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Electrophoretic Patterns of Storage Proteins in Phaseolus Prone to Cotyledonal Cracking

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ELECTROPHORETIC PATTERNS OF STORAGE PROTEINS IN

PHASEOLUS PRONE TO COTYLEDONAL CRACKING

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

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in

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DEDICATION

To my father

my aunts

Nooria and Layla

ACKNOWLEDGEMENTS

Zahra N. Hash

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ABSTRACT

Electrophetic Patterns of Storage Proteins in

Phaseolus Prone to Cotyledonal Cracking

by

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Utah State University, 1984

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Cotyledonal- or transverse-cracking (TVC) in certain cultivars of
snapbeans, Phaseolus vulgaris L., seeds, clearly evident during
germination, seriously places affected seedlings at a competitive
disadvantage. TVC is an inherited trait and occurs across cell walls of
cotyledons rather than along cell walls. Thus, it might be hypothesized
that internal pressure resulting from swelling of storage proteins
during imbibition might account for cellular rupture. To further
elucidate this possibility, experiments were designed to compare
electrophoretic patterns of storage proteins from seeds of snapbeans
resistant and susceptible to TVC, and to correlate the different
patterns of polyacrylamide gel electrophoretograms of these proteins to
TVC.

One hundred seeds were selected randomly from a bulk sample of 225 g
from each of 17 seed lots representing 15 cultivars, seed coats removed
and cotyledons finely ground (60 mesh). Seed flours were defatted twice
with hexane (50 mL/g) at 4^0C and defatted flours reground with a mortar

and pestle. Seed proteins were extracted in 0.5 M NaCl (solvent to four ratio of 10:1) at pH 7.5 for 1 h at 4^oC with centrifugation at 10,000 g for 30 min. Separated proteins were subjected to electrophoresis under denaturing and non-denaturing conditions and molecular weight of different protein brands determined. Different protein banding patterns were identified and correlated to the TVC phenomenon. The data showed visual differences between banding patterns of resistant and susceptible cultivars.

While the electrophoretic technique shows observable differences in cultivars expressing differential TVC, it is not clear which protein bands are associated with the TVC phenomenon. For plant breeders to employ this tool in screening for TVC resistant snapbean cultivars, further requirements are needed.

(87 pages)

INTRODUCTION

Snapbeans, Phaseolus vulgaris L., are an important protein food crop
in many parts of the world. As with any other crop, there are problems
encountered in their production. One problem of interest, cotyledonal-
or transverse-cracking (TVC), has been recognized as a major problem in
some white-seeded cultivars since their release in the early 1950's.
The TVC is quite distinctive from embryo fractures. In embryo frac-
tures, one or both cotyledons become separated from the embryo, or the
radicle remains attached to one cotyledon of a pair and the plumules to
the other. By contrast, TVC ranges from hairline fractures to deep-
seated cracks that cause the cotyledons to shatter under slight pressure
or during imbibition and subsequent germination (Morris et al. 1970).
The young seedling is then deprived of part or all of its initial food
supply and is at a competitive disadvantage with seedlings having two
full cotyledons. Research has shown that germination, early seedling
growth, maturity and yield of snapbeans are influenced proportionally to
the amount of cotyledonary tissue broken and/or missing (Hollis 1964,
Peat et al. 1981, Schweitzer 1972, Waters 1960, Waters and Atkin 1959).
These researchers observed that cotyledonal cracking in P. vulgaris
could reduce the yield as much as 88% under unfavorable environmental
conditions. Seeds of many TVC cultivars did not germinate due to a
deficiency in essential food, or to microbial invasion and subsequent
decay of the fractured seeds.

Cotyledonal cracking has been studied in relation to many factors
as: (1) operating speed of mechanical threshers (Anonymous 1949, Atkin

1958, Green et al. 1966, Toole et al. 1951), (2) seed moisture content and seed coat permeability (Dickson et al. 1973, Hoffman and Kanapaux 1952, Honma and Denna 1962, James 1949, Morris et al. 1970, Ott and Ball 1943, Pollack and Manalo 1970, Shull and Shull 1932, Wijuandi and Copeland 1974), (3) seed coat color (Kannenberg and Allard 1964), (4) seed coat thickness (Korban et al. 1981, Ott and Ball 1943, Watson 1948), and (5) calcium (Ca) and magnesium (Mg) level in both seed coats and cotyledons (de Kock 1964, Dickson et al. 1973, Echandi et al. 1970, Powrie et al. 1960, Puhkalbskaya 1964).

Morris et al. (1970) observed that TVC occurred across cell walls of the cotyledons rather than along cell walls. Thus, it might be hypothesized that internal pressure resulting perhaps from swelling of storage proteins during imbibition might account for cellular rupture. Schweitzer (1972) demonstrated a reduction in the activity of amylases and succinatecytochrome C reductase during early seedling growth in TVC affected snapbeans as compared to those not affected by TVC. Moreover, there is substantial evidence that TVC is an inherited trait (Dickson 1975; Dickson and Boettger 1977; Dorrell 1968; J. L. Morris 1983, personal communication, Rogers Brothers Company, Twin Falls, ID). Should the quality of storage proteins be correlated with this trait, then the electrophoretic technique becomes a powerful tool for plant breeders and geneticists to screen, select and develop newer, higher yielding cultivars that do not exhibit this characteristic.

Based on these observations, the overall objective was to study electrophoretic patterns of storage proteins in snapbeans and correlate them to the TVC trait. More specifically, the objectives are:

1. To compare electrophoretic patterns of storage proteins from the seeds of snapbeans resistant and susceptible to cotyledonal cracking.

2. To correlate the different patterns of polyacrylamide gel electrophoretograms of these proteins to cotyledonal cracking.

LITERATURE REVIEW

Transverse cotyledonal cracking (TVC) was first observed in peas (Shull and Shull 1932). Shull and Shull (1932) observed that pea seeds with TVC exhibited a more rapid rate of water uptake than those without, and attributed this increased rate to the developed cracks. Later, during the early 1950's many new white-seeded snapbean cultivars were released that also exhibited the TVC phenomenon.

Observations by plant breeders in the Seed Industry indicated that TVC in snapbeans was an inherited character (Dickson 1975; Morris 1984, personal communication). Dickson (1975) observed that TVC inheritance was complex and appeared to involve overdominance. However, he reported that broad-sense heritabilities varied from 37.8% to 57.5% and narrow-sense heritabilities varied from 26.9% to 46.6%.

Germination percentage was decreased in newly released cultivars due to the increase in cotyledonal abnormality. Atkin (1964) observed a positive relationship between the productivity of bean plants and the amount of cotyledonal tissue remaining on the emergence of such morpho-logically defected seeds was reduced by as much as 50 to 62%. Moreover, Waters (1960) observed that when one-half of the cotyledonal tissue was missing from emerging bean seedlings, the dry weight yield was reduced by 50%. When 75% of the cotyledonal tissue was missing, yield dropped to 5.6% of normal. Other studies conducted by McAlister and Krober (1951) and Moore (1964) have shown that removal of cotyledonal tissue at, or prior to, emergence of bean seedlings, delayed flowering and reduced plant yield. By contrast, McAlister and Krober (1951) reported

there was no effect on plant development when cotyledonal tissue was removed after emergence. Also, Ndunguru and Summerfield (1975) showed that cotyledons of soybean can make an important contribution to seedling growth. This observation was also supported by Peat et al. (1981), who found that the removal of both cotyledons from soybean seedlings 10 days after planting, reduced their leaf production, stem height, branching, and dry weight of flowering.

Toole and Toole (1951), and Green et al. (1966) stated that mechanical threshers operating at high speeds affected seed quality and increased the injuries that lowered germination. Several workers (Atkin 1958, Dickson et al. 1973, Dorrell 1968, Hoki and Pickett, 1973, McCollum 1953, Morris et al. 1970, Pollack and Manalo 1970, Schweitzer 1972, Silbernagel and Burke 1973, Wijuandi and Copeland 1974) emphasized the importance of seed moisture content at threshing time in relation to mechanical damage of the seed, with high injury occurring as seed moisture decreased. Barriga (1961) conducted experiments on the relation between moisture level and mechanical abuse in 41 cultivars of navy beans. He observed considerable differences in the degree of injury and in an inverse relationship between moisture content of the cultivar and percentage of injury. In other words, the higher the moisture content of the seed, the lower the injury. McCollum (1953) and Pollack et al. (1969) observed more cracking in snapbean cultivars when seeds were planted in wet soil with an insufficient amount of oxygen.

Some of the early research showed that temperature influenced severity of cotyledonal cracking. McCollum (1953) planted seeds of the Rival cultivar of snapbeans at three temperatures, 10, 20, and 30 C. He observed that seeds imbibed at cooler temperatures showed more severe

cracking than those at 30 C. Hoki and Pickett (1973) noticed that
internal cotyledonal cracking increased rapidly as imbibition tempera-
ture was lowered to 10 C or below. However, Clark and Kline (1965)
stated that cold water treatment appeared to reduce germination
slightly, but there was no evidence that it increased the amount of
cotyledonal cracking.

Farooqui and McCollum (1954) noted a high rate of cotyledonal
abnormality in susceptible cultivars for seed coat rupture. They also
observed that seed coat rupture increased even under the most favorable
conditions for growth. Their data indicated an inverse correlation
between high yields and increased rupture.

With few exceptions, researchers demonstrated that white-seeded
snapbeans were more susceptible to TVC than seeds with dark-colored seed
coats (Atkin 1958, Dickson 1975, Dickson and Boettger 1976, Kannenberg
and Allard 1964). Anderson (1956) and Atkin (1958) observed that
Streamliner, a white-seeded snapbean, was more resistant to TVC than
dark-colored snapbeans. Dickson (1975) observed that bean lines with a
seed coat greater than 9.5% of the weight of the cotyledons and one that
adhered tightly to them were more resistant to TVC and mechanical damage
than those cultivars with loosely bound, lighter seed coats. He further
noted that dark-seeded beans were more resistant than white-seeded ones,
but the white-seeded ones that survived were superior to most of the
cultivars tested.

Dorrell (1968), Dorrell and Adams (1969), and Hoki (1971) reported
that round seeds were more tolerant to mechanical abuse and cotyledonal
cracking than seeds of other shapes. Schweitzer (1972) reported that
density, weight and shape influenced seed quality.

McCollum (1953) showed that seed coat permeability had an important effect on TVC and that susceptible beans imbibed water rapidly. He also reported that removing the seed coat from resistant beans prior to germination increased TVC damage. Using a refractometric method, Morris et al. (1968) showed that seed coat permeability ranged from 0.78 $g/mm^2/h$ to 1.82 $g/mm^2/h$ in 11 bean cultivars tested. Furthermore, they generalized that dark-seeded beans were less permeable than white-seeded ones.

Korban et al. (1981) determined seed-coat cracking injury in beans by using three testing methods, the Vogel small plot thresher (field), seed dropping and a controlled rotating impact disk machine. Moreover, they demonstrated that uniform seed-coat thickness of a thick seed-coat increased resistance to seed-coat cracking and TVC.

Dorrell and Adams (1969) stated that tolerant bean cultivars generally had a thinner parenchymatous layer underlying osteosclerids. Dickson et al. (1973) also observed a positive relationship between cotyledonal cracking and cell wall material in the cotyledons.

Snapbean seeds contain variable amounts of calcium (Ca) and magnesium (Mg). Bonner (1936), Echandi et al. (1970), and Snyder (1936) noted that the seed coat of Great Northern beans contained a 2.47% Ca while the cotyledons contained only 0.037% Ca. However, Aqil and Boe (1975) stated that TVC resistant bean cultivars had higher seed Ca, Mg and N contents that susceptible ones. This is in agreement with Dickson et al. (1973), who reported low Ca and Mg in the cotyledonary cell walls of bean cultivars exhibiting TVC susceptibility.

Seed Proteins

Schweitzer (1972) suggested that the cracking injury in snapbean seeds might involve protein synthesis, since activity of some enzymes was reduced in the seedlings of TVC susceptible plants.

Generally, legume seeds contain a higher level of protein than those of other plants. They have at least two to three times more protein than cereal grains. Therefore, they are principal sources of proteins in many parts of the world, especially in mixed diets (Adams 1973, Millerd 1975, Roberts 1972). In developing countries where animal proteins are in limited supply, legumes have been labeled as "meat for poor people" (Mosse and Pernollet 1983).

Due to the importance of legume proteins, research has been conducted to improve their quality and increase their quantity. It has been reported that protein content of legumes differs from one species to another, and also from one cultivar to another (Blixt 1979). Silbernagel (1968) noted that P. vulgaris contained from 17 to 30% protein. Jaffe and Brucher (1974) studied the protein content of 100 pure lines of beans and found significant differences in total protein among them. Bressani (1969), de Moraes and Angelucci (1971), Rutger (1970) and Tandon et al. (1957) reported that snapbean seeds contained protein quantities ranging from 16 to 31%. Bressani et al. (1961) observed that red and black beans that were grown in Guatemala contained an average of 22.3% protein. Kelly and Bliss (1975a) stated that the amount of protein in four different cultivars of beans used in their experiments ranges from 21.5% to 31.9%. In 150 lines of Cicer areitinum, the amount of protein ranged from 15 to 29.6% (Singh and

Jambunathan 1980), while Ortega et al. (1974) reported that the protein content of bean cultivars in Mexico varied from 17.9 to 37.6%.

Increasing protein percentages in beans is desirable since there is an increasing need for more plant protein as world population increases. This could be done by increasing protein quantity per plant or per hectare. In other words, in breeding programs, relations between seed yield and protein percentage must be considered. Leleji et al. (1972) selected five lines of dry beans, P. vulgaris, studied the inheritance of crude protein and correlated it with seed yield. They established a broad-sense heritability ranging from 30.7 to 63.7% and a narrow-sense heritability of 20.1% for backcrosses and 5.0% and 12% on F_3/F_2 regression. They concluded that high-yielding cultivars tended to produce low protein percentages. This conclusion agreed with the earlier results of Rutger (1970), who stated that bean yield was negatively correlated with percent protein. Porter (1972) also observed an inverse correlation between seed yield and protein content in dry beans. Kelly and Bliss (1975b) reported a low negative correlation ($r = -0.30$) between seed yield and protein percentage of four bean cultivar. They suggested that selection should be made initially for high yield and then protein content. Brim (1973) and Payne (1977) noted that it was possible to obtain genotypes with high protein contents, but this was commonly achieved at the expense of total yield of dry matter.

Since dry beans are poor nitrogen (N) fixers, N fertilizers are required to improve the field. Edge et al. (1975) studied the influence of six different levels of N fertilizers on dry bean yield components. They noted that yield per plant and pods per plant as well as seed size were increasing with increasing N. In addition, the found that crude

protein percentage and crude protein yield were related to the amount of N added. Tolla (1978) found that N, phosphorus (P), potassium (K), and sulfur (S) solutions that were applied to the roots of three P. vulgaris cultivars increased seed proteins in controlled environmental conditions but not in the field.

In view of protein distribution in developing and germinating bean seeds, Racusen and Foote (1980) noted that glycoprotein II was accumulated very rapidly when bean seeds reached their maximal length and fresh weight. Moreover, they stated that this protein decreased rapidly between 7 to 10 days during germination. However, Racusen and Foote (1971) had earlier reported that germination for up to 114 hours had very little influence on a glycoprotein of 130,000 daltons or on the total soluble protein.

Studying the variation in protein content of seeds from different parts of the bean plant and within individual seeds, Leleji (1974) noted that the seed position within the pod had no influence on protein content. However, in the greenhouse, but not in the field, the pod position on the plant influenced the amount of protein. Also, he stated that the distal end of the seed contained a slightly less protein than did the proximal end, with a high correlation between the distal end and the average protein content of the seed. In addition, Barker et al. (1976) observed that outer and central parts of cotyledons contained different amounts of protein fractions. Earlier data of Leleji (1971) showed that the seed position within pods had no significant influence on the percentage of crude protein, but in greenhouse grown plants, position influenced the percentage of protein slightly. Tulman (1975) noted that there was no influence of either position on the plant or

seed position within the pod on total protein. Baudet et al. (1977) conducted a systematic analyses on single seeds. They randomly picked 50 single seeds within a seed sample of the same cultivar grown and harvested under the same conditions. They showed that protein contents ranged from 23 to 40.5%. Moreover, in samples obtained from two different plants, protein content of single seeds ranged from 21.6 to 32% for one plant, and from 23 to 34% for the other plant. Wolff (1975a,b) observed that individual seed weight and protein content of different seeds from the same pod or pods located on the same node were very similar. Yet as the distance from the roots increased, seed weight as well as protein content per seed diminished. Also, a linear correlation was found between seed weight and protein content per seed. This relation has been shown to be due to genetic characteristics of the cultivars (Moose and Pernollet 1983).

In general, composition of storage proteins varies in different legume species (Boulter et al. 1967). It has been found that 80% of legume seed proteins is considered as a reserve protein that accumulates in cotyledons during seed development. This storage protein is made-up of a small number of salt soluble proteins called globulins (Brohult and Sandegren 1954, Derbyshire et al. 1975). In spite of difficulty in isolating seed protein bodies from beans, several investigators have overcome the problem and succeeded in characterizing these storage proteins (Barker et al. 1976, Bollini and Chrispeels 1979, Murray and Crump 1979).

Osborne (1894) first noted that most of the extractable proteins in beans were globulins. This observation has also been emphasized by many other authors. Waterman et al. (1923) earlier found three globulins in

beans. Usually the globulin fraction of beans is regarded as consisting of two major components, namely vicilin and legumin (Danielsson 1949). Pusztai (1966) and Pusztai and Watt (1970) isolated two different kinds of globulins with carbohydrate portions; glycoproteins I and II. Derbyshire et al. (1976) suggested that glycoprotein II is a vicilin-like protein. McLeester et al. (1973) and Racusen and Foote (1971) isolated a protein similar to glycoprotein II that was named globulin-1 (G_1). Later, Ma and Bliss (1978) stated that G_1 protein was the major storage protein fraction in seeds of common beans. The G_1 protein is a single protein soluble at relatively high salt concentration (Sun and Hall 1975). Sun et al. (1974) reported that at pH 7 this protein has a sedimentation coefficient of 7 S and at pH 4.5 associated with an 18 S fraction.

Estimating the molecular weight of globulin subunits, Pusztai and Watt (1970) reported that glycoprotein II was made of a single subunit of 40,000 daltons. However, McLeester et al. (1973) stated that G_1 consisted of three subunits of 43,000; 47,000; and 50,000 daltons. Barker et al. (1976) used several methods of extraction and fractionation to isolate major storage protein of bean seeds. They found that there were three major proteins with a molecular weight of 32,000 and 47,000 and 50,000 daltons. These proteins were soluble at pH 4.7. In addition, they isolated another protein that was insoluble at 4.7 and its fractions had molecular weights of 20,000 and 60,000 daltons. These results were confirmed by Bollini and Chrispeels (1978) and Murray and Crump (1979) who named these protein vicilin.

Bollini and Chrispeels (1978) proposed that glycoprotein II, G_1, and the major storage protein along with euphaseolin of Kloz and Klozova

(1974) are the same and all homologous to the same 7 S vicilin of P. vulgaris.

Landsteiner and Raubitschek (1908) found that beans contain a soluble hemagglutinin. This was later proved to be a protein, and many investigators have extracted active fraction that they considered to be pure (Jaffe and Gaede 1959, Rigas and Osgood 1955). Several years later, Jaffe and Hannig (1965) extracted protein from black kidney bean seeds and they found that two fractions were salt soluble and nine fraction were water soluble. Moreover, they reported that four of these fractions had hemagglutinating activity. Hence, only two different hemagglutinin proteins were positively identified. Bollini and Chrispeels (1978) stated that phytohemagglutinin was another glyco-protein that was regarded as a storage protein. It is about 10% of the seed protein content. This glycoprotein corresponds to the protein named G_2, globulin, by McLeester et al. (1973) and also known as lectin. It consists of two subunits of 34,000 and 36,000 daltons that are capable of associating into five tetramers (Mosse and Pernollet 1983). Each subunit has specific binding sites for sugar residues. A similar pattern of five lectin proteins have been described by Reisfeld et al. (1962). These five proteins represent isometric tetramers consisting of two different subunits of variable amounts, one leucoagglutinating and mitogenic, the other erythroagglutinating (Felsted et al. 1975, Leavitt et al. 1977, Miller et al. 1973).

Finally, seed beans contain legumin, which is an 11 S protein fraction and consists of at least three subunits of 20,000; 34,000; and 37,000 daltons (Derbyshire and Boulter 1976). It seems to be a minor storage protein in P. vulgaris.

MATERIALS AND METHODS

Plant Material

Mature, dry seeds of snapbeans, Phaseolus vulgaris L., cvs Early
Gallatin Earliwax and White-seeded Tendercrop-1 were obtained from
Rogers Brothers Company, Twin Falls, Idaho 83301. These cultivars were
known to represent a range of cotyledonal cracking (Transverse Cracking
- TVC) (Figures 1 and 2). Cracking severity was derived according to
Morris (1967), i.e., on a 1 to 5 crack index scale: 1) cotyledons with
only a slight crack, 2) for those with one definite crack, 3) for those
with two definite cracks, 4) for those with three definite cracks and 5)
for cotyledons having four or more cracks. Initial studies to establish
whether cultivars of different known TVC exhibited similar electro-
phoretograms were conducted with these cultivars. After differences in
electrophoretograms were shown, an additional listing of cultivars was
requested from Rogers Brothers Company. At this time, it was requested
that the identity and degree of TVC of the cultivars be coded and remain
unknown to us until after all studies were completed.

One hundred seeds were selected randomly, regardless of size or
shape from a bulk sample of 225 g from each cultivar. Seeds were
immersed in deionized distilled water for 3-5 h at 25 C. Seed coats
were then removed, seeds air-dried at 25 C and finely ground (60-mesh)
in a Stein Laboratories Mill (Model L), with care taken to prevent an
increase in temperature. Seed flour of each cultivar was defatted twice
with hexane (50 mL/g) at 4 C (Matta et al. 1981). Defatted flours were
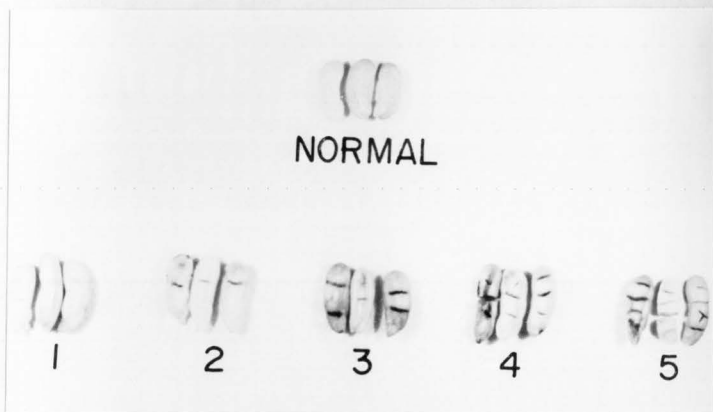
Figure 1. Examples of 5 crack index classifications used to rate cracking severity in this study.
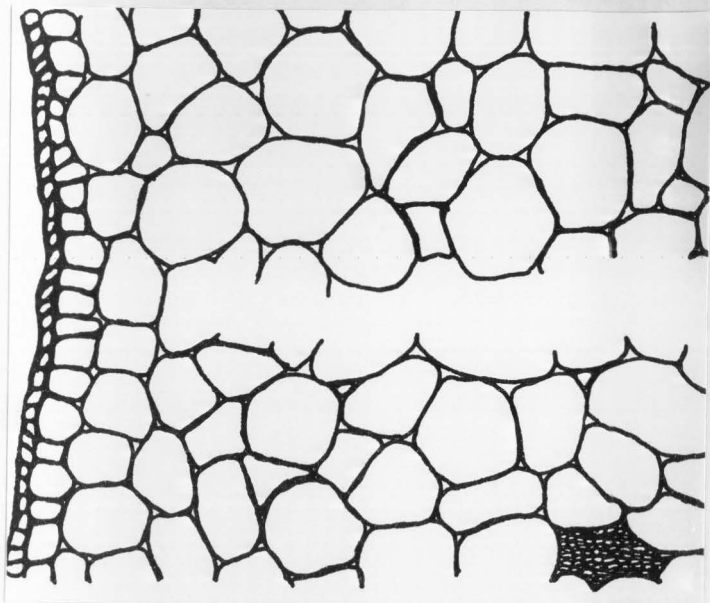
Figure 2. Camera lucida drawing of a longitudinal section of bean cotyledonal showing a typical cotyledonal crack. Drawing made at 100X magnification (approximately 425X). (After Morris et al. 1970).

then re-ground with a mortar and pestle, with suitable washing of instruments between grindings to prevent sample contamination.

Protein Extraction

Seed protein was extracted with a modified protocal of Ishino and Ortega (1975). Defatted flour was stirred in 0.5 M sodium chloride (NaCl) (solvent to flour ratio, 10:1) at pH 7.5 for 1 h at 4 C. The mixture was then centrifuged at 10,000 g for 30 min. Insoluble materials were discarded and supernatants mixed with a sample buffer in a 1:1 ratio and used for loading onto gels. In the denaturing electrophoresis system, samples were mixed with the sample buffer, heated in a boiling water bath for 90 s and then cooled before they were loaded onto gels.

Electrophoresis

Non-denaturing discontinuous electrophoresis was performed on a 12% polyacrylamide separating gel, pH 8.8, with a 5% polyacrylamide stacking gel, pH 8.37, at 4 C (Davis 1964). Electrophoresis was conducted on a Hoefer Scientific Instrument, Model SE 500, vertical gel slab (Figure 3). The upper buffer was Tris-ethylenediamintetra-acetic acid (EDTA) - boric acid (7:1:2) g/L, pH 8.37 and the lower buffer was Tris-HCl, pH 8.5. Initial studies were done using the cultivars Early Gallatin, Earliwax and White-seeded Tendercrop-1 as they represented a range of TVC. The protein extract of these cultivars was mixed with the sample buffer that consisted of the upper buffer: glycerol: distilled water (1:1:1) in a 1:1 ratio. Twenty-five µL of the above mixture were loaded onto gels. After loading the samples, electrophoresis was carried out at
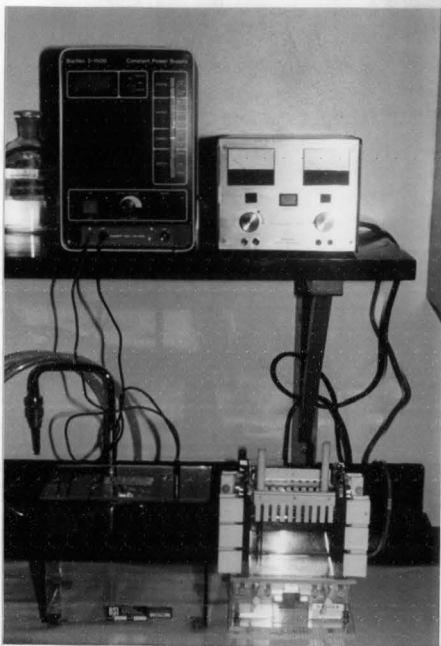
Figure 3. Vertical slab gel: standard fabricated unit of Hoefer
Scientific Instruments model SE 500.

a constant 100 V for 1 h, at which time the voltage was increased to 220 V for 10 h. After electrophoresis, gels were fixed with 40% methanol and 10% acetic acid solution for 2 h and stained for several with 0.125% Coomassie Brilliant Blue R-250 in 50% methanol and 10% acetic acid. Finally, gels were destained by diffusion against several changes of the fixative solution (Appendix A).

Molecular weights of separated proteins were determined by following the protocol of Bryan (1977) and Davis (1964) as outlined in Sigma Technical Bulletin No. MKR-137 (1983). Unknown proteins, along with known standard proteins, were electrophoresed in 7, 8, and 9% polyacrylamide separating gels. The relative mobility (R_f) of the protein in each gel relative to the tracking dye (bromophenol blue), which was mixed with the sample buffer, was determined. The 100 log (R_f x 100) values were plotted against the three gel concentrations as percent on standard graph paper for each protein. Negative slopes from these graphs were plotted against known molecular weights of the standards on 2 cycle log-log paper. This produced a linear plot from which the molecular weight of the unknown proteins were determined. With this protocol, gels were calibrated with the following standard proteins: α-lactalbumin, bovine milk, MW 14,200, carbonic anhydrase, bovine erthyrocytes MW 29,000, albumin chicken egg MW 45,000 and albumin, bovine serum MW 66,000 (monomer) and 132,000 (dimer) (Appendix B).

In the denaturing discontinuous system, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following

Laemmli's (1970) protocol. The separating gel was 10% T^1, 2.7% C^2, pH 8.8, and the stacking gel was 4% T, 2.7% C, pH 6.8. The running buffer, which was used for both lower and upper buffer chambers, consisted of 0.25 M Tris, 0.192 M glycine, pH 8.3, and 0.1% SDS. The protein extract of all seventeen cultivars was mixed with a sample buffer that consisted of 0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 10% 2-mercaptoethanol (ME) in a ratio of 1:1, which then was heated in a boiling water bath for 90 s and stored in a freezer until used. After the gels were prepared, 10 μL of each sample were loaded onto gels, and the samples were electrophoresed for 4.75 h at a constant voltage of 100 V. The gels were stained immediately upon termination of the electrophoresis with 0.125% Coomassie Blue R-250 in 50% methanol and 10% acetic acid for several h. After staining, the gels were transferred to destaining solutions of 50% methanol and 10% acetic acid for 2 h, followed by further destaining in 7% acetic acid and 5% methanol for several h. The second solution was changed several times (Appendix C).

The discontinuous system of Laemmli (1970) was used to determine molecular weights of separated polypeptides. Gels were loaded with standard proteins along with the unknown polypeptides. α-lactalbumin, bovine milk, MW 14,200, albumin, chicken egg, MW 45,000 and albumin, bovine serum MW 66,000 (monomer) were used as standard proteins. A drop of 0.1% bromophenol blue was added to the upper buffer chamber as a tracking dye. By measuring the R_f for the standard proteins, a standard curve was generated. The standard curve showed the R_f of standard proteins and the log of their molecular weights. R_f of the unknown

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[1] T = Total acrylamide %
[2] C = Total N,N'-methylenebisacrylamide %

polypeptides were measured and their molecular weights were determined from the standard curve (Appendix D).

Drying the Gels

The destained gels were immersed in a solution of 1% glycerol, 10% acetic acid for at least 1 h. Then they were dried on a sheet of filter paper using a slab gel dryer (Hoefer Scientific Instrument, Model SE 1150) (Figure 4).

Figure 4. Gel slab dryer - Hoefer Scientific Instrument Model SE 1150.

RESULTS AND DISCUSSION

Transverse cotyledonal cracking (TVC) index for the P. vulgaris
cultivars, Earliwax, Early Gallatin-1, and White-seeded Tendercrop-1,
was 0.12, 1.70, and 3.45, respectively (Table 1). Because of their
range in TVC index, these cultivars were utilized in initial experiments
to compare electrophoretic patterns of storage proteins from the seeds
of snapbeans resistant and susceptible to TVC and to correlate the
different patterns of PAGE electrophoretograms of the protein bands to
the TVC phenomenon. Staining patterns of PAGE gels containing
polypeptides from cotyledonal cells are shown in Figure 5.
Electrophoretograms for these three cultivars indicated that major
protein bands were qualitatively and quantitatively very similar, with
the exception of band A in lane 2 that belonged to the White-seeded
Tendercrop-1 cultivar. This band did not appear in the other two
cultivars. However, being thin, this band was easily affected by
destaining processes and sample concentrations.

Appearance of band A only in the sample with the highest cracking
index number in the three cultivars tested suggests a correlation
between storage protein patterns and the TVC trait. Since inheritance
of the TVC trait has already been demonstrated in snapbeans (Dickson
1975, Dickson and Boettger 1977, Dorrell 1968, Morris 1984, personal
communication), the use of protein markers have shown that enzyme
electrophoresis is a useful method for identification of cultivars of
soybeans (Gorman and Kiang 1978) and cereals (Kahler and Allard 1970,
Menke et al. 1973). Moreover, Marchylo and LaBerge (1980) used

Table 1. Cotyledon cracking index of seventeen cultivars of Phaseolus
vulgaris L.

Cultivar	Crack Index
Early Gallatin-1	1.70
Early Gallatin-2	1.70
White-seeded Tendercrop-1	3.45
White-seeded Tendercrop-2	3.45
Earliwax	0.12
R. Kinghorn Wax	2.60
Provider	1.40
Slim Green	4.20
Pure Gold	0.50
Vita Green	2.35
Green Pod 103	4.40
Green Pod	1.20
Dandy	2.40
Cascade	4.70
Roma II	0.80
Lancer	0.30
Contender	0.00
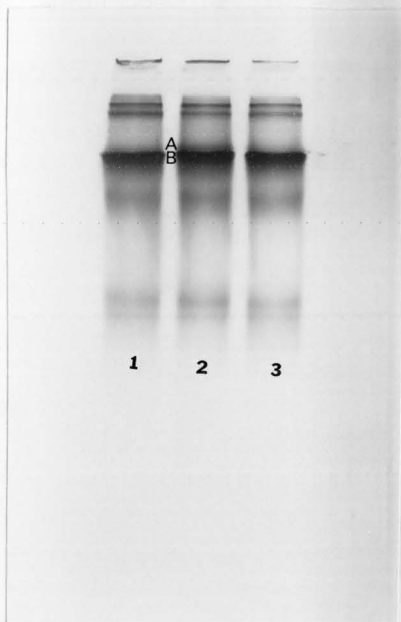
Figure 5. Polyacrylamide gel electrophoresis (12%) of the storage proteins of three Phaseolus vulgaris L. cultivars.

Legend: (1) Early Gallatin-1, (2) Tendercrop-1, (3) Earliwax.

electrophoresis for identification of the major protein, hordein, in barley cultivars. Quiros (1980) used the starch gel electrophoresis technique to identify mother plants from contaminants in an alfalfa nursery. Torres and Tisserat (1980) used leaf isozymes on starch gel electrophoresis technique to identify mother plants from contaminants in an alfalfa nursery. Torres and Tisserat (1980) used leaf isozymes on starch gel on electrophoresis as genetic markers in date palms, while Krasnuk et al. (1976, 1978) applied this tool in cold tolerance studies of alfalfa cultivars. Menendez et al. (1982) also used protein electrophoresis to distinguish genotypes within the same species of apple.

To identify protein bands of bean cultivars using the non-denaturing system, three different gel concentrations were used (Figures 6, 7, and 8). These gels were loaded with unknown samples along with four standard proteins. The relative mobility (R_f) of standard protein bands and major unknown bands were measured from the three gels in Figures 6, 7, and 8 and are listed in Table 2. Results were calculated according to the following equation: $100 \, (\log R_f \times 100)$ and plotted as a function of gel concentration percentage for both standard proteins (Figure 9) and the unknown (Figure 10). A standard curve was then obtained by plotting the negative slope of data in Figure 9 against the molecular weight of the related standard protein (Figure 11). Similarly, negative slopes were measured on unknown protein bands from data in Figure 10 and results compared to the standard curve in Figure 11. Molecular weights of proteins in the three unknown samples ranged from 14,000 to more than 390,000 daltons (Table 2). The most abundant, distinguishable band in all of the samples has a molecular weight of about 148,000 daltons (Figure 5, band B). This band is located in the region closely related
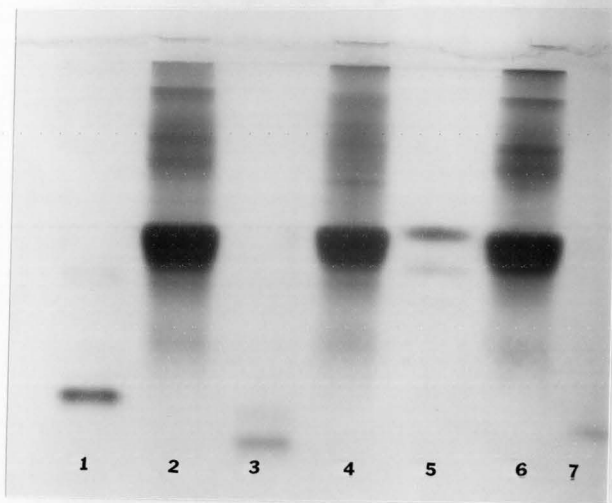
Figure 6. Polyacrylamide gel electrophoresis (7%) of the standard proteins and the storage proteins of three Phaseolus vulgaris L. cultivars.

Legend: (1) Bovine Serum Albumin, (2) Earliwax, (3) Chicken Egg Albumin, (4) Tendercrop-1, (5) Carbonic Anhydrase, (6) Early Gallatin-1, (7) α-Lactalbumin.
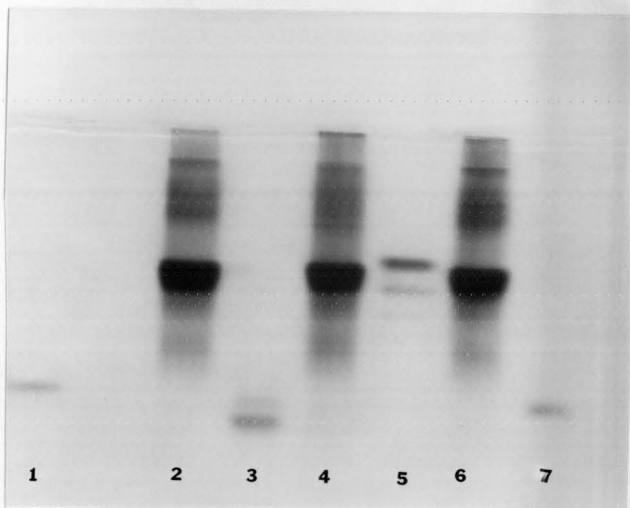
Figure 7. Polyacrylamide gel electrophoresis (8%) of the standard
proteins and the storage proteins of the three Phaseolus vulgaris L.
cultivars.

Legend: (1) Bovine Serum Albumin, (2) Earliwax, (3) Chicken Egg
Albumin, (4) Tendercrop-1, (5) Carbonic Anhydrase, (6) Early Gallatin-1,
(7) α-Lactalbumin.
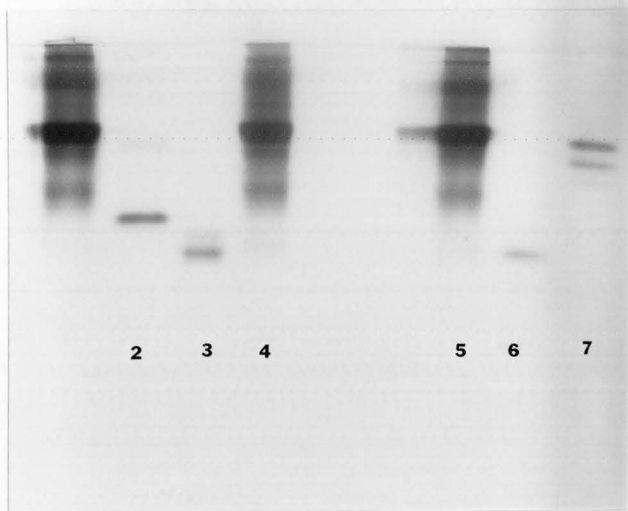
Figure 8. Polyacrylamide gel electrophoresis (9%) of the standard
proteins and the storage proteins of three Phaseolus vulgaris L.
cultivars.

Legend: (1) Earliwax, (2) Bovine Serum Albumin, (3) Chicken Egg
Albumin, (4) Tendercrop-1, (5) Early Gallatin-1, (6) α-Lactalbumin, (7)
Carbonic Anhydrase.

Table 2. Molecular weights and relative mobilities (R_f) of the standard proteins and the unknown proteins of Phaseolus vulgaris L. cultivars on three different polyacrylamide gel concentrations.

Protein	R_f 7%	8%	9%	-Slope	MW
α-Lactalbumin	0.66	0.58	0.53	4.76	14,200
Carbonic Anhydrase	0.29	0.25	0.22	6.00	29,000
Albumin, Chicken Egg	0.74	0.61	0.54	6.84	45,000
Albumin, Bovine Monomer	0.64	0.51	0.43	8.64	66,000
Albumin, Bovine Dimer	0.41	0.30	0.23	12.56	132,000

Unknown Protein	R_f 7%	8%	9%	-Slope	Approximate MW
A	0.28	0.20	0.14	15.06	290,000
B	0.34	0.26	0.20	11.53	148,000
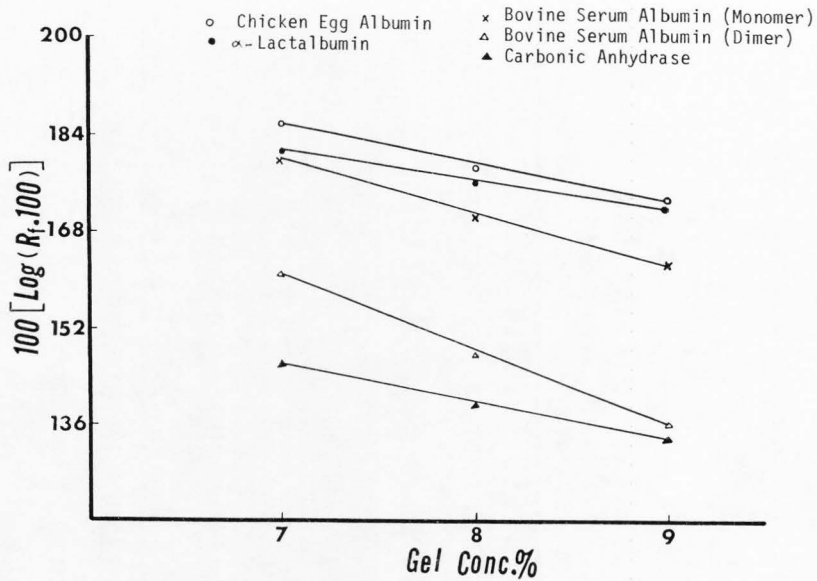
Figure 9. The 100 [Log (R_f x 100)] values of the standard proteins as a function of three different polyacrylamide gel concentrations.
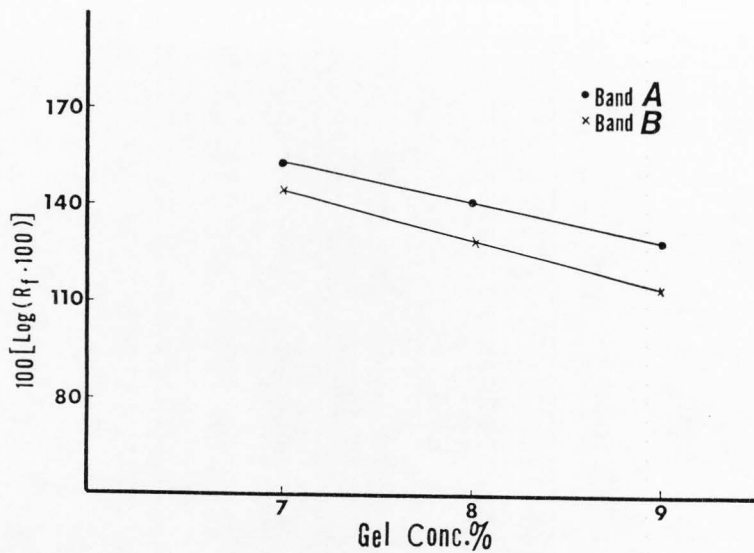
Figure 10. The 100 [Log (R_f x 100)] values of the unknown proteins of Phaseolus vulgaris L. cultivars as a function of three different polyacrylamide gel concentrations.
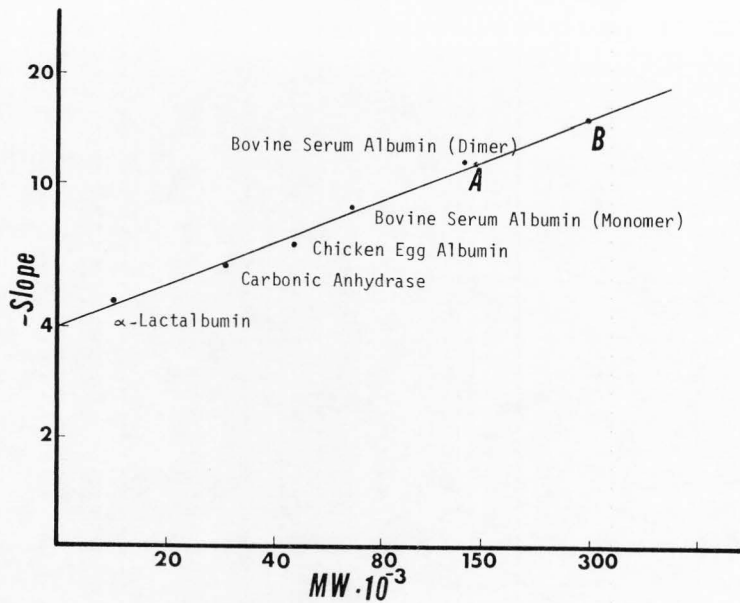
Figure 11. The standard curve for the molecular weight determination under non-denaturing system polyacrylamide gel electrophoresis.

to G_1, globular protein, the most common storage protein in snapbeans (Ma et al. 1980). Band A of White-seeded Tendercrop-1 located in lane 2 of Figure 5 has a molecular weight of approximately 290,000 daltons.

To further substantiate the evidence that the TVC trait was associated with storage proteins, additional cultivars (15 cvs), representing a wide range in the TVC index, were obtained from Rogers Brothers Company, Twin Fall, Idaho (Table 1). The new listing of cultivars also contained different samples of two of the original cultivars. The same method was used to test these fifteen cultivars along with the previous three cultivars. Staining patterns of PAGE gels containing proteins from cotyledonal cells are shown in Figure 12. The electrophoretogram shows distinct differences in protein banding patterns between different cultivars. Data, summarized in Table 3, indicated that all cultivars with a cracking index ranging from 2.35 to 4.70 contained three distinguishable bands, namely B, D, and F. The Dandy cultivar with a TVC index of 2,40, was missing bands B and F, but possessed the bands C and G, along with band D.

Cultivars with a cracking index ranging from 0.00 to 1.70 are considered as resistant or moderately resistant to TVC. Data in Table 3 show that these cultivars contain different banding patterns. Electrophoretograms of gels of cultivars Early Gallatin-1, Early Gallatin-2, Pure Gold and Contender shown in lanes 1, 8, 9, and 17 in Figure 12, respectively, all contained four well defined protein bands, A, B, D, and F. Other cultivars, representing a range of TVC indices, lacked one or more of these bands, but did contain additional bands. For example, Earliwax, with a TVC index of 0.12 was missing bands A and D but contained band G (Figure 12, lane 3). Moreover, bands A and B

Table 3. Banding patterns of seventeen cultivars of Phaseolus vulgaris
L. in non-denaturing polyacrylamide gel.

Cultivar	Band Pattern							Crack Index
Contender	A	B	-	D	-	F	-	0.00
Earliwax	-	B	-	-	-	F	G	0.12
Lancer	-	-	C	D	-	F	-	0.30
Pure Gold	A	B	-	D	-	F	-	0.50
Roma-II	-	B	-	D	-	F	-	0.80
Green Pod 103	-	B	-	D	-	F	G	1.20
Provider	A	B	-	-	E	F	-	1.40
Early Gallatin-1	A	B	-	D	-	F	-	1.70
Early Gallatin-2	A	B	-	D	-	F	-	1.70
Vita Green	-	B	-	D	-	F	-	2.35
Dandy	-	-	C	D	-	-	G	2.40
R. Kinghorn Wax	-	B	-	D	-	F	-	2.60
Tendercrop-1	-	B	-	D	-	F	-	3.45
Tendercrop-2	-	B	-	D	-	F	-	3.45
Slimgreen	-	B	-	D	-	F	-	4.20
Green Pod	-	B	-	D	-	F	-	4.40
Cascade	-	B	-	D	-	F	-	4.70

were absent in Lancer (lane 16) but bands C, D, and F were present. Band A was missing from Green Pod 103 (lane 12), yet bands B, D, F, and G were present. Bands A, B, E, and F were present in Provider (lane 5) while band D was missing. Interestingly, Roma-II (lane 15), which had a cracking index of only 0.80, had a banding pattern similar to the patterns of the cultivars exhibiting a relatively high TVC index. Bands B, D, and F were also present in this cultivar. Other bands were not distinguishable.

The SDS-polyacrylamide gel (PAGE) system allows separation of storage proteins according to their molecular weight. Protein extracts were mixed with sample buffer in the presence of mercapto-ethanol (ME) (reducing agent). This treatment increased the number of minor bands and revealed many variations between samples. Banding patterns of the fifteen cultivars utilized in this experiment are shown in Figure 13. These data summarizing the different banding patterns among the samples are shown in Table 4.

Except for track 17 in Figure 13, which belongs to the Contender cultivar, it is very clear that major bands are alike in all samples. This cultivar shows a decrease in the intensity of major bands relative to all other samples. The A band, which seems to be a very common band in the cultivars with low cracking index, is missing in this cultivar. The B, E, and H bands are extremely weak and hardly distinguishable. Also only one band is identified in the C group band, which consists of three thin bands. However, the D, I, K. and L bands are well defined.

The Green Pod 103, Provider, Early Gallatin-1, and Early Gallatin-2 cultivars (Figure 13, lanes 12, 5, 1, and 8, respectively) that have cracking indices of 1.20, 1.40, 1.70, and 1.70, respectively, show very

Table 4. Summary of the banding patterns on SDS-PAGE of seventeen cultivars of _Phaseolus_ _vulgaris_ L.

Cultivar	Band Pattern	Crack Index
Contender	- B C D E - - H I - K L - - -	0.00
Earliwax	A B C D E F - - I - K L - N O	0.12
Lancer	A B C - E F - H - J K L - N -	0.30
Pure Gold	A B C D E F G H I - K L - N O	0.50
Roma-II	A B C D E - - - I - - - M - O	0.80
Green Pod 103	A B C D E F - H I - K L - N O	1.20
Provider	A B C D E F - H I - K L - N O	1.40
Early Gallatin-1	A B C D E F - H I - K L - N O	1.70
Early Gallatin-2	A B C D E F - H I - K L - N O	1.70
Vita Green	A B C D E F - H I - K L - N O	2.35
Dandy	- - C - E F - H - J K - M - -	2.40
R. Kinghorn Wax	- B C D E - G H I - K - M - -	2.60
Tendercrop-1	- B C D E - G H I - K L - N O	3.45
Tendercrop-2	- B C D E - G H I - K L - N O	3.45
Slimgreen	A B C D E F - H I - K - M N O	4.20
Green Pod	A B C D E F - H I - K L - N O	4.40
Cascade	A B C D E F G H I - K L - - O	4.70

cultivars, is replaced by two very thin bands in the Green Pod 103
cultivar only. Also, the J and M bands disappeared in these cultivars.
Banding patterns of Earliwax and Roma-II (Figure 13, lanes 3 and 15,
respectively) are similar. The L band disappeared and was replaced by a
distinct, fast moving band M. The F band is also not clear in lane 15
in Figure 13. The Pure Gold cultivar (Figure 13, lane 9) has a unique
banding pattern, all bands are present except the J and M band. In
addition, the Lancer cultivar (Figure 13, lane 16) lacks the D, I, M,
and O bands, and the G band is replaced by two thin bands. Yet this
sample contains a new band J and H band is wide and extremely sharp.

Although the cracking index for Green Pod is very high (4.40), that
for the Vita Green relatively high (2.35) and that for Green Pod 103
relatively low (1.20), they show the same banding patterns (Figure 13,
lanes 11, 10, and 12, respectively) (Table 2). Since the H band is
extremely weak in the Green Pod 103 cultivar, it might not be considered
as a distinguishable band. Moreover, the D, E, and F, bands are
relatively darker in the Green Pod 103 sample than these same bands in
Green Pod and Vita Green.

Tendercrop-1 and Tendercrop-2 cultivars (Figure 13, lanes 2 and 7)
show analogous banding patterns. The A, F, J, and M bands were absent,
while other bands were notable. Slimgreen and Cascade cultivars (Figure
13, lanes 6 and 14, respectively) show a few variations in their banding
patterns. The Slimgreen cultivar lacks the G, J, and L, bands.
However, the Cascade cultivar lacks the J and M as well as the N bands.
In the R. Kinghorn Wax cultivar (Figure 13, lane 4), the A, F, J, L, N,
and O bands were missing. Yet, the M band that is located just under
the L band position is very sharp in this sample. The Dandy cultivar

(Figure 13, lane 13) seems to have a very distinct banding pattern among those cultivars with the high cracking indices. The H and M bands are well defined and the G band is substituted for by two thin bands. Also, a new minor band J appeared just under the I band position that is missing in this sample.

Although several distinct patterns were detected, a number of cultivars with similar cracking indices, shared common patterns. A few patterns were unique among the cultivars that were used throughout this experiment such as that for the Contender and Dandy cultivars.

Figures 14 and 15 show two SDS-PAGE electrophoretograms that were loaded with the standard proteins along with the unknown samples. These gels were used to estimate the molecular weight of the polypeptides under a denaturing scheme (Table 5). Figure 16 represents the standard curve that was determined from the measurement of the R_f value of each standard protein. The molecular weight of unknown bands were identified from the standard curve.

Since there are more than one banding pattern in resistant and susceptible cultivars, this might indicate that there are more than one gene responsible for the appearance of the TVC trait. These results are in agreement with those of Dickson (1975) who reported that TVC is a complexly inherited trait. In his study, he found that resistant parents have dominant genes for the TVC while the most susceptible parents have recessive genes.

It is well known that the storage proteins are genetically controlled. Also, the electrophoretic results from a fairly large number of snapbeans cultivars, exhibiting a wide range of TVC index, indicate that storage proteins from TVC resistant cultivars are visually

Figure 14. SDS-PAGE of proteins: (1) Tendercrop-1, (2) Earliwax,
(3) Bovine Serum Albumin, (4) R. Kinghorn Wax, (5) Provider, (6) Urease,
(7) Chicken Egg Albumin, (8) Tendercrop-2, (9) Pure Gold, (10)
α-Lactalbumin.
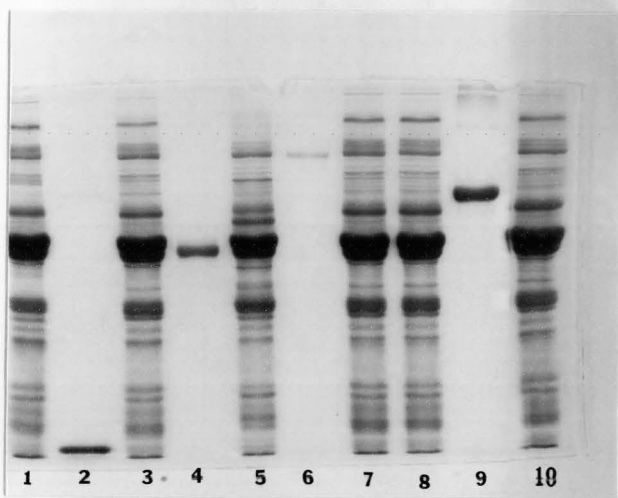
Figure 15. SDS-PAGE of proteins: (1) Roma-II, (2) α-Lactalbumin, (3) Cascade, (4) Chicken Egg Albumin, (5) Dandy, (6) Urease, (7) Green Pod 103, (8) Green Pod, (9) Bovine Serum Albumin, (10) Vita Green.

Table 5. Molecular weights and the relative mobilities of the standard proteins on a 10% SDS-PAGE-1.

Protein	Molecular Weight	$Log_{10}MW$	R_f
α-Lactalbumin	14,200	4.15	0.94
Chicken Egg Albumin	45,000	4.65	0.43
Bovine Serum Albumin	66,000	4.82	0.22
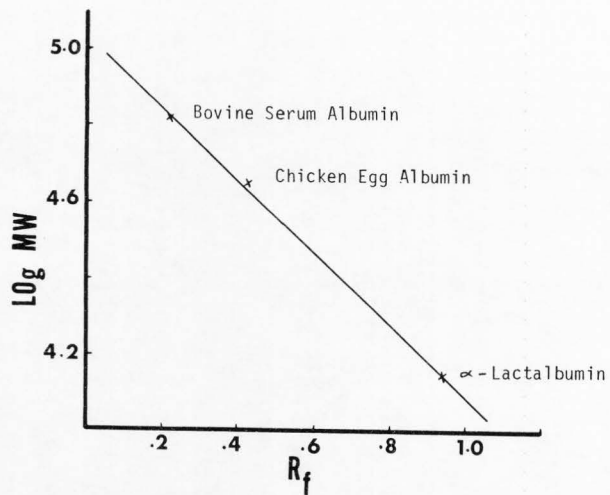
Figure 16. The standard curve for the determination of the molecular weights of polypeptides by SDS-polyacrylamide gel.

different from TVC susceptible ones. Based on these data, plant breeders should be able to use electrophoretic techniques to screen for TVC resistant cultivars in a breeding program.

SUMMARY AND CONCLUSIONS

Cotyledonal- or transverse cracking (TVC) has been recognized as a major problem in some white-seeded snapbean cultivars since their release in the early 1950's. This inherited character has been studied in relation to factors such as: 1) mechanical damage from threshers, 2) seed moisture content and seed coat permeability, 3) seed coat color, 4) seed coat thickness, and 5) calcium (Ca) and magnesium (Mg) level in both seed coats and cotyledons.

Morris et al. (1970) reported that TVC occurred across cell walls of the cotyledons rather than along the cell walls. Thus, it might be hypothesized that internal pressure resulting perhaps from swelling of storage proteins during imbibition might account for cellular rupture. To test whether storage proteins were correlated with cellular rupture, one hundred seeds were selected randomly from each of seventeen snapbean cultivars. Storage proteins were extracted from defatted, finely ground fours by 0.5 M NaCl (solvent to four ratio of 10:1) at pH 7.5 for 1 h at 4 C. The mixture was centrifuged at 10,000 g for 30 min and aliquots of resulting supernatant solutions were electrophoresed.

Non-denaturing discontinuous electrophoresis was performed on a 12% polyacrylamide separating gel, pH 8.8, with a 5% polyacrylamide stacking gel, pH 8.37 at 4 C. The denaturing discontinuous SDS-PAGE was performed on a 10% polyacrylamide separating gel, pH 8.8, with a 4% stacking gel, pH 6.8. Molecular weights were determined in both methods for the intact proteins and the polypeptides, respectively. Gels were stained immediately upon termination of the electrophoresis with 0.125%

Coomassie Blue R-250 in 50% methanol and 10% acetic acid for several
hours.

Electrophoretic results from the snapbean cultivars utilized in this
study, exhibiting a wide range of TVC index, indicated that storage
proteins from TVC resistant cultivars were visually different from TVC
susceptible ones. Although TVC of initial cultivars tested, White-
seeded Tendercrop-1, Earliwax and Early Gallatin-1, were known, the
second group of 15 cultivars was coded as to name and TVC index until
after the study was completed. A visual comparison of protein bands
(Figures 12 and 13) did, however, show similarities and differences.
Cultivars were subsequently identified as to name and TVC index and
these similarities and differences noted. While the electrophoretic
technique shows observable differences in cultivars expressing
differential TVC, it is not clear which protein bands are associated
with the TVC phenomenon (Tables 3 and 4). For plant breeders to employ
this tool in screening for TVC resistant snapbean cultivars, further
refinements are needed.

At this point, the author would like to suggest some research ideas
for further study.

1. Start with well known genetic background plant material.

2. Extract specific enzymes such as analyses and succinate-
cytochrome c reductase.

3. If a band or bands that are correlated with the TVC are
identified, elute that bands from gels and study their amino acid
sequences.

4. Measure the amount of protein in that specific band and their
molecular weights.

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APPENDIXES

Appendix A

Non-Denaturing System Electrophoresis

Preparation of Reagents

Note: Use deionized, distilled water and filter all reagents.

Separating Gel Buffer

Dissolve 11.47 g Tris and 28.92 ml 1 N HCl in water. Dilute to 100 ml with water and adjust to pH 8.8 with HCl.

Stacking Gel Buffer

Dissolve 7 g Tris, 1 g EDTA and 2 g boric acid in water. Dilute to 1 liter with water and adjust to pH 8.37. Use this buffer for the upper chamber also.

Acrylamide Solution of Separating Gel

Dissolve 11.3268 g acrylamide and 0.6742 g N,N'-methylenebis-acrylamide in 100 ml of separating gel buffer.

Acrylamide Solution for Stacking Gel

Dissolve 4.72 g acrylamide and 0.28 g N,N'-methylenebisacrylamide in 100 ml of stacking gel buffer.

Lower Buffer

Dissolve 22.7 g Tris and 150 ml 1 N HCl in water and dilute to 3 liters.

Sample Buffer

Combine 1 ml stacking gel buffer, 1 ml glycerol, and 1 ml water.

Fixative Solution

Combine 400 ml methanol, 100 ml glacial acetic acid and 500 ml water.

Ammonium Persulfate Solution (APS)

Dissolve 1 g ammonium persulfate in 10 mL of water.

Gel Preparation

Assemble the vertical slab gel unit in the casting mode. In a 100 mL flask mix 50 mL of acrylamide solution for separating gel with 0.3 mL APS solution. Add a magnetic spin bar, and place it on a magnetic stirrer. Add 2-3 drops of N,N,N,N'-Tetramethyl-ethylenediamine (TEMED) and gently swirl the flask to mix. Using a greased plastic syringe (50 mL) with a 20 guage needle, fill the glass sandwich up to 1 cm below the lower end of the comb (that will produce one cm stacking gel). Before gel polymerizes, layer 1-2 mL of water on top of each gel, being careful not to disturb the surface of the gel solution. When a sharp interface between the gel and the water layer forms, pour the liquid from the surface of the gels. In a 50 mL flask, mix 20 mL of stacking gel solution with 1 mL of APS solution. Add a magnetic stirring bar, and place the flask on a magnetic stirrer for about 5 min. Add 1 drop of TEMED and gently swirl the flask to mix. Add 1-2 mL of this solution to each sandwich to rinse the surface of the gels. Pour the solution and fill each sandwich with stacking gel solution. Insert a comb into each sandwich. Take care not to introduce any bubbles below the teeth of the comb. Allow gels to polymerize and slowly remove the comb from gels. Pull the comb straight up to avoid disturbing well dividers. Rinse each well with distilled water then drain wells and fill each well with the upper buffer solution.

Loading and Running the Gels

Combine equal parts of protein sample and sample buffer. Using a Hamilton syringe (50 L), underlayer the sample in each well. Remove lower cams and place the unit in the lower buffer chamber. To get a constant, low temperature place the unit with the lower buffer chamber into an ice chest filled with ice. Fill the upper buffer chamber with the upper buffer solution and the lower buffer chamber with the lower buffer solution. Put the lid on the unit and connect to the power supply. Set the power supply to constant voltage. Turn the power supply on and adjust the voltage to 100 V for 1 h then 200 V for 10 h. Let it run for the required time, then turn the power supply off and disconnect the power cable.

Staining and Destaining the Gels

Disassemble glass sandwiches and put gels into the fixative solution for at least two h. Remove gels and put them into Coomassie Blue R-250 staining solution for several h. Finally, destain them with the fixative solution by changing solution several times.

Appendix B

Determination of Molecular Weight by Non-Denaturing

System: Preparation of Reagents

Separating Gel Buffer

Dissolve 36.3 g Tris and 0.23 mL TEMED in water. Dilute to 100 mL with water and adjust to pH 8.9 with HCl.

Stacking Gel Buffer

Dissolve 5.98 g Tris and 0.46 mL TEMED in water. Dilute to 100 mL with water and adjust to pH 6.7 with HCl.

Acrylamide Solution for Separating Gel

Dissolve 28 g acrylamide and 0.74 g N,N'-methylenebis acarylamide in water and dilute to 100 mL.

Acrylamide Soltuion for Stacking Gel

Dissolve 10 g acrylamide and 2.5 g N,N'-methylenebisacrylamide in water and dilute to 100 mL.

Ribofalvin Solution

Dissolve 4 mg riboflavin in 100 mL of water.

Sucrose Solution

Dissolve 5 g of sucrose in 100 mL of water.

Ammonium Persulfate Solution

Dissolve 40 mg of ammonium persulfate in 5 mL of water.

Running Buffer

Dissolve 1.2 g Tris and 5.76 g glycine in water and dilute to 2 liters. The pH should be approximately 8.3 at 25° C.

Fixative Solution

Mix 400 mL of methanol, 70 mL of acetic acid and 530 mL of water.

Staining Reagent

Dissolve 0.5 g Coomassie Brilliant Blue in 500 ml of fixative solution. Store tightly capped container at room temperature (25°C). This reagent is stable for several months.

Sample Buffer

Combine 1 mL stacking gel buffer, 1 mL glycerol and 1 mL water containing 0.25 mg bromophenol blue.

Preparation of Sample

Dilute the unknown protein with an equal volume of sample buffer.

Preparation of Molecular Weight Markers

Reconstitute each of the protein standard vials (Table 6) with 1 mL of 50 mM NaCl and 1 mM sodium phosphate and adjust to pH 7.0. If urease is used, it should be mixed with 5.0 mL of distilled water.

Avoid repeated freezing and thawing. Stock solutions may be dispensed into working aliquots, frozen, then discarded after 2-3 uses. Solutions may be frozen at -20° C for future use.

Immediately before use, dilute standards with an equal volume of sample buffer.

Preparation of Gels

Mix separating gel buffer, acrylamide solution for separating gel, and sucrose solution according to Table 7. Add a magnetic stirring bar and place the mixture on a magnetic stirrer. Add APS to the mixture and mix solution carefully to avoid introducing air. Fill the glass sandwich with this mixture and layer water on top of gel solution as explained before in Appendix A. In a 50 mL flask, combine 4 mL

stacking gel buffer, 8 mL acrylamide solution for stacking gel, 4 mL riboflavin solution, and 16 mL sucrose solution. Mix as before (Appendix A). Remove water layer from polymerized separating gels and wash the top of each of them with approximately 1-2 mL of stacking gel solution. Carefully dispense stacking gel solution into each glass sandwich and insert combs as in Appendix A. Allow gels to sit for at least one-half hour to be polymerized. Treat gels as in Appendix A.

Loading and Running the Gels

Underlayer sample on gels according to amounts listed in Table 8 and as explained before (Appendix A). Fill both the upper and the lower buffer chamber with the running buffer solution. Electrophorese samples at a constant voltage of 100 V for one hour and then at 200 V until the marker dye (bromophenol blue) is one centimeter from the anodic end of the gel. Disassemble the unit and mark the center of the bromophenol blue dye front with a piece of fine wire or a fine needle.

Staining and Destaining

Immerse gels in fixative solution for two h. Stain gels in staining solution for at least six h. Overnight staining is preferred. Destain gels in fixative solution by diffusion against several changes of fixative solution. Finally, transfer gels into 7% acetic acid solution for storage. Allow gels to stand in acetic acid solution for at least three h before reading migration distances. Record migration distances of the tracking dye and of the blue protein bands from the top of the separating gel. On gels with standard proteins exhibiting charged isomers, measure the migration distance of the darkest band.

To determine the relative mobility (R_f) of a protein, divide its migration distance from the top of the separating gel to the center of the protein band by the migration distance of the bromophenol blue tracking dye from the top of the separating gel:

$$R_f = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

100 (Log (R_f x 100)) values (ordinate) are plotted against the gel concentration as percent (abscissa) on standard graph paper for each protein (Text, Fig. 9). Negative slopes from these graphs (ordinate) are plotted against known molecular weights of standards (abscissa) on two cycle log-log paper (Text, Fig. 11). Determine the molecular weight of the unknown protein from the graph.

Table 6. Standard proteins and their corresponding molecular weights.

Proteins	Molecular Weights
α-Lactalbumin, Bovine Milk	14,200
Carbonic Anhydrase Bovine Erythrocytes	29,000
Albumin Chicken Egg	45,000
Albumin, Bovine Serum	66,000 (monomer) 132,000 (dimer)

Table 7. Reagent amounts (mL) for three different separating gel
concentrations.

mL of Reagent	% Gel Concentration		
	7.0	8.0	9.0
Separating Gel Buffer	3.00	3.00	3.00
Acrylamide Solution for Separating Gel	6.00	6.86	8.58
Sucrose Solution	13.50	12.64	10.94
Ammonium Persulfate Solution	1.00	1.00	1.50

Table 8. Amount (μL) of the standard proteins and the unknown sample applied to gel.

Protein	Amount of Sample Applied to Gel (L)
∝-Lacalbumin	15
Carbonic Anhydrase	20
Albumin, Chicken Egg	20
Albumin, Bovine Serum	15
Unknown	25

Appendix C

SDS Gel Electrophoresis: Preparation of Reagents

Monomer Solution (30% Tris 2.7 Bis)

Dissolve 58.4 g acrylamide and 1.6 Bis in Water. Dilute to 200 mL with water. Store at 4°C in the dark.

Running Gel Buffer (1.5 M Tris-Cl pH 8.8)

Dissolve 36.3 g Tris in water. Dilute to 200 mL with water and adjust to pH 8.8 with HCl.

Stacking Gel Buffer (0.5 M Tris-Cl, pH 6.8)

Dissolve 3.0 g Tris in water. Dilute to 50 mL with water and adjust to pH 6.8 with HCl.

Ten Percent SDS

Dissolve 50 g SDS in 500 mL water.

Initiator (10% ammonium persulfat)

Dissolve 0.5 g ammonium persulfate in 5.0 mL of water.

Running Gel Overlayer (0.375 M Tris-Cl, pH 8.8, 0.1% SDS)

Combine 25 mL of solution 2 and 1.0 mL of 10% SDS solution. Dilute to 100 mL with water.

2X Treatement Buffer (0.125 M Tris-Cl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethano)

Combine 2.5 mil of solution 3, 4.0 mL of solution 4, 2.0 mL of glycerol and 1.0 mL 2-mercaptoethanol (ME). Dilute to 10.0 mL with water. Divide in aliquots and freeze.

Tank Buffer (0.25 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS)

Dissolve 12 g Tris and 57 glycine in water. Add 40 mL of 10% SDS solution to the mixture and dilute to 4.0 liters with water. The pH of this solution need not be checked.

Staining Stock (1% Coomassie Blue R-25)

Dissolve 2.0 g Coomassie Blue R-250 in water. Dilute to 200 mL with water, stir and filter.

Staining Solution (0.125% Coomassie Blue R-250, 50% methanol, 10% acetic acid)

Combine 62.5 mL of staining stock, 250 mL methanol, and 40 mL acetic acid. Dilute to 500 mL with water.

Destaining Solution I (50% methanol, 10% acetic acid)

Combine 500 mL methanol and 100 mL acetic acid and dilute to 1.0 liter with water.

Destaining Solution II (7% acetic acid, 5% methanol)

Combine 70 mL acetic acid and 50 mL methanol x and dilute to 1.0 liter with water.

Sample Preparation

Combine equal parts of protein sample and 2X treatment buffer in a test tube, and put the tube in a boiling water bath for 90 seconds. Then remove the sample and put it on ice until ready to use. This treated sample can be put in the freezer for future runs.

Gel Preparation

Assemble the vertical slab gel unit in the casting mode. Mix 60 mL of separating gel solution according to Table 9. Leave out ammonium persulfate and TEMED. Add a small magnetic stirring bar and place the flask on a magnetic stirrer. Then, add TEMED and ammonium persulfate and gently swirl the flask to mix, being careful not to generate bubbles. Fill the glas sandwich with this solution as explained in Appendix A. When the gel polymerizes, pour off the water layer and rinse surfaces with distilled water. Finally, add about 1.0 mL of running gel overlayer solution and allow the gel to sit for several hours. Mix 20 mL of stacking gel solution according to Table 1. Leave out the ammonium persulfate and the TEMED. Deaerate the solution as before. After that, add ammonium persulfate and TEMED. Pour the liquid from the surface of the separating gels and add 1-2 mL of stacking gel solution to each sandwich to rinse the surface of the gel. Pour off the liquid and fill each sandwich with stacking gel solution. Insert combs as in Appendix A and allow the gel to set for at least one-half hour.

Loading and Running the Gels

Remove combs from gels and rinse the wells as before in Appendix A. Fill each well with tank buffer and underlayer the sample as before. Fill the lower buffer chamber with tank buffer until sandwiches are immersed in buffer. If bubbles get trapped under ends of the sandwiches, coax them away with a pipette. Fill the upper buffer chamber with tank buffer also and add one drop of 0.1% bromophenol blue as a tracking dye. Put the lid on the unit and connect to the

Table 9. Amounts of stock solutions for separating and stacking SDS-PAGE preparation.

Stock Solution	Separating gel 10% T 2.7% C	Stacking gel 4% T 2.7% C
30% T 2.7% C	20 ml	2.66 ml
Separating gel buffer	15 ml	--
Stacking gel buffer	--	5.0 ml
10% SDS	0.6 ml	0.2 ml
H_2O	24.1 ml	12.2 ml
Ammonium Persulfate	300 l	100 l
TEMED	20 l	10 l

power supply. Set power supply to a constant voltage. Turn power supply on and adjust the voltage to 100 V. Electrophores gels for 5 h. By this time, the tracking dye will reach the bottom. Turn the power supply off and disconnect power cables.

Staining the Destaining Gels

Disassemble sandwiches and put gels into stain solution for 4-8 h. Remove gels and put them in destaining solution I for one h. Finally, transfer gels into destaining solution II for several h.

Appendix D

Determination of the Molecular Weights

by SDS Gel Electrophoresis

Prepare gels and the unknown sample as in Appendix C. Reconstitute standard proteins as in Appendix B, and mix equal parts of standard proteins and 2X treatment buffer (Appendix C). Underlayer the unknown samples onto gels along with standard proteins. Electrophores samples as in Appendix C. Before the staining and the destaining processes, mark the tracking dye (bromophenol blue) on the gels. Then treat the gels as in Appendix C. After the last destaining step, measure the R_f, i.e., the ratio of the distance from the top of separating gel to the polypeptide divided by the distance from the top of the separating gel to the dye front, and generate a standard curve. This curve will show the R_f of the polypeptides and the log of their molecular weights. The R_f of the unknown polypeptides are determined in the same way, and the log of its molecular weight read directly from the standard curve.