AN ABSTRACT OF THE THESIS OF

Janet M. Ferguson for the degree of Master of Science

in Crop Science presented on October 11, 1984

Title: SDS-PAGE OF SEED PROTEINS FOR IDENTIFICATION OF VARIETIES AND SPECIES OF RYEGRASS (LOLIUM SPP.)

Abstract Approved: Don F. Grabe

The number of varieties of ryegrass (Lolium spp.) has increased greatly in recent years. An accurate and rapid laboratory technique to identify these varieties would benefit the consumer as well as protect an organization's Plant Variety Protection rights. There is also a need for a technique to complement the seedling fluorescence test to differentiate annual (L. multiflorum Lam.) from perennial (L. perenne L.) ryegrass. Electrophoresis has been successful in species and variety identification of ryegrass, but often differences are based only on band intensity or require analysis of 100 plants or more. The purpose of this study was to develop electrophoretic procedures which would identify varieties of perennial ryegrass, differentiate between annual and perennial ryegrass species and detect seed mixtures of these two species.

Proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Extractions were made on ground seed from bulk samples and from single seeds.
In the characterization study of perennial ryegrass, 28 varieties were tested. Many of the protein bands were common for all of the varieties. Individual varieties were characterized by presence or absence of specific bands and by band intensity ratios calculated from densitometer scans. Most of the varieties were differentiated by unique banding patterns. The varieties 'Pennant' and 'Premier', however, were not successfully differentiated from each other nor was 'Omega' found to be different from the variety 'Birdie.'

In a study to determine the feasibility of detecting mixtures of species, SDS-PAGE was conducted on 17 annual, 3 intermediate, and 28 perennial ryegrass varieties. The annual and intermediate varieties possessed protein bands at $R_f$.71 and .73 that were not found in any of the perennial varieties. Bands were present in the perennial varieties at $R_f$.80 and .88 that were absent or very faintly stained in the annual and intermediate varieties. The intermediate species could not be differentiated from the annual species.

Attempts were made to use SDS-PAGE to detect contamination of perennial ryegrass seed lots with small percentages of annual ryegrass seed. Annual and perennial ryegrass seeds were mixed together in different proportions to make concentrations of 0, 1, 3, 5, 10, 25, 50, 75, and 100% annual seed. Visible detection of the annual bands was possible in the mixtures of 25% or more annual seed. Densitometer scans could detect the presence of these annual bands in mixtures of 10%, but not in lower concentrations.
Protein extracts of individual seeds were electrophoresed to determine whether species mixtures can be detected on an individual seed basis. When individual seeds were used, the resulting banding patterns were different than those produced from bulk seed extracts from the same variety. Furthermore, no two seeds within a variety showed the same banding patterns. However, the characteristic annual bands at $R_f$ .71 and .73 were still evident in most single seeds from annuals. Likewise, the characteristic perennial bands at $R_f$ .80 and .88 were normally present in individual seeds of the perennials.

Banding patterns of SDS-PAGE of seed proteins were not affected by year and location grown, class of certification or viability or vigor of the seed. This one procedure can be used to differentiate varieties as well as species of ryegrass, making this SDS-PAGE system adaptable to seed testing needs.
SDS-PAGE OF SEED PROTEINS FOR IDENTIFICATION
OF VARIETIES AND SPECIES OF RYEGRASS (LOLIUM SPP.)

by

Janet M. Ferguson

A Thesis
submitted to
Oregon State University

In partial fulfillment of
the requirements for the
degree of
MASTER OF SCIENCE

Completed October 11, 1984
Commencement June 1985
APPROVED:

Redacted for Privacy
Professor of Agronomy in charge of major

Redacted for Privacy

Head of Department of Crop Science

Redacted for Privacy

Dean of Graduate School

Date thesis is presented: October 11, 1984
Typed by Lynn O'Hare for: Janet M. Ferguson
ACKNOWLEDGEMENTS

I would like to thank Dr. Don Grabe, my major professor, for his help, time, and continued encouragement during the past two years. Thanks also to my committee, Dr. Norman Bishop for his help on laboratory techniques, and Dr. Willis McCuistion for his support and encouragement in my studies.

I would also like to express my appreciation to Dr. Te May Ching, who found time to answer my many questions.

I am grateful to Ms. Lynn O'Hare for typing my thesis and for helping to keep my thoughts and ideas in line with requirements.

I would also like to thank Agricultural Service Corporation and Western Seed Company for their financial support and Rodger Danielson and the staff of the Oregon State University Seed Testing Laboratory for supplying the samples used in my research.

Last, but not least, I would like to thank my fellow graduate students for their support during my stay here. A special thanks to Tom Chastain who many times gave me the encouragement and confidence I needed to pursue my goals.
DEDICATION

I would like to dedicate this thesis to my family and friends. Especially to my parents who encouraged me to return to my studies and offered their complete support during my work. To my friends Rich, Cynthia, Joan, and Daryl who never let me lose my sense of direction, my sense of balance, but most of all my sense of humor.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>MANUSCRIPT I:</strong> IDENTIFICATION OF VARIETIES OF PERENNIAL RYE-GRASS BY SDS-PAGE OF WATER SOLUBLE SEED PROTEINS</td>
<td>3</td>
</tr>
<tr>
<td>Abstract</td>
<td>4</td>
</tr>
<tr>
<td>Introduction</td>
<td>5</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>9</td>
</tr>
<tr>
<td>Results</td>
<td>11</td>
</tr>
<tr>
<td>Discussion</td>
<td>23</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>26</td>
</tr>
<tr>
<td><strong>MANUSCRIPT II:</strong> SEPARATION OF ANNUAL AND PERENNIAL SPECIES OF RYEGRASS BY SDS-PAGE OF WATER SOLUBLE SEED PROTEINS</td>
<td>30</td>
</tr>
<tr>
<td>Abstract</td>
<td>31</td>
</tr>
<tr>
<td>Introduction</td>
<td>33</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>36</td>
</tr>
<tr>
<td>Results</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>52</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>54</td>
</tr>
<tr>
<td><strong>BIBLIOGRAPHY</strong></td>
<td>56</td>
</tr>
<tr>
<td><strong>APPENDICES</strong></td>
<td></td>
</tr>
<tr>
<td>Literature Review</td>
<td>59</td>
</tr>
<tr>
<td>Electrophoresis Theory</td>
<td>59</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>60</td>
</tr>
<tr>
<td>Starch</td>
<td>62</td>
</tr>
<tr>
<td>Special Techniques</td>
<td>63</td>
</tr>
<tr>
<td>Variety Identification</td>
<td>64</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>66</td>
</tr>
<tr>
<td>Reagents and Methods</td>
<td>68</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.3</td>
<td>Densitometer scans of protein bands of 14 perennial ryegrass varieties. Scans taken from gels illustrated in Figure 1.1.</td>
</tr>
<tr>
<td>1.4</td>
<td>Densitometer scans of protein bands of 14 perennial ryegrass varieties. Scans taken from gels illustrated in Figure 1.2.</td>
</tr>
<tr>
<td>1.5</td>
<td>Banding patterns of SDS-PAGE of proteins from seed of different generations of perennial ryegrass. (1) Certified Pennant, (2) Registered Pennant, (3) Certified Premier, (4) Registered Premier, and (5) Foundation Premier.</td>
</tr>
<tr>
<td>1.6</td>
<td>Banding patterns of SDS-PAGE of proteins from perennial ryegrass seeds of different vigor levels. (1) Elka high vigor, (2) Elka low vigor, (3) Elka dead, (4) Barry high vigor, (5) Barry low vigor, (6) Barry dead, (7) Linn high vigor, (8) Linn low vigor, (9) Linn dead.</td>
</tr>
<tr>
<td>2.1</td>
<td>Banding patterns of SDS-Page of seed proteins. Gel a: lanes 1-9 are annual ryegrass and lanes 10-19 are perennial ryegrass. Varieties are (1) Ninak, (2) Carumba, (3) Tetrone, (4) Billion,</td>
</tr>
<tr>
<td>Figure</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
</tr>
</tbody>
</table>

2.2 Banding patterns of SDS-PAGE of proteins from mixtures of annual and perennial ryegrass. Percent annual seed (by weight) in each mixture is (1) 0% Gulf (100% Manhattan), (2) 1% Gulf, (3) 3% Gulf, (4) 5% Gulf, (5) 10% Gulf, (6) 25% Gulf, (7) 50% Gulf, (8) 75% Gulf, (9) 100% Gulf, (10) 0% Marshall, (100% Manhattan), (11) 1% Marshall, (12) 3% Marshall, (13) 5% Marshall, (14) 10% Marshall, (15) 25% Marshall, (16) 50% Marshall, (17) 75% Marshall, (18) 100% Marshall. MW = molecular weight standard. |

2.3 Densitometer scans of proteins from mixtures of Manhattan perennial ryegrass with Marshall annual ryegrass seed. Scans are from the gel illustrated in Figure 2.2. Arrows point to annual bands appearing in Zone E in the mixtures of 10% and above. |

2.4 Banding patterns of SDS-PAGE of proteins from single seeds of annual ryegrass. Gel a, single seeds of Gulf; gel b, single seeds of Marshall. |

2.5 Banding patterns of SDS-PAGE of proteins from single seeds of perennial ryegrass. Gel a, single seeds of Pennant; gel b, single seeds of Premier. |
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>Banding patterns of SDS-PAGE of proteins from single seeds of Oregreen intermediate ryegrass.</td>
<td>49</td>
</tr>
</tbody>
</table>
INTRODUCTION

The production of annual (Lolium multiflorum Lam.) and perennial (L. perenne L.) ryegrass seed for domestic and foreign markets is an important industry in Oregon. Annual and perennial ryegrass rank number one and two for the number of acres and total value of Oregon's seed crop.

Laboratory techniques to accurately describe perennial ryegrass varieties are needed not only for organizations seeking plant variety protection of new varieties, but also for consumer protection. Since new varieties are often morphologically similar, their separation and identification would normally have to be made on the basis of chemical or biochemical differences. Electrophoresis of enzymes and proteins has been used successfully to identify varieties of several crop species such as wheat, barley, oats and corn. Perennial ryegrass varieties have been identified using several enzyme systems. Extraction procedures, especially temperature, are critical when analyzing enzymes. The instability of some enzyme systems during storage can result in altered banding patterns. An electrophoretic procedure applicable to seed proteins that were stable during storage and less influenced by extraction methods would be of use in seed.

An improved method of distinguishing between annual and perennial ryegrass species would also be beneficial to seed testing
laboratories. A replacement for the current technique to separate these species, the fluorescence test, would be of interest to both the Association of Official Seed Analysts and International Seed Testing Association. The new procedure would need to be accurate, rapid and easily performed.

Studies were conducted to determine if sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) could: (1) provide separation and characterization of perennial ryegrass varieties, (2) distinguish between annual and perennial ryegrass species, and (3) detect mixtures of annual and perennial ryegrass species.

The results are presented in the form of two manuscripts. The first describes methods of identifying varieties of perennial ryegrass and the second describes methods of distinguishing between annual and perennial ryegrass species.
MANUSCRIPT 1
IDENTIFICATION OF VARIETIES OF PERENNIAL RYEGRASS BY
SDS-PAGE OF SEED PROTEINS
ABSTRACT

The number of varieties of perennial ryegrass (*Lolium perenne* L.) continues to grow. Accurate and rapid laboratory techniques to differentiate between varieties has become increasingly important for both consumer protection and plant variety protection. The purpose of our work was to develop an electrophoretic procedure for ryegrass seed which would differentiate varieties of perennial ryegrass.

Proteins were extracted from ground seeds of 28 perennial ryegrass varieties. Extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedures. A total of 27 bands were present with many of the bands common for the 28 varieties. Varieties were characterized by presence or absence of specific bands and by band intensity ratios calculated from densitometer scans. The varieties 'Pennant' and 'Premier' were not successfully differentiated from each other nor was 'Omega' found to be different from the variety 'Birdie.' All other varieties were differentiated by unique banding patterns.

Banding patterns from SDS-PAGE of proteins were not affected by year and location of production, class of certified seed, or viability and vigor of the seed.

Additional index words: *Lolium perenne* L., sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
IDENTIFICATION OF VARIETIES OF PERENNIAL RYEGRASS BY SDS-PAGE OF SEED PROTEINS

INTRODUCTION

Currently in Oregon, 46 varieties of perennial ryegrass (Lolium perenne L.) are entered under the certification program and over 220 varieties are eligible for production under regulations of the Organization for Economic Cooperation and Development. Many of the varieties are phenotypically and genetically similar and the task of identifying these varieties becomes increasingly more difficult.

Electrophoresis has recently become a popular research tool for variety identification in numerous agricultural crops. Differences in general proteins and specific isoenzymes has led to the successful identification of varieties of wheat (Bietz and Wall, 1972; Shewry et al., 1978a), barley (Shewry et al., 1978b), oats (McDonald, 1980), broad beans (Bassiri and Rouhani, 1977; Barratt, 1980), bluegrass (Wilkinson and Beard, 1972; Wehner et al., 1976; Spoor and Hay, 1979; Wu et al., 1984), alfalfa (Quiros, 1980), fine fescue (Villamil et al., 1982), soybeans (Larsen, 1967; Singh et al., 1969; Gorman and Kiang, 1977; Blogg and Imrie, 1972), and subterranean clover (Dalling et al., 1979).

In contrast to self-pollinating crops, the outbreeding grasses show an acceptable degree of variation due to their heterozygous nature. Hayward and McAdam (1977) demonstrated that this variability could be taken into consideration and used in identifying varieties where the isoenzyme patterns are known to be simply
inherited. Staining for glutamate oxaloacetate-transaminase (GOT) and phosphoglucoisomerase (PGI), they found the PGI/2 region to be the most diagnostic for perennial ryegrass varieties. The use of $\chi^2$ analysis of allelic frequencies of the PGI/2 locus allowed them to differentiate the nine varieties included in their study. Their work also detected a change in allelic frequency during multiplication of four generations of two varieties, thus suggesting the use of this system to detect genetic shift.

Several other studies have been conducted using PGI isoenzyme. Nielsen (1980) separated 35 genotypes of tetraploid ryegrass. In this study, Nielsen found that individual band intensity is primarily a function of gene dosage, and that if two or three different alleles are present in a plant, the intensity of the bands depends on the number of each allele. Ostergaard and Nielsen (1981) used 11 varieties of annual and eight varieties of perennial ryegrass to study the effect of several factors on PGI/2. No deviations in banding patterns were found within varieties because of seed size or seed source. They also concluded that ryegrass varieties could be distinguished on the basis of only 100 plants and one enzyme locus, and that the PGI/2 locus does not appear to be linked to genes which determine heading date. Also using PGI/2, Gilliland et al. (1982) identified 23 early maturing varieties of perennial ryegrass. They found that PGI/2 genotype frequencies were unchanged by stress conditions in the growing season, but cautioned that use of some other isoenzymes may be influenced by environmental conditions.
Jones (1983), however, found that storage of leaf extracts of ryegrass can lead to alterations in some of the isoenzyme banding patterns of PGI/2. The study indicated a gradual reduction in the relative amount of the original form of PGI/2 along with a corresponding increase in the level of a second form, resulting in a new band formation. Jones suggests that caution should be used when interpreting banding patterns of isoenzymes because of this proteolytic action.

Payne and Koszykowski (1983) evaluated the use of esterase, peroxidase and glutamate dehydrogenase enzyme systems. They harvested leaf tissue from field-grown plants before heading, during heading, and after regrowth. The results revealed (1) variation among individual plants and no difference among varieties for peroxidase, (2) only one glutamate dehydrogenase band was present for all plants regardless of harvest date or variety, and (3) variation among individual plants for esterase was consistent regardless of stage of growth and there were relationships between certain banding patterns and specific varieties. They concluded that peroxidase and glutamate dehydrogenase isoenzymes were of little value for identifying varieties of perennial ryegrass, but that esterase isoenzymes could be used for identification purposes.

De Prins and Van De Weghe (1983) successfully identified eight varieties of perennial ryegrass using electrofocusing on polyacrylamide gels. By staining for seed proteins and esterase, they found the banding patterns of the varieties were unaffected by soil type in which the seed were produced or by the year they were harvested.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used successfully to identify varieties of wheat (Shewry et al., 1978a) and barley (Shewry et al., 1978b). The purpose of this study was to investigate the feasibility of using SDS-PAGE of seeds to identify varieties of perennial ryegrass and to determine advantages of SDS-PAGE over existing methods.
MATERIALS AND METHODS

Seed lots of 28 perennial ryegrass varieties were obtained from the Oregon State University Seed Testing Laboratory. All seed lots were produced under the certification program.

Approximately 4 g (about 2000 seeds) of whole seed were ground in a Wiley Mill until all material passed through a 20-mesh screen. Proteins were extracted by adding 1.5 ml of extraction buffer (10 ml H₂O, 2.5 ml .5 M Tris-HCl pH 6.9, 2.0 ml glycerol, 3.75 ml 10% SDS, 1.0 ml 2-mercaptoethanol) to .2 g of ground seed. After 1 hr, samples were placed in water at 95-100 C for 4 min. Samples were allowed to cool to room temperature, liquid was decanted and centrifuged for 10 min at 11,600 x g. The supernatant was then stored at 4 C until electrophoresed.

SDS-PAGE was conducted using a Biorad dual slab system. Vertical slab gels 0.75 mm thick, consisted of a 7% lower resolving gel and 2.5% upper stacking gel. Reservoir buffer contained .025 M Tris, .133 M glycine and .1% SDS at a pH of 8.3. Fifteen μl of protein supernatant were placed in the stacking gel sample wells, followed by 30 μl of reservoir buffer containing bromophenol blue which served as the tracking dye. Electrophoresis was carried out at 10 mA through the stacking gel and then 25 mA until the tracking dye was approximately 1 cm from the gel bottom (approximately 6 hrs). The system was maintained at 8-10 C throughout the duration of electrophoresis. Protein staining using Coomassie blue R250 followed procedures described by Chrambach et al. (1967) except gels were stained overnight and destained with 10% TCA. Electrophoresis
was conducted on at least three extractions of each sample in separate runs to assure repeatability.

Densitometer scans of the stained gels were obtained with a Biorad Scanning Densitometer Model 1650 operating at 150 nm. $R_f$ values were calculated from these charts. Intensity ratios for bands located between $R_f$ .53 and .64 were calculated for each variety. These ratios were obtained by dividing the absorbance value of each band in the $R_f$ .53-.64 region by the absorbance value of the lightest band in that region. These ratios were compared for at least three extracts and then ranked from lowest to highest to obtain relative intensity rankings.

Seed lots grown at different locations in 1981, 1982, and 1983 were chosen to determine if the environment during seed development affected banding patterns of the 28 varieties. Additional samples of Registered 'Premier' and Registered and Foundation 'Pennant' were provided by Agriculture Service Corporation for studies on the effect of class of certified seed on the SDS-PAGE results.

Three varieties were selected to determine if seed viability affected the protein banding patterns. Seed moisture content was increased to approximately 21% and seeds were placed in sealed containers at 35 C for 12 and 26 days. Germination tests were conducted to determine the effect of these treatments on seed viability and vigor. Seeds were allowed to air dry and seed proteins were extracted and electrophoresis conducted as described above.
RESULTS

A total of 27 bands were present for the 28 varieties of perennial ryegrass (Figures 1.1 and 1.2). Densitometer scans, shown in Figures 1.3 and 1.4, indicate both location and intensity of these bands. For ease of visual examination, the gels and densitometer scans were divided into six zones. Zone A represents $R_f$ values of 0 to .35, zone B from .36 to .45, zone C from .46 to .52, zone D from .53 to .64, zone E from .65 to .74, and zone F from .75 to 1.0.

Although differences between varieties were often apparent throughout the gels, the protein bands in zone D were of most diagnostic value. No attempt was made to regulate the amount of total protein electrophoresed for each variety used, therefore comparison of peak heights (band intensity) between varieties is not possible. However, ratios of intensity of specific bands in zone D were characteristic for each variety.

By using a combination of band number and location and differences in intensity ratio of bands in this zone, most of the 28 varieties could be identified. The number and intensity rankings of the zone D bands are presented in Table 1.1. As typical with electrophoresis, some drifting of bands was seen between runs, but the patterns and intensity ratios remained stable.

Some groups of varieties can be distinguished by the number of bands in zone D (Table 1.1). 'Caravelle' was the only variety studied that had six zone D bands. Thirteen varieties had five
Figure 1.3. Densitometer scans of protein bands of 14 perennial ryegrass varieties. Scans taken from gels illustrated in Figure 1.1.
Figure 1.3

Barry  Caravelle
Citation  Cropper
Linn  Grimalda
Princess  Derby
Elka  Pelo
Manhattan  Palmer
Regal  Birdie

RELATIVE ABSORBANCE

A  B  C  D  E  F
0.2  0.4  0.6  0.8

A  B  C  D  E  F
0.2  0.4  0.6  0.8

Zone
Figure 1.4. Densitometer scans of protein bands of 14 perennial ryegrass varieties. Scans taken from gels illustrated in Figure 1.2.
Figure 1.4

Dasher

Omega

Premier

Belle

Blazer

Yorktown

Ranger

Game

Pennant

Pennfine

Diplomat

Fiesta

Yorktown II

Prelude

RELATIVE ABSORBANCE

A | B | C | D | E | F
0.2 | 0.4 | 0.6 | 0.8 | Rf
Table 1.1

Zone D

<table>
<thead>
<tr>
<th>Variety</th>
<th>No. bands</th>
<th>Intensity rank</th>
<th>Variety</th>
<th>No. bands</th>
<th>Intensity rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barry</td>
<td>4</td>
<td>3,4,2,1\textsuperscript{1}</td>
<td>Dasher</td>
<td>4</td>
<td>1,2,4,3</td>
</tr>
<tr>
<td>Caravelle</td>
<td>6</td>
<td>1,4,2,6,5,3</td>
<td>Game</td>
<td>4</td>
<td>3,4,2,1</td>
</tr>
<tr>
<td>Citation</td>
<td>5</td>
<td>1,2,4,5,3</td>
<td>Omega</td>
<td>5</td>
<td>1,2,4,5,3</td>
</tr>
<tr>
<td>Cropper</td>
<td>4</td>
<td>1,4,3,2</td>
<td>Pennant</td>
<td>3</td>
<td>1,3,2</td>
</tr>
<tr>
<td>Linn</td>
<td>4</td>
<td>2,4,3,1</td>
<td>Premier</td>
<td>3</td>
<td>1,3,2</td>
</tr>
<tr>
<td>Grimalda</td>
<td>5</td>
<td>2,2,4,3,1</td>
<td>Pennfine</td>
<td>5</td>
<td>1,2,3,4,3</td>
</tr>
<tr>
<td>Princess</td>
<td>4</td>
<td>3,2,4,1</td>
<td>Belle</td>
<td>5</td>
<td>2,1,3,1,1</td>
</tr>
<tr>
<td>Derby</td>
<td>5</td>
<td>1,2,5,4,3</td>
<td>Diplomat</td>
<td>5</td>
<td>4,2,5,3,1</td>
</tr>
<tr>
<td>Elka</td>
<td>3</td>
<td>2,3,1</td>
<td>Blazer</td>
<td>4</td>
<td>3,2,4,1</td>
</tr>
<tr>
<td>Pelo</td>
<td>5</td>
<td>3,4,5,2,1</td>
<td>Fiesta</td>
<td>4</td>
<td>1,2,4,3</td>
</tr>
<tr>
<td>Manhattan</td>
<td>5</td>
<td>3,2,4,2,1</td>
<td>Yorktown</td>
<td>5</td>
<td>3,4,5,2,1</td>
</tr>
<tr>
<td>Palmer</td>
<td>5</td>
<td>3,3,4,2,1</td>
<td>Yorktown II</td>
<td>5</td>
<td>3,2,4,1,1</td>
</tr>
<tr>
<td>Regal</td>
<td>4</td>
<td>1,2,3,4</td>
<td>Ranger</td>
<td>4</td>
<td>1,2,3,2</td>
</tr>
<tr>
<td>Birdie</td>
<td>5</td>
<td>1,2,4,5,3</td>
<td>Prelude</td>
<td>4</td>
<td>3,4,2,1</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Numbers indicate relative intensity ranking of zone D bands within each variety with 1 being the lowest intensity.
bands, 11 varieties had four bands and three varieties had three bands.

The varieties that had three bands in zone D were 'Elka', Pennant, and Premier. Elka has a distinct pattern throughout the gel as shown in the densitometer scan (Figure 1.3). Results in Table 1.1 show the intensity ranking of Elka's three bands to be 2, 3, 1. Pennant and Premier both have intensity rankings of 1, 3, 2. No other distinguishing characteristics can be seen for these two varieties.

Of the varieties with four bands in zone D, 'Barry', 'Game', and 'Prelude' have the same intensity ranking of 3, 4, 2, 1. Barry can be distinguished from Game and Prelude by a characteristic band in zone C (refer to densitometer scans in Figures 1.3 and 1.4 and gel photographs, Figure 1.1 and 1.2) that is absent or very faint in Game and Prelude. To separate Game and Prelude, a band in zone E (Figures 1.2 and 1.4) is present in Prelude that is absent in Game. 'Dasher' and 'Fiesta' have intensity rankings of 1, 2, 4, 3, but Fiesta has a band in zone E that is absent in Dasher (Figures 1.2 and 1.4).

'Birdie', 'Citation', and 'Omega' have five bands in zone D, each with a ranking of 1, 2, 4, 5, 3. Citation has bands in zone F that are equal in intensity (Figures 1.1 and 1.3) while Birdie and Omega (Figures 1.1 through 1.4) show different intensities of the four bands in this zone. Birdie and Omega can not be differentiated from each other. The varieties 'Yorktown' and 'Pelo' have five bands in zone D with intensity rankings of 3, 4, 5, 2, 1. To
distinguish between the two varieties, Pelo has an additional band in zone E. All other varieties can be distinguished by distinct zone D patterns.

With the exception of Grimalda, the three seed lots of each variety grown in 1981, 1982, and 1983 at different locations showed no differences in variety banding patterns. The seed lot of Grimalda from 1982 was denied certification because of contamination with annual ryegrass seed. However, the 1981 and 1983 lots had identical banding patterns.

To determine if the generation of the seed lot tested would affect banding patterns, Foundation and Certified seed of the variety Pennant and Foundation, Registered and Certified seed of the variety Premier were analyzed and results shown in Figure 1.5. No differences were seen for the different generations of either variety.

Questions on the effect of seed viability on the banding patterns of the proteins were investigated (Figure 1.6). The intensity of the bands appears to increase after aging the seeds, but no differences were seen for location of bands of high vigor, low vigor or dead seeds.
Figure 1.5. Banding patterns of SDS-PAGE of proteins from seed of different generations of perennial ryegrass. (1) Certified Pennant, (2) Registered Pennant, (3) Certified Premier, (4) Registered Premier, and (5) Foundation Premier.
Figure 1.6. Banding patterns of SDS-PAGE of proteins from perennial ryegrass seeds of different vigor levels. (1) Elka high vigor, (2) Elka low vigor, (3) Elka dead, (4) Barry high vigor, (5) Barry low vigor, (6) Barry dead, (7) Linn high vigor, (8) Linn low vigor, (9) Linn dead.
DISCUSSION

The choices of electrophoretic methods of identifying varieties of perennial ryegrass are numerous (Hayward and McAdam, 1977; Nielson, 1980; Ostergaard and Nielsen, 1981; Gilliland et al., 1982; Jones, 1983). Investigators must keep in mind that application of the research on variety identification will ultimately be applied by seed testing laboratories and, therefore, test procedures must be practical as well as effective.

The choice of seed versus plant material is one consideration. McDonald (1980) states that compared to mature field-grown plants, unimbibed seeds have a major advantage because they are of the same stage in their life cycle and are relatively stable physiologically. Seed laboratories are usually not equipped to grow plant material needed for many of the enzyme analyses. The time and space needed to grow seedlings makes the choice of techniques lean toward those that utilize seed rather than plant material.

Slab gels are preferred over tube gels for variety identification (Stegemann, 1983) because unequivocal comparison of banding patterns is possible only in slabs.

Using allelic frequencies, for example the PGI isoenzyme, to identify varieties of perennial ryegrass requires analysis of a minimum of 100 individual plants (Hayward and McAdam, 1977). This approach to variety identification is more applicable to identifying existing fields of ryegrass rather than routine variety testing of seed lots. Hayward and McAdam were able to detect a genetic shift in varieties using the frequency of specific alleles. SDS-PAGE
procedures in this study are applicable to bulk seed only. In a separate study (Ferguson and Grabe: in preparation), we discussed the use of the SDS-PAGE system is discussed for analyzing single seeds. Of the varieties being evaluated, no two seeds (out of 80 single seeds) from a given seed lot had the same banding pattern. Therefore, the above-mentioned techniques could not be used, as the isoenzymes can, to detect a genetic shift unless the bulk seed banding pattern were affected. In this investigation using single seed, it was possible to detect contamination of annual and intermediate ryegrass seed in perennial seed lots.

A major advantage of SDS-PAGE of proteins is the stability of the proteins themselves. The banding patterns were found to be unaffected by storage time in the extraction buffer and by extraction temperature. It is well understood that extraction procedures (temperature especially) are critical for enzyme analysis. Jones (1983) demonstrated the instability of the PGI enzyme where proteolytic activity during storage resulted in different banding patterns. Other characteristics of the SDS-PAGE system are that seed source (year and location grown), seed viability, and seed certification class had no effect on variety banding patterns.

Because of the methods of breeding perennial ryegrass varieties, it is doubtful if any one specific test will successfully separate all varieties. Many varieties are closely related, often with common parents in their breeding background. The SDS-PAGE procedures used in this study successfully separated all but two sets of two varieties. Pennant and Premier are varieties developed
from similar parentage and, although they are morphologically different in the field, no distinguishing characteristics were evident when using this procedure. Birdie and Omega were found to have banding patterns that were identical and, therefore, separation of these two varieties was not possible. Perhaps other systems of electrophoresis or morphological characteristics could be used to separate them.

The need for standardization is well established in all aspects of seed testing. The ability to standardize each electrophoresis gel with a commercially available molecular weight standard makes it possible to compare SDS-PAGE results between laboratories. This, plus the fact that SDS-PAGE has been used successfully to identify varieties of other crop species, makes this procedure more adaptable for routine variety testing. A single procedure could possibly be developed to identify varieties of several different species, thereby eliminating the need for a different electrophoretic method for each crop.
LITERATURE CITED


MANUSCRIPT 2

SEPARATION OF ANNUAL AND PERENNIAL SPECIES OF RYEGRASS

BY SDS-PAGE OF SEED PROTEINS
ABSTRACT

There is a need for a rapid and effective technique that would complement the seedling fluorescence test to differentiate annual (Lolium multiflorum Lam.) from perennial (L. perenne L.) ryegrass. Electrophoresis procedures have been successful in separating the two species, but differences between the two are often based only on band intensity. The purpose of this work was to develop an electrophoretic procedure that would, by presence or absence of distinct bands, differentiate between annual and perennial ryegrass species and detect mixtures of annual and perennial ryegrass.

Proteins were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Seventeen annual, three intermediate (L. hybridum Hausslen), and 14 perennial varieties were tested. The annual and intermediate varieties possessed characteristic protein bands at Rf .71 and .73 that were not found in any of the perennial varieties. Moreover, bands were present in the perennial varieties at Rf .80 and .88 that were absent or very faintly stained in the annual and intermediate varieties. The banding patterns for annual and intermediate species were similar.

Attempts were made to use SDS-PAGE to detect contamination of perennial ryegrass seed lots with small percentages of annual seed. Mixtures of annual and perennial ryegrass seed were made in concentrations of 0, 1, 3, 5, 10, 25, 50, 75, and 100% annual seed. Annual bands were visible in the samples containing 25% or more annual seed. Densitometer scans could detect the annual bands in
the mixtures containing 10% annual seed, but lower percentages of annual seed could not be detected in the bulk seed mixtures.

Protein extracts of individual seeds were electrophoresed to determine whether species mixtures can be detected on an individual seed basis. When individual seeds were used, the resulting banding patterns were different than those produced from bulk seed extracts from the same variety. Furthermore, no two seeds within a variety showed the same banding patterns. However, the characteristic annual bands at Rf .71 and .73 were still evident in most single seeds from annuals. Likewise, the characteristic perennial bands at Rf .80 and .88 were normally present in individual seeds of the perennials.

The SDS-PAGE procedures used in this study were successful in differentiating between annual and perennial ryegrass. Bulk seeds and single seeds of the two species show distinct species dependent banding patterns. Perennial ryegrass seed lots contaminated with 10% or more annual ryegrass seed could be detected by analyzing bulk seed.

SEPARATION OF ANNUAL AND PERENNIAL SPECIES OF RYEGRASS BY SDS-PAGE OF SEED PROTEINS

INTRODUCTION

When ryegrass seed production began in Oregon, few problems of mixing the seed of annuals (Lolium multiflorum Lam.) and perennials (L. perenne L.) occurred. Few varieties of each species existed at that time and annuals flowered about 2 weeks later than the perennials (Jensen, 1963). Since then, numerous varieties of each species have been introduced. Cross pollination of the two species occurs because of overlapping of their pollination periods. This problem, along with mechanical mixing of seed of the two species, led to the need for laboratory tests to distinguish annual from perennial ryegrass.

The seedling fluorescence test developed by Gentner (1929) provided a simple test to distinguish the two species. Since Gentner's discovery, it has been shown that plants possessing perennial growth habit could exhibit seedling fluorescence as well as those with an annual growth habit. Justice (1946) published a review of literature on ryegrass seedling fluorescence.

The search for alternative methods of distinguishing annual from perennial ryegrass has led to several studies on the use of electrophoresis. Larsen (1966) investigated the use of seed proteins to differentiate the two species. He found a unique protein band present in perennials that was absent in the annuals. Nakamura (1979), using a similar technique, also reported differences in
annual and perennial protein banding patterns. He reported differences between diploid and tetraploid annuals although his work was based on a limited number of varieties. Nakamura reported the presence of different seed esterase bands in annuals and perennials. The procedure for extracting esterase was reportedly simpler and the differences between the species were more distinct than when using proteins. Nakamura also investigated other enzyme systems, but these systems showed either no reaction or no differences between the species.

Payne et al. (1980) expanded the use of seed esterase and successfully separated 16 annual ryegrass varieties from 35 perennial varieties. They, as Nakamura, found the annuals exhibited a dark band that was absent in the perennials. Mixtures of 0, 25, 50, and 75% annual ryegrass in perennial ryegrass were successfully detected using the intensity of this band. Upon increasing the sensitivity of the densitometer, they were able to detect mixtures of only 5% annual ryegrass.

Electrofocusing to distinguish annuals from perennials was investigated by De Prins and Van De Weghe (1983). They found a characteristic protein band present in annuals that was absent in perennials when a gel of 3.5-9.5 pH gradient was used. A second gel consisting of a gradient of pH 4.0-6.5 showed a protein band on the anodic side characteristic for annuals. This second procedure also allowed for distinction of individual varieties. Esterase patterns in a gel of pH gradient 3.5-9.5 revealed still another difference
between annuals and perennials. Soil type and harvest year did not affect banding pattern.

The use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is widely accepted as a method to determine molecular weights of proteins and their subunits (Bietz and Wall, 1972; Hames, 1981). SDS-PAGE has been used successfully to identify varieties of wheat (Shewry et al., 1978a), barley (Shewry et al., 1978b), and broad beans (Barratt, 1980).

The objectives of this study were to determine if SDS-PAGE can be used to (1) distinguish species of ryegrass, (2) detect specific mixtures of annual and perennial ryegrass, and (3) detect ploidy level of annual ryegrass varieties.
MATERIALS AND METHODS

Seed lots of 17 annual, three intermediate (L. hybridum Hausslen), and 14 perennial varieties of ryegrass were obtained from the Oregon State University Seed Testing Laboratory and commercial seed sources. All seed lots were produced under the certification program.

Annual-perennial seed mixtures were obtained by mechanically blending seed of the perennial variety 'Manhattan' with seeds of the annual 'Marshall' and again separately with the annual 'Gulf'. Mixtures (by weight) were made to represent 0, 1, 3, 5, 10, 25, 50, 75, and 100% annual seed in perennial ryegrass.

Approximately 4 g (about 2000 seeds) (1 g for mixed annual-perennial) of whole seed were ground in a Wiley Mill until all material passed through a 20-mesh screen. Proteins were extracted from ground seed (.2 g/1.5 ml) with extraction buffer consisting of 10 ml H₂O, 2.5 ml of 0.5 M Tris-HCl pH 6.9, 2.0 ml glycerol, 3.75 ml 10% SDS, and 1.0 ml 2-mercaptoethanol. After 1 hr, samples were placed in a hot water bath (95-100 C) for 4 min. Samples were cooled and centrifuged at 11,600 x g for 10 min. The supernatant was stored at 4 C until electrophoresed.

Single-seed proteins were extracted by crushing each seed in a 1.5 ml plastic micro-centrifuge tube using a tapered glass rod. To each seed, 100 µl of extraction buffer was added, the seed was further crushed and the mixture allowed to stand for 1 hr before centrifuging at 11,600 x g.
SDS-PAGE was conducted using a Biorad dual slab system. Vertical slabs, 0.75 mm thick, consisted of 7% resolving gel and 2.5% stacking gel. Reservoir buffer containing 0.25 M Tris, 0.01% SDS, and enough glycine to bring the pH to 8.3 was used and the system maintained at 8-10 °C during electrophoresis. Fifteen µl of protein supernatant were placed in the stacking gel sample wells followed by 30 µl of reservoir buffer containing bromophenol blue (tracking dye). Electrophoresis was carried out at 10 mA through the stacking gel and then 25 mA until the tracking dye was 1 cm from the bottom of the gel (approximately 6 hrs.). Coomassie blue staining procedures described by Chrambach et al. (1967) were used, except gels were stained overnight and destained with 10% trichloroacetic acid (TCA). Densitometer readings were obtained with a Biorad Model 1650 scanning densitometer at 150 nm.

The results reported are representative of at least three separate extractions and electrophoretic runs.
RESULTS

Characteristic patterns for annual, perennial and intermediate ryegrass can be seen in Figures 2.1a and 2.1b. For ease of identification, the gel patterns were divided into specific zones. Zone A represents bands from $R_f$ 0-.35, B from .36-.45, C from .46-.52, D from .53-.64, E from .65-.74, and F from .75-1.0.

The intermediate ryegrass varieties (Figure 2.1b) have banding patterns similar to the annual species. Many of the bands are common for the annuals and perennials, but some are distinct. The most obvious differentiating characteristic is the appearance of double bands ($R_f$ .71 and .73) in zone E. These bands appear in each annual variety tested and were absent from all perennial varieties except 'Grimalda'. Upon further investigation, the seed lot of Grimalda used in this study was determined to have a fluorescence value of 30.75%. The appearance of the double band in this sample is assumed to be contamination of annual seed or crossing of Grimalda with an annual.

Other diagnostic characteristics for annual species appear in zones A and C. In zone A, the perennial varieties lack the uppermost band ($R_f$ .24) and the double bands appearing at $R_f$ .29 and .30. Not every annual variety has these bands, but none of the perennials included in this study showed bands in these areas. Though not as diagnostic, bands in zone C are more intensely stained in annuals than in perennials.

Figure 2.1

Variety

(a)

Variety

(b)
Additional bands were seen in the perennial varieties in zone F. These bands were much more intensely stained than corresponding bands of the annual varieties at Rf .80 and .88.

Banding patterns of samples consisting of the mixtures of 0, 1, 3, 5, 10, 25, 50, 75, and 100% of the annuals Marshall or Gulf with the perennial Manhattan are shown in Figure 2.2. Densitometer analysis of the patterns of the mixtures of Marshall and Manhattan can be seen in Figure 2.3. The mixtures of Gulf and Manhattan showed similar results. Visual detection of the annual double bands in zone E is possible in mixtures of 25% or greater while densitometer scans can detect these bands in the mixtures of 10% or more.

Results of single seed extracts can be seen in the gels of the annual varieties Gulf (Figure 2.4a) and Marshall (Figure 2.4b), the intermediate variety 'Oregreen' (Figure 2.6), and the perennial varieties 'Pennant' (Figure 2.5a) and 'Premier' (Figure 2.5b). Additional annual varieties 'Ninak', 'Tetrone', and 'Carumba' were also analyzed and similar results were obtained. For the varieties Pennant and Premier, 80 single seeds of each were analyzed. No two single seeds within a variety have the same banding pattern and the patterns seen in single seeds differ widely from the patterns seen in the bulk samples. Annual and perennial varieties show some characteristic patterns, with intermediate varieties again similar to the annuals. At least one, if not both, of the annual double bands in zone E of the annual bulk samples is present in single seeds of annual (Figures 2.4a and 2.4b) and intermediate (Figure 2.6) varieties. The intensely stained perennial bands in zone F
Figure 2.2. Banding patterns of SDS-PAGE of proteins from mixtures of annual and perennial ryegrass. Percent annual seed (by weight) in each mixture is (1) 0% Gulf (100% Manhattan), (2) 1% Gulf, (3) 3% Gulf, (4) 5% Gulf, (5) 10% Gulf, (7) 50% Gulf, (8) 75% Gulf, (9) 100% Gulf, (10) 0% Marshall (100% Manhattan), (11) 1% Marshall, (12) 3% Marshall, (13) 5% Marshall, (14) 10% Marshall, (15) 25% Marshall, (16) 50% Marshall, (17) 75% Marshall, (18) 100% Marshall. MW = molecular weight standard.
Figure 2.3 Densitometer scans of proteins from mixtures of Manhattan perennial ryegrass with Marshall annual ryegrass seed. Scans are from the gel illustrated in Figure 2.2. Arrows point to annual bands appearing in Zone # in the mixtures of 10% and above.
Figure 2.4. Banding patterns of SDS-PAGE of proteins from single seeds of annual ryegrass. Gel a, single seeds of Gulf; gel b, single seeds of Marshall.
Figure 2.4
Gulf Single Seeds

Marshall Single Seeds
Figure 2.5. Banding patterns of SDS-PAGE of proteins from single seeds of perennial ryegrass. Gel a, single seeds of Pennant; gel b, single seeds of Premier.
Figure 2.5
Pennant Single Seeds

Premier Single Seeds
Figure 2.6. Banding patterns of SDS-PAGE of proteins from single seeds of Oregreen intermediate ryegrass.
seen in the bulk seed can also be seen in all but two single seeds of the perennials. These bands are not as pronounced in the annuals and intermediates.

Banding patterns of several varieties of diploid and tetraploid annual ryegrass are shown in Figure 2.7. It is not possible to determine ploidy level of ryegrass varieties using this method.
DISCUSSION

The detection of mixtures of annual ryegrass present in perennial ryegrass seed lots is important to the seed industry. Payne et al. (1980) compared the fluorescence test to electrophoretic methods for distinguishing annual from perennial ryegrass. They reported that esterase electrophoresis is quicker (1 day compared to 14 days for fluorescence tests), that esterase banding patterns were not dependent on seed viability (fluorescence tests evaluate only normal seedlings), and that esterase is linked to growth habit (perennial plants have been known to fluoresce).

The SDS-PAGE procedures described in this study show distinct characteristic bands for both annual and perennial ryegrass varieties. The presence or absence of these specific bands from bulk samples makes identification of these two species simple. In another study (Ferguson and Grabe: in preparation), it was shown that the SDS-PAGE methods used in this study were not affected by seed source (year and location grown) or seed viability.

The determination of small percentages of annual seed present in perennial seed lots is not possible by the SDS-PAGE techniques used in this study for bulk seed. Ten percent annual ryegrass is the minimum contamination level detectable, while certification standards demand detection at levels as low as 1%. The possibility of detecting specific mixtures of annual and perennial ryegrass by using single seeds instead of bulk samples was investigated and it was found that the single seed extracts of annual, perennial, and intermediate varieties show characteristics that differ from bulk
seed patterns of their variety. The fact that no two seeds show the same pattern is an indication of the wide variability present in the cross-pollinating ryegrasses. The results presented in Figures 2.4, 2.5, and 2.6 offer no guarantee that all varieties of different ryegrass species will exhibit the characteristic species-dependent bands, but the possibility exists that single seed extracts using these procedures could detect specific annual-perennial mixtures of a given seed lot.

The methods used in this study showed no characteristic banding patterns that would allow distinction of ploidy level among annual ryegrass varieties.

Different patterns were observed in the individual varieties of annual ryegrass, indicating the potential use of these procedures for identifying varieties. Several seed lots of each of the perennial varieties used were analyzed in a previous study (Ferguson and Grabe: in print), and consistency of bands among seed lots of the different varieties was verified. Three seed lots each of Marshall, Gulf, Oregreen, Tetrone, and Billion were tested for this study and no differences were seen between the seed lots for each of the varieties.
LITERATURE CITED


Bibliography


Electrophoresis Theory

Electrophoresis provides a simple method for separation of ionic mixtures. Proteins are ionic, known to carry a net charge at any pH other than their isoelectric point (Lehninger, 1982). The application of an electric field to a protein mixture causes the proteins to migrate toward the oppositely charged electrode. The rate of migration will depend on the ratio of charge to mass of the proteins; the higher the ratio, the faster the migration (Names, 1981).

With zone electrophoresis, molecules to be separated are placed as a narrow zone at a suitable distance from the electrodes such that, during electrophoresis, proteins of different mobilities travel as discrete zones which gradually separate from each other (Hames, 1981). Zone electrophoresis can be carried out in free solution, but satisfactory separation may not be achieved. The effects of heating in free solution could cause zone disturbance and diffusion in free solutions broadens bands even after electrophoresis is terminated (Davis, 1964). For these reasons, zone electrophoresis is usually carried out in a solution, stabilized within a supporting medium such as paper, silica gel, alumina or cellulose, or in gels of starch, agarose or polyacrylamide (Hames, 1981). A gel matrix, unlike other media, is a latticed structure with pores of molecular dimensions that can impose a frictional resistance on migrating ions. As they migrate through the gel,
proteins of different sizes will be retarded to degrees proportional to their dimensions. This separation of mixtures, known as molecular sieving, is achieved through size as well as charge differences (Davis, 1964). The extent of molecular sieving depends on how close the gel pore size approximates the size of the migrating protein molecules (Hames, 1981).

Starch and acrylamide gels provide a relatively non-ionic framework for electrophoretic migration (Peirce and Brewbaker, 1973). Both are popular media and both have advantages and disadvantages for their use.

**Acrylamide**

Polyacrylamide gels are generated by the free radical polymerization of acrylamide monomer and the crosslinking co-monomer, bisacrylamide (N,N'-methyl-bis-acrylamide). Variation in the concentrations of monomer and co-monomer, along with varying the degree of polymerization (chain length), will lead to polyacrylamide gels with a wide range of pore sizes (Hames, 1981).

Reproducibility in the formation of polyacrylamide gels is one of its major advantages over starch gels. Optimal polymerization temperatures, removal of polymerization inhibitors such as oxygen, and use of high purity reagents can insure gels of equal quality (Chrambach and Rodbard, 1971; Hames, 1981). Some other advantages of acrylamide gels are their transparency, mechanical strength, and ease of generating different pore sizes (Davis, 1964). Ornstein (1964) reports that acrylamide gels are also thermostable, rela-
tively inert and are completely non-ionic (starch can carry a slight charge).

One major disadvantage in choosing acrylamide is that prior to polymerization acrylamide is highly toxic. This toxicity, however, disappears once gels are polymerized (Hames, 1981).

**Slab vs Tube**

Polyacrylamide gel electrophoresis (PAGE) can be carried out in either tubes or slabs. The choice would depend upon intended use. Slab gels are preferred when many samples, including molecular weight markers, are to be compared. In a single slab, identical conditions are maintained, producing banding patterns that are directly comparable (Hames, 1981). Tube gels, in contrast, even of the same sample, are rarely identical. According to Hames, additional advantages of slab gels are that heat is more rapidly dissipated, densitometer readings are easier and they take less time to prepare. Tube gels are preferred when determining the optimum pH of a system, when automatic fractionation is used, and when procedures call for slicing gels for radioactive proteins.

**Discontinuous vs Continuous**

Disc electrophoresis derived its name from the dependence of the technique "on discontinuities in the electrophoretic matrix and, coincidentally, from the discoid shape of the separated zones of ions" (Ornstein, 1964). Ornstein's system was used in tube and the term "disc" has come to be more closely associated with tube discontinuous systems rather than slabs (Gordon, 1969).
Discontinuous (or multiphasic) buffer systems use different buffer ions in the gel and electrode vessel reservoir (Chrambach and Rodbard, 1971; Hames, 1981). The major advantage of discontinuous systems, as explained by Hames, is that large amounts of dilute protein samples can be loaded onto the gels, but good resolution can still be obtained because proteins are concentrated (or stacked) into narrow zones in a large-pore stacking gel prior to separation in the resolving gel.

Zone electrophoresis systems in which the same buffer ions are present throughout the sample, gel and electrode vessel reservoirs at constant pH are referred to as continuous buffer systems (Hames, 1981).

**Starch**

Starch gels were first developed by Smithies (1955) for use on serum proteins. The gels are formed by carbohydrate chains branching and intertwining. Pore size can be varied simply by altering the starch concentration (Gordon, 1969). Some protein separate equally as well on starch as on acrylamide gels. Comparison of the sharpness of bands formed by proteins in both systems, however, shows that the bands formed by larger proteins in certain acrylamide gels are sharper than the same proteins in starch (Gordon, 1969). Starch gels, according to Gordon, have some advantages over acrylamide. With starch gels, the only additional reagents required are those needed in the buffer solutions and also starch is completely non-toxic. An additional advantage of starch over acrylamide is that starch can be sliced into thin slabs which allows several
different staining systems to be used from only one electrophoretic run.

**Special Techniques**

In addition to separating proteins according to their size and charge, electrophoresis techniques can be modified to estimate protein molecular weights, to separate proteins on the basis of their isoelectric point, and to carry out two-dimensional electrophoresis.

For molecular weight estimation, excess sodium dodecyl sulfate (SDS) can be used to mask the native charge of the proteins. SDS, an anionic detergent, binds to the molecules, making all the proteins equal in charge density and, therefore, separation is based only on size or molecular weight (Chrambach and Rodbard). The use of a reducing agent such as 2-mercaptoethanol or dithiothreitol (DTT) maintains the protein sulfhydryl groups in the reduced state (Hames, 1981).

In the technique of electrofocusing, proteins are allowed to migrate through a pH gradient and are separated at their isoelectric points (An der Lan and Chrambach, 1981). The isoelectric point is the pH at which the net protein charge is zero; therefore, migration towards an electric charge will cease (Lehninger, 1982).

Two-dimensional electrophoresis adds still another technique for separating proteins. In conventional electrophoresis there is a possibility that two or more polypeptides may migrate to the same spot, creating an image of a single band. Two-dimensional sepa-
ration incorporates separation in one direction followed by a second separation at right angles to the first (Sinclair and Rickwood, 1981).

The first directional separation is frequently carried out on tube gels; once separation is completed, the tube is sliced and laid across the top of a slab gel for the second separation. Frequently, the two separations are different; for example, one direction by molecular weight and the other along a pH gradient for electro-focusing (Sinclair and Rickwood, 1981).

**Variety Identification Using Electrophoresis**

Morphological differences between varieties must have an accompanying biochemical difference, but all biochemical differences are not necessarily expressed in morphological characteristics (Larsen, 1969). McKee (1973) pointed out that since varieties should differ from each other by one or more protein or enzyme constituents, then electrophoresis could be a valuable tool to help characterize and identify varieties.

Electrophoresis has indeed been successful in identifying varieties of many crop species, using both starch and PAGE. Varieties of crops such as wheat (Bietz and Wall, 1972; Shewry et al., 1978a), barley (Shewry et al., 1978b) and oats (McDonald, 1980) are just a few of the many crops studied.

Ryegrass species and varieties have been investigated by several researchers. Techniques of starch and PAGE using enzyme systems, proteins and isoelectric focusing have resulted in successful separation of varieties. Hayward and McAdam (1977), Neilson
(1980), Ostergaard and Neilson (1981), and Gilliland et al. (1982) investigated differences in phosphoglucoisomerase enzyme systems in perennial ryegrass plants. Other enzymes in ryegrass plants esterase, peroxidase and glutamate dehydrogenase were evaluated by Payne et al. (1980) and Payne and Koszykowski (1983). Seed protein differences were seen by Larsen (1966) and Nakamura (1979) using poly acrylamide gels and by DePrins and Van De Weghe (1983) using electrofocusing on polyacrylamide gels.
LITERATURE CITED


REAGENTS AND METHODS

Stock Solutions

Acrylamide Stock

CAUTION: ACRYLAMIDE IS NEUROTOXIC - USE GLOVES AND FACE MASK WHEN PREPARING STOCK SOLUTION.

28.0 g acrylamide
0.735 g bis-acrylamide
Makes 100 ml

Add 28 g acrylamide to about 40-50 ml distilled water. Allow to dissolve. Add .735 g bis. Mix until all crystals are dissolved. Bring to 100 ml volume with distilled water. If sigma acrylamide is used, impurities must be removed. This is done by adding a small amount (approximately .2 g/100 ml) of activated charcoal to the acrylamide stock. Allow to mix about 10-15 minutes; then, filter through Whatman No. 1 filter paper. Follow this with another filtration through a .45 micron millipore filter system.

Store in dark bottle 4 C. Remains stable for several months.

Gel Buffer A

36.3 g Tris (or Trizma Base)
PH to 8.8-9.0 with HCl
0.23 ml TEMED
To 100 ml with distilled water

Dissolve 36.3 g Tris in approximately 20 ml of 2N HCl. Check pH - continue adding HCl (2N) until pH is between 8.8 and 8.9. Add .23 ml TEMED and bring to 100 ml with distilled water. Filter through .45 micron millipore filter system.

Store at room temperature for several weeks.

Gel Buffer B

5.98 Tris (or Trizma Base)
To pH 6.6-6.8 with HCl (2N)
0.46 ml TEMED
To 100 ml with distilled H$_2$O

Dissolve 5.98 g Tris in approximately 30 ml 2N HCl. Check pH - continue adding HCl (2N) until pH is between 6.6 and 6.8. Add .46 ml TEMED. Bring to 100 ml with distilled water. Filter through .45 micron millipore filter system.

Store room temperature for several weeks.
10% SDS
Dissolve 10 g SDS in approximately 50 ml distilled water. Bring to 100 ml with distilled water. Filter through .45 micron millipore filter system.

Store room temperature for 1-2 weeks.

Riboflavin
Dissolve 0.004 g riboflavin in 80 ml distilled water. Bring to 100 ml with distilled water. Filter through .45 micron millipore filter system.

Store at 4 C for several months.

Reagents Made Fresh

1.5% Ammonium Persulfate
Add .15 g ammonium persulfate to 10 ml distilled water.

Extraction Buffer

.5M Tris-HCl pH 6.8

Follow procedure for making gel buffer B except exclude .46 ml TEMED. No filtration required.

For approximately 36 samples at 1.5 ml per sample, mix the following:

30 ml distilled water
7.5 ml .5M Tris-HCl pH 6.8
6.0 ml glycerol
11.25 ml 10% SDS

Mix these well - then, transfer solution to fume hood and add 3.0 ml of 2-mercaptoethanol.

Keep mixture under hood until extraction procedure is completed and samples secured (lids closed) in micro-centrifuge tubes.

Reservoir Buffer

Stock
30.3 g Tris (or Trizma Base)
100 g glycine (or enough to bring pH to 8.3-8.4)
10 g SDS

Dissolve 30.3 g Tris and 100 g glycine in approximately 700 ml
 distilled water. Adjust pH to 8.3-8.4 with glycine. Dissolve 10 g SDS in the solution. Bring to 1000 ml with distilled water.

For reservoir mixtures, dilute stock 10X - that is for 4000 ml (600 in top reservoir and 3400 in bottom) - dilute 400 ml stock with 3600 ml distilled water). This dilution should be made the day before electrophoresis is performed and stored at 4 C overnight.

To make 7% PAGE: (4 gels 0.75 mm thick)

Resolving gel
14 ml acrylamide stock
7.5 ml gel buffer A
0.6 ml 10% SDS
3.0 ml 1.5% ammonium persulfate
34.9 ml distilled water

Polymerize 20 minutes

Stacking Gel

2.5 ml acrylamide stock
5.0 ml gel buffer B
0.2 ml 10% SDS
2.5 ml riboflavin stock
9.8 ml distilled water

Polymerize under UV light for 30 minutes.

Water Soluble Protein Extraction Procedure Using Bulk Ground Seed

.2 g ground seed is weighed into a glass test tube. Under the hood, add 1.5 ml extraction buffer. Shake tubes to assure all material is wetted. Allow to stand for 1 hour. Place tubes in hot water (95-100 C) for 4 minutes. Remove and allow to cool to room temperature. Decant liquid into 1.5 ml plastic micro-centrifuge tube. Secure lid. Centrifuge at 11,600 x g for 10 minutes. Store at 4 C until electrophoresed.

(Use 15 µl samples for .75 mm thick gels.)

Using Single Seed

Place a single seed in a 1.5 ml micro-centrifuge tube. Crush the seed using a taper glass rod. Under the hood, add 200 µl of extraction buffer. Further crush the seed and allow the samples to stand for 1 hour. Centrifuge for 10 minutes at 11,600 x g. Store at 4 C until electrophoresed.

(Use 15 µl samples for .75 mm thick gels.)
Coomassie Blue R-250 Staining Procedure

Fix gel protein band for 40 minutes in 12.5% TCA.

Stain in a 1:20 dilution of 1% aqueous CB R250 in 12.5% TCA (i.e. 1 ml of 1% aqueous CB to 20 ml of 12.5% TCA).

After electrophoresis, remove stacking gel. Place gels in 12.5% trichloro acetic acid (TCA). Allow gels to fix for 40 minutes (preferably with agitation).

When gels are placed in fixative TCA, prepare the 1% aqueous CB solution - for 4 gels .75 mm thick - weigh .4 g CB - add this to 40 ml distilled water. This solution requires about 30 minutes to dissolve.

Also prepare 800 ml of 12.5% TCA - (Do not mix aqueous CB and 12.5% TCA until the last minute).

After 40 minutes, remove 12.5% TCA from gels. Combine 1% aqueous CB to 800 ml 12.5% TCA. Mix for several seconds then add 200 ml of the stain to each gel.

Stain gels for 2 hours (or overnight as we do then distain with 10% TCA).

Store gels in the dark in 10% TCA.

(To remove residue from gel surface, wash gels briefly (few seconds) in a 50% methanol solution.)