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Two-Dimensional Electrophoretic Analysis of Wheat Seed Proteins[1]

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ABSTRACT

High resolution two-dimensional (2-D) electrophoresis with isoelectric focusing in the first dimension and electrophoresis in sodium dodecyl sulfate in acrylamide gradient gels in the second dimension has been used to produce maps of proteins, including glutenins and gliadins, extracted from wheat seeds. Amino acid substitutions producing single or multiple charge changes, and additions or deletions altering protein mass by approximately 2% are visible on these gels; the former produce horizontal displacements in gel patterns, the latter vertical displacements. The thesis that mutations in storage proteins produce grid patterns of variants on 2-D gels has been examined experimentally, and confirmatory results have been obtained using 14 varieties of wheat. The results suggest that 2-D analysis will be useful for varietal identification, for classification of storage proteins of wheat, and for studies on the geneology of wheats.

***Additional index words:* *Triticum aestivum* L., Iso-Dalt, Chinese Spring, Maris Freeman, Carbamylation, Gliadins.**

THE complex of proteins present in the seeds of various wheat cultivars (*Triticum aestivum* L.) has proven difficult to resolve analytically, to interrelate functionally and genetically, and to name in a useful manner. Given a hexaploid genome, gene duplication, and the existence of numerous varieties, the core analytical problem appears to be that of identifying families of related genes and their protein products.

High resolution two-dimensional (2-D) electrophoresis, with isoelectric focusing in urea under either equilibrium or nonequilibrium conditions in the first dimension and electrophoresis in the presence of sodium dodecyl sulfate in the second is capable of resolving several thousand proteins in one analysis (O'Farrell, 1975; Anderson and Anderson, 1979, 1984; Taylor et al., 1983), and has been previously applied to the analysis of wheat endosperm proteins in several laboratories (Brown and Flavell, 1981; Brown et al., 1979, 1981; Holt et al., 1981; Jackson et al., 1983; Miflin and Shewry, 1979; Moonen and Zeven, 1984; Payne et al., 1984a, 1984b; Stegemann and Pietsch, 1983).

Approximately one-third of single amino acid substitutions produce a charge shift, and single charge differences can be observed in 2-D gels as horizontal displacements over the molecular mass range of approximately 10 000 to 200 000 daltons (Anderson and Anderson, 1984). Mass differences due to additions or deletions of approximately 2% or larger can be detected over the same molecular mass range. Comparative studies of protein amino acid sequences suggest that during evolution an average of one addition or deletion occurs per 42 amino acid replacements, and that an average of 1.8 amino acids is added or deleted (Dayhoff and Barker, 1975). Therefore, nearly all mutations involving the more-numerous charge shifts would be detected in 2-D patterns, but only those rare mass changes (gap events) involving addition or deletion of more than 2% of the protein mass would be seen.

Mutuations in related endosperm protein genes (i.e., genes derived from one ancestral gene) may be expected to produce both charge and mass modifications. We propose that in composite 2-D patterns the protein products of related genes should appear as charge trains with spots which are equally spaced along horizontal lines. Mutations altering mass should produce new spots which are vertically displaced, and each mass mutation should in turn give rise to a new series of charge mutations producing new charge trains. If all of the proteins derived from one ancestral gene were analyzed on one 2-D gel, the result predicted would be a square grid pattern of spots.

In this paper we ask whether such a family of spots can be seen experimentally either in maps from single analyses, or in composite maps prepared from analyses of the proteins from related varieties. We also consider whether posttranslational modifications (PTMs) might produce the same pattern since PTMs may produce both charge and mass modifications (Anderson et al., 1984; Uy and Wold, 1977).

METHODS

2-D Electrophoresis. High-resolution 2-D electrophoresis was performed in acrylamide with recently modified versions of the ISO-DALT system previously described (Anderson and Anderson, 1979; Tollaksen et al., 1984). Isoelectric focusing and nonequilibrium pH gradient electrophoresis (O'Farrell et al., 1977; Willard et al., 1979) were done in groups of 20 simultaneously cast cylindrical gels. To resolve the more basic proteins, gels were loaded from the acid end and run for 14 000 volt hours, which was almost to equilibrium. Gradient slab gels (10 to 20%) were used for the second dimension separations and were stained with Coomassie Brilliant Blue.

Sample Preparation. Fourteen wheat cultivars were analyzed which were Armada, Avalon, Chinese Spring, Fenman, Flanders, Galahad, Longbow, Mardler, Maris Freeman, Maris Huntsman, Mission, Norman, Rapier, and Timmo. Individual wheat kernels, or groups of 5 to 10 kernels, were ground in a specially designed stainless steel grinder, transferred to tared 1.5-μL microfuge tubes and weighed. A volume of solubilization mix equal to four times (in more recent experiments six times) the weight of the group sample was added. The suspension was briefly sonicated and allowed to extract for 1 h at room temperature. The extraction mixture contained 3 *M* urea, 2% ampholytes (pH 3 to 10), 1% dithiothreitol, and 4% FSN 100 (a fluorocarbon-based detergent supplied by duPont). The suspension was resonicated briefly and centrifuged for 2 min in a Beckman microfuge. The supernatants were carefully removed with a disposable polyethylene pipette and frozen at −70 °C in 400-μL microfuge tubes until they were analyzed.

Internal Standards. Internal standards for charge (Anderson and Hickman, 1979) were prepared by the sequential carbamylation of creatine phosphokinase (CPK). Stan-

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dards for molecular mass were prepared from rabbit muscle and used as described (Giometti et al., 1980).

RESULTS

Analysis of Chinese Spring Wheat. A 2-D pattern of Chinese Spring wheat proteins with an included CPK charge standard train is shown in Fig. 1. The pattern is not random as is the case with most tissue proteins. Rather the proteins in the upper two-thirds of the pattern in Fig. 1 appear to be associated with charge trains, as predicted. Orienting studies showed that no major interfering storage proteins were found in the area where CPK carbamylated charge standards run and hence these standards are suitable for the left (acid) portion of the pattern. For more basic proteins, however, a new series of standards will be required.

Internal molecular mass standards were used to prepare the map shown in Fig. 2, which also indicates landmark areas (employed for descriptive purposes only) that may contain sets of related proteins. Those lying along horizontal lines may be different by one or more charges, while those lying fairly close together along a vertical line may be related mass variants involving additions or deletions.

Proteins lying in landmark areas I-III have a high probability of being related. Those that are in IV, however, involve many overlapping trains, and include high molecular mass gliadin subunits and alcohol-soluble subunits of glutenin having masses

Fig. 1. High-resolution two-dimensional electrophoresis pattern of endosperm proteins extracted from Chinese Spring wheat grains. Gels are oriented with the acid end of the isoelectric focusing separation to the left and the basic end to the right. The carbamylated charge stand. .d train, indicated by an arrow, is derived from creatine phosphokinase.

around 44 000 daltons (Bietz and Wall, 1980) and low molecular mass gliadins ranging in mass from 33 000 to 37 000 daltons (Beitz et al., 1977).

Landmark area I and II proteins are glutenins. The proteins of landmark area III are soluble in 70% ethanol and are identified as ω-gliadins on the basis of solubility and molecular mass (Woychik et al., 1961; Bietz and Wall, 1972).

Demonstration of relatedness will ultimately require genetic studies and sequencing of either individual proteins or the genes that give rise to them. A preliminary indication of relatedness or its lack may be gained, however, by recovering the protein from individual spots, subjecting them to partial proteolysis, and analyzing the products by one-dimensional (1-D) electrophoresis (Giometti and Anderson, 1984). This technique has been applied to spots 1 to 5 indicated in Fig. 2. The results (not shown) indicate identical patterns for spots 1 and 2; spots 3 and 4 likewise give identical patterns, which are different from the patterns for 1 and 2. The cleavage pattern of spot 5 differs from both the patterns for 1 and 2, and that for 3 and 4. If spot 5 is related to spots 3 and 4, it has been modified in ways that affect proteolytic cleavage of it.

Comparison of Different Strains. To search for proteins in different strains which might be related, and hence if mapped together might fall in the postulated grid patterns, 14 different varieties of wheat were analyzed and the results for the high-molecular mass region are shown in Fig. 3. For orientation, landmark areas I, II, and III are indicated for the Armada variety. In landmark area I, candidate molecular mass variants (vertical displacements) are seen for the Avalon and Maris Freeman varieties, while major charge variants appear for Avalon, Maris Freeman, Chinese Spring, and especially Timmo.

Charge differences, or major molecular mass differences, are easily distinguished when two variants are present in the same sample, and thus, appear together. However, it is more difficult to determine whether the major landmark area I protein of Galahad is a charge variant of the area I protein of Mardler, for example, because only one protein is present in this area in each of the samples. The questions to be asked are first, whether all of the major variants of landmark area III, for example, would fall in two molecular mass groups (i.e., would fall along just two horizontal lines), and hence may be descended from two ancestral proteins having different molecular masses produced by a major addition or deletion. The second question is whether the major proteins in this area appear at equal intervals in a horizontal direction and hence may be charge variants produced by point mutations of a single gene ancestral to the group.

Using landmark area III proteins as a model, extracts from Maris Freeman and Chinese Spring were mixed in three different ratios and analyzed with the results shown in Fig. 4. The major spots fall on two isomass lines indicated as 1 and 2, and form discrete horizontal charge trains, which differ in mass by approximately 8 kilodaltons. The interrelationships of the spots are shown diagrammatically in Fig. 5, to-

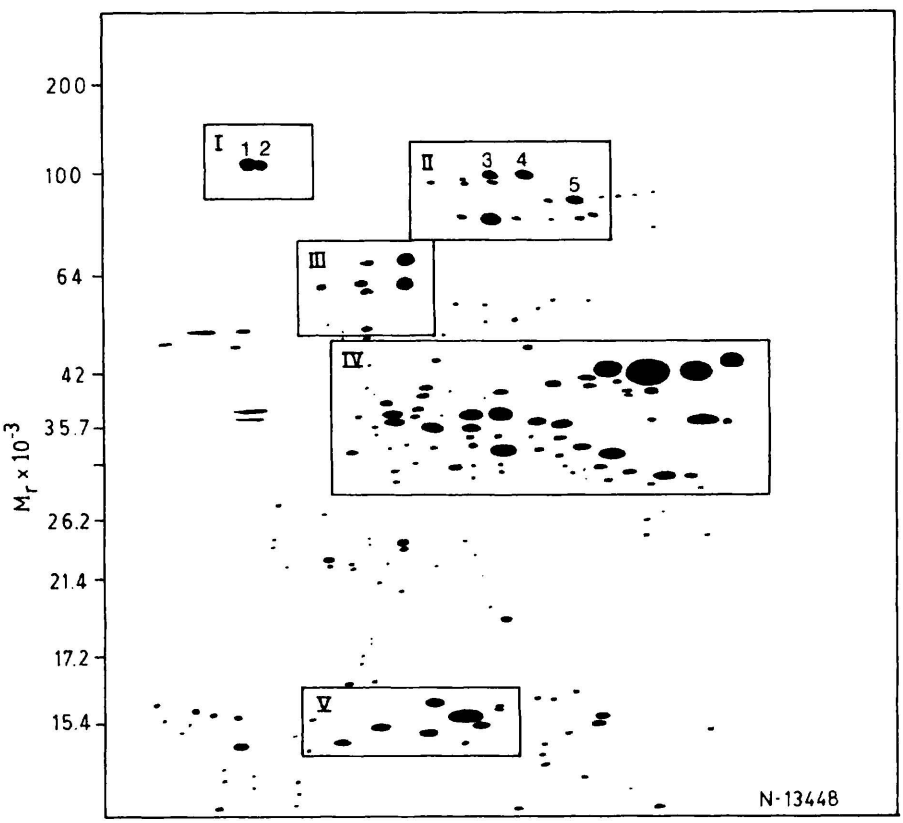

Fig. 2. Reference map for Chinese Spring wheat with molecular mass values derived from internal mass standards shown to the left. Landmark areas are delineated for reference. Proteins marked 1-5 were recovered for the limited proteolysis experiments described in the text.

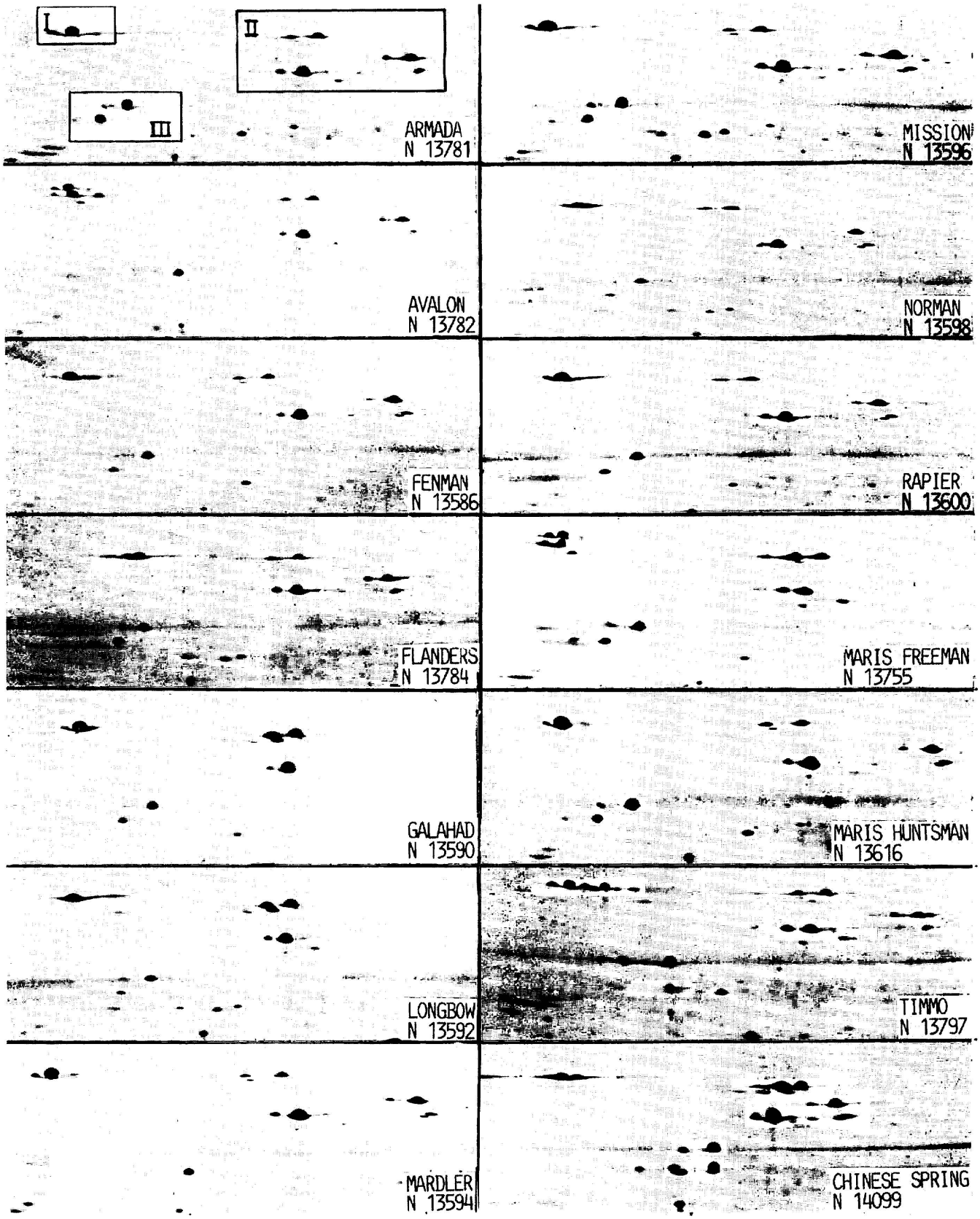

Fig. 3. High molecular mass proteins from fourteen varieties of wheat. Landmark areas are indicated in the Armada variety for orientation.

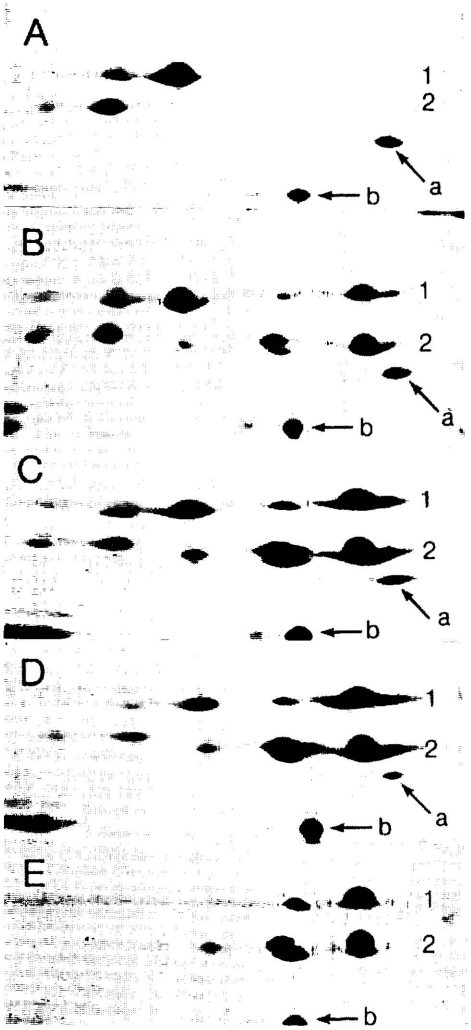

Fig. 4. Landmark area III from high-resolution 2-D patterns of proteins from Maris Freeman (A) and Chinese Spring (E) showing charge variant proteins found along horizontal iso-mass lines 1 and 2. Mixtures of proteins from these two varieties are shown in B-D. The ratios of Maris Freeman proteins to Chinese Spring proteins were 2:1 (B), 1:1, (C), and 1:2 (D). A marker protein from Maris Freeman is shown at *a*, while *b* is found in both varieties.

gether with grid lines indicating where the spots should be from the theoretical considerations presented in the introduction of this paper. Note that the spots are not quite evenly spaced in a horizontal direction in Fig. 5. On the top row the space between the Maris Freeman spot farthest to the right and the Chinese spring spot farthest to the left is larger than the rest. If the CPK charge train in Fig. 1 is carefully examined, it is seen that a wider space is also seen in the charge standards in this area. This difference in the spacing of the charge standard suggests that the unevenness seen in Fig. 4 and 5 is due to discontinuities in the ampholytes and not due to an absence of an average and uniform charge unit difference.

If the spacings in Fig. 5 are indeed due to single charge differences, they should match the spacings seen when the same proteins are modified by carbamylation to produce charge trains, as has been

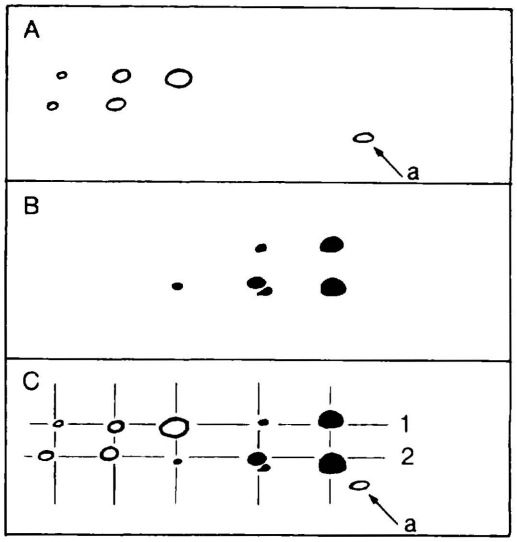

Fig. 5. Drawing of landmark area III proteins from Maris Freeman, A; and Chinese Spring, B; and a mixture of the two (derived from Figure 10), C. Grid markings show positions expected for sequential charge modifications, and for a mass difference between charge trains 1 and 2 of approximately 8000 daltons. The protein indicated by an arrow is from Maris Freeman and may be unrelated to other proteins in this area.

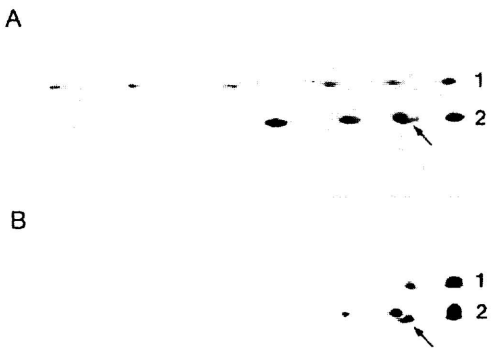

Fig. 6. Comparison of carbamylated (A) and uncarbamylated (B) landmark area III proteins of Chinese spring. Upper and lower carbamylation trains (A) are extensions of the small trains normally seen (B). Protein shown by arrow serves as a reference marker.

demonstrated with CPK. Extracts of Chinese Spring wheat were therefore carbamylated and the results for area III shown in Fig. 6. Comparison of the experimental and control samples demonstrates that the spacing observed in the composite maps of Chinese Spring and Maris Freeman after carbamylation closely corresponds to that observed for uncarbamylated samples.

Endosperm proteins have been analyzed by 1-D electrophoresis in starch or acrylamide gels in the presence or absence of urea, by electrophoresis in SDS, or by isoelectric focusing (Wrigley et al., 1982; Stegemann and Pietsch, 1983). Figure 7 illustrates diagrammatically the relationships between three of these methods, and their relationshp to high resolution 2-D electrophoresis. The 2-D pattern of the composite of proteins from Fig. 5 are shown in Fig.

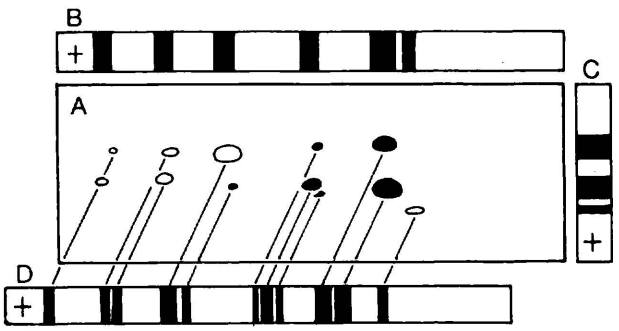

Fig. 7. Diagrammatic representation of relationship between 2-D pattern of wheat proteins and 1-D patterns of the same proteins obtained by three different methods. A, Drawing of 2-D pattern of proteins from landmark area III from composite of Chinese Spring and Maris Freeman (taken from Fig. 5C); B, pattern of same proteins obtained by isoelectric focusing under the same conditions used in the first dimensional separations in 2-D electrophoresis; C, pattern of the same proteins obtained by SDS electrophoresis in acrylamide; and D, diagrammatic pattern of the same proteins separated by electrophoresis in starch or acrylamide. Proteins in the upper horizontal row in A have a higher molecular mass than those of the second row, and hence are retarded (sieved) during electrophoresis through a microporous gel such as acrylamide, giving the resolution seen in D.

7A. The isoelectric focusing pattern for the same mixture is shown diagrammatically in Fig. 7B, and the pattern given by SDS electrophoresis is given in Fig. 7C. The type of pattern obtained by 1-D electrophoresis in a microporous gel in which the separation is based on both electrophoretic mobility and sieving is shown diagrammatically in Fig. 7D. One reason for the popularity of 1-D electrophoresis in starch or acrylamide is that under favorable conditions more complex mixtures can be resolved than with either isoelectric focusing or SDS electrophoresis because of sieving. The exact relationship between a 2-D pattern as shown in Fig. 7A and a 1-D electrophoretic pattern such as is illustrated in Fig. 7D will depend on the precise conditions used for the 1-D separations and may be quite different from those illustrated. The principle of sieving in 1-D electrophoresis remains the same, however, and molecules having the same mobility (charge-to-mass-ratio) in free solution but which differ in mass may be separated.

DISCUSSION

We have adapted the ISO-DALT system for high resolution two-dimensional protein electrophoresis, previously developed for clinical and basic research (Anderson and Anderson, 1979, 1982), to the analysis of whole wheat grains. The patterns suggest that charge modifications often occur in the major storage proteins of wheat and that mass modifications occur less frequently. The former appear as spots that are horizontally aligned and evenly spaced, thus indicating a similar mass for members of a charge modification series. Modifications involving change in mass (gap events), if sufficiently large, may produce a variant protein whose spot position is vertically displaced. With time, charge modifications of this mass variant may occur, giving rise to a new charge train parallel with the parent one. The initial results presented here support our thesis that mutations of storage protein genes would give rise to spots on 2-D gels that fall into grids representing both charge modifications and mass modifications.

Charge modifications may be due to (i) base substitution mutations yielding amino acid substitutions in the proteins coded for, (ii) additions, deletions, inversions, or frame shifts which change the number of acid or basic amino acids, (iii) posttranslational modifications (PTMs) programmed to occur in the cell producing the protein (Beevers, 1982; Uy and Wold, 1977), or (iv) age-related changes such as deamidation, which can occur in proteins during storage. It is essential to distinguish among these four processes both for genetic studies and for attempts to develop a storage protein nomenclature similar to that proposed for mammalian cell proteins (Anderson and Anderson, 1984).

Whether mutations which change mass produce a charge shift depends on whether the added or deleted segment contains acidic or basic amino acids. Repeating sequences of gliadins (Kasarda et al., 1983), with one exception, do not appear to contain charged groups (Rafalski et al., 1984), and the addition or deletion of most repeats would therefore be expected to alter mass but not charge.

In several instances we see a small row of spots, which decrease rapidly in intensity from right to left. The members of this decreasing series, excluding the first (right hand) spot, may be due to enzymatic PTMs or to aging. To distinguish between these two possibilities, it will be necessary to follow the course of synthesis to see which form in a charge train appears first and to find out whether the PTMs involve sialation, phosphorylation, deamidation, or one of the other numerous known PTMs (Uy and Wold, 1977). For storage proteins, especially, it is important to see whether modifications increase with storage time and thus may be age related. Archeologically derived samples of wheat do not yield useful 1-D electrophoretic maps (Zeven et al., 1975) suggesting that modifications do occur with age, but may occur slowly. No convincing evidence for PTMs has been presented previously in part because sensitive methods for detecting them have not been available.

The number of charge and mass modifications seen in wheat storage protein 2-D maps is surprisingly large, and would not be expected from previous comparative studies on cellular proteins. The possibility that some mass modifications are produced by proteolytic cleavage must be examined experimentally, and can be approached by analyzing grains which have been pulse radiolabeled at various stages during the synthesis of storage proteins, or by mapping the translation products from endosperm mRNA. However it is unlikely that interstrain differences seen in related proteins are caused by posttranslational modification.

We propose the following explanation for the large numbers of single amino acid substitutions (as evidenced by charge shifts) and gap events seen in wheat storage proteins. Previous studies on the incidence of protein variants detectable by 2-D electrophoresis suggest that proteins that are involved in cell structures are highly constrained, and few variants of them are found (McConkey, 1982). Soluble cell proteins,

which have fewer structural constraints because they do not physically interact with other proteins, constitute a second class of proteins that show increased variability. Storage proteins constitute a third class of proteins that appear to have even fewer structural constraints and that can be widely varied without affecting survival and natural selection. If this postulate is true, it has profound implications for wheat breeding and for radiation biology because it suggests that in storage proteins we may be able to observe a very large fraction of original mutational events, with decreased or minimal selection.

It has been proposed that blocks of proteins that always appear together on 1-D electrophoretic patterns, and hence are coinherited, may be the products of tightly linked genes (Metakovsky et al., 1984). Bands in a single "block" that are close together on 1-D gels may be due to charge modification of a single protein, and more widely spaced bands in a block may be due to proteolytic cleavage of a protein that was originally much larger. Alternatively, the members of a "block" may fall in one grid pattern, and may be the products of tandem gene duplication with subsequent mutation.

Preliminary conclusions from 2-D maps studies (and also from 1-D electrophoresis), which suggest that two or more proteins are related, require confirmation by sequencing either entire proteins or the genes producing them, by genetic analysis to show which proteins are actually allelic, and by biochemical studies to differentiate genetic differences from post-translational modifications. The major advantage of 2-D maps is that they provide the highest resolution analytical separations currently available, can provide quantitative data, and indicate which proteins may be interrelated and should be intercompared. Our strategy for mapping is to produce maps containing as many spots as possible initially. In subsequent studies the classical endosperm protein fractions will be mapped. This data together with accreting protein identification data (including enzyme identifications) will provide a series of descriptors to be associated with each spot listed in a wheat protein data base (Anderson and Anderson, 1982) and accessible through electronically displayed maps.

Annotated 2-D maps of wheat protein may constitute the best description and definition of a variety for patent or varietal protection purposes since patent or registration protection is for the whole plant, and the status of protection for parts of plants (whether genes, proteins, or sets of proteins) is unclear (Williams, 1984). It is evident that routine 2-D mapping of half grains will be useful in plant breeding studies, and may serve to define varieties objectively.

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