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Identification of cultivar-specific proteins of winter wheat (*T. aestivum* L.) by high resolution two-dimensional polyacrylamide gel electrophoresis and color-based silver stain

The polypeptide patterns obtained from individual caryopses (kernels) of two wheat cultivars were analyzed using high resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) methods. While few polypeptides were detected with Coomassie Blue staining methods, over two hundred peptides were easily distinguished with the color-based silver stain. The relative isoelectric points of the constituent polypeptides ranged from pH 3.8 to 9 and the relative molecular weights from 5000 to 200 000. The majority of the proteins of the two wheat varieties analyzed with 2-D PAGE were similar although distinct proteins having molecular weights of 23 000, 30 000, 37 000 and 70 000 were identified, which have molecular properties unique to each variety. The use of the color-based silver stain makes it possible to identify and characterize hundreds of proteins in individual wheat seeds. These methods are, therefore, adaptable for the rapid analysis and characterization of specific gene products of single kernels of wheat.

1 Introduction

In recent years, many attempts have been made to identify specific proteins associated with wheat cultivars [1–5]. A variety of electrophoretic methods have been used to analyze specific wheat proteins. Many of these methods have been used for the verification of cultivars used in research projects as well as to identify and characterize new cultivars [2]. It has been previously pointed out that one of the major problems associated with the identification of wheat proteins is the variation in methodology and equipment used from laboratory to laboratory [1]. The use of diversified methods has arisen in part from the rapid development of electrophoretic system in human and animal research [6, 7], as well as in plant research. Previous studies using one-dimensional polyacrylamide gel electrophoresis (PAGE) analysis have demonstrated that there are protein differences among different wheat cultivars [3]. Although one-dimensional PAGE provides a rapid analysis of protein patterns, the limited resolution could mask differences which are vital for detailed characterization.

Methods for the identification and characterization of proteins using high resolution two-dimensional(2-D) polyacrylamide gel electrophoresis (2-D PAGE) have become increasingly popular during the past decade. These methods have proven invaluable in basic research in the characterization and identification of proteins [8–13]; see reviews [6, 7]. They have been developed for application in clinical research and have been used extensively in the identification of proteins associated with genetic diseases [14, 15]. Methods have now been developed to simplify and "automate" as well as to standardize the analysis of proteins using 2-D PAGE [16–20]. These methods have been developed primarily for the identification and characterization of human proteins but are now easily adaptable to studies on plant proteins. Recent advances in protein detection in polyacrylamide gels have also been made, including a variety of sensitive silver stains that are 200 times more sensitive than the Coomassie Brilliant Blue R-250 [21–24]. One of these silver stains has been standardized to stain different proteins different colors [23, 24]. Although the molecular basis of this staining is not clearly understood, this method provides a third dimension in protein detection.

In the present studies, we have adapted commonly used 2D PAGE methodology and the color-based silver stain to identify over a hundred polypeptides present in individual wheat seeds. These methods have demonstrated that the protein patterns in wheat caryopses are complex and that distinct polypeptides are associated with different wheat cultivars. These methods provide more detailed analysis of specific wheat proteins than has been previously described and should prove invaluable in agricultural as well as basic research.

2 Materials and methods

2.1 Sample preparation

Wheat samples of two hard red winter wheat cultivars (Wichita and Centurk) were obtained from the Colorado State University seed certification program and are representative of cultivars that have been grown extensively in the Central Great Plains area. Individual kernels were ground in a mortar and pestle until the resulting flour would pass a 1 mm sieve. Kernels were solubilized in 500 µl solubilization buffer containing 9 M urea, 2 % Ampholines (pH range 3.5–9.5, LKB, Hicksville, N.Y.), 2 % β-mercaptoethanol, and 4 % Nonidet P-40 (Serva Chemical Co.) as described by O'Farrell [8]. High speed centrifugation (200 000 g) was carried out for 2 h to remove insoluble material which would interfere with isoelectric focusing (IEF). Multiple seeds were analyzed and replicate gels of each were run in two separate laboratories (USDA-ARS, Akron, CO and Department of Cell Biology, Baylor College of Medicine, Houston, TX).

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Abbreviations: **PAGE**, polyacrylamide gel electrophoresis; **2-D**, two-dimensional; **IEF**, isoelectric focusing; **SDS**, sodium dodecyl sulfate

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2.2 High-resolution two-dimensional polyacrylamide gel electrophoresis

Electrophoresis in the first dimension (IEF) and second dimension (sodium dodecyl sulfate, SDS) gel electrophoresis was carried out using modifications of the methods previously described [8, 9, 10]. Isoelectric focusing gels were cast using the apparatus described by Dunbar [20] which consists of two plastic 50 ml conical centrifuge tubes, which have had the conical portion removed. The two tubes are stacked together and a flat-bottomed cap is screwed onto the bottom. The acrylamide-urea mixture [20, 25] containing wide range Ampholines is poured into the plastic cylinder and the glass tubes (200 µl glass pipets which have had their tips removed) are placed into the solution. Water is then poured gently onto the surface of the acrylamide to force the tubes to be filled by capillary action. Following polymerization, the samples are added to the gels; electrofocusing was carried out for 17 h at 600 V (25 °C). After equilibration in SDS sample buffer for 10 min, the IEF gels were applied to 10–20 % gradient polyacrylamide gels prepared using the Anderson DALT casting system (Electronucleonics, Inc. Oakridge, TN) as described by Anderson and Anderson [16]. This system has the advantage that multiple gradient slab gels can be cast and run simul-

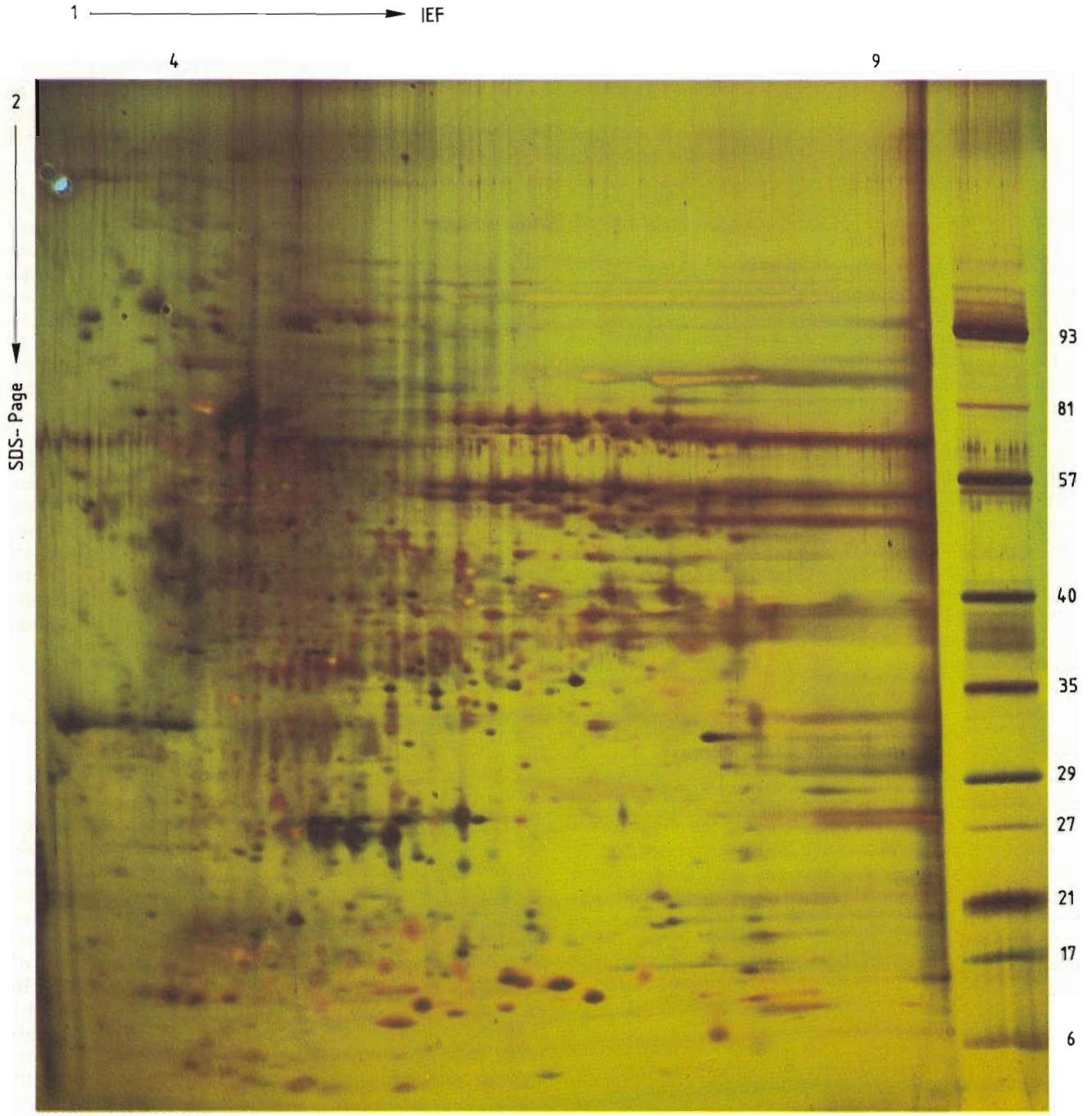

Figure 1. High-resolution two-dimensional analysis of proteins from a single wheat kernel. IEF was carried out in the first dimension using wide range Ampholines (pH 3.5–9.5) and second-dimension electrophoresis was carried out using 10–20 % gradient gel electrophoresis. Proteins were stained using the color-based silver stain and molecular weight markers standardized for silver staining (Health Products, Inc.) were used.

taneously such that identical gel patterns are easily obtained. Molecular weight standards developed for the color-based silver staining (Health Products Inc., South Haven, Mich. 49090) were placed on one side of each gel for reference and for determination of relative molecular weights. These standards were compared with the low molecular weight standards from Pharmacia Fine Chemicals (Piscataway, NJ 08854). One of the replicate gels in each set was stained with Coomassie Brilliant Blue R-250. The other gels were fixed and silver-stained with the procedure described by Sammons *et al.* [23, 24], or with the Gelcode color-based silver stain kit (Health Products Inc.).

3 Results

The 2-D PAGE protein polypeptide pattern obtained from a single kernel of wheat (Centurk) is shown in Fig. 1. It is apparent from this protein pattern that the protein composition of a single wheat seed is complex, since many polypeptides can easily be identified. These peptides have relative molecular weights ranging between 5000 and 200 000 and relative p*I*'s ranging from 3.8–9. The relative molecular weights were determined using two molecular weight standards (Fig. 2). The numbers indicate the area of the gels where unique peptides between the two cultivars are routinely observed (see Fig. 3). Sources of the standards designed for the color-based silver stain were used for most samples since more proteins are included as reference markers and since these are also standardized to stain different colors, a property which aids in comparing gels.

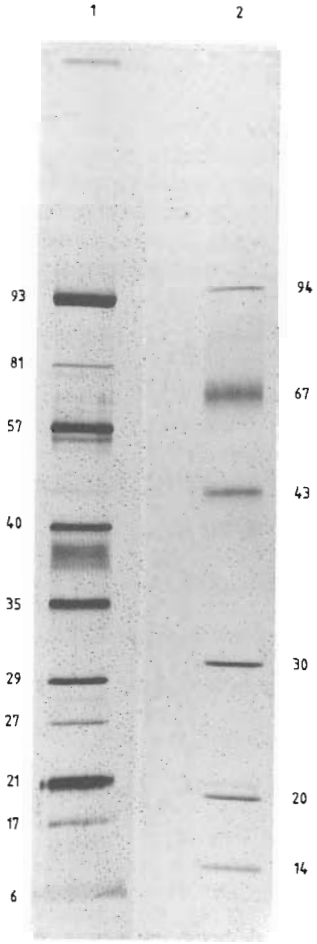

Figure 2. Comparison on the sides of 2-D PAGE gels of molecular weight markers (1) standardized for color of staining with (2) conventional markers (Pharmacia).

Fig. 3 illustrates a black and white enlargement of a portion of the 2-D PAGE gels which compares the protein patterns of the two wheat varieties, Centurk (2A) and Wichita (2B). The majority of identifiable peptides are identical between these varieties. There are, however, distinct protein differences which were always observed between the two varieties of individual seeds. Those portions of the patterns are shown, since these are the only areas of the gel in which there were distinct protein variation between the two varieties. These protein differences were apparent in all wheat kernels examined at all protein concentrations used. The group of low molecular weight proteins (M_r 25 000–28 000), which stain gray in color, vary between the two varieties according to relative charge and molecular weight. This group of proteins appear to be storage proteins since they correspond in color or staining, relative p*I* and molecular weight to a purified gliadin fraction obtained from D. Kasarda (USDA, Berkeley, CA; data not shown). The two peptides 1 (M_r 25 000) and 3 (M_r 27 000) were always present in the Centurk wheat variety while the peptide 2 (M_r 27 000) was present only in the Wichita variety. The acidic protein 4, which has a relative molecular weight of 32 000, appeared to be more abundant in the Centurk variety. The proteins labeled 5 (M_r 35 000; black) and 7 (M_r 35 000; rust) were consistently present in the Centurk variety while these were never apparent in the protein pattern of Wichita even when increased concentrations of protein were loaded on the gels. The group of proteins enclosed in the brackets labaled 8 in Fig. 3A appear to be a related group of proteins because their molecular weights are identical and because they stain the same shade of red. Although a similar molecular weight and color staining group of proteins is present in the Wichita variety (3B), there were always fewer charge species than were present in Centurk. Finally, a subtle but distinctive difference was always observed in the group of red staining proteins enclosed in brackets, labeled 9, having a relative molecular weight of 72 000. Two distinct molecular weight families were always present in the Centurk protein family while there was no distinct separation in the molecular weights of these two molecular weight groups in the Wichita protein family. Again, this difference was consistent in multiple kernels of wheat and was easily detected in gel patterns containing differing concentrations of proteins.

4 Discussion

The studies presented here demonstrate that high-resolution 2-D PAGE gel electrophoresis and silver staining can be used to identify and compare many polypeptides from individual wheat seeds. This method demonstrates the complexity of wheat proteins and allows the identification of specific gene products characteristic of different wheat varieties. The majority of proteins identifiable with the silver stain exhibit minimal molecular weight and charge heterogeneity. This is in contrast to many animal cellular proteins which exhibit extreme charge and molecular weight heterogeneity due to post-translational modification (*i. e.* glycosylation, sulfation, and phosphorylation [12, 20, 26]. Since the majority of metabolic proteins in wheat seeds would not be targeted for cell secretion as are many mammalian proteins, they would not be expected to have extensive glycosylation.

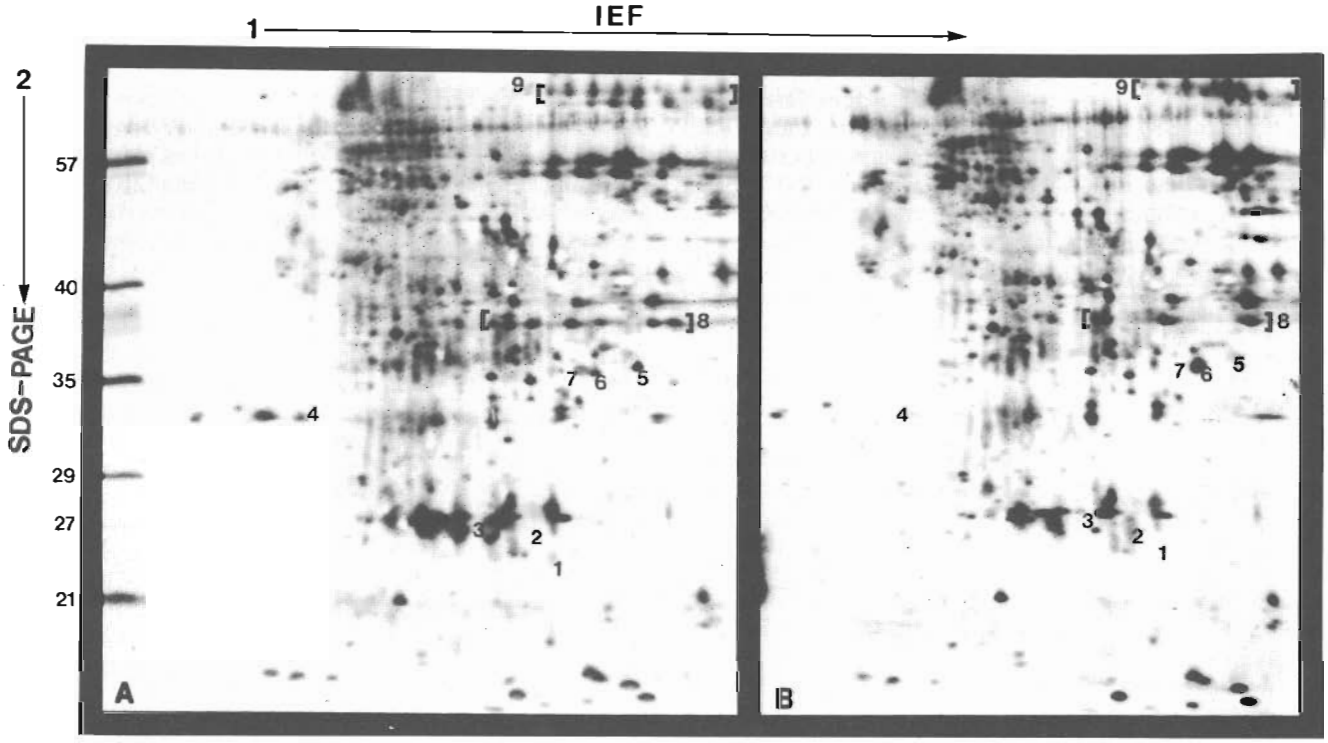

Figure 3. Comparison of protein patterns of two wheat varieties analyzed by high-resolution two-dimensional polyacrylamide gel electrophoresis. (A) Centurk; (B) Wichita. Numbers indicate protein which were routinely found to vary between the kernels of the two wheat varieties.

To date, the majority of detailed analyses of wheat proteins have been carried out on isolated storage proteins [14, 27]. Although these storage proteins are abundant in wheat seeds, this abundance of individual storage proteins is not reflected by the procedures used in these studies. One explanation for this is that some proteins such as the protein calmodulin are not readily detected with the silver stains (B. S. Dunbar, unpublished observations). A second explanation is that related proteins exhibiting charge and molecular weight heterogeneity such as is observed with immunoglobulin light chains [29] would result in the dispersion of proteins over a wide area of the gel. This heterogeneity would make abundant proteins appear to be less concentrated than they would on a one-dimensional PAGE gel.

While many polypeptides can be analyzed with this method it is apparent that the vast majority of these have identical patterns when analyzed with high resolution 2-D PAGE. Although it has been speculated that the recognition of multiple biotypes within a single wheat variety might be detected if more discriminating forms of electrophoretic methods were used [30], no differences were apparent in the present studies which would indicate major variations between individual wheat seeds, even at the level of resolution and sensitivity of detection by the silver-based stain. Although subfractionation of proteins may demonstrate that there are protein differences, it is apparent that the hundreds of polypeptides identified using these methods are similar within individual seeds of a single cultivar. Since the silver stain methods are currently the most sensitive protein detection methods available, it is difficult to imagine the more detailed analysis of subtractionated proteins isolated from a single kernel prior to electrophoretic analysis.

The methods described in this study should prove to be valuable methods for the analysis of hundreds of specific proteins of individual wheat seeds. The use of such high resolution protein analysis methods is critical in the standardization of wheat varieties for both basic and agricultural research as well as in developing a better understanding of gene expression in plants.

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