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FEATURES OF AMYLASE INHIBITORS IN WHEAT GRAINS

The grains of cereal are able to synthesize and store various protein inhibitors of proteases and amylases. Despite some scientific breakthroughs, there is insufficient information about º-amylase/subtilysin protein inhibitor of wheat grains. Furthermore, when baking bread from germinated wheat grains, the excess activity of α-amylase, which contributed to the restoration of its quality, is suppressed by an inhibitor added from the outside.

In our work, the localization of the α-amylase protein inhibitor (PI) in the endosperm and peripheral parts of the grain (shell, aleurone) was determined. In the endosperm, the protein may be free or associated with starch granules. No inhibitor detected in the germ of grain. PI inhibits the catalytic center of Amy1α-amylase of wheat grain, having little influence on the center responsible for the binding of the enzyme to starch granules. The activity and composition of α-amylase in nine samples of wheat meal with high autolytic activity were analyzed (the number of falls is from 248 to 72). FN 91 sec grist showed the most heterogeneous spectrum to the effect of mixtures of wheat's inhibitor and raw extract from grain bran that were taken from the previous studies on α-amylase enzyme. The results of the experiment showed that exposure to the addition of two drugs of purified and raw inhibitors to grist extract with a low number of drops led to a decrease in the activity of α-amylase (Amy1). The novelty of the work was found to inhibit the localization of protein inhibitors and the catalytic center of wheat α-amylase.

Key words: wheat, germination, growth, protein inhibitor, α-amylase, aleurone, Falling number.

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Бидай дәндеріндегі амилаза ингибиторларының ерекшеліктері

Астық дәндері протеазалар мен амилазалардың әртүрлі ақуыз ингибиторларын синтездеуге және жинақтауға қабілетті. Кейбір ғылыми жетістіктерге қарамастан, бидай дәнінің ақуыз ингибиторы α-амилаза/субтилизин туралы ақпарат жеткіліксіз. Сонымен қатар, өнген бидай дәнінен нан пісірген кезде оның сапасын қалпына келтіруге ықпал еткен α-амилазаның артық белсенділігін сырттан қосылған ингибитор арқылы басады.

Біздің жұмысымызда эндоспермдегі және дәннің перифериялық бөліктеріндегі (қабық, алейрон) α-амилаза ақуызды ингибиторының (АИ) локализациясы анықталды. Эндоспермде ақуыз бос күйінде немесе крахмал түйіршіктерімен ассоциацияланған болуы мүмкін. Ұрық бөлігінде ингибитор анықталмады. АИ крахмал түйіршіктерімен ферменттің байланысуына жауапты орталыққа аз ықпал ете отырып, бидай дәнінің Amy1 α-амилазасының каталитикалық орталығын тежейді. Автолитикалық белсенділігі жоғары бидай ұнының тоғыз үлгісіндегі α-амилазаның белсенділігі мен компоненттік құрамы талданды (Түсу саны 248-ден 72-ге дейін). Алдыңғы зерттеулерде бөліп алған бидай ингибиторының қоспалары және дән кебегінен алынған шикі сығындының α-амилазасына ферментіне әсеріне ТС 91 сек шрот ең гетерогенді спектр көрсетті. Тәжірибе нәтижелері төмен Түсу саны бар шрот сығындысына тазартылған және шикі ингибиторлардың екі препаратын қосу арқылы әсер ету α-амилаза (Amy1) белсенділігінің төмендеуіне әкелді. Жұмыстың жаңалығы ақуызды ингибиторлардың локализациясы мен бидайдың α-амилазасының каталитикалық орталығын тежейтіні анықталды.

Түйін сөздер: бидай, өну, пісіп жетілу, ақуызды ингибитор, α-амилаза, алейрон, түсу саны.

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Особенности ингибиторов амилазы в зерне пшеницы

Зерновки злаковых способны синтезировать и накапливать разнообразные белковые ингибиторы протеаз и амилаз. Несмотря на некоторые научные достижения, информации об ингибиторе белка зерна пшеницы α-амилазе/субтилизине недостаточно. Кроме того, избыточная активность α-амилазы, способствовавшая восстановлению ее качества при выпечке хлеба из пророщенных зерен пшеницы, подавляется внешне добавляемым ингибитором.

В нашей работе установлена локализация белкового ингибитора (БИ) α-амилазы в эндосперме и в периферийных частях зерновки (оболочки, алейрон). В зародышевой части ингибитор не обнаружен. БИ ингибирует каталитический центр α-амилазы Ами1 зерна пшеницы, при этом мало затрагивая центр, ответственный за связывание фермента с гранулами крахмала. Проанализированы активность и компонентный состав α-амилазы в девяти образцах пшеничного шрота с повышенной автолитической активностью (ЧП от 248 до 72). Все образцы, но в разной степени, содержали обе изогруппы – Ами1 (α-амилазы «прорастания» и Ами 2 (α-амилазы «созревания»). Результаты эксперимента показали, что добавление обоих препаратов ингибиторов, как очищенного так и грубого, к экстракту шрота с низким ЧП приводило к подавлению α-амилазной (Ами1) активности. Новизной работы стала локализация белковых ингибиторов и ингибирование каталитического центра α-амилазы пшеницы.

Ключевые слова: пшеница, всхожесть, созревание, белковый ингибитор, α-амилаза, алейрон, число падения.

Introduction

Inhibitors are different substances of a protein nature. They have the ability to inhibit the proteolytic activity of certain enzymes. That is why they are also called as protease inhibitors. Furthermore, we can name protease inhibitors as digestive enzymes. This group includes enzymes such as α-amylase, chymotrypsin, trypsin, pepsin [1].

Substances of protein nature make up the largest group of enzyme inhibitors, and the most studied in terms of its composition are proteins that inhibit the activity of proteases [2]. Protein inhibitors form complexes with stable proteolytic enzymes under physiological conditions, in which the enzyme completely or partially loses its activity. In addition, several dozen protein inhibitors that suppress the activity of trypsin, chymotrypsin, carboxypeptidase, kallikrein, elastase, plasmin and other proteolytic enzymes have been identified and studied. Most of them were obtained in a homogeneous crystalline state. The molecular masses of protein inhibitors range from several thousand to several hundred thousand, but they are mainly composed of proteins with a molecular weight of about 6000 Da. Many enzyme inhibitor proteins are glycoproteins. At the moment, the initial structures of several dozen inhibitors have been discovered. Among them, we can mention trypsin inhibitors I and II of the pancreas of pigs and cattle, soy, peanut and bean inhibitors isolated from snake venom, lima beans and pineapple proteinase inhibitors [3,4]. Cereal grains are able to synthesize and accumulate various protein inhibitors of proteases and amylases. These proteins are usually divided according to their structure and ability to inhibit certain classes of enzymes. To date, serine-proteinase inhibitors are the best studied, most of which have several inhibitory domains. In storage organs such as seeds and tubers, inhibitors accumulate during embryogenesis and maturation. It is assumed that in the event of damage or attack by the pathogen and insects, they can perform a protective function [5].

Protein inhibitors against endogenous α-amylase have been relatively little studied. The first group of inhibitors usually refers to the components of the plant defense system. The physiological role of inhibitors of the second group is to regulate the activity of the endogenous enzyme during the ripening period of the grain [6,7]. Among the α-amylase inhibitors of cereals, the most popular is the bifunctional α – amylase/subtilisin inhibitor (BASI), which was first found in barley grain [8]. The inhibitor is able to suppress the activity of barley α-amylase and serine protease of microorganisms. Further, BASI-like inhibitors have been found in the seeds of some other cereals. Currently, the functioning of these proteins and their regulation for practical use are being inten-

sively studied [9,10]. Similar BASI inhibitors were later found in the seeds of several other cereals, including wheat (Wasi) [11]. These two functional inhibitors are currently combined into one group of proteins – ASI [12].

Despite some progress, research on the wheat grain α-amylase protein inhibitor remains very insufficient. Kazakhstan, being one of the main producers of high-quality wheat grain in the world, annually loses from 10 to 30% of the crop due to the uncontrolled synthesis of α-amylase, which causes PHS [13].

Data on inhibitor properties and regulation, as well as methodological approaches in the study of inhibitors from other cereals, were useful for our work with the wheat α-amylase inhibitor. Analysis of general literature sources allows us to draw conclusions about the prospects of the chosen direction of research.

One of the main defects of flour is high autolytic activity (AA), which indicates an increase in the activity of enzymes, especially α-amylase[14]. Most often, such flour is obtained from sprouted or frozen grain. In the production of bread, AA is usually reduced by methods such as hydrothermal processing to inactivate excess α-amylase, acidification of dough with liquid yeasts, lactic enzymes, lactic acid, etc. [15]. These methods have both advantages and certain disadvantages. In addition, the cereal itself is rich in various substances – regulators of enzyme activity. These include substances of protein, carbohydrate and phospholipid nature [16,17].

Bread baked from such flour will have a sticky crumb, reduced in size, indistinct in shape. In this regard, it is very important to develop ways to improve the quality of bread made of defective flour.

Based on the above data, we determined the localization of the α-amylase protein inhibitor in the endosperm and peripheral parts of the grain (shell, aleurone, germ part). Moreover, we also determined the effect of the protein inhibitor on the binding of the enzyme to starch granules. The number of drops of different wheat varieties grown in Kazakhstan has been determined as well. The activity and component composition of α-amylase inhibitors in nine samples of wheat flour with high autolytic activity were analyzed (the number of drops is from 248 to 72).

Materials and methods

As objects of research, wheat grains in a dormant state, various wheat varieties (*Triticum aestivum* L.) bran, aleurone, embryonic part, wheat meal, starchy endosperm were obtained.

Isolation of the α-amylase inhibitor

Wheat grains in a dormant state were planted in petri dishes. Further, on day 4, the sprouted part were removed separately. Plant material (wheat grains, grinded grain, bran, flour, germ part, etc.) was extracted at pH 5.0 with 0.05 m acetate buffer with 5mm $CaCl_2$ in a 1:3 ratio. After stirring for 1 hour at +4°C, the mixture was centrifuged for 15 minutes at 3000 rpm. The supernatant was used as a source of the α-amylase inhibitor. To remove the associated α and β amylases, the extract was heated at 75° C for 10 minutes, then quickly cooled and centrifuged [18].

Determination of inhibitory activity

The determination of antiamylase activity was carried out by adding 1 mm $CaCl_2$ at pH-8.0 (50 mm phosphate buffer) [19]. Then, the inhibitor and α-amylase were reacted in a 1: 1 ratio. 1 ml of starch was used for each sample as a substrate. After 10 minutes of incubation, 100ml of Iodine solution (0.005% J_2 / 0.05% KJ) was added. The spectrofotometer carried out measurements with a length of 320 nm [20].

Insulation of starch granules

Starch granules (SG) were separated from finely grinded wheat flour by repeated decanting and centrifugation in distilled water. The resulting raw SG preparation was washed three times with ethanol, dried at 35 ° C for 48 hours and stored at room temperature [21].

The experiments and measurements of enzyme activity were performed three times. Furthermore, the experiment data were statistically calculated in Microsoft Excel and their standard deviations were shown.

Results and discussion

Amount of α-amylase inhibitor in different parts of wheat grain

At the first stage of the work, we studied the localization of the protein inhibitor in various anatomical parts of the wheat grain. According to barley grain research data, the synthesis of the endogenous α-amylase inhibitor is maximum during the period of full ripening. In this regard, the analysis of the quantitative content of the α-amylase inhibitor of wheat was carried out on the mature, dormant grain ("Kazakhstan 10").

Several grain fractions were obtained using the SD1 roller mill (Chopin, France). Fraction 1 had mainly shells and aleurons, fraction 2 had germ particles, germ shield and subaleyronic layer of the endosperm, and fraction 3 had white flour consist-

ing only of starchy endosperm. Whole grain powder (grist) was obtained using a laboratory mill ML 3100 (Perten, Sweden). The resulting grist consists of homogeneous particles, the size of which does not exceed 80 micrometre.

The content of inhibitors in isolated grain fractions was determined by its antiamylase activity. The highest inhibitory activity was seen in the starchy endosperm (2700 enzyme unit/ mg protein); in the peripheral parts of the endosperm and in the germ part, the inhibitor was present in a slightly smaller amount (2330 enzyme unit/mg protein). The smallest amount of it is contained in grain shells and in the aleurone layer (1980 enzyme unit/ mg protein) was. Inhibitory activity in whole grains 2680 enzyme unit/ mg made up protein. (Figure 1).

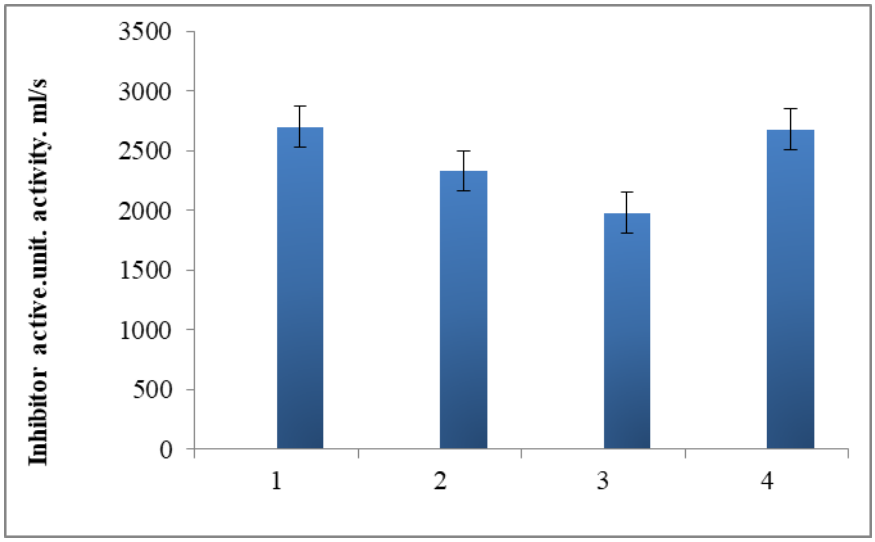

Figure 1 – Amount of α-amylase inhibitor in different parts of wheat grain (1-starchy endosperm, 2 – germ and subaleurone layer, 3 – grain shell and aleurone, 4-whole grain)

However, it remained unclear whether the inhibitor protein was present in the germ part, since the 2-bran fraction contains the outer layer of the endosperm in addition to this tissue. For this purpose, the embryos were isolated in organic solutions by flotation method, the extracts from which did not show inhibitory activity. Consequently, the activity of 2 bran fractions is expressed by an inhibitor derived from the endosperm.

Currently, it is not clear whether the presence of an inhibitor affects the binding of α-amylase to starch granules. For more information content, the study conducted a comparative experiment using two other known carbohydrate-based α-amylase inhibitors, β-cyclodextrin and acarbose.

First, the effect of 3 types of inhibitors on the hydrolysis of soluble starch α-amylase Amy1 of wheat was studied. To do this, equal aliquots of the purified enzyme were pre-incubated for 10 minutes at a temperature of 30°C with different concentrations of inhibitors. determination of α-amylase activity was carried out according to the following standard procedure [22]. From the graph (Figure 2A) we can see that the inhibitory effect of β-cyclodextrin on substrate decomposition is very weak. In contrast, a protein inhibitor derived from grain and acarbose showed a high inhibitory effect. And the oligosaccharide had a fairly high effect, especially in the highest concentrations (almost 100%).

A different result was observed when using starch granules as a substrate. To do this, α-amylase Amy1 was pre-incubated with the studied inhibitors (1 µg each) at 30°C for 10 minutes. Then, commercial granulated wheat starch (Sigma-Aldrich) with a pH of 5.1 50 mg in 2 ml of 0.05 m acetate buffer was added to the samples. The mixture was incubated for 10 hours by slowly whisking in a shaker at 30°C. the degree of sorption (binding) of α-amylase was estimated by the amount of protein measured using the Lowry method every 2 hours remaining in the medium.

From the graph shown in Figure 2B, it can be seen that β-cyclodextrin significantly (up to 90%) inhibited the binding of α-amylase to starch gran-

ules. In contrast, acarbose and grain inhibitor did not show such an inhibitory effect. It should be noted that in the last hours of incubation (8-10 hours), there was no increase in sorption of the α-amylase inhibitor, which may be due to partial breakdown of the enzyme-inhibitory complex.

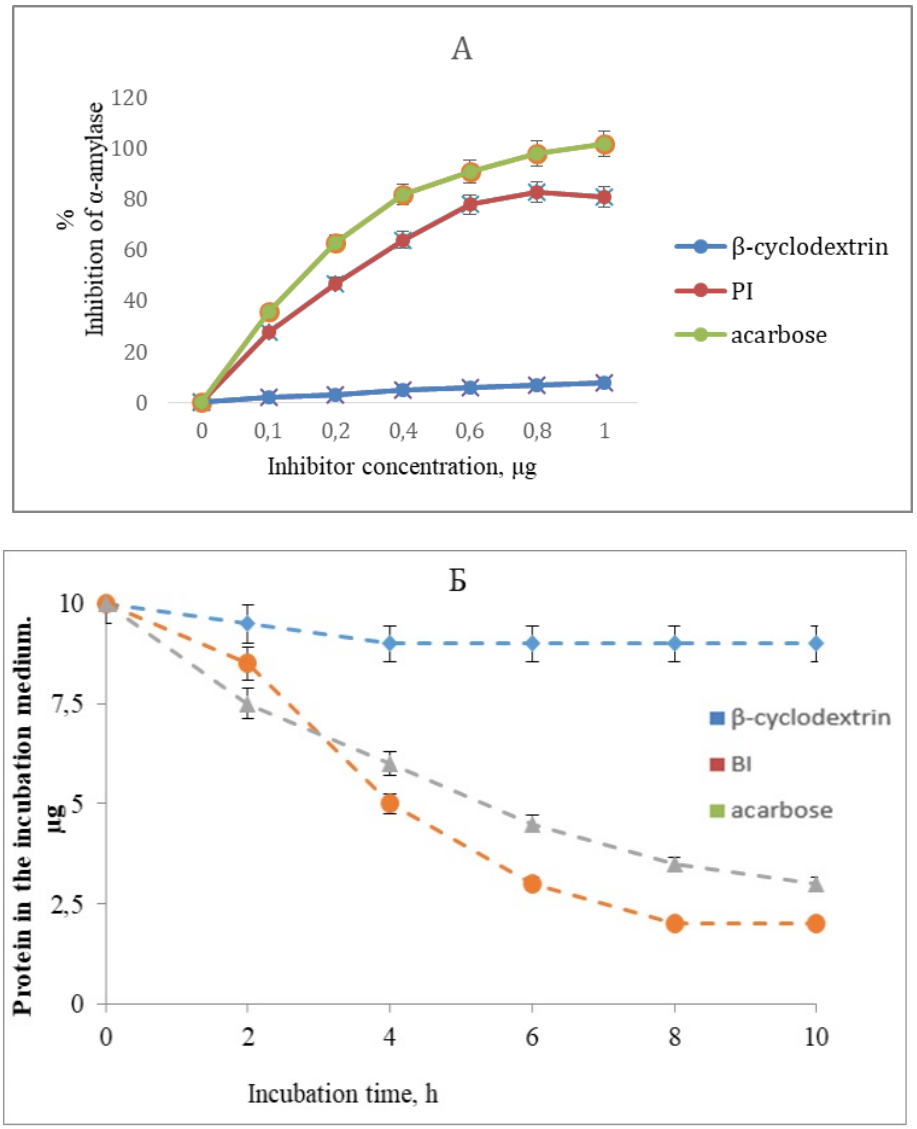

Figure 2 – The effect of various inhibitors on the hydrolysis of soluble starch (A) and the binding of α-amylase Amy1 to granular starch (B)

The data obtained indicate differences in the action of different types of α-amylase inhibitors. β-cyclodextrin prevents the enzyme from binding to starch granules, but has not inhibited the hydrolysis of soluble starch. Acarbose and a protein inhibitor from wheat grain suppressed hydrolysis of the soluble substrate with α-amylase, but did not affect its sorption in starch granules.

As noted above, there are at least 2 active centers in the structure of α-amylase, one of which carries out the hydrolysis reaction (catalytic center), the other is responsible for the interaction of the starch granules of the enzyme (binding center). With this in mind, we can conclude that a protein inhibitor of wheat blocks the catalytic site of α-amylase without significantly affecting the binding site. In terms of its effect on the enzyme, the bifunctional inhibitor is similar to acarbose oligosaccharide, but differs from another carbohydrate inhibitor – β-cyclodextrin.

Effect of α-amylase inhibitor on flour amylase activity

One of the most important indicators of wheat flour in baking is autolytic activity (AA), which varies widely depending on the weather, climatic and soil conditions of grain cultivation[23]. Unfavorable conditions for the ripening or preservation of the grain provoke its germination, accompanied by an increase in the activity of enzymes, mainly α-amylase. To assess the autolytic activity of flour, the Hagberg method is used, in which the Falling Number (FN) is determined – the viscosity indicator [24,25]. The higher the autolytic activity of the flour, the lower the viscosity of the slurry and, accordingly, the lower the value of the number of falls (in seconds). For wheat flour, the optimal value for the number of falls is considered to be 250 s. When the numbers decrease for less than 250 seconds, the bread turns out to be of poor quality, low in shape, sticky and shapeless. We analyzed the activity and component composition of α-amylase in nine samples of wheat flour with high autolytic activity (FN 248 to 72). In previous studies, the effect of isolated wheat inhibitor supplements and raw extract from grain bran on the α-amylase of FN 91 sec grist with the most heterogeneous spectrum was studied. Taking into account the thermal stability of the inhibitor, a bran extract without amylase activity was obtained by preliminary 10-minute processing at a temperature of 80°C. The results of the experiment led to a decrease in the activity of α-amylase (Amy1) by adding two drugs of purified and raw inhibitors to the grist extract with a reduced number of drops (Figure 3).

Figure 3 – Activity of the non germinated part of wheat grain (aleurone) and total wheat meal (grist) inhibitor number of drops:1–248s,2–237s,3–220s,4–204s,5–179s,6–175s,7–171s,8–91s, 9-72s

Thus, the data obtained make it possible to use a protein inhibitor of endogenous α-amylase, as well as processing residues of grains consisting of an inhibitor (for example, bran) to correct flour with high autolytic activity and improve the quality of bread.

Conclusion

The localization and content of the α-amylase protein inhibitor in wheat grain, as well as the participation of various endogenous factors in the regulation of its activity, were studied. It has been found that the inhibitor is present both in the starchy endosperm and in the peripheral parts of the caryopsis (shell, aleurone). In the endosperm, the protein can be in a free state or bind to starch granules. No inhibitor was found in the germinal part.

In a comparative plan, the effect of carbohydrate inhibitors (β-cyclodextrin, acarbose) and the protein inhibitor α-amylase on granular starch bind-

ing and hydrolysis was studied. It has been found that the protein inhibitor inhibits the catalytic center of α-amylase Amy1 in wheat grains, while having little effect on the center responsible for binding the enzyme to starch grains.

Both refined and raw preparation of the inhibitor effectively suppress the Amy1 isoenzymes of wheat flour with a number of drops. The results obtained make it possible to use protein inhibitors of endogenous α-amylase, as well as grain processing residues consisting of them (for example, bran) to correct flour with high autolytic activity and improve the quality of bread.

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