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TECHNICAL BRIEF

Staining efficiency of specific proteins depends on the staining method: Wheat gluten proteins

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To analyze gluten proteins involved in celiac disease (CD) by proteomic analysis, prolamins extracted from hexaploid wheat varieties were analyzed by SDS-PAGE and 2-DE. Differences between staining methods (CBB, silver nitrate, SYPRO Ruby, and CyDye) were analyzed in comparison to immunoblotting. Staining efficiency varied *per* protein across methods, and complete staining of all gluten proteins could not be achieved by one of these methods. Care should be taken in the selection of staining method especially if one wants to relate the results to data obtained by immunoblotting.

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Hexaploid wheat (*Triticum aestivum*) is worldwide grown and used for preparation of baked products, such as bread, due to the presence of gluten proteins. Gluten proteins form an elastic matrix when wheat flour is mixed with water and give volume to bread during rising. Wheat gluten extracts are also increasingly used as an additive in many food products such as soups, sauces, candies, *etc.* Gluten proteins from wheat are composed of monomeric gliadins and polymeric glutenins. Gliadins are subdivided into α/β -, γ -, and ω -gliadins. The individual polypeptides of glutenins are subdivided into high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS). These subunits are linked by intermolecular disulfide bonds. Gliadins and glutenins together are called prolamins and contain large amounts of the amino acids proline and glutamine and only small amounts of arginine, lysine, and histidine. A negative aspect of prolamins is that they can contain

immune responsive peptides that can cause celiac disease (CD; gluten intolerance) in genetically susceptible individuals [1]. After consumption of gluten-containing foods, specific peptides from prolamins trigger an immune response that causes damage to the small intestine in these patients. This leads to a range of symptoms including altered bowel habits, malnutrition, and weight loss [2].

In wheat breeding programs a lot of effort has been put in analyzing allelic codes for gliadins and glutenins in order to come to high yield, high resistant wheat cultivars with good baking quality [3]. These allelic codes that are used in breeding to predict dough properties are not always suitable for the identification of prolamins containing CD immune responsive peptides because of the low abundance of some of these proteins. To study the presence and variance of these immune responsive prolamins peptides in different modern wheat varieties, a proteomics study was initiated.

As prolamins have an atypical amino acid composition, the staining behavior of these proteins was expected to deviate from standard. We therefore investigated the effect of different staining procedures after gelelectrophoretic separation of total prolamins extracts in comparison to immunoblotting using antibodies that visualize prolamins containing CD immune responsive peptides. Prolamins from bread wheat varieties were analyzed by SDS-PAGE and 2-DE. Separate SDS-PAGE gels were stained with CBB R-250 (traditional), G-250 (colloidal, PageBlue™), silver nitrate, and SYPRO Ruby.

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Abbreviations: CD, celiac disease; HMW-GS, high molecular weight glutenin subunits; LMW-GS, low molecular weight glutenin subunits

Because of similar molecular masses, many gliadins share a mobility region with LMW-GS if analyzed by SDS-PAGE. 2-DE results in increased resolution of these proteins. One variety was selected for 2-DE, for which proteins of one gel were visualized with CBB R-250, SYPRO Ruby, and CyDye labeling. In the CyDye experiment, 2-DE was performed using Cy3, one of the CyDye DIGE fluor minimal dyes (Cy2, Cy3, and Cy5) used in DIGE [4]. In DIGE, CyDyes are used for labeling different protein samples prior to analyzing them on the same gel. The CyDyes have very similar molecular masses and are positively charged to match the lysine residues of the protein to avoid shifting from the unlabeled protein. Cy2 is frequently used to label a reference sample that can be included as an internal standard on each gel. DIGE makes it possible to detect and quantitate differences between experimental pairs of samples resolved on the same gel and between gels. This method is very sensitive, 0.25–0.95 ng can be detected [5], and by using an internal standard sample, comparison between gels is highly facilitated. For further downstream characterization methods, such as MS, 2-DE gels can be poststained by SYPRO Ruby or CBB.

The total prolamin content was extracted from six modern hexaploid wheat varieties: Bovictus, Combi, Sperber, Rektor, Toronto, and Ambras, as described (van den Broeck *et al.*, submitted for publication). Fifty milligrams of wheat flour was extracted with 50% isopropanol with continuous mixing (MS1 Minishaker, IKA Works) at 1000 rpm for 30 min at room temperature, followed by centrifugation at 10 000 rpm for 10 min. The residue was re-extracted twice with 50% isopropanol/1% DTT/50 mM Tris-HCl (pH 7.5) for 30 min at 60°C. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep® FP220A Instrument for 10 s at speed 6.5 m/s followed by sonication for 10 min in an ultrasonic cleaner bath (Branson 3510, Branson Ultrasonics Corporation). Extracts were combined and proteins were analyzed on SDS-PAGE gels (10% acrylamide) using the Hoefer SE 260 mighty small II system (GE Healthcare). Separate SDS-PAGE gels were stained with traditional CBB R-250 (0.1% w/v in 45% methanol/10% acetic acid, for 1 h), with colloidal CBB G-250 (PageBlue, Fermentas, for 16 h), silver nitrate [6], and SYPRO Ruby Protein gel stain (1/20 dilution in 20% ethanol, BioRad Laboratories, according to manufacturer's instructions). For CBB R-250 staining, 15 µg protein was loaded of each sample, and for CBB G-250 staining, 2 µg protein was loaded of each sample. For staining with silver nitrate and SYPRO Ruby, 1 µg protein was loaded. Gels stained with CBB R-250 were destained for 2 h or alternatively for 16 h in 10% ethanol/7.5% acetic acid. From one gel, proteins were blotted onto NC (0.2 µm, BioRad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (BioRad Laboratories) at 100 V for 1 h. Blots were incubated and visualized as described [7] using monoclonal antibodies (mAbs) specific for stimulatory T-cell epitope Glia-α20 [8].

Prolamins from wheat variety Rektor were also analyzed by 2-DE and staining properties were compared by applying CBB R-250 staining, SYPRO Ruby staining, and Cy3 label-

ing. For 2-DE, proteins were separated in the first dimension on Immobiline Drystrip pH 3–10 of 24 cm (GE Healthcare). Protein (50 µg) was labeled with Cy3 (GE Healthcare) according to manufacturer's instructions. For overnight rehydration of IPG strips, Cy3 labeled protein was combined with unlabeled protein (100 µg) to a final volume of 450 µl in rehydration buffer (6 M urea, 2 M thiourea, 2% CHAPS, 20 mM DTT) complemented with 0.5% IPG buffer pH 3–10 (GE Healthcare) according to manufacturer's instructions. The rehydrated strips were focused on an IPGphor (GE Healthcare) until 50 000 V·h. Prior to second dimension, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris-HCl, pH 8.8) containing 1% w/v DTT, followed by 15 min in equilibration buffer containing 2.5% w/v iodoacetamide. Separation in the second dimension was performed on SDS-PAGE gels (10% acrylamide) using the Hoefer ISO-Dalt System (GE Healthcare). After electrophoresis, a gel image was obtained for Cy3 with an FX-scanner (BioRad) using an excitation wavelength of 532 nm and an emission wavelength filter of 555 nm. Following the Cy3 scan, the gel was poststained with SYPRO Ruby or with CBB R-250.

SDS-PAGE and 2-DE analysis of prolamins from wheat flour using the different staining procedures resulted in different staining intensities for individual proteins depending on the dye used (Figs. 1 and 2). Each method resulted in a slightly different protein pattern. Traditional CBB R-250 staining has the limitation of a low detection sensitivity (8–16 ng) as compared to PageBlue (5 ng), silver nitrate (4–8 ng), and SYPRO Ruby staining (1–2 ng) [9]. This was compensated by loading more protein on these gels (Figs. 1A, 1B, and 2A). CBB R-250 interacts with lysine and arginine residues [10]. All CBB staining methods are easy and compatible with characterization methods such as MS. However, it was observed that the length of destaining appeared to be very important for the resulting protein pattern. All CBB R-250 stained prolamins destained to some extent. They were destained in water, as was already shown by Hurkman and Tanaka [11]. Proteins in the ω-gliadin region (66.3–55.4 kDa) destained to a greater extent than other proteins and even diffused out of the gel. We determined that destaining also occurs in 10% ethanol/7.5% acetic acid. Comparing Fig. 1A with 1B shows that not only proteins in the particular ω-gliadin region diffused out of the gel, but also proteins with lower molecular masses disappeared from the gel (boxed proteins in Fig. 1). Some of these proteins were identified with immunoblotting to contain the specific T-cell epitope Glia-α20 (Fig. 1F) and are therefore important in the analysis of variation in prolamin protein patterns among different wheat cultivars in relation to CD.

Colloidal staining with PageBlue (CBB G-250) does not require a destaining step and is much more sensitive than traditional CBB R-250 staining (compare Fig. 1A with 1C). It is an endpoint staining and it interacts with lysine, arginine, histidine, and tyrosine residues [10]. The linear dynamic range extends over two orders of magnitude. Figure 1C

Figure 1. SDS-PAGE (10%) analyses of prolamin extracts from hexaploid wheat varieties. 1, Bovictus; 2, Combi; 3, Sperber; 4, Rektor; 5, Toronto; 6, Ambras. (A) Protein (15 μ g) stained with CBB R-250 and 2 h destaining. (B) Protein (15 μ g) stained with CBB R-250 and 16 h destaining. (C) Protein (2 μ g) stained with PageBlue. Arrows indicate poorly stained proteins. (D) Protein (1 μ g) stained with silver nitrate. (E) Protein (1 μ g) stained with SYPRO Ruby. (F) Western blot analysis with mAb against T-cell epitope Gli-a20.

Figure 2. 2-DE of the prolamin extract from hexaploid wheat variety Rektor, 50 μ g protein labeled with Cy3 combined with 100 μ g unlabeled protein. IEF performed on 24 cm linear IPG strip pH 3–10, followed by SDS-PAGE (10%). (A) Stained with CBB R-250. (B) Stained with SYPRO Ruby. (C) Cy3 signal. Arrowheads represent spots selectively visualized with CBB R-250 and SYPRO Ruby. Circles represent spots only detected with Cy3. The spot indicated with the square is detected with SYPRO Ruby and Cy3, but not with CBB R-250. (D, E, and F) Enlargements of boxes in A, B, and C, respectively.

shows that some HMW-GS could be visualized more clearly compared to silver staining (Fig. 1D) but some proteins were poorly stained (indicated with arrows in Fig. 1C). The boxed proteins shown in Fig. 1 also diffused out of the gel when kept in water (result not shown). Gels should be kept in 20% ammonium sulfate to prevent this loss [10].

Silver staining provides high sensitivity and interacts with lysine residues [10]. However, the procedure is quite complex and overdevelopment can occur, as is seen with staining of prolamins (Fig. 1D). Because HMW-GS stained less with silver nitrate than other prolamins, the duration of development had to be increased, which led to overdevelopment of gliadins and LMW-GS (Fig. 1D, 45–31 kDa

range). As a result, the intensities of protein bands or gel spots do not reflect the true abundance of different proteins, which implies that silver staining cannot be used in quantification [4].

SYPRO Ruby is an end-point stain with a high sensitivity and is quantitative over three orders of magnitude. It is reported to detect lysine, arginine, as well as histidine [10]. It is an easy one-step staining of proteins without long destaining steps and is compatible with MS. Figure 1E shows that some HMW-GS (the same as with Page Blue™) could be visualized more clearly compared to silver staining. Gliadins and LMW-GS in the 45–31 kDa region were more discrete due to end-point staining. A disadvantage of SYPRO Ruby is the speckled background staining.

In DIGE, CyDye DIGE fluor minimal dyes (Cy2, Cy3, and Cy5) are used for labeling of protein samples prior to gel electrophoresis. Labeling only 1–2% of all the lysine residues with CyDye was found to be optimal [4]. However, prolamin proteins contain only small amounts of lysine, arginine, and histidine [12]. Therefore, the efficiency of CyDye labeling of prolamins may be affected as well. Indeed, some prolamins were not labeled (Fig. 2F, indicated with arrowheads). On the other hand, some proteins were labeled that were not visualized with SYPRO Ruby (compare Fig. 2E with 2F, encircled spots), or CBB R-250 (compare Fig. 2D with 2F, encircled spots).

Immunoblotting using specific antibodies against CD immune responsive peptides is also a very sensitive detection method, but only for the detection of specific gliadins and glutenins (Fig. 1F). Prolamins from different varieties appearing on the same height in a SDS-PAGE gel display differences in antibody binding capacity. The mAbs staining indicates that similarly abundant proteins of the same molecular weight, which may have been identified by the same allelic code for dough properties [3], can be very different in immune staining, and that some low abundant proteins have a very strong immune response. Our results show that in identification of proteins containing immune responsive peptides in relation to CD it is imperative to detect also low abundant proteins that are not used in band identification for dough properties.

In conclusion, for visualizing gluten proteins all staining methods described here are applicable, though prominent selective staining is observed for each method. As a consequence, no single staining method provides complete staining of the prolamin profile on SDS-PAGE and 2-DE gels, but the methods show an inconsistent pattern for individual spots on 2-DE gels. For traditional CBB R-250 staining, a higher amount of protein has to be loaded on a gel and destaining for more than 2 h should be avoided because of the selective disappearance of proteins. Colloidal PageBlue (CBB G-250) staining is very sensitive compared to traditional CBB staining, but unfortunately, some proteins are poorly stained (Fig. 1C). Silver staining may result in overdevelopment, but if one attempts to visualize lower abundant proteins this is a suitable method. SYPRO Ruby is a very

sensitive stain and it displays better staining of HMW-GS compared to silver staining. Wheat varieties can be compared using SYPRO Ruby staining on 2-DE gels. However, small shifts in protein patterns (e.g., due to allelic differences) can be difficult to detect without the use of an internal reference. In this sense, the use of DIGE is preferable. CyDye labeling in DIGE analysis is suitable, although some proteins cannot be visualized, compared to CBB and SYPRO Ruby staining. On the other hand, some prolamins are labeled with CyDye that are not visualized by CBB and SYPRO Ruby. In terms of efficiency, the possibility of running three samples on the same gel, including a reference sample, reduces the number of gels and this makes DIGE a very useful method for the analysis and comparison of large numbers of wheat varieties.

Our study shows that especially for gluten proteins, due to their aberrant amino acid composition and their solubility, the staining method used to visualize the proteins in 1-D or 2-DE affects the resulting protein pattern. In CD research, the most important criterion in the selection of the staining method is that as many proteins as possible should be visualized, including proteins present in small amounts, as all of them may be recognized by specific antibodies against CD immune responsive peptides.

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