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Review

Gel electrophoresis for the identification of plant varieties

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Abstract

The use of various types of gel electrophoresis for distinguishing between and identifying plant varieties is well established. This review describes how different electrophoretic techniques [native polyacrylamide gel electrophoresis (PAGE), sodium dodecyl sulphate PAGE, isoelectric focusing and two-dimensional methods] have been utilised in a wide range of crop species. The fact that there are different types of plant variety, varying in genetic structure, is emphasised, and the way in which this influences the choice of technique is discussed. Other factors which need to be borne in mind when discussing identification in its broad sense are also outlined. Some practical applications of electrophoresis in this area are mentioned and finally future trends for the use of electrophoresis in plant variety identification studies are considered.

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1. Introduction

Most agriculturally important crop species exist as a number of genetically distinct, but related, varieties. These varieties are either indigenous, locally adapted ecotypes (landraces) or, increasingly commonly in the modern world, cultivated varieties (cultivars), actively produced via the efforts of plant breeders. The ability to discriminate between and identify varieties of crops is crucial to the seed and related industries. All sectors of these industries benefit from plant variety identification and there are various approaches which can be taken to the problem. This article is concerned with the use of gel electrophoresis of proteins and enzymes as a means of variety identification. It will concentrate on the application of electrophoresis to cultivars, although much of the information is equally applicable to landraces.

1.1. Types of plant variety

There are generally four categories of cultivated variety which can be recognised, arising primarily from the reproductive system of the crop species in question. Hence there are (1) varieties comprised of a line or lines of self-pollinated (autogamous), highly inbred plants; (2) varieties which consist of a series of cross-pollinated (allogamous) plants; (3) varieties which are re-constituted at each cycle of seed production by crossing inbred parental lines (F1 hybrids); (4) varieties which are clones, or groups of clones. The genetic structure and degree of uniformity (homogeneity) of each of these categories of variety differ. For instance, all individual plants from a self-pollinated variety will be homozygous at many loci and be uniform phenotypically, whereas cross-pollinated varieties are populations, whose individuals can be either homozygous or heterozygous at a particular locus. Again, whilst all plants within a F1 hybrid variety are uniformly heterozygous and within a clonal variety are either homozygous or heterozygous, both types of variety are extremely uniform phenotypically. These differences in genetic structure are important when considering the use of electrophoresis for identification.

1.2. Plant variety identification

There are various different approaches that can be taken to plant variety identification, ranging from straightforward phenotypic (morphological) examination to an assessment of DNA polymorphisms [1,2]. The method of choice depends to some extent on the type of variety in question, but more importantly on the particular identification requirements of the job in hand. Because variety identification is important to a number of different elements of the agricultural seed sector, the method of choice will vary from situation to situation. Also, there are varying interpretations of the concept of identification, i.e. (1) identification in its true sense —what variety is this? (2) variety distinctness —is this variety different from those? (3) varietal purity —is there more than one variety in this sample? (4) variety description —can we obtain data that can be used to describe varieties and hence assist in their characterisation and classification? Thus clearly the requirements of a grain miller, who needs to be able to identify varieties of wheat suitable for processing into particular types of flour product, differ greatly from those of someone involved in variety registration work, where a detailed description of the variety is required, along with data that will distinguish it from other varieties of the same species. Having said that, gel electrophoresis of proteins and enzymes has been successfully applied to many different identification situations.

2. Electrophoresis and variety identification

The fact that proteins are direct products of gene translation and transcription makes them ideally suited for plant variety identification purposes. Analysis of protein composition can be considered to be an analysis of gene expression and a comparison of the composition of a particular set of proteins becomes a comparison of the genetic differences between individuals.

Since all varieties are different from one another, and the differences must be at least in some part genetically based, protein composition forms an ideal means of variety discrimination. In plants, there are many proteins that are highly polymorphic, particularly the seed storage proteins but also many other seed and vegetative proteins and enzymes. Also, there are many types of electrophoresis method that can be used to separate plant proteins. Hence it should not be altogether surprising that gel electrophoresis should be so useful for variety identification work.

2.1. Seed proteins

Much of what follows is concerned with the analysis of seed proteins and thus a brief review of their classification is worthwhile. A useful nomenclature for seed proteins is the Osborne fractionation [3], which is based on the solubility properties of different fractions of seed proteins. Although molecular analysis has called certain aspects of this fractionation into doubt, it provides a convenient system for laboratory use. In the Osborne classification, there are four types of seed protein: (1) water-soluble albumins, comprising mostly enzymes; (2) salt-soluble globulins, which occur in membrane-bound protein bodies and are seed storage proteins in sensu stricto; (3) alcohol-soluble prolamins, which are also true storage proteins; (4) acid- or alkali-soluble glutelins, which are probably mostly structural proteins, but may have some metabolic functions. The proportion of each type of protein varies from species to species. For instance, the seeds of cereals such as wheat, barley, maize or rye contain high levels of prolamin-type proteins, whereas in other cereals (oats, rice) higher levels of globulins are found. Leguminous seeds (e.g. beans, peas, lentils) also have a large proportion of their proteins in the form of globulins.

2.2. Approaches to the use of electrophoresis

Because of the differences in the genetic structure of the various types of variety (Section 1.1.), the ways in which electrophoresis can be used to distinguish between and identify varieties vary depending on the species under consideration. Two principal approaches have been recognised [2,4]: (1) the direct comparison of protein compositions between varieties, which usually (but not necessarily) requires the analysis of polymorphic proteins which are encoded at multiple loci. The prolamins of cereal seeds provide good examples. These seed storage proteins are encoded at several multi-genic loci, and the products of one locus comprise a group of electrophoretically separable protein bands. With this approach, the criterion for a difference between varieties is taken as the presence or absence of a particular band or group of bands at a defined position on the gel; (2) the indirect comparison of the frequency of occurrence of protein band phenotypes in different varieties. This usually (but again not exclusively) necessitates the analysis of proteins (enzymes) derived from a single locus, i.e. isozymes. It is generally true that the first approach is more suited to inbred, F1 hybrid and clonal varieties whereas the second, or variants of it, can be utilised for cross-pollinated varieties.

With this background in mind, the remainder of this review considers the various types of gel electrophoresis method that have been used for plant variety identification. This topic has been reviewed previously in recent years (for instance [5–7]) and it is not intended to repeat such efforts. Rather, each group of methods is considered in turn, and the objective is not to compile a comprehensive list of references and applications, but rather to highlight particular problems and successes, in the context of the different identification situations.

3. The use of "native" gel electrophoresis methods

For this review, "native" electrophoresis methods are taken to include those methods in which no dissociating agent which affects protein charge is present and in which the biological activity of the proteins is largely preserved.

Hence this definition specifically excludes methods involving the use of sodium dodecyl sulphate (SDS) and also isoelectric focusing (IEF) methods, both of which are dealt with subsequently. For convenience, the section is divided into consideration of methods involving specifically defined and/or "total" protein fractions, and those involving particular enzymes.

3.1. Analysis of proteins

3.1.1. Acid polyacrylamide gel electrophoresis of prolamins

One of the most well-documented and widely used native electrophoretic approaches to plant variety identification is the analysis of the cereal prolamin storage proteins at acid pH. Prolamins from cereal seeds are usually known by their trivial names, e.g. gliadins in wheat, hordeins in barley, zeins in maize, avenins in oats. Their analysis serves as a useful model for discussing general problems in the uses of electrophoresis for variety identification and so will be considered in some detail.

The first reported analyses of wheat gliadins utilised starch gel electrophoresis (SGE) and a lactic acid–aluminium lactate buffer system at pH 3.2 (see refs. [5,7–9]) although this has now been almost entirely superseded by the use of polyacrylamide gel electrophoresis (PAGE). There are many variants of acid PAGE methods which have been applied to a range of cereal crop species. For instance, Cooke [5] listed 17 published acid PAGE methods for the analysis of gliadins, differing mainly in the buffer and gel compositions, and more have been produced since the date of that survey (1988). This wide range of methodologies has caused problems, particularly for the comparison of results from laboratories and for variety registration work, and various organisations have sought to rationalise the situation by adopting "standard" methods. Prominent amongst these internationally have been the International Association of Cereal Science and Technology (ICC) and the International Seed Testing Association (ISTA), both of which have standard reference methods for the identification of wheat varieties by electrophoresis. The methods are similar, involving the use of acid PAGE to separate gliadins [4,7,10], but they do differ in detail and can produce somewhat different separations of certain gliadins in some cases. However, it should be noted that the actual discrimination between different varieties achieved by the two methods is similar, i.e. the results produced from analysis of a particular sample have equivalent meaning. The Royal Australian Chemical Institute (RACI) also has a standard method for wheat, employing gradient pore acrylamide gels at acid pH. All of the methods can be applied to the separation of prolamins from other cereals [5,7–9]. Typical results for varieties of wheat, barley, triticale, rye and oats analysed using the ISTA acid PAGE method are shown in Fig. 1. These methods have also been used, with modifications, to distinguish between varieties of rice, maize, and sorghum [5–7]. Discontinuous versions of the acid PAGE procedure, using an alanine–formic acid buffer at pH 4.25, have also been published, and seem to improve the resolution of certain gliadins [11]. This method can also be applied to study varieties of barley, oats, triticale and other cereals.

Acid PAGE of prolamins is clearly capable of revealing extensive protein polymorphism. To be able to use this polymorphism for identification purposes, it is necessary to employ a system for recording the data from the gels ("scoring") and then devise a classification scheme based on the recorded data. There are various ways of scoring the data from acid PAGE gels. Fig. 2 illustrates some of the systems that can be used for recording wheat gliadin profiles, for instance. Again, White and Cooke [13] compared six schemes of nomenclature for barley hordeins and proposed a standard method for seed testing and similar uses. It remains to be seen whether or not these proposals will become widely adopted. Regardless of the detailed system used, authors from many countries have published catalogues of prolamin electrophoretic patterns, particularly for wheat and barley varieties [5–10]. Such relatively straightforward acid PAGE techniques can be impressively discriminating. For example, a study of 353 European barley varieties found

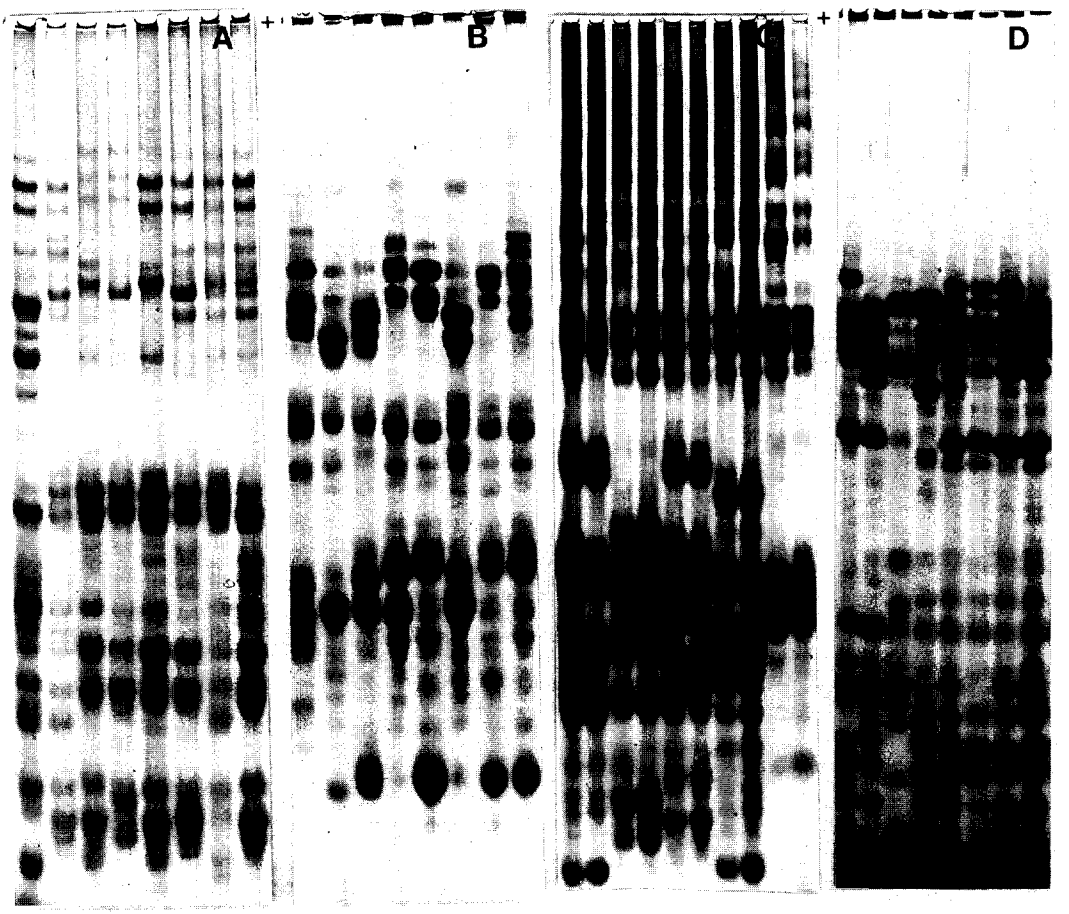

Fig. 1. The use of the ISTA standard reference acid PAGE method [10] to analyse the alcohol-soluble prolamins from varieties of: (A) wheat (*Triticum aestivum*), (B) barley (*Hordeum vulgare*), (C) triticale (× *Triticosecale*) (tracks 1 to 8) and rye (*Secale cereale*) (tracks 9 and 10), (D) oats (*Avena sativa*). In all cases, each track represents the prolamin profile of a single seed of a different variety (in C, the varieties are in pairs). Note the polymorphism of the prolamins and the variety-specific profiles. For oats (D), the ISTA method was modified by the use of 12.5% (T) acrylamide gels —for all other crops the gels are 9% (T) acrylamide. In all cases the tank buffer is glycine–acetic acid, pH 3 and the anode and origin are at the top. (Unpublished data of Cooke et al.).

that they could be divided into 70 different groups, based solely on the hordein composition [13]. Even higher levels of discrimination are generally reported for collections of wheat varieties following acid PAGE of gliadins, e.g. 137 out of 155 varieties grown in the UK were uniquely identified [14].

A point of some interest and importance, apparently not always appreciated by authors, is that it is essential to assess the uniformity (homogeneity) of the protein profile of a variety. This can only be achieved by analysing individual seeds from within varieties. Even though modern varieties of wheat and other cereals are highly self-pollinated and often produced by single plant descent techniques, it is still the case that a proportion of them contain so-called biotypes, that is they consist of more than one electrophoretically identifiable line [5,8–10,13–15]. Biotypes are not a major obstacle to the use of electrophoresis for variety identification, but they must be recognised and catalogued.

Acid PAGE methods have proved to be extremely useful for variety identification of self-

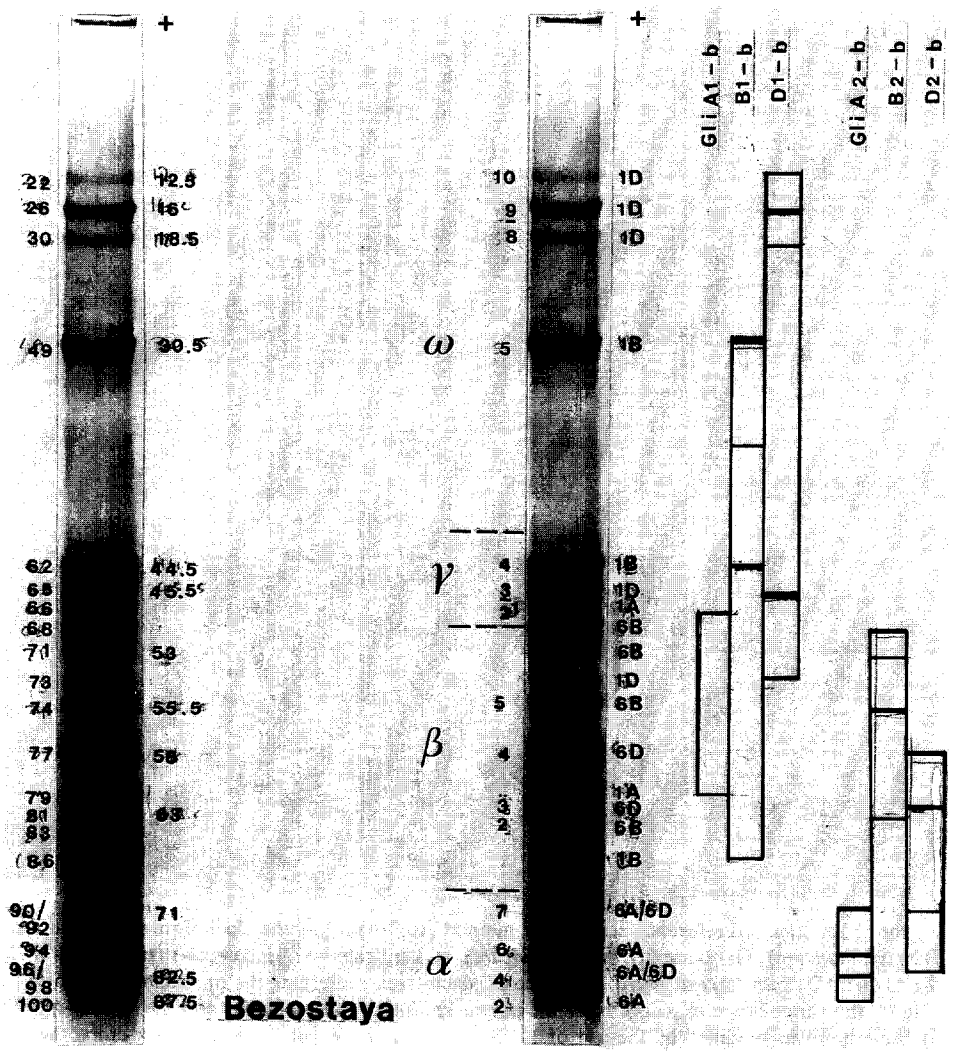

Fig. 2. Some of the nomenclature systems that have been used to describe gliadin profiles, with the variety Bezostaya as an example. From the left the systems are: (i) that originally used by Autran et al. (see ref. [9]) for gliadins separated by SGE, but since applied by various laboratories for PAGE analyses [14]; (ii) that proposed by Wrigley et al. [9] and used extensively in Canada; (iii) that utilised by Konarev and co-workers for cataloguing the collection of the Vavilov Institute (see refs. [5,14]); (iv) the chromosomal locations and alleles at the *Gli-1* and *Gli-2* loci, originally proposed by Sozinov et al. and recently modified and extended by Metakovsky [12]. Not all of the bands have been assigned in all cases. Acid PAGE (ISTA method [10]) was used for analysis of gliadins. Sample of Bezostaya kindly provided by Dr. Eugeny Metakovsky (ISPC, Milan). Photographs from unpublished data of Dida and Cooke.

pollinated cereals. New developments continue to be made. For instance, Hsam et al. [16] recently reported the use of commercially available horizontal gels and buffer kits for the analysis of barley hordeins, which clearly could be useful in some of the more routine laboratory testing situations. There are, however, many other electrophoretic methods and polymorphic protein systems in plants that can be exploited for identification purposes. The problems that were outlined above —standardisation of methodology and gel scoring, cataloguing of profiles

and the need to assess uniformity— all apply, in varying degrees, to the techniques discussed in the remainder of this article.

3.1.2. Other proteins

The analysis of other seed or vegetative proteins by native electrophoresis methods has received less attention than the cereal prolamins, but there is still abundant literature on the subject (refs. [4–6,8,17] for instance). Acid PAGE (as for cereal prolamins) has been used for identification of varieties of peas [18] and of the pasture legume *Centrosema* [19], in each case by analysing acid-soluble seed proteins. However, PAGE at alkaline pH is more normally used. A particularly well-established example of the use of native PAGE is the work carried out by Stegemann's group in Braunschweig, using PAGE at pH 7.9 and 8.9 to separate the soluble proteins of potato tubers. A somewhat similar procedure has been used to produce the gel shown in Fig. 3. This work has resulted in the publication of catalogues of potato protein profiles, as well as in the examination of the entire potato germplasm collection from the International Centre in Lima [20]. Similar procedures have been used for identification of other tuberous crops, such as sweet potato, mashua, oca and ulluco [21,22].

The reviews by Cooke [5] and Smith and Smith [6] contain details and references to the use of SGE or PAGE techniques for variety identification in a wide range of crops, including legumes (peas, *Phaseolus* beans, *Vicia* beans, soybeans, peanuts), forage species (lucerne, various grasses), *Brassicas* (both vegetable and oilseed), vegetables (peppers, lettuce, cucumber, onions) and fruits (peaches, grapes, olives). The number of varieties examined in these cases varies enormously and not all authors address the question of how to use the data to achieve identification per se, often relying on visually apparent differences between tracks on a gel as evidence of discrimination. Nonetheless, it is clear that native PAGE of seed and vegetative proteins can be successfully applied to distinguish between varieties of many crops of agricultural importance and these methods continue to be of considerable significance.

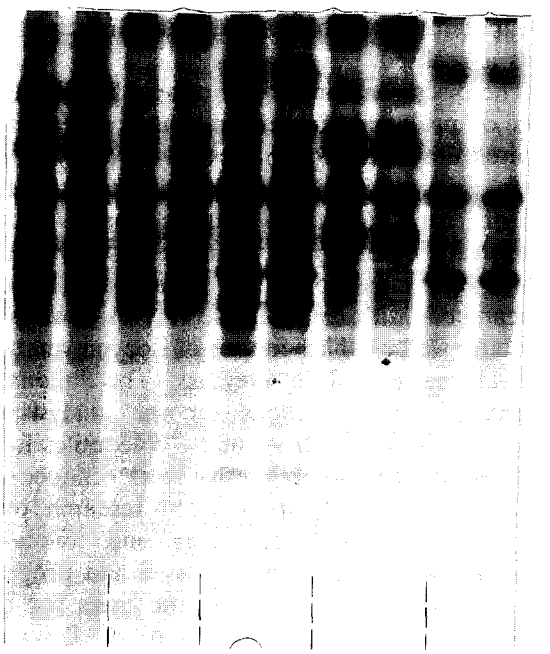

Fig. 3. The separation of the total soluble proteins from individual tubers of potato (*Solanum tuberosum*) varieties by a discontinuous PAGE procedure at pH 8.3. Each track is the profile from a single tuber and the varieties are in pairs. Note the identical profiles obtained from the different tubers of the same variety and the variety-specific differences between varieties. Such gels can also be stained for various isozyme activities, e.g. esterases. (Unpublished data of Cooke et al.)

3.2. Analysis of enzymes

Instead of staining gels for "total" protein, as in the methods above, it is possible to use staining reagents specific for particular enzymes. Such isozyme analysis has proved to be extremely useful for plant variety identification and has been comprehensively reviewed [5,6,8,23,24]. Much of the early work on isozyme analysis used SGE, and indeed much still does, since starch gels can be more readily sliced, for multiple enzyme staining, than can polyacrylamide gels. However, PAGE has also been extensively used and is perhaps becoming more popular, as the possibilities of buying pre-cast gels increase and

the potential of "blotting" to obtain copies of gels is realised.

The discriminatory power of acid PAGE analysis of prolamins in self-pollinated cereals has limited the extent to which isozyme analysis has been utilised for variety identification in the major crops such as wheat and barley [5,6]. However, other species, and especially those which are cross-pollinated, have been widely researched, with considerable success. Two particularly well established uses of isozyme analysis are in maize and in ryegrass. The "industry standard" SGE methods for the analysis of numerous isozymes from maize coleoptiles have arisen from the comprehensive work of Stuber et al. [25] from North Carolina. This research, over a period of about 15 years and involving thousands of experimental crosses and progeny analyses, determined the genetic basis for the control of various isozymes (see ref. [25] and references therein) and has proved invaluable for maize identification and purity analyses. An example of SGE used to separate malate dehydrogenase (MDH) isozymes from maize coleoptiles is shown in Fig. 4. Similar careful analyses in ryegrass (reviewed in refs. [5,23]) have established the genetic control of a number of enzymes, with SGE of phosphoglucoisomerase (PGI)-2 being a favoured system for variety identification work, coupled with acid phosphatase (ACP) and isocitrate dehydrogenase (IDH) [26].

The cross-pollinating nature of ryegrass species results in varieties being heterogeneous (see Section 1.2.) and so identification or discrimination relies on comparisons of the frequency of occurrence of isozyme phenotypes between varieties (Section 2.2.). This in turn requires the analysis of a sufficient, statistically valid, number of individuals from each variety and then pairwise analysis of the phenotype frequencies. When conducted properly, such analysis can be very powerful. A particularly thorough recent study [27] of diploid and tetra-

Fig. 4. The use of SGE to separate isozymes of MDH from maize (*Zea mays*) coleoptiles [25]. The gel shows four different maize inbred lines, with five (or seven in one case) individuals of each. At least six loci are involved in the expression of MDH in maize, making gel interpretation somewhat complex. However, each band on the gel can be identified as the product of a particular locus [25]. The alleles at all of the loci have been coded (for instance, band 3–16 indicates that the band derives from locus 3 and is allele 16). The alleles marked on the gel are: a = 2–3; b = 2–6; c = 2–3/6 (heterodimer); d = 2–6/3–16 (heterodimer); e = 4–12 and 5–12; f = 3–16; g = 3–16/8 (heterodimer), h = 3–18. Other less well resolved alleles are also present but not marked. Photograph kindly supplied by Dr. Stephen Smith (Pioneer, Johnston, IA, USA) and reproduced with permission.

ploid ryegrasses using PAGE to separate leaf isozymes found that 500 varieties could be classified into at least 54 groups, on the basis of PGI-2 frequencies from 48 plants alone. The use of more plants per variety, or other isozymes, increased the level of discrimination still further. Improved electrophoretic conditions, which increase the number of easily recognisable alleles and hence phenotypes, can also help to achieve better varietal discrimination [28].

Isozyme analysis has been applied to the identification of a large number of different crops —Nielsen [23] listed 36 species and more have been reported since (e.g. refs. [29–31], amongst many others). Detailed genetic analysis has not been carried out in all of these cases, and is not absolutely essential, although clearly it adds to the confidence that can be placed in the data if the genetic basis of a particular isozyme system is known. However, it is important to demonstrate the reproducibility of the analysis, the variability within varieties and the effects of the environment ("site and season") on the isozyme profiles, as in Booy et al. [31], for instance.

An interesting variation on native methods of isozyme analysis is the use of cellulose acetate electrophoresis (CAE). CAE has a number of potential advantages over both SGE and PAGE in terms of speed, ease of use and the small volumes of extracts, buffers and staining reagents required. Recent work [32,33] has demonstrated that CAE coupled with a range of isozyme stains can readily be used to identify and differentiate between varieties of sunflowers, sorghum and tomatoes. Provided that the resolution of the isozymes is adequate, CAE is an approach that may well merit further investigation for some variety identification applications.

4. The use of SDS-PAGE

Denaturing gel electrophoresis, generally involving the analysis of reduced proteins in the presence of SDS, has been extensively used for plant variety identification. Proteins derived from seeds have been widely utilised, with variants of the well-known "Laemmli" procedure (see [10]) being commonly applied, i.e. a discontinuous system, using Tris–HCl gel buffers and a Tris–glycine tank buffer, with varying acrylamide separating gel concentrations. For convenience, two different major approaches can be recognised —those involving the analysis of "total" buffer- or SDS-soluble seed proteins and those in which the analysed proteins constitute a more or less well-defined fraction. This is a somewhat artificial distinction, as will become clear. However, both approaches have been very successful with a wide range of crops of all types, both self-pollinating and cross-pollinating.

4.1. Analysis of "total" seed proteins

One very simple way of detecting seed protein polymorphisms is to analyse "total" seed protein extracts by SDS-PAGE. This can be extremely effective and has been used for variety discrimination and identification in many species, including oats and other cereals, peas, beans of various types, soybeans, chickpeas, lentils, groundnuts, many grasses and forage crops, coffee and cotton (see [2,5,6,8] for references). Some examples are shown in Fig. 5. For self-pollinating crops such as peas, SDS-PAGE of "total" seed proteins is particularly useful and has been adopted by ISTA as a standard reference method [10]. Intravarietal uniformity has been investigated in some cases. As with prolamin analysis, electrophoretic lines or biotypes do exist within some varieties [15] and their presence needs to be determined before the methods can be used with a high degree of certainty. Many of the crops listed above, however, are cross-pollinated and in these cases it is necessary to exercise care in the evaluation of data. As with isozymes, the seed protein profiles of a cross-pollinated species are heterogeneous within a variety, which can be readily demonstrated by single seed analysis (refs. [2,5,17,34] and Fig. 6). Although protein phenotype frequencies could be determined (cf. isozyme analysis above), in practice the complex nature of the profiles generally precludes this. However, since the inherent variability within a cross-pollinated variety is genetically fixed (or at

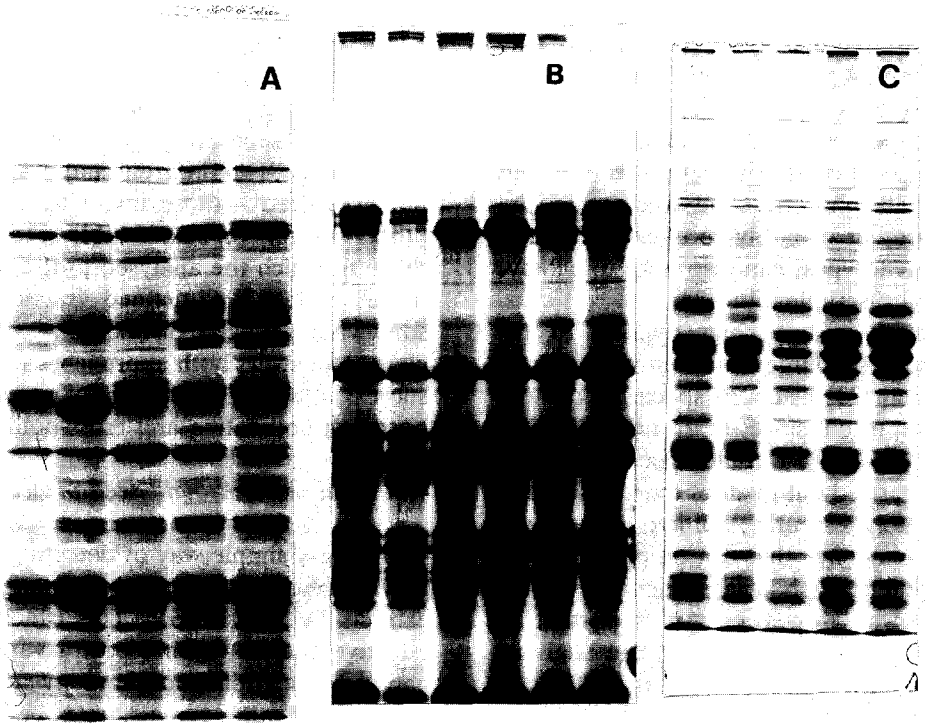

Fig. 5. SDS-PAGE of "total" seed proteins is a useful means of distinguishing between varieties of various crops, e.g. (A) peas (*Pisum sativum*), (B) carrots (*Daucus carota*), (C) *Phaseolus* beans. In A and C, 10% (T) acrylamide gels were used, as in the ISTA standard reference SDS-PAGE method [10] and each track is the profile of a single seed of a different variety. In B, 7.5% (T) gels were used and in addition to buffer, SDS and reducing agent, the extractant contained 0.5% dimethylformamide. Three varieties are shown in B (in pairs), with each track representing the profile of 20 seeds, bulked and extracted together. In all cases the gel buffer is tris–HCl and the tank buffer tris–glycine. (Unpublished data of Cooke et al.)

least maintained in equilibrium), by taking a sufficient number of single seeds and bulking them together for extraction and analysis, it is possible to obtain a characteristic overall protein profile for a variety. Such bulked (or pooled) profiles have been found to be consistent, reproducible and independent of generation and environmental factors [5,10,17,34,35]. Moreover, it now becomes possible, in effect, to treat cross-pollinating species in the same way as self-pollinated ones, and distinguish between varieties on the basis of the presence or absence of bands at a particular point on the gel (see Figs. 5 and 6). This represents a very convenient approach to the confirmation of variety identity in cross-pollinated species and has been recommended by ISTA as a standard reference method for ryegrass species and varieties [10]. The major disadvantage is that it is practically impossible to conduct any kind of varietal purity analysis using bulked samples.

Recent work utilising SDS-PAGE has demonstrated the potential of horizontal electrophoresis systems and commercially available, premade, gradient pore gels [e.g. 36–38]. This is representative of a trend which is evident throughout all areas of electrophoresis practice, i.e. the movement towards "off-the-shelf" products and smaller, thinner gels, which require shorter running times and lower volumes of buffers, stains, etc. It is certainly true that SDS-PAGE with gradient pore gels can improve the separation between some proteins and hence increase the opportunities for the discovery of useful polymorphisms. Commercially available gels are also (at least in theory) more likely to be

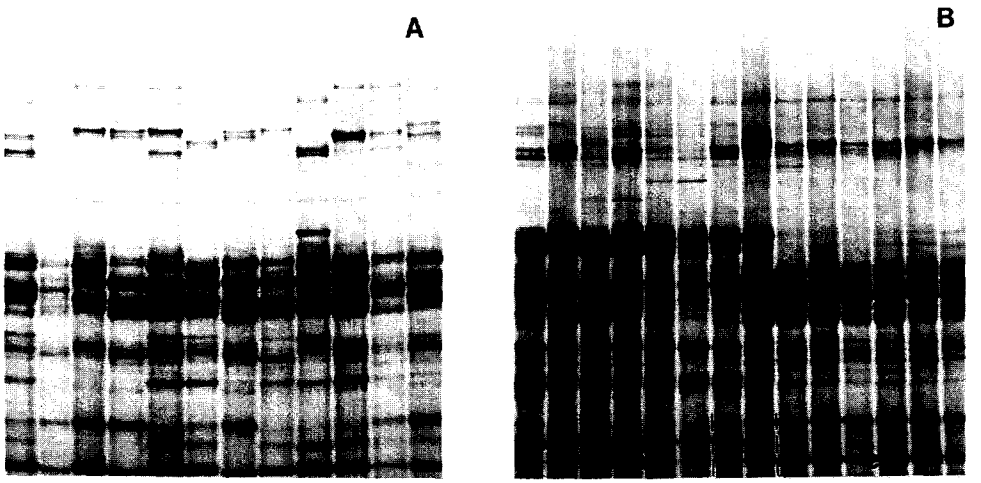

Fig. 6. SDS-PAGE [12.5% (T) acrylamide concentration] analysis of seed proteins of ryegrass (*Lolium* spp.). (A) Individual seeds of the variety Grasslands Rui. Note the extensive seed-to-seed variability in the protein profiles, characteristic of cross-pollinating species. Analysis of bulked samples of seeds allows the profiles to be used for variety identification and discrimination, as in (B). Here, each track represents the "total" seed protein profile of a bulked (0.5 g) seed sample of different varieties of Italian ryegrass (Ital.-*L. multiflorum*), perennial ryegrass (Per.-*L. perenne*) or hybrids (Hy.) between the two species. Note also that the hybrid varieties contain groups of bands clearly characteristic of the Italian/perennial parental material. Photographs kindly supplied by Dr. Sue Gardiner (DSIR, New Zealand) and reproduced with permission.

of uniformly good quality. However, it can be an expensive operation to buy the large number of gels often utilised by laboratories and the costs of this have to be balanced against the costs of, and flexibility gained from, employing staff to make gels. This is a difficult equation and one which is heavily influenced by the circumstances and requirements of different types of laboratory. Another interesting development is the detection of electro-blotted glycoproteins of various types, following SDS-PAGE, which again can improve the discrimination between varieties in some cases. For instance, barley varieties with identical hordein proteins could be distinguished by the presence of polymorphic glycoproteins of different types [36].

4.2. Analysis of specific protein fractions

Almost certainly the most thoroughly researched example of the use of SDS-PAGE to analyse a specific seed protein fraction is provided by the high-molecular-mass (HMW) glutenins of wheat. These constitute a quantitatively minor, but functionally very important, group of seed proteins that are involved in the determination of bread-making quality in hexaploid varieties of wheat (see ref. [39] for review). The genetic basis for their composition is well understood [5,39] and there is considerable polymorphism in the HMW glutenin sub-unit composition of bread wheat varieties (see Fig. 7). This polymorphism can be used for variety identification purposes and authors in many countries have published surveys of the HMW glutenins present in collections of varieties (much of this work has been summarised by Morgunov et al. [40]). The vast majority of this work has used a discontinuous Laemmli procedure, with a separating (resolving) gel concentration of between 10 and 17.5% acrylamide. Improvements to this procedure, which increase the resolution between certain of the sub-units, have been used by some workers. For instance, Brzezinski [41] has devised a technique using two stacking gels at pH 7.85 (4% acrylamide) and pH 8.3 (6% acrylamide) above a resolving gel of 10% acrylamide. This resolves the 2 and 2* pair and the 9 and 10 pair of sub-units (see Fig. 7), which otherwise can require the use of two separate

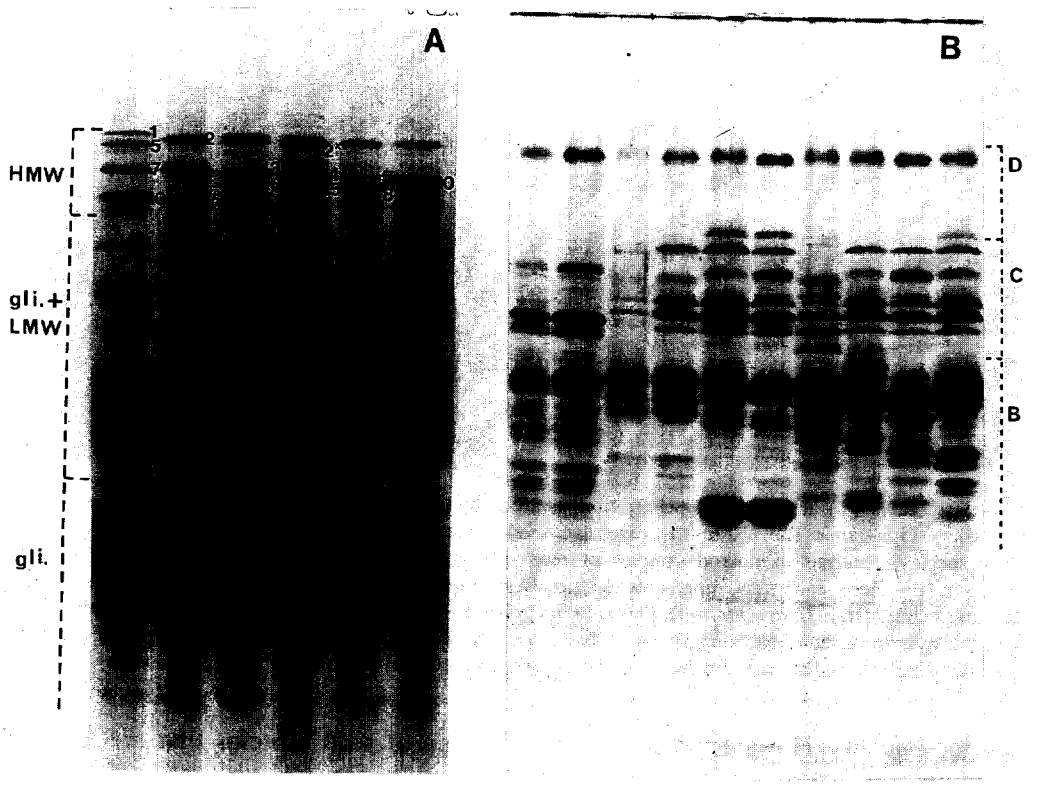

Fig. 7. SDS-PAGE used to analyse the HMW glutenins of wheat (A) and the hordeins of barley (B) varieties. In A, a 10% (T) acrylamide gel was used, whereas in B the gel was 17.5% (T) concentration. In both cases, extraction was carried out with tris–HCl buffer containing SDS and a reducing agent, the gels contained tris–HCl and the tank buffer was tris–glycine. Some of the HMW sub-units (HMW) are marked in A and the gel also shows the position of the LMW glutenins (LMW) and gliadins (gli). In gel B, the B, C and D hordein fractions are marked. In both cases, each track represents the profile of a single seed of a different variety. The polymorphism of the various protein fractions, and consequent potential for discrimination and identification, are clear. (Unpublished data of Cooke and White.)

electrophoretic runs with gels of differing acrylamide concentration for definite identification.

Low-molecular-mass (LMW) glutenins of wheat have also attracted attention, particularly for their possible relationship to bread-making quality but also as a possible means of discrimination between lines and varieties. Unfortunately, in most SDS-PAGE separations, the LMW glutenins overlap with gliadins (Fig. 7) and determining their composition is not easy. This problem can be addressed by extracting seed proteins with 70% ethanol and then using a two-step SDS-PAGE procedure, which involves a preliminary separation under non-reducing conditions [42]. The LMW glutenins are well resolved and relatively free from other interfering proteins under such conditions. No attempts have yet been made to utilise LMW glutenin sub-unit composition for variety identification purposes, but there are clearly differences between varieties which could be exploited [42].

SDS-PAGE can also be successfully used to analyse hordeins from barley (see Fig. 7), as an alternative to acid PAGE. Since the same proteins are analysed in both cases, the degree of discrimination achieved between a given collection of varieties is broadly equivalent and the choice of method largely relies on individual preference and the needs of a particular laboratory [5,13]. SDS-PAGE does resolve D-hordeins

more readily than acid PAGE, which may be of benefit even though the degree of polymorphism of these proteins is small. Recent reports have utilised different extraction techniques and commercially available horizontal gradient pore gels in conjunction with silver staining to attempt to improve the resolution of hordeins by SDS-PAGE [36,43], but it is arguable that the benefits gained are sufficient to warrant the more complex and timely analysis and staining protocols needed.

SDS-PAGE has been widely used to analyse globulin proteins from legumes and other seeds [5,8,15]. Although for most identification purposes it is more convenient to use a total SDS-soluble fraction for analysis (as in Fig. 5), in some cases, the removal of other "interfering" proteins allows the globulin composition to be determined more precisely. This is true for sunflower seeds, where cryo-precipitation from the salt-soluble seed protein fraction allows purified 11S globulin (helianthinin) to be isolated, analysed and used for variety identification and description [44].

5. The use of isoelectric focusing

Although IEF has long been recognised as an extremely powerful technique, which provides a very high degree of protein resolution, it has not been particularly widely used for plant variety identification work [5,6,8]. However, this situation is beginning to change, as IEF techniques evolve and become more reliable (and also cheaper) and more attention is paid to crops other than the major self-pollinating cereals.

There are principally three methods of producing the pH gradients in gels, necessary for IEF —(1) the use of carrier ampholytes (CA), (2) the use of immobilised pH gradients ("Immobilines") (IPG), (3) the combined use of CAs and "Immobilines" (sometimes called ICAPG). The discovery and commercial exploitation of IPGs (see ref. [45]) is one of the two factors that has substantially altered the way in which researchers regard IEF. The other is the increasingly widespread use of ultrathin-layer (UTL) gels. Prior to about 1980, IEF was somewhat restricted, requiring the use of relatively thick slab gels or more commonly tube gels, which in turn involved a considerable investment in ampholytes (which were expensive) and long running and staining times. However, nowadays it is possible to prepare gels down to 0.1 mm in thickness with no real difficulties and to have either very broad (pH 2–11) or narrow (1 pH unit) pH gradients. The gels can also be purchased ready-made and can be rehydrated in a medium of choice, so that IEF has become an extremely flexible set of techniques. Accordingly, the use of IEF for more general applications, including plant variety identification, has increased. Previously, the tendency was to use IEF when there was no alternative. For instance, the prolamins of maize (zeins) consist of a number of polypeptides of similar molecular mass but with considerable charge heterogeneity and hence IEF was the method of choice for analysis [5,6,8]. There are other species in which PAGE or SGE of proteins or enzymes does not reveal sufficient polymorphism, whereas IEF does and so is more useful (e.g. seed esterases from onions and other crops, Fig. 8 and also see ref. [5] for other examples). Alternatively, IEF can be used to enhance the discrimination between certain varieties, as a secondary or supplementary technique. Good examples are the analysis of buffer-soluble proteins from oats varieties, to sub-divide groups of varieties with similar avenin profiles (Fig. 9) and IEF of peroxidases from potatoes to extend the identification achieved by PAGE of total proteins [46]. Increasingly though, IEF is being used as the method of choice, either because it represents the best and most convenient method of analysis ([47] and Fig. 10) or because it offers an improvement over previously used techniques. As an example of this, Weiss et al. [48] reported that IEF-IPG of hordeins is superior to SDS-PAGE when used to discriminate between a collection of 55 European barley varieties. More groups of varieties could be recognised following IEF-IPG analysis, even if the SDS-PAGE gels were silver-stained. IEF-IPG is without doubt an extremely powerful

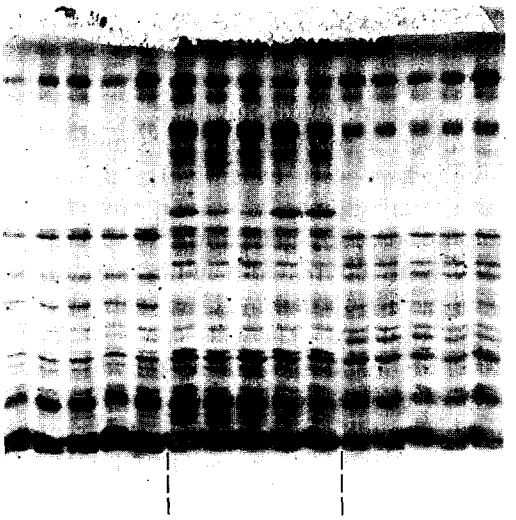

Fig. 8. The use of IEF to analyse the water-soluble esterases from seeds of pearl millet (*Pennisetum typhoides*) and distinguish between lines and varieties. Each track represents the esterases extracted from a different bulked sample of 25 seeds. The three genotypes shown, with five separate extractions of each, are (from left to right) a restorer line, a male-sterile line and a hybrid variety. Note the consistency of profile within the genotypes and the differences between them. The gel was a Pharmacia "PAG" plate, pH 4–9. (Adapted from Varier and Cooke [47].)

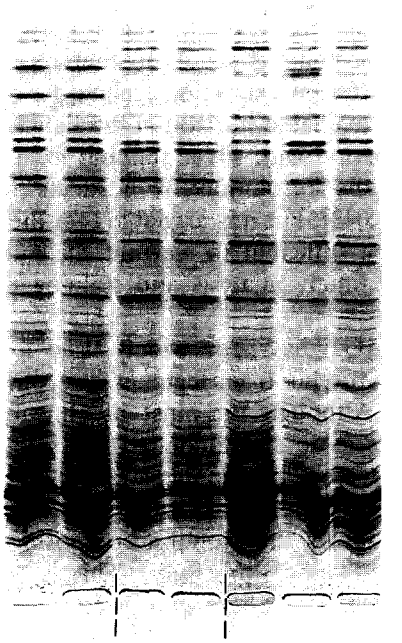

Fig. 9. UTLIEF of the urea-soluble seed proteins can be used to distinguish between species and varieties of oats. The first four tracks (in pairs) are profiles of *Avena ludoviciana* and *A. sterilis* (two species of wild oats) and the remaining three tracks are different varieties of *A. sativa* (cultivated oats). Individual seeds were analysed in each case. The gel was 0.1 mm thick, containing CAs giving a pH gradient from approximately 4 to 10, with the anode at the bottom. (Unpublished data of Cooke and Draper.)

technique, giving very narrow protein bands and high resolution (Fig. 10). It can be used for both total protein and isozyme analysis [45] and is being increasingly utilised for commercial applications (see Section 7 below). IEF gels of all types can also be successfully blotted and the blots stained for proteins or isozymes [49], which further adds to the attractions of the technique and removes some of the previously perceived disadvantages. Hence it is reasonable to predict that greater use will be made of IEF for plant variety work in the future. However, those practitioners rushing to utilise IEF should remember that the principles for the use of electrophoresis techniques, as noted above, apply equally to IEF. There must be consideration of the type of variety being investigated and due regard given to how this affects the use and interpretation of the data.

6. Two-dimensional electrophoresis

Two-dimensional (2D) electrophoresis techniques have, in theory, several advantages over the previously discussed one-dimensional methods and hence should be of considerable use for assessing genetic variation, including distinguishing between plant varieties. Since in 2D methods proteins are separated on the basis of two independent criteria (commonly charge and molecular mass), a high number of products can be analysed in one experiment. The range of proteins that can be analysed is large and autoradiography, coupled with automated gel analysis, makes it possible to assess quantitative, as

Fig. 10. The buffer-soluble seed proteins of 11 varieties of beans (*Vicia faba*) separated using IEF-IPG (pH 4–10) in a gel also containing CAs, urea and glycerol. Note the very narrow protein bands and the extremely fine resolution obtained with this technique, and the differences between the profiles. Photograph kindly supplied by Professor Angelika Görg (München, Germany) and reproduced with permission.

well as qualitative, variation. However, despite these potential advantages, 2D methods have not generally found great favour for variety identification studies. It is true that 2D methods, usually but not exclusively IEF combined with SDS-PAGE, can be successful, even when closely related varieties are examined. Thus 2D methods have been shown to distinguish between varieties of wheat, barley, maize, potatoes, peas, beans, peanuts, coffee and various fruit species, amongst others (see [5,6,8,50] for references). However, with the exception of the reported work on wheat gliadins and glutenins [5,50], most of these studies have done little more than demonstrate a difference in the 2D "maps" of pairs or small numbers of varieties. Very few, if any, attempts have been made to assess varietal homogeneity or to develop broadly based gel recording systems. The use of IEF-IPG is beginning to have an impact here also, though, with the more widespread use of the technique known as IPG-Dalt (2D-electrophoresis using IPG gels in the first dimension and horizontal, gradient pore SDS-PAGE in the second dimension, followed by silver staining). This technique, including miniaturised versions, has been well described [51] and used to examine the polymorphism of seed proteins in barley ([52] and Fig. 11), pepper [53] and other crops including carrots, cucumbers, cauliflowers and tomatoes [54]. IPG-Dalt represents an interesting approach, since it can begin to alleviate many of the difficulties normally associated with 2D analysis, such as the standardisation and reproducibility. The ability to compare several 2D maps side by side on the same second dimension gel [55] is particularly useful. The recently reported work on pepper seed proteins [53] is an ideal example of the careful approach needed to use 2D electrophoresis successfully for variety identification, and also clearly illustrates how 2D analysis can be used to demonstrate sufficient polymorphisms between closely related varieties to make estimations of genetic distance both possible and valid. This kind of problem has also been addressed by Burstin et al. [56], who compared various ways of minimising experimental variations in the 2D (IEF–SDS-PAGE) analysis of maize inbred lines. This is particularly important if quantitative variations are going to be assessed for their genetic basis and if a protein database based on 2D analysis is to be constructed, as has been suggested for barley seed proteins [57].

7. Practical applications

It should be evident from the foregoing discussions that gel electrophoresis of proteins and/or enzymes provides a powerful set of techniques for distinguishing between and identifying plant varieties. The methods have a wide range of practical applications in the seed and allied trades, which have been well described previously [1,2,5–10,17,58] and will not be repeated

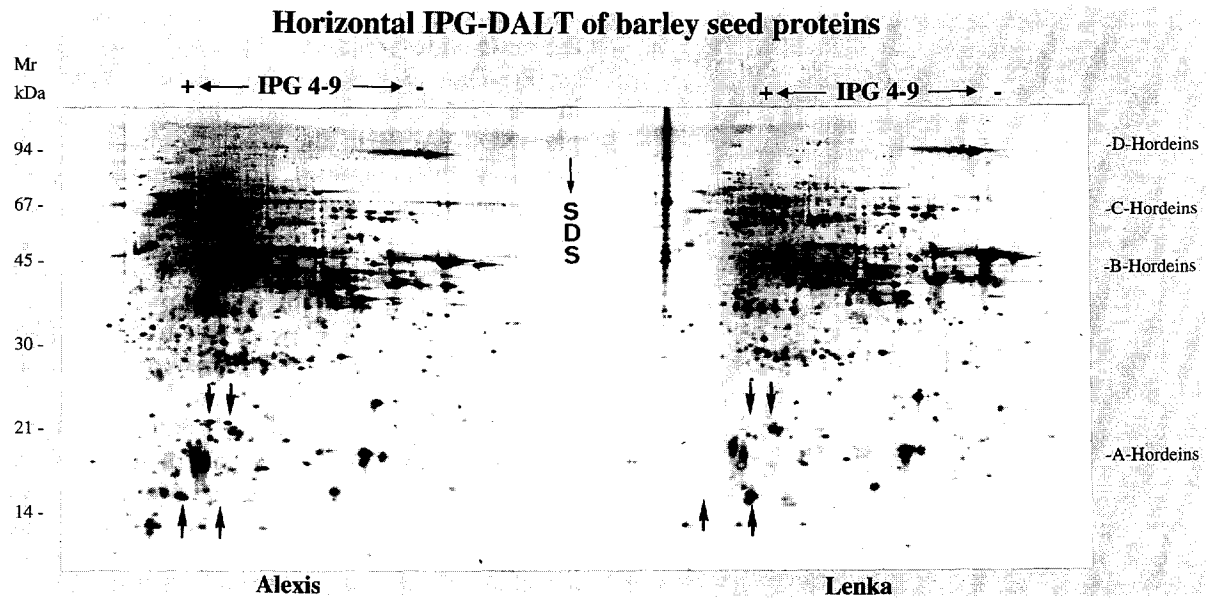

Fig. 11. Horizontal IPG-Dalt analysis of seed proteins, coupled with silver staining, can be used to demonstrate a range of differences between varieties. The arrows indicate polymorphisms in the so-called A-hordeins that were investigated for their relationship to malting quality. First dimension, IEF with IPG, pH 4–9 (separation distance 110 mm); second dimension, SDS-PAGE with a 12–17% (T) acrylamide concentration pore gradient gel. The seed proteins were extracted in urea–dithiothreitol–Triton X-100. From ref. [55], reproduced with permission. Photograph kindly supplied by Professor Angelika Görg (München, Germany).

here. Briefly, these applications include (1) plant breeding, as a source of genetic markers, (2) variety registration, through distinctness, uniformity and stability testing and awards of Plant Breeders' Rights, (3) seed production and certification, as a means of monitoring varietal identity and purity, (4) documentation of genetic resources, in gene-banks and other collections and monitoring the purity and identity of multiplied accessions, (5) quality control in processing and retail industries and (6) the measurement of F1 hybrid purity.

These last two areas are interesting, both scientifically and economically. The issue of varietal identity and purity is important to those industries where mechanised processing requires the use of only certain, "quality" varieties (e.g. malting, flour milling, bread making) and many companies in these areas use electrophoresis as part of their quality control mechanism. Identification is also important where there is a premium payable for certain varieties, either for processing or for sale to the public. A good example is provided by the potato industry, where again electrophoresis is used to check the material being traded. However, for the seed industry, the question of the genetic purity of F1 hybrid seed lots is undoubtedly of the utmost significance. The use of SGE to analyse a range of isozymes from maize and hence to estimate purity is a long-established procedure, particularly in the USA (see refs. [5,6,58] for discussion). IEF of zeins can be utilised in a similar manner and there is now much increasing interest in the use of UTLIEF and IEF-IPG for this and similar work. For instance, Van den Berg [59,60] has reported the use of UTLIEF to analyse both seed proteins and alcohol dehydrogenase (ADH) and assess F1 hybrid purity in tomatoes and alludes to its use for other horticultural crops such as various *Brassicas*. His group has also developed equipment for the rapid homogenisation of high numbers of single seeds or other tissues simultaneously in less than 1 min

[61]. This "production line" kind of approach to electrophoresis allows the analysis of many thousands of seeds in a single working day, and probably points the way ahead for certain areas of application. There is at least one newly founded commercial company, from the Netherlands, whose activities are based almost solely on analyses carried out for F1 hybrid purity assessment in high-value crops, using UTLIEF procedures. Other companies are promoting IEF in pre-cast agarose gels for variety identification and purity testing in a wide range of crops such as cereals, oil crops, grasses, maize, tomatoes, potatoes and various vegetables [62].

8. Future trends

In common with all active areas of science, electrophoresis techniques are continually developing and it can be predicted with reasonable confidence that their application to plant variety testing will evolve and expand over the next few years. We are already seeing reports of the use of capillary electrophoresis for the identification of varieties [63] and the examination of DNA profiling techniques of various kinds will surely increase (see [2,6] for references). However, gel electrophoresis of proteins and enzymes in its several forms still has much to offer in this particular field and it is possible to discern various trends for the future of its use.

One such trend, which has been mentioned several times previously in this review, is the move towards smaller, thinner and, usually, pre-cast gels. It is now possible to buy ready-made gels in a range of formats, for all of the types of gel electrophoresis that one would normally wish to carry out for variety identification purposes. This phenomenon is liable to have its largest impact in the areas of IEF and 2D-electrophoresis, offering simplicity, convenience, high resolution and, crucially, a guaranteed quality of gel. However, there is potential even in the more "traditional" areas. For instance, Wrigley et al. [64] have described the use of pore gradient gels of only 25 mm length for both acid PAGE of gliadins and SDS-PAGE of glutenins. The time for electrophoresis can be reduced to between 10 and 20 min with such gels. However, it must be borne in mind that often, particularly in more routine testing situations, what is most critical is the total throughput of samples that can be achieved, rather than the time for one particular analysis. It is often the case that the sample preparation time is the rate-limiting step, rather than the electrophoresis itself, and this is still going to be an essential (and timely) stage of the operation, whatever the gel running time. Nevertheless, there are situations where it is advantageous to have rapid running times, and the development and use of more miniature systems will no doubt continue.

On the question of high resolution in gels, it should be borne in mind that this may not always the ideal that should be sought. It is possible that improved resolution actually complicates the differentiation between varieties, by revealing a multitude of non-polymorphic protein bands. Again, inadequately resolved gel patterns may not always be a result of poor technique, but could rather reflect an aggregation of proteins that is functionally important (see Wrigley [65], for an example of this). Thus whilst usually desirable for identification work, higher band resolution of itself is not always essential.

It must also be remembered that the availability of pre-cast gels, although convenient analytically, does not solve all of the problems of variety identification. Whilst such gels allow the comparison of unknown and standard samples relatively easily, for a definitive identification it is still necessary to have assessed factors such as intra-varietal homogeneity, to have devised a gel scoring and classification system and to have established a database of protein profiles in some form. There are no easy short-cuts to achieving these objectives and they must always be remembered when discussing identification per se. The recently advanced claims for agarose IEF, for instance [62] need to be judged against this background.

Another area which has seen much interest recently and where progress can be expected is in the evaluation of gels. In plant variety work, most gel scoring is done by visual observation

and comparison. Whilst this has served us well, it is inevitably somewhat subjective and it is difficult to believe that more information could not be obtained from gels by a more automated approached to evaluation. The widespread availability and power of PCs has provided the possibility of processing gels or images using software packages that only a few years ago would have been unimaginable. Images of gels obtained by densitometry, video cameras or desk-top scanners can now be analysed using a range of commercial software packages and examples of the use of such systems for protein and isozyme analyses of varieties of various crops have been published [64,66,67]. This kind of approach has re-kindled research interest in areas such as gliadin analysis. Although IEF of gliadins has long been recognised to have some potential for wheat variety identification, the success of acid PAGE in this area and the relative ease with which such gels can be scored did much to stifle the use of IEF. However, if automated gel imaging, processing and comparisons are possible, then IEF becomes attractive again. Hence recent reports of gliadin analysis by IEF in agarose gels [67], and particularly the use of neural networks to classify the protein patterns [68] may well stimulate interest. A further interesting feature of some of these software packages is their ability to handle data not only from protein gels, but also from HPLC profiles or DNA polymorphism analysis [64]. This offers the prospect of a planned and structured approach to variety identification, using the most suitable technique or combination of techniques for a given situation, with an objective, computer-assisted evaluation of the data.

It is to be hoped that this review indicates that there is still considerable "life in the old dog" of gel electrophoresis for plant variety identification. I have tried to concentrate on more recent work, updating previous reviews, highlighting areas of progress and emphasising some general principles. I hope I can be forgiven for any omissions of favourite papers and will plead lack of space and personal ignorance. The literature associated with electrophoresis and variety identification is expanding enormously, as more researchers become aware of the elegance and simplicity of many of these techniques and begin to apply them to their own problems. The requirements of modern crop production and the desire for more genetically based, yet cost-effective testing procedures will ensure that gel electrophoresis has a continuing and expanding role to play in plant variety identification studies for some time yet.

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