Separation of Water-Soluble Proteins from Cereals by High-Performance Capillary Electrophoresis (HPCE)

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ABSTRACT

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Most research concerning grain proteins has concentrated on the gluten storage proteins. The albumins and globulins are the water- and salt-soluble proteins that contain biologically active enzymes and enzyme inhibitors. A free-zone capillary electrophoresis method was developed to separate these proteins. Optimization included sample extraction method, capillary temperature, buffer composition, and additives. The optimal conditions for separation of these proteins was 50 µm i.d. × 27 cm (20 cm to detector) capillary at 10 kV (with a 0.17 min ramp-up time) and 25°C. The optimum buffer was 50 m*M* sodium phosphate, pH 2.5 + 20% acetonitrile (v/v) (ACN) + 0.05% (w/v) hydroxypropylmethyl-cellulose (HPMC) + 50 m*M* hexane sulfonic acid (HSA). Sample stability was an issue that was addressed by lyophilizing fresh extracts and redissolving in aqueous 50% ethylene glycol and 10% separation buffer. This method was successfully used in both wheat flour and whole meal samples. Comparisons were made of several wheats of different classes as well as several cereal grains. This methodology could be useful in screening cereal grains for important enzymes and their impact on end-use quality such as food functionality, food coloration, and malting quality.

Considerable research is conducted to develop methods for separating food proteins by high-performance capillary electrophoresis (Bean et al 1998; Bean and Lookhart 2001a; Frazier 2001; Reico et al 2001). HPCE has been used to check for adulteration, to explore protein-food quality relationships, and to identify food sources and ingredients based on protein profiles (Gallardo et al 1995; Chou et al 1998; Alam et al 2000). HPCE has been used to successfully separate meat, dairy, and cereal proteins from a wide variety of materials (Bean and Lookhart 2001a; Reico et al 2001).

Several methods have been reported for the separation of cereal proteins by HPCE (Bean et al 1998; Bean and Lookhart 2001a). Most of these methods have focused on separating the storage proteins of cereal grains. The primary purpose of storage proteins is to store nitrogen for use during growth of the emergent plants. Storage proteins are important sources of nutrition and have many important functional roles in foods, for example the role of wheat storage proteins, gluten proteins, in breadmaking. HPCE separations of cereal storage proteins have been used to fingerprint cereal cultivars and to study the role of storage proteins in food and feed functionality (Bean et al 1998; Bean and Lookhart 2001a).

Although functionally important, storage proteins are not the only class of proteins found in grain kernels. Classical fractionation divides cereal kernel proteins into four broad solubility classes: 1) the water-soluble or albumin proteins; 2) the salt-soluble or globulin proteins; 3) the alcohol-soluble or prolamins; and 4) the acid- or base-soluble the glutelins (Osborne 1907). In most cases, the latter two classes are the storage proteins, while the albumin and globulin proteins are generally considered to be metabolic or structural proteins (Wrigley and Bietz 1987). In certain cases, some albumin and globulin proteins may also be storage proteins (Shewry et al 1995).

The albumin and globulin fractions represent ≈15% of the flour protein. The proteins present in these groups are nutritionally significant due to higher content of the essential amino acids lysine and methionine as compared with the gluten proteins. The albumins and globulins are functionally diverse as many of the albumin-globulin proteins are enzymes or enzyme inhibitors and thus have several functions that relate to plant germination and are involved in quality parameters. The majority of albumin-globulin proteins are α-amylase inhibitors, including the CM family of inhibitors that are present mainly in the globulin fraction. Although, in general, albumin proteins exhibit a low degree of polymorphism, several groups have demonstrated the utility of isoelectric focusing analysis of distinct albumin proteins as biochemical markers for varietal identification (Singh et al 2001).

Recent work by Singh et al (2001) using antibodies, HPLC, N-terminal, and internal amino acid sequencing has identified several main components in the water- and salt-soluble protein fractions from the wheat cultivar Chinese Spring. Additional components identified in this fraction were α-amylase, β-amylase, lipid transfer protein, and serine carboxypeptidase III. Several proteins were not identified due to blocked N-termini.

The water-soluble fraction may also contain important oxidative enzymes such as polyphenol oxidase (PPO) and peroxidase, which can be involved in discoloration of food products, especially whole grain products, as PPO is located mainly in the bran. Interestingly, a number of these proteins have been identified as allergens that contribute to baker's asthma (Sanchez-Monge et al 1992; Sanchez-Monge et al 1997; Weiss et al 1997). Functionally, the amylase and amylase inhibitors in these fractions are important in the breakdown of the starch and their respective amounts may have roles in preharvest sprouting damage of cereal grains such as wheat and sorghum (Murty et al 1984; Henry et al 1992; Dicko et al 2002). Levels of both PPO and peroxidase enzymes have been linked to food quality of sorghum lines (Dicko et al 2002).

While considerable work has been done developing methods for separating cereal storage proteins, very little research has focused on methods for separating the albumin and globulin proteins of cereals. Lookhart and Bean (1995a) identified these proteins extracted from wheat in early free-zone capillary electrophoresis (FZCE) separations under separation conditions optimized for separating storage proteins. Capelli et al (1998) theorized that the albumin and globulin proteins may have a higher tendency to bind to the inner walls of fused silica capillaries due to higher amounts of basic amino acids. Subsequent work by Bean and Lookhart (1998) showed that better reproducibility of storage protein separations could be obtained if the albumin and globulin proteins were removed from the storage protein extracts. It is also possible that other compounds, such as free amino acids, DNA, carbohydrates that are co-extracted with the albumin, and globulin proteins interfere with the separations of the storage proteins. Indeed, direct extracts of ground grain samples, particularly whole meal, should be regarded as "dirty" samples (Bean and Lookhart 2001b).

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Because of the issues noted above in analyzing the albumin and globulin proteins by FZCE, this project focused on developing methods to produce high-resolution, reproducible separations of the water-soluble proteins in cereal grains. A reliable FZCE method for separating water-soluble proteins of cereals would allow routine characterization and screening of these proteins to determine their role in food and feed quality.

MATERIALS AND METHODS

High-Performance Capillary Electrophoresis

A Beckman P/ACE 2050 with 50 μm i.d. × 27 cm (20 cm to detector) fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) was used for all separations in this study. Optimum separation conditions were 10 kV (with a 0.17-min ramp-up time) and 25°C. These conditions were used unless conditions were varied for optimization procedures. Samples were injected for 2 sec at 20 psi and were detected by UV at 200 nm. Capillary ends were cut with a diamond wheel cutter (Bean and Lookhart 2001b).

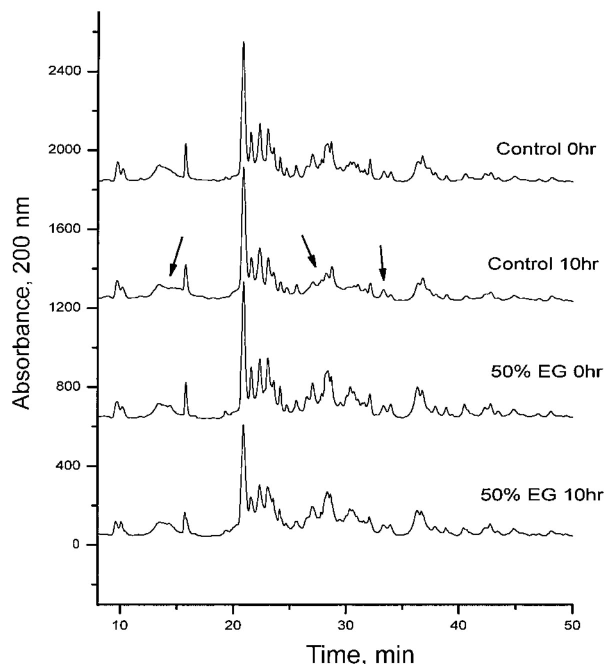

Fig. 1. RP-HPLC chromatogram of WS proteins of wheat (from whole meal) with and without (control) the addition of 50% EG at 0 and 10 hr of incubation at room temperature. Arrows point to areas that exhibit significant change over the time period stabilized by addition of EG.

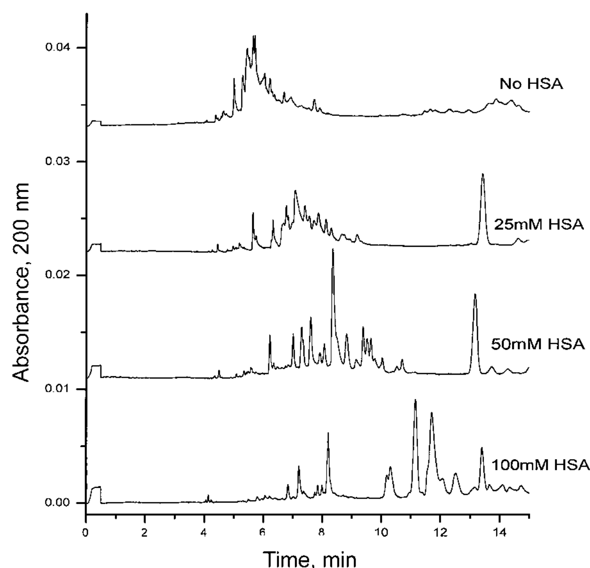

Fig. 2. Effects of increasing concentrations of HSA in separation buffer on WS protein separations. Separation buffer was 50 m*M* sodium phosphate, pH 2.5 + 20% ACN (v/v) + 0.05% HPMC (w/v) at 10 kV and 25°C.

The optimum buffer was 50 m*M* sodium phosphate, pH 2.5 + 20% acetonitrile (v/v) (ACN) + 0.05% (w/v) hydroxypropylmethylcellulose (HPMC) + 50 m*M* hexane sulfonic acid (HSA). This buffer provided the optimum separations; other conditions were used during the optimization process as noted in above.

Before each data set, on both old and new capillaries, capillaries were rinsed with separation buffer for 15 min followed by preconditioning at 20 kV (740 V/cm) for 5 min (Ross 1995). Between each separation, capillaries were rinsed with separation buffer for 2 min.

Reversed-Phase HPLC

Separations were made using an Agilent 1100 HPLC system with a Zorbax SB300 C8 column. Separation conditions were as described by Singh and Skerritt (2001), except flow rate was 0.5 mL/min.

Sample Preparation

Water-soluble (WS) proteins were extracted from 250 mg of flour or ground whole meal with 1 mL of deionized water for 5 min with continual vortexing for 5 min (Bean and Lookhart 1998). Samples were centrifuged and an aliquot of 250 μL was removed. The extraction was then repeated and a second aliquot of 250 μL was removed. Pooled aliqouts were then lyophilized to dryness. Lyophilized samples were redissolved in 500 μL of 50% ethylene glycol + 10% of the optimum separation buffer described above. In some cases, protein samples were not lyophilized and were mixed 1:1 with a solution of 50% ethylene glycol and 10% CE buffer before analysis.

Salt-soluble (SS) proteins were extracted from flour or ground whole meal after WS proteins were extracted as described above. A tris-HCl buffer at pH 7.8 containing 50 m*M* KCl and 5 m*M* ethylenediaminetetraacetic acid (EDTA) was used as solvent (Bean and Lookhart 1998). Two 5-min extracts were made and the individual extractions were pooled 1:1 as described for the WS proteins. SS proteins were also lyophilized and redissolved as described for the WS proteins.

RESULTS AND DISCUSSION

Sample Stability

During initial attempts to develop a FZCE method for separating WS proteins of wheat, the protein profiles were not stable and changed over time. Before a FZCE method could be successfully developed, it was necessary to stabilize the WS extracts. Water-soluble proteins of cereals are generally considered to be metabolic proteins (enzymes). Thus, when extracted with water, these enzymes could potentially become active, which may have been one cause of the instability of the WS protein samples.

It was also noted that samples extracted from flour rather than whole ground meal were more stable. During the milling of flour, the outer layers (the bran) are removed. Many of the WS and SS proteins are concentrated in the outer layer of the kernels (Wrigley and Bietz 1987) as well as material such as cell wall remnants, sugars, etc., which could contribute to sample instability.

Several different methods were attempted to stabilize the WS extracts by denaturing the proteins, thus rendering any enzymes inactive. Samples were mixed after extraction with a number of different solvents including ACN, 1-propanol, 8*M* urea, ethylene glycol (EG), ethanol, dimethyl sufloxide (DMSO), and a protease inhibitor cocktail (Sigma P-9599). Heating samples (100°C for 5 min to render enzymes inactive) as well as mixing samples 1:1 with a pH 2.5 buffer and a pH 10.0 buffer, respectively, were also attempted. Sample stability was analyzed by separating samples

repeatedly by RP-HPLC over a 15-hr period. The most effective treatment was mixing the sample 1:1 with EG (final concentration 50% EG), mixing samples 1:1 with 8*M* urea or 1:1 with DMSO. Examples of the results obtained when mixing a sample 1:1 with EG are shown in Fig. 1. Changes can be seen in the untreated samples, but the sample profiles remained constant in the samples containing 50% EG for ≈15 hr. Slight differences between the control extract (no additive) and the samples containing 50% EG were noted (see peaks at 35–38 min), however, RP-HPLC patterns were constant in the 50% EG samples for at least 15 hr, after which small changes were noted in the profiles (data not shown). Though slightly different from the control patterns, the patterns were stabilized by the addition of 50% EG, which was the goal of this experiment. Samples mixed with urea and DMSO also showed good stability. However, the use of EG in sample buffers for FZCE improved the resolution and reproducibility of protein mixtures separated by FZCE (Gordon et al 1991), which is an advantage of using EG over urea or DMSO. Furthermore, urea can modify proteins under certain conditions, making it unfavorable for use. Heated samples also showed improved stability. However, a precipitate was formed during heating and HPLC profiles of heated samples differed from those of the control, suggesting changes to the proteins during heating (data not shown). Also, lyophilized samples were more stable than samples analyzed directly after extraction (data not shown).

The recommended sample preparation for analysis of WS and SS proteins is to lyophilize samples after extraction and redissolve the samples in a mixture of 50% EG + 10% separation buffer. The need for the addition of separation buffer is explained below. Samples that were not lyophilized could be mixed 1:1 with 50% EG + 10% separation buffer and successfully analyzed. However, as noted above, unlyophilized samples were not as stable, and the need to add the EG solution diluted the sample, thereby reducing sensitivity. Lyophilzed and nonlyophilized samples did give the same electropherograms when separated by CE, however. This indicated that all of the proteins in the lyophilized sample were resolubilized (data not shown).

Optimization

The majority of methods developed to separate cereal proteins have used low pH buffers with one or more additives to improve protein solubility and reduce protein-capillary wall interactions (Bean et al 1998). Thus, the initial buffer used to separate cereal WS proteins was a sodium phosphate buffer at pH 2.5. HPMC (0.05%) was used as a dynamic coating to reduce protein-capillary wall interaction (Bean and Lookhart 1998). Several buffer concentrations at 25–100 m*M* were tested; 50 m*M* was optimum (data not shown).

Several additives were tested to improve the resolution of WS proteins. Organic modifiers such as ACN have improved the separation of cereal storage proteins (Bean and Lookhart 1998). Because of this, a range of solvents (MeOH, EtOH, 1-PrOH, EG, and ACN) were tested at concentrations of 5–20%. As for cereal storage proteins (Lookhart and Bean 1996; Bean and Lookhart 2000), 20% ACN provided the best resolution of the WS proteins (data not shown).

Next, spermine, hexane sulfonic acid (HSA), and lauryl sulfobetaine were tested for their effect on the separation of WS proteins. Spermine has been effective in improving protein separations and preventing protein-capillary wall interactions (Verzola et al 2000); lauryl-sulfobetaine has improved the resolution of cereal storage proteins and prevented protein-capillary wall interactions (Lookhart and Bean 1996); and HSA has improved resolution of protein separations (Moring and Nolan 1990). Unlike previous work with wheat storage proteins (Lookhart and Bean 1996), HSA was the most effective additive for WS proteins. Figure 2 shows the impact of increasing concentrations of HSA. The optimum concentration was 50 m*M*.

The impact of the buffer ion type was also investigated. There is an inverse relationship between buffer conductivity and analyte mobility in FZCE. Therefore, using buffer ions with low conductivity should result in faster separations (Hjerten et al 1995). Lower conductive buffers also generate less current during a separation, resulting in less joule heating (Reijenga et al 1996). Bean

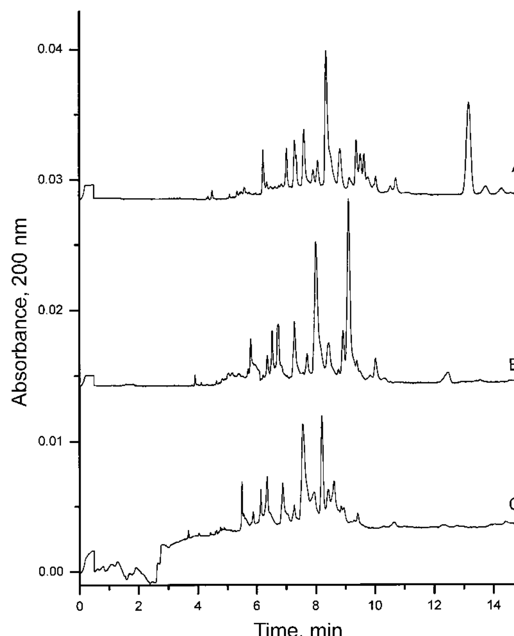

Fig. 3. Effects of buffer type on separation of WS proteins of wheat. A, 50 m*M* sodium phosphate, pH 2.5; B, 50 m*M* phosphate-glycine, pH 2.5; and C, 50 m*M* iminodiacetic acid, pH 2.7. All buffers contained 0.05% HPMC and 20% ACN. Separation conditions as in Fig. 2.

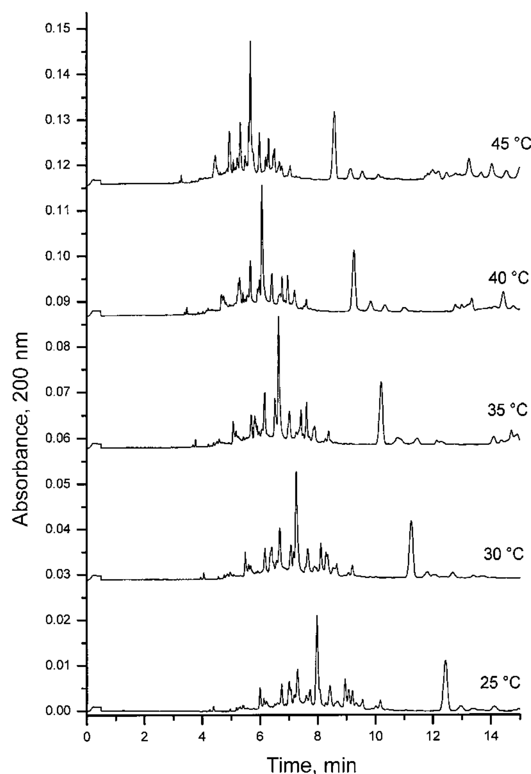

Fig. 4. Effects of capillary temperature on separation of WS proteins from wheat. Separation buffer was 50 m*M* sodium phosphate, pH 2.5 + 20% ACN (v/v) + 0.05% (w/v) HPMC, 50 m*M* HSA. Separation conditions as in Fig. 2.

and Lookhart (1998) demonstrated that replacing the high-conductive sodium phosphate with lower conductive glycine in FZCE buffers resulted in faster separations of cereal storage proteins with minimal loss in resolution. The isoelectric buffer iminodiacetic acid (IDA), which is a special type of very-low-conductive buffer, has also been used to produce rapid separations of peptides (Bossi and Righetti 1997) and cereal storage proteins (Bean and Lookhart 2000).

Because of the potential for low-conductive and isoelectric buffers to reduce separation times, both a 50 m*M* phosphate-glycine buffer (pH 2.5) and a 50 m*M* IDA buffer (pH 2.7) containing 20% ACN and 50 m*M* HSA were compared with a 50 m*M* sodium phosphate buffer (pH 2.5) containing the same additives (Fig. 3). Phosphate-glycine and IDA buffers produced slightly faster separations than the sodium phosphate buffer. However, the sodium phosphate buffer produced higher resolution separations, particularly at 9–10 min. Considerable differences in selectivity were noted between the different buffer types that could potentially be exploited to optimize separations for a particular protein. The sodium phosphate buffer was chosen as optimum for this application, even though the separation time was somewhat longer than the other buffers.

Finally, separation temperature and voltage were optimized. The best temperature was 25°C (Fig. 4) with the optimum voltage at 10 kV (data not shown). These separation conditions (i.e., low temperature, Fig. 4) were different from the optimum conditions reported for the separation of cereal storage proteins using a similar sodium phosphate buffer (Lookhart and Bean 1995b; Lookhart and Bean 1996). This may be due to solubility; that is, the storage proteins required higher temperatures during separation to help maintain solubility. Note that low levels of some proteins were observed in the wheat extracts that migrated much later than the majority of the WS and SS proteins (data not shown). These proteins migrated in the time frame where gliadins typically migrate (Bean et al 1998) and were therefore considered to be gliadin or gliadin precursors, both of which can be extracted with water (Singh and Skerrit 2001), though this remains to be verified.

Reproducibility

As mentioned previously, initial attempts at FZCE separations of WS proteins resulted in poor reproducibility due to sample instability. Once samples were stabilized and separation conditions were optimized, it was possible to evaluate the reproducibility of the method. Several factors were investigated for impact on separation reproducibility. During preliminary tests, migration times drifted widely from run to run (data not shown). Examination of the current profiles showed that large fluctuations in the initial stages of the separation were noticed and were not reproducible from run to run. This was overcome by adding a small amount of separation buffer (10%) to the sample buffer. This greatly smoothed the current profile (data not shown). Sample injection amount was also optimized (Bean and Lookhart 2001c). Injections (2 sec and 4 sec) were tested over consecutive injections to test migration time repeatability. The 2-sec injections were much more reproducible than the 4-sec injections (data not shown). The 4-sec injections again showed large current fluctuations during the initial current ramp-up. This may have been due to a larger plug of sample buffer that contained 50% EG and therefore disturbed the current. Contrary to previous research on storage protein separations (Bean and Lookhart 2001c), a short voltage ramp-up time of 0.17 min (vs. 0.68 min) was more reproducible than longer times. This demonstrates the need to fully optimize all conditions when developing new separation methods.

Capillary rinsing protocols were also investigated. Capillaries were rinsed with buffer only, 1*M* HCl followed by buffer, 1*M* NaOH followed by buffer, and a combination of 1*M* NaOH, 1*M* HCl, and buffer. The use of a voltage preconditioning step was also investigated (Ross 1995). Migration time reproducibility was best with the buffer-only rinse between separations and an initial voltage preconditioning step (20 kV for 5 min) before the first injection of a data set. Figure 5 shows 10 consecutive injections of the same sample. Migration times varied by only 0.4% relative standard deviation, showing good reproducibility.

Application

Once samples were stabilized and separation conditions optimized, this methodology was applied to separations of several different wheat classes and different types of cereal grains. The first comparison made, however, was between wheat flour and whole meal. As mentioned previously, whole meal contains the entire kernel, whereas flour is composed mainly of endosperm. Because albumin proteins are concentrated in the bran, which is removed during milling, WS proteins from flour and whole meal were compared

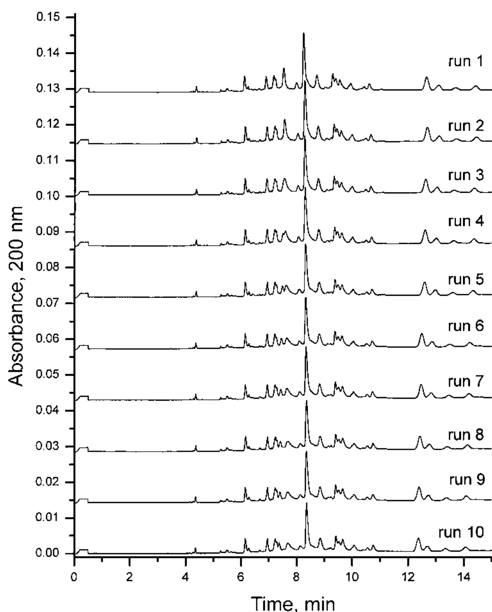

Fig. 5. Reproducibility of 10 successive runs of WS proteins of wheat using optimized conditions (50 m*M* sodium phosphate buffer, 50 m*M* HSA, 0.05% HPMC, and 20% ACN). Separation conditions were 25°C and 10 kV.

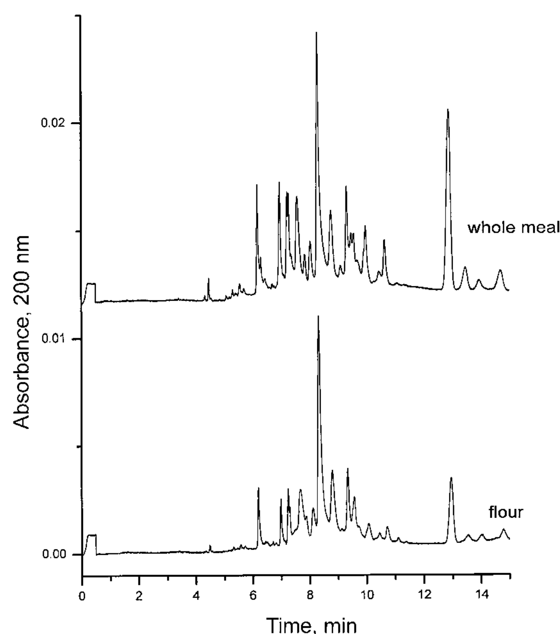

Fig. 6. Comparison of water-soluble (WS) proteins of wheat from whole meal and flour. Separation buffer as in Fig. 5.

(Fig. 6). Several differences were observed between the two extracts, with the major differences appearing quantitative. The early migrating peaks at 6–8 min were present at higher percentages (data not shown) in the whole meal than in the flour extract. Qualitative differences were also visible between 9 and 10 min, with additional peaks seen in the whole meal sample. For these reasons, we used whole meal for all separations.

WS proteins from cultivars of different wheat classes were analyzed using the optimized method (Fig. 7). The most notable difference was between the durum wheat Jori 69 and the other wheat classes. The large peak between 8 and 9 min was missing from the durum wheat. This same pattern was also seen in several other durum lines analyzed; that is, the large peak between 8 and 9 min was missing (data not shown). It is possible that this methodology could be used to check for adulteration of durum wheat with other classes of wheat. Further research in progress to identify the peaks present in these separations will provide more information on the differences seen in the CE electropherograms.

The methodology developed in this study was used to separate WS proteins from several additional cereal grains (Fig. 8). Good separations were noted for each of the cereal grains. Patterns from each grain were distinct from each other. Wheat, oats, barley, and rye showed several peaks were resolved from the WS proteins. Rice and sorghum however had much lower levels, and fewer peaks were resolved. In addition, the patterns for rice and sorghum were very similar. As for wheat, research is in progress to identify peaks present in the CE electropherograms of the various cereals tested.

Finally, SS proteins were extracted from several different grains and analyzed using the methodology developed in this study. Salt-soluble proteins, usually referred to as globulins, have been separated by previous FZCE methods, although good resolution was not obtained (Lookhart and Bean 1995; Shomer et al 1995). One reason for this may be the high levels of salt used to extract globulins, often 0.5*M* NaCl (Shomer et al 1995). However, good resolution was obtained in this study (Fig. 9). The buffer used to extract globulins for this study used a much lower salt level; the total level was 150 m*M* (Bean and Lookhart 1998; Marion et al 1994). The use of HSA in the buffer, which has a relatively high conductance, may have also improved the sample stacking for the samples analyzed here. Note that globulins were extracted directly from whole meal; albumins (WS) were not removed first, thus these samples likely contain WS proteins as well as SS proteins (Wrigley and Bietz 1987; Singh et al 2001). For most of the cereals, more peaks were seen in the direct SS extracts, which is consistent with both albumins and globulins extracted with this solvent. Some samples, such as rice and barley, showed several peaks in the SS proteins that were not present in the WS extracts, while other cereals such as wheat, oats, and sorghum showed very similar patterns between the WS and SS extracts. Of all the cereals, rice showed the most difference between the two types of extracts. The best separations of SS proteins were from the barley samples. This methodology might be useful in screening barley enzymes (once identified in the electropherograms) for relationship to malting quality.

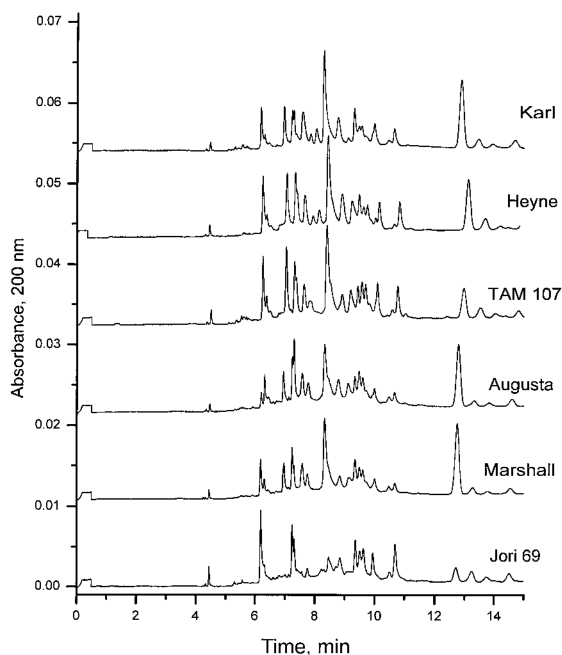

Fig. 7. Water-soluble (WS) protein separations from different U.S. wheat classes: Karl (hard red winter), Heyne (hard white winter), TAM 107 (HRW with rye translocation 1A:1R), Augusta (soft wheat), Marshall (hard red spring), and Jori 69 (durum wheat).

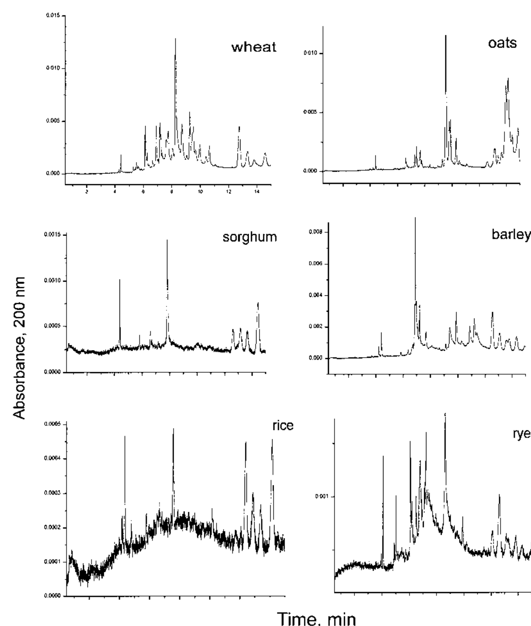

Fig. 8. Separation of water-soluble (WS) proteins from various cereal grains. Separation conditions as in Fig. 5.

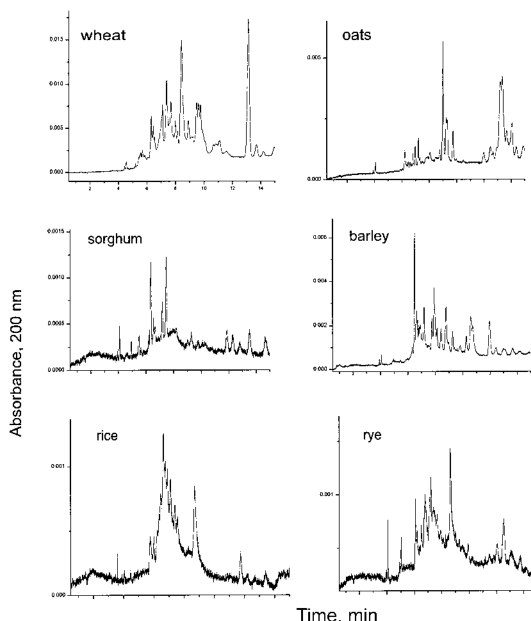

Fig. 9. Separation of SS proteins from various grains. Separation conditions as in Fig. 5.

CONCLUSIONS

Methods for separating WS proteins of cereals were optimized using wheat proteins as a model for optimization. Excellent separations were achieved using a 50 m*M* sodium phosphate buffer (pH 2.5) containing 50 m*M* HSA and 20% ACN. For optimum reproducibility, samples had to be stabilized by lyophilization and subsequent resuspension in 50% EG. Under these conditions, highly repeatable separations could be achieved for WS proteins. This methodology was applied to the separation of WS proteins from several U.S. wheat classes as well as to WS proteins from other cereal grains. The methodology was also applied to the separation of SS proteins from several cereal grains. This method could be used to study the WS and SS proteins during kernel development, to study their role in end-use quality, and screen breeder's samples for presence or absence of important WS or SS proteins. Work is in progress to identify the peaks in the electropherograms of WS and SS extracts from several cereals. Once completed, this methodology could be used to rapidly screen lines for types and amounts of proteins present and their relationships to food and feed quality, as well as studies on the synthesis of cereal kernel proteins.

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