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Journal of Chromatography B, 849 (2007) 344–350
JOURNAL OF CHROMATOGRAPHY B
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Extraction of wheat endosperm proteins for proteome analysis☆,☆☆

William J. Hurkman*, Charlene K. Tanaka

U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, 800 Buchanan St., Albany, CA 94710, United States

Received 2 June 2006; accepted 27 November 2006
Available online 11 December 2006

Abstract

Total protein extracts of wheat endosperm are widely used for the analysis of the highly abundant gliadins and glutenins. In this review, the most popular total endosperm extraction methods are compared for their effectiveness in proteome coverage. A drawback of total endosperm extracts is that the enormous dynamic range of protein abundance limits the detection, quantification, and identification of low abundance proteins. Protein fractionation is invaluable for improving proteome coverage, because it reduces sample complexity while enriching for specific classes of less abundant proteins. A wide array of techniques is available for isolating protein subpopulations. Sequential extraction is a method particularly suited for subfractionation of wheat endosperm proteins, because it takes advantage of the specific solubility properties of the different classes of endosperm proteins. This method effectively separates the highly abundant gliadins and glutenins from the much less abundant albumins and globulins. Subcellular fractionation of tissue homogenates is a classical technique for isolating membranes and organelles for functional analysis. This approach is suitable for defining the biochemical processes associated with amyloplasts, specialized organelles in the endosperm that function in the synthesis and storage of starch. Subproteome fractionation, when combined with 2-DE and protein identification, provides a powerful approach for defining endosperm protein composition and providing new insights into cellular functions.

Keywords: 2-DE; Albumins; Amyloplasts; Endosperm; Gliadins; Globulins; Glutenins; Proteome; Wheat

1. Introduction

Wheat flour is the main ingredient in most types of breads, pastries, and pastas worldwide, because of its unique protein composition (reviewed: [1]). All-purpose flour is the finely ground endosperm of the wheat grain that is separated from the bran (aleurone, seed coats, pericarp) and germ (embryo) during the milling process. Flour contains predominantly starch (approximately 70–80% dry weight) and protein (approximately 10–15% dry weight). Approximately 80% of the endosperm protein is comprised of the gluten proteins, which have unique elasticity and extensibility properties that determine flour functionality. The gluten proteins consist of the monomeric gliadins and polymeric glutenins that, in turn, are comprised of high molecular weight and low molecular weight-glutenin subunits. Wheat grain research has focused on detailed analysis of the gluten proteins to better understand those aspects of protein composition that account for the unique properties of flour [2]. Although the non-gluten protein classes, the albumins and globulins, are a smaller percentage of endosperm protein, they have important roles in cellular metabolism, development, and responses to environment. The unparalleled resolving power of 2-DE has made it the method of choice for analysis of the complex protein populations of the endosperm [2]. Proteomic approaches utilizing 2-DE have provided new insights into protein composition of the endosperm [3–6], processes involved in grain development [7,8], effects of environment on grain fill [9–13], chromosomal locations of genes [14–16], and potential markers for genotype identification and stress tolerance [17–19]. The majority of these studies utilized total protein extracts, which are appropriate for the analysis of the abundant gliadins and glutenins that dominate the endosperm proteome. In this review, the most popular total endosperm extraction methods

Abbreviations: 2-DE, two-dimensional polyacrylamide gel electrophoresis; IEF, isoelectric focusing; IPG, immobilized pH gradient; PAGE, polyacrylamide gel electrophoresis

☆ This paper is part of a special volume entitled "Analytical Tools for Proteomics", guest edited by Erich Heftmann.

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* Corresponding author. Tel.: +1 510 559 5720; fax: +1 510 559 5818.
E-mail address: bhurkman@pw.usda.gov (W.J. Hurkman).

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doi:10.1016/j.jchromb.2006.11.047

are compared to illustrate their effectiveness in the analysis of the endosperm proteome. Since the gliadins and glutenins mask all but a few of the more abundant non-gluten proteins in total protein extracts, two approaches, depletion of high abundance proteins and cell fractionation, are described that extend endosperm proteome coverage respectively to the albumins and globulins and amyloplast proteins.

2. Total protein extraction methods

Extraction of proteins from plant samples is challenging. Plant cells often contain proteases that, if active in the extraction buffer, reduce and alter protein populations. Plant cells also contain various non-protein components that interfere with protein separation during electrophoresis, causing streaking and smearing of the 2-DE patterns. Among these components are cell wall and storage polysaccharides, lipids, phenolics, salts, nucleic acids, and a broad array of secondary metabolites [20]. The optimal extraction procedure must minimize protein degradation and eliminate non-protein components that interfere with protein separation during electrophoresis. The most common methods used to prepare total protein extracts from plant tissues are urea, SDS, TCA, and phenol. In this section, proteins were extracted from flour by each of these methods and the 2-DE patterns compared (Fig. 1). Proteins were precipitated from each sample and quantified by the procedure of Lowry et al. [21] as described in Hurkman and Tanaka ([22,23]; see also Section 3.1]. Protein extracts were solubilized in urea buffer (9 M urea, 4% NP-40, 1% DTT, and 2% ampholytes) and centrifuged at 16,000 × *g* for 10 min (Eppendorf Centrifuge 5415C, Brinkman Instruments, Inc., Westbury, NY) to remove insoluble material. Equal amounts of protein (18 μg) were loaded onto the IEF gels and 2-DE carried out according to Hurkman and Tanaka [22,23].

Although urea buffers are most often used to solubilize proteins for IEF, they can also be used to extract proteins directly from wheat flour [25–27]. For Fig. 1A, flour was extracted essentially by the method of Payne et al. [24] as summarized in Fig. 2. Fifty milligram of flour was suspended in 200 μl of urea buffer (2 M urea, 10% glycerol, 65 mM DTT, and 20 mM Tris, pH 8.0), the suspension incubated at room temperature for 1 min, and insoluble material removed by centrifugation at 16,000 × *g* for 10 min (Fig. 2). The resulting 2-DE pattern (Fig. 1A), like that of Payne et al. [24], contains four protein regions. The proteins in region 1 are the high molecular weight-glutenin subunits, in region 2 the ω-gliadins [28], in region 3 the α- and γ-gliadins and low molecular weight-glutenin subunits, and in region 4 the low molecular weight-albumins and globulins. The albumins and globulins consist of many more proteins (compare Figs. 1A

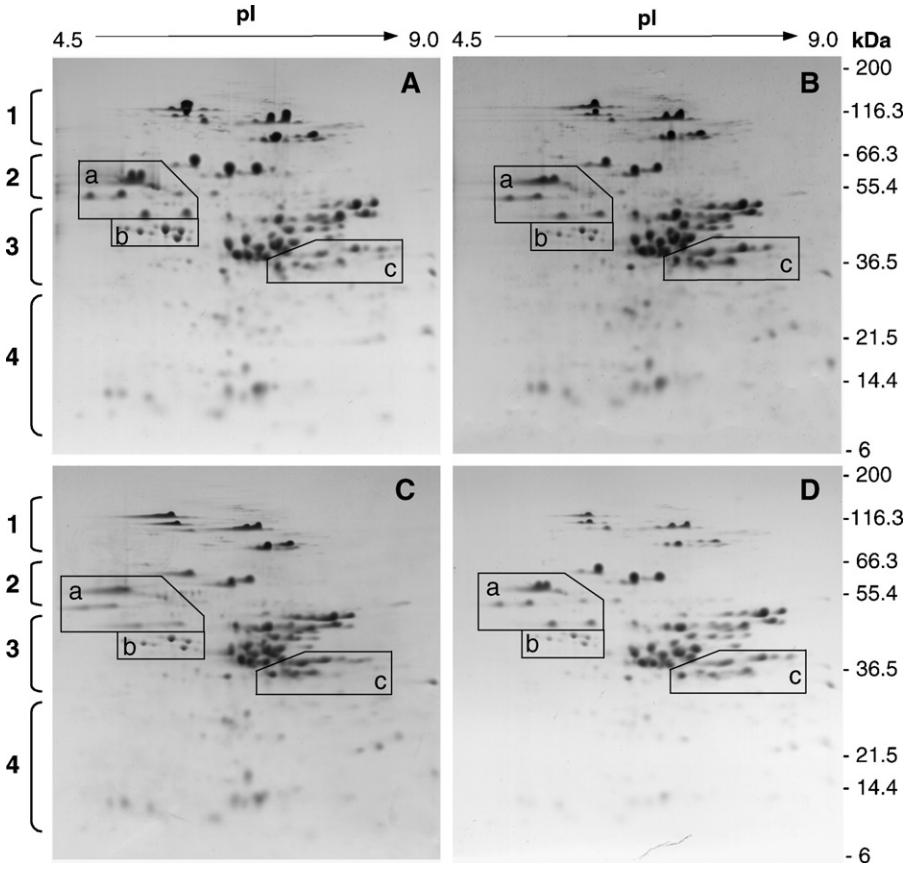

Fig. 1. 2-DE comparison of wheat flour proteins extracted with urea, SDS, TCA, or phenol. (A) Urea-soluble proteins. (B) SDS-soluble proteins. (C) TCA/acetone-insoluble proteins. (D) Phenol-soluble proteins. Numbered brackets indicate region 1, high molecular weight-glutenin subunits; region 2, ω-gliadins; region 3, the α- and γ-gliadins and low molecular weight-glutenin subunits; and region 4, low molecular weight-albumins and globulins. Boxes a–c highlight differences and similarities in the gel patterns.

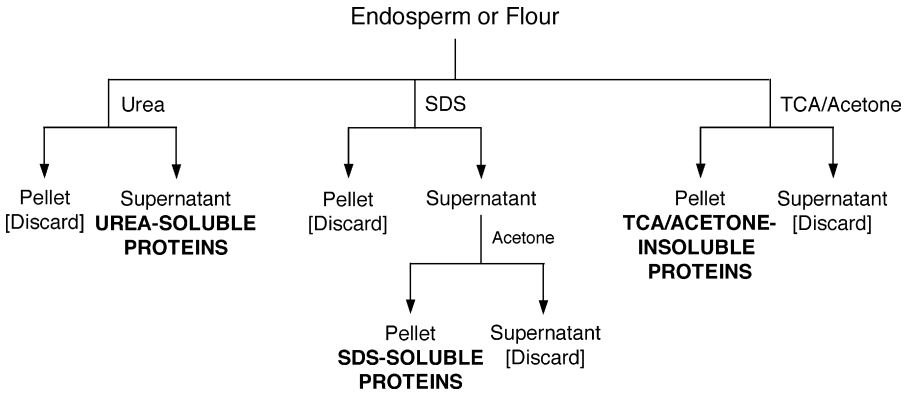

Fig. 2. Extraction of wheat endosperm or flour proteins with urea, SDS, or TCA/acetone.

and 5B), but their presence is masked by the highly abundant gliadins and glutenins. The 2-D gels of proteins extracted by SDS (Fig. 1B), TCA (Fig. 1C), and phenol (Fig. 1D) also exhibit this phenomenon.

SDS can be used to solubilize wheat endosperm proteins for analysis by 2-DE [29,34]. However, proteins extracted with SDS are negatively charged and cannot be separated by IEF. We found, as outlined in Fig. 2, that the SDS can effectively be removed by precipitating the proteins with acetone prior to solubilization in urea buffer. For this method, 50 mg of flour was suspended in 800 μl of SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris–Cl, pH 6.8), incubated for 1 h at room temperature, and insoluble material removed by centrifugation at 16,000 × *g* for 10 min. The proteins were then precipitated by addition of 4 volumes (vol.) of cold acetone and recovered by centrifugation. The most noticeable differences between the 2-DE patterns of proteins prepared with SDS (Fig. 1B) and those prepared with urea (Fig. 1A) are found in region 3. A group of acidic proteins is proportionately less in amount (compare box b in Fig. 1A and B) and a group of basic proteins is proportionately more in amount (compare box c in Fig. 1A and B) in the gel of the SDS solubilized proteins.

Extraction of plant proteins with a mixture of TCA and acetone is a widely used method for 2-DE analysis [7,29]. For Fig. 1C, flour was extracted by the procedure of Granier [29]. As summarized in Fig. 2, 100 mg of flour was suspended in 10 ml of acetone containing 10% TCA and 0.07% 2-mercaptoethanol, incubated for 1 h at room temperature, and insoluble material removed by centrifugation at 16,000 × *g* for 10 min. The proteins were rinsed with acetone and recovered by centrifugation. Like the pattern for proteins extracted with SDS, region 3 acidic proteins are proportionately less (compare box b in Fig. 1A and C) and basic proteins proportionately more in amount (compare box c in Fig. 1A and C) compared to those prepared with urea. However, proteins prepared with TCA were not as well resolved as those prepared with urea or SDS. Horizontal streaking of the proteins and accompanying loss of resolution is noticeable in regions 1 and 2, but is especially prominent for the acidic proteins in regions 2 and 3 (compare box a in Fig. 1A–C).

The phenol partitioning method has proven useful for 2-DE of proteins from a wide range of plant tissues, as well as difficult to analyze proteins such as membrane proteins [22,30] and proteins synthesized *in vitro* [31]. For Fig. 1D, flour was extracted as described previously [31,32], except that the protein in the phenol phase is recovered rather than the RNA in the aqueous phase (Fig. 3). Seventy-five milligram of flour was suspended in 750 μl of phenol [450 μl PCI (phenol/chloroform/isoamyl alcohol 25:24:1)] plus NTES buffer (300 μl 10 mM NaCl, 10 mM Tris, 1 mM EDTA, 1% SDS) for 10 min at 4 °C and the phases were separated by centrifugation. The phenol phase was recovered and re-extracted with an equal vol. of NTES buffer. Proteins were precipitated by the addition of 5 vol. of 0.1 M ammonium acetate in methanol. The precipitate was washed three times with the ammonium acetate in methanol and once with acetone. Compared to the other three methods, the proteins that partitioned into phenol (Fig. 1D) have proportionately less of the region 3 acidic proteins (compare box b in Fig. 1A and D). Region 3 basic proteins are proportionately similar in amount to those extracted with urea (compare box c in Fig. 1A and D) and proportionately less than those extracted with SDS or TCA (compare box c in Fig. 1B–D). In addition, the high molecular weight-glutenin subunits in region 1 are proportionately less in

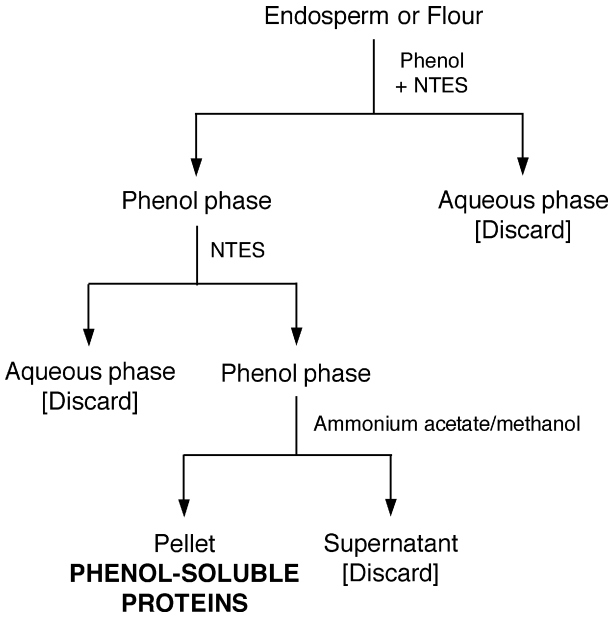

Fig. 3. Extraction of wheat endosperm or flour proteins by phenol partitioning.

amount compared to those in the gels of proteins prepared by the other three methods and proteins in region 4 are also proportionately less in amount. Because the phenol method relies on NaCl buffer partitioning and methanol precipitation, many of the region 4 albumins and globulins are not recovered since they are soluble in NaCl and methanol (see Section 3.1).

In summary, the 2-D gel patterns are similar qualitatively for the different extraction methods, but differ quantitatively. Effectiveness of protein extraction is reagent dependent, which is not surprising given the highly heterogeneous nature and structural complexity of proteins. The extraction method of choice depends on whether the goal is to identify as many proteins as possible or to quantify protein levels. Quantitative extraction is not necessarily required for protein identification, but is essential for comparative analysis of protein populations. The extraction methods compared in this section demonstrate that phenol partitioning, an effective procedure for many plant samples, is not the method of choice for analysis of wheat flour proteins. This result highlights the importance of comparing extraction procedures with respect to protein yields and number of proteins recovered. It should also be pointed out that the composition of the solubilization buffer, which is not addressed in this review, is equally important in protein recovery [33].

3. Subfractionation methods

One often cited disadvantage of 2-DE is that entire proteomes cannot be visualized in a single gel. Cellular protein populations have enormous diversity with respect to function, sequence, physical properties, and relative abundance, making it difficult to obtain the entire proteome. Perhaps more importantly, the high dynamic range of protein abundance, which varies by six or more orders of magnitude in eukaryotic cells [34,35], makes display of low abundance proteins problematic. One strategy for improving proteome coverage is the use of multiple, overlapping, narrow-range, first dimension IPG gels [36–38]. While this approach increases the number of proteins that can be displayed, entire proteome coverage is still not possible. The most viable strategy for increasing proteome coverage is to isolate subproteomes by exploiting protein properties. The advantages of fractionation are that it reduces sample complexity while enriching for specific protein classes. In this section, fractionation methods based on the specific solubility properties of different classes of wheat grain proteins and on cellular location are described.

3.1. KCl and methanol solubility

Osborne's [39] solubility fractionation techniques for plant proteins provide an effective way to isolate the major classes of seed proteins: albumins (soluble in water or dilute salt), globulins (soluble in dilute salt, but insoluble in water), gliadins (soluble in aqueous alcohol), and glutenins (insoluble in aqueous alcohol). A method that we routinely use to fractionate wheat endosperm proteins takes advantage of the solubility properties of wheat endosperm proteins in KCl, methanol, SDS, and acetone [23] is summarized in Fig. 4. Fifty milligram of flour was suspended in 200 μl of cold (4 °C) KCl buffer (50 mM Tris–HCl, 100 mM KCl, 5 mM EDTA, pH 7.8). The suspension was incubated on ice for 5 min with intermittent mixing and centrifuged at 14,500 × *g* for 15 min at 4 °C (Tomy MRX-151; Peninsula Laboratories, Inc., Belmont, CA). The pellet or KCl-insoluble fraction was suspended in 800 μl of SDS buffer (2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris–Cl, pH 6.8), incubated for 1 h at room temperature, and insoluble material removed by centrifugation at 16,000 × *g* for 10 min at room temperature. The proteins were precipitated from the SDS buffer by the addition of 4 vol. of cold (−20 °C) acetone and incubation overnight at −20 °C. Following centrifugation, the pellet was rinsed by pipetting cold acetone onto the pellet, centrifuging at 16,000 rpm for 10 min at room temperature, and pipetting the acetone off of the pellet. The pellet (gluten proteins) was dried by vacuum centrifugation (Speed Vac DNA 110; Savant Instruments, Inc., Farmingdale, NY) and solubilized in urea buffer (9 M urea, 4% NP-40, 1% DTT, and 2% ampholytes) to a final concentration of 3 μg protein/μl. The KCl-

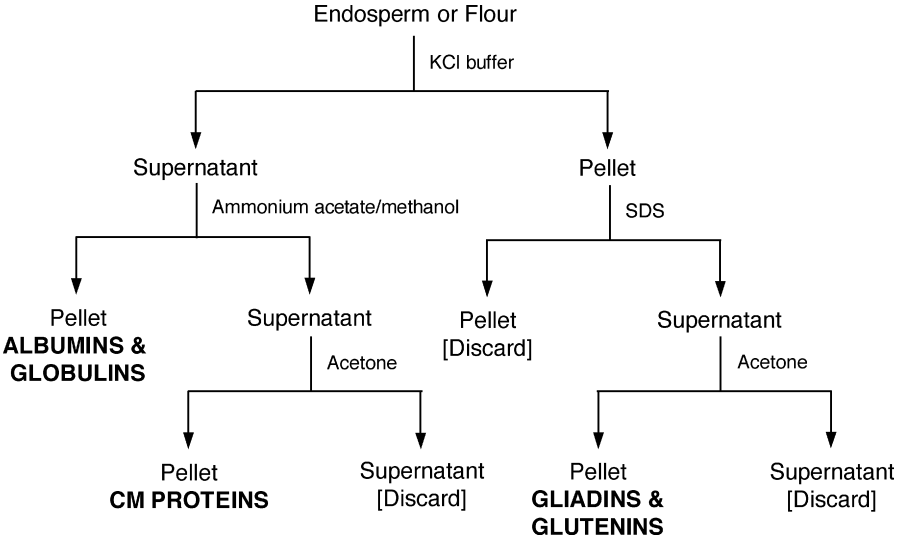

Fig. 4. Isolation of protein fractions enriched in gliadins and glutenins, albumins and globulins, or CM proteins by sequential extraction of wheat endosperm or flour.

soluble fraction was collected and 5 vol. of 0.1 M ammonium acetate in methanol was added at room temperature. Following incubation overnight at −20 °C, the methanol-insoluble fraction was pelleted by centrifugation at 14,500 × *g* for 15 min at 4 °C. The pellet (albumins and globulins) was rinsed with cold acetone and solubilized in urea buffer. The proteins in the methanol-soluble fraction were precipitated with acetone, and the pellet (CM proteins) was rinsed, and solubilized in urea buffer. For protein determination, triplicate samples were removed from the SDS solubilized KCl-insoluble fraction (5 μl), the KCl-soluble fraction (10 μl), and the KCl-soluble/methanol-soluble fraction (25 μl) for protein analysis. Following precipitation of the fractions with the appropriate solvent, protein was quantified by the procedure of Lowry et al. [21]. Equal amounts of protein (18 μg) were loaded onto the IEF gels and 2-DE was performed according to Hurkman and Tanaka [23].

The 2-DE patterns for the gliadin and glutenin, albumin and globulin, and CM protein fractions are distinctive (Fig. 5A–C). The pattern for the gliadin and glutenin fraction (Fig. 5A) has relatively few low molecular weight-albumins and globulins. The pattern for the albumin and globulin fraction (Fig. 4B) is considerably different from that for the gliadin and glutenin fraction. With the depletion of the gliadins and glutenins, the many proteins that make up the albumin and globulin fraction are unmasked. Identification of these proteins by mass spectrometry revealed that the majority of the proteins are enzymes functional in biochemical processes ranging from carbohydrate metabolism and protein synthesis/assembly to storage and stress/defense [8]. Only 6 of the 254 proteins identified in this fraction were glutenin proteins, confirming the effectiveness of the KCl separation procedure. The 2-DE pattern of the CM protein fraction (Fig. 5C) is characterized by the presence of abundant proteins with molecular weights less than 20 kDa. Identification of the proteins in this fraction by mass spectrometry [40] revealed that they are primarily α-amylase or α-amylase/trypsin inhibitors that are traditionally called CM proteins [41,42]; some α- and γ-gliadins (neutral proteins with molecular weights greater than 40 kDa) are also prominent in this fraction.

3.2. Cellular origin

Cell fractionation techniques are central to increasing our understanding of protein composition and metabolic functions of subcellular compartments. In recent studies [5,6], proteomic analyses of amyloplasts isolated from wheat endosperm was undertaken to define the biochemical processes taking place in these organelles. Amyloplasts were isolated for Fig. 5D using

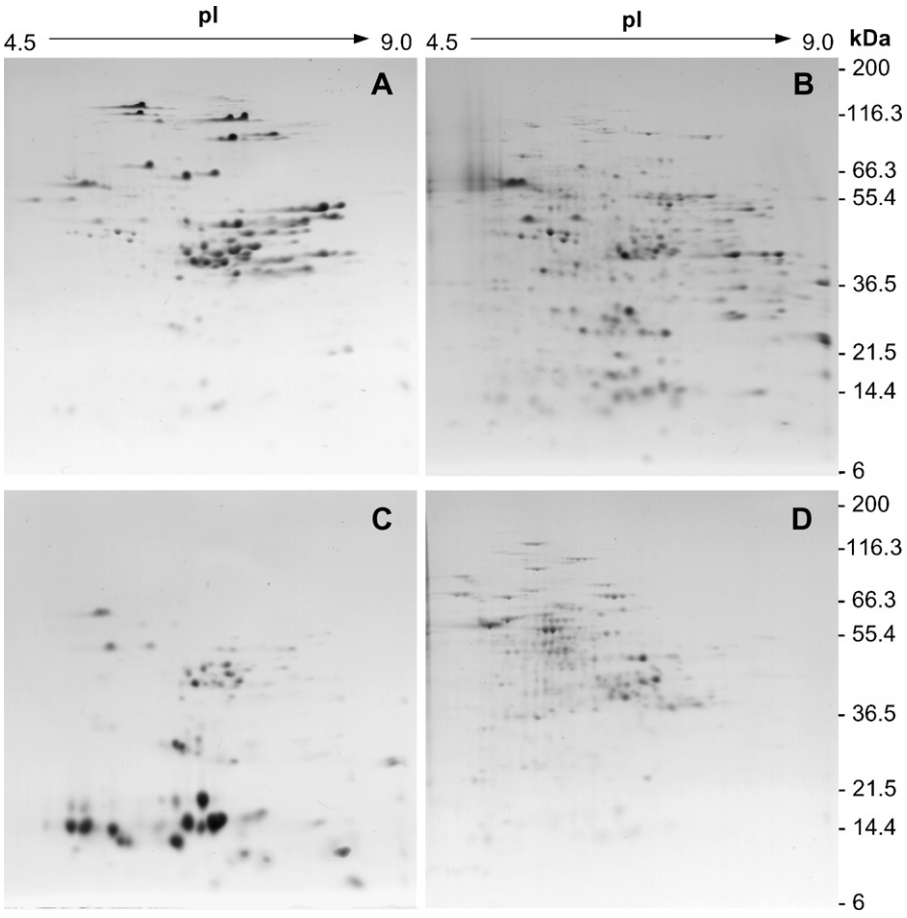

Fig. 5. 2-DE comparison of wheat endosperm subproteomes. (A) KCl-insoluble/acetone-insoluble, gliadin and glutenin fraction. (B) KCl-soluble/methanol-insoluble, albumin and globulin fraction. (C) KCl-soluble/methanol-soluble, CM protein fraction. (D) Amyloplast fraction.

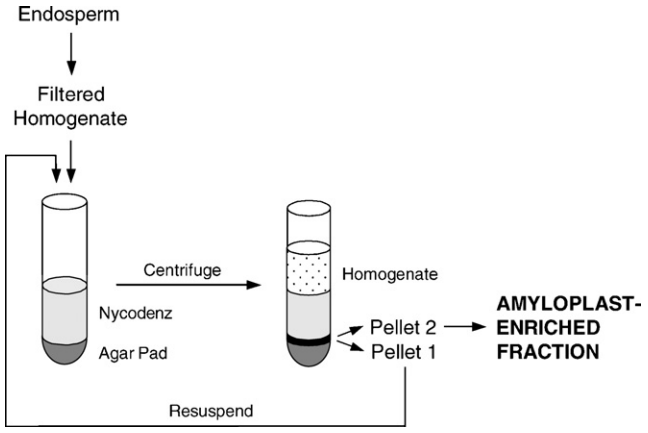

Fig. 6. Isolation of an amyloplast-enriched fraction from wheat endosperm.

the procedure of Tetlow et al. [43] as summarized in Fig. 6. Grain was harvested at 10 dpa from 20 wheat heads. Embryos were cut from the grain, endosperm was squeezed through the opening created, and collected in ice-cold buffer (0.5 M sorbitol, 50 mM HEPES pH 7.5). The endosperm was transferred to plasmolysis buffer (0.8 M sorbitol, 50 mM HEPES pH 7.5, 1 mM EDTA, 1 mM KCl, 2 mM $MgCl_2$) and incubated for 1 h on ice. Plasmolyzed endosperm was chopped twice for 30 s with an electric knife, the blades of which were replaced with holders fitted with single-edge razor blades. The resulting homogenate was filtered through two layers of Miracloth and gently pipetted onto a 4-ml cushion of 2% Nycodenz (Nycomed, Oslo, Norway) in plasmolysis buffer in a 15 ml conical tube containing a 2 ml 1% agar pad at the bottom. Following centrifugation for 10 min at 30 × *g* and 4 °C (Eppendorf Centrifuge 5810 R, Brinkman Instruments, Inc., Westbury, NY), the supernatant fraction was removed by aspiration and discarded. The pellet containing the amyloplasts was gently suspended in plasmolysis buffer and the Nycodenz procedure repeated once more. Amyloplasts were solubilized in urea buffer (9 M urea, 4% NP-40, 1% DTT, and 2% ampholytes), centrifuged to remove insoluble material, and separated by 2-DE according to Hurkman and Tanaka [22,23].

The 2-D pattern for amyloplast proteins (Fig. 5D) is quite different from the patterns for protein fractions prepared by the other methods in that the majority of proteins have acidic to neutral p*I*s and molecular weights greater than 40 kDa. Identification of amyloplast proteins by mass spectrometry revealed that only 46 of the 289 proteins identified were found among the 254 proteins identified in the albumin and globulin fraction [6,8]. This result illustrates the advantage of organelle isolation in the proteomic analysis of less abundant proteins. The protein identifications demonstrate that, in addition to carbohydrate metabolism, amyloplasts are involved in a range of processes including cytoskeleton/plastid division, energetics, nitrogen and sulfur metabolism, nucleic acid-related reactions, synthesis of various building blocks, protein-related reactions, transport, signaling, and stress/defense [6]. Based on these findings, Balmer et al. [6] concluded that amyloplasts have broader metabolic activities than previously recognized and that they may participate in extra-organellar biosynthetic processes.

4. Concluding remarks

The extraction methods covered in this review coupled with protein identification by mass spectrometry have been used to establish proteome maps for the gliadins and glutenins, albumins and globulins, CM proteins, and amyloplast proteins. These maps have been invaluable in developing a more comprehensive picture of endosperm protein composition and developmental processes. The albumins and globulins function in a wide variety of metabolic processes ranging from carbohydrate metabolism and protein synthesis/assembly to storage and stress/defense [8]. A comparison of early (10 dpa) and late stages (36 dpa) of grain fill revealed that carbohydrate metabolism, transcription/translation, and protein synthesis/assembly were the principal endosperm functions at 10 dpa [8]. Carbohydrate metabolism and protein synthesis/assembly were also major functions at 36 dpa, but the predominant activities were the accumulation of stress/defense and storage proteins. Specific albumins and globulins targeted by thioredoxin, a widely distributed small regulatory protein, were identified [44] and unique sets of targets were found at early (10 dpa) and late (36 dpa) developmental stages [45]. Proteomic studies of amyloplasts isolated from wheat endosperm demonstrate that, in addition to starch biosynthesis and degradation, these organelles are involved in a wide range of metabolic activities [5,6]. Like the albumins and globulins, amyloplast proteins are also targeted by thioredoxin [6,46]. Taken together, these findings present an overview of proteins and metabolic processes operating in the developing wheat grain and provide new insights into their regulation. Many more questions remain to be answered about wheat endosperm. The relationship of protein composition to flour quality, nutritional quality, and allergenicity continue to be important research areas. Changes in endosperm protein populations during grain development and in response to environmental fluctuations remain to be determined to understand effects of environment on flour quality. Identification of markers for flour quality, cultivar identification, and agronomic traits remain important goals. Proteomic approaches remain a promising way to obtain answers to these questions.

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