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COMPARABLE EFFICIENCY OF DIFFERENT EXTRACTION PROTOCOLS FOR WHEAT AND RYE PROLAMINS

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ABSTRACT

The identification and quantification of cereal storage proteins is of interest of many researchers. Their structural or functional properties are usually affected by the way how they are extracted. The efficiency of extraction process depends on the cereal source and working conditions. Here, we described various commonly used extraction protocols differing in the extraction conditions (pre-extraction of albumins/globulins, sequential extraction of individual protein fractions or co-extraction of gluten proteins, heating or non-heating, reducing or non-reducing conditions). The total protein content of all fractions extracted from commercially available wheat and rye flours was measured by the Bradford method. Tris-Tricine SDS-PAGE was used to determine the molecular weights of wheat gliadins, rye secalins and high-molecular weight glutelins which are the main triggering factors causing celiac disease. Moreover, we were able to distinguish individual subunits (α/β-, γ-, ω-gliadins and 40k-γ-, 75k-γ-, ω-secalins) of wheat/rye prolamins. Generally, modified extraction protocols against classical Osborne procedure were more effective and yields higher protein content in all protein fractions. Bradford measurement led into underestimation of results in three extraction procedures, while all protein fractions were clearly identified on SDS-PAGE gels. Co-extraction of gluten proteins resulted in appearance of both, low-molecular weight fractions (wheat gliadins and rye secalins) as well as high-molecular weight glutelins which means that is not necessary to extract gluten proteins separately. The two of three extraction protocols showed high technical reproducibility with coefficient of variation less than 20%. Carefully optimized extraction protocol can be advantageous for further analyses of cereal prolamins.

Keywords: extraction; prolamins; wheat; rye

INTRODUCTION

Cereal baked products are predominantly manufactured from wheat or rye flours. Storage non-enzymatically active proteins (prolamins), namely gliadins in wheat and secalins in rye, together with glutelin polymers represent the main triggering factor of celiac disease **(van den Broeck et al., 2011)**. Celiac disease is an inflammatory disorder that mainly affects the small intestine with typical gastrointestinal or extraintestinal symptoms **(Kaukinen et al., 2014)**. So far, the only therapy for celiac disease is lifelong gluten-free diet avoiding any products from wheat, rye, barley, their crossbred varieties and possibly oats **(Zingone et al., 2010)**. Majority of patients following strict gluten-free diet continue to suffer from symptoms, therefore to avoid contamination of gluten-free products by gluten and tighten labeling of such products is a priority.

According to the mobility in polyacrylamide gels, wheat gliadins are subdivided into α/β-, γ- and ω-subunits **(Wieser, 2007)** while rye secalins comprise from γ- and ω-subunits **(Shewry, 2004)**. Wheat α/β- and γ-gliadins as well as rye 40k-γ-secalins belong to the group of monomeric polypeptides with low molecular weight of approx. 28-45 kDa. Wheat ω-gliadins, rye 75k-γ-secalins and rye ω-secalins have molecular weight of approx. 50-80 kDa **(van Eckert et al., 2010)**. Glutelin polymers of wheat are generally subdivided into low-molecular weight glutenin subunits (LMW-GS) and high-molecular weight glutenin subunits (HMW-GS) **(van den Broeck et al., 2009)**, and glutelins of rye are represented by the HMW secalins **(Wieser, Koehler, 2008)**. These proteins have elevated content of two amino acids, glutamine (35% in wheat) and proline (15% in wheat), which makes them highly resistant to degradation by gastrointestinal proteolytic enzymes **(Gregorini et al., 2009)**. Therefore, analysis of structural or functional properties of wheat/rye prolamins requires an appropriately optimized extraction protocol.

Generally, based on different solubility, cereal proteins can be classified into water/salt-soluble albumins and globulins, alcohol-soluble prolamins, and high-molecular weight glutelins soluble in diluted acid/base solutions **(Osborne, 1924; Mamone et al., 2011)**. Various extraction protocols usually consisted initially of removing albumins and globulins **(Kruger et al., 1988)** or exploited co-extraction of gluten proteins (wheat gliadins and glutenins) without pre-extraction of salt soluble proteins **(van den**

Broeck et al., 2009). The differences among prolamin extraction methods involved various temperature conditions as well. Extractability of prolamins is almost unaffected by heating up to 75-80 °C **(Wieser, 1998)** which is the major problem of heat-processed foods. One way how to increase the extractability of prolamins is using a reducing agent to the extractant. However, reducing conditions are more suitable for low-molecular weight α/β- and γ-gliadins in wheat bearing 3-4 intramolecular disulphide bonds comparing to cysteine-free ω-gliadins **(Wieser, 1998)**.

Here, we investigated various extraction protocols of wheat/rye flour proteins involving different sequential extraction steps (pre-extraction of albumins and globulins, co-extraction of gluten proteins, sequential extraction of gliadins/secalins and glutelins) as well as different conditions (heating and non-heating, reducing and non-reducing).

MATERIAL AND METHODOLOGY

Biological Material

Commercially available wheat and rye flours were obtained from mill house Vitaflora (Kolarovo, Slovakia). Gliadin standard was purchased from Sigma-Aldrich (St. Louis, USA).

All extraction protocols were optimized for milligram quantities in Eppendorf tubes and extractions were performed in technical duplicates.

Extraction of cereal proteins according to Osborne (1924)

Cereal proteins were extracted using 1.5 ml of solvent per 50 mg flour by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 1 hour at room temperature. Albumin and globulin fractions were extracted with 0.5 M NaCl, the salt was then removed by distilled water, and finally, prolamins were extracted with 70% (v/v) aqueous ethanol. After each step, supernatant was centrifuged at 9000 x *g* for 15 min at room temperature.

Extraction of cereal proteins according to Osborne (1924) and further modified by Weiss et al. (1993)

To obtain salt soluble protein extract, flours (375 mg) were firstly extracted with 1.5 ml 50 mM Tris-HCl (pH 8.8) containing 1.5% (w/v) polyvinylpolypyrrolidone for 1 hour at 4 °C with vortexing at 15-min intervals. Centrifugation was carried out at 20,000 x *g* for 20 min at 4 °C. The extraction step for salt soluble proteins was repeated at the same conditions. Supernatants were pooled and referred to as "albumin/globulin" fraction. To remove buffers, pellet was re-suspended and washed in distilled water. Alcohol soluble proteins were extracted twice with 1.5 mL of 75% (v/v) aqueous ethanol by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 2 hours at room temperature. After centrifugation at 20,000 x *g* for 20 min at 4 °C, both supernatants referred to as "prolamin" fraction were pooled. The rest of ethanol was removed by re-suspending the pellet in distilled water. Finally, the "glutelin" extract was obtained by addition of 1.5 mL of SDS-DTT buffer (50 mM Tris-HCl, pH 8.8, 1% SDS 0.5% DTT) and extracted for 1 hour at room temperature with vortexing at 15-min intervals, followed by centrifugation at 20000 x *g* for 20 min at 4 °C.

Extraction of cereal proteins according to van den Broeck et al. (2009)

The two-step gluten extraction procedure was carried out at protein sample/extraction buffer ratio 1:10 (w/v). Pre-extraction of wheat gliadins and rye secalins was performed with 50% aqueous iso-propanol (v/v) by continuous mixing (Roller Mixer SRT9D, Stuart, Staffordshire, UK) at 60 rpm for 30 min at room temperature, followed by centrifugation at 10,000 x *g* for 10 min at room temperature. The residual pellet was extracted twice with 50% aqueous iso-propanol, 50 mM Tris-HCl, pH 7.5 containing 1% (w/v) DTT (ratio 1:10) for 30 min at 60 °C with vortexing every 5-10 min, followed by centrifugation at 10,000 x *g* for 10 min at room temperature. After each step, samples were properly re-suspended by mixing and sonicated for 10 min in an ultrasonic bath (Sonorex Digitec, Bandelin, Berlin, DE). Supernatants were pooled and referred to as "two-step gluten extract".

Measurement of total protein content

All protein fractions from each extraction protocol were divided into few aliquots (300 µL) and precipitated with 5 volumes of ice-cold 1 M ammonium acetate in methanol incubated at -30 °C overnight. The next day precipitate was centrifuged at 5,000 x *g* for 10 min at 4 °C, washed 2-times with ice-cold 1 M ammonium acetate in methanol, and pellet was dried using vacuum concentrator (Concentrator Plus, Eppendorf, Hamburg, DE). One aliquot was reconstituted in 100 µL of solubilisation buffer (8 M Urea, 50 mM DTT) and used to determine the protein concentration using Bradford Solution for Protein Determination (Applichem, Darmstadt, DE) according to manufacturer´s instructions with BSA as a standard. Protein quantification was performed in technical duplicate (n = 2) using BioDrop DUO spectrophotometer (Biochrom Ltd, Cambridge, UK).

SDS-PAGE analysis

The second aliquot after ammonium acetate precipitation was reconstituted in 100 µL of buffer for electrophoresis (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol) and analyzed by Tris-Tricine SDS-PAGE under reducing conditions according to the Schägger-von Jagow method **(Schägger and von Jagow, 1987)**. Proteins (10 µg/lane) were separated using BioRad MiniProtean Tetra Cell system (Bio-Rad Laboratories, Hercules, USA), followed by silver staining **(Blum et al., 1987)**. Gels were scanned using a Bio-Rad GS-800 Densitometer (Bio-Rad Laboratories, Hercules, USA) and saved as TIFF format.

RESULTS AND DISCUSSION

The aim of our work was to assess the efficiency of various extraction protocols focusing on wheat and rye flour prolamins. Both flours are routinely used in Slovak bakery industry. Many extraction protocols have recently been developed **(Singh et al., 1991; Weiss et al., 1993; DuPont et al., 2005; van den Broeck et al., 2009)** for cereal proteins, mainly wheat gliadins.

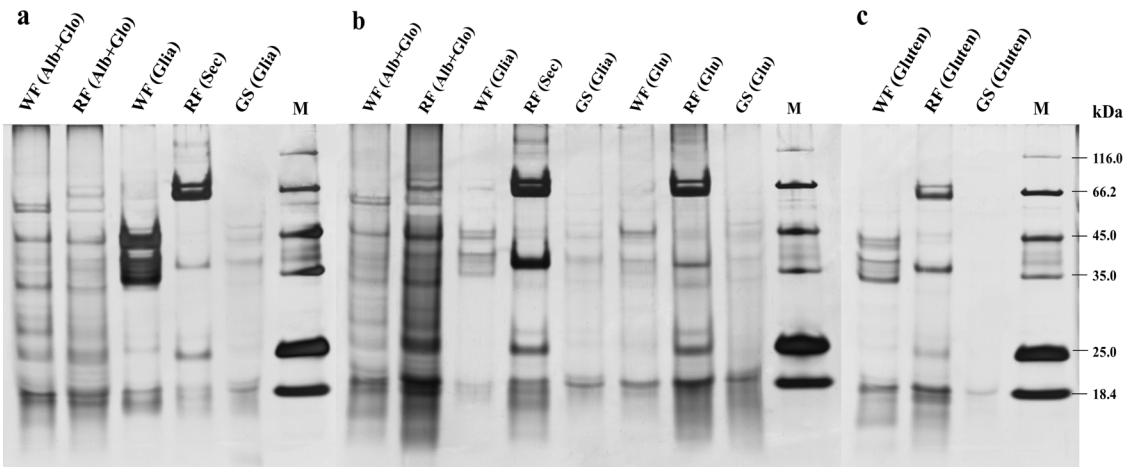

Figure 1 SDS-PAGE analysis of wheat (WF) and rye (RF) flour protein fractions followed by silver staining using different extraction protocols (a) Osborne, 1924; (b) Osborne, 1924 further modified by Weiss et al., 1993 and (c) van den Broeck et al., 2009. Gliadin standard (GS) was used as a control. Abbreviations in parenthesis refer to the protein fractions of wheat and rye flour as well as gliadin standard after each step of extraction: Alb+Glo – albumins and globulins; Glia – gliadins; Sec – secalins; Glu – glutelins; Gluten – gluten extract; M – marker.

However, pilot study of **Osborne (1924)** based on different solubility of proteins is most widely used extraction protocol. Generally, albumin and globulin fractions are soluble in water/salt solutions, prolamins are soluble in alcohols and high-molecular weight glutelins are soluble in diluted acid/base solutions. According to **Osborne (1924)** procedure the average protein content (n = 2) of wheat gliadins was only 0.03 mg.mL^{-1} (±0.01) and rye secalins 0.03 mg.mL^{-1} (±0.00) after removal of salt soluble albumins/globulins (Figure 2). To evaluate the efficiency of gliadin/secalin extraction we assigned gliadin standard as a control sample for measurement. Ethanol extraction of gliadin standard resulted in average protein content (n = 2) of 2.89 mg.mL^{-1} (±0.01) suggesting incomplete extraction of wheat/rye prolamins from flours.

SDS-PAGE analysis revealed that Osborne procedure was successful in extraction of prolamins (Figure 1a) indicating few strong bands of approx. 30-45 kDa (α/β- and γ-gliadins) and 66.2 kDa (75k-γ-secalins) in wheat and rye flours, respectively. Electrophoretic profile of gliadin standard is poorly visible (Figure 1a), most likely due to the incomplete solubility in SDS-PAGE buffer. The average protein content (n=2) of albumins/globulins was 0.06 mg.mL^{-1} (±0.02) and 0.18 mg.mL^{-1} (±0.00) in wheat and rye flours, respectively. The obtained results suggested that Bradford determination underestimates real amount of protein content comparing to SDS-PAGE analysis. Contrasted differences between Bradford measurement and electrophoretic profiling could also be assigned to very low technical reproducibility (e.g. 23% error between wheat gliadins duplicates in Bradford measurement).

The Osborne procedure was further modified **(Weiss et al., 1993)** by separated extraction of low-molecular weight subunits (gliadins in wheat, secalins in rye) and high-molecular weight glutelins, as well as by the addition of reducing agent at non-heated conditions. The average protein content (n = 2) after two sequential extraction steps was 1.76 mg.mL^{-1} (±0.01) and 0.13 mg.mL^{-1} (±0.01) of wheat gliadins and rye secalins, respectively. Albumins/globulins were also sequentially extracted; after pooling the average protein content (n = 2) was 1.08 mg.mL^{-1} (±0.06) in wheat and 0.76 mg.mL^{-1} (±0.02) in rye flours. These results indicated that salt soluble albumins/globulins in rye flour are more abundant comparing to secalins (Figure 2). Glutelins were most represented in both, wheat and rye flours (Figure 2) with average protein content (n=2) of 2.32 mg.mL^{-1} (±0.07) and 0.87 mg.mL^{-1} (±0.04), respectively.

However, SDS-PAGE analysis revealed that glutelins extracted at reducing conditions have similar molecular weights (Figure 1b) but with less intensity of α/β- and γ-gliadins (35-40 kDa) as well as 40k-γ-secalins (one band of approx. 37 kDa). These results are in agreement with general statement that glutelins are high-molecular weight subunits **(van den Broeck et al., 2009)** and are co-extracted together with monomeric gliadins and secalins. After glutelin extraction using reducing agent, rye 75k-γ-secalins (two bands of approx. 66.2 kDa) represented dominant fraction (Figure 1b) with highest protein content, 0.87 mg.mL^{-1} (±0.04). The same conclusions were also achieved in a study of **Gellrich et al., (2003)**. While the average protein content (n = 2) of gliadin standard was very high, 8.03 mg.mL^{-1} (±0.37) in gliadin extract and 2.44 mg.mL^{-1} (±0.01) in glutelin extract, electrophoretic profile indicated its impaired solubility in SDS-PAGE buffer (Figure 1b).

The last protocol used in our study **(van den Broeck et al., 2009)** differs from previous in simultaneous two-step co-extraction of gluten proteins (gliadins/secalins and glutelins) under reducing conditions at higher temperature (60 °C) and without removal of albumins/globulins. The average protein content (n = 2) in wheat gluten extract was markedly lower, 0.77 mg.mL^{-1} (±0.01), comparing to **Weiss et al., (1993)** modification protocol (Figure 2), probably due to the underestimation of results using Bradford measurement. In case of rye gluten extract, the average protein content (n = 2) was 0.50 mg.mL^{-1} (±0.08).

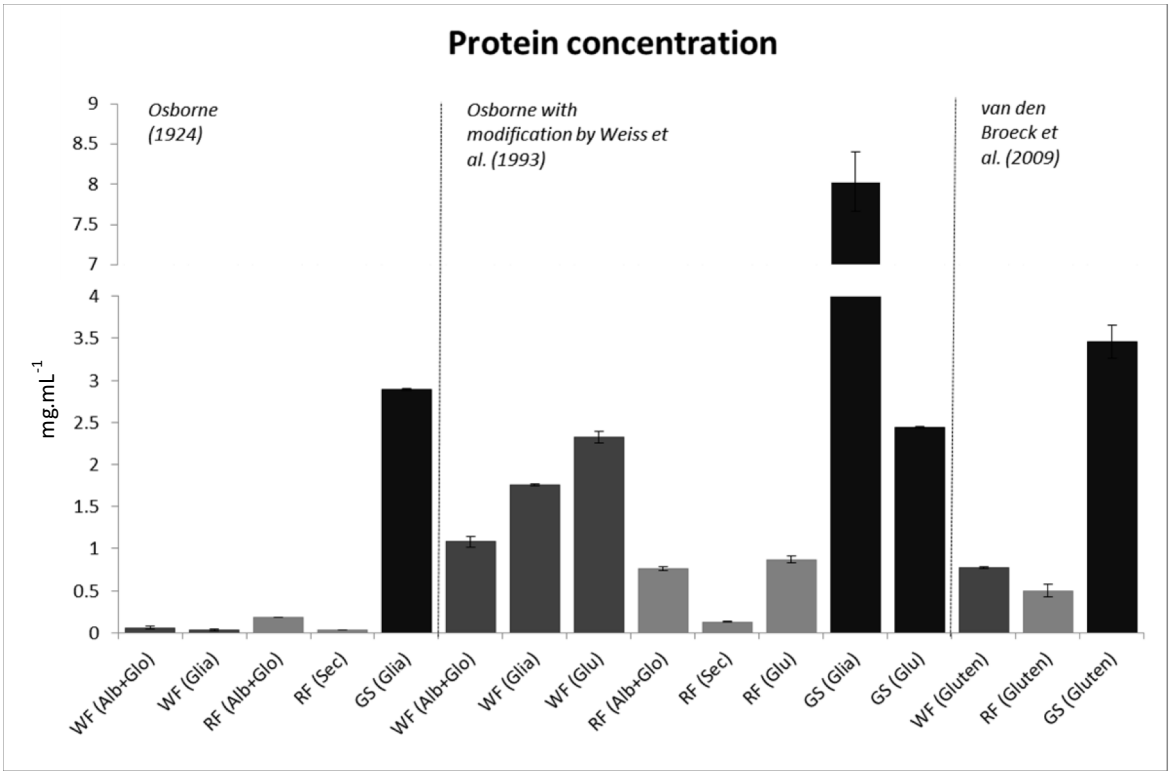

Figure 2 Protein concentration (mg.mL^{-1}) of wheat (WF) and rye (RF) flour protein fractions using Bradford solution for protein determination in three different extraction protocols. Gliadin standard (GS) was used as a control. Abbreviations in parenthesis refer to the protein fractions of wheat and rye flour as well as gliadin standard after each step of extraction: Alb+Glo – albumins and globulins; Glia – gliadins; Sec – secalins; Glu – glutelins; Gluten – gluten extract. Data presented is averages ± standard deviation (n = 2). All error bars are included.

SDS-PAGE analysis revealed typical bands of approx. 30-45 kDa (α/β- and γ-gliadins) and 37 kDa (40k-γ-secalins) in wheat and rye flours, respectively (Figure 1c). Moreover, 75k-γ-secalins (two bands of approx. 66.2 kDa) were also detected (Figure 1c) in rye gluten extract. The average protein content (n = 2) of gliadin standard in gluten extract was 3.46 mg.mL^{-1} (±0.20). Similarly, to previous protocols, separation of gliadin standard on polyacrylamide gel was insufficient due to the incomplete solubility in SDS-PAGE buffer (Figure 1c).

Precisely optimized extraction protocol is a critical step to analyze cereal proteins many of which causing allergies or food intolerances. In our study we aimed to compare different extraction protocols for wheat/rye prolamins as a main triggering factor in celiac disease. Generally, wheat contain higher amount of prolamins than rye which was proved by e.g. fractionation of protein complex **(Mickowska et al., 2012)**. Both, wheat and rye prolamins has highest content of two amino acids, glutamine and proline **(Mickowska et al., 2012)** suggesting their poor digestibility by gastric enzymes. As a result, Glu- and Pro-rich peptides containing T-cell stimulating epitopes are occurred that can cause celiac disease. Ancient varieties of wheat and rye could also be harmful **(Ciclitira et al., 2005; Hybenova et al., 2013)** as they are genetically similar with amino acid composition comparable to modern varieties. However recent studies **(van den Broeck et al., 2010)** revealed that e.g. presence of the Glia-α9 epitope was lower in the wheat landraces.

In our study we aimed to analyze prolamin extract from commercially available wheat and rye flours. According to Bradford method, the total protein content was slightly lower comparing to other studies **(van den Broeck et al., 2009)**. The differences could be attributed by using the different wheat varieties. Moreover, wheat/rye flours used here were milled during different conditions (procedure not described) which probably resulted in a loss of proteins, for instance, wheat ω-gliadin fractions with molecular weight of 50-80 kDa were almost unable to detect in SDS-PAGE gels. Contrary, **van den Broeck et al., (2009)** described that ω-gliadins/D-type LMW-GS fractions were abundantly presented in all wheat varieties. In summary, the efficiency of extraction protocol depends not only on the cereal protein source, but also on working conditions and analytical method of their identification/quantification.

CONCLUSION

Various extraction protocols with different working conditions examined here were generally efficient in extraction of wheat/rye flour prolamins. Although, the pilot Osborne procedure yields in lower protein content using Bradford measurement comparing to SDS-PAGE analysis, it is still considered as an effective method due to its rapid and simple nature. Up to date, several modifications of extraction conditions are under investigation using multiple extraction steps or reducing agents. These protocols are usually time consuming, however, carefully optimized conditions can reduce not only time but also can increase the protein content in extracts. In some cases, the huge amount of starting material is required for analysis. Therefore, in our study we optimized all extraction

protocols for milligram quantities using Eppendorf tubes. Except the Osborne procedure, the two protocols used here showed high technical reproducibility according to Bradford with coefficient of variation less than 20%. Assuming above mention facts, van den Broeck protocol is a good choice for simultaneous co-extraction of gluten proteins from wheat/rye flours.

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