

## Chitinases of wheat seedling and their biochemical properties

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### Abstract

Chitinases are important protective enzymes in plants and belong to a special group of pathogenesis-related (PR) proteins. The main target of these enzymes is various pathogenic fungi that contain chitin in their cell walls. Under the influence of fungi, chitinases produced by plants, hydrolyze chitin, which leads to suppression of the growth and death of the pathogen. Considering their important protective role, much attention is paid to the studies of these enzymes. Individual isozymes of chitinase are often used in testing and breeding agricultural plants for resistance to fungal diseases. In this work, we studied the isoenzyme composition and a number of physicochemical properties of wheat chitinases, which are important for a better understanding of their functioning in this grain crop. It was found that in seedlings of 7 days old, chitinases are localized both inside and outside the cells. Intracellular chitinase is represented only by basic components, whereas extracellular - by acidic ones. Using an affinity sorbent, the forms of the enzyme with a chitin-binding domain (CBD) were determined, which had isoelectric points (pI) of 9.3, 9.0, 8.6, 8.2, 8.0, 7.6, 5.7 and 4.6. According to SDS-PAGE electrophoresis, the molecular weight (M.w.) of these enzymes corresponded to the values of 33, 35 and 56 kDa and they were not the glycoproteins. In the spectrum of isoelectric focusing (IEF) of chitinases, exochitinases were identified, which had pI values exclusively in the acidic range of 4.3-5.2. Significant differences were revealed in the thermal stability of chitinases. The acidic components were most resistant to elevated temperatures.

**Keywords:** *Triticum aestivum* L., Seedling, Chitinase, Isoenzymes, Chitin-binding domain

### Introduction

Chitinolytic enzymes are widespread and are found in bacteria, fungi, plants and animals. In plants, chitinases are involved in some physiological processes - germination, embryogenesis, ethylene synthesis, digestion (carnivorous plants, that feed on insects), as well as in the fight against environmental stresses, that is, cold, drought, and salinity (Grover, 2012). Nevertheless, the main function of chitinases is to protect the plant organism from phytopathogens - bacteria, fungi, nematodes and arthropods, the integuments of which contain chitin. Many of the chitinases have potentially high antifungal activity and are produced in response to a pathogenic attack (Kumar et al., 2018). The secreted enzymes hydrolyze the chitin of the cell walls to chitooligosaccharides and N-acetylglucosamine. Due to the important protective and defense role, the study of plant chitinolytic enzymes has received much attention recently, as evidenced by a number of reviews (Sharma et al., 2011; Kumar et al., 2018; Iqbal and Anwar, 2019). Chitinases are part of the PR (pathogenesis-related) protein group and form 4 families out of 17 (PR-3, -4, -8 and -11) (Ebrahim et al., 2011; Sharma, 2013). Plant chitinase, like many polymer hydrolases, has multiple molecular forms and encoded by a family of genes. Additional difficulties in the study arise due to the presence of constitutive and inducible forms of the enzyme, as well as the tissue and organ specificity of their expression.

The significant polymorphism of chitinases is primarily due to the complex organization of natural substrates - chitin and its various derivatives of oligosaccharides, which suggests differences in the structure of enzymes, their substrate specificity, kinetic characteristics, and other properties (Kasprzewska, 2003; Kaczmarek et al., 2019). By the type of catalytic action, chitinases are divided into endochitinases (EC 3.2.1.14), which cleave internal bonds in chitin, forming chitotriose and chitobiose, and exochitinase - chitobiosidases (EC 3.2.1.29), cleaving off terminal diacetylchitobiose and N-acetyl- $\beta$ -1,4-glucosaminidases (EC 3.2.1.30), which decompose the products of endo- and exochitinases to N-acetylglucosamine. On the basis of the amino acid composition and structure of the catalytic center, chitinases were grouped into families of 18, 19, and 20 glycoside hydrolases (GH). Families 18 and 19 are considered to be chitinases, since they catalyze the degradation of chitinous

polymers. Family 20 includes chitobiosidase and N-acetyl- $\beta$ -1,4-glucosaminidase. Family 18 mainly consists of bacterial and fungal chitinases, while family 19 is represented exclusively by plant chitinases (Rathore and Gupta, 2015).

According to the primary structure, plant chitinases are divided into 7 classes (I-VII). Chitinases of classes III and V belong to the GH18 family, while chitinases of classes I, II, and IV belong to the GH19 family. In cereals, the first three classes of enzymes are most commonly found. It was shown that there is no definite correlation in the distribution of chitinases across plant species, their organs and tissues, but it was found that only some of them have antifungal properties (Taira, 2010; Oyeleye and Normi, 2018).

Class I chitinases are found only in plants and are induced in response to a pathogen. They have complete N- and C-terminal domains, during biosynthesis, most of them are directed to the vacuole (Gijzen M., 2001; Taira et al., 2002). These chitinases are generally highly antifungal. In their structure, they contain a chitin-binding domain (CBD), due to which a strong antifungal effect is provided. Nearby is the spacer (hinge) region and the catalytic domain. Chitinases of this class are divided into 2 subclasses - I a (acidic forms) and II b (basic forms). Class II chitinases have been found not only in plants, but also in bacteria and fungi. They are structurally similar to class I chitinases, but lack CBD and spacer regions (Taira, 2010). Enzymes of this class have acidic properties. Class III chitinases have a unique structure and differ from all other chitinases. They are multifunctional and exhibit lysozyme, exochitinase activity and are similar to bacterial chitinase. There is no CBD in their structure. These enzymes have a wide range of optimum pH values and are thermostable at 60-70°C. Chitinases of classes IV, V, VI, and VII are hardly found in cereals (Sharma et al., 2011).

To date, chitinases have been most studied in tobacco, and barley, rice, and rye among cereals. Wheat chitinase contains about 10 isoforms with a wide range of isoelectric points (pI) - in the range of acidic, basic and neutral pH from ~ 3.1 to 9.7 with a molecular weight of ~ 20 to 40 kDa (Ride and Barber, 1990; Mohammadi et al., 2001 ; Moravčíková et al., 2017). This suggests differences in physicochemical properties and pH optimum of action, which is very important for display of activity in a pathogenic attack. Despite some success, the wheat chitinase complex remains poorly studied.

Kazakhstan is one of the major producers and exporters of wheat. In this regard, the country pays great attention to increasing the yield and resistance of this grain crop to phytopathogens. The article presents some new data on the composition and some physicochemical properties of wheat chitinases, which contribute to a better understanding of the functioning of these defense enzymes in this grain crop.

## **Materials and Methods**

### **Plant materials**

Wheat grain, variety Shortandy 98 (*T. aestivum* L.), was obtained from A.I. Barayev Scientific and Production Center of grain farming, Shortandy, Kazakhstan.

### **Growing wheat seedlings**

10 g of grain were soaked in distilled water for 1 hour, sterilized with 0.5% sodium hypochlorite for 5 minutes, washed with several portions of running water and rinsed with distilled water. The grains were planted on moistened filter paper in plastic containers with lids and germinated in a thermostat in the dark at 22°C for 7 days. The obtained seedlings were placed in a glassful and washed with distilled water. Grains, roots, and shoots were detached from the seedlings, which were used for preparation of the enzyme extracts.

### **Preparation of the enzyme extract**

The plant material was homogenized in 0.05 M sodium acetate buffer pH 5.0 at a ratio of 1:3. The homogenate was infused at 4°C for 1 hour, then centrifuged at 10000 g for 15 minutes. The supernatant was used as a source of enzymes.

### **Assay of chitinase activity**

The chitinase activity was assayed spectrophotometrically (Fink et al., 1988) with some modification. The reaction mixture contained 0.1 ml of 5% colloidal chitin (Sigma) in 0.05 M sodium acetate buffer pH 5.0, 0.1 ml of 0.5 M sodium acetate buffer pH 5.0, 0.7 ml of distilled water, and 0.1 ml enzyme

extract. The mixture was incubated at 37°C for 5 hours, after which the reaction was stopped by adding 1 ml of 3,5-dinitrosalicylic acid (DNS) reagent, followed by boiling at 90°C for 10 minutes. Then the mixture was cooled on ice for 5 minutes and centrifuged at 8000 g for 10 minutes. The absorbance of the samples was measured at a wavelength of 540 nm against a control prepared in the same way, but with the addition of DNS before adding the enzyme extract. The specific activity of chitinase was expressed in mg /ml<sup>-1</sup> N-acetyl-D-glucosamine (NAG) per 1 hour<sup>-1</sup> per 1 mg<sup>-1</sup> protein. The chitinase activity of the samples was measured in triplicate, from which the average value was determined. The protein content in plant extracts was determined by the method (Lowry et al., 1951).

### **Isolation of apoplastic and vacuolar chitinases**

Isolation of apoplastic fluid was performed from whole wheat shoots by infiltration-centrifugation method (O'Leary et al., 2014) with some modifications. Shoots of 7 days old seedlings were cut off at the base, washed three times with distilled water, and slightly dried on filter paper. Plant material in the amount of 8 g was soaked for 2 minutes in a solution of 0.05% Triton X-100, washed with distilled water and dried. The seedlings were placed in a Bunsen flask with 150 ml distilled water cooled to 4°C and containing 100 mM KCl. The plant material was infiltrated at a pressure of 10 mbar for 30-40 minutes using a PC3001 Vario vacuum pump (Vacuubrand, Germany). As a result of this procedure, the shoots acquired a deep dark color, after which they were washed with distilled water and dried with filter paper. After that, the shoots were placed in 50 ml test-tube and centrifuged at 4°C for 15 minutes at a speed no exceeding 3000 g. The liquid accumulated at the bottom of the test-tube contained extracellular apoplastic chitinase, whereas the plant extract contained an intracellular vacuolar enzyme. To isolate intracellular chitinase, infiltrated 7 days old shoots (8 g) without intercellular fluid were homogenized in 24 ml of 0.05M sodium acetate buffer, pH 5.0. The homogenate was incubated at 4°C for 1 hour and centrifuged at 8000g for 15 minutes. The enzyme supernatant was dialyzed against 0.05 M sodium acetate buffer pH 5.0 at 4°C and stored at -20°C until use.

### **Chitinase purification by substrate affinity chromatography**

At the first stage of chitinase purification, the supernatant proteins were precipitated with ammonium sulfate within the saturation range of 30-80% at 0°C for 1 hour. The protein precipitate was collected by centrifugation at 10000 g for 10 minutes at 4°C. The precipitate was dissolved in 4 ml of 0.05 M sodium acetate buffer, pH 5.0, and centrifuged to remove insoluble impurities. The protein solution was desalted using a CentriPure P10 Column (Serva, Germany) with a volume of 1.5 ml according to the manufacturer's protocol. All protein fractions were stored at -20°C until use.

Chitinases with chitin binding domain (CBD) of shoots, roots, germinating and dormant wheat grains were purified by column chromatography on chitin resin (New England Biolabs, USA) according to the method described in (Sørensen et al., 2010). Chromatographic procedures were carried out at 8°C using a glass column of 0.8×4 cm. The chitin resin sorbent in 2 ml of 0.05 M sodium phosphate buffer, pH 7.4 was placed in the column. The column was equilibrated with the same buffer. After precipitation with ammonium sulfate 0.5 ml of a protein solution was dialyzed against 0.05 M sodium phosphate buffer, pH 7.4 and loaded onto the column. The binding of chitinase to the affinity sorbent was carried out slowly over 40 minutes. Unbound proteins were removed first with starting buffer and then with 0.05 M sodium acetate buffer pH 5.1. Protein fractions were collected by 0.5 ml. The proteins not bound to chitin were concentrated to a volume of 1.5 ml in an Amicon cell (Millipor, USA) on a PM-10 filter. The bound enzyme was eluted with 20 mM acetic acid, pH 3.0. The protein solution was neutralized to pH 6.5-7.0 by adding 0.5 M sodium phosphate buffer pH 7.4 immediately after elution. Buffer was added dropwise to each fraction and the pH was monitored using indicator paper. Fractions containing chitinase were pooled and dialyzed against 0.05 M sodium acetate buffer, pH 5.0 on a CentriPure P10 column. The enzyme solution was concentrated to a volume of 1.5 ml. The chitin resin column chromatography procedure was performed three times for each organ. Enzyme preparations were stored at -20°C until use.

### **Isoelectrofocusing (IEF) and chitinases detection in PAG**

Separation of chitinase isozymes was performed by native isoelectric focusing (IEF) technique using a Multiphor II horizontal electrophoresis chamber (GE Healthcare, Sweden). A polyacrylamide gel (PAG) with size 9×12×0,1 cm contained 5% acrylamide and 2% Servalyt pH 3.0-10.0 (Serva,

Germany). Enzyme samples with a volume of 15 µL were applied to the surface of the horizontal gel in the center using paper applicators 10×5 mm in size (Serva, Germany). First, the IEF was carried out at a voltage of 100 V for 1 hour, then every hour the voltage was increased by 100 V, bringing it to a final voltage of 500 V. During the IEF, the gel was cooled at 6°C.

Endochitinase isozymes were detected using a gel replica with copolymerized glycol chitin according to the method (Pan et al., 1989). Glycol chitin was obtained by the method (Molano et al., 1979). After IEF, the gel was incubated in 0.05 M sodium acetate buffer pH 5.0 for 10 minutes. Then a 5% polyacrylamide coating gel (replica) 1 mm thick containing 0.04% glycol chitin was applied to the separating gel. The overlaid sandwich gels were incubated at 37°C for 2 hours in a glass container under humid conditions. Then the replica was removed and placed in a freshly prepared 0.01% fluorescent bridgethener 28 (Sigma, USA) in 0.5 M Tris-HCl buffer pH 8.9 at 25°C for 15 minutes. The replica was incubated in distilled water at + 8°C for 24 hours. The bands of activity were visualized on a Quantum ST5 Gel Doc instrument (Vilber Lourmat, France) under UV light with a wavelength of 365 nm. A kit of proteins (Sigma, USA) was used as markers of isoelectric points (pI): amyloglucosidase - 3.6; trypsin inhibitor - 4.6; β-lactoglobulin - 5.1; carbonic anhydrase 5.9, 6.6; myoglobin - 6.8, 7.2; lectin - 8.2, 8.6, 8.8; trypsinogen - 9.3.

Exochitinase isozymes (N-acetylglucosaminidase) were detected in PAG according to the method (Dušková et al., 2011) with some modifications. After IEF, the gel was incubated in 0.05 M sodium acetate buffer pH 5.0 for 10 minutes at 37°C. Filter paper impregnated with the chromogenic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUF-NAG) (Sigma, USA) at a concentration of 0.02% was applied to the separating gel. The gel was incubated at 37°C for 30-40 minutes in a closed glass container under humid conditions. The zones of activity were visualized on a Quantum ST5 Gel Doc instrument (Vilber Lourmat, France) under UV light with a wavelength of 365 nm.

#### **Sodium dodecyl-sulfate (SDS) PAG electrophoresis of proteins**

The electrophoretic separation of proteins was carried out in an 8×8,5×0,1 cm plate with 12% separating and 5% concentrating PAG with sodium dodecyl-sulfate according to the method (Laemmli et al., 1970) using a Compact Dual Mini TV-100Y vertical electrophoresis chamber (England). Protein extracts of 20 µl were mixed with 20 µl Sample Buffer 2X (Serva, Germany) and boiled at 95°C for 5 minutes. Electrophoresis was carried out for 30 minutes at a voltage of 40V, and then for 2 hours at a voltage of 150V. To staining total protein, the gel was fixed for 1 h in 20% trichloroacetic acid at room temperature. The gel was stained for 2 hours at 37°C with 0.1% Coomassie Brilliant Blue R250 solution (Sigma, USA) freshly prepared in 7% acetic acid and 20% ethanol. Excess dye in the gel was removed by repeated washing in 7% acetic acid. A kit of proteins (Pharmacia Biotech, Sweden) was used as molecular weight (M.w.) markers: α-lactalbumin - 14.4, trypsin inhibitor - 20.1, carbonic anhydrase - 30.0, ovalbumin - 43.0, albumin - 67.0, and phosphorylase b - 94.0.

#### **Detection of chitinase activity after SDS-PAG electrophoresis**

Zones of chitinase activity after SDS-PAG electrophoresis were revealed according to the method (Trudel and Asselin, 1989). After electrophoresis, SDS was removed from the gel by incubation at 37°C in 0.05 M sodium acetate buffer pH 5.0 containing 1% Triton X-100 for 2 h. The gel was re-soaked for 1 hour in 0.05 M sodium acetate buffer pH 5.0 at 25°C without Triton X-100. The gels were then incubated in freshly prepared 0.01% Fluorescent Bridgethener 28 in 0.5 M Tris-HCl pH 8.9 at 25°C for 15 minutes. After that, the gels were left in distilled water at 8°C for 24 hours. Zones of enzyme activity were revealed in the same way as in the case of the IEF.

#### **Staining of glycoproteins after SDS-PAG electrophoresis**

Staining of glycoproteins after SDS-PAG electrophoresis was performed using a reagent kit (Pierce, no. 24562, USA). In short, the principle of the method consists in the oxidation of the carbohydrate residue of glycoproteins by periodate to aldehydes, followed by staining of glycols with Schiff reagent. The preparation of working reagents and the procedure for staining glycoproteins were carried out according to the instructions attached to the kit. After electrophoresis, the gel was immersed in 100 ml of 50% ethanol and fixed for 30 minutes. The gel was transferred into 100 ml of 3% acetic acid, then washed with gentle stirring for 10 minutes. We repeated this step once again. The gel was transferred into 25 ml of an oxidizing solution and stirred gently for 15 minutes. The gel was washed by gently

shaking in 100 ml of 3% acetic acid for 5 minutes. This step was repeated two more times. The gel was transferred into 25 ml of glycoprotein dye and mixed gently for 15 minutes. The gel was then transferred into 25 ml of a recovery solution and gently mixed for 5 minutes. The gel was thoroughly washed with 3% acetic acid and distilled water. The glycoproteins appeared as purple streaks. The gel was stored in 3% acetic acid. After staining, the gel was photographed.

### Effect of temperature on chitinase activity

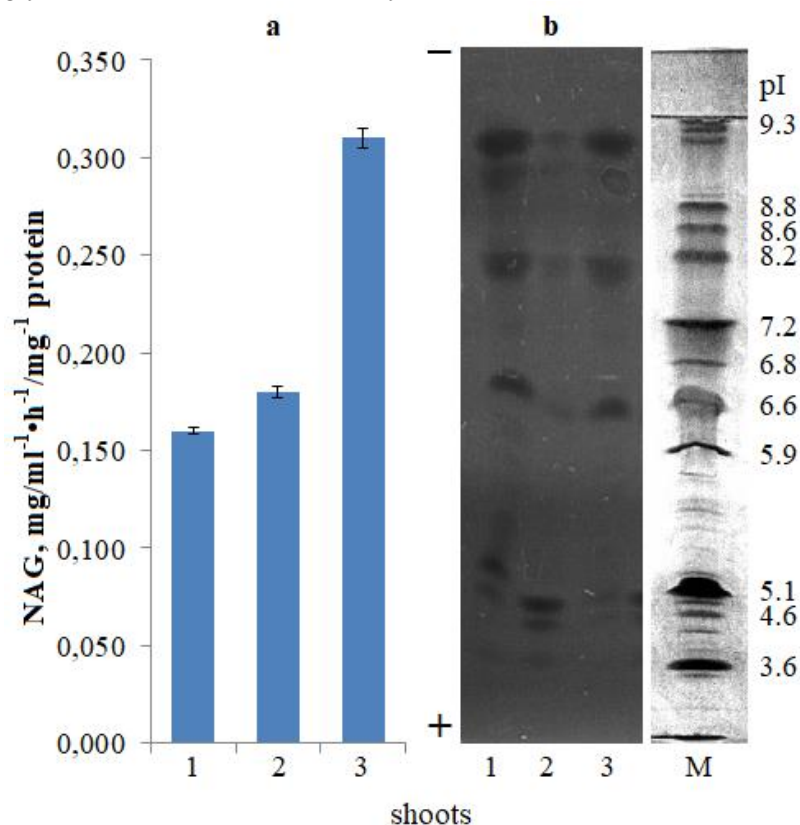
Samples of the enzyme (0.4 ml each) after precipitation with ammonium sulfate were heated at temperatures of 30 (control), 60, 63, 66 and 69°C for 10 minutes in a water bath, sharply cooled in ice water and centrifuged for 10 minutes at a speed of 8000 g. The supernatant was collected and used for the analysis of the isozyme composition of chitinases by the IEF technique.

## Results

### Cellular localization and detection of endo- and exochitinases of wheat seedlings in the IEF spectrum

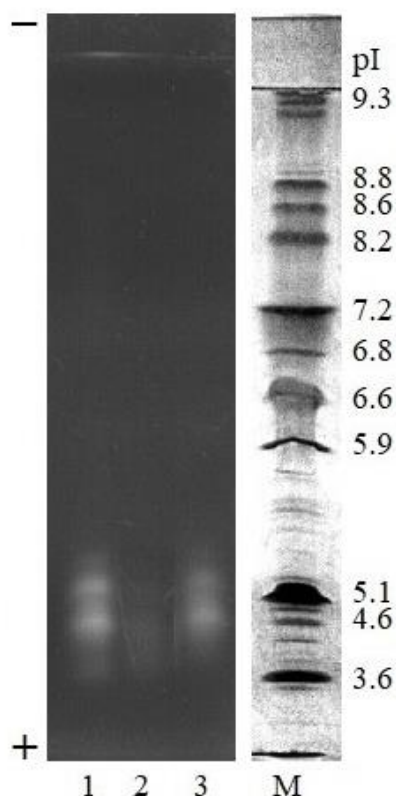
Chitinases differ in their localization, among which there are intracellular and extracellular forms. According to some data, intracellular (vacuolar) chitinase can quantitatively exceed the extracellular (apoplastic) enzyme by several times. Moreover, in some plants, apoplastic chitinase is hardly found in the norm. The accumulation of the enzyme in the intercellular fluid was observed under external influences, in particular, during fungal pathogenesis (Anand et al., 2004; Aleandri et al., 2008). These features of cell localization and quantitative distribution suggest differences in the defense functions of chitinase.

In our studies, the levels of extracellular (apoplastic) and intracellular (vacuolar) chitinase activity of shoots of 7 days old wheat seedlings were determined. Quantitatively the vacuolar enzyme significantly exceeded the apoplastic one, but the specific (per unit of protein) activity of the latter was almost 2 times higher (Fig. 1, a). According to the IEF, the cells contained only acidic forms of chitinase with pI from ~ 3.6 to 5.1, while inside the cells - neutral and alkaline components with pI from ~ 6.6 to 9.3 (Fig. 1, b). The zones of chitinase activity in the gel after IEF were identified using the substrate of glycol chitin, therefore these enzymes were endochitinases.



**Figure–1. Activity (a) and IEF (b) of chitinase of the wheat shoots: 1- total chitinase, 2- extracellular (apoplastic) chitinase, 3- intracellular (vacuolar) chitinase, M- pI markers**

According to the mode of action on the substrate, chitinases are divided into two types - endochitinases and exochitinases. In addition, chitinolytic enzymes can exhibit the activity of N-acetyl- $\beta$ -glucosaminidase, which are referred to the exo-action chitinases. It is well known from the literature, that the endo/exo type of action largely determines the participation or degree of participation of the enzyme in the defense against pathogenic attack. To detect exochitinase in the gel after IEF we used a specific chromogenic substrate 4-methylumbelliferyl-N-acetyl-glucosaminide. From the presented electrophoregram, it follows that N-acetyl- $\beta$ -glucosaminidase of wheat seedling is represented exclusively by acidic components in the pH range ~ 4.3-5.2 (Fig. 2).



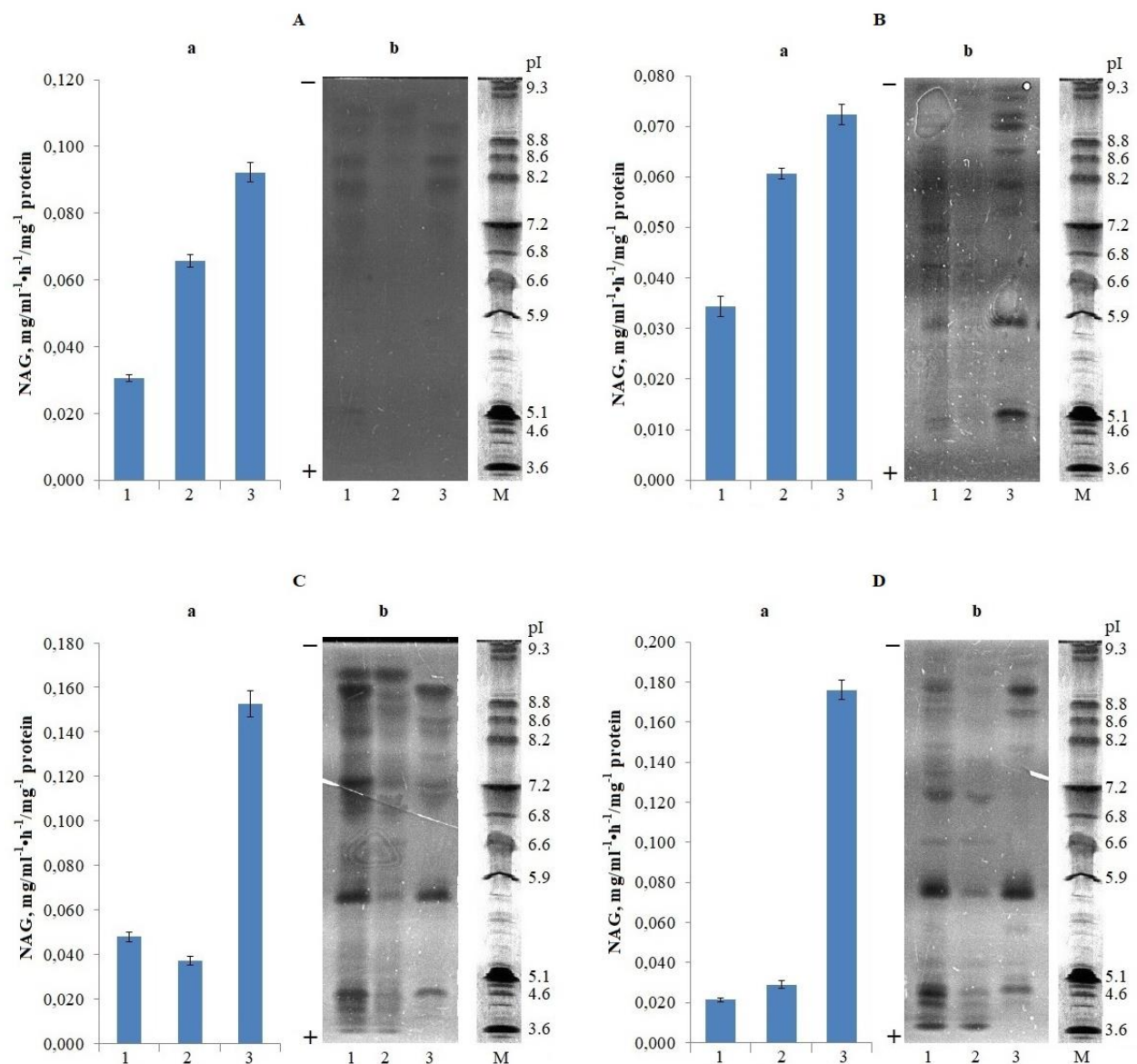
**Figure–2. IEF of exochitinase (substrate 4-MUF-NAG) of the wheat seedlings: 1- germinating grains, 2- roots, 3- shoots, M- pI markers**

#### **Purification of chitinase with CBD substrate by affinity chromatography and determination of its isozyme composition**

By their structure, chitinases are subdivided into forms containing a chitin-binding domain (mainly chitinases of class I) and those not containing this domain (classes II and III). The presence or absence of CBD is an important characteristic and plays a critical role in the display of the enzyme's antifungal properties.

To identify and purify chitinases with CBD from various seedling organs, we used a column with insoluble chitin (chitin resin), as described above in the “Materials and Methods” section. Analysis of the distribution of chitinase activity over various organs of the seedling showed that the specific activity of the enzyme with CBD is highest in roots and shoots as compared with dormant and germinating grains (Fig. 3, a). According to the IEF data, chitinases with CBD, exhibiting specificity for the affinity sorbent, were present in the acidic, neutral and alkaline regions of spectrum (Fig. 3, b). Most isoforms of this class of chitinases had alkaline pI values (~ 9.3, 9.0, 8.6, 8.2, 8.0, 7.6). Components with pI ~ 9.1, 8.8, 4.0, 3.6 did not bind to the affinity sorbent, which indicated the absence of CBD in their structure, i.e. they do not belong to class I chitinases. It should be noted that the IEF spectrum of chitinases with CBD in vegetative organs (root and shoot) and in grains as a whole is similar, and isoforms with pI ~ 9.0, 8.6, 7.6, 5.7 were common. However, among chitinases with CBD in

germinating grain, basic isoforms prevailed in the pH range of ~ 7.2-9.3. The main (major) chitinases with and without CBD in wheat seedlings are components with pI ~ 9.0, 5.7, 4.6 and 9.1, 3.6, respectively.



**Figure–3. Activity (a) and IEF (b) of chitinases of the wheat seedlings: 1- total chitinase, 2- chitinase without CBD, 3- chitinase with CBD, M- pI markers. A-dormant grains; B-germinating grains; C-shoots; D-roots.**

For greater clarity, the data on chitinases and their isoelectric points from grain and seedling organs were summarized in Table 1.

**Table-1. Isoforms of chitinases with and without CBD and their isoelectric points**

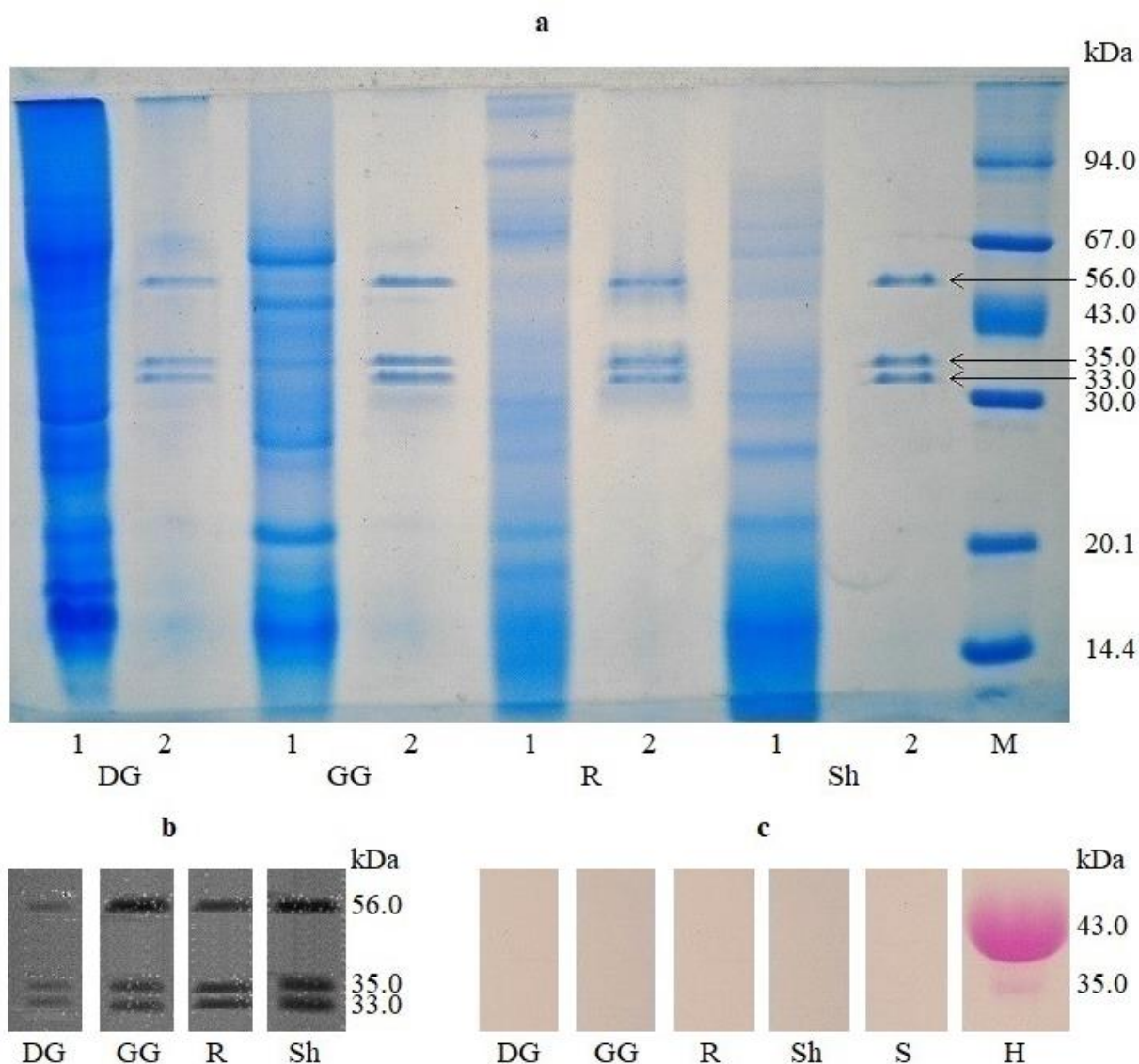
Plant organ	Chitinase without CBD			Chitinase with CBD		
	Basic	Neutral	Acid	Basic	Neutral	Acid
Dormant grain	9.1	-	-	9.0, 8.6, 8.2	-	-
Germinated grain	-	7.0	-	9.3, 9.1, 9.0, 8.6	7.6, 6.9	5.7, 5.1
Root	-	7.0, 6.6	4.0, 3.6	9.0, 8.6	7.6	5.7, 4.6

Shoot	9.1, 8.8	-	4.0, 3.6	9.0, 8.6, 8.4	7.6, 7.2	5.7, 4.6
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The presented data show the similarities and differences in the quantitative distribution and the presence in the IEF spectrum of chitinase isoforms with CBD and without CBD in the organs of the wheat seedlings. It should be noted that dormant and germinating grains are characterized by a high presence of chitinases with CBD (almost all components of the spectrum are adsorbed on the chitin column). Unlike grains in shoots and especially in roots, chitinases without CBD are significantly higher.

#### Determination of the molecular weight of chitinases with CBD by SDS-PAG electrophoresis

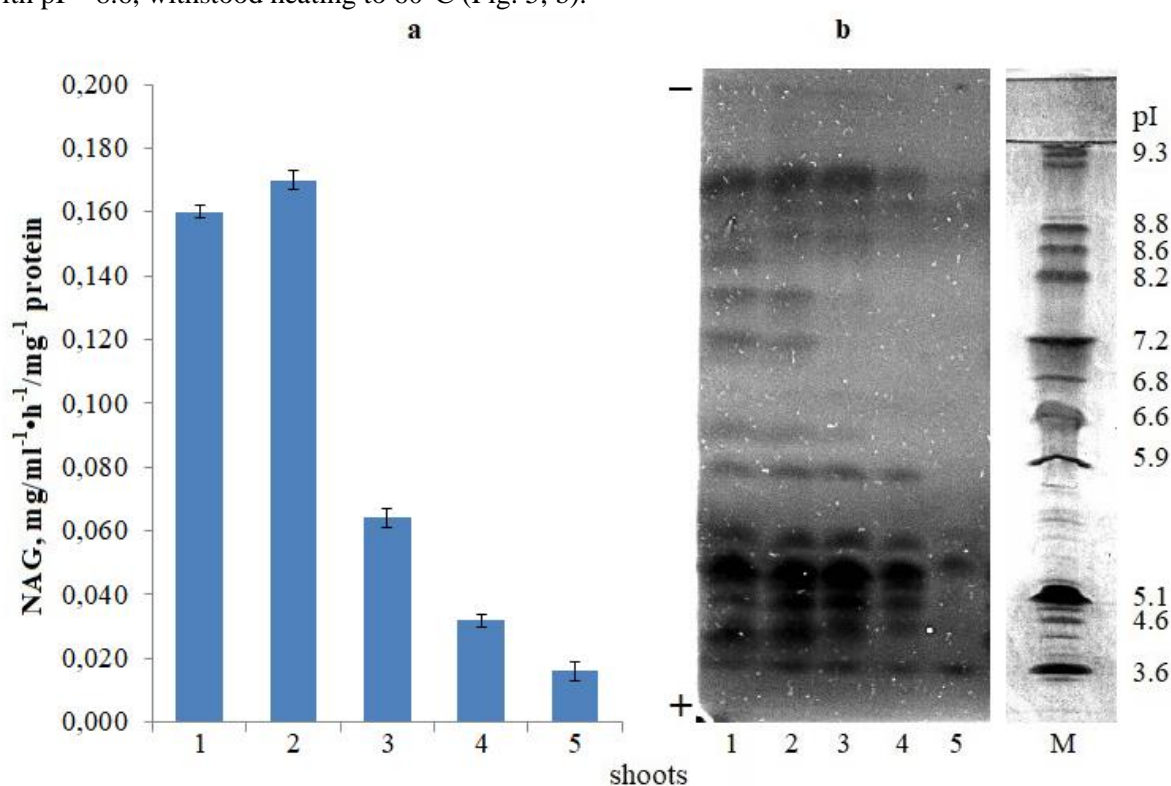
To determine the molecular weight of chitinase with CBD, small portions of extracts from resting grains and various seedling organs were bound to chitin resin and subjected to denaturing SDS-PAG electrophoresis. It was found that chitinase with CBD is represented by three proteins with molecular weights of about 33, 35, and 56 kDa (Fig. 4, a). Renaturation of the enzyme after SDS-PAG electrophoresis and staining of the gel for chitinase activity confirmed the data obtained on the component composition and molecular weight of chitinase bound by an insoluble polymer (Fig. 4, b). An important characteristic of a protein is the presence or absence of a carbohydrate residue in its structure. As it is known, glycosylation gives a protein a number of properties, for example, increased resistance to proteolysis and high temperature, promotes its targeted transport, etc. To determine whether chitinase is a glycoprotein, SDS-PAG electrophoresis was performed followed by specific staining of PAG for the presence of a carbohydrate residue. As a result, it was found that chitinase bound by chitin does not give a positive reaction, i.e. not glycosylated (Fig. 4, c).



**Figure–4. SDS-PAGE electrophoresis of chitinases of the wheat seedlings: 1- total chitinase, 2- chitinase with CBD, M- M.w. markers (a), SDS-PAGE electrophoresis with renaturation (substrate glycol chitin) (b) and SDS-PAGE electrophoresis for glycoprotein staining (c): DG- dormant grains, GG-germinating grains, R-roots, Sh-shoots, S-negative control (soybean trypsin inhibitor), H-positive control (horseradish peroxidase)**

### Thermal stability of chitinases in wheat seedlings

The thermal stability of chitinase and its individual components has been studied. For this, the enzyme extract from the shoots was heated for 10 minutes at temperatures of 60, 63, 66 and 69°C. After heating, the samples were rapidly cooled and centrifuged to remove denatured proteins. The control was an unheated enzyme. Analysis of activity and native IEF showed the presence of relatively thermostable and thermolabile chitinase components. At 60°C heating the sample for 10 minutes, the chitinase activity was slightly (5%) higher than in the control. With further heating, the enzyme activity decreased by more than 90% (Fig. 5, a). Almost all chitinases, with an exception of one main component with pI ~ 8.6, withstood heating to 60°C (Fig. 5, b).



**Figure–5. Activity (a) and IEF (b) of chitinase of the wheat shoots: 1- 30°C (control), 2- 60°C, 3- 63°C, 4- 66°C, 5- 69°C, M-pI markers**

Heating at 63°C led to a disappearance from the spectrum of two weakly basic chitinases with pI ~ 7.2 and 8.0, and at 66°C - of one neutral component with pI ~ 6.6, and basic component with pI ~ 8.6. Only two components: acidic with pI ~ 3.6 and weakly acidic with pI ~ 5.4 withstood a temperature of 69°C.

### Discussion

A review of the literature has shown that among cereals, the chitinase complex in wheat remains one of the poorly studied. Data on the physicochemical properties, isoenzyme composition, cellular localization, and organ specificity of wheat chitinases are scarce. There is no information on the identification of individual components of the electrophoretic spectrum, their belonging to one or another class of chitinases. Meantime, this knowledge is necessary for a better understanding of the functioning of the wheat chitinase system in the normal and stress conditions, as well as for assessing

the defense potential against pathogens. Molecular weight, isoelectric point, thermal stability, kinetic parameters, the presence or absence of a chitin binding domain, and cell localization are very important characteristics for the classification of chitinases.

For display of the physicochemical properties and functional features of chitinases, an important factor is their cellular localization. Our study revealed a strong distribution of individual chitinases in the shoots of wheat seedlings: basic and neutral isoforms are concentrated inside the cell (vacuolar enzymes), and acidic isoforms are concentrated outside the cell in the extracellular fluid (apoplastic enzymes). This is in good agreement with the available literature data on the localization of different isoforms of chitinases in certain other plant species. Native IEF analysis of chitinases showed the presence of one component with pI ~ 4.3 in the apoplast, and the rest with pI ~ 9.0, 8.2 and 6.6 in the vacuole. All of these enzymes are endochitinases, since they hydrolyze polymeric chitin. In addition to endochitinases, N-acetyl- $\beta$ -glucosaminidase, which belongs to exochitinases, was also identified among the chitinolytic enzymes of wheat seedlings. This enzyme was represented by 2-3 exclusively acidic proteins with a pI in the range from ~ 4.3 to 5.2.

The differences in the location of chitinases with different isoelectric points are not accidental and depend on the pH within each compartment of the secretory pathway. For example, the pH of the cytosol is 7.3, the endoplasmic reticulum is 7.1, the Golgi apparatus is 6.3, the vacuole is 5.2, while in the peroxisomes and apoplast it is 8.2, and the pH of the apoplast is not stable and can increase in response to salt stress, drought, and also when interacting with pathogens (Shen et al., 2013; Geilfus, 2017; Savchenko et al., 2019). The same patterns in the localization of acidic and alkaline isoforms are also typical for some other enzymes, for example,  $\beta$ -1,3-glucanase, protease, peroxidase, superoxide dismutase (Ignacio et al., 2015; Maksimović et al., 2019).

In the present study, a high degree of heterogeneity of chitinases in wheat seedlings with more than 10 components located in a wide pH range from ~ 3.6 to 9.3 was established using IEF in a gradient of ampholines pH 3-10. According to the data presented in a large review (Malik, 2018), purified basic chitinases of arabidopsis, barley, beans, peas, and some other species had a pI of around 9.0, neutral chitinases in beans and cotton were close to 7.0, and acidic chitinases from beans, peas, ficus and tomatoes were about 4.0. In our study, we found a great similarity in the IEF spectra of chitinase from germinating grains, roots, and shoots of wheat seedlings in terms of the set of components and their distribution in the gel. On the other hand, the resting grains contained a small amount of isozymes (about 5), which located mainly in the alkaline pH range. In germinating grains, the component composition of chitinases was much richer, however, in the acidic region of the spectrum, there was only one isozyme with pI ~ 5.1. High polymorphism of the enzyme is typical for both germinating grains and vegetative organs.

Chitinases of class I play a very important role in plant protection against fungal pathogens, since they have a direct antifungal effect due to the presence (in addition to the catalytic) chitin binding domain. CBD provides binding of the enzyme to the chitinous membrane of the fungus, lysis of the polymer, and suppression or death of the pathogen. A detailed study of the structure of class I chitinase from *Vigna unguiculata*, purified by affinity chromatography, showed the content of 8 cysteine residues in the chitin-binding domain at the same positions as in hevein. Analysis of the crystallographic model and molecular calculations of coupling using chitooligosaccharides revealed the presence of residues involved in sugar binding and catalysis, as well as a possible mechanism of antifungal action (Landim et al., 2017). Recently, researchers have paid close attention to the identification, study of the properties and application of class I chitinases and their genes to increase the resistance of agricultural plants to phytopathogens (Toufiq et al., 2018; Bartholomew et al., 2019).

For the purification of chitinases with CBD, a substrate affinity precipitation by batch method or chromatography on a colloidal or regenerated chitin matrix is usually used. In our work, a chitin resin column was used as a specific sorbent, the effectiveness of which has been shown in other studies (Singh et al., 2007; Sørensen et al., 2010). Using this sorbent, chitinases from grain and wheat seedling organs were separated into isoforms with and without CBD. Both forms of chitinase were identified in the IEF spectrum; isoforms with CBD from shoots had pI values around ~ 9.0, 8.6, 8.4, 7.6, 7.2, 5.7 and 4.6. In many ways, similar spectra of this enzyme were observed in roots and germinating grains with insignificant differences in individual components. In particular, germinating grain is characterized by the presence of additional strongly basic isozymes with pI ~ 9.3 and 9.2. Acid chitinases, except for components with pI ~ 4.6 and 5.7, did not bind to the affinity sorbent, i.e., they

did not contain CBD in their structure. The data obtained indicate a noticeable predominance of isoforms with CBD in the chitinase complex in wheat, which is generally typical for plants.

Chitinases are a very diverse group of enzymes. They vary in activity, temperature and pH optima, and in size. Their molecular weight usually ranges from ~ 20 to 120 kDa, whereas the most bacteria has a range of ~ 20-60 kDa, and in plants this value is ~ 25-40 kDa (Kisiel and Jęckowska, 2019). In our work, using SDS-PAGE electrophoresis, the M.w. for chitinases with CBD of wheat seedlings was determined and it corresponded to ~ 33, 35 and 56 kDa. The sizes of the first two proteins are well within the indicated limits for plant chitinases. The third protein was slightly larger than the usual sizes for plant chitinases (56 kDa), however, isoforms with ~ 20 kDa or  $\geq 40$  kDa were also previously described (Chang et al., 2014). When studying the enzyme activity in tetra- and hexaploid wheat genotypes, gel detection analysis revealed up to four fractions (~ 20, 30, 42, and 95 kDa) of proteins with chitinase activity in relation to the long chain of polymers (Moravčíková et al., 2017). In the gel after SDS-PAGE electrophoresis of chitinases from triticale seedlings, there were five separate protein bands with low (18, 23, 26, and 33 kDa) and relatively high M.w. (51 and 55 kDa) (Zur et al., 2013). Most of the low M.w. chitinases exhibited antifungal activity in vitro as well as in vivo, while high M.w. isoforms were involved in protection from abiotic (cold) stress. A number of studies have investigated the structure and enzymatic properties of chitinases with CBD from other cereals. The molecular weight of the enzyme from rye and rice was ~ 26 kDa, and from barley and corn ~ 32 kDa (Ohnuma et al., 2012; Tanaka et al., 2017; Toufiq et al., 2018; Dowd et al., 2018). All these chitinases belong to class I of family 19 glycosyl hydrolases (GH19).

Among plant chitinases both glycosylated forms, i.e. enzymes having carbohydrate residues in the structure and not glycosylated forms are found. As it is known, the carbohydrate component gives the protein molecule greater resistance, for example, to elevated temperature or the action of proteases. The multifunctional enzyme ICChI with the activity of chitinase/lysozyme/exochitinase which is glycosylated (14-15%) and has M.w. 35 kDa and pI 5.3 was purified from the latex of *Ipomea carnea*. The enzyme is stable at 80°C, and the glycoprotein nature of the protein may well be a factor of high thermal stability (Patel et al., 2009). Glycosylation is usually performed by enzymes designed for secretion outside the cells, but vacuolar class I chitinase in tobacco leaves is not glycosylated. As shown in this work, chitinases with CBD from wheat seedlings did not give a positive reaction with Schiff's reagent, i.e. do not contain carbohydrate residues. This chitinase is mainly represented by intracellular basic and neutral isoforms. There is very little information in the literature on glycosylated chitinases in plants, but these secreted enzymes are better described in various animals and microorganisms, which are characterized by a serine/threonine-rich glycosylated domain (Hamid et al., 2013; Le and Yang, 2019).

Plants do not contain very heat-labile chitinases, but highly thermostable enzymes have been isolated from pomegranate, which remain active up to 65°C for several hours, enzymes from beans retained 70% of the initial activity at 70°C after 60 minutes of exposure, whereas chitinases from garden bean remain stable only up to 60°C (Kopparapu et al., 2011; Sharma and Hooda, 2018; Wang et al., 2012). Our study showed that the components of chitinase from wheat seedlings have different sensitivity to elevated temperature. The acidic components exhibited the highest thermal stability, three of which with pI ~ 3.6, 5.1, and 5.4 withstood heating at 69°C for 10 minutes. Neutral and basic isoforms are more thermolabile and lose activity at 66°C. An interesting fact is a slight increase in the activity of chitinase relative to the control after heating to 60°C, which is probably associated with a disappearing of inhibitory proteins. In general, in comparison with other plant species, wheat has a moderate thermal stability of chitinase, especially its acidic isoforms.

The data obtained make it possible to classify wheat seedling chitinases, which bind to insoluble chitin, to class I, since they have CBD in their structure. These enzymes are represented by basic, neutral and acidic isoforms. Their molecular weight is greater than 30 kDa and they are not glycosylated. Chitinases not bound by chitin do not contain CBD and are classified as class II or possibly class III, which are also found in cereals. These isoforms are represented by isozymes with different pI ~ 9.1, 7.0, 4.0, and 3.6. Chitinases of class II are mainly acidic apoplastic proteins, for example, in sweet potatoes, the enzyme has a M.w. 26.3 kDa and pI 5.0 (Liu et al., 2020). Extracellular acidic chitinases of class III with bifunctional lysozyme/chitinase activity have been found, for example, in hevea, cucumber, and arabidopsis (Malik, 2019). Among the chitinolytic enzymes of wheat seedlings, N-

acetylglucosaminidase was also identified, which is represented by 2-3 exclusively acidic proteins with pI ranging from ~ 4.3 to 5.2.

## Conclusion

The data obtained in this study show that chitinase in wheat seedlings is represented by multiple molecular forms. Among them, intra- and extracellular enzymes were found, as well as isoforms with a chitin-binding domain and without CBD. Their isoelectric focusing and SDS-PAGE electrophoresis pattern was established. The results expand knowledge about the wheat chitinase complex and can be useful in the search and identification of enzymes (and individual isozymes) as markers of resistance to fungal pathogens.

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## Contribution of Authors

Khakimzhanov A: Data Analysis, Literature Review, Manuscript Writing

Kuzovlev V: Data Collection, Designed Research Methodology

Abaildayev A: Data Collection, Statistical Analysis, Manuscript Writing

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