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The MS^E-proteomic analysis of gliadins and glutenins in wheat grain identifies and quantifies proteins associated with celiac disease and baker's asthma☆

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ABSTRACT

Precise content of gliadin (Glia) and glutenin (Glu) proteins in wheat grain are largely unknown despite their association with celiac disease, various allergies, and physical processing properties of wheat. Developing methods to quantitatively measure clinically relevant proteins could support advancement in understanding exposure thresholds and clinical study design. The aim of this study was to use a data-independent mass spectrometry (MS^E) approach for quantifying gliadin and glutenin proteins in wheat grain. The biologically replicated analysis yielded concentrations for 34 gliadin and 22 glutenin proteins. The primary focus of this survey was on measuring celiac disease proteins and baker's asthma associated proteins along with the proteins associated with viscoelastic properties of wheat flour and grain texture. The technical coefficients of variation ranged from 0.12 to 1.39 and indicate that MS^E proteomics is a reproducible quantitative method for the determination of gliadin and glutenin content in the highly complex matrix of protein extracts from wheat grain. This article is part of a Special Issue entitled: Translational Plant Proteomics.

1. Introduction

Wheat (*Triticum aestivum* L.) is a widely used cereal for human consumption. Wheat grain has some unique properties when processed into wheat flour that depend on protein composition [1]. Based on solubility, wheat grain proteins can be divided into the following: i) water soluble albumin, ii) salt soluble globulin, and iii) alcohol soluble gluten proteins [2]. Gluten proteins represent up to 80% of wheat proteins [3] and are divided into gliadins (Glia) and glutenins (Glu) based on electrophoretic mobility [4,5]. Glia proteins are mostly monomeric proteins with molecular weight 28–55 kDa and can be divided either based on electrophoretic mobility into α/β-, γ-, and ω-gliadins or based on amino-acid sequence into ω5-,

Abbreviations: Glia, protein fraction containing mostly gliadins; Glu, protein fraction containing mostly glutenins; MSE, data-independent mass spectrometry; emPAI, exponentially modified protein abundance index; GFP, Glu-1-fibrinopeptide B; CV, coefficient of variations; HMW GS, high molecular weight glutenin subunits; LMW GS, low molecular weight glutenin subunits.

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ω1,2-, α/β- and γ-gliadins [6,7]. Glu proteins can be sorted according to their content of high (HMW 70–90 kDa) and low (LMW 20–45 kDa) molecular weight (MW) glutenin subunits [8,9]. In addition to Glia and Glu, wheat flour contains a number of minor storage proteins, such as "avenin-like" proteins that are similar to oat storage proteins called avenins [10].

In wheat, Glia and Glu proteins are often associated with celiac disease and various allergies [for review see 11–13]. The development of methods for quantitative determination of clinically relevant Glia and Glu proteins is important in order to understand the exposure thresholds and to support clinical allergy study designs. Presently, quantitative methods for the determination of clinically relevant Glia and Glu proteins are mostly based on immunology techniques [14–16]. However, techniques based on mass spectrometry are likely to provide more robust characterization of Glia and Glu proteins [16,3]. Among these, a novel data-independent MS^E acquisition method takes advantage of a data collection approach that focuses on maximizing the amount of data collected for a proteome by rapid switching between low and elevated collision energies to assess multiple target proteins simultaneously [17]. Evaluation of the data output during MS^E analysis of tomato root microsoms showed that MS^E is a reproducible and reliable method for protein identification [18]. In combination with data-dependent MS/MS approaches, MS^E proteomics has also provided reliable identification of cytosolic ribosome proteins isolated from the model plant, *Arabidopsis thaliana* [19]. In this work by Hummel et al. [19], MS^E was used in combination with peak alignment to measure the relative abundance for several ribosomal proteins in a multiplex (proteomic) approach. Previous reports using non-plant systems have shown that MS^E is also capable of providing absolute quantitative analysis by analyzing the signal response of a known quantity of internal standard that has been spiked into the sample [20,21].

The aim of this study was to develop a reliable and robust method based on MS^E that will provide reproducible, quantitative measures of the wheat grain proteins, Glia and Glu, some of which are relevant for wheat food allergy.

2. Material and methods

2.1. Plant material

Bread wheat cultivar "Viginta" was provided by SELEKT Ltd., Bučany. To avoid seed-to-seed variability, 1 kg of wheat grain was divided into 4 equal parts. Each part was then divided into five 50 g portions that were each milled by electric grinder (50 g of grains three times for 5 s to avoid heat). Milled grain portions were stored at −20 °C until use. Each portion of ground grain was considered a replicate sample.

2.2. Extraction of alcohol soluble proteins from wheat grain

Gliadin (Glia) and glutenin (Glu) proteins were extracted from 200 mg of a milled grain sample according to Broeck [22]. Glia proteins were obtained after extraction with 1 ml of 50% (v/v) aqueous iso-propanol by continuous mixing (Thermomixer comfort, Eppendorf) at 1000 rpm for 30 min at room temperature, followed by centrifugation at 2500×*g* for 15 min at room temperature (RT). The procedure was repeated one time. To obtain Glu proteins, the procedure above was repeated and the pellet was further extracted with 1 ml of 50% (v/v) aqueous iso-propanol, 50 mM Tris–HCl (pH 7.5) containing 1% (w/v) DTT for 30 min at 60 °C with mixing every 5 to 10 min, followed by centrifugation at 10,000×*g* for 10 min at RT (Fig. 1).

2.3. Protein "in-solution" digestion in alcohol

Because alcohol interferes with total protein determination according to the Bradford method [23], wheat grain extracts produced by the method described in Section 2.2 were divided into two equal aliquots. The first aliquot was then used to determine the protein concentration and second for further analysis by mass spectrometry. Protein from the first aliquot were precipitated with 5 volumes of 0.1 M ammonium acetate in 100% methanol and washed with 0.1 M ice cold ammonium acetate in 100% methanol twice. After centrifugation at 5000 *g* at 4 °C for 10 min, the liquid phase was removed. Precipitated proteins were reconstituted in 100 μl of solubilization solution (8 M urea, 2 M thiourea, 2%CHAPS, 50 mM DTT). Total protein concentration was then measured according to Bradford [23].

One hundred micrograms of the total protein preparation (second aliquot) was reduced with DTT (final concentration 10 mM) and alkylated with IAA (final concentration 40 mM), gently mixed, and incubated at RT for 45 min in the dark. Trypsin (Promega, Madison, WI, USA) in 1 mM NH_4HCO_3 was added to protein solution (1:25), gently mixed, and incubated overnight at 37 °C (16–20 h). The reaction was stopped with formic acid (final concentration 5%). The resulting tryptic digest was concentrated and desalted using Peptide Cleanup C18 spin columns (Agilent Technologies) according to the manufacturer's instructions and stored at −80 °C until mass spectrometry (MS) analyses.

2.4. Mass spectrometry

Prior to MS analysis, the trypsin digested extracts were concentrated under vacuum to 20 μl using Concentrator plus

Fig. 1 – The workflow. Gliadins (Glia) and glutenins (Glu) were extracted from milled wheat grain. Gliadins and glutenins were then digested with trypsin, peptides were spiked with internal standard (predigested bovine hemoglobin, 186002327), separated by nanoAcquity ultraperformance liquid chromatography (UPLC), and analyzed by MS^E. For protein identification alternate scans between low and elevated energies were used to generate fragmentation data without precursor selection. For protein quantification, MS signals for the three most intense tryptic peptides were calculated for internal standard, bovine hemoglobin. Then, the average MS signal response from the standard was used to determine a universal signal response factor (USRF, counts/mol of protein). The USRF was then used to determine the concentration of the target proteins. This approach resulted into quantification of 34 Glia and 22 Glu proteins in at least biological triplicate.

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