Effect of Phytase Addition to Buckwheat Wort on the Selected Fermentable Sugars, Polypeptide Profile and Nitrogen Content from Free Aminoacids

Robert DULIŃSKI*, Marek ZDANIEWICZ2, Aneta PATER2

1 Department of Biotechnology and General Food Technology, Faculty of Food Technology, University of Agriculture in Krakow, Balicka Street 122, 30-149 Krakow, Poland
2 Department of Fermentation Technology and Microbiology, Faculty of Food Technology, University of Agriculture in Krakow, Balicka Street 122, 30-149 Krakow, Poland
* Corresponding author: Robert DULIŃSKI e-mail: r.dulinski@ur.krakow.pl

Abstract
Relatively high levels of phytates in buckwheat malt and the low activity of endogenous phytases that limit the effective use of substrates for fermentation and yeast metabolism (starch, proteins, minerals) are an argument for using phytases in beer production technology. Two mash-in programs were applied: (1) the Congress program, typical for basic raw materials, (2) a program with temperature optimized for phytase activity. Commercial preparations of 3-phytase (Finase P) and 6-phytase (Ronozyme) were used in the study. Monitored levels of selected fermentable sugars indicates a statistically significant effect of phytase addition on the glucose content in both mash-in programs used. The SEC-HPLC chromatography allowed to select a key polypeptide with an estimated molecular weight of 40 kDa, whose relative peak area decreases as a result of the applied mash-increase treatment with phosphorolytic enzymes, although this relation was not statistically confirmed in the analysis of free amino acids content. The analyses carried out also indicate that apart from the target molecules, namely phytate and inositol, the use of phytases in the process of buckwheat wort preparation slightly changes the profile of fermentable sugars and causes significant changes in the polypeptide profile of the final mash.

Keywords: phytases; buckwheat beer; fermentable sugars; polypeptide profile.

INTRODUCTION
Buckwheat (Fagopyrum esculentum), a pseudo-cereal, is an important component of functional food, which due to its favourable amino acid profile can be used in a gluten-free diet, as a starting material for bread production or as a raw material in brewing (Pragya, Singh; Rita, 2016, Kumari and Chaudhary, 2020, Świeca et al., 2020). Bioactive and functional properties of beer intended for a special group of consumers (gluten-free beer) can be improved by using such unconventional ingredients in the malting process. An additional aspect of using buckwheat is the high content of bioactive inositols, namely D-chiro-inositol (DCI) and myo-inositol (MI), which have potential positive effects on the glycaemic index, as well as immunomodulatory, signalling, and cell-growth stimulating properties (Gillaspy 2011, Yao et al., 2011, Rendle et al., 2016, Owczarczyk-Saczonek et al., 2018). Phosphoglycans P-type that contain D-chiro-inositol and galactosamine may act as secondary relay mimicking insulin or amplifying the signal generated by this blood sugar regulator (Larner et al., 2010).
Enzymes and buckwheat beer

Beer produced from components other than barley malt will have a higher price level due to the supply and cost of the initial raw material, as well as the activity of internal enzymes that hydrolyse starch, which in the case of buckwheat means that they must be added during mash-in. It is possible to reduce the cost of buckwheat beer production by optimizing the composition and dose of enzymes (Duliński et al., 2019), adding phosphorolytic enzymes other than amylases that allow to loosen the structure of the so-called super-glucans present in buckwheat seeds and to eliminate the influence of unfavourable proportion of amylose to amylopectin in buckwheat starch (Ola et al., 2012).

Phytate-enzymatic hydrolysis

Phytic acid (myo-inositol 1,2,3,4,5,6-hexaphosphate) is the main phosphorus reservoir in plant tissues, which due to its ability to form complexes with proteins and minerals is considered as an antinutrient (Bohn et al., 2008). In food products of plant origin, such as bread or pseudocereal seeds, the relatively high level of this substance may be reduced by way of enzymatic hydrolysis. Due to their specificity, the following enzymes that hydrolyse myo-inositol phosphate esters should be distinguished: 3-phytase A (EC.3.1.3.8), 6-phytase A (EC.3.1.3.26), and phytase B (EC.3.1.3.2) (Konietzny and Greiner 2002). The catalytic action of 3-phytase A starts from the phosphate group at the C3 carbon of the myo-inositol ring, while 6-phytase A starts from the C6 carbon. Phytases A are not able to hydrolyse the bond at the C2 carbon due to its axial conformation. This bond, however, can be hydrolysed by phytase B, i.e., non-specific acid phosphomonoesterase (Konietzny and Greiner 2002). Phytic acid can also be dephosphorylated during cooking, as well as on an alternative enzymatic pathway to that presented for bread. This is possible due to the activity of phytases produced by filamentous fungi or endogenous plant enzymes active during soaking of the grains and germination (Azeke et al., 2011, Avendano et al., 2016).

Dephosphorylation of phytate as a result of the catalytic action of endogenous plant enzymes used as raw materials in brewing industry is limited due to their low activity and the fact that they operate beyond the optimum pH and temperature for phytases (Greiner and Konietzny 2006). Free amino nitrogen (FAN) is a very important indicator quantified for malt wort (Hill and Stewart 2019). In case of its deficiency, the capacity of fermentation declines significantly and the process may even stop. This is due to the reduced supply of such FAN components as amino acids and small peptides, which obviously affects the metabolism of yeast.

The aim of the project of which this work is a part was an attempt to use commercial phytase preparations for the production of functional beer based on buckwheat malt and to optimize the extraction of biologically active forms of D-chiro-inositol and myo-inositol (Duliński et al., 2019, 2020).

An additional effect associated with the introduction of exogenous phytases at the mash-in stage may result from the release of starch, proteins, as well as Ca and Zn ions from phytate complexes. An increase in the content of these substances was expected to significantly improve the efficiency of both mash-in and yeast fermentation. Therefore, the basic element of the study was to observe changes that occur in the polypeptide profile, selected saccharides, and amino acids during the process of raw material mashing. The observation was conducted by means of ion chromatography, SEC-HPLC, and spectrophotometric techniques.

MATERIALS AND METHODS

Materials

Buckwheat malt was purchased from the local Castle Malting® (Belgium) malting plant.

Enzymes

Amylase

In the study, the MATS® L CLASSIC preparation (DSM Nutritional; TE Heerlen, the Netherlands) was used; it is a liquid alpha-amylase obtained from genetically unmodified strain of Bacillus licheniformis. The enzyme was added in a dose of 0.4 kg-ton-1 at the beginning of the mash-in process according to the manufacturer’s recommendations.

Phytases

Commercial enzyme preparations: 3-phytase A (Finase®P) BASF, Ludwigshafen, Germany, manufacturer’s declared activity 5.1 kFTU/g, and 6-phytase A (Ronozyme® NP) Novozymes, Bagsværd, Denmark), declared activity 4.7 kFTU/g. One unit of phytase A preparation (FTU) was defined as the amount of enzyme that releases 1 μM of inorganic phosphorous from 2 mM of sodium phytate in 1 minute at 40°C, pH=4.5. Finase P was added in the amount of 1 mg/g of buckwheat malt; Ronozyme was added in the amount of 30 mg/g.
Preparation of laboratory wort

Mash-in

Buckwheat malt wort was prepared in a similar way to barley malt wort (ECB 4.5.1). For this purpose, 50 g of buckwheat malt ground in a laboratory grinder (Laboratory mill type WZ-1 (Poland)) was weighed into the mashing cups, then the containers were placed in a water bath of the mashing apparatus (Mash Bath R12 with connection to PC (1-CUBE, Czech Republic)) heated to 45°C, and stirrers were mounted: (1) Congress program—Then distilled water at temperature of 45°C was poured into the cups in 200 ml portions. The apparatus maintained this temperature for 30 minutes, then raised it at a rate of 1°C · min⁻¹ to 70°C, constantly stirring the samples. When the samples reached 70°C, 100 ml of distilled water of the same temperature was added to the cups and the temperature was kept for 1 hour. Next, the cups were cooled down to 20°C and their contents were filled with distilled water to obtain the weight of 450.0 g.

(2) The mash-in program developed to optimize temperature ranges for phytase activity—The apparatus maintained the temperature of 35°C for 15 minutes, 45°C for 15 minutes, 65°C for 40 minutes, 72°C for 30 minutes, and 78°C for 10 minutes. Next, the temperature was raised at a rate of 1°C · min⁻¹ until it reached 70°C, with constant stirring of samples. When the samples reached 70°C, 100 ml of distilled water warmed to the same temperature was added to the cups, and then the set temperature was maintained for 1 hour. Then, the cups were cooled to 20°C and filled up with distilled water to the mass of 450.0 g.

Analysis of fermentable sugars

Fermentable sugars were determined according to the recommended Analytica-EBC 9.27 method with slight modifications using the HPLC Ultimate-3000 (Thermo-Dionex, Sunnyvale, CA, USA) apparatus equipped with an autosampler with eluent pre-conditioning and an Aminex HPX-87C column (300 x 7.8 mm, Bio-Rad Laboratories, Hercules, California, USA) and a refractometer (Knauer, model 2400i). The chromatographic conditions were as follows: injection volume-5 μl; mobile phase-deionised water (18 MOhm), flow rate-0.5 ml/min, thermostatisation-85°C, positive (Breu et al., 2008).

SEC-HPLC analysis

An UltiMate 3000 HPLC chromatograph (Thermo-Dionex, Sunnyvale, CA, USA) with a TSK-GEL® GMPWXL column (7.8 mm ID x 30.0 cm), 10 μm (Tosoh Bioscience, Berlin, Germany) was used for the analyses. Working conditions: type of elution-isocratic, eluent-200 mM sodium phosphate buffer, pH 6.8, flow rate-0.6 mL/min, thermostat-25ºC, detection-UV-Vis 214/280 nm (PDA 3000 Dionex detector).

Free amino nitrogen

Free amino nitrogen (FAN) was measured using ninhydrin-based methods with the use of the absorbance measurement at 570 nm (Beckman DU-650 UV-Vis) according to the method: 8.10 Free Amino Nitrogen in wort by Spectrophotometry (IM) (Anon 1998).

Statistical analysis

Experimental data were subjected to the one-way analysis of variance (ANOVA) to detect significant differences among means and expressed as a mean ± standard deviation (SD). Differences among means were checked with the Tukey test at p<0.05 using Statistica for Windows, version 12.5 (Statsoft Inc., Tulsa, OK, USA) statistical software.

RESULTS AND DISCUSSIONS

Fermentable sugars

The contents of glucose and fermentable sugars in the wort are 30–50% lower than levels reported in the scarce literature (Deželak et al., 2014), which can partly be explained by changes in the composition of raw materials and at the stage of wort mashing.

In the first series of experiments, 3-phytase and 6-phytase (Ronozyme and Finase P, respectively) were added to malt. As compared to the control wort (10 831 mg/L), a statistically significant increase in glucose levels was observed when both 6-phytase and 3-phytase preparations were used in the Congress mash-in programme (13 294 mg/L and 12 555 mg/L for Ronozyme and Finase P, respectively) (Figure 1). No similar correlation was found in the case of the phytase-activity-optimised programme where glucose levels determined in control samples with added alpha-amylase (8 479 mg/L) did not differ statistically significantly from sugar content in buckwheat wort supplemented with phosphorolytic enzymes (8791 mg/L and 8979 mg/L of 6- and 3-phytase, respectively) (Figure 1).
Figure 1. Glucose content in buckwheat wort subjected to mashing with added phosphorolytic enzymes (3- and 6-phytase) in two programmes: the Congress method and the method with temperature optimised for phytase activity. Means ± standard deviation are presented. Mean values denoted by different letters within bars differ significantly at $p \leq 0.05$.

An analogous observation was made with respect to another fermentable sugar, namely maltose, the content of which did not change significantly in samples of malt mashed with added phytases; in this case, both the Congress mash-in variant (12,042 mg/L and 13,444 mg/L for 3- and 6-phytase, respectively) compared to the corresponding control (11,682 mg/L) and the programme optimised for phytase activity (12,985 mg/L and 11,469 mg/L for 3- and 6-phytase, respectively) compared to the control without added phosphorolytic enzymes (11,416 mg/L) (Figure 2).

Figure 2. Maltose content in buckwheat wort subjected to mashing with added phosphorolytic enzymes (3- and 6-phytase) in two programmes: the Congress method and the method with temperature optimised for phytase activity. Means ± standard deviation are presented. Mean values denoted by different letters differ significantly at $p \leq 0.05$. 

**Polypeptide profile and SEC-HPLC chromatography**

Polypeptides from buckwheat wort were separated by size-exclusion chromatography (SEC) and chromatographic spectra were recorded in UV-Vis detection mode with 214 and 280 nm wavelengths. Although the polypeptide profile of buckwheat wort compared to SEC-HPLC profiles obtained for barley beer allows to note some analogies, such as the presence of five main peaks and a similar shape of the spectra, the proportions and approximate molecular weight (40 and 14–16 kDa) of the two main peaks differ significantly (Vieira, Elsa; Moura Cristina 2012).

Five main peaks were identified on the chromatograms (Figure 3), and changes in the relative peak area were statistically analysed. The interpretation of these data in the qualitative and semiquantitative analysis allows to conclude that polypeptides represented on chromatograms by four main peaks with a retention time of 16–12 min undergo dynamic evolution during the process, which leads to a reduction in the total peak area of the peptides quantified.

A protein with approximate molecular weight of 40 kDa, that occurs in all mash-in programs as peak No. 2 (retention time 12.9 min) and is present both in control samples and in samples with added phytases (Figure 3) was selected as the most representative polypeptide for the whole series of analyses.

![Figure 3. Buckwheat wort elution profile (control without added phosphorolytic enzymes, Congress mash-in method) obtained using the SEC-HPLC technique, separation in a TSK GMPWXL column, and detection with UV 280 nm wavelength.](image)

The analysis of free amino acid nitrogen content did not confirm differences in FAN levels for wort enriched with phytases compared to control in statistical tests (Figure 5.). However, the tendency to release slightly more amino acids and small peptides observable in the Congress program for the variant of mash enriched with phytases is correlated with the decrease in the peak area (from 49 relative units to 31) of the key polypeptide mentioned above (estimated molecular mass 40 kDa) recorded in SEC-HPLC chromatography and statistically significant in this case (Figure 4). In the studies of Mikulski et al., (Mikulski et al., 2015) a slight increase in FAN (3.85 mg/L on average) was recorded in the high viscosity mashed maize wort with added phytases and proteases. In the quoted studies, where phytase group enzymes were added to the model system at a dose of 8 U/g of malt, the total hydrolysis of phytate was also noted (Mikulski et al., 2015). This favoured the release of the maximum amount of proteins and peptides from phytate complexes, while the maximum rate of phytate reduction determined in our study was 65% (Duliniński et al., 2020). However, it is worth noting that in the work mentioned above the conditions of hydrolysis, i.e. not only the temperature (55°C), but also the pH value (5.5) and time, were optimised for phytase activity. In our study, the pH remained within the range typical of a standard mash-in program (pH 5.8), which could reduce the efficiency of the phytases but at the same time improve the efficiency of amylases, especially in the Congress variant. The beneficial effect of adding phytase (0.8–1.2 U/g) may also result from reducing the mash-in time (from 104 to 96 h), as shown by Qiu et al. (Qiu and Lu 2017).
Figure 4. Analysis of the relative peak area (SEC-HPLC) of the main polypeptide fraction in buckwheat wort subjected to mashing with added phosphorolytic enzymes (3- and 6-phytase) by way of two programs: the Congress method and the method with temperature optimised for phytase activity. Means ± standard deviation are presented. Mean values denoted by different letters differ significantly at $p \leq 0.05$.

Figure 5. The free amino acid content in buckwheat wort mashed with added phosphorolytic enzymes (3- and 6-phytase) in two programmes, the Congress method and the method with temperature optimised for phytase activity. Means ± standard deviation are presented. Mean values denoted by different letters differ significantly at $p \leq 0.05$.

In the mash prepared by way of the Congress method, the key protein peak area decreased by 25% (6-phytase) to as much as 37% (3-phytase), as compared to the control sample without phytases, and the mean values of this parameter are lower for the Congress method (39 relative area units, RAU) than for the program optimised for phytase activity (58 RAU).

It is probable that the effect of key changes in the structure of polymers (polypeptides and polysaccharides) in the mash-in process facilitated by the addition of phosphorolytic enzymes is even greater in the standard mash-in method. This is because the Congress method promotes optimal amylase activity, while phytases potentially release an additional pool of substrate (starch) and Ca ions (cofactors) from phytate complexes. Consequently, the
synchronization of the activity of amylases and phytases has additional effects, namely an increased release of fermentable sugars (glucose) (Figure 1).

CONCLUSIONS

In this work, which is a part of a larger project, an attempt was made to optimize the composition of enzymatic preparations used in the buckwheat beer production process in order to obtain increased recovery of biologically active forms of inositol, D-chiro-inositol and myo-inositol, and reduce the level of phytate, which is an antinutrient. The change in the profile of inositol phosphates was noted in our latest published studies. The additional effect, that is the decline of phytate content, was confirmed by monitoring the profiles of saccharides and peptides at subsequent stages of mash-in. The content of selected fermentable sugars (glucose) in wort shows statistically significant differences when using 6-phytase (Ronozyme) and 3-phytase (Fianse P) preparations, as well as in the Congress method. On the basis of the results obtained, it can be concluded that the application of the preparation studied has a favourable effect on the extraction of sugars from buckwheat malt, increasing the recovery of glucose and maltose by 8–10%, as compared to the classic amylolytic preparation Mats L Classic (control). SEC-HPLC chromatography allows to notice significant changes in the polypeptide fractions in the product, as well as an expected but not statistically confirmed increase in free amino acids and a notable decrease in the proportion of higher molecular weight peptides due to the phytase addition impact on polymers present in the raw materials.

Author Contributions: R.D. Conceived and designed the analysis; wrote the paper. M.Z. Performed the analysis, collected the data; A.P. Contributed data or analysis tools.

Funding Source: This research was funded by the Polish National Center for Research and Development, LIDER 46/0185/L-9/17/NCBR/2018 grant.

Conflicts of Interest

The authors declare that they do not have any conflict of interest.

REFERENCES