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The effect of an enzyme preparation containing phytase on the microstructure and some indicators of the composition of wheat grain

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ABSTRACT

With the growing demand for products fortified with biologically active substances, consumer interest in whole-grain products is also growing. Whole grains are rich in fiber, vitamins, minerals, and antioxidants. However, biotechnological solutions are required to increase the nutritional value of grain products, including the use of enzyme preparations such as carboxylases and phytases. The purpose of this study was to study the effect of a complex enzyme preparation containing cellobiohydrolase, xylanase, beta-glucanase, and phytase on changes in the microstructure of the wheat grain surface and the aleurone layer, trace element, carbohydrate composition, fatty acid profile, and antioxidant activity. The study's objects were whole grains and the aleurone layer of Moskovskaya 39 wheat. After fermentation, cross sections of the grain surface and the aleurone layer were examined using a scanning electron microscope. The study of the aleurone layer revealed incorrect packaging of protein balls and fuzzy boundaries. It was shown that the content of phosphorus, sulfur, calcium, and copper in the grain after fermentation was significantly lower than in the native grain, by an average of 1.5 – 2 times. The rate of phosphoric acid release from wheat grain treated with the enzyme preparation was higher than under the action of the endogenous phytase of the grain when soaked in water. The total sugar content in grains soaked in the enzyme solution increased significantly by 22.22% ($p < 0.05$) compared to native grains. After soaking, the levels of unsaturated fatty acids in wheat increased, including linoleic acid, α-linolenic acid, γ-linolenic acid, and erucic acid. At the same time, the content of oleic acid decreased. The increase in unsaturated fatty acid content is probably due to their synthesis during the grain swelling period. The antioxidant activity of grain soaked in an enzyme preparation solution was significantly ($p < 0.001$) higher than that of native grain and grain soaked in water by 3.0 and 1.6 times, respectively. Thus, the experimental data indicate that treating wheat grain with a complex enzyme preparation containing carboxyhydrazes and phytase is an effective means of increasing the grain's nutritional value.

Keywords: grain, wheat, phytase, microstructure, composition

INTRODUCTION

The global increase in consumption of refined grain products may lead to health problems, since whole grains are richer in nutrients than their refined counterparts **[1]**. Nutrients in cereal grains are unevenly distributed: trace elements and dietary fiber are mainly concentrated in the outer layers **[2]**, **[3]**. The beneficial properties of whole-grain products are largely explained by the synergistic effect of wheat bran and germ and their biologically active ingredients **[4]**. Consumption of bran has risen significantly with the popularity of whole-grain foods and

awareness of the need for moderate processing **[5]**, **[6]**. Among the outer grain layers, the aleurone layer is of greatest interest to nutrition physiologists, as it contains the highest concentrations of biologically active compounds **[7]**.

In mature wheat grains, the aleurone layer consists of a single layer of cells located between the endosperm and the seed coat. The main component of the aleurone cell wall is arabinoxylan, with β-glucan and proteins also present **[8]**, **[9]**, and **[10]**. Wheat has modified aleurone cells that differ cytologically from typical aleurone cells. This modified aleurone layer, located near the grain furrow, serves as the main transport tissue **[11]**. In cross-section, aleurone cells are well-organized, tightly packed, and cuboidal. They contain thick cell walls and inclusions such as protein–carbohydrate bodies, aleurone grains, and lipid droplets **[9]**, and **[12]**. Aleurone cells contain numerous spherical particles known as aleurone grains, 2–4 μm in diameter **[13]**. The wheat aleurone layer is a concentrated source of minerals and vitamins, containing about 50% of total dietary fiber and most of the antioxidants accumulated in the seed **[3]**, while the endosperm consists mainly of carbohydrates **[14]**. Phenolic compounds in the aleurone layer are highly esterified with arabinoxylans (98.5%), with small amounts present in conjugated or free forms **[15]**.

The aleurone layer contains biogenic mineral elements calcium, iron, zinc, magnesium, and potassium **[16]**. Antinutritional factors such as phytic acid, which binds minerals and prevents their absorption, are also concentrated in the aleurone layer **[17]**. Biologically active compounds are not always readily absorbed into the bloodstream. The microstructure of the food matrix influences the bioavailability of bioactive substances and trace elements **[18]**. Wheat bran and the aleurone layer are matrices with highly complex structures. At the molecular level, dietary fibers and other bioactive compounds are mainly present in bound forms. The cell wall matrix is primarily composed of polysaccharides, while other bioactive compounds, including minerals, are adsorbed or bound at the molecular level **[19]**.

To release bound bioactive compounds, enzymatic treatment has been applied to wheat fractions **[20]**, and **[21]**. Phytase enzymes are increasingly used in food processing, as they break down phytates and increase mineral bioavailability. Phytases are already applied in baking and bran fractionation **[22]**. Their use in cereal products improves nutritional profiles by enhancing the availability of vital trace elements, flavonoids, and antioxidant activity **[23]**, and **[24]**. For phytase to penetrate the aleurone layer, seed coats must be modified by hemicellulase preparations, especially those containing endoxylanase **[25]**, and **[26]**.

Scientific Hypothesis

Enzymatic treatment modifies seed coat/aleurone microstructure and promotes phytate hydrolysis leading to redistribution of minerals within the grain and changes in biochemical indicators.

Objectives

The aim of this study was to investigate the effect of a complex enzyme preparation containing cellobiohydrolase, xylanase, β-glucanase, and phytase on changes in the microstructure of the wheat grain surface and aleurone layer, carbohydrate composition, fatty acid profile, lipid groups, and antioxidant activity.

MATERIAL AND METHODS

Samples

Samples description: We analyzed the microstructure and quality indicators of soft winter wheat (*Triticum aestivum*) of the Moskovskaya 39 variety.

Samples collection: The samples were collected and stored at room temperature.

Samples preparation: The samples were collected and freed from visible impurities, then rinsed with plenty of tap water. For the examination, 1 kg of the average sample was taken from each sample.

Number of samples analysed: To determine the microstructure and content of quality indicators in *Triticum aestivum* grains, 6 grain samples were analyzed.

Chemicals

All chemical reagents were supplied by Merck (Germany).

Animals, Plants and Biological Materials

The aim of the study is to analyze the quality indicators of wheat grain (*Triticum aestivum*) of the Moskovskaya 39 variety after enzymatic hydrolysis.

Instruments

Agilent 1100 liquid chromatograph with ESA Coulochem III electrochemical detector (Agilent Technologies Inc., USA), Silufol plates (Czech Republic), Carlo Erba Strumentazione gas chromatograph, HRGC 5300 Mega Series (Italy), Specord M40 spectrophotometer (Carl Zeiss Industriel Messtechnik GmbH, Germany), Hitachi 170-70 (Japan), the JEOL JSM 6390 electron microscope (JEOL, Japan).

Laboratory Methods

Microstructural studies were performed using a JEOL JSM-6390 scanning electron microscope (JEOL, Japan). Prepared samples were placed on copper disks, coated with platinum using a JEOL JEE 44E vacuum evaporator, and examined at an accelerating voltage of 15 kV. Analysis of mineral element distribution in anatomical parts of the grain and relative content in washings was performed using an EMF miniCup X-ray detector system attached to the JEOL JSM 6390 microscope. The microscope's resolution is 4 nm at an accelerating voltage of 15 kV, with a scaling factor of x10 to x10000. During the elemental analysis, the working distance (WD) is 10 mm. Quantitative X-ray microanalysis with a defined point area of analysis was performed. The X-ray microanalysis data were obtained according to standard protocols, which include a micrograph of the sample structure, a data weighting table, atomic correlations, spectra, and histograms.

Analysis of spectral line intensities enabled the determination of the relative concentration of the desired element in mass%. The accuracy of the analysis is distributed as follows: with an element concentration of 1 to 5%, the accuracy is less than 10%; from 5 to 10%, the accuracy is less than 5%; with an element concentration of more than 10%, the accuracy is less than 2%. 30 points of each sample were studied. The local analysis is 3 mm, and the scanning area is at least 12 microns. Statistical software Statgraphics Centurion XVII (StatPoint Inc.USA) was used for statistical evaluation.

The concentration of mineral elements in morphological parts was determined after dry combustion in a Nabertherm muffle furnace (Germany) at 450 ° C and dissolution of ash in a mixture of 10% hydrochloric and nitric acids by atomic absorption spectrophotometry in an air-acetylene flame on a Hitachi 180-80 apparatus (Japan), with a deuterium background corrector.

Phytase activity was assessed indirectly by the rate of release of phosphate from the substrate spectrophotometrically. To a 1 cm3 of fluid was poured keyhole 1 cm^3 of 10% trichloroacetic acid solution and 2 cm^3 reagent "C" (3.66 g iron sulfate (II) was dissolved in a solution of ammonium molybdate (2.5 g of ammonium molybdate was dissolved in pre- 8 cm^3 of sulfuric acid and adjusted to 250 cm3 with distilled water). Absorbance of the test solution after 30 minutes of soaking at room temperature for the SF-56 spectrophotometer (OKB SPEKTR, Russia) for a wavelength of 750 nm in a cuvette with a distance of 1 cm between the faces against distilled water. A calibration curve was obtained for phosphorus mass concentration using standard aqueous solutions of known KH2PO4 concentration. Phytase activity was calculated using the formula:

$FA = ([PO_4] * 106 * Rrs * Rs) / (M * 103 * t_p)$, (1)

where Rrs – dilution of the enzyme preparation in the reaction mixture;

Rs – pre-dilution of the enzyme preparation (before adding to the reaction mixture);

M – molecular weight phosphate;

tp – the reaction time.

Sugar concentration in grain samples was determined chromatographically with electrochemical detection using an Agilent 1100 liquid chromatograph equipped with an ESA Coulochem III detector. Separation of sugar mixtures was achieved on an anion-exchange column with a bonded amino phase, followed by electrochemical detection. Grain samples were crushed and sieved. Flour portions were suspended in an acetate buffer (0.1 M, pH 5.0) at a dry-matter concentration of 100 g/L. Suspensions were incubated in a thermostated shaker (40 °C, 250 rpm) for 2 hours. After centrifugation (20 min), supernatants were collected, diluted tenfold, and analyzed for sugar concentration.

Fatty acid composition was determined by gas chromatography of methyl esters prepared from extracted lipids. Lipids were extracted, then transesterified with methanol in the presence of hydrogen chloride. Methyl esters were dissolved in hexane and analyzed on a Carlo Erba HRGC 5300 Mega Series chromatograph (Italy) with a Chrompack CP 7420 capillary column. Temperature program: 110 °C (10 min), ramp 5°C/min to 175 °C (15 min), 4 °C/min to 200 °C (5 min), 3 °C/min to 225 °C (60 min). Injector at 260 °C, detector at 240 °C, nitrogen as carrier gas.

Antioxidant activity was determined spectrophotometrically in ethanol extracts following Silva et al. **[27]**, based on percentage inhibition of the DPPH radical (2,2-diphenyl-1-picrylhydrazyl). Optical density was measured at 515 nm using a Specord M40 spectrophotometer (Carl Zeiss Industriel Messtechnik GmbH, Germany).

Glutathione was determined using the following method. After soaking the grain, 4 g of plant material is taken away and grown in a mortar with 2 ml of 0.3 n mercury acetic acid solution and 2 ml of 30% sodium acetic acid solution. The homogenate from the mortar is transferred to a centrifuge tube, and the remaining 10 ml of distilled water is washed off. Stir with a glass stick and leave for 10 minutes for complete precipitation. Then they are centrifuged, the solution is discarded, and the precipitate is washed 2 times with water in 10 ml portions while

stirring. Together with other substances, glutathione is found in the sediment, which is dissolved in hydrochloric acid. To do this, 10 ml of 1 N hydrochloric acid solution is added to the precipitate and mixed for 5 minutes. Then 1 ml of 20% potassium iodide solution is added, mixed and centrifuged. The centrifugate is transferred to a 100 ml titration flask. The precipitate is washed with 10 mL of distilled water while stirring. After centrifugation, the solution is added to the first centrifuge. 0.5 ml of starch solution is added to the resulting solution, and the mixture is titrated with 0.001 n KIO_3 solution until a non-vanishing blue color appears. 1 ml of 0.001 n KIO_3 solution corresponds to 0.307 mg of glutathione. The glutathione content is calculated by the formula (1):

$$X\ (\%) = 30700 \times a \times K/n \quad (1)$$

where a is the volume of 0.001 n of the KIO_3 solution,
K is the normality of the potassium iodate solution,
n is the normal glutathione titer (mg) multiplied by 100 to calculate per 100 g of substances.

Glutathione with acetic acid mercury gives a precipitate of mercury glutathionate in a slightly alkaline medium. The precipitate is washed with water and dissolved in hydrochloric acid. The solution is separated from water-insoluble substances and the glutathione transferred to the solution is filtered with a solution of KIO_3 in the presence of potassium iodide and starch. Potassium iodide is added in excess to bind mercury ions to form a soluble, colorless complex. The experiment was repeated three times. The reference was a standard glutathione sample **[28]**.

Flavonoid content was determined by photocolormetric analysis **[29]**. 1 g of the sample was treated with 70% ethanol and heated in a reflux flask in a boiling water bath for 30 minutes, with periodic shaking to wash the raw material particles from the walls. The flask was cooled and adjusted to its original mass with the same solvent. The extraction was filtered into a 100 ml volumetric flask and labeled with 70% ethanol. The contents of the flask were thoroughly mixed, and the optical density of the solution was measured on an SF-56 spectrophotometer (OKB SPEKTR, Russia) at 338 nm in a 10 mm cuvette. 70% ethyl alcohol was used as the reference solution. The standard was a 0.1% alcohol solution of a standard luteolin sample. The standard range of the curve was 0.01-0.1% luteolin. Total flavonoids were calculated as 2-O-arabinoside isovitexin equivalents according to the formula (2):

$$X\ (\%) = D \times 100/353 \times m \quad (2)$$

where D is the optical density of the test solution,
m is the grain weight, g.

Description of the Experiment

Study flow: For the experiment, 6 grain samples were collected from the laboratory of breeding and primary seed production of winter wheat (Federal Scientific Center Nemchinovka, Moscow, Russian Federation), which developed the Moskovskaya 39 variety. Three wheat grain samples represented elite seeds (selected varietal seeds obtained from the subsequent propagation of the original seeds (super-elite). The other three samples were elite seedlings from the first generation. The wheat grain was cleaned of impurities and washed with cold water. The dry complex enzyme preparation was mixed with citrate buffer (pH 4.5) at the rate of 0.6 g/l for 0.2 hours in a 2-liter container using a magnetic stirrer, then wheat grains were added, grain ratio:The solution was 1:2. The complex enzyme preparation contained cellulase, α-glucanase, xylanase and phytase (produced by *Penicillium canescens*). The enzyme activity was: cellulase – 58.711 ncat/g, xylanase – 12.135 ncat/g, beta-glucanase – 51.317 ncat/g, phytase – 205.268 ncat/g. The drug was provided by the Laboratory of Physico-chemical Transformation of polymers of the Chemical Faculty of Lomonosov Moscow State University **[30]**. The grains were incubated for 8 hours at 50 ± 2 °C in a TS-1/80 dry-air thermostat (Smolenskoye SKTB SPU, Russia). The concentration of the enzyme preparation corresponds to the optimum for bread production from whole-grain flour. The manufacturer specifies 50 °C and pH 4.5 as optimal for achieving maximum enzyme complex activity. The duration of hydrolysis is 8 hours, and the grain ratio is:A 1:2 soaking solution was established experimentally and corresponds to a wheat grain moisture content of more than 41%, which is the optimal indicator for grain dispersion in the production of grain bread **[31]**, **[32]**. The control was native dry grain and grain soaked in water under the same conditions as in the variant with the enzyme preparation. No enzyme inactivation was performed after incubation. Aleurone-rich product (up to 90% aleurone cells) was obtained according to a Russian patent **[33]**.

Quality Assurance

Number of repeated analyses: Six grain samples were taken for analysis.

Number of experiment replication: Samples were analyzed three times.

Reference materials: The equipment manufacturer has provided instructions for checking its operability.

Calibration: Each instrument was calibrated before each experiment, and calibration checks were performed regularly to ensure measurement accuracy. Each instrument was calibrated before each experiment, and calibration checks were performed periodically to ensure measurement accuracy.

Laboratory accreditation: The experiments were conducted in a laboratory accredited to ISO 17025.

Data Access

The data confirming the results of this study are not publicly available.

Statistical Analysis

Statistical analysis was performed using STATISTICA 13.3 (TIBCO Software Inc., USA). The samples were tested for normality using the Shapiro-Wilk test. To assess the differences between the compared indicators of native grain, grain soaked in water, and Grain soaked in an enzyme preparation solution, a one-factor dispersion analysis was performed, followed by a posteriori Tukey test. The uniformity of the variances was evaluated using Fisher's F-test. The results are presented as the mean ± standard deviation (M ± SD). The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The grain surface is the structural unit that first responds to the action of water and enzyme solutions based on cellulases. The main building block of cellulose molecules are microfibrils, which are long, narrow fibrillar structures containing both crystalline and amorphous regions. This microfibril structure provides strength through hydrogen bonds, as confirmed by electron microscopy and X-ray crystallography **[34]**.

Hemicelluloses are crosslinked with cellulose microfibrils, helping to create strong plant cell walls **[35]**.

The fibrillar structure of the surface layers of the grain complicates enzymatic hydrolysis and requires the use of special enzymes for cleavage of the material - endoglucanases and cellobiohydrolases. The availability of cellulose is limited by a complex matrix, and the crystalline parts reduce the amount of enzymes adsorbed on the substrate **[36]**.

Before the phytase enzyme, which is part of the complex of the studied drug, penetrates into the aleurone layer to catalyze the hydrolysis of phytin, it needs cellulose hydrolysis to occur. Cellulase must penetrate the cell wall matrix. Cellulase is an enzyme resource consisting of subunits of enzymes such as endoglucanases, cellobiohydrolases, and β—glucosidases, which specialize in the cleavage of the matrix. The process begins with endoglucanase, which randomly cleaves β-1,4 bonds, releasing both reducing and non-reducing ends of cellulose. Then cellobiose is released, which, when hydrolyzed by cellobiohydrolases, is converted into sugar molecules **[37]**. Application of the complex enzyme preparation containing cellulase, hemicellulases, and phytase results in preferential degradation of hemicelluloses in deeper tissues. Our earlier studies showed that phytate degradation is largely due to the presence of xylanase and β-glucanase in the enzyme complex; the intensity of phytin hydrolysis reaches its maximum when the grain substrate is treated with a preparation containing cellulase, β-glucanase, and xylanase **[26]**. Changes in the microstructure of the grain surface under the action of the enzyme complex containing phytase were studied in comparison with native grain (untreated) and grain soaked in tap water under identical conditions (50 °C, 8 hours).

Figure 1 presents micrographs of wheat grain surfaces: native grain, grain soaked in water, and grain soaked in the enzyme solution containing carbohydrases and phytase.

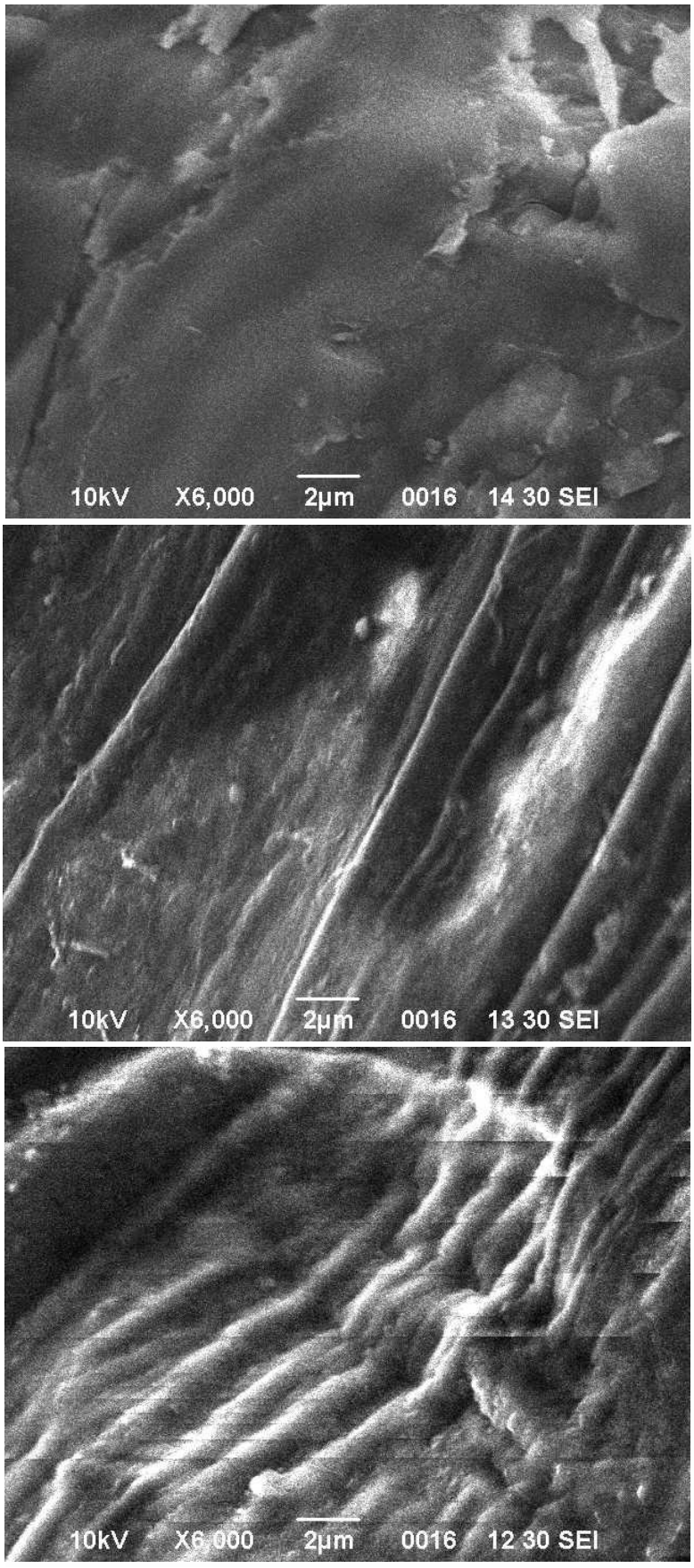

Figure 1 Micrographs of wheat grain surfaces.
Note: 1 – native grain, 2 – grain soaked in water, 3 – grain soaked in the enzyme solution containing carbohydrases and phytase. Magnification: 6000×.

Native wheat grain at 6000× magnification exhibits a characteristic microrelief of parallel cellulose fibrils covered by a cuticular layer composed of hemicellulose molecules /1/. Under the action of water (control), soaking at 50 °C for 8 hours altered the surface relief of wheat grain /2/. In the cuticular part of the microrelief, visible bundles of long, exposed cellulose microfibrils appeared. Hydrolysis of the hemicellulose matrix occurred due to the grain's own endogenous enzymes. Treatment with the complex enzyme preparation containing cellulase, hemicellulases, and phytase resulted in preferential destruction of hemicelluloses, penetrating deeper into the tissue /3/.

The folds of the grain surface relief in this version of the experiment are deep; the parallel microfibril texture prevails, with practically no transverse sections formed by hemicellulose molecules. The exposed interfibrillary paracrystalline areas become more accessible to water and the enzymes that make up the complex preparation. Modification of surface structures occurs both longitudinally and radially. An article by Haraldsson et al. also highlights the possibility of combining beta-glucan degradation with the action of phytase and beta-glucanase during malting, which is of interest for the production of grain products with high nutritional value **[38]**. Kuznetsova et al. reported **[39]**, **[40]** changes in the surface microstructure of wheat, rye, and triticale grains under the action of complex enzymatic preparations based on cellulases.

Using a scanning electron microscope, the microstructure of the surface of the fruit coat of the grain on a longitudinal section was examined (Figure 2). It is known that pore sizes in plant cell walls range from 4 – 5 to 13 nm. According to the literature, biological treatment of plant cell walls increases pore size **[41]**

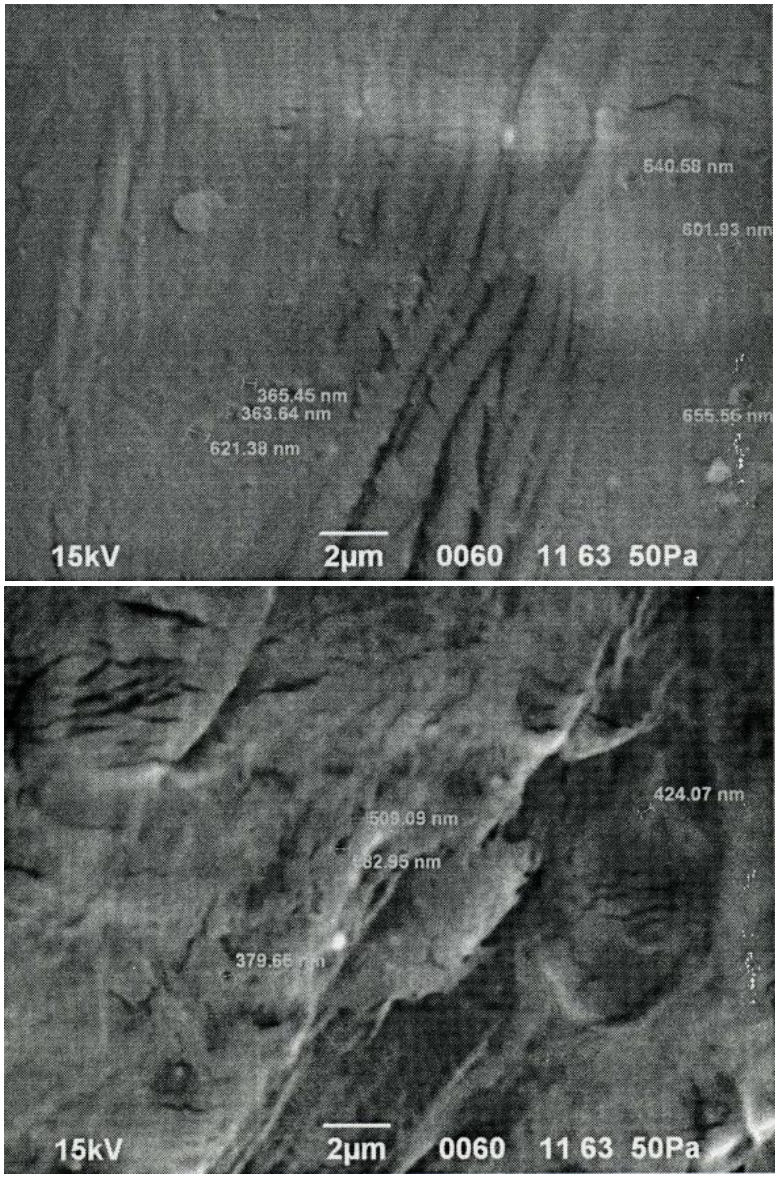

Figure 2 Micrographs of the surface of longitudinal sections of fruit shells of wheat grain.
Note: 1 – grain soaked in water, 2 – grain soaked in a solution of a complex enzyme preparation based on phytase). Magnification 5500x.

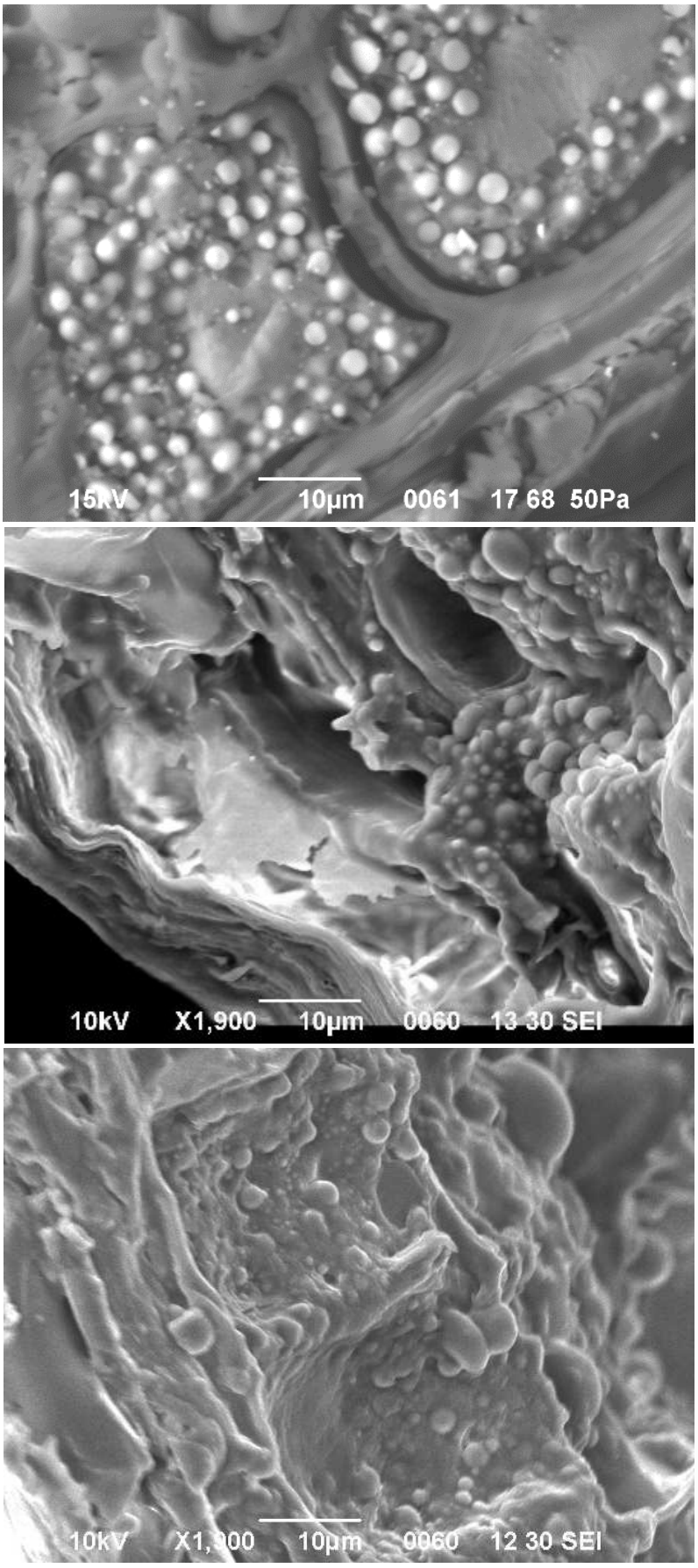

Figure 3 Micrographs of the surface of the cross-section of the aleurone layer of wheat grain.
Note: 1 – native grain, 2 – grain soaked in water, 3 - grain soaked in a solution of a complex enzyme preparation. Magnification of 1900x.

Cracks in the coats are visible in the micrographs. As a result of the coordinated action of the enzyme complex, the preparation alters the surface relief of the fruit coats of the grains and increases cracks size, facilitating the penetration of phytase into the aleurone layer to the site of phytin hydrolysis and leading to increased phytase activity.

After soaking wheat grain in water for 8 hours at a temperature of 50 °C, the cracks size in the fruit coats ranged from 321.3 to 555.5 nm. After treatment of wheat grain with a complex enzyme preparation containing phytase, the diameter of cracks in the fruit coat increased to 379.6 – 582.9 nm.

Figure 3 shows microphotographs of the surface of a cross-section of the aleurone layer of wheat grain.

The fruit and seed coats of wheat grains in two experimental samples swelled after soaking. The microphotographs show a swollen hyaline layer of the seed coat with elongated longitudinal cells and a clearly distinguishable aleurone layer. This is likely related to the increased permeability of cell membranes under the influence of water and enzyme solution. The results on changes in the structure of the fruit and seed coats of wheat grain during water soaking are consistent with previous findings **[42]**.

Microphotographs of the aleurone layer surface of native wheat grain and grain after enzymatic hydrolysis in a solution of the complex enzyme preparation, taken at 1900× magnification, revealed textural features of aleurone grains. The contents of aleurone cells in native wheat grain were represented by properly packed protein globules with a diameter of 1 – 2 μm. These results are consistent with the literature, which used high-resolution images. Globoids up to 2 mkm in size, varying in density, were found in the aleurone layer, filling aleurone cells **[43]**. During soaking in water, disruption of the proper packing of protein globules was observed, with empty spaces appearing and globules becoming indistinct. After enzymatic hydrolysis of the aleurone layer, it was clear that some grains were destroyed and had blurred boundaries.

The results obtained are consistent with the literature, indicating that during grain soaking under increased moisture and temperature, awakening processes begin, followed by swelling, the precursor of germination. These biochemical processes probably lead to the activation of endogenous enzyme systems, the breakdown of high-molecular-weight storage substances in the endosperm, and the synthesis of new compounds in the embryo, all of which contribute to changes in the kernel's chemical composition **[44]**.

On the surface of the aleurone layer of wheat grain, points were selected for microanalysis of chemical composition using energy-dispersive X-ray spectrometry. Data were obtained on the diversity of mineral composition of the aleurone layer and shells of native grain and grain after enzymatic treatment (Table 1).

As for cell walls, their physico-chemical properties affect the mineral nutrition of plants and the distribution of elements, since metal ions do not just pass through the apoplast to the plasmalemma, but can also be adsorbed or fixed on the components of the cell wall. The movement of ions in the cell walls also partially depends on electrostatic interactions, leading to the accumulation of cations in the apparent free space of the wall **[45]**. The shells accumulate mainly calcium, potassium, magnesium, and copper. After enzymatic hydrolysis, the amount of mineral elements decreases.

Analysis of our research results showed that the globoids of the wheat aleurone layer are particularly rich in phosphorus, calcium, potassium, magnesium, zinc, iron, and manganese, which are essential for enzymatic activity and human health. The presence of phosphorus in the globoids indicates the presence of phytic acid. The data obtained on the content of macro- and microelements in the aleurone layer of wheat grain are consistent with the results of other studies that demonstrated notable concentrations of phosphorus, magnesium, and manganese in the globoids of the aleurone layer of wheat grain and other plants **[46]**. Elemental microanalysis of aleurone cells showed that Zn, Fe, Na, Mg, Al, and P appear to be distributed in a similar way: their greatest amount is contained in the globoids of the aleurone cell symplastic region. It is especially clear that globoids are places of relatively high phosphorus concentration, and there is little phosphorus in the cell walls. The same can be observed for Mg. Thus, elementary cartography of P and Mg, due to their higher fluorescence signals, demonstrates a positive correlation in their distributions, especially within globoids **[47]**, and **[48]**.

After soaking the grain in water for 8 hours, the content of all chemical elements in the aleurone layer decreased, with particularly large changes observed during soaking in an enzyme preparation solution. So, after soaking in water, the magnesium content in the shell and globoids of the aleurone layer of grain was lower than that of the native grain by 40.00% ($p < 0.05$) and 5.5 times ($p < 0.001$). The globoids showed a 3.5–fold decrease in manganese ($p < 0.01$), a 2.0-fold decrease in phosphorus ($p < 0.01$), and a 37.93% decrease in calcium ($p < 0.01$). In addition, the iron content in the globoids decreased by 37.50%, but this difference was not statistically significant ($p > 0.05$). At the same time, no statistically significant differences were identified between water-soaked grain and native grain in the contents of sulfur, potassium, nickel, and copper in either the shell or globoids of the aleurone layer, nor in the levels of phosphorus and calcium in the shell, or cobalt in the globoids.

Soaking the grain in the enzyme preparation had a more pronounced effect on the mineral composition of the wheat aleurone layer than soaking in water. In particular, in the aleurone layer coat of grain soaked in the enzyme

preparation, the phosphorus content was significantly lower than that of native grain and water-soaked grain by 3.0-fold ($p < 0.01$) and 2.5-fold ($p < 0.05$), respectively; sulfur by 2.7-fold ($p < 0.05$) and 2.3-fold ($p < 0.05$); potassium by 10-fold ($p < 0.001$) and 8-fold ($p < 0.01$); calcium by 2.0-fold ($p < 0.05$) and 1.8-fold; copper by 1.7-fold ($p < 0.05$) and 1.4-fold; and zinc by 6.0-fold ($p < 0.05$) and 3.0-fold, respectively.

In the globoids of the aleurone layer of grain soaked in the enzyme preparation, a significant decrease was observed compared to the corresponding values in native and water-soaked grain: phosphorus decreased by 3.3-fold ($p < 0.01$) and 1.6-fold ($p < 0.05$); sulfur by 2.7-fold ($p < 0.01$) and 2.2-fold ($p < 0.01$); potassium by 35.42% ($p < 0.05$) and 26.19%; calcium by 3.1-fold ($p < 0.001$) and 1.9-fold ($p < 0.001$); manganese by 7.0-fold ($p < 0.01$) and 2.0-fold; and iron by 3.2-fold ($p < 0.01$) and 2.0-fold ($p < 0.05$), respectively. It should also be noted that magnesium were absent in both the globoids and the coat of the aleurone layer of grain soaked in the enzyme preparation.

Table 1 Distribution of chemical elements in the shell and aleurone layer of wheat grain, mass %.

The chemical element	Native grain		Grain soaked in water		Grain soaked in an enzyme preparation	
	Shell	Globoids	Shell	Globoids	Shell	Globoids
Magnesium	0.10 ±0.010	0.44 ±0.020	0.06 ± 0.006^{a}	0.08 ± 0.015^{c}	–	–
Phosphorus	0.06±0.006	4.11± 0.294	0.05± 0.006	2.04±0.187^{c}	0.02±0.006^{bA}	1.25±0.192^{bA}
Sulfur	0.08±0.010	0.16±0.015	0.07±0.012	0.13±0.015	0.03±0.006^{aA}	0.06±0.010^{bB}
Potassium	0.10±0.006	0.48±0.035	0.08±0.010	0.42±0.030	0.01±0.006^{cB}	0.31±0.025^{a}
Calcium	0.10±0.010	0.58±0.035	0.09±0.015	0.36±0.026^{b}	0.05±0.006^{a}	0.19±0.025^{cC}
Manganese	–	0.14±0.015	–	0.04±0.010^{b}	–	0.02±0.006^{b}
Iron	0.02±0.010	0.16±0.017	0.02±0.006	0.10±0.015	0.01±0.006	0.05±0.006^{bA}
Cobalt	–	0.02±0.010	–	0.01±0.006	–	0.01±0.006
Nickel	0.02±0.006	0.03±0.012	0.01±0.006	0.03±0.010	0.01±0.006	–
Copper	0.12±0.015	0.05±0.012	0.10±0.015	0.05±0,006	0.07±0.006^{a}	0.04±0.006
Zinc	0.06±0.010	0.24±0.025	0.03±0.017	0.18±0.025	0.01±0.006^{a}	0.14±0.021^{a}

Note: a ($p < 0.05$); b ($p < 0.01$); c ($p < 0.001$) – differences are statistically significant when compared with native grain; A ($p < 0.05$); B ($p < 0.01$); C ($p < 0.001$) – differences are statistically significant when compared with indicators of grain soaked in water.

The mass concentration of trace elements is a relative value that characterizes their presence in the grain. Table 2 presents the results of the analysis of mineral content by atomic absorption spectrophotometry.

Table 2 Distribution of chemical elements in the shell, aleurone layer, and endosperm with wheat germ, mg/kg abs. dry matter.

Elements	Native grain			Grain soaked in water			Grain soaked in an enzyme preparation		
	Shell	Globoids	Endosperm +germ	Shell	Globoids	Endosperm +germ	Shell	Globoids	Endosperm +germ
Mg	2.83	5.40	1.05	1.68	4.85	2.18	0.85	1.22	8.05
K	4.91	15.45	3.22	3.85	13.08	7.14	1.54	6.74	14.32
Ca	3.42	13.60	0.88	3.00	11.60	3.27	1.63	6.31	11.12
Mn	0.11	0.30	0.07	0.08	0.21	0.16	0.04	0.10	0.36
Fe	0.63	2.34	0.20	0.40	2.08	0.96	0.21	1.20	2.24
Co	-	0.36	0.12	0.02	0.28	0.24	0.01	0.18	0.30
Ni	0.10	0.10	0.06	0.06	0.08	0.14	0.04	0.05	0.19
Cu	0.22	0.16	0.05	0.17	0.14	0.15	0.06	0.10	0.25
Zn	1.75	2.56	0.50	1.28	2.05	1.10	0.60	0.95	3.24

These results confirm the patterns of migration of trace elements shown in Table 1. The action of a complex enzyme preparation during soaking of millet grain increases the amount of trace elements in the endosperm and embryo.

The observed changes in the content of macro- and microelements in the aleurone layer of wheat grain during soaking in the enzyme preparation solution are attributed to phytase hydrolysis of phytin. During the breakdown of the metal-phytin complex, the chemical elements migrate to other morphological parts of the grain, where enzymes and other biologically active compounds are synthesized during germination.

All the considered elements are transferred to the soaking medium to some degree. Potassium, nickel, and cobalt are the most widely removed from the grain during the soaking process. (removal averages 3.2%), cobalt and nickel (removal of these elements averages 1.6 and 1.8%, respectively). When considering the relative redistribution of these elements between the soaking medium and the washing waters, an increase in their concentration to 20–26.4 wt% was observed. However, the content of potassium, nickel, and cobalt in the washing waters, determined by the AAC method, showed that potassium was removed by 3.2%, nickel by 1.8%, and cobalt by 1.6%. The removal of the most important chemical elements – calcium, iron, copper, and zinc is insignificant. The content of these elements in the medium after soaking ranges from 0.02 to 0.09 mg/kg of absolutely dry matter. The content of the studied elements in the soaking medium increases or decreases slightly relative to the control (soaking in water without an enzyme preparation). It is known that some organic acids (citrate, malate, oxalate) are capable of forming strong bonds with heavy metal ions. All metals were arranged by Pfeiffer in a descending order of stability constants of the complex compounds formed **[49]**.

Weak chelators are strongly bonded to copper, cobalt, nickel, and zinc. It has been shown that citrate forms complexes with cobalt, nickel, and zinc; therefore, citrates are involved not only in the neutralization of heavy metals in the cytoplasm but also in the processes of their transport **[50]**. The soaking medium is a citrate buffer solution of an enzyme preparation. It can be assumed that citrate forms complexes with some metals, and this makes it difficult for them to transition from the grain to the aqueous phase.

Phytic acid is considered an anti-nutritional factor due to its ability to form insoluble and indigestible complexes with metal ions. Nevertheless, nutrition experts currently recognize the positive properties of phytic acid, as it exhibits antioxidant activity **[51]**.

Figure 4 shows the change in phytase activity in wheat grain during soaking in water and in a solution of a complex enzyme preparation.

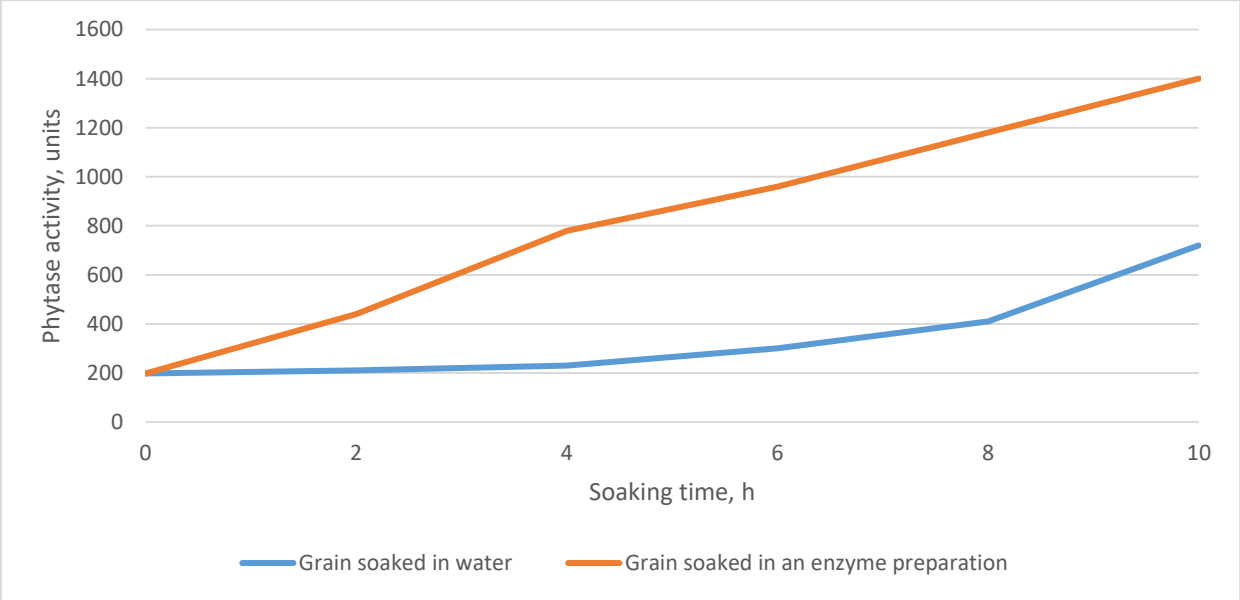

Figure 4 Change of phytase activity in wheat grain during soaking.

Studies have examined the rate of phosphoric acid release from substrates under the action of a complex enzyme preparation containing phytase. This indicator characterizes the activity of phytase. Phytase activity in native wheat grain is 198 units. It was found that when using a complex enzyme preparation, phytase activity was 2.9-fold higher than when soaking grain in water, where biochemical processes begin under high humidity and temperature, including the activation of endogenous phytase.

All the above results indicate that processes related to grain germination were initiated at 50 °C for 8 hours. The beginning of grain germination during soaking in water leads to a change in the microstructure of the surface and the redistribution of trace elements within the anatomical parts of the grain. The activation of enzyme systems is indicated by an increase in phytase activity from 200 to 410 units in 8 hours. However, when the grain was soaked in a buffer solution of a complex enzyme preparation, the processes associated with germination proceeded faster. Micrographs of the grain surface show that the drug's enzymes caused intensive hydrolysis of cell wall components, which contributed to the formation of microcracks in the shells and accelerated the penetration of the soaking solution into the grain. Our research has shown that wheat grain soaked in an enzyme preparation solution gains optimal moisture content of more than 41% for the production of grain bread in 8 hours, and when

soaked in water in 9 – 10 hours. When studying the glucose-amylase and protein-proteinase complexes of grain after soaking, it was found that when soaked at a temperature of 50 °C for 8 hours, "The number of falls" indicator, characterizing the amylase activity of grain, increased by 4.4%, proteinase activity – by 28% compared with native wheat grain, the presence of an enzyme preparation This led to an increase in this indicator by 6% and 34%, respectively. These results indicate an intensification of the grain germination process in the presence of a complex enzyme preparation **[52]**, and **[53]**.

Since the enzyme preparation contains carbohydrases that catalyze the hydrolysis of non-starch polysaccharides in the wheat grain coat, the carbohydrate composition of native grain and grain soaked in water or in the enzyme preparation solution was determined (Table 3).

Table 3 Carbohydrate composition of wheat grain, g/l.

The carbohydrate	Native grain	Grain soaked in water	Grain soaked in an enzyme preparation
Arabinose	0.00±0.006	0.01±0.006	0.04±0.006^{bA}
Galactose	0.00±0.006	0.00±0.006	0.01±0.010
Glucose	0.34±0.021	0.36±0.025	0.40±0.020
Sucrose	0.11±0.015	0.13±0.020	0.13±0.015
Xylose	0.00±0.006	0.00±0.006	0.04±0.010^{a}
Fructose	0.26±0.036	0.22±0.031	0.23±0.038
Raffinose	0.01±0.010	0.01±0.006	0.03±0.006
Cellobiosis	0.00±0.006	0.00±0.006	0.00±0.006
Maltose	1.74±0.079	1.97±0.091	2.18±0.085^{a}
The sum of sugars	2.50±0.142	2.70±0.161	3.04±0.115^{a}

Note: a ($p < 0.05$); b ($p < 0.01$) – the significance of differences when compared with the indicators of native grain; A ($p < 0.05$) – the significance of differences when compared with the indicators of grain soaked in water.

After soaking, the carbohydrate composition of the wheat grain changed. The most pronounced changes were observed when the grain was soaked in the enzyme preparation solution. The total sugar content increased significantly by 22.22% ($p < 0.05$) compared to the native grain. In grain soaked in water, the total sugar content was 12.82% higher than in native grain, but this difference was not statistically significant ($p > 0.05$). Partial hydrolysis of arabinoxylans in grain soaked in the enzyme preparation led to an increase in arabinose and xylose content. An increase in glucose and maltose content was also observed, likely due to the hydrolysis of both non-starch polysaccharides and starch during swelling. Glucose content in grain soaked in the enzyme preparation exceeded that of native grain by 17.64% and that of water-soaked grain by 11.11%. For maltose, the increases were 15.29% ($p < 0.05$) and 10.66%, respectively. Žilić et al. observed a similar composition of reducing sugars in the wheat grain **[54]**.

The fatty acid composition of wheat grain lipids was determined after soaking for 8 hours at 50 °C in water and in the enzyme preparation solution (Table 4). In native grains, polyunsaturated fatty acids predominated over saturated fatty acids, and their content increased after soaking. Among the unsaturated fatty acids in the studied grains, linoleic and oleic acids were present in the highest amounts.

The most important saturated fatty acid in wheat grain is palmitic acid, whose content increased by 3.91% after soaking in water and by 7.55% ($p < 0.05$) after soaking in the enzyme preparation solution. At the same time, the stearic acid content decreased by 2.59% and 11.02% ($p < 0.05$), respectively, compared to native grain.

After soaking, the content of unsaturated fatty acids in wheat grain increased, including linoleic, α-linolenic, γ-linolenic, and erucic acids. At the same time, oleic acid content decreased. These changes in the fatty acid composition of wheat grain lipids were most pronounced when the grain was soaked in the enzyme preparation solution. Specifically, the linoleic acid content in grain soaked in the enzyme preparation exceeded that of native grain and water-soaked grain by 3.59% ($p < 0.05$) and 2.83%, respectively; α-linolenic acid by 13.95% ($p < 0.05$) and 7.71%; γ-linolenic acid by 2.0-fold ($p < 0.05$) and 1.8-fold ($p < 0.05$); and erucic acid by 17.46% ($p < 0.05$) and 5.71%, respectively. Oleic acid content in grain soaked in the enzyme preparation was 3.78% lower than in native grain and 3.34% lower than in water-soaked grain, though these differences were not statistically significant ($p > 0.05$).

Table 4 Changes in the fatty acid composition of wheat grain lipids after soaking in water and an enzyme preparation, %.

Fatty acid	The fatty acid index	Native grain	Grain soaked in water	Grain soaked in an enzyme preparation
capric	10:0	0.01±0.006	0.01±0.006	0.01±0.006
lauric	12:0	0.02±0.006	0.02±0.006	0.03±0.010
myristic	14:0	0.24±0.020	0.15±0.015	0.14±0.015
pentadecanoic	15:0	0.09±0.015	0.10±0.017	0.09±0.010
pentadecenic	15:1	0.03±0.006	0.02±0.006	0.02±0.010
palmitic	16:0	15.62±0.201	16.23±0.444	16.80±0.324[a]
hexadecenic	16:1	0.08±0.012	0.09±0.010	0.10±0.015
palmioleic	16:1 9- cis	0.13±0.020	0.15±0.021	0.15±0.025
margarine	17:0	0.09±0.010	0.10±0.013	0.12±0.010
heptadecenic	17:1	0.03±0.006	0.03±0.012	0.03±0.010
stearic	18:0	1.27±0.031	1.16±0.046	1.13±0.025[a]
oleic	18:1 9-cis	15.06±0.565	14.99±0.542	14.49±0.522
vaccenic	18:1 11- trans	0.87±0.042	0.85±0.030	0.88±0.036
iso-octadecadienoic	18:2i	1.21±0.045	0.18±0.040	1.19±0.026
linoleic	18:2	59.02±0.582	59.46±0.597	61.14±0.393[a]
γ- linolenic	18:3 ω-6	0.08±0.015	0.09±0.012	0.16±0.020[aA]
ά- linolenic	18:3 ω-3	3.80±0.120	4.02±0.231	4.33±0.107[a]
arachidonic	20:0	0.19±0.025	0.18±0.020	0.18±0.021
gontoic	20:1	0.77±0.046	0.70±0.041	0.70±0.056
begenic	22:0	0.10±0.015	0.10±0.017	0.10±0.012
erucic	22:1	0.63±0.030	0.70±0.042	0.74±0.020[a]

Note: a ($p < 0.05$) – the significance of differences when compared with the indicators of native grain; A ($p < 0.05$) – the significance of differences when compared with the indicators of grain soaked in water.

The action of lipase and lipoxygenase enzymes at 50 °C, with a 12-hour soaking time for wheat grain, can initiate hydrolysis and oxidation. However, it is known that the activity of grain lipases depends on the substrate's moisture content. The optimal moisture content for grain lipase is 13 – 15%. An increase in grain moisture to 41% reduces lipase activity. The optimal pH value of the activity of grain lipases (wheat, rye, barley, and oats) is 7.0 – 8.2 **[55]**. The optimum pH for lipase from wheat seed germs is 7.9–8.1, and the temperature optimum is 37 ± 2 °C **[56]**. The optimal temperature of action of grain lipoxygenase is in the range of 20-40 ° C, and the optimal pH value is in the range of 6.2 – 7.5 **[55]**. The optimum pH for lipoxygenase from wheat seed germs is 6.9–7.1, and the temperature optimum is 30±2 °C. A temperature rise to 40°C or higher results in a significant increase in the inactivation rate constant, especially at high H+ ion concentrations **[56]**.

There are conflicting results and opinions regarding changes in the content of saturated and unsaturated fatty acids in grains of various crops after germination and various technological treatments. It is known that during wheat germination, the content of linoleic and palmitic acids increases, while oleic acid decreases **[57]**. Similarly, in **[58]**, after three days of wheat germination at 20 °C, the content of palmitic and linoleic acids in the sprouted wheat increased, whereas oleic acid decreased. However, **[59]** reported no changes in fatty acid content in wheat after 48 hours of germination. Kang et al. noted that the content of oleic acid decreased by 50%, while the content of linoleic and linolenic acids increased by 1.3 and 5.4 times, respectively, after germination of buckwheat seeds **[60]**. Hahm et al. reported that sprouted sesame seeds without peel are rich in linolenic acid **[61]**. Hassan et al. reported that pretreatment methods (ultrasound and microwave radiation) do not affect the oil's quality, including its essential fatty acid content **[62]**.

It is likely that the increase in the amount of unsaturated fatty acids may be due to the enzymatic hydrolysis of polysaccharides of grain cell walls and the redistribution of lipids in the anatomical parts of the grain. A decrease in the level of oleic acid may be associated with its decomposition by lipolytic enzymes **[62]**. A slight increase in

unsaturated fatty acids may result from free fatty acids failing to be converted into carbohydrates, leading to an increase in fat content during germination **[63]**.

Differences in fatty acid composition in wheat grains after soaking and swelling may have varying effects on human health upon consumption. Future studies should focus on determining dynamic changes in fatty acid content during grain swelling and germination.

The observed changes in the fatty acid composition of the aleurone layer of grains soaked in an enzyme preparation solution are probably due to the action of xylanase, beta-glucanase and cellulase, which modify cellulose and sequentially cleave pentosans such as arabinoxylan into low molecular weight oligosaccharides. In **[64]**, it was shown that enzymatic biotreatment increased free fatty acid content by 6 times compared with treatments without biotreatment. The increase in free fatty acid levels differed depending on the enzyme composition used.

When using a mixture of cellulase, xylanase, and α-amylase, the content of free fatty acids increased to a greater extent than when using xylanase alone **[65]**.

Changes in the fatty acid and lipid class composition of the grain may be related to the production of lipids required for seed growth and to structural changes that occur after the breakdown of other chemical components.

A key objective of our study was to investigate the antioxidant content and antioxidant activity of wheat grain after soaking in water and in the enzyme preparation solution. The results are presented in Table 5.

Table 5 Changes in the content of antioxidants and the level of antioxidant activity of wheat grain after soaking in water and an enzyme preparation.

Indicator	Native grain	Grain soaked in water	Grain soaked in an enzyme preparation
The content of glutathione, mg/100g	0.0155±0.0008	0.0306±0.0011^{c}	0.0614±0.0016^{cC}
Flavonoid content,%	0.030±0.0042	0.048±0.0035^{a}	0.059±0.0031^{b}
Antioxidant activity, % inhibition of the DPH radical	8.21±0.316	15.50±0.490^{c}	24.82±0.748^{cC}

Note: a ($p < 0.05$); b ($p < 0.01$); c ($p < 0.001$) – the significance of differences when compared with the indicators of native grain; C ($p < 0.001$) – the significance of differences when compared with the indicators of grain soaked in water.

It was found that soaking the grain increased its antioxidant content, including glutathione and flavonoids, and enhanced antioxidant activity compared to the corresponding values in native grain. These changes were more pronounced when the grain was soaked in the enzyme preparation solution. Specifically, glutathione content in the grain soaked in the enzyme preparation exceeded that in native grain and water-soaked grain by 4.0-fold ($p < 0.001$) and 2.0-fold ($p < 0.001$), respectively, while flavonoid content increased by 96.67% ($p < 0.001$) and 22.92%, respectively. Wheat is known to contribute significantly to the antioxidant status, having a beneficial effect on human health **[66]**. The antioxidant activity of grain soaked in the enzyme preparation was significantly higher ($p < 0.001$) than that of native grain and water-soaked grain, by 3.0-fold and 1.6-fold, respectively.

Limitations

The main limitation of this study is the small number of wheat samples (six from a single variety, Moskovskaya 39), which may limit the generalizability of the findings to other wheat genotypes or growing conditions. Additionally, the experiment was conducted under controlled laboratory soaking conditions (50 °C, pH 4.5), which may not fully reflect industrial-scale processing or real germination environments.

Another limitation is that mineral redistribution was assessed primarily through microanalysis and atomic absorption spectrophotometry without direct bioavailability testing in vivo or in vitro digestion models. Furthermore, no enzyme-inactivation step was performed after incubation, which may affect the interpretation of biochemical changes during subsequent processing.

Future studies involving multiple wheat varieties, larger sample sizes, and bioaccessibility assessments under simulated gastrointestinal conditions would provide a more comprehensive evaluation of the nutritional impact of enzymatic treatment.

CONCLUSION

The conducted studies demonstrated that the coordinated action of the enzyme complex containing cellulase, xylanase, and β-glucanase modifies the surface relief of wheat grains and increases the size of cracks in the fruit coat, facilitating the penetration of the enzyme phytase into the aleurone layer to the site of phytic acid hydrolysis. The microstructure of the aleurone layer undergoes significant changes under the influence of the enzyme preparation, with partial destruction of aleurone grains. Energy-dispersive X-ray spectrometry revealed a decrease in the content of all studied chemical elements in the aleurone layer. In the aleurone layer globoids of grains soaked in the enzyme preparation, a significant reduction was observed compared to native grain and water-soaked grain in the levels of phosphorus by 3.3-fold ($p < 0.01$) and 1.6-fold ($p < 0.05$), sulfur by 2.7-fold ($p < 0.01$) and 2.2-fold, potassium by 35.42% ($p < 0.05$) and 26.19%, calcium by 3.1-fold ($p < 0.001$) and 1.9-fold ($p < 0.001$), manganese by 7.0-fold ($p < 0.01$) and 2.0-fold, and iron by 3.2-fold ($p < 0.01$) and 2.0-fold ($p < 0.05$), respectively. These results indicate phytic acid hydrolysis, migration of mineral elements within the grain, and increased availability for biochemical processes. The distribution of trace elements in the grain obtained by the AAC method confirms that when the grain was soaked in a solution of a complex enzyme preparation, the studied chemical elements migrated to the endosperm and the embryo. These results indicate the hydrolysis of phytic acid and the migration of mineral elements inside the grain. The study of the rate of phosphoric acid release from the substrate under the action of a complex enzyme preparation based on phytase showed that the phytase activity of wheat grain treated with the preparation was 2.9-fold higher than that of endogenous phytase in grain soaked in water. Soaking wheat grains in the enzyme preparation also altered their carbohydrate composition. The total sugar content in grains soaked in the enzyme solution increased significantly by 22.22% ($p < 0.05$) compared to native grains. Partial hydrolysis of arabinoxylans in enzyme-treated grains led to an increase in arabinose and xylose content. After soaking, the levels of unsaturated fatty acids in wheat increased, including linoleic acid, α-linolenic acid, γ-linolenic acid, and erucic acid. At the same time, the content of oleic acid decreased. The increase in unsaturated fatty acid content is probably due to their synthesis during the grain swelling period. Furthermore, soaking the grains increased antioxidant content, including glutathione and flavonoids, and antioxidant activity compared to native grains. The antioxidant activity of grains soaked in the enzyme preparation was significantly higher ($p < 0.001$) than that of native grains and water-soaked grains, by 3.0-fold and 1.6-fold, respectively. Thus, the experimental data indicate that treating wheat grains with a complex enzyme preparation containing carbohydrases and phytase is an effective means of enhancing the grain's nutritional value.

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