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INTRODUCTION

At the time of the Second International Workshop on Gluten Proteins in 1984, reversed-phase high-performance liquid chromatography (RP-HPLC) of wheat gluten proteins (1) was a new method. Polypeptides were separated on microporous silica-based supports derivatized with hydrophobic ligands, resulting in high-resolution separations based on differences in surface hydrophobicity (2). RP-HPLC was shown to have significant advantages in speed, sensitivity, reproducibility, resolution, preparative capabilities, and ability to provide quantitative data. RP-HPLC is now used in many laboratories to analyze proteins of all cereal grains. The method is still being refined and optimized, however, and new applications are being found.

Since many wheat proteins influence or relate to functionality, a promising application of RP-HPLC is determination of quality. "Quality" may be any desirable or undesirable grain, flour, or product characteristic, such as loaf volume, elasticity, or hardness. This paper summarizes current efforts to analyze wheat quality by RP-HPLC, and discusses the future of the method.

RP-HPLC: PRINCIPLES AND METHODS

Derivatization of uniform 5-10 micron microporous (ca. 300 Angstroms) silica particles with hydrophobic ligands (usually alkyl groups) led to wide-pore RP-HPLC columns, to which proteins associate hydrophobically under relatively polar conditions. Solvents for protein RP-HPLC contain water, an organic component (usually acetonitrile), and an organic modifier (usually trifluoroacetic acid). As hydrophobicity increases by raising the percentage of nonpolar solvent, bound proteins selectively elute in order of increasing surface hydrophobicity. Columns prepared from such treated silicas...
combined with solvent delivery systems that generate reproducible gradients at accurate flow rates, an automatic sample injector, and a UV detector sensitive to absorbance of peptide bonds easily and rapidly generate a great deal of excellent data. RP-HPLC is especially useful because it separates proteins on the basis of surface hydrophobicity. This protein characteristic complements separations of most other chromatographic and electrophoretic methods that are based on size or charge. RP-HPLC also has the potential of relating the chemical nature of proteins to their interactions and functionality. Detailed descriptions of RP-HPLC methodology have been published (2-9).

PREVIOUS STUDIES

Early RP-HPLC studies achieved good separations of wheat albumins plus globulins, gliadin, and glutenin subunits (2,4,5). Analysis of total protein extracts shows that each major protein class has distinct characteristics, eluting in the order of omega gliadins, high-molecular weight (MW) glutenin subunits, low-MW alpha and beta gliadins, low-MW ethanol-soluble glutenin subunits, and gamma gliadins (10). RP-HPLC was also applied to varietal identification (4,11).

IMPROVEMENTS IN RP-HPLC METHODS

RP-HPLC is still evolving and improving. Higher column temperature significantly enhances resolution, presumably by disrupting the hydrogen bonds that cause proteins to aggregate, even while remaining soluble (6), and by permitting more rapid movement of proteins through the porous silica. Newer columns also may exhibit useful differences in selectivity. In addition, resolution is usually better than that of earlier studies (2,4): 40-70 peaks and shoulders now frequently result from fractionation of whole gliadin (Fig. 1). This is about twice that of early studies. Faster separations are now also possible (6). Without doubt, further improvements will occur: for example, good separations of non-cereal proteins on small diameter columns may take less than 1 min.

RP-HPLC procedures are also still being optimized. Huebner and Bietz (12) discussed factors that may affect HPLC results and prediction of quality. Baselines were improved by varying TFA concentrations of solvents used to generate gradients. Gliadins may be extracted at a lower ethanol concentration, enhancing binding of large samples to columns for preparative RP-HPLC. For long-term storage, low temperature improves sample stability. Resolution of high-MW glutenin subunits differs with state of reduction and alkylation, and with different denaturants or detergents. For example, inclusion of SDS in RP-HPLC solvents (2) is possible, and may improve resolution of cereal proteins. SDS maintains protein solubility, prevents protein aggregation, and may modify the column, affecting selectivity and resolution.

QUALITY PREDICTION BY HPLC

Several studies have already demonstrated that RP-HPLC can predict wheat
FIGURE 1. RP-HPLC separations of Coteau gliadin on Vydac C18 and C4 columns, using the same gradient conditions.

Quality. One way that this is possible is varietal identification by analysis of gliadin fingerprints (4,11). Knowing the identity of a variety reveals much about its end-use characteristics. Sozinov and Poperelya (13) demonstrated correlations between gliadin blocks and quality. We also found that cultivars of all wheat classes were differentiated by RP-HPLC analysis of gliadins. In some cases, the method may be preferable to polyacrylamide gel electrophoresis (PAGE), since it has good resolution, is rapid and automatic, and permits good quantitation. Since it separates proteins by a complementary method, it may also differentiate some genotypes that appear identical upon PAGE.

Quality may also relate to the presence or absence of specific proteins, as first shown for RP-HPLC by analysis of durum wheats (14). All varieties had one of two patterns, differing in peaks corresponding to gamma-gliadins 42 and 45, which relate to poor or good pasta quality, respectively. A rapid RP-HPLC test for these polypeptides was developed, and may be useful to screen for quality potential early during breeding.

Wheat quality can also be inferred by analysis of glutenin subunits (5). Glutenin's unique high-MW subunits, which differ among classes and varieties, relate to the potential for desirable functional properties (15). This relationship was extensively investigated by Payne et al. (16), and proved to be valid. These high-MW subunits also differ significantly in RP-HPLC
elution characteristics (5), making them useful for identification.

Early studies using size-exclusion HPLC (17) demonstrated that quality also relates to size distributions of unreduced wheat proteins. Later studies, however, suggested caution with this method (18), because native glutenin is difficult to extract reproducibly and contains many species varying in MW and solubility. Glutenin also tends to aggregate, even in the presence of SDS; it is thus important to analyze samples rapidly, or stabilize them before analysis. Nevertheless, size-exclusion HPLC has significant potential as a rapid test of breadmaking quality.

Size-exclusion HPLC can also indicate quality potential by analyzing the ratio of high- to low-MW reduced glutenin subunits (18). This relationship is also apparent upon RP-HPLC, because high-MW subunits characteristically elute first (5). Among varieties differing in mixing time, loaf volume, baking quality, and general score, the percentage of high-MW glutenin subunits correlates highly with general score and mixing time.

Thus, quantitative relationships among unreduced wheat proteins or their subunits may correlate significantly with breadmaking quality. Through analysis of these proteins, HPLC provides ways to rapidly and reproducibly estimate quality of samples for processing or during marketing.

NEW QUALITY FACTORS DETECTED BY RP-HPLC

Many of these studies confirm previously-known relationships of wheat quality to specific proteins. In some cases, however, RP-HPLC demonstrates new relationships. For example, analysis of wheats varying in breadmaking quality suggested that the amount of a late eluting gliadin fraction in hard red spring wheats correlates inversely and significantly with general baking (breadmaking) scores. This "baking quality gliadin factor", if consistently applicable, could be a useful early indicator of baking quality during breeding.

Samples of French wheats recently analyzed further support the concept that specific gliadins related to breadmaking quality can be measured by RP-HPLC (Fig. 2). For these wheats, the amount of these specific peaks (eluting between 50 and 60 min) is high in a poor wheat, and nearly zero in a strong wheat. Within these fractions, only specific proteins may relate to quality. Specific gliadins related to quality do occur in bread wheats (13, 19-20), as in durums (21). Such relationships may be indirect, however, if genes coding specific proteins are closely linked to other genes which directly influence quality.

VARIETAL AND BIOTYPE ANALYSIS BY RP-HPLC

RP-HPLC also provides insights into wheat biotypes and their relationships. For example, single-kernel analyses of many varieties reveals more than one fingerprint (4,6). Such observations can be extremely important if a variety is important in breeding; for example, Nap Hal has both high protein and high lysine potential. Varietal identification may also be necessary during marketing, or for quality control. Also, if protein fingerprinting is used to predict quality, RP-HPLC may help assure constancy of genotype for a variety.
FIGURE 2. RP-HPLC separations of strong and poor French wheat varieties, demonstrating quantitative differences in late eluting components which correlate with strength.

RP-HPLC and PAGE can both analyze biotypes (22). When 100 kernels of the variety 'Newton' were characterized, PAGE revealed differences among slow-moving omega gliadins, while HPLC revealed differences among early-eluting components. The methods agreed completely in dividing the kernels into two distinct sets of biotypes. Components of interest were also easily isolated by HPLC.

RP-HPLC can provide new insights into wheat biotypes and purity. Figure 3 shows results for some French wheat varieties. The durum cultivar Durox has two biotypes, which differ slightly upon PAGE. Durox A has a unique electrophoresis pattern, but Durox B is identical to the variety Tomclair, which has poor quality.

Half-kernels of Durox were characterized by J.-C. Autran by PAGE; the other halves were analyzed by RP-HPLC (Fig. 3). Upon HPLC, all kernels of the Durox-B biotype were identical; also, all Durox A biotypes were similar. Biotypes A and B differed from each other, however, both quantitatively and by a peak at about 29 min, which may correspond to differences apparent upon PAGE. Among the A biotype kernels, however, one (Durox A2) displayed a significant quantitative difference from the predominant Durox A type (Durox A1) at about 24 min. This difference was not apparent by PAGE, and may be an additional biotype or exemplify extreme quantitative variation within a variety. It is most significant that Durox B and Tomclair, which appear the same by PAGE, differ upon HPLC. Qualitative differences occur at 29 and 35
FIGURE 3. RP-HPLC analysis of gliadins from the French wheat varieties Tomclair, of the two major biotypes (A1 and B) of the variety Durox, and of a kernel exhibiting an atypical Durox A genotype (A2).

Thus, these genotypes are easily differentiated by HPLC, but not by PAGE.

ANALYSIS OF HIGH-PROTEIN WHEATS

RP-HPLC may also be well-suited for identifying useful proteins for subsequent incorporation into bread wheats, as seen in analysis of Triticum dicoccoides gliadins (Fig. 4). *Tr. dicoccoides* is a wild species containing 14-28% protein; breeders hope to incorporate this characteristic into commercial bread or durum wheats. Figure 4 shows RP-HPLC analyses of gliadins of Langdon durum, of *Tr. dicoccoides*, and of substitution lines, developed by L. Joppa, in which *dicoccoides* chromosomes substitute for durum chromosomes. The presence or absence of specific peaks in the substitution lines, compared to those of Langdon and *dicoccoides*, reveals chromosomal locations of genes coding major gliadins. These results confirm knowledge of gliadin coding in durum wheat (10), and show that many *Tr. dicoccoides*
FIGURE 4. RP-HPLC separations of gliadins extracted from Langdon durum, Tr. dicoccoides, and the disomic 1B, 1A, and 6A substitution lines of Tr. dicoccoides chromosomes into durum. Chromosomal control of major durum and dicoccoides peaks, inferred from significant differences (*) in peaks in the substitution lines, is shown.

Gliadins are also coded by genes on chromosomes 1A and 1B. One major 1B-coded peak in dicoccoides is similar in elution time to gamma-gliadin 45, present in strong durum varieties; dicoccoides lacks a component similar to gliadin 42. These results suggest that 1B-coded gliadins (or closely linked glutenin polypeptides) may partially explain the high protein content of Tr. dicoccoides.

We also examined glutenin subunits of durum/dicoccoides substitution lines (Fig. 5). Langdon durum has two high-MW subunits (coded by 1B) (23), while dicoccoides has four high-MW subunits. Analysis of the substitution
FIGURE 5. RP-HPLC analysis (early portions of chromatograms) of high-molecular weight glutenin subunits of Langdon durum, *Tr. dicoccoides*, and disomic substitution lines of *Tr. dicoccoides* LA and LB chromosomes into Langdon durum.

lines revealed that the two minor subunits of *dicoccoides* are controlled by genes on chromosome LA. High-MW subunits of the LB substitution line exhibit slight but significant differences in retention times from the high-MW subunits of Langdon durum. These subunits from *Tr. dicoccoides* may be allelic variants of those from Langdon durum, but may more closely correlate with quality or high protein content.

RELATION OF HIGH-MW GLUTENIN SUBUNITS TO QUALITY

When glutenin's high-MW subunits were first characterized by SDS-PAGE (15,24), their unique chemical properties, combined with coding (of some) by D genome chromosomes and presence only in glutenin, wheat's "strength" protein, suggested that high-MW glutenin subunits are related to quality. Payne et al. later showed that allelic variation at the Glu-1 loci does influence quality (16), and suggested standard varieties which exhibit all known allelic variation (25).

Glutenin's high-MW subunits can also be clearly visualized and identified by RP-HPLC (5). These subunits, being larger and less hydrophobic, elute earlier than lower-MW, ethanol-soluble subunits (Fig. 6). We have now used
RP-HPLC to examine standard varieties which exhibit allelic variation for high-MW glutenin subunits (Fig. 7). Differences between allelic gene products were revealed; relationships are especially apparent in terms of absolute elution times. Thus, RP-HPLC can be an excellent alternative to SDS-PAGE for identifying genotypes having high-MW subunits associated with quality. HPLC may also be faster for such analyses. One drawback to the use of HPLC is that glutenin sample extraction and preparation is somewhat laborious. Better methods are being devised for glutenin isolation and genotype analysis by RP-HPLC, which should make RP-HPLC analysis of glutenin increasingly valuable for breeding and marketing.

COMPUTER EVALUATION OF RP-HPLC DATA

HPLC rapidly generates too much data to fully evaluate visually and empirically. Computer analysis of RP-HPLC data is therefore becoming as important as the separations themselves. Computer-assisted data analysis may identify quality related proteins, which can then be isolated and characterized. Rapid analytical procedures may result, and information may help in varietal improvement. Computers should also be able to identify varieties automatically through comparison of data to that of stored standards. The procedure may be simpler than automated varietal identification through electrophoresis, since variability of HPLC retention times should be less.
FIGURE 7. RP-HPLC analyses of high-molecular weight (early eluting) subunits of glutenin from varieties exhibiting most known allelic variation (25) at the Glu-1 locus.

HPLC also reveals quantitative relationships not obvious by electrophoresis. This has many advantages, and new types of data interpretation become possible. However, such information could complicate varietal identification. For example, RP-HPLC is revealing that there is sometimes relatively significant quantitative variation among proteins within a variety. For example, Fig. 8 shows significant quantitative variation among gliadins from a variety grown on soils differing in sulfur level. Differences occur in relative amounts of early eluting omega-gliadins, which lack sulfur, as compared to later-eluting alpha-, beta-, and gamma-gliadins, which contain sulfur amino acids.

A variety grown across a normal range of environments may also exhibit significant quantitative differences in proteins upon RP-HPLC (Fig. 9), resulting from soil or environmental factors. Thus, gliadin fingerprints are not as constant as generally believed, even though genotypes are qualitatively similar upon HPLC. Accurately defining the normal quantitative variation for individual proteins within genotypes should improve the
FIGURE 8. RP-HPLC analysis of gliadins from the wheat variety Egret, grown under conditions of low, medium, and high soil sulfur.

The objectivity of varietal identification. Such knowledge should also lead to a better understanding of protein synthesis, accumulation, and regulation, and may suggest opportunities to improve protein composition.

ANALYSIS OF MIXTURES AND HYBRIDS

Computers may also help evaluate wheat quality through analysis of proteins extracted from mixtures and hybrids. The computer program "Chromatographic Comparison Plot" (CHROCP) permits addition and subtraction of chromatograms in any ratio. As illustrated previously (8), a chromatogram of a known or probable component, when subtracted from that of a mixture,
FIGURE 9. RP-HPLC comparison of gliadin from Butte wheat grown at Williston and Langdon, ND. The difference plot, generated by the computer program CHROCP, emphasizes quantitative differences in protein composition at the two test sites.

yields a pattern characteristic of the other component, and reveals the amount of each component. Such analyses could be valuable during marketing.

RP-HPLC may also useful for determining pedigrees of wheat hybrids. Since endosperm proteins of a hybrid represent a 2/1 contribution of the maternal and paternal parents, adding chromatograms of any two varieties, in an appropriate ratio, gives a computer-derived chromatogram nearly identical to that of the genuine hybrid (Fig. 10). These results confirm that there is little regulation of expression of gliadins. Any differences from expected patterns offer important clues concerning protein synthesis, composition, and regulation.

In the reverse experiment (data not shown), a chromatogram of a known or suspected parent can be subtracted from that of a hybrid. The resulting
FIGURE 10. Computer analysis, using program CHROCP, of a wheat hybrid. RP-HPLC patterns of gliadins from the varieties Yolo and Sunbird, added in a 2:1 ratio, give a computer-derived chromatogram similar to that of the actual hybrid. Computer-derived pattern closely resembles the other parent. Such analyses may be useful to register hybrids, and to trace pedigrees. It may also be possible for RP-HPLC to estimate hybrid purity by comparing data for mixtures or bulk samples to that of pure samples.
FIGURE 11. Gaussian deconvolution of RP-HPLC data. Gliadin from the variety Coteau was fractionated on a C4 column. Stored chromatographic data for early eluting minor components were then analyzed by computer program GAUSDC.

GAUSSIAN DECONVOLUTION OF RP-HPLC DATA

RP-HPLC data often contain much more information than is readily apparent. Computers can further analyze such data. Expansion of a relatively minor portion of a typical chromatogram (Fig. 11, top left) reveals additional components. Gaussian curve deconvolution may quantitate these components, and provide additional information. The operator first estimates the number of components, elution positions, and approximate heights. Approximate peak widths and baseline are specified. The computer then performs a reiterative process to match the sum of individual peaks to the original curve (Fig. 11, right). It then reports areas for each peak (Fig. 11, lower left). Resulting peak widths, indicated by standard deviations, may further indicate peak purity. For example, since peak 1 (at 12.51 min) is considerably wider than other peaks, it probably contains two or more components.

COMPUTER IDENTIFICATION OF QUALITY-RELATED PROTEINS

Until now, visual analysis has been used to reveal most relationships of proteins to quality. We need more objective ways to identify non-obvious
relationships of HPLC data to quality. Also, as amount of data increases, adequate visual evaluation becomes impossible. An example of how computers might identify additional quality-related proteins was presented previously (12). Statistical analysis revealed a high correlation between (a) areas or heights of peaks known to be associated with quality and (b) independently-measured quality characteristics. More advanced statistical techniques may identify groups of proteins which affect quality, or indicate gene linkages. Identification of such proteins will make it possible to isolate and characterize them, and determine how they influence quality. It should then be possible to use such information in breeding improved varieties, in screening germplasm populations, or for quality control. Such knowledge may also give insights about how protein synthesis, expression, and regulation affect yield and performance.

CONCLUSIONS

RP-HPLC of proteins is still a recent and rapidly developing method, but is already widely used for many applications. Its use is certain to increase. New applications of RP-HPLC to analysis of wheat proteins have already been described; computer evaluation of data may be especially valuable in giving new insights between protein composition and quality. Since RP-HPLC separates proteins on the basis of surface hydrophobicity, results may potentially relate to processing and product characteristics (26). RP-HPLC will find wide use for optimization of processing, for quality assurance, and in marketing, and will become increasingly valuable to the cereal chemist.

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