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INFLUENCE OF PHYTOLYTIC AND PROTEOLYTIC ENZYMES ON CONVERSION OF WHEAT AND CORN GRAIN POLYMERS

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Abstract

Grain, in addition to starch, hemicelluloses and protein, is known to be phytic acid and its salts. It has been shown that the use of phytolytic enzymes promotes the release of phosphorus, improves the digestibility of feed nutrients and increases the meat productivity of livestock and poultry. However, the catalytic effect of phytases and their combinations with proteases on degradation of polysaccharides and protein polymers in grain raw materials, on the release of cations and anions, especially in grain wort for the production of alcohol, has been little studied. This work shows for the first time that phytolytic enzymes improve qualitative estimates, rheological properties and change the ionic composition of grain wort by not only releasing phosphates and metal cations, but also by increasing the concentration of organic salt anions. The combined action of phytolytic and proteolytic enzymes on polymers of grain raw materials provides their catalytic degradation to soluble carbohydrates, nitrogenous substances, cations and anions to produce the enriched grain wort. This work aimed to assess the degree of phytolytic and proteolytic conversion of high-molecular-weight polymers in grain wort. For grain wort, 50 g portions of wheat (*Triticum* sp.) or corn (*Zea mays*) grain flour put into Erlenmeyer flasks were added with 150 cm^3 of water and incubated in a water bath. At grain batching for starch dextrinization, a thermo-stable α-amylase was added (0.6 units/g starch). For starch conversion to sugars and hydrolysis of non-starch polysaccharides in the control, we used glucoamylase (9.0 units/g starch) and xylanase (0.15 units/g raw material). Phytase (from 1.0 to 2.5 units/g raw material) and a proteolytic enzyme preparation (0.1 units/g raw material) were also added. The control was added neither with phytase, nor the proteolytic enzyme. The profiles of the main polymers of grain raw materials, the grain wort concentration, the content of reducing carbohydrates (RC) were determined according to the techno-chemical instructions for the control of alcohol production, the amine nitrogen (NH_2^+) concentration was measured by a method based on the ability of amino acids to form soluble copper compounds with a suspension of copper phosphate. The dynamic viscosity of the grain wort was evaluated by vibrational viscometry. The study of the ionic composition of the grain mix and wort was carried out using a PrinCE-560 series capillary electrophoresis system (PrinCE Technologies B.V., Netherlands) equipped with a conductometric detector. The optimal dosage of phytase was 1.5 units/g raw material which ensures the maximum release of ions identified by capillary electrophoresis and the effective conversion of polysaccharides, protein and phytin substances of the grain. It was found that the phytolytic enzymes contributed to a decrease in the viscosity of wheat and corn wort of more than 20 %, a 9.5-11.3 % increase in the concentration of reducing carbohydrates, and a 2.1-2.4-fold increase in the concentrations of released ions. The concentration of amino nitrogen in the wort did not change significantly. It was shown that as a result of the phytolytic action, the concentration of not only metal phosphates and cations, but also anions of organic salts, such as oxalates, malates, citrates, and succinates (in wheat wort) and oxalates, malates, citrates, and lactates (in corn wort) increased. A more significant effect of phytase for corn wort was revealed: the concentration of phosphates in the nutrient medium increased 3.9 times vs. 1.6 times for wheat wort, the levels of

potassium and magnesium ions were 12 % and 22 % higher, respectively, as compared to the control in which the phytase was not used. The optimal composition of the enzyme complex is proposed which ensures the effective hydrolysis of polysaccharides, phytin and protein substances of processed raw materials. The synergistic effect of phytolytic and proteolytic enzymes enhances the catalytic hydrolysis of high molecular weight polymers of plant origin and enriches the grain wort with carbohydrates, nitrogenous substances, phosphates, and minerals in bioavailable form. Biocatalytic treatment of grain with the developed enzyme complex which, along with amylases and xylanase, contains phytase and proteases, provides a 16.8 % and 18.8 % increase in the concentration of reducing carbohydrates in wheat and corn wort, a 1.7-fold and 1.9-fold increase in amine nitrogen and a decrease in wort viscosity by 41.7 % and 44.7 %, respectively.

Keywords: phytase, protease, wheat raw material, corn raw material, biocatalysis, ions, cations, phosphorus, grain wort

The agro-industrial complex of the Russian Federation annually processes more than 100 million tons of agricultural raw materials. Alcohol, starch, brewing and feed manufacturers are large consumers of grain. Innovative technologies assist in increasing commercial profitability of grain processing and are also targeted to solve environmental problems and to produce competitive food and feed products [1-3]. These technologies are effective due to broad substrate specificity of biocatalysts, providing deep hydrolysis of high molecular weight polymers of grain raw materials [4-6]. It was previously shown that the synergism of amylolytic, proteolytic and hemicellulolitic enzymes improves grain quality, enriches the wort with carbohydrates, nitrogenous substances, and increases the yield of the target product [4].

Grain contains, in addition to starch, hemicelluloses, cellulose, and proteins, phytic acid and its salts — phytates which are the principal storage form of mineral phosphorus in plants [7-9]. The amount of phytic compounds in grain differs between crops and also depends on seed quality and growing conditions. For example, the amount varies from 0.4 to 3.9 % for wheat and from 0.7 to 2.8 % for corn [10, 11]. Phosphorus in plants is approximately 80 % phytic acid, or *myo*-inositol-1,2,3,4,5,6-hexakisphosphate ($InsP_6$) in which inositol-bound phosphorus is not bioavailable [12, 13]. In addition, phytates, exhibiting a high negative charge, bind metal cations [9, 14, 15] and also form rather strong complexes with proteins and carbohydrates [16, 17]. Phytases allows the release of valuable components of raw materials and increases its bioavailability [13].

Phytase which is present in plants during their growth promotes the catalytic degradation of phytic acid [18], but its amount is insufficient for the complete release of phosphorus. Phytases synthesized by microorganisms are attracting more and more attention. The most promising producers are fungi of the genus *Aspergillus* (6) and recombinant strains of yeast and bacteria [19-21].

In recent investigations which focus on raw grain processing for various purpose, the catalytic efficiency of phytases is attracting increasing interest. Considerable attention is paid to phytases as tools to improve the digestibility of feed nutrients. It is shown that phytases promote the breakdown of phytic compounds, the release of phosphorus and other trace elements, which leads to an increase in the growth and meat productivity of animals and poultry [22-24]. The interaction of phytic acid and its salts with substances that make up food raw materials and food products has been under consideration [16, 17, 24-26].

Investigations of sorghum and maize lager beer brewing using phytases have shown the potential for improving the nutritional value of yeast during grain wort fermentation [8, 10, 27, 28]. It has been confirmed that, under conditions of anaerobic fermentation, phosphorus is assimilated by yeast, mainly in the initial phase. Young actively multiplying yeast cells contained 2 times more phosphorus compared to the end of fermentation. However, the catalytic efficiency of phytases in raw grain processing for alcohol, especially the composition of a complex of

hydrolytic enzymes with different substrate specificity have practically not been studied [4]. There are only preliminary data on the positive effect of the *Aspergillus awamori* phytase on yeast growth and alcoholic fermentation of grain wort [28].

In the presented work, we showed for the first time that in grain wort, due to phytase activity, the concentration of anions of organic salts increases in addition to a release of phosphates and metal cations. The combined action of phytases and proteolytic enzymes increases the degree of catalytic destruction of grain biopolymers resulting in soluble forms of carbohydrates, nitrogenous substances, cations and anions. This contributes to the production of enriched grain wort with good rheological properties. Hereof, the optimal enzymatic complex is proposed to ensure effective hydrolysis of polysaccharides, phytic and protein substances in grain and to maximum accumulate reducing carbohydrates, amine nitrogen, phosphates and minerals in a bioavailable form.

This work aimed to assess effects of phytase and proteolytic enzyme preparations on the conversion of wheat and corn high molecular weight polymers in preparing grain wort.

Material and methods. Alcoholic fermentation was performed in 2019-2020 using traditional enzyme preparations (EP) of different substrate specificity. These were Amylex® 5T (Genencor, USA; heat stable α-amylase, 2000 AAU/cm^3), Diazyme® X5 (Genencor, USA; glucoamylase, 8000 GlAU/cm^3, 140 AAU/cm^3), Tegazyme RT 75L (Lyven SA, France; xylanase, 3600 XAU/cm^3), Protoorizin (VNIIPBT, Russia; proteases, 620 PAU/cm^3), Phytaflow (Novozymes, Denmark; phytase, 30,000 PhAU/cm^3).

For wheat (*Triticum* sp.) or corn (*Zea mays* L.) grain wort, 750 cm^3 Erlenmeyer flasks, each with 50 g of grain flour and 150 cm^3 of water (1:3) were mixed and incubated in a PE-4300 water bath (Ekros, Russia) according to the "soft" enzymatic-hydrolytic processing, i.e., the batches were allowed for 30 min at 40-50 °C, then kept for 120 min at 85 °C, stirring occasionally. After cooling to 60 °C, the batches were treated with EP for 60 min. At mixing flour and water, the thermostable α-amylase was used (0.6 AAU/g starch) for starch dextrinization. In the control batches, glucoamylase (9.0 GlAU/g starch) and xylanase (0.15 XAU/g raw material) were added for saccharification of starch and hydrolysis of non-starch polysaccharides. In the experimental batches, along with amylase, glucoamylase and xylanase, phytase (1.0, 1.5, and 2.5 PhAU/g raw material) and proteases (0.1 PAU/g raw material) were added. The concentration of soluble solids in wheat wort was 21.1 %, in corn 21.8 %.

The major grain polymers, wort gravity, and reducing carbohydrates (RC) were measured as described [29], the amine nitrogen (NH_2^+) quantifying was based on the ability of amino acids to form soluble copper complexes with copper phosphate suspension [30]. Dynamic viscosity of wort was assessed by vibrational viscometry (an SV-10 sinusoidal vibration viscometer, A&D Co., Ltd., Japan) with Win-CT Viscosity software. The ionic composition of batches mix and wort was studied using a PrinCE-560 series capillary electrophoresis system (PrinCE Technologies B.V., Netherlands) equipped with a conductometric detector [31].

Statistical processing of the data obtained in at least three replicates was carried out using the Student's *t*-test at $p < 0.05$ (Statistica 6.0, StatSoft, Inc., USA). The mean values (*M*) and standard errors of the means (±SEM) were calculated.

Results. To study the effect of phytase on wheat and corn wort quality (Fig. 1-3), the phytase was used at a dosage of 1.0 PhAU/g raw material, previously established for rye wort [28]. The phytase contributed to a significant ($p < 0.05$)

increase in the concentration of reducing carbohydrates in grain wort, by 9.5-11.3 % compared to the control, but practically did not affect the protein hydrolysis (see Fig. 1, A, B). The concentration of amine nitrogen in the wort significantly increased ($p < 0.05$) only when proteases were part of the enzymatic complex, i.e., 1.7-fold for wheat wort and 1.9-fold for corn wort (see Fig. 1, B).

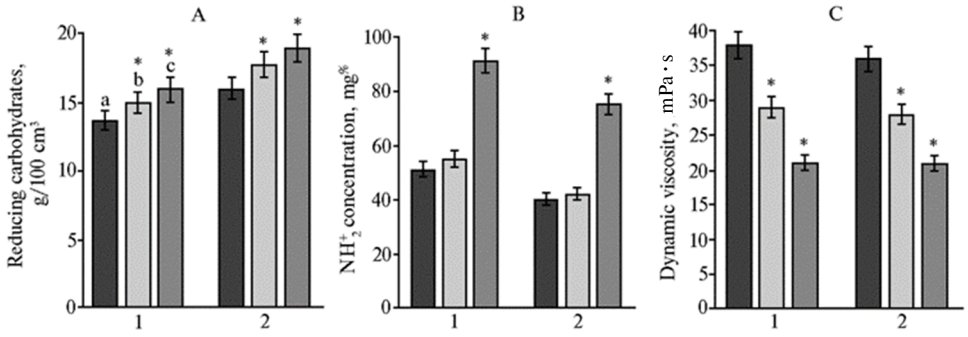

Fig. 1. Reducing carbohydrates (A), NH_2^+ **concertation** (B) **and dynamic viscosity** (C) **of wheat** (1) **and corn** (2) **wort as influenced by different enzyme preparations:** a — control, b — control + phytase (1.0 PhAU/g raw material), c — control + phytase (1.0 PhAU/g raw material) + protease (0.1 PAU/g raw material). See *Materials and methods* section for details.
* Differences form the control (a) are statistically significant at $p < 0.05$.

The rheological properties of the grain wort revealed in this study confirmed our earlier data for rye wort [28]. Phytase caused a 23.7 % decrease in viscosity for wheat wort and a 22.2 % decrease for corn wort ($p < 0.05$) (see Fig. 1, B). A complex of enzymes, including amylase, glucoamylase, xylanase, phytase, and protease, provided a deeper hydrolysis of polysaccharides. The RC level was 16.8 % higher in wheat wort and 18.8 % higher in corn wort as compared to the control ($p < 0.05$) (see Fig. 1, A). The wort viscosity decreased more significantly, by 41.7-44.7 % ($p < 0.05$) (see Fig. 1, C).

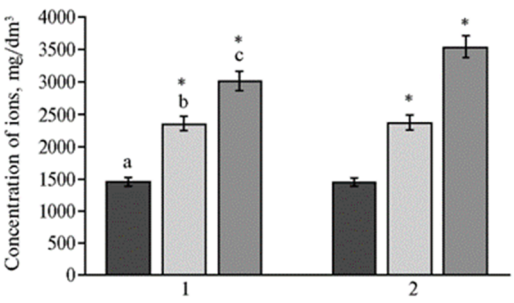

Fig. 2. Total ions in wheat (1) **and corn** (2) **wort as influenced by different enzyme preparations:** a — grain batch, b — control, c — control + phytase (1.0 PhAU/g raw material). See *Materials and methods* section for details.
* Differences b—a, c—a, and c—b are statistically significant at $p < 0.05$.

It is known that salts of phytic acid, being strong chelating agents, form stable protein-phytate complexes with proteins and also bind metal cations, which can negatively affect the functions of hydrolytic metal-dependent enzymes [32]. In our experiment, phytase had a significant effect on the increase in the total concentration of ions in the wheat and corn wort (Fig. 2). Ion concentrations increased 2.1-2.4-fold ($p < 0.05$) as compared to flour-water mix and 1.3-1.5-fold as compared to the control ($p < 0.05$).

Enzyme complexes containing phytase significantly ($p < 0.05$) increased the proportion of released phosphates in the pool of identified ions as compared to the control, 40.8 % vs. 31.2% for wheat and 30.8 % vs. 11.3 % for corn (Fig. 3). The proportion of potassium and magnesium ions remained practically unchanged.

We also investigated the effect of the dosage of phytase in the enzyme complex (1.0; 1.5; 2.5 PhAU/g raw material) on the quality parameters of the wheat and corn wort (Fig. 4).

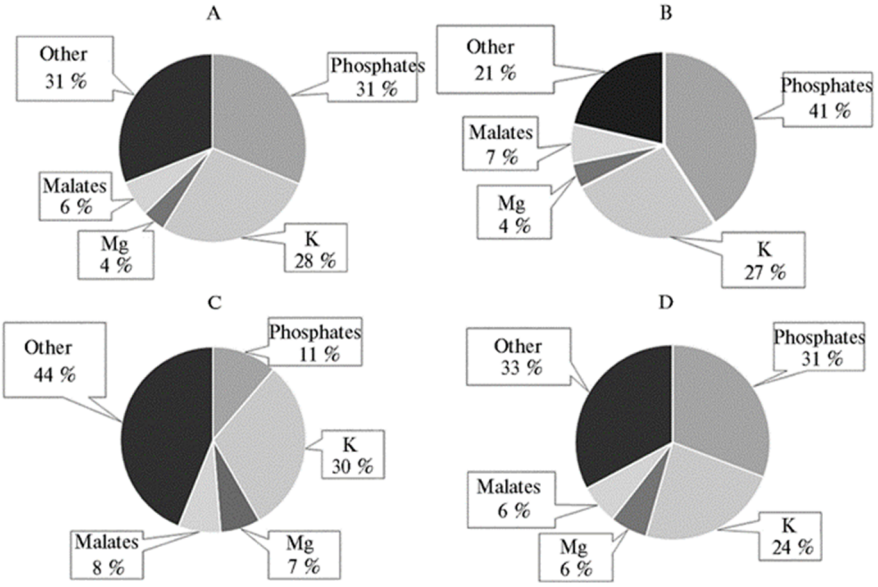

Fig. 3. Content of principal identified ions (% of total pool) **in wheat** (A, B) **and corn** (C, D) **wort as influenced by different enzyme preparations:** A, C — enzyme complex without phytase (control), B, D — enzyme complex with phytase (control + phytase 1.0 PhAU/g raw material). See *Materials and methods* section for details.

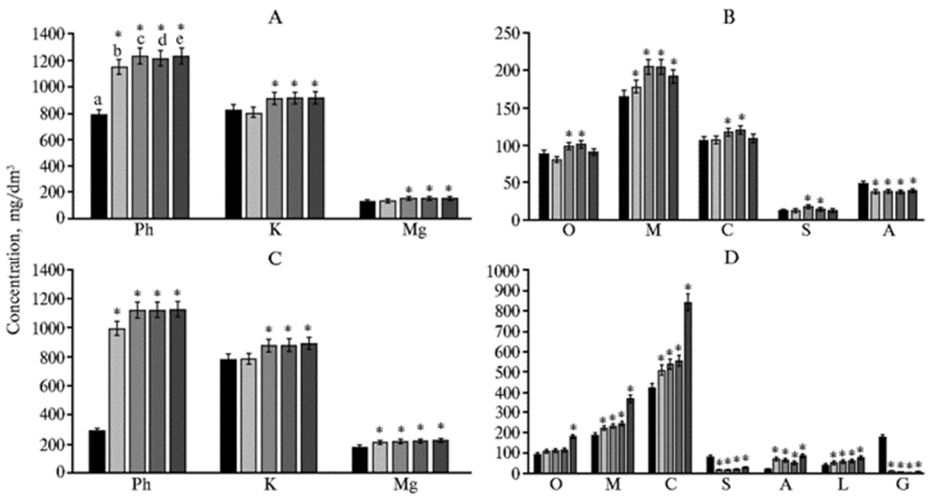

Fig. 4. Profiles of inorganic and organic ions in wheat (A, B) **and corn** (C, D) **wort as influenced by different enzyme preparations:** a — control, b — control + phytase (1.0 PhAU/g raw material), c — control + phytase (1.5 PhAU/g raw material), d — control + phytase (2.5 U PhA/g raw material), e — control + phytase (1.0 PhAU/g raw material) + protease (1.0 PAU/g raw material); Ph — phosphates, K — potassium, Mg — magnesium, O — oxalates, M — malates, C — citrates, S — succinates, A — acetates, L — lactates, G — glycolates. See *Materials and methods* section for details.
* Differences form the control (a) are statistically significant at $p < 0.05$.

Phytase promoted the release of phosphorus (see Fig. 4). Thus, in wheat wort upon treatment with phytase-containing enzyme complex, the phosphate concentration varied from 1145 to 1232 mg/dm^3 depending on the phytase dosage, which exceeded 1.5-1.6-fold ($p < 0.05$) the control (see Fig. 4, A). In addition, there was a slight increase in the concentration of potassium and magnesium ions, by 11 % and 17 % ($p < 0.05$) for a dosage of 1.5 1.5 PhAU/g raw material. Phytase had a more pronounced effect on the corn wort: the concentration of phosphates

in the nutrient medium increased ($p < 0.05$) 3.4-3.9-fold (991-1124 mg/dm^3 vs. 290 mg/dm^3) (see Fig. 4, C). The concentration of potassium and magnesium ions increased by 12 and 22 %, respectively ($p < 0.05$). The data obtained showed that the optimal dosage of phytase was 1.5 PhAU/g raw material. Further increase in its concentration to 2.5 PhAU/g practically had no effect on the grain wort quality parameters.

Phytase and an increase in its dosage influenced not only the concentration of phosphorus, potassium and magnesium ions in the wort, but also other identified ions. As compared to the control, the content of anions of organic salts (see Fig. 4, B, D) increased. Particularly, malates (succinic acid salts) amounted to 178.2-205.1 vs. 165.4 mg/dm^3 for wheat and 224.1-367.8 vs. 189.66 mg/dm^3 for maize. Citrates amounted to 510.1-844.4 vs. 422.8 mg/dm^3 in the corn wort whilst practically did not change in the wheat wort. The concentration of lactic acid salts (lactates) in the corn wort increased 1.4-2.0 times ($p < 0.05$) depending on the dosage of phytase. However, phytase led to a decrease ($p < 0.05$) in the glycolic acid salts in corn wort (6.3-10.9 mg/dm^3 vs. 178.4 mg/dm^3 in the control). When processing wheat, lactates and glycolate in the wort were practically absent.

With addition of proteases, we observed a definite tendency to an increase in the concentration of phosphorus, potassium and magnesium ions in the grain wort (see Fig. 4, A, C).

The potential of phytases to increase the level of bioavailable phosphorus and feed and food digestibility has been actively investigated [13, 16, 22, 23]. Nevertheless, the catalytic action of phytases has not been sufficiently studied, as well as their synergism in terms of conversion of polysaccharides and protein polymers of grain and the release of cations and anions, especially in fermented grain wort.

Here we have shown the positive effect of phytase and proteolytic enzymes on wheat and corn wort composition and ion profiles. Apparently, not only phosphorus but also carbohydrate and protein polymers were released due to biocatalytic hydrolysis of phytates which are strong chelating agents capable of forming stable protein-phytate complexes with proteins [33]. This contributes to a better access of hydrolytic enzymes to substrates. Also, the release of metal cations from the active centers of these enzymes may increase their catalytic activity and, therefore, destruction of grain wort polymers.

Our findings indirectly confirm reports which proved the role of phytic acid in the inhibition and regulation of the catalytic ability of metalloprotein enzymes. Of these, xanthine oxidase has been more studied [33, 34]. We have shown that the complex of enzymes which, along with carbohydrases, contains phytase and proteases promotes a deeper hydrolysis of polysaccharides and protein substances. In wheat and corn wort, there was a significant ($p < 0.05$) increase in RC concentration (by 16.8 %and 18.8 %), amine nitrogen (1.7-fold and 1.9-fold) and a decrease in viscosity (by 41.7 % and 44.7 %).

Thus, a positive effect of phytases on rheological properties, ion profiles and bioavailable phosphorus in wheat and corn wort has been established. The use of phytase contributes to a significant release of ions, up to 2 times as much as in grain mix. Due to phytase, there was an increase in organic salts, the oxalates, malates, citrates, succinates for wheat wort and oxalates, malates, citrates, lactates for corn wort. In wheat and corn wort, phosphates increased 1.6 times and 3.9 times, potassium by 11 % and 12 %, magnesium by 17 % and 22 % as compared to the control. Phytase decreased the viscosity of grain wort by more than 20 %. For catalytic conversion of wheat and corn biopolymers, the optimal dosage of phytase, ensuring maximum accumulation of ions, is 1.5 PhAU/g raw material.

The synergistic action of phytases and proteases enhances catalytic hydrolysis of high-molecular-weight polymers and enriches grain wort with carbohydrates, nitrogenous substances, bioavailable phosphates and minerals. Our findings indicate that further experiments are necessary to assess effects of phytase complex on amylolytic and proteolytic processing of grain raw materials, on yeast metabolism, growth and reproduction during ethanol fermentation, and on digestibility of grain feeds fed to farm animals.

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