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REGULAR ARTICLE

PROTEOMICS ANALYSIS OF WHEAT AND BARLEY GRAIN

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ABSTRACT

Plant biotechnological research is focused on research and application of objective methods for determination of quality production. Cereals are main part of human nutrition and strategic resources. It is very important to expand knowledge of cereal proteome by usage of new methods for protein detection. Our work was focused on wheat and barley proteome analysis by 2-D electrophoresis not only in wide range of pH (pH 3-11), which is suitable for analysis of whole seed proteome, but also in narrow range of pH (pH 6-11) which is suitable for detection of gluten proteins, mainly. Results show that storage proteins are the main part of cereal grain proteins. Inhibitor of alpha-amylase and inhibitor of alpha-amylase/trypsin were the most represented. Group of barley storage proteins showed similarity to wheat prolamin proteins, but there were observed some differentiations in amount of protein. Similarity was found in pH range 7-10 between proteins with molecular weight 45-55 kDa.

Keywords: wheat, barley, 2-D electrophoresis, protein maps, coeliacdisease

INTRODUCTION

Cereals grains are one of the most important sources of energy and nutrients in human and animal beings. Mainly, wheat and bread are essential to human life and are the basic food in many countries.

The tribe *Triticeae*includes genotypes of wheat and barley which have many related genetic and biochemical features **(Shewry, 2004/Rev. 2006)**. The most important impacts on the nutritional quality and functional properties of grains have storage proteins. Amount of storage proteins is about 50% or more of the total proteins in wheat and barley mature grains **(Shewry and Halford, 2002)**.

The prolamin fractions of wheat and barley consist of complex mixtures of components with M_r values by SDS/PAGE ranging from about 30,000 to 100,000. Despite this high level of polymorphism, the individual components can be classified on the basis of their amino acid compositions and sequences into only three groups: the sulphur-rich (S-rich), sulphur-poor (S-poor) and high molecular-weight (HMW) prolamins **(ShewryandTatham, 1990)**.

Only the wheat grain contains gluten proteins, which are able to form elastic dough which is necessary for production of leavened bread. These gluten proteins are also needed to make the great variety of foods that are associated with wheat around the world **(Wrigley, 2009)**.

Gluten-sensitive enteropathy is also known as coeliac disease (CD) and is one of the most frequent intolerances of food. It is an inflammatory disease of the upper small intestine (jejunum, duodenum) caused by consumption of cereal products **(Mowat, 2003)**.

CD appears to represent a spectrum of clinical features and presentations, including malabsorbtion of nutrients generally, diarrhoea, and loss of appetite. In children, there may be abdominal distension, vomiting, and muscle wasting, accompanied by impaired ability to thrive **(Anderson and Wieser, 2006)**.

Two–dimensional gel electrophoresis has frequently been used to characterize the diversity of protein components in wheat and barley for example, in relation to genetic analysis of gluten proteins **(Jackson *et al.*, 1983)**. However, only since the end of 1990s have large–scale high-throughput proteomic approaches been applied to wheat and barley grain proteins. Proteomic analyses exploit the availability of genome sequences to identify proteins based on the use of mass spectrometry to determine the masses of peptide fragments resulting from the tryptic digestion of small amounts of components separated by electrophoresis. Unfortunately, the genome of wheat has not been fully sequenced **(Goff *et al.*, 2002)**.

The objectives of our study wasthetwo-dimensional gel electrophoresis separation of wheat and barley seed proteins focused on preparation protein maps of wheat and barley from the point of view the Coeliac disease. There was also realized the comparative study of wheat and barley proteome with the aim of differences.

MATERIAL AND METHODS

Materials

Seeds of wheat (cv. BREA) and barley (cv. EXPRES) were obtained from the Gene Bank of the Research Institute of Plant Production, Piešťany in the Slovak Republic and were milled by CU Mill, (Lionhill Company a.s.) to a homogenous flour.

Methods

Two - dimensional gel electrophoresis (2-DE)

Samples preparation

Proteins were extracted from the flour by adding 1 ml of buffer [250 µl DTT (28 mg/ml), 12.5 µl IPG buffer (carrier ampholytes) and 237.5 µl ultra-pure water to 2 ml IPG rehydration buffer (7 M urea, 2 Mthiourea, 2 % CHAPS)] to 50 mg of flour. The samples were then wheel-mixed for 1 h, RT and then centrifuged 3 min, 9, 000 x g, RT. The protein content of the supernatant (SN) was estimated by Coomassie Plus protein assay (Thermo Scientific, Pierce, UK) (based on the Bradford assay) and samples were stored at -20 ºC until use. The protein content of the oat extract was insufficient, so the Compact-Able™ Protein Assay Preparation Reagent Set (Thermo Scientific, Pierce, UK) was used to precipitate the protein, which was then re-suspended in the extraction buffer as described previously.

1st Dimension - Isoelectric focussing (IEF): Immobilised pH gradient (IpG) strips (GE Healthcare, Amersham UK), 7 cm, pH 3-11 NL and pH 6-11, were used for the first dimension. Strips were hydrated O/N at 20°C 125 µl rehydration buffer [7 M urea; 2 Mthiourea, 2 % w/v CHAPS; 0.5 M DTT; relevant pH range IPG buffer; 0.001 % w/v bromophenol blue] containing ~ 40 µg protein of sample. Focussing was performed at 20 ºC,

current 50 µA *per* strip (300V 30 min 0.2 kVh; 1000 V 30 min, 0.3 kVh; 5000 V, 1 h 20 min, 4.0 kVh; 5000 V, 25 min, 2.0 kVh). Focussed IPG strips were stored at -80 ºC until required.

2nd Dimension - SDS PAGE: Focussed IPG strips were equilibrated in tris-acetate equilibration buffer [0.122 M tris-acetate containing 0.5 % w/v SDS; 6 M urea; 3 % w/v glycerol; 52 mM DTT; 0.01% w/v Bromophenol blue]. After 30 min strips were derivatised in the dark with 0.14 Miodoacetamide in equilibration buffer for a further 30 min. Strips were then transferred to 1 mm, 4-12 % Bis-Tris Zoom™ gels for the second dimension. Gels were run at 200 V and 100 W per gel for 35 min using 1 x MES SDS Running Buffer. Gels were fixed O/N in 40 % v/v methanol containing 10 % w/v TCA before staining with SYPRO Ruby Stain (Invitrogen, UK) in the dark O/N. After de-staining O/N with 10 % v/v methanol and 6 % TCA, gels were imaged using a high-resolution molecular imager (PHAROS FX™ Plus, Bio-Rad, UK). Imaged gels were returned to de-stain solution and stored in the dark at 4 °C until required.

RESULTS AND DISCUSSION

Protein maps of Wheat

Many proteins were focussed over pH 3-11 (figure 3) between 6,000 – 120,000 Da and the most abundant proteins were observed in the basic region of the gel; including three abundant proteins visible at approximately pI 7, 14,000 Da (figure 1ii). These corresponded to trypsin/alpha-amylase inhibitors previously identified from the soluble fraction of wheat dough **(Salt *et al.*, 2005)**, as well as in immature wheat-grain endosperm proteins **(Skylas *et al.*, 2000)**. A larger abundant protein group was visible between 36-55,000 Da (figure 1i) and these proteins were resolved at the correct mass and pI range for gluten, previously identified by **Skylas *et al.*, (2000)**; where a group of high molecular weight glutenin subunits with pI 5.5-6.5, 66-100,000 Da and a group of omega gliadins with pI 5.0-6.0, 45-55, 000 Da were observed using 2-DE. This group is particularly important for celiac disease, so these basic proteins were separated over a narrower range of pH 6-11 (figure 1 iii). It was obvious that these proteins were highly abundant as they were much better resolved and well-defined spots were achieved over this basic pH range (pH 6-9). These proteins formed a distinct pattern of spots allowing comparison with 2-D maps of other cereal proteins. It was also observed, that the alpha-amylase/trypsin inhibitors were better resolved in the basic region (figure 1iv). Although these proteins are not linked to celiac disease, they have been reported to trigger

baker's asthma and have been linked to food allergy **(James *et al.*, 1997)**. The overall spot pattern from wheat (cv. Brea) over pH 6-11 (figure 1) was partially similar to those reported by **Skylas *et al.* (2000)** and **Akagawa*et al.* (2007)**. These included proteins identified as gamma-gliadins (~ pI 8-10, M_rs 28 - 30, 000 Da), alpha/beta-gliadins (~pI 6.5-7.5, M_rs 25 - 35, 000 Da) and gluten proteins (pI 6-10, M_rs 35 - 60, 000 Da). A cluster of polypeptides resolved at the end of pH 6-11 gel may be tritin (~pI 9.5-10, M_rs 25, 000 Da) (figure 1v), a protein synthesis inhibitor, which was previously identified by MALDI-MS **(Salt *et al.*, 2005)**.

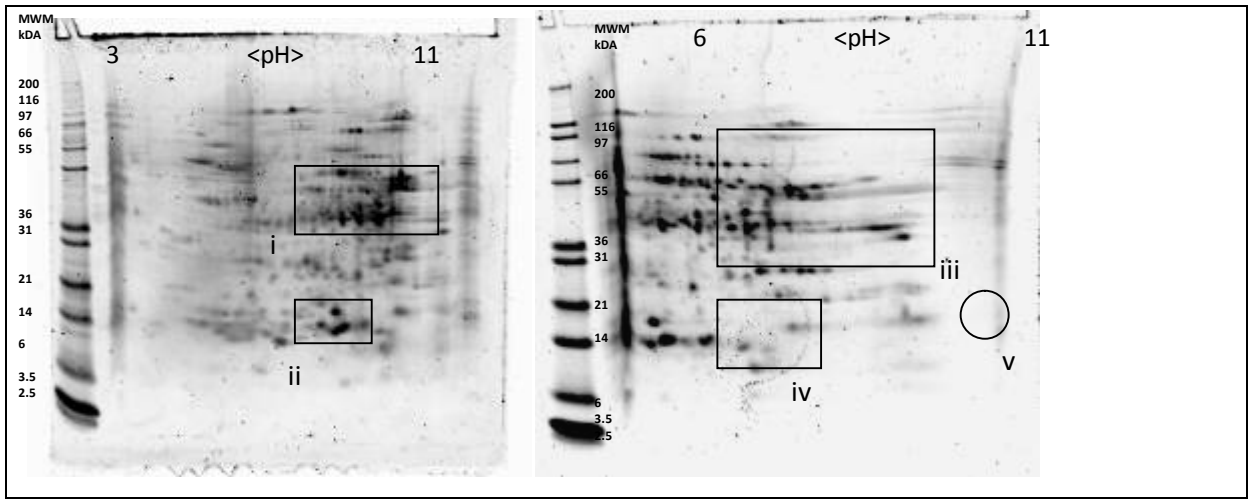

Figure 1 Protein maps of wheat (cv. Brea), **MWM –** molecular weight marker

Protein mapsof Barley

As with the wheat proteins, many spots were focussed between pH 3-11, and the apparent molecular weights of the resolved proteins were between 6-200,000 Da (figure 2). A group of intense spots of low molecular mass (6-21,000 Da) and pI 4.5 of 5.5 (figure 2i), were observed which corresponded to alpha-amylase/trypsin inhibitor proteins previously identified in developing barley seeds by **Finnie *et al.*, (2002)** and **Perrocheau *et al.*, (2005)**. A second group of alpha-amylase/trypsin inhibitor proteins have been previously identified **(Bak-Jensen *et al.*, 2004)** with a low molecular weight range from 12-16,000 Da and pI 5-7 and could be the group of proteins highlighted between 12-21,000 Da (figure 2iv and figure 2v).

Perrocheau *et al.*, (2005) identified the hordeins and 12 S seed storage proteins, which were observed on both the pH 3-11 gel (figure 2ii) and 6-11 gels (figure 2iii) in the range of 35-45,000 Da, in the pI 6-8 range. Specifically, spots observed at approximately 45,000 Da, pI 8 should correspond to the B3 Hordein.

Bak-Jensen *et al.* (2004) and **Perrocheau *et al.*(2005)** identified beta-glucosidase (45,000 Da, pI 6.5), alcohol dehydrogenase (11,000 Da and pI 8) and non-specific lipid-transfer protein (60-62,000Da, pI 6-8). Glyceraldehyd-3-phosphate dehydrogenase (42,000 Da and pI 7) could also be present according to identifications made by **Bak-Jensen *et al.* (2004)**; these proteins may be present in highly populated areas of the gels making it difficult to determine by eye.

A protein visible at the very basic region on the pH 6-11 gel (figure 1vii) was also observed at a similar pI and mass on the wheat gel (~pI 9.5-10, M_rs 25, 000 Da - figure 1v). This protein was focussed and resolved at the pI and mass corresponding to tritin (as described for wheat).

On the pH 3-11 gel (figure 2ii) an abundant region of spots was observed ranging from pI 6-9. Some protein spots appeared not-well defined with an irregular shape, or two spots very close to each other, indicating that at least two proteins were resolved at similar mass and pI, resulting in overlapping making it difficult to distinguish between proteins. Due to their position on the gel it was assumed that these proteins were the hordeins and were then separated over a pH range of 6-11 (figure 2iii). This separation allowed the visualisation of many more storage proteins than were visible over a broad range, and resolved the issue of proteins overlapping. The gluten proteins appeared as isoelectric streaks suggesting that they may have undergone post-translational modifications (PTM). The spot patterns of the gluten proteins from barley had similarities to the prolamins from wheat (figure 1iii).

We could not assign any identifications to the group of intense unidentifiable protein spots of low molecular mass (10-20,000 Da) and pI 6 of 8 (figure 2vi).

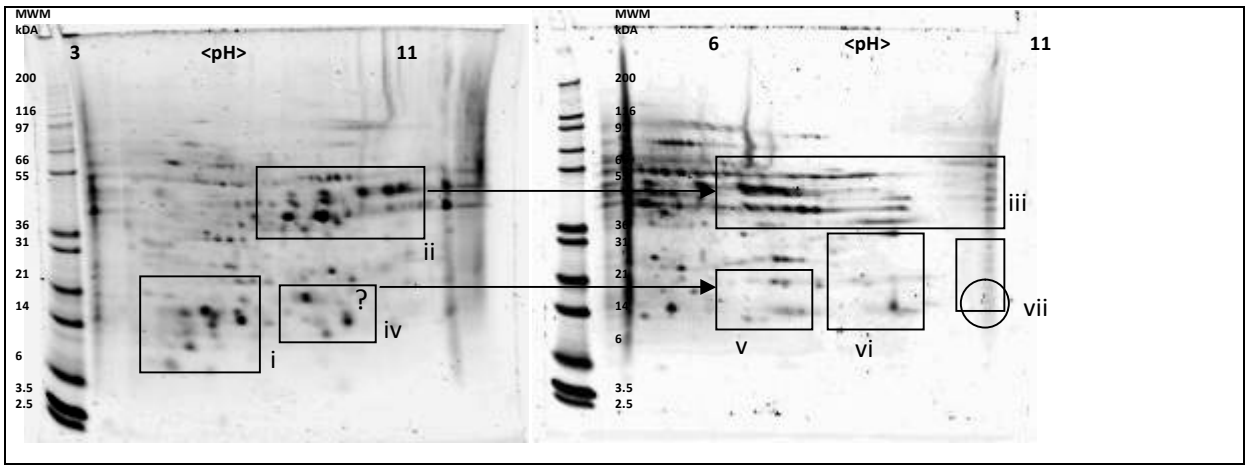

Figure 2 Protein maps of barley (cv. Expres), **MWM –** molecular weight marker

The most abundant endosperm proteins from wheat identified in this work belong to the seed storage gliadin and glutenin families, alpha-amylase inhibitor and alpha-amylase/trypsin inhibitor families.

A larger abundant group of storage proteins was visible at the very basic region on the pH 6-11 gels. The spot patterns of the gluten proteins from barley had similarities to the prolamins from wheat although some spots did differ in their abundance. These spots were, in general, more intense in fractions of wheat cv. Brea seeds. Similarities were found mainly in range of pI 7-10 and Mrs 45-55,000 Da that is matched to the region of gluten proteins, that are one of the Coeliac disease triggers. Accordingly, spots of higher intense are visible on the bottom of the gels at approximately pI 5-9 and Mrs 14,000 Da these corresponded to trypsin/alpha-amylase inhibitors.

These proteins have different roles in storage, folding, synthesis of proteins, nitrogen metabolism, glycolysis, pathogen defence, carbohydrate metabolism, stress, and detoxification **(Bak-Jensen *et al.,* 2004).** The function of a few of identified sequences is yet unknown. Since the complete barley genome is not determined, the fraction of basic proteins of the proteome is not known. In 2-D gels, numerous spots were identified as being proteins of the alpha-amylase/trypsin inhibitor family, also called chloroform/methanol (CM) soluble proteins due to their extractability by these mixtures of organic solvents. Noticeably, the inhibitory action of these proteins is probably not directed against barley enzymes, but against enzymes from insects and other pathogens **(Franco *et al.,* 2002)**. These inhibitory proteins were found in the pI 4-7 range and M_rs range of 12-16,000 Da of barley mature seed.

Although the hordeins are very abundant in the endosperm of the mature seed, **Bak-Jensen *et al.,* (2004)** identified only one spot of them, because majority was not readily soluble in the low salt buffer, which they had used in their study. Hordeins were also identified on barley 2-D gels. Hordeins are prolamins, a major family of cereal seed storage proteins. These major proteins of barley endosperm generally display poor solubility in water. They consist of a complex polymorphic mixture, with major groups B, C, D and gamma designated on the basis of their apparent molecular mass by electrophoresis. They are characterized by a high proline and glutamine content, and a fraction of the hordein polypeptide fragments. The B hordeins, which are relatively sulfur-rich, are present as polymers stabilized by interchaindisulfide bonds, and are the major hordein fraction present in barley grain, accounting for about 80% of the total hordeins. The D hordeins, which are also relatively sulphur-rich, only account for a few percent of the fraction, and the gamma hordeins are minor components **(Evans andSheedan, 2002)**.

CONCLUSION

Proteomics help us to fill and understand the space between expression of genes and grain composition. Knowledge of it allows us to identify genome parts which determine not only composition of protein and enzymatic activities, but also relationship between gene expression and growth conditions. Usage of proteomics in the future will include the exploration of protein markers which will be responsible for significant aspects of genotype or genotype-environment interactions.

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