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A computer-assisted examination of the storage protein genetic variation in 841 accessions of *Triticum dicoccoides* *

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Summary. *Triticum turgidum* L. var. *dicoccoides* (wild emmer) is an important genetic resource for increasing the protein content of common wheat (*Triticum aestivum* L.). Many studies have shown that the presence or absence of bands in sodium dodecyl sulfate polyacrylamide (SDS-PAGE) electrophoregrams of wheat storage proteins to be of a purely genetic character. A total protein extraction and SDS-PAGE technique was used to estimate the storage protein genetic variability among 841 accessions of wild emmer collected from various ecological regions in the Middle East. In addition, a computer data bank was developed, recording the one-dimension electrophoregram bands for each accession by molecular weight (MW) and relative Coomassie Blue staining intensity as determined from densitometer scans. Analyses of this information are being used to identify specific accessions for further study by two dimension electrofocusing-electrophoresis and breeding and genetic analyses. The computer-assisted analyses indicated that the greatest genetic variability occurs for proteins in the high MW region (above 70,000 MW) followed by those in the medium range (70,000 to 33,300 MW). Comparatively little variability was revealed for protein subunits of below 33,300 MW.

Key words: Electrophoresis – Endosperm – Proteins – Emmer – Wheat

Introduction

A major portion of the world's population depends on wheat (*Triticum aestivum* L. em. thell.) as their primary source of nutrients. Because of this, and the known deficiency of lysine in wheat protein, higher levels of protein and lysine are needed, especially to improve the low income wheat consumer's diet. Wild emmer, *T. turgidum* L. var *dicoccoides* (Körn. in litt. in Schweinf.) Bowden, a close relative of cultivated wheats, was found to contain relatively high kernel protein content. Some of the wild emmer accessions also have large grain size (Avivi 1979; Grama et al. 1984). Accessions of this species are now being used as germplasm sources for improving wheat grain protein content and quality. The potential for exploiting this exotic material opens new possibilities to achieve the goal of increased genetic capacity for protein accumulation and improved quality provided sufficient useful new genetic variability can be identified in the wild emmer gene pool.

Electrophoregrams of proteins can be used to assess genetic variability. Many studies have shown that the structure, production (presence or absence), and dosage of kernel storage proteins are controlled almost entirely by the genotype of the plant, and are therefore virtually unaffected by the growing conditions (Auriau et al. 1976; Fullington et al. 1980, 1983; Orth and Bushuk 1973; Zillman and Bushuk 1979; for a review see Kasarda et al. 1976; Konzak 1977). Furthermore, electrophoresis of wheat proteins has been used to identify varieties. This work also provides evidence that the presence or absence of bands is largely independent of environmental influences.

Relatively few accessions of *T. dicoccoides* have previously been examined electrophoretically for kernel protein variability. Although earlier work had provided evidence of genetic variation among storage proteins in *T. dicoccoides,* so few accessions were examined that they could hardly represent the extent of variability present in this species. Johnson et al. (1967) examined only 12 accessions of *T. dicoccoides* and studied 43 accessions representing eight subspecies of tetraploid

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wheats (Johnson 1967). Recently, however, Nevo et al. (1982) examined soluble protein allozyme patterns of 457 individuals representing 12 Israeli *T. dicoccoides* populations. He found considerable genetic variability for these proteins, as revealed by their differential mobility in non-denaturing starch gel electrophoresis. The aim of the present work was not only to confirm the results of previous research but to expand their scope. The use of a microcomputer enabled us to compare hundreds of accessions (most of which previously unexamined) and to compile an easily accessible record of the proteins present in each. Further analyses may identify accessions having protein types and protein contents of interest for genetics and breeding. As an example, electrophoresis studies have implicated protein bands of specific high MW as critical to the processing properties of wheat (Payne et al. 1979). This suggest that other high MW proteins may have high value for improving wheat quality. Our computerized system has facilitated the selection of accessions having similarly high MW proteins as a basis for studies of their potential value for improving wheat processing properties (Mansur-Vergara 1984). Further analysis of the proteins in different regions of densitometer scans of the gels (SDS-PAGE) and the interrelationships among protein bands are presented in another report (Mansur-Vergara et al. 1984).

Materials and methods

Wheat samples

Mature seeds of 841 accessions of *Triticum dicoccoides*, which ranged in protein content from 15 to 25%, were used in this study. The accessions originated from plant explorations conducted in Israel, Turkey or Lebanon and were representative of various environments, including those with different climatic and soil characteristics. The accessions studied included seeds collected in nature as well as seeds increased in nurseries at the Volcani Institute, Bet-Dagan, Israel. Some accessions were bulk collections, whereas others were single-plant selections or even reselections carried on for several years.

Extraction of proteins from kernels

Total protein can be extracted from wheat kernels using buffers containing sodium dodecyl sulfate and 2-mercaptoethanol (Cole et al. 1981; Fullington et al. 1980). In this study total proteins were extracted from the brush half of the kernel of the *T. diccocoides* accessions using the buffer system of Fullington et al. (1980).

The half seeds were crushed using pliers with jaws covered by a small piece of filter paper. The volume of buffer used for extraction was adjusted to 325 µl of buffer/10 mg seed in all cases. The mixture was ground and homogenized for 1 min using a tissue-grinding pestle and mortar attached to a slow speed electric motor. The samples were then heated in boiling water for 5 min to aid solubilization and reduction. The insoluble material was spun down for 10 min at 8,699 × g and a 10 µl aliquot of the supernatant was used for electrophoresis.

Electrophoresis

The discontinuous high resolution sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system used was similar to that devised by Laemmli (1970) and modified later by Fullington et al. (1980, 1983) and Cole et al. (1981) for wheat storage proteins. Some minor but significant differences in apparatus, molecular weight standards, gel size, staining and destaining procedures were necessary for the present assays.

Apparatus

A Hoeffer SE 500 vertical slab gel unit was used for SDS-PAGE thrughout this work. A constant current of 15 mA per gel for about 3.2 h was applied to 18 samples plus two molecular weight standards in each gel. The voltage varied from 70–80 V at the start of the run to 200–220 V at the end. The first 440 test samples were loaded in duplicate. However, as experience was gained, the gels proved sufficiently uniform and clear that duplicate samples were not necessary. Unusual samples were confirmed by two or more later runs.

Molecular weight standards

Molecular weight standards (MWS) obtained from Bio-Rad Laboratories, Richmond, California, consisted of the following proteins: lysozyme 14,000 MW, soybean trypsin inhibitor 21,500 MW, carbonic anhydrase 31,000 MW, ovalbumin 45,000 MW, bovine serum albumin 66,200 MW, and phosphorylase B 92,500 MW. The MWS were prepared by mixing one part of the standard proteins with 19 parts extraction buffer that included 5% 2-mercaptoethanol, as with the wheat samples. The MWS were included in two slots of all the gels, at the center and at the end. Faint impurities were observed in the MWS but they caused no difficulties in the determination or calculations.

Acrylamide gels

Gel slabs with 20 slots were used. The separating gel (10 cm × 13.5 cm × 0.75 mm) contained 17.5% (w/v) acrylamide, (0.08% (w/v) bis (N,N′-methylene-bis-acrylamide), 25% of 1.5 M tris (tris hydroxymethyl aminomethane) buffer made to pH 8.8 with HCl. Of this solution, 47.2 ml were polymerized by adding 246 µl of 10% ammonium persulfate freshly prepared 10 min before pouring the gels. In addition, immediately before pouring the gels, 16 µl of TEMED (tetramethylenediamine) and 0.48 ml of a 10% SDS solution were added. The stacking gel (3 cm × 13.5 cm × 0.75 mm) contained 3.85% acrylamide (w/v), 0.104% bis (w/v), 25% of 0.5 M tris buffer adjusted to pH 6.8 with HCl. A 20.17 ml aliquot of this solution contained, in addition, 100 µl of 10% ammonium persulfate, 10 µl TEMED, and 0.2 ml of 10% SDS.

Staining and destaining

Gels were stained using a solution of 2.5 g Coomassie Blue R-250 in 500 ml methanol, 150 ml glacial acetic acid, and 1,850 ml glass-distilled water for at least 12 h, but no more than 18 h. The destaining solution was made up by mixing 500 ml methanol, 160 ml glacial acetic acid and 1,340 ml glass-distilled water. The destaining solution was changed daily until no color was visible in the background. The gels were subsequently dried on porous cellophane following the procedure described in the Bio-Rad manual for the Gel Slab Dryer Model 224.

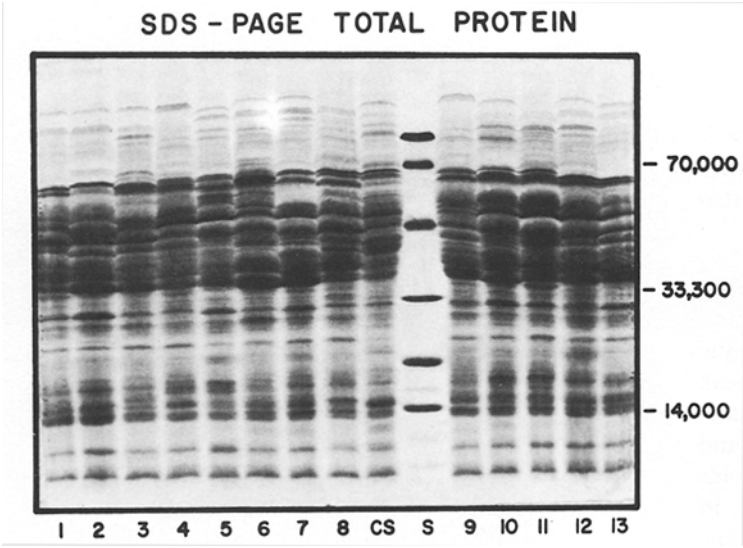

Fig. 1. *SDS-PAGE* of 13 selected *T. dicoccoides* accessions chosen on the basis of uniqueness from several different gels and reproduced in a single run. The variety 'Chinese Spring' is included for comparison. *Slot 1* = BP11, *2* = BP13, *3* = BP17, *4* = BP150, *5* = BP156, *6* = BP374, *7* = BP376, *8* = BP388, *CS* = 'Chinese Spring', *S* = Molecular weight standards, *9* = BP462, *10* = BP574, *11* = BP573, *12* = BP783, *13* = BP1010

Densitometry and computer analysis

A Beckman densitometer Model R-112 was interfaced to a Radio Shack TRS-80 Model II 64K microcomputer equipped with an 8 mega bytes memory hard disk accessory. Gels were scanned at 570 nm, in the low speed (densitometer) setting (about 2 min/scan) with the slit size 0.3×2 mm. This information was directly digitized by an A-D converter and transferred to the microcomputer. A set of computer program software for data acquisition and analysis in TRS-80 Model II[1] BASIC was written. These programs include "Dense" to read and then store all the raw data in a file. Another program, designated "Geldump", produces a graph of each scan as well as numbers, and assigns a MW and a relative intensity value (relative to average intensity) to each peak and transfers all the data to a storage file for later access. This program also normalizes all graphs to equivalent areas for comparisons independent of the protein amount loaded onto the gel.

Program "Gel STD" was used to calculate the MW of each peak. The computer input information was the following: distance of migration from the origin to a high MW band (the first darkly stained band was regularly used) and a low MW band (usually the last band), the gel length, and the distance migrated by each MWS. Other computer programs were written to carry out qualitative analyses for assessing the genetic variability for protein subunit composition of the accessions.

A description of these programs follows:

"Gel Fuzz". This program smoothed graphs to remove shoulders and very small peaks that could not be distinguished from background noise. All analyses were carried out on both smoothed and raw data. Results (not presented) indicated that the smoothed data would be more reliable.

"Gel Blob" searched through each scan and then printed a dot in a graph of relative intensity (RI) vs. MW for every peak. Other program options included the ability to specify the MW range, relative intensity range, and to select samples for further analyses. This made it possible to focus on a particular area of the gels. Processing options allowed the use of the raw data or smoothed data, i.e., with shoulders and very small peaks removed. This program was the basis for the qualitative analysis discussed below.

Qualitative analysis. The output that resulted from the processing of the gel scan data with the "GelBlob" program was a relative intensity (RI) vs. MW graph representing all the scans, thus a composite of all the samples. This is herein designated as a "dot graph". The dot graph was subdivided into three regions: the high MW (140,000 to 70,000), the medium MW (70,000 to 33,300), and the low MW region (33,300 to 8,800). These divisions were chosen at the natural break points for grouping bands in the gels. These groups were further subdivided into their individual MW ranges which correspond to dot clusters or spaces between clusters. The high MW region was subdivided into seven MW ranges decreasing in MW by about 10% each time. This was done because no clusters or spaces between clusters existed within this region, unlike the case for the medium and low MW regions which were divided in terms of their clusters and spaces. The program "GelBar" was designed to analyze the make-up of these MW ranges. Three graphs of frequency vs. MW were produced, one for the high, one for the medium, and one for the low MW regions. For each MW range the graphs produced show the number of peaks and their frequencies.

"Gel Freq". This program was used to graph or produce tables of MW vs. frequency (presence or absence of bands) over specified MW regions, relative intensities, of any number of samples. Either the raw data or the data file previously processed through "GelFuzz" was used.

Results

The results illustrated by the protein profiles of selected samples shown in the gel of Fig. 1 indicate that the lower MW region (33,300 and under) was the least variable, while the high MW region (140,000 to 70,000) had the greatest protein subunit variability, and the medium MW region (70,000 to 33,300) in between.

[1] Trade name of Tandy Radio Shack Corp., Fort Worth, Texas

Other lines of evidence also support these conclusions:

1 Observations

Visual observations, and the review of the gels over many times suggested to the experienced observer that the order of variation was as stated above.

2 Dot graph

Figure 2 is a computer-generated RI vs. MW composite of the 841 samples, with each dot representing a peak from a single scan. In the low MW region, the dot/peaks (representing protein subunits) cluster around definite MWs. A relatively greater spread of the dots/peaks occurs in the medium MW region. However, in the high MW region, no dot/peak clustering occurs. Complementing the observations made of the gels, it was deduced from this graph that the clustering of dots/peaks occurs around a given MW when a band was present in most samples at the MW in question. If the MW measurements were 100% accurate and the same band were to appear in all the samples, differing only in intensity, then the dot graph (Fig. 2) would show this

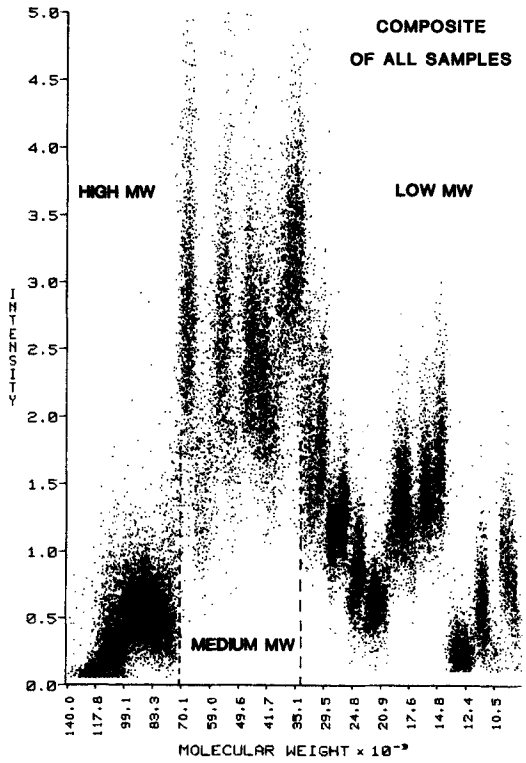

Fig. 2. Composite of all samples showing divisions for high (70,000 MW and above), medium (70,000 MW to 33,300 MW), and *low* MW regions (33,300 to 8,800 MW)

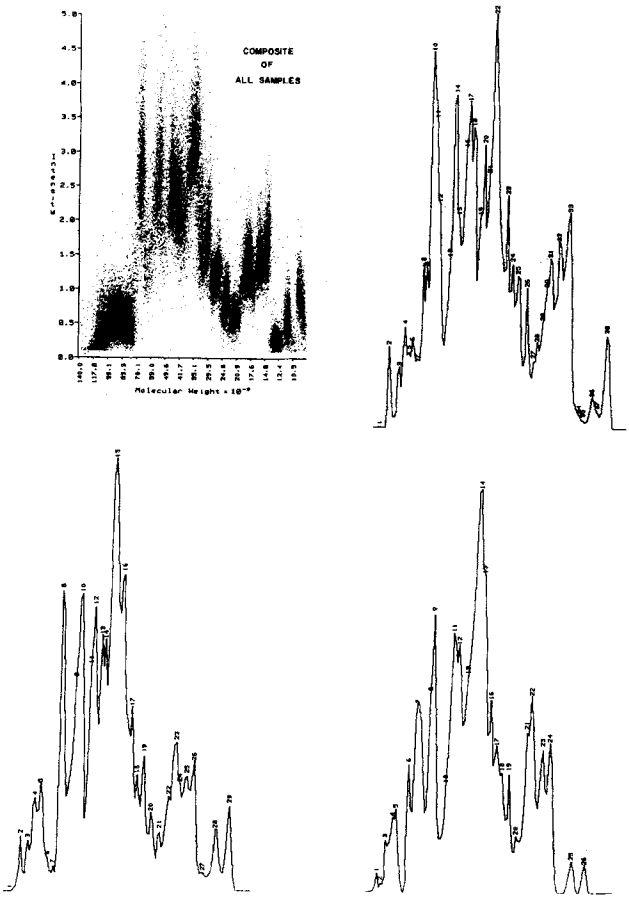

Fig. 3. Composite of all samples and three individual scans

as a line made up of dots at the corresponding MW. Given that SDS-PAGE MW calculations from gel to gel carry a certain error, a cluster rather than a line of dots appears around, rather than exactly at, a given MW. Figure 3 illustrates how the composite represents individual scans.

3 A final line of evidence is the computer-generated graphs of Figs. 4–6. These graphs were obtained by dividing the dot graph (Fig. 2) into the MW ranges that make up its clusters and the spaces between them which had fewer dot peaks (Fig. 7). A close examination of Figs. 4–6 reveals the heterogeneity in the make-up of each cluster, and hence the protein subunit variability in the 841 accessions of *T. dicoccoides* studied. In the low MW region (Fig. 4) one band predominates in most of the MW ranges. However, in the medium and high MW regions (Figs. 5 and 6) ranges almost always have a diverse composition.

As an example, the variation in protein composition in the 47,800 to 39,600 MW range differs markedly from that in the 8,800 to 10,000 MW range. In the 47,800 to 39,600 MW region 4% of the samples had one

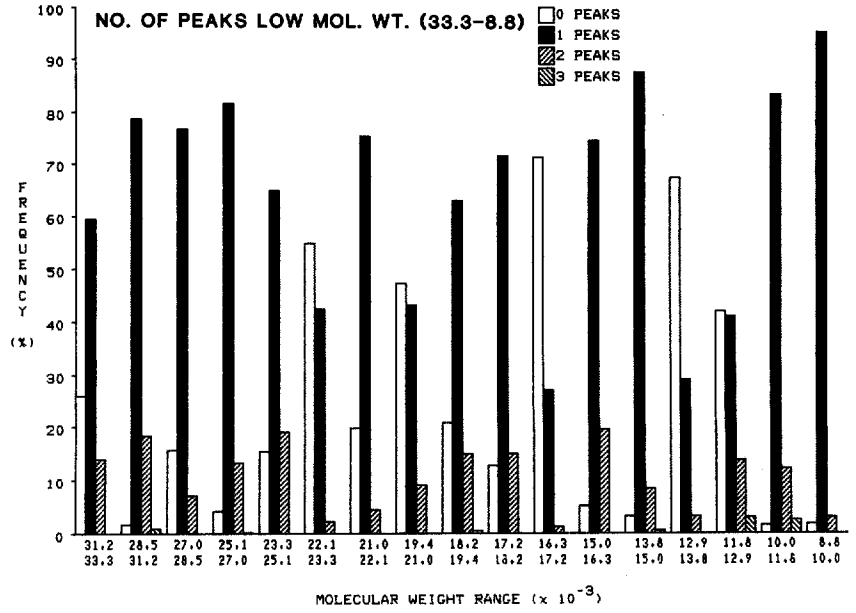

Fig. 4. Computer-generated graph of frequency (presence or absence) for peaks in the low MW region

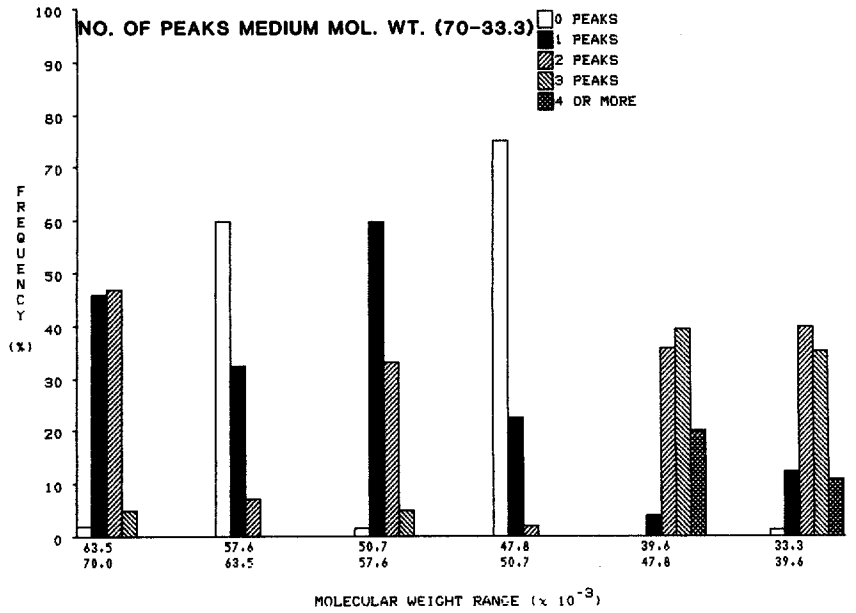

Fig. 5. Computer-generated graph of frequency (presence or absence) vs. MW for peaks in the medium MW region

peak in this region, 36% had two peaks, 40% had three peaks, 20% had four peaks or more in this range. In contrast, in the range between 8,800 and 9,800, 95% of the samples had one peak and only 2% of the samples had no peak. The less compact cluster in this higher MW range is thus more heterogeneous compared to the compact cluster between 8,800–9,800 MW. Consequently, these results indicate that the low MW region (33,300 and under) is highly conserved while extensive protein subunit variability is present in the medium (70,000 to 33,300 MW) and in the high MW region (140,000 to 70,000 MW).

Discussion

The data show that proteins of the low MW region of SDS-PAGE gels are the least variable, probably because this region is mostly composed of low MW albumin and globulins, many of which are essential enzymes or structural proteins. One would not expect such proteins to vary much in their size because a slight change in size could render them nonfunctional. However, it is entirely possible that greater genetic variation may be revealed for low MW soluble proteins using another electrophoretic system that detects protein on

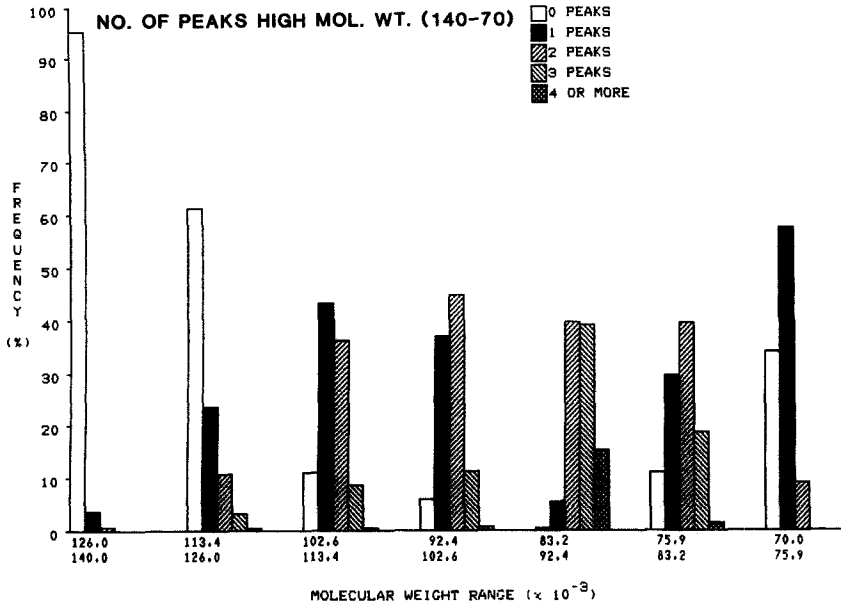

Fig. 6. Computer-generated graph of frequency (presence or absence of peaks) vs. MW range for peaks in the high MW region

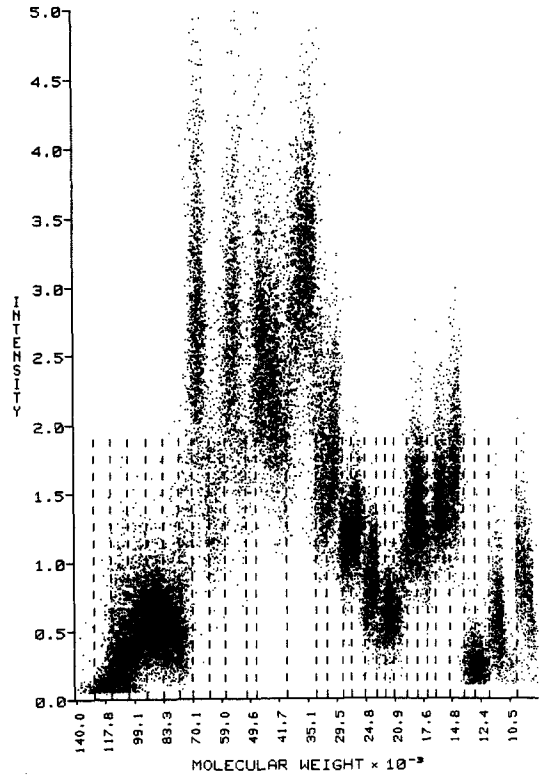

Fig. 7. Composite of all samples partitioned into individual clusters and spaces between clusters

the basis of charge or other property than MW. The work of Nevo et al. (1982) supports this hypothesis. They found considerable allozyme variation for soluble enzymes extracted from leaf tissue of seedlings of *T. dicoccoides* using nondenaturing starch gel electrophoresis. It also must be noted that SDS-PAGE of total protein probably masks part of the genetic variation existing in a population, because protein subunits of different pH and amino acid composition, but of the same MW, would migrate to the same place in the gel. Moreover, the relative amount of low MW proteins also is less compared to those of higher MW. These factors may explain the relatively little variation observed for the low MW subunits in this study.

In contrast, the medium and high MW regions of the total protein scans showed extensive protein subunit variability in their migration in SDS-PAGE gels. These are mostly storage proteins, i.e., gliadins and glutenins (Cole et al. 1981; Fullington et al. 1980). As the function of these proteins is to serve as a nutrient reserve for the young seedling, it is reasonable to speculate that variation in the size of these proteins would not be deleterious to the plant. Therefore, great structural variability can exist.

The nature of this variation is not yet certain, but a number of explanations have been offered. Lawrence and Shepherd (1980) suggested that variations in high MW glutenins, for bands which appear as alternatives of each other (as may be the case in some of the *T. dicoccoides* accessions), can be explained in simplest terms as due to alternative forms of the same allele. Another possibility is that post-translational cleavage of a protein precursor causes variation in size, provided that variation occurs on the location of the point of cleavage (Lawrence and Shepherd 1980). Regarding this point, Larkins and Hurkman (1978) presented evidence in maize that some of the storage proteins are synthesized as precursors which are later processed by proteolytic cleavage of a small sequence of amino acids. Recent work by Greene (1981) provides evidence that the same phenomena occur in wheat. He observed that gliadin polypeptides synthesized in vitro were larger than their in vivo counterparts. He suggested that the former may be precursors which, in vivo, undergo processing to yield the smaller final product.

Another explanation is that variation in protein subunit migration in SDS-PAGE may be due in part to variation from the assumed constant ratio of SDS binding to the proteins (1.4 g SDS/g protein). If considerable variation occurs from this theoretical value, then proteins of the same size can vary in mobility. Fortunately, Bietz and Wall (1972) found that gluten subunits (mostly glutenins and gliadins) bind SDS in proportions close to the theoretical value.

Phosphorylation, glycosylation or conjugation also are known to cause anomalous behavior of proteins in SDS-PAGE (Chrambach et al. 1976), so these types of modification must be taken into consideration as possibilities. However, the low MW region was so constant among the accessions analyzed that it was used as an internal control. Thus, it is certain that most of the protein subunit variability observed in the high and medium MW weight regions is due to genetic factors, with the possibilities that: 1) the presence or absence or the size of a carbohydrate moiety attached to a protein could have caused some of the variability and 2) although results indicated that the HMW region had the greatest protein subunit variability, it is possible that part of the variability may be more apparent than real. The relatively high concentration of acrylamide used (17%) tends to concentrate the HMW bands near the top of the gel, therefore, slight variability even within single gels relative to the MWs can result in appreciable variation in apparent mobility of bands with identical mobilities. This may have contributed to the apparent absence of peaks in the HMW region of the dot graph.

Unequal crossing-over of chromosomes containing repeated DNA sequences is another possible genetic factor accounting for the protein subunit variability. This event would give rise to allelic genes or gene complexes whose end products are polypeptides of varying length. The occurrence of repeated DNA sequences in wheat is well documented (Peacock et al. 1981). However it is not yet certain that such regions code for storage proteins.

The examples offered above can be considered as events that may have occurred in the early stages of diploid wheat progenitor evolution. Therefore, the variation in protein subunits observed for *T. dicoccoides* may be a product of differential diploid parent contribution to the formation of the tetraploid *T. dicoccoides*. Kasarda (personal communication) favors this idea. His view is that the variable contribution could have occurred either through multiple formation of tetraploids involving interactions of different A and B genome donors or through introgressive events at a later stage, mainly with the independently formed AB combinations. Zohary and Feldman (1962) explained genetic variability in morphology and other characters of the polyploid species of *Aegilops-Triticum* by suggesting that the formation of initial amphidiploids was followed by extensive hybridization between them.

On the other hand, ample time has elapsed for a major portion of the variability resulting from the recombinations of genes from polyphyletic origins to have evolved within *T. dicoccoides* as races become established in separated local environments. More research on protein molecular evolution and wheat genetics will be needed to ascertain the true nature of the variability observed in the protein subunits. Also, an extensive analysis needs to be made of the diploid progenitors, and comparisons made with those from locations nearby the sites of natural *T. dicoccoides* swarms. Moreover, if appreciable variation is observed in SDS-PAGE gels, which differentiate proteins solely on the basis of size and not charge, isoelectric point or amino acid sequence, then the variability observed in 2-D and other electrophoretic systems must be even greater. Future uses of our computerized data retrieval system will include: 1) selecting accessions which differ greatly in their protein composition to conduct two-dimensional electrophoresis studies; 2) selecting potentially valuable accessions for genetic analyses of known variation in *T. dicoccoides;* 3) studying correlations of the proteins with location and pathology data; 4) selecting those accessions with highest relative intensity in the low MW regions for their possible contribution of genes coding for proteins containing high percentage of essential dibasic amino acids; 5) selecting those accessions with high MW proteins of interest in breeding to improve bread processing quality. Efforts are now under way to select and exploit those accessions of potential value for breeding to increase the quantity and quality of proteins in high yielding modern wheats.

The breeding of bread wheat cultivars of high protein content by transferring genes from one *T. dicoccoides* accession is already well advanced (Grama et al. 1982).

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