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Optimization of High Intensity Ultrasound Treatment of Proso Millet Bran to Improve Physical and Nutritional Quality[§]

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SUMMARY

Millet is an unexploited cereal with potential in the food industry due to its nutritional value and resistance to harsh climate conditions. Nutritious millet byproducts have a potential application in the development of functional cereal products, but require processing in order to improve their physical and nutritional quality. Therefore, we investigated high intensity ultrasound as a pretreatment to increase the amount of freely available bioactives from proso millet bran. We also analysed the effect of high intensity ultrasound on enzymatic browning, water retention and protein digestibility, which are crucial for the utilization in the bakery and pasta industry. A 15 % millet bran suspension in water was treated with 400-W ultrasound probe for 5, 12.5 or 20 min, with the 60, 80 or 100 % amplitude. High intensity ultrasound treatment with 80 % amplitude for 12.5 min improved most significantly the nutritive value; the antioxidant activity measured by FRAP test increased by 15 % ($p<0.05$), and total phenolic content by 16 % ($p<0.05$). Still, the impact on the increase of water-soluble and ethanol-insoluble dietary fibre by 38 % was evident after the treatment for 20 min at 100 % amplitude. High intensity ultrasound treatment at 100 % amplitude for 5 min caused the largest improvements in water retention and limited browning of the sample. High intensity ultrasound treatment activated polyphenol oxidase, regardless of the applied heating of the sample. Due to its ambiguous impact on proso millet bran characteristics, the treatment required an optimization, which showed that the optimal pretreatment of a 15 % millet bran suspension in water is at 100 % amplitude for 9.3 min.

Key words: antioxidant activity, enzymatic browning, high intensity ultrasound, millet bran, water retention

INTRODUCTION

Millet is an unexploited cereal with great potential in the agro-food industry due to its resistance to drought and harsh climate conditions (*1*), while bran is a byproduct with potential health benefits due to its content of protein, essential fatty acids, minerals, vitamins and components with antioxidant capabilities, such as polyphenols and phytosterols. Additional nutritional value of bran is its high content of dietary fibre (*2*), so the leading authorities are constantly urging its daily consumption due to its proven health benefits. According to the Academy of Nutrition and Dietetics (Chicago, IL, USA), intake of 14 g of dietary fibre per 1000 kcal consumed can decrease the risk of obesity, cardiovascular disease and type 2 diabetes (*3*). In spite of that, a fibre-rich product like bran is underutilized and mostly used as animal feed (*4*). Unfortunately, application of bran to bakery products can have a deteriorating effect on its structure and sensory properties, causing a drop in volume and a bitter taste (*2*). Mechanism responsible for deleterious effect of bran on bakery products is not completely clear but is partially caused by higher water retention due to the ability of bran for dynamic hydration (*2*). Dynamic hydration is characteristic for dietary fibre from bran, and enables adsorption of water through two different mechanisms, one caused by external stress, and the other by hydrogen bonds resulting in excess water intake. The absorbed water is available for starch gelatinisation during baking, which lowers starch gelatinisation temperature and consequently shortens the time of loaf volume

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growth (*5*). In order to exploit the potential of bran as a raw material for production of cereal functional products, some novel processes are needed to modify its properties to make them suitable for the bakery industry.

Ultrasound is a robust non-thermal green technology which generates mechanical energy by sound waves, causing series of alternating contractions and expansions. The deteriorating effect of high intensity ultrasound on cell walls is caused by collapses of cavitation bubbles which cause strong shear forces that form microfractures in plant tissues and help mass transfer, penetration of the solvent, and diffusion and release of extractable compounds (*6*). Besides its effect on the availability of compounds with high nutritive value, high intensity ultrasound can affect rheological properties of food and cause a change in appearance (colour) or applicability (water retention, protein digestibility) of a product. Change in colour, specifically occurrence of browning, that develops as a result of the use of high intensity ultrasound is possibly explained by activation of enzymes. Polyphenol oxidase (PPO) (1,2-benzenediol:oxygen oxidoreductase) is a generic name for a group of enzymes catalysing the oxidation of phenolic compounds, which results in the generation of melanin pigments (*7*). It is actively involved in plant defence and can be used as a marker of resistance to downy mildew infections in pearl millet (*8*). Because of its protective role, it is highly concentrated in the bran fraction of the grain (*2*). Browning of food products includes first a catalytic reaction of oxidation of mono- and diphenolic compounds to quinones by PPO, and second a non-enzymatic condensation process of forming complex brown polymers – melanins. The catalytic reactions of PPO do not only affect the colour, but also the flavour, texture and nutritional value of the material (*9*). They result in the oxidation of phenolics and also a decrease in available amino acids. Felton *et al.* (*7*) reported that the presence of PPO caused a decrease of lysine content in casein.

With the aim of making bran an asset for production of cereal functional products, we investigated application of high intensity ultrasound to proso millet bran. The hypothesis of this research is that improvement in the nutritive value of millet bran could be achieved through a pre-treatment with high intensity ultrasound due to its potential of releasing dietary fibre and compounds with antioxidant activity form their insoluble/bound to soluble/free forms, while customizing proso millet bran physical characteristics for requirements of bakery industry by limiting enzymatic browning and water retention capacity.

MATERIALS AND METHODS

Proso millet bran

Millet bran was obtained after decortication of proso millet (*Panicum miliaceum*) Sonček variety grown in 2017, and given as a gift from Mlinopek (Murska Sobota, Slovenia). The bran was sieved through a 500-μm pore size sieve to achieve a product without foreign matter and of high nutritive value.

Chemical composition analysis

Chemical composition of the proso millet bran fraction smaller than 500 μm was determined according to the standard AACC and ICC methods. Protein content was determined by the AACC method 46-12.01 (*10*), which included acid digestion of the sample, distillation of ammonia in 4 % boric acid (Gram-Mol, Zagreb, Croatia) and titration of the distillate with 0.1 M HCl (Gram-Mol) until neutrality. Protein content was calculated using factor 6.25. Total fat content was determined according to the ICC Standard No. 136 (*11*), which included the extraction with hexane (95 %; J.T.Baker, Avantor, Radnor, PA, USA), removal of the solvent and weighing the obtained residue. Dry matter content was determined with AACC method 44-19.01 (*12*), by drying the samples in an air-oven (ST-01/02; Instrumentaria, Zagreb, Croatia) at 135 °C for 2 h.

Ultrasound treatment

Forty-five grams of proso millet bran fraction were suspended in 300 mL of distilled water in a 600-mL glass beaker. The sample was treated with the UP400S ultrasonic processor (Hielscher GmbH, Hamm, Germany) at 400 W and 24 kHz, using a 22-mm titanium probe (H22D; Hielscher GmbH). Treatments were conducted according to the central composite design generated with Design Expert v. 11.0.3 software (*13*) as shown in **Table 1**. Temperature change was monitored using a temperature probe immersed into the sample during high intensity ultrasound treatment. Predicted specific heat capacity of the sample, amounting to c_p=3886 J/(kg·K), was calculated from the determined chemical composition of the sample and values for specific heat capacity of individual food components according to the following equation (*14*):

$$c_p = \Sigma w_i \cdot c_{pi} \qquad /1/$$

where c_{pi} is the specific heat capacity of a major food component and w_i is the mass fraction of the major food component.

Table 1. Central composite design of high intensity ultrasound treatments of proso millet bran fraction smaller than 500 μm

Run	Sample code	Amplitude %	Amplitude (coded value)	Time min	Time (coded value)
1	60A12.5M	60	−1	12.5	0
2	100A20M	100	+1	20	+1
3	100A5M	100	+1	5	−1
4	80A5M	80	0	5	−1
5	80A12.5M	80	0	12.5	0
6	100A12.5M	100	+1	12.5	0
7	80A12.5M	80	0	12.5	0
8	80A12.5M	80	0	12.5	0
9	80A12.5M	80	0	12.5	0
10	60A20M	60	−1	20	+1
11	60A5M	60	−1	5	−1
12	80A20M	80	0	20	+1
13	80A12.5M	80	0	12.5	0

Together with the change in temperature during time, it served to calculate the acoustic power (*P*) of high intensity ultrasound according to the following equation (*15*):

$$P = m \cdot c_p \cdot \left(\frac{dT}{dt} \right) \qquad /2/$$

where m is the mass of the sample (kg), c_p is the specific heat capacity of the sample (J/(kg·K)) and $\frac{dT}{dt}$ is the change in temperature (K) over time (s).

Control sample (0A20M) was the same water suspension of proso millet bran fraction, but stirred for 20 min at room temperature on a magnetic stirrer (C-MAG HS-7; IKA® Works, Inc., Wilmington, NC, USA) at 500 rpm. Then, the samples were frozen to –80 °C and freeze dried (Alpha 1-4 LSCplus; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) for 42 h.

Polyphenol oxidase activity

The measurement of PPO was carried out using AACC method 22–85.01 (*16*) with some modifications. Sample mass was adjusted to 10 mg and the quantity of the L-DOPA (Sigma-Aldrich, Merck, Steinheim, Germany) sample solution was adjusted to 0.5 mL and diluted with distilled water to a final volume of 1 mL. Afterwards, the samples were mixed on vortex (IKA® MS 3 basic shaker; IKA® Works Inc.) for 55 min at 1000 rpm and centrifuged (MicroCL 21; Thermo Fisher Scientific GmbH, Dreieich, Germany) for 5 min at 6164×*g* prior to spectrophotometric measurement (Specord 50 Plus; Analytik Jena, Jena, Germany) at 475 nm. According to the applied method (*16*), activity of PPO is expressed as the absorbance.

Browning index

The colour of proso millet bran fraction was measured using the colour space values (L^*, a^* and b^*) of the CIELab system, using a colorimeter (spectrophotometer CH-3500 D; Konica Minolta, Basildon, UK). Browning index (BI) was calculated according to (*17*):

$$BI = \frac{(a + 1.75L)}{(5.645L + a - 0.3012b)} \qquad /3/$$

Dietary fibre

Dietary fibre can be divided according to its solubility and molecular mass to: insoluble dietary fibre, high-molecular-mass dietary fibre soluble in water that precipitates in 78 % aqueous ethanol (SDFP), and low-molecular-mass dietary fibre soluble in water that remains soluble in 78 % aqueous ethanol (SDFS). They were determined with the integrated total dietary fibre assay kit (Megazyme, Bray, Ireland) according to AOAC method 2011.25 (*18*), using Shimadzu HPLC system with MetaCarb 67C column (Agilent Technologies, Santa Clara, CA, USA) and LC solution v. 1.22 SP1 software (Shimadzu, Tokyo, Japan).

Water retention

Water retention capacity (WRC) of proso millet bran fraction was determined according to the AACC method 56-20.01 (*19*) as modified by Jacobs *et al*. (*20*). Sample (2 g proso millet bran fraction) was soaked in 20 mL of distilled water for 60 min, and then centrifuged (Rotina 35; Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 2813×*g* for 10 min. The supernatant was discarded and the pellet drained for 15 min by placing the tubes at a 45° angle. The residue was weighed. The WRC capacity is expressed as percentage of sample mass on dry mass basis.

Protein digestibility

In vitro protein digestibility (IVPD) was determined following the multi-enzyme method of Hsu *et al*. (*21*) modified according to Tinus *et al*. (*22*). Briefly, 500 mg of proso millet bran fraction (mass equivalent to 62.5 mg protein) was rehydrated in 10 mL of distilled water at 37 °C for 1 h before adjusting the pH of the sample to 8.0. Drop in the pH value (ΔpH) of the sample 10 min after adding 1 mL of the multi-enzyme solution was measured with a pH/mV temperature meter (model 3510; Jenway, Cole-Parmer, Wertheim-Mondfel, Germany) and used to calculate the IVPD (%) according to the following equation:

$$IVPD = 65.66 + 18.1\Delta pH \qquad /4/$$

The multi-enzyme solution consisted of 16 mg trypsin form porcine pancreas type IX-S, 31 mg α-chymotrypsin from bovine pancreas type II, and 13 mg protease from *Streptomyces griseus* Type XIV, all from Sigma-Aldrich (Merck).

Extraction of phenolic compounds

Freeze-dried samples were used for the extraction of free phenolic compounds according to a modified HEALTHGRAIN method (*23*). Precisely 250 mg of sample were weighed into a 2-mL Eppendorf safe lock tube. To each sample, 1 mL of 80 % ethanol (*V/V*, HPLC grade; Fisher Chemical, Fisher Scientific, Hampton, NH, USA) was added, vortexed for 0.5 min at 1500 rpm and placed into ultrasonic bath (Bandelin Electronic RK 100 H; Sonorex, Berlin, Germany) for 10 min, after which the samples were centrifuged (MicroCL 21; Thermo Fisher Scientific) at 6164×*g* for 15 min, and the supernatant was decanted into new tubes. Extraction process was repeated two more times and the combined supernatants were evaporated under a nitrogen stream. Samples were stored at –20 °C. Prior to further analysis of total phenolic content and antioxidant activity, the samples were dissolved in 0.5 mL of methanol (HPLC ultra gradient; J.T.Baker), centrifuged at 6164×*g* (MicroCL 21; Thermo Fisher Scientific) and transferred into new tubes.

Total phenolic content

Total phenolic content was determined according to Yu *et al*. (*24*) with modifications. Distilled water (0.4 mL), extract (0.015 mL) and Folin-Ciocalteu reagent (0.1 mL; Sigma Aldrich, Merck)

were pipetted into a cuvette and put aside for 3 min after which 20 % Na_2CO_3 (0.3 mL; Sigma-Aldrich, Merck) and distilled water (1.185 mL) were added. The solution was mixed and incubated in the dark for 2 h. The absorbance was measured at 765 nm (Specord 50 Plus; Analytik Jena). Measurements were done in triplicate and the results expressed in mg galic acid equivalent (GAE) per gram of sample, using a calibration curve constructed from the standard of galic acid (Sigma-Aldrich, Merck).

Antioxidant activity

Antioxidant activity was determined by DPPH radical-scavenging capacity, ferric reducing antioxidant power (FRAP) and ABTS (2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) assay as described by Belščak *et al*. (*25*) with some modifications. For the DPPH assay, 0.02 mL of the extract was added to 0.95 mL or 0.06 mM DPPH (2,2-diphenyl-picrylhidrazyl; Sigma Aldrich, Merck) in methanol (HPLC ultra gradient, J.T.Baker). The free radical-scavenging capacity was evaluated by measuring the absorbance at 517 nm (Specord 50 Plus; Analytik Jena) after a 30-minute reaction in the dark. For FRAP assay, 0.01 mL of methanol extract was mixed with 1 mL of FRAP reagent. Absorbance was measured at 593 nm after incubation for 4 min. For the ABTS assay, 0.02 mL of extract was mixed with 2 mL of ABTS reagent (Sigma-Aldrich, Merck), mixed, and the absorbance measured at 734 nm after 6 min of incubation in the dark. Measurements were done in triplicate and the results were expressed in μmol Trolox equivalent (TE) per gram of sample, using a calibration curve constructed from the standard solution of Trolox (>97 %; Sigma-Aldrich, Merck).

Statistical analysis

One-way ANOVA with *post-hoc* Tukey's test was applied in order to determine significant differences between mean values, followed by correlation analysis, using Prism v. 7.04 (*26*). All analyses were performed at 95 % confidence level. The optimization of high intensity ultrasound treatment of proso millet bran fraction was done with Design Expert v. 11.0.3 (*13*), utilizing the parameters with a significant change in the output values after the treatment. The required parameters were set as following: maximization of TPC, FRAP and SDFS, and minimization of WRC, PPO and BI. Power of the high intensity ultrasound, DPPH, ABTS, insoluble dietary fibre, SDFP and IVPD were not included in the optimization since they only showed significant change compared to the control sample (0A20M), but were not affected by the amplitude and time of high intensity ultrasound treatment.

RESULTS AND DISCUSSION

Millet bran fraction containing only particles smaller than 500 μm was primarily chosen in order to remove the larger impurities that are part of the proso millet bran raw material due to an underdeveloped technology for small grain purification. Other than that, a fraction of smaller particles was separated due to preliminary analysis results that showed that this fraction is richer in protein (13.9 g/100 g; results not shown), which is an advantage considering that the chosen proso millet bran fraction has a mediocre protein digestibility (**Fig. 1**). Bran fractionation also resulted in a product that was high in fibre content, especially insoluble dietary fibre (34.2 g/100 g, **Table 2**). Higher fat content of the proso millet bran fraction (9.98 g/100 g, data not shown) is considered an advantage since proso millet contains a high amount of unsaturated fatty acids, particularly linoleic fatty acid (*27*), which has potential health benefits.

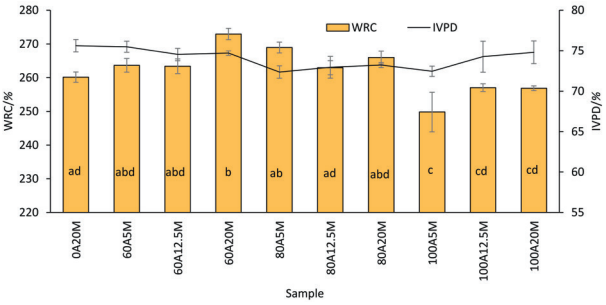

Fig. 1. Water retention capacity (WRC) and *in vitro* protein digestibility (IVPD) of the high intensity ultrasound-treated proso millet bran fraction smaller than 500 μm (mean value±standard deviation) in comparison with the control (0A20M). Mean values of WRC with different letters differ significantly at $p<0.05$

Table 2. Mass fractions (g/per 100 g dry mass) of insoluble and soluble dietary fibre of the high intensity ultrasound-treated proso millet bran fraction smaller than 500 μm compared to the control

Sample	*w*(IDF)/%	*w*(SDFP)/%	*w*(SDFS)/%
0A20M (control)	(34.2±0.5)^{a}	(1.0±0.3)^{a}	(0.54±0.01)^{a}
60A5M	(39.1±0.0)^{bc}	(1.4±0.0)^{a}	(0.33±0.01)^{b}
60A12.5M	(34.3±0.4)^{a}	(2.2±0.3)^{a}	(0.40±0.00)^{c}
60A20M	(35.5±0.3)^{ac}	(1.0±0.3)^{a}	(0.37±0.01)^{b}
80A5M	(32.7±0.6)^{a}	(1.0±0.3)^{a}	(0.39±0.00)^{c}
80A12.5M	(34.6±1.5)^{a}	(1.6±0.6)^{a}	(0.32±0.02)^{b}
80A20M	(35.7±0.4)^{ac}	(1.5±0.0)^{a}	(0.33±0.01)^{b}
100A5M	(32.5±1.0)^{a}	(1.5±0.1)^{a}	(0.45±0.00)^{c}
100A12.5M	(34.1±0.9)^{a}	(1.3±0.0)^{a}	(0.35±0.00)^{b}
100A20M	(34.6±0.1)^{a}	(1.6±0.1)^{a}	(0.75±0.00)^{d}

Results are presented as mean value±standard deviation ($p<0.05$). Mean values with different superscript within the same column differ significantly at $p<0.05$. IDF=insoluble dietary fibre, SDFP=dietary fibre soluble in water that precipitates in 78 % aqueous ethanol, SDFS=soluble dietary fibre in water and 78 % aqueous ethanol

Treatment with high intensity ultrasound is mostly utilized for extraction procedures and further development of its application as a pretreatment of raw material in the food industry is needed. In addition, although the positive effect of high intensity ultrasound treatment of wheat bran on extraction of compounds with antioxidant activity has already been shown and the mechanism explained (*28,29*), its effect specifically depends on the material and therefore needs to be optimized for millet bran.

Acoustic power and temperature change are useful descriptive properties of the high intensity ultrasound treatment

which simplify the understanding of the effect of cavitation and temperature during the treatment. Power of high intensity ultrasound was proportional to the temperature gradient (r=0.811, p<0.001), which was the highest in the first few minutes of the treatment and therefore, the shortest treatments had the highest intensity ultrasound power (**Fig. 2**). Time of the treatment is the determining factor for the total change in temperature (r=0.923, p<0.001). In all treatments lasting 5 min, the final sample temperature did not rise above 40 °C, indicating that in these treatments the cavitation effect was the dominant one, while in other treatments (12.5 and 20 min) the rise of temperature possibly overpowered the effect of cavitation (*30*).

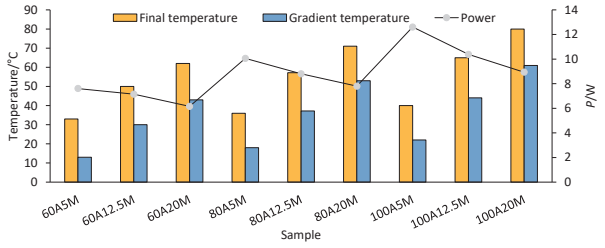

Fig. 2. Final temperature, temperature gradient and power of high intensity ultrasound of proso millet bran fraction smaller than 500 µm

High intensity ultrasound can have both an activating and inactivating effect on enzymes (*31*,*32*), depending on its power, but also on the properties of the product (pH, water activity, *etc.*) (*30*), as well as on the enzyme type, its amino acid composition, and conformation (*33*). In this research, the activity of PPO was higher in all treated samples than in the control (0A20M) (**Fig. 3** and **Table 3**). This was surprising for treatments in which sample temperature at the end of the treatment was above 60 °C (60A20M, 80A20M, 100A12.5M and 100A20M; **Fig. 2**). The longer treatments with a higher final temperature (**Fig. 2** and **Fig. 3**) caused the largest increase in the PPO activity (30–83 % augmentation compared to the control (0A20M)). Indeed, the activity of PPO correlated positively with the time of treatment (r=0.770, p=0.001) and with the temperature increase (r=0.849, p<0.0001). It is possible that the energy generated by the high intensity ultrasound accelerated the reactions catalysed by the PPO by promoting the collision of enzyme and substrate (*32*). Also, longer exposure to high intensity ultrasound could result in the rupture of the cell wall, which could consequently improve the accessibility of the membrane-bound PPO (*34*). Browning of the samples correlated well with the activity of PPO (r=0.853, p<0.0001) (**Fig. 3**). Enzymatic browning occurs in two reactions of which the first depends on the PPO activity and results in the formation of quinones. The second is a non-enzymatic reaction of quinones with amino acids and phenols in which the reddish-brown coloured melanin is formed (*9*). Therefore, the potential mechanism by which high intensity ultrasound enhances browning of the sample is not only the acceleration of the reactions by PPO, but also making more of the second substrate (phenols) available for the reaction by freeing it from its insoluble form.

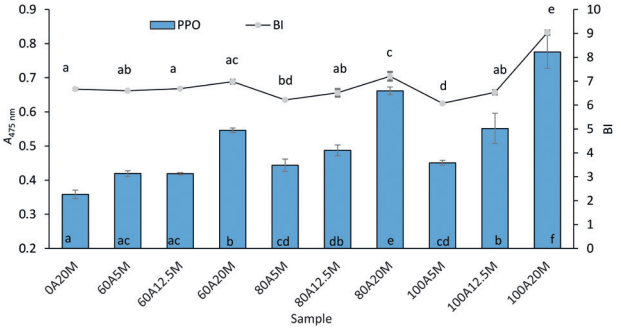

Fig. 3. Browning index (BI) and polyphenol oxidase (PPO) activity expressed as absorbance at 475 nm of high intensity ultrasound-treated proso millet bran fraction smaller than 500 µm (mean value±standard deviation), in comparison with the control (0A20M). Mean values of PPO activity with different letters differ significantly at p<0.05

Table 3. Significant coefficients (p<0.05) of researched parameters during high intensity ultrasound treatment of proso millet bran fraction smaller than 500 µm

Parameter	Intercept	A (amplitude)	B (time)	AB	B^2
TPC	1.8277	ns	0.0176	ns	– 0.1803
FRAP	2.7325	ns	0.0796	ns	– 0.3839
WRC	262.6	– 6.0439	ns	ns	ns
BI	6.8	ns	0.7215	0.64729	ns
PPO	0.4866	0.0656	0.1116	0.049601	0.0629

TPC=total phenolic content, FRAP=ferric reducing antioxidant power, WRC=water retention capacity, BI=browning index, PPO=polyphenol oxidase activity, ns=not significant at p<0.05

The most successful treatments of proso millet bran fraction with high intensity ultrasound, as measured by FRAP and TPC, were the ones that lasted 12.5 min (**Table 3** and **Table 4**). The best results were achieved with 80 % amplitude and 12.5 min of treatment, which resulted in 15.2 % higher TPC than in the control sample and 16.3 % improved antioxidant activity measured by FRAP test (**Table 4**). The positive correlation between FRAP and TPC test (r=0.884, p<0.001) further confirmed the link between the growing antioxidant activity and the amount of free phenolics. Heating of samples with high intensity ultrasound could cause degradation of phenolic compounds and result in reduced TPC, as confirmed by Chandrasekara *et al.* (*35*). In that research, temperature increase of the samples during treatment did not correlate negatively with the TPC. Our results are in agreement with those of Luo *et al.* (*36*). Additionally, it is possible that the effect of high intensity ultrasound on TPC is concealed by the activating effect which high intensity ultrasound has on PPO. Consequently, the freed phenolics could have been used up in the oxidation reactions catalysed by PPO, making them inaccessible again. Except for that, it is possible that high intensity ultrasound enhanced the activity of other phenol-degrading enzymes, *e.g.* peroxidase (*7*), which would contribute to this effect. Although the change in the FRAP antioxidant activity of proso millet bran fraction was positive, DPPH and ABTS did not change, except for the treatment with 80 % amplitude for

5 min. Different results among various antioxidant tests are a consequence of different reagents and, therefore, the reaction with compounds of various chemical composition.

Table 4. Change in the antioxidant capacity measured by FRAP, DPPH, ABTS and total phenolic content of high intensity ultrasound-treated proso millet bran fraction smaller than 500 μm compared to the control

Sample	FRAP as TE/(μmol/g)	DPPH as TE/(μmol/g)	ABTS as TE/(μmol/g)	TPC as GAE/(μg/g)
0A20M (control)	$(2.3\pm0.1)^{ac}$	$(1.6\pm0.1)^{ac}$	$(2.6\pm0.1)^{ab}$	$(1.6\pm0.2)^{ac}$
60A5M	$(2.6\pm0.3)^{c}$	$(1.6\pm0.0)^{ac}$	$(2.9\pm0.1)^{a}$	$(1.7\pm0.1)^{abc}$
60A12.5M	$(2.6\pm0.0)^{c}$	$(1.7\pm0.1)^{c}$	$(2.6\pm0.0)^{ab}$	$(1.7\pm0.1)^{abc}$
60A20M	$(2.6\pm0.0)^{c}$	$(1.6\pm0.1)^{ac}$	$(2.9\pm0.1)^{a}$	$(1.8\pm0.1)^{ab}$
80A5M	(2.0±0.1)a	$(1.4\pm0.0)^{b}$	$(2.3\pm0.0)^{b}$	$(1.5\pm0.1)^{c}$
80A12.5M	$(2.8\pm0.1)^{bd}$	$(1.6\pm0.1)^{ac}$	$(2.7\pm0.1)^{ab}$	$(1.9\pm0.1)^{b}$
80A20M	$(2.5\pm0.2)^{cde}$	$(1.4\pm0.1)^{ab}$	$(2.9\pm0.0)^{ab}$	$(1.7\pm0.1)^{abc}$
100A5M	$(2.3\pm0.2)^{ce}$	$(1.6\pm0.1)^{ac}$	$(2.4\pm0.2)^{ab}$	$(1.7\pm0.1)^{abc}$
100A12.5M	$(2.6\pm0.0)^{cd}$	$(1.5\pm0.0)^{ac}$	$(2.4\pm0.2)^{bc}$	$(1.7\pm0.1)^{abc}$
100A20M	$(2.1\pm0.2)^{ae}$	$(1.5\pm0.1)^{abc}$	$(2.6\pm0.1)^{ab}$	$(1.5\pm0.1)^{c}$

Results are presented as mean value±standard deviation ($p<0.05$). Mean values with different letters in superscript within same column differ significantly at $p<0.05$. FRAP=ferric reducing antioxidant power, DPPH=radical-scavenging capacity, ABTS=(2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) assay, TPC=total phenolic content, TE=Trolox equivalent, GAE=galic acid equivalent

Health benefits of daily consumption of whole grain cereals are mostly attributed to a higher dietary fibre intake (*3*). Soluble fibre seems to have a more significant effect on health than insoluble dietary fibre (*37*). Since high intensity ultrasound has the potential to cause physical changes and increase diffusion of soluble compounds (*38*), it could have an impact on the quantity of soluble fibre by making them more available to the solvent. Indeed, Zhang *et al*. (*39*) showed that high intensity ultrasound impacts the degree of polymerization, resulting in better solubility of the fibre. Generally, high intensity ultrasound treatment did not significantly affect insoluble dietary fibre, except for the treatment at 60 % amplitude for 5 min, which caused a significant 14.2 % increase of insoluble dietary fibre compared to the control (**Table 2**). High intensity ultrasound treatment did not significantly affect the SDFP either, but changed the mass fraction of SDFS. Treatments with 60 or 80 % amplitude caused a drop in the mass fraction of SDFS, while only the treatment at 100 % amplitude for 20 min caused a 23.6 % increase of SDFS compared to the control. Despite the increase of SDFS, the mass fraction of insoluble dietary fibre remained unchanged.

Compared to the control, only high intensity ultrasound treatment at 100 % amplitude resulted in a decrease of WRC (**Fig. 1** and **Table 3**). All other treatments increased WRC, which is undesirable for bran intended for bakery products. Possible explanation for the isolated effect of this treatment is the high mass fraction of starch (26 % dry mass) of proso millet bran fraction. To a certain point, high intensity ultrasound enhances starch water swelling ability (*40*), but as the temperature and the intensity of the treatment increase, the treatment damages and brakes down the starch granules (*38*), causing gelatinisation and a lower WRC. Further on, high fibre content of proso millet bran fraction certainly influenced the WRC. Elleuch *et al*. (*41*) explained that mixing of a sample could result in opening up of the fibre structure, which would in turn make cellulose hydroxyl groups more accessible to the interaction with water. Since vigorous mixing is a result of high intensity ultrasound application, this is a possible reason for the increase of WRC of the treated samples.

Although not statistically significant, IVPD decreased during the high intensity ultrasound treatment compared to the control (**Fig. 1**). It is possible that this effect is a consequence of a higher concentration of polyphenols after the treatment since some polyphenols can bind to proteins, making them inaccessible (*42*). The effect could also be caused by the temperature increase during high intensity ultrasound treatment. Our results agree with the finding that digestibility of proso millet proteins decreases after cooking due to the formation of hydrophobic aggregates (*43*). This effect is specific for proso millet proteins as high intensity ultrasound treatment usually leads to partial defolding of protein structure (*44*).

CONCLUSIONS

Enhancement of antioxidant activity and total phenolic content of proso millet bran fraction was most successful with the application of 80 % amplitude for 12.5 min, while the improvement of physical properties, browning and water retention were most pronounced at 100 % amplitude for 5 min. Besides that, dietary fibre solubility was significantly higher after the treatment at 100 % amplitude for 20 min. High intensity ultrasound treatment enhanced the polyphenol oxidase activity regardless of the temperature of the sample, but the effect was not statistically significant at shorter treatment times. Therefore, utilization of high intensity ultrasound as an asset for improving millet bran intended for the bakery industry requires optimization. According to our results, the best pretreatment (desirability 0.62) to acquire an improved proso millet bran fraction is a 15 % water solution of the fraction treated with a 400-W high intensity ultrasonic processor and a 22-mm probe at 100 % amplitude for 9.3 min. Taking into consideration its effect on both physical and nutritive characteristics, further research should focus on the possibility of application of high intensity ultrasound for the deactivation or activation of specific enzymes of proso millet bran fraction, such as polyphenol oxidase.

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