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PURIFICATION AND BIOCHEMICAL PROPERTIES OF WHEAT ENDOCHYTINASE

Abstract. Chitinolytic enzymes are the most important components of the plant defense system against various pathogens. Chitinases hydrolyze the N-acetyl-β-glucosamine-containing polymer substrates (chitin, chito-oligosaccharides), which are part of the cell walls of fungi, nematodes and insects. The high polymorphism of chitinases in cereals, including wheat, the poor knowledge of their biochemical properties and activity regulation is one of the main obstacles in understanding the functioning of this enzyme complex.

The aim of the work was the study of some physico-chemical characteristics of wheat endochitinase. Using chromatography on a specific chitin affinity sorbent, endochitinase was purified from shoots, roots and seeds of wheat seedlings. The enzyme was represented by several isoforms with a molecular weight of about 30 kDa and pI in the acidic, neutral, and alkaline regions. There were no significant differences in the isoenzyme composition of endochitinase from different organs of the wheat seedlings. Some physico-chemical properties of wheat endochitinase were determined - pH and temperature optimum, thermal stability, the effect of different 2-valent metal cations on activity. The results can be used in the enzymology of the interaction of plants and phytopathogenic fungi.

Key words: wheat, endochitinase, isoenzymes.

Introduction. To date, a large amount of factual material has been accumulated on the induction in plants in response to the lession of specific pathogenesis related (PR) proteins by viruses, bacteria and fungi. These proteins are classified into 17 families according to their structure and properties [1]. Special attention in connection with the study of plant protection mechanisms against phytopathogens is given to chitinases (EC 3.2.1.14), capable of destroying the cell walls of fungi [2-4]. As part of the PR proteins, these enzymes form 4 families. In plants, chitinase, like other polymer hydrolases, is represented by several isoenzymes and is encoded by a family of genes. Chitinases are subdivided into constitutive and inducible forms, differ in tissue specificity of expression [5]. The significant polymorphism of the enzyme is due to the complex organization of natural substrates - chitin and its various oligosaccharide derivatives, suggesting differences in their substrate specificity and structural features of the isoenzymes [6].

According to the type of action on the substrate in the composition of chitinases, endochitinases and exochitinases are distinguished. The first enzymes cleave chitin randomly inside the polymer, producing soluble low molecular weight N-acetylglucosamine multimers, such as chitotriose, chitotetraose, and diacetylchiobiose dimer. The last enzymes are capable to cleave only the terminal carbohydrate residue of the polymer [7, 8]. Based on the primary structure, plant chitinases are divided into 7 classes (I – VII). It is shown that there is no definite correlation in the distribution of chitinases by plant species, their organs and tissues. However, it was found that only some chitinases have antifungal properties [9, 10].

The chitinase complex and its functioning are most studied in tobacco, and among cereals - in barley and rye. In wheat, the composition of this enzyme has about 10 isoforms having a wide range of pI in the acidic, alkaline, and neutral pH from 3.1 to 9.7. It has been shown that some isoforms to some extent may be involved in protecting the plant from pathogenic attack [11-13]. Despite certain successes, wheat chitinases are still relatively poorly studied, especially their physicochemical properties and activity

regulation. The main difficulties in their study is the relatively high polymorphism of the enzyme. Additional difficulties are imposed by the existence of constitutive and inducible forms of the enzyme, the tissue specificity of their expression, as well as hormonal and metabolic control of their activity.

In the present work, we studied some biochemical properties of wheat endochitinase purified by affinity chromatography on chitin.

Materials and methods of research. The objects of study were wheat (*T.aestivum* L.) seedlings and their individual organs.

Determination of chitinase activity. To determine the activity, 1 ml of colloidal chitin (5 mg/1 ml of 0.05 M acetate buffer pH 5.2) was added to a 0.1 ml sample and incubated for 4 h at T 37°C on a shaker at a speed of 120 rpm. After incubation, the reaction was stopped with 1 ml 3,5-dinitrosalycilic acid (DNS), the mixture was boiled in a water bath for 5 min, then centrifuged for 5 min at 8000 rpm. After centrifugation, the optical density was measured at a wavelength of 545 nm [14]. The resulting amount of N-acetyl-D-glucosamine was found using the calibration curve for N-acetyl-D-glucosamine. The enzyme activity was expressed in mg of N-acetyl-D-glucosamine per 1 h in 1 ml.

Substrate Chitinase Affinity Chromatography. The endochitinase was purified by affinity chromatography on a chitin column at a temperature of +4°C. For this purpose, the shrimp chitin that was previously swollen in water (for 12 h) was placed in a column of 1.0 x 10.0 cm and equilibrated with 20 mM sodium bicarbonate pH 8.0. The extract proteins obtained after precipitation of $(NH_4)_2SO_4$ (20-80%) were transferred to the same buffer after dialysis. The sample was introduced into the column with the sorbent and washed with buffer, until the complete absence of protein at the exit. Protein fractions were collected in 5 ml at a flow rate of 30 ml/h of buffer. After washing the column with starting buffer, 20 mM sodium acetate pH 5.3 was passed. The chitinase bound to the sorbent was eluted with 75 mM acetic acid pH 3.0. 0.2 M NaOH was immediately added to the enzyme fraction and the pH was adjusted to 6.0-7.0. To obtain the maximum amount of purified chitinase, affinity chromatography was performed repeatedly. The fractions containing the enzyme were combined and concentrated at 4°C in an Amicon cell with a PM-10 filter.

Protein electrophoresis. The electrophoresis of proteins under denaturing conditions with sodium dodecyl sulfate (SDS-Na_2) was carried out in slabs of 10% polyacrylamide gel (PAG) with a size of 8x10 cm and thickness of 1 mm according to the method of Laemmly. Coomassie brilliant blue G-250 was used for staining PAG for total protein.

Isoelectric focusing and detection of chitinase in PAG. Native IEF was performed in a 6% PAG plate 9x15 cm and 1 mm thick with 1% Servalyt pH 3-10. 10 μl of the preparation was applied to each well of the applicator. Enzyme separation was performed at 600 W for 5 h on a Multiphor II (LKB instrument). Identification of chitinase activity zones was performed using a gel replica with a polymerized substrate of 0.02% glycol chitin. After IEF, the working gel and replica were incubated for 15 min in 0.05 M acetate buffer pH 5.0. Then the two gels were tightly pressed to each other and incubated as a sandwich for 2 h in an thermostat at 42°C. After that, the gel replica was transferred to 0.5 M Tris buffer pH 8.8 with 0.01% Fluorescent bridgetener 28 and held for 10 min. The gel was left overnight in water at room temperature. Bands of activity were visualized with the help of a Gel-doc Quantum ST5 (Wilber Lourmat) with a UV length of 254 nm [15].

Results and discussion. *Purification and isozyme composition of chitinases.* By their structure, chitinases are divided into forms containing a chitin-binding domain (chitinase class I) and not containing this domain (classes II and III). The presence or absence of ChBD is an important biochemical characteristic and plays a crucial role in the demonstration of the protective properties of the enzyme. A distinctive feature of class I chitinases is their ability to bind with an insoluble substrate – polymeric chitin. Chitinases that bind to chitin (ChB chitinases) are typical endochitinases.

For purification and identification of wheat seedlings chitinases, a shrimp chitin column was used. The extract proteins were preliminarily concentrated by precipitation with ammonium sulfate within the saturation range from 20 to 80%. As a result of column chromatography, it was established that all organs of the 5-day seedlings - shoots, roots and seeds contain chitinases that have an affinity for the natural polymer substrate. The results of denaturing electrophoresis in the presence of SDS-Na_2 and native isoelectrofocusing using ampholytes in the pH range of 3-10 indicate the complex polymorphism of chitinolytic enzymes in wheat and, in particular, endochitinases.

Polymer-bound chitinases from all organs of the seedling are represented by several proteins with a molecular mass of around 30 kD (figure 1A). In shoots and roots, the composition of ChB chitinases was similar and included three proteins each with masses of approximately 28, 33, and 35 kD. In contrast, two additional proteins with masses of 26 and 30 kD were present in the germinating seed.

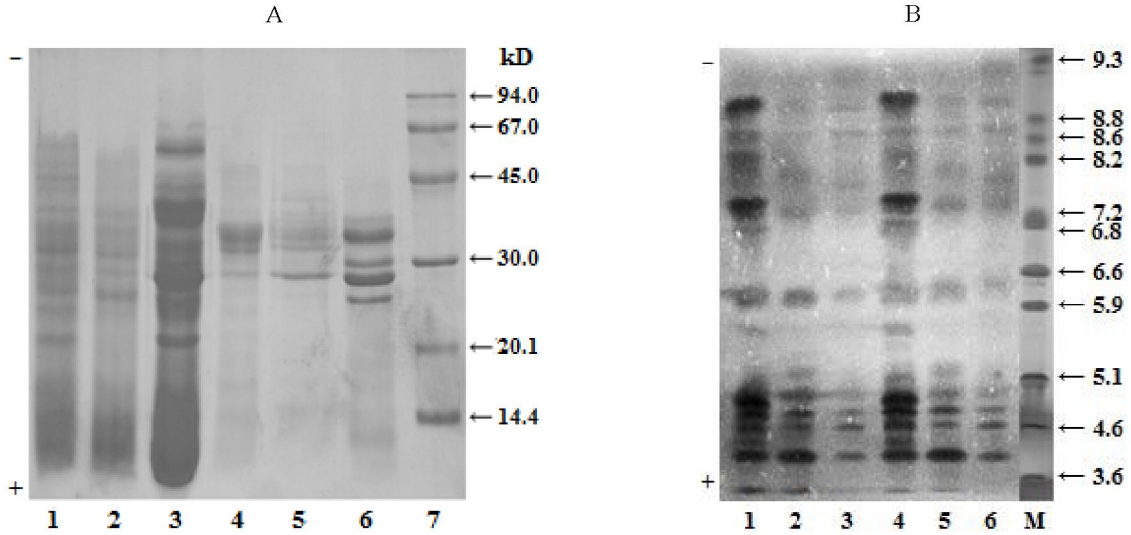

Figure 1 – SDS electrophoresis of purified ChB chitinases from different seedlings organs (A) and IEF spectra of seedlings chitinases, containing and not containing ChBD (B):
A – gel staining of the Coomassie G-250: 1-3 – shoot, root and seed protein prior to application to the chitin column; 4-6 – ChB chitinases of shoot, root and seed respectively; 7 – protein markers m.v. B –1-3 shoot, root and seed chitinase prior to application to the chitin column, 4-6 – chitinase with ChBD of shoot, root and seed respectively, м – IEF markers

The spectra of the native IEF fractions of the enzyme bound and not bound to chitin is shown in Figure 1B. Chitinases with ChBD, exhibiting affinity for the sorbent, were present in both the acidic and alkaline regions of the gel. Some of the isoenzymes had pI in the alkaline region (9.0, 8.7, 8.2, 8.0, 7.6), and in the acidic region there were components with pI 6.0, 5.0, 4.6, 4.0. It should be noted that the spectra of chitinases with ChB centers in the vegetative organs (root, shoot) and in the seed as a whole is similar. These are isozymes with pI 9.0, 8.7, 8.2, 5.0, 4.6, 4.0.

Biochemical properties of chitinases. Environmental conditions - temperature, pH, metal cations and their concentration, are among the most important factors influencing the activity of the enzyme and its interaction with the substrate. The effect of different pH values on the chitinase activity of wheat seedlings was studied. The enzyme showed catalytic activity in a broad wide range of pH - from 3.5 to 9.5 with an optimum in the range of 5-5.5 (figure 2). The wide pH effect of the enzymes on the substrate is obviously explained by the considerable heterogeneity of the isoenzyme composition, including acid, neutral and alkaline forms. As can be seen from the IEF spectra (figure 1B), ChB chitinases located in a wide range of isoelectric points. It should be noted that chitinase from germinating seeds, as compared with those of shoots and roots, retained greater activity in the alkaline region of pH.

The effect of different positive temperatures (30,40,50,60 and 70°C) on the activity of purified wheat seedlings chitinase was studied. The optimum temperature of the medium for the display of the catalytic activity of chitinase was 40°C (figure 3).

The effect of temperature pretreatment (thermo stability) on the activity of purified wheat seedlings chitinase was investigated. The enzyme samples were heated at 40, 50, 60 and 70°C for 10 min, cooled sharply, centrifuged, and the activity in the supernatant was measured. From the graphs presented in figure 4, it can be seen that chitinase is resistant to elevated temperature and partially showed activity at a maximum value of 70°C. The relative heat resistance within 60°C was characterized by the enzyme from the roots.

A very important factor in the regulation of enzyme activity are metal cations of the medium. In our work, we studied the effect of different concentrations of divalent cations Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+}, Ba^{2+} on the activity of ChB chitinases of wheat.

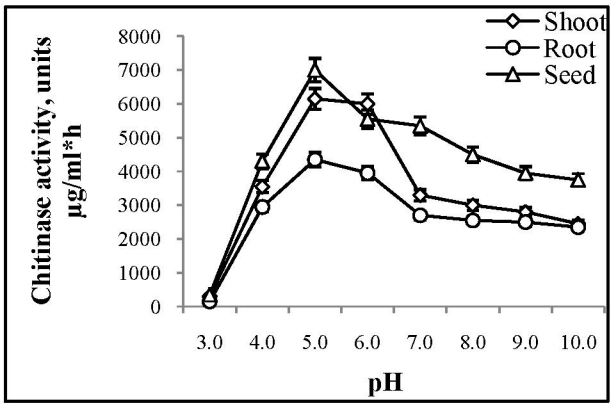

Figure 2 – The effect of pH on chitinase activity from different organs of a 5-day seedlings

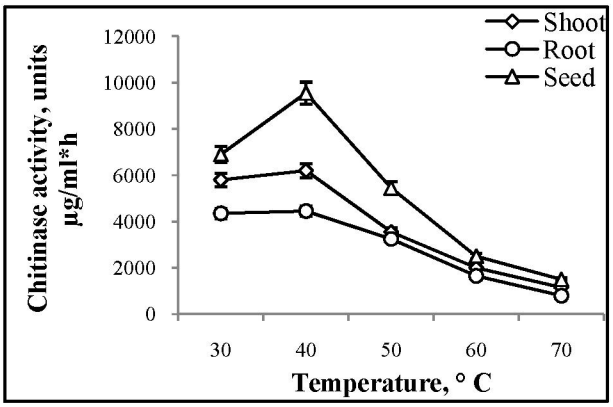

Figure 3 – The effect of temperature on the activity of chitinases from different organs of a 5-day seedlings

For this, metals were introduced into the incubation medium of the enzyme with the substrate in the form of chloride salts at a concentration of cations of 1, 5, and 10 mM. In addition, the enzyme itself was preincubated with the cation for 10 min. The data presented in figure 5 indicate significant differences in the effect of different metals on the chitinase activity of wheat seedlings.

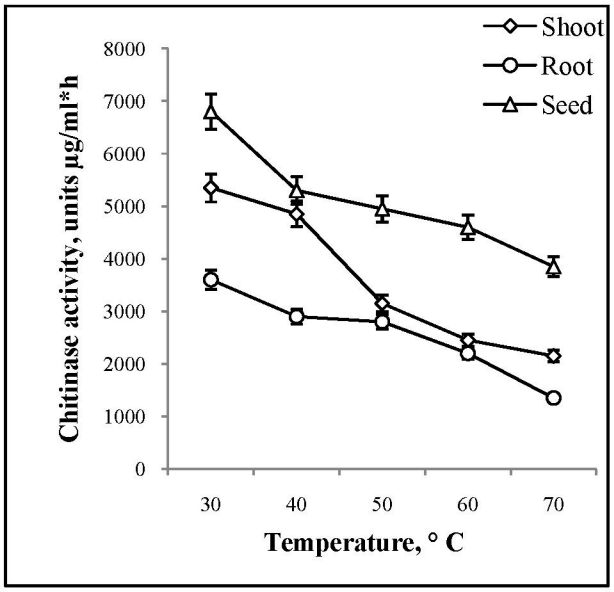

Figure 4 – Thermal stability of chitinases from different organs of a 5-day seedlings

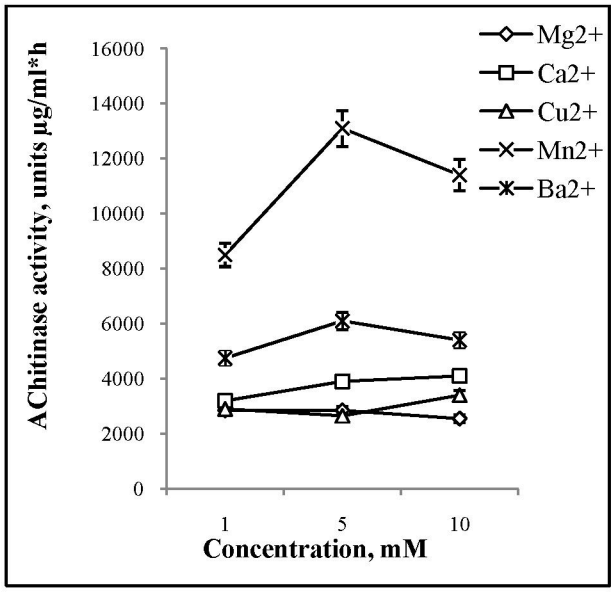

Figure 5 – The effect of metal cations on the activity of seedling chitinase

The highest inhibitory effect was observed for the Cu^{2+} cations and in the metal concentration of 10 mM the enzyme was almost completely inactivated. In contrast, the Mn^{2}+ cations increased chitinase activity (at 5 mM concentration). Ba^{2+} cations and slightly less Mg^{2+} had a similar activating effect.

Conclusion. Purification of wheat chitin-binding chitinase was carried out using substrate affinity chromatography, their composition and some physicochemical properties were determined. According to the SDS-Na_2 electrophoresis of ChB chitinases, seedlings were represented by several proteins with molecular masses in the region of 30 kD. In shoots and roots, the composition of the enzyme was similar and included three proteins with masses of 28, 33 and 35 kD, and two additional components with masses of 26 and 30 kD were present in the germinating seeds. The presence of acidic, neutral and alkaline isoforms has been established using native IEF as part of ChB chitinase. The spectrum of chitinases in the vegetative organs (root, shoot) and in the seed as a whole is similar. Major components were pI 9.0, 8.7, 8.2, 5.0, 4.6, 4.0.

A number of other physicochemical properties ChB chitinases of wheat, which are important for the demonstration of activity, have been studied. The enzyme was active in a wide pH range - from 3.5 to 9.5 with an optimum in the range of 5-5.5. The optimum temperature for the demonstration of chitinase catalytic activity is 40°C. Differences in the thermal stability of the purified enzymes were revealed. It was established that chitinase retained significant activity at 60°C for 10 min, however, heating at 70°C almost completely inactivated the enzyme. As part of chitinases, acidic isoforms are most sensitive to temperature increases. Established significant differences in the action of different metals on the activity of chitinases. The greatest inhibitory effect was exerted by the Cu^{2+} cations. In contrast, Mn^{2+} cations stimulated the activity of the enzyme. Ba^{2+} and a little less Mg^{2+} had a similar activating effect.

Authors' contributions. Zh.D. Beskempirova and A.O. Abaildaev participated in the preparation of plant material, extracts and measurement of the enzyme activity, V.A. Kuzovlev - in the protein electrophoresis and IEF, A. A. Khakimzhanov - in general guidance and preparation of the article.

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БИДАЙ ЭНДОХИТИНАЗАСЫНЫҢ БИОХИМИЯЛЫҚ ҚАСИЕТТЕРІ ЖӘНЕ ТАЗАРТУ

Аннотация. Хитинолитикалық ферменттер әртүрлі патогендерге қарсы өсімдік қорғау жүйесінің маңызды компоненттері болып табылады. Хитиназалар саңырауқұлақтардың, нематодтардың және жәндіктердің жасушалық қабырғасының құрамына кіретін полимерлі субстраттары (хитин, хито-олигосахаридтер) бар N-ацетил-β-глюкозаминді гидролиздейді. Астық тұқымдастылардағы, оның ішінде бидайдағы хитиназалардың жоғары полиморфизмі, олардың биохимиялық қасиеттерін және белсенділіктерін реттеудің нашар зерттелгендігі - бұл фермент кешенінің жұмыс істеуін түсінудегі негізгі кедергілердің бірі болып табылады.

Жұмыстың мақсаты бидай эндохитиназасының кейбір физико-химиялық ерекшеліктерін зерттеу болды. Арнайы хитинді аффинді сорбенттегі хроматография көмегімен бидайдың өскіндерінен, тамырынан және дәндерінен эндохитиназа тазартылды. Фермент молекулалық салмағы шамамен 30 кДа және ИЭН қышқылдық, бейтарап және сілтілік аймақтардағы бірнеше изоформалар көрсетті. Бидайдың әр түрлі мүшелеріндегі эндохитиназаның изоферменттік құрамында айтарлықтай айырмашылықтар анықталмады. Бидай эндохитиназасының кейбір физико-химиялық қасиеттері - pH және температура оңтайлылығы, термиялық тұрақтылығы, белсенділікке әртүрлі 2-валентті металл катиондарының әсері анықталды. Нәтижелер өсімдіктер мен фитопатогендік саңырауқұлақтардың өзара әрекеттесу энзимологиясында қолданылуы мүмкін.

Түйін сөздер: бидай, эндохитиназа, изоферменттер.

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ОЧИСТКА И БИОХИМИЧЕСКИЕ СВОЙСТВА ЭНДОХИТИНАЗЫ ПШЕНИЦЫ

Аннотация. Хитинолитические ферменты являются важнейшими компонентами защитной системы растений против различных патогенов. Хитиназы гидролизуют N-ацетил-β-глюкозамин содержащие полимерные субстраты (хитин, хитоолигосахариды), входящие в состав клеточных стенок грибов, нематод и насекомых. Высокая полиморфность хитиназ у злаковых, в том числе пшеницы, слабая изученность их биохимических свойств и регуляции активности является одним из основных препятствий в понимании функционирования этого ферментного комплекса.

Целью работы явилось исследование некоторых физико-химических особенностей эндохитиназы пшеницы. С помощью хроматографии на специфичном аффинном сорбенте хитине была очищена эндохитиназа из ростков, корней и зерновок проростков пшеницы. Фермент был представлен несколькими изоформами с молекулярным весом около 30 кД и ИЭТ в кислой, нейтральной и щелочной области. Существенных различий в изоферментном составе эндохитиназы из различных органов проростка пшеницы не выявлено. Определены некоторые физико-химические свойства эндохитиназы пшеницы – pH- и температурный оптимумы,

термостабильность, влияние разных 2-валентных катионов металлов на активность. Результаты могут быть использованы в энзимологии взаимодействия растений и фитопатогенных грибов.

Ключевые слова: пшеница, эндохитиназа, изоферменты.

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