on the activity and stability of the purified BFI were examined. The inhibitor displayed the highest activity in weakly alkaline medium (pH 7.8–8.0), whereas its activity dropped almost to zero at a pH of 5.2, especially in combination with heat treatment. However, the inhibitor displayed a very high thermostability at a pH of 8.0, retaining its activity even at 90°C during a short (10 min) treatment (Fig. 6).

This property distinguished the studied inhibitor from a relatively thermolabile, BASI-related protein from barley, [7] but was similar to the high thermostability of several protein inhibitors that are close in properties, such as 0.19 α-amylase inhibitor from wheat grain [28] and papaya Kunitz-type trypsin inhibitor [29].

BFI activity strictly demanded the presence of Ca^{2+} cations in the medium. The removal of these cations resulted in a loss of BFI ability to inhibit α-amylase. Calcium ions also supported BFI thermostability. As was shown, an increase in calcium ions from 0.05 to 20 mM enhanced manifold growth in BFI stability during heating for 10 min at 80°C (Fig. 7).

An important characteristic of wheat flour for bread-baking quality is α-amylase (**AA**) activity. Adverse conditions during grain maturation or storage (humidity and decreased temperature) can induce grain germination, accompanied by a most undesirable increase in enzyme activities, mainly, α-amylase activity. The AA in flour is commonly assessed by the Hagberg method to determine the FN, which reflects viscosity. The higher the flour AA is, the lower is the viscosity of suspension and, correspondingly, the lower is the FN. The optimal FN value for wheat flour is 250 s. Flour with an FN below 250 s gives low-quality, poorly shaped bread with sticky crumbs.

The effects of purified and crude (after heating for 15 min at 80°C) BFI on grist α-amylase with an FN number of 91 s containing a heterogeneous set of enzymes accumulated during maturation were studied. Before the inhibitor was added, grist water extract was heated for 15 min at 80°C to remove thermolabile β-amylase and α-AMY2. The results (Fig. 8) demonstrate that addition of the inhibitor to the grist extract with a low FN completely inhibited the activity of an AMY1 α-amylase.

As was shown earlier [30], BFI is synthesized in ripening grain starting from the milk–wax stage to reach the maximum quantity at full maturity. The inhibitor activity during grain germination rapidly decreases because of several factors, such as a weakly acid pH (leading to inactivation of the inhibitor and enhancing its dissociation from the complex with enzyme) and its cleavage by proteases. BFI is presumably involved in the control (inactivation) of excess α-amylase activity in the ripening or mature grain that appears during preharvest sprouting or activation of LMA form of the enzyme.

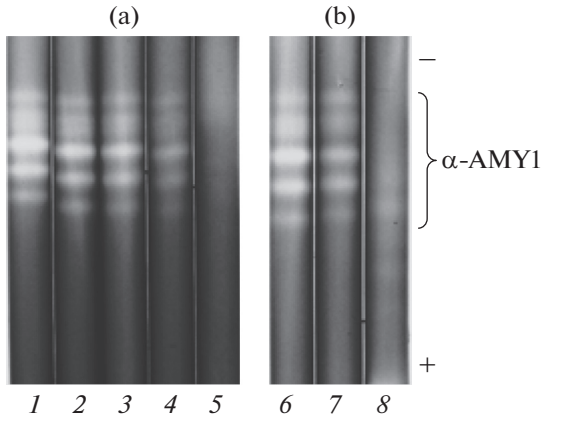

Fig. 8. Gel electrophoresis of water extracts of the grist with a low FN after the addition of 0, 10, 20, 40, and 80 μg of (a, *1–5*) crude and (b, *6–8*) purified wheat BFI.

Thus, the properties (molecular weight, isoelectric point, and high specificity towards the isozymes with a high pI value) of the wheat grain BFI of endogenous α-amylase and the protease subtilisin were significantly similar to the properties of the "two-headed" BASI. A high thermostability and strong dependence on Ca^{2+} cations are specific features of the isolated protein inhibitor. The obtained data demonstrate the ability of BFI to efficiently inhibit the α-amylase formed during grain ripening in grain with a low FN value.

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