Chapter 9

Application of High-Resolution, Two-Dimensional Electrophoresis to the Analysis of Wheat, Milk, and Other Agricultural Products

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High resolution two-dimensional electrophoresis allows hundreds of proteins to be separated and characterized in submilligram samples of complex protein mixtures. Applications of this method to the analysis of agriculturally important products, including milk, meat, and wheat are reviewed. In a model study we analyzed 100 individual kernels of the wheat cultivar Newton (Triticum aestivum L.) for electrophoretic variants. One variant protein was found in 47 kernels, while three variant proteins occurred together in two of the kernels. The implications of two-dimensional electrophoresis for cultivar identification and the problem of relating electrophoretic protein variants to genetic variants are discussed.

High resolution two-dimensional electrophoresis with computerized image analysis and data reduction is the highest resolution method currently available for the analysis of complex protein mixture. In this discussion we review the potential of the method for the analysis of agricultural food products including milk, meat products, and wheat, and present one representative study on the analysis of Newton wheat for electrophoretic variants.

While 2-D electrophoresis is fundamentally a very simple technique, numerous technical problems arise if the method is to give high resolution results which are reproducible and intercomparable, and if quantitative results are to be obtained. The interpretation of data posses additional challenges because the vast majority of proteins in both plant and animal tissues have been neither described nor named, and therefore have functions which remain to be discovered.

Our objective is to develop analytical systems and protein data bases for agricultural products which allow proteins to be identified with confidence by position, to identify as many "known" proteins as possible in the patterns, and to develop methods for quickly and reliably finding both quantitative and qualitative differences

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New Directions in Electrophoretic Methods

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between samples. The data bases are linked electronically to the patterns displayed in color on a CRT. This arrangement allows rapid recovery and display of all available information relative to each protein resolved, and differences between one or more patterns may be discovered. New multi-windowing techniques allow the same section of several dozen patterns to be seen and intercompared in one image.

Fundamentals of 2-D Electrophoresis

High resolution 2-D electrophoresis is done in acrylamide gels using either isoelectric focussing or non-equilibrium pH gradient electrophoresis in the first dimension, and electrophoresis in the presence of sodium dodecyl sulfate in the second (1-4). Denaturing and reducing conditions are used in both dimensions so that multimeric proteins are dissociated into subunits, and the tertiary structure of subunits is largely lost. The resulting separation is based on charge (which reflects the ratio between acid and basic amino acids in each protein), and mass. Since the proteins are unfolded, all amino acids contribute to the overall charge.

The resolving power of 2-D electrophoresis is very high. When complex mixtures of proteins are carbamylated to produce charge shift trains for proteins having a large range of molecular weights, it is found that single charge differences can be easily detected over the mass range of approximately 10,000 daltons to over 100,000 daltons (5). Carbamylated proteins have therefore been used to produce charge trains which appear on 2-D patterns as horizontal rows of spots which are useful for calibration in the first dimension (6,7).

For calibration in the second dimension, use is made of proteins of known molecular mass which give horizontal streaks across the final gel which each define a different and known mass (8). Mass differences of greater than 2% may be detected over the entire mass range resolved, which means that single amino acid deletions cannot ordinarily be seen (5).

Resolution may be defined in terms of the total number of spots of the sizes seen on a particular gel which could be packed into a 2-D pattern and still be resolved (9). This is a theoretical number, and it is independent of the actual number seen in a gel. It accurately reflects differences in resolution seen between batches of gels. Experimentally we have produced gels with theoretical resolutions as high as 38,000 using our ISO-DALT system.

Recently the ISO-DALT system has been extensively modified so that 40 isoelectric focussing gels may be run in parallel, the slab gels are cast using a computerized gel casting system, and larger second dimension slab gels are used (N.L.Anderson, unpublished studies).

A number of image analysis and data base systems have been developed to allow intercomparison of gels, and for spot quantitation. The TYCHO system allows images to be rapidly acquired, streaks and other imperfections removed, and images matched using non-linear stretching algorithms (10). The reliability of this process is demonstrated by systematically removing one at a time each spot from one of the two patterns being intercompared, regenerating the removed spot from the remaining data, and then comparing the regenerated spot position with the original true position. The average error between the original and the regenerated spots was a

small fraction of a millimeter (9). Using Coomassie Blue staining, more than 100 proteins may be measured in liver samples with coefficients of variation of less than 15% (11). Using specific antiserum staining individual proteins may be identified in entire 2-D patterns transferred to nitocellulose or other suitable binding supports (12).

Analysis of Milk

The analysis of milk illustrates the power of 2-D methods to resolve interesting problems. While the major components of human milk and cows milk have been identified on 2- D gels (14), hundreds of minor ones which can be resolved on silver-stained gels remain to be characterized. The contribution of 2-D methods to the solution of a central problem in milk coagulation important to cheesemaking is not well known and, since it has only appeared in the literature of human clinical chemistry, it is briefly reviewed here. Caseins, including kappa casein, have isoelectric points around pH 4.5, and all may be isoelectrically precipitated. The terminal peptide region of Kappa casein contains acidic amino acids and is multiply post-translationally modified by the addition of negatively charged groups including phosphate and sialic acid, to give the characteristic acid isoelectric point. However, when the negatively charge-modified region (termed the macropeptide) is cleaved off by chymosin, the remaining larger molecule (para kappa casein) bears a net positive charge. Para kappa casein, which was discovered to be very basic using 2-D gel analysis, then precipitates with the remaining caseins at neutral pH. Experimentally other similarly basic proteins will also precipitate alpha and beta caseins (14).

This discovery of the mechanism of the first step in cheese making is based on 2-D analysis where the native kappa casein was found at the acid end of the 2-D gel (i.e., to the left), while the larger cleavage product, para kappa casein, was found to be so basic that is was not on a regular 2-D pattern, but was only found when non-equilibrium first dimension conditions, specifically designed for the analysis of very basic proteins, were employed. Once it was realized how very basic para kappa casein is, the mechanism of protein precipitation in cheesemaking became apparent and was confirmed using other similarly basic proteins. 2-D analysis is therefore of great use in the study of protein processing, and offers many interesting opportunities to study protein changes during the ripening of cheese, and in the alteration of proteins in foods during processing and storage.

Muscle Proteins

Muscle proteins have been extensively studied (see review in ref. 15) and this work provides the basis for future research on various meats. While many muscle proteins have been identified in samples of human and rabbit origin, we have found no published systematic study of meat proteins of various food animals, nor of the changes which occur during aging, storage, and spoilage. Such studies would be useful for the identification of adulterants, for studies on curing and aging, and in the evaluation of storage conditions.

Analysis of Wheat Endosperm Proteins

High resolution two-dimensional electrophoresis has been applied to the analysis of endosperm proteins (16-22) and used to determine the chromosomal location of a number of different proteins in aneuploid strains of wheat. The method has not previously been used to look for electrophoretic protein variants within one strain. In this paper we describe individual analyses of 100 grains of Newton wheat (Triticum aestivum L.) with the procedures previously described (22). Newton (CI 17715) is a hard red winter wheat and is an increase of a single F4 plant derived from six parent strains (23). One dimensional electrophoresis has been used to detect variants of endosperm proteins, commonly termed biotypes (24,25), coexisting in one strain of wheat. They have not been systematically studied by two-dimensional electrophoresis however. The questions raised here are: (1) Can high-resolution 2-D electrophoresis using non-equilibrium conditions in the first dimension resolve reproducibly endosperm proteins in a series of 100 samples, (2) are electrophoretic proteins variants observed in a widely planted strain with this method, (3) will the method prove useful in strain characterization, and (4) will molecular inventories provided by 2-D maps prove useful and possibly necessary for patenting or registering new varieties.

Methods. Individual wheat kernels were ground in a small stainless steel mortar and extracted with a mixture containing urea, a nonionic detergent, and a reducing agent as previously described (22). Using a modification of our ISO-DALT system (3,4) we cast and ran both the first dimension non-equilibrium pH gradient gels and second-dimensional slab gels in batteries of 20. All gels were fixed and stained in Coomassie Brilliant Blue as described, and analyses were done on 8x10 inch high contrast prints. A Bausch and Lomb zoom transfer scope, model ZT4-H, was used to compare prints and to prepare maps of patterns.

Results. A map of Newton wheat proteins is shown in Fig. 1. Of the 100 grains analyzed, 47 maps contain the landmark area IV electrophoretic variants shown in Fig. 2, while two maps exhibited all three of the landmark area II electrophoretic variants shown in Fig. 3.

Discussion. The results indicate that electrophoretic variants exist in one sample of an inbred strain of wheat, that these variants are easily detected by high resolution 2-D electrophoresis, and that reproducible results may be obtained during an extended series of analyses. One major electrophoretic variant was found in 47% of the grains, while three minor electrophoretic variants occurred together in 2% of the grains analyzed. It appears that the genes for the three minor variants may be tightly linked, or that all may be the products of one gene, with the lower-molecular-mass variants produced by successive cleavage of two peptides off a larger variant.

It is important to distinguish between electrophoretic variants, posttranslational modification, physiological variants, and genetic variants. The electrophoretic variants described here could be true genetic variants produced either by mutations of existing genes in Newton, or by the introduction into the strain of new genes allelic

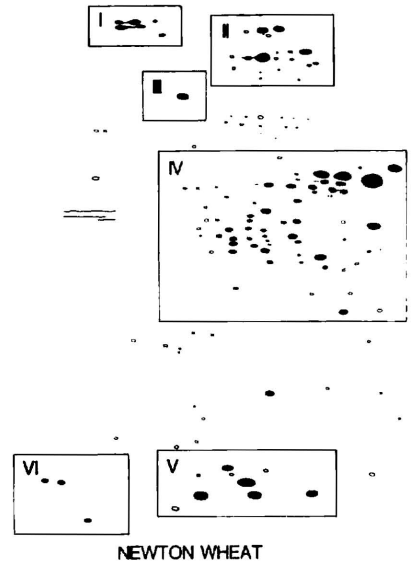

Figure 1. Map of major endosperm proteins of Newton wheat. The landmark areas indicated by rectangles are those previously described (9). The electrophoretic variants in this study occurred in landmark areas II and IV.

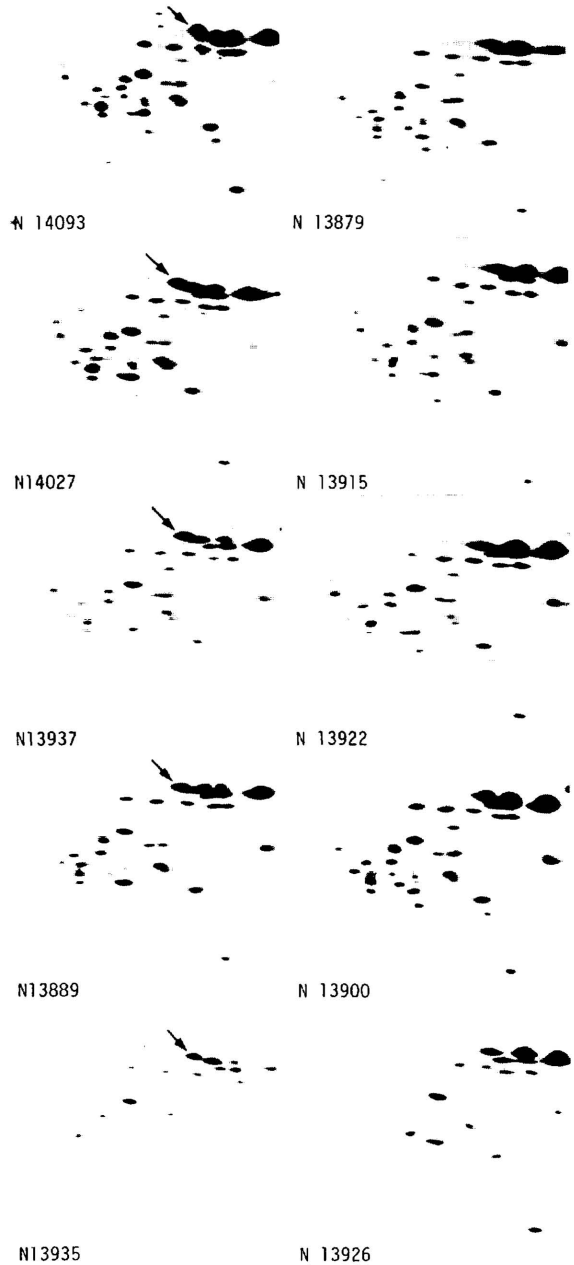

Figure 2. Photographs of portions of Coomassie blue stained gels showing landmark area IV. Representative wild type patterns are shown on the right, and patterns containing electrophoretic variants are shown on the left. Arrows identify variant protein.

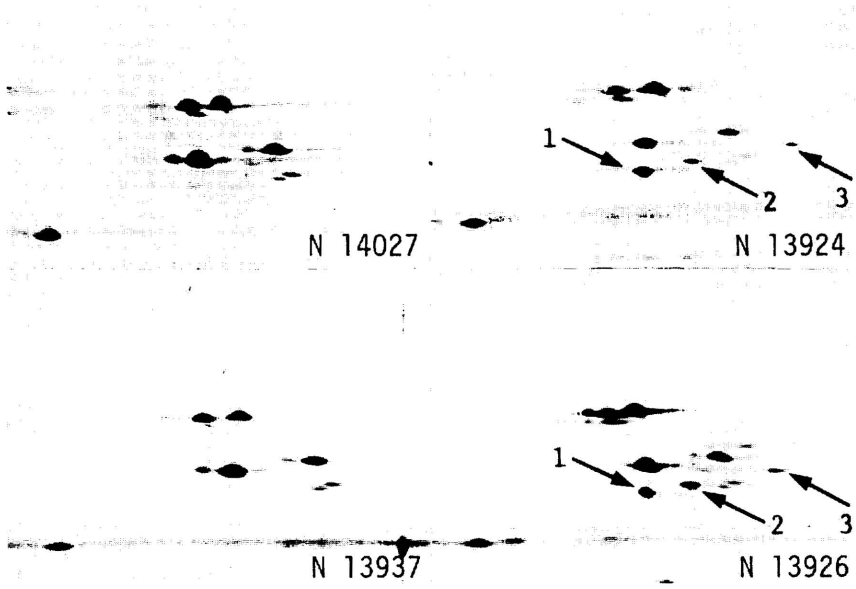

Figure 3. Photographs of landmark area II showing wild type patterns (left) and the two patterns exhibiting three coexpressed electrophoretic variants identified by arrows (right).

to those already present. In either case, breeding studies would show whether or not the gene products (proteins) behaved in a Mendelian fashions, whether a variant and a candidate wild type protein are allelic, and whether any of the genes are tightly linked. If an electrophoretic variant is a physiological variant, then the gene for that protein may behave in a Mendelian manner, but the appearance of the protein gene product would be controlled by a physiological or environmental variation as is the case, for example, with temperature mutants. New posttranslational modifications that are produced by a new or altered protein-modifying enzyme may also yield new spots that are not the direct product of altered genes. Sequencing of the gene responsible for a "wild type" and for a candidate genetic variant provides the definitive answer to the question of whether a given electrophoretic variant is indeed a direct genetic one. In the practice of clinical chemistry, electrophoretic variants that are not direct genetic variants are so rare that it is nearly always assumed that an electrophoretic variant is a genetic variant. A similar assumption has often been made in the interpretation of 1-D electrophoretic patterns of endosperm proteins. However, with the high resolution made possible by 2-D electrophoresis it is important to reexamine all possible sources of electrophoretic variants and to demonstrate experimentally the molecular and genetic basis of observed variants. In experimental studies of wheat endosperm proteins previously reported, altered electrophoretic patterns have not been seen in response to different environmental conditions (26-30), but have been noted in cases of extreme sulfur deficiency in wheat (31).

Since only a fraction of a grain is actually required for analysis, the seed embryo and part of the endosperm may be retained and germinated. Thus, 2-D electrophoresis may be used to screen several hundred individual kernels and the results used to select embryos that will produce uniform strains which may be crossed to demonstrate that candidate variants and wild-type pairs are indeed allelic. Whether the variants described here contribute in any important way to flour and baking quality can best be discovered by producing substrains homozygous for each.

If 2-D maps are to be used for varietal identification and for inclusion in patent and registration descriptions as we have suggested (22), then either residual variability should be eliminated from the strain, or variants should be included in varietal maps and descriptions. This suggestion arises because entire organisms and not genes or gene products are patented or registered under present interpretations of the relevant law in the United States (32).

When an electrophoretic variant is observed, it is important to discover which other protein in a pattern is the product of the allelic wild type gene. The positive identification of variant and wild-type pairs will require partial sequencing of the proteins involved and/or breeding studies in which plants homozygous for each gene of a pair are obtained. The most likely candidate for the wild-type protein for the polymorphism shown in Fig. 2 is the protein immediately to its right, which appears to be diminished in quantity when the polymorphism is present. For the three variants in Fig. 3, the protein immediately above variant number 1 may be its wild-type allele; however for variants 2 and 3 it is not possible to assign candidate wild-type spots. Methods for identifying posttranslational modification have been previously discussed (22).

Genetic Purity

In any analytical series such as the one described, the questions of strain definition and of cultivar purity arise. For strains to be used in the Great Plains area of the United States, many breeders consider genetic heterogeneity necessary for best agronomic results. In contrast, homogeneous types are usually preferred in Europe and in Canada. In either case a description of the original strain as developed by the breeder is required if purity during extended cultivation is to be assessed. Any wheat grown on a large scale will inevitably become genetically contaminated with other germ plasm, or a batch of seed may be physically contaminated with a few kernels from another variety. Therefore the variants observed may have been present in the original plant and, if unrelated to any undesirable property of the cultivar, may never have been bred out of the strain. Alternatively the minor variant may have appeared later and may be present in a small fraction of existing stores of Newton wheat. The possibility also exists that one or both of the electrophoretically variant spots could be physiological variants what are expressed under some environmental conditions and not others. While only extreme growing conditions have been found to alter 1-D electrophoretic patterns of wheat endosperm proteins (31), the unlikely possibility of physiological variability can only be ruled out experimentally.

The basic questions relating to the design of experiments to settle these problems are whether the electrophoretic variants described were present in the original single parent plant from which the strain was derived, whether mew mutations have occurred during bulking and in production, whether new genetic material has been introduced by accidental crossing during the years since the variety was first produced, or whether the sample we have analyzed is unique and contaminated. Obviously a more extended series of studies is required to answer these questions concerning the past history of the variety Newton..

Prospectively, 2-D mapping of the parent strains and the cultivar registered would define the variety at the outset and would suggest the parental origin of each electrophoretic variant seen. Should electrophoretic variants be observed, they can be bred in or out at the very outset, or heterozygosity may be deliberately maintained using 2-D electrophoretic techniques to guide breeding. In addition, strains homozygous for each variant may be produced to determine whether any of the variant proteins are linked to a desired property, for example to baking quality. Once a cultivar is defined by 2-D mapping, then it is a simple matter to follow changes occurring with time by mapping small numbers of kernels obtained from many different locations. For retrospective studies, carefully randomized sampling of many different seed stocks during successive years will be required to answer some of the questions raised.

When a data base of 2-D maps of wheat proteins becomes available, it will be easier to identify contaminating kernels or their genes, to discover new proteins arising from adventitious crosses, and to identify the sources of such accidentally introduced genes. To maintain a new variety, it may be necessary to continually

monitor each stage in seed production from breeder's seed through foundation seed, registered seed, on to the certified seed supplied to farmers. The studies reported here therefore constitute only a first step in the application of high-resolution two-dimensional electrophoresis to wheat cultivar identification and to wheat breeding.

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