Separation of alcohol soluble sorghum proteins using non-porous cation-exchange columns☆

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ABSTRACT

Kafirins, the storage proteins and major protein of the cereal grain sorghum, play an important nutritional role for millions of people in parts of Africa and Asia. Kafirins are non-water soluble, being soluble only in the presence of detergents or aqueous alcohol mixtures and are among the most hydrophobic of the cereal proteins. Limited M_w heterogeneity of kafirins reduces their resolution when separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Charge based separation techniques have been shown to have improved resolution of kafirins, but due to the nature of their solubility, ion-exchange (IE)-HPLC has not been widely used to separate these proteins. To overcome issues of solubility, two different mobile phases were evaluated. The first mobile phase was based on 60% acetonitrile at acidic pH using guanidine-hydrochloride (Gdn-HCl) gradients to elute the proteins from a non-porous cation-exchange column. The second mobile phase tested consisted of 60% acetonitrile using an increasing concentration gradient of a triethylamine phosphate (TEAP) buffer at pH 3.0. The type of alkylation reagent used to stabilize kafirin extracts prior to analysis was found to have an impact on the IE-HPLC separations with the reagent 4-vinylpyridine providing the best resolution. Separations of kafirins in the TEAP mobile phase system resulted in 10 major peaks being resolved. Combining IE-HPLC with reverse phase (RP)-HPLC into 2D separations revealed that the α-kafirins clustered into three major groups not readily apparent in either 1D separation.

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1. Introduction

Cereal grains are a major source of protein for humans worldwide and play important roles in animal feeds. Grain proteins also have important functional roles in food products; the most widely known example of which is wheat gluten. Sorghum grain proteins are no exception and play an important role in the utilization of sorghum and its nutritional properties. The most heavily researched topic with regards to sorghum proteins has been the issue of protein digestibility in both raw flour and cooked sorghum products. Research has shown that while protein digestibility in raw sorghum tends to be close to that of other cereals such as maize, digestibility decreases upon cooking while that of other cereal proteins tends to increase [1,2]. Reduced digestibility of sorghum proteins may in turn influence starch digestibility [3], which impacts sorghum applications in nutrition, human or animal, and biofuel production.

The major proteins in sorghum are the prolamins. Prolamins are characterized by their solubility in aqueous alcohols and high levels of the amino acids proline and glutamine [4]. Kafirins, sorghum prolamins, have been divided into four subclasses: α, β, γ, and δ based on various factors including solubility and molecular weight [5,6]. Because kafirins subclasses have been partially defined by differences in their M_w, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been a major tool used to study sorghum proteins. SDS-PAGE typically resolves kafirins into 4–5 major bands spanning a relatively narrow apparent molecular weight range of ∼15–28 kDa [6].

While SDS-PAGE has been useful in studying kafirin proteins, compared to other analytical methods, it has low resolution in separating the kafirin subclasses. This is especially true for the α-kafirins, which are 80–84% of the total kafirins [6]. Recently, it has been reported that there are 19 α-kafirins expressed in sorghum [7] all within a very narrow M_w range of ∼2–3 kDa [6], which would limit the ability of SDS-PAGE to fully characterize the α-kafirin

family of proteins. What role, if any, the composition and content of these various α-kafirins may have on sorghum nutritional and functional properties are unknown.

Analytical methods that do not rely on M_w to separate proteins may be useful in further study of the kafirins, especially the α-kafirins. In fact, other analytical methods have been used to separate kafirin subclasses with improved resolution including, isoelectric focusing (IEF) [8], reverse-phase high-performance liquid chromatography (RP-HPLC) [8–11], and free zone capillary electrophoresis (FZCE) [11]. The highest resolution of kafirins has been achieved with IEF and FZCE, which have resolved α-kafirins into numerous peaks/bands [8,11]. FZCE separates proteins based on differences in charge density while IEF on the basis of differences in isoelectric point, which is related to charge. Thus, techniques that utilize charge differences offer the highest resolution of kafirins.

One charge based separation technique that has not been widely used to separate cereal proteins is ion-exchange high-performance liquid chromatography (IE-HPLC). This is due to properties of prolamins, the first of which is the fact that prolamins are not water soluble [4]. Detergents, chaotropes, or aqueous organic solvent mixtures are required to solubilize cereal prolamins, all of which complicate the use of IE-HPLC. Detergents can add charges to proteins which would alter their chromatographic behavior as well as interfere with protein binding to ion-exchange columns [12,13]. Despite these challenges, there have been a few successful reports of IE-HPLC separations of cereal prolamins, such as wheat and barley [12]. Most of these methods have used low levels (1–2 M) of urea in the IE-HPLC mobile phases to maintain solubility of the proteins during separation. However, kafirins have been reported to have reduced solubility in urea even at high concentration (8 M) relative to their solubility in organic solvents such as 70% acetonitrile (ACN) or 70% ethanol [11].

Non-ionic detergents have been used to maintain solubility of membrane proteins during ion-exchange separations, though the detergents have been used at lower levels (e.g. 0.05–0.1%) [14,15] than typically used to solubilize sorghum proteins (generally 1–2%). In any case, the anionic detergent, SDS, has been found to be the most effective at solubilizing sorghum proteins [16] which would not be suitable for use in ion-exchange chromatography. Mixtures of organic solvents can also be problematic due to the low solubility of many salts in organic solvents [13,17] and salts such as NaCl can precipitate sorghum proteins from aqueous organic solvents such as 70% ethanol [18].

Despite the poor solubility of many salts in organic solvents, there have been successful IE-HPLC separations of proteins under these conditions. For example, $NaClO_4$, which has good solubility in organic solvents, has been used in combination with up to 70% of ACN to separate proteins by cation-exchange chromatography [13]. Triethylamine phosphate (TEAP) buffers have been used in the presence of high levels of organic solvents in RP-HPLC [19], hydrophilic interaction liquid chromatography (HILIC) [20,21], size-exclusion chromatography (SEC) [22], and ion-exchange chromatography [21]. Guanidine-hydrochloride (Gdn-HCl) also has good solubility in organic solvents [23] and Gdn-HCl gradients have been used to elute wheat proteins during preparative ion-exchange separations [24]. Gdn-HCl also has the advantage of being a strong chaotrope and may help maintain the solubility of non-water soluble proteins during IE-HPLC in the presence of organic solvents.

IE-HPLC has the potential to provide high resolution separations of sorghum proteins on a platform that can easily be adapted for use as a preparative technique. This is a distinct advantage over both FZCE and IEF. Thus, the goals of this project were to evaluate IE-HPLC mobile phase combinations which would maintain the solubility of the kafirins during separation. Secondary goals were to identify the kafirin subclasses in IE-HPLC separations and compare the resolution to RP-HPLC separations of

kafirins as well as the potential for two-dimensional IE × RP-HPLC separations.

2. Experimental

2.1. Samples and chemicals

Sorghum samples used in this study were grown in either Lane County, Kansas in 2008 or Nebraska in 2003. The maize sample was from a collection of cereal grains at the USDA-ARS, Center for Grain and Animal Health Research (Manhattan, KS). All sorghum flour samples were ground with a UDY cyclone mill 181 (UDY Corporation, Fort Collins, CO) using a 0.5 mm screen. The maize sample was ground using a commercial coffee grinder due to its higher lipid content. HPLC grade acetonitrile was purchased from Fisher Scientific (Fair Lawn, NJ). All other chemicals were purchased from Sigma (St. Louis, MO).

2.2. Kafirin preparation for HPLC analysis

Kafirin samples were extracted and prepared as described in Bean et al. [10]. Briefly, albumins and globulins were pre-extracted using a 50 mM Tris–HCl buffer pH 7.8 containing 100 mM KCl and discarded prior to extracting the kafirins. Kafirins were then extracted from 100 mg of ground whole meal using two 5 min extractions of 1 mL of solution containing 60% tert-butanol (t-BuOH) (v/v), 2% β-mercaptoethanol (β-ME) (v/v), and 0.5% sodium acetate (NaOAc) (w/v). The supernatants of these two extractions were pooled in a 1:1 ratio and then alkylated using 4-vinylpyridine (4-VP) [25].

2.3. Instrumental and data analysis

All HPLC separations were conducted using an Agilent 1100 series instrument (Agilent, Santa Clara, CA). IE-HPLC was performed on a non-porous Agilent Bio SCX NP1.7 SS (4.6 cm i.d. × 50 mm, 1.7 μm) analytical column equipped with an Agilent Bio SCX NP1.7 SS guard column. Various mobile phases were used for the IE-HPLC separations. RP-HPLC was done using a surface porous Agilent Poroshell 300 SB C18 analytical (2.1 mm i.d. × 75 mm, 5 μm) and guard column as described previously [10]. Peaks from IE-HPLC separations between 8 and 30 min as well as the solvent peak (1–6 min) were collected by an Agilent fraction collector and lyophilized. Lyophilized samples were resuspended in 100 μL 60% t-BuOH/2% β-ME/0.5% NaOAc solvent, and then injected (5 μL) on the RP-HPLC column. Origin (MicroCal Software, Inc., Northhampton, MA) was used to plot all chromatograms.

3. Results and discussion

3.1. Mobile phase comparisons

To the best of our knowledge there has only been one report of the use of organic solvent mixtures in IE-HPLC separations of cereal proteins. Gliadins, wheat prolamins, were previously separated using IE-HPLC [26] with a mobile phase containing 30–35% ACN and 0–250 mM NaCl at low pH. While the IE-HPLC method reported in Bietz [26] that was successful for wheat proteins would appear promising, this method is not applicable for sorghum (or likely maize) proteins. The method used for wheat proteins contained only 30% ACN and utilized NaCl to elute the proteins from the ion-exchange column. Kafirins have been found to have poor solubility at this level of ACN and were found to have optimum solubility in 60% ACN [11]. Thus, two alternative mobile phase conditions were tested for separating kafirins by IE-HPLC.
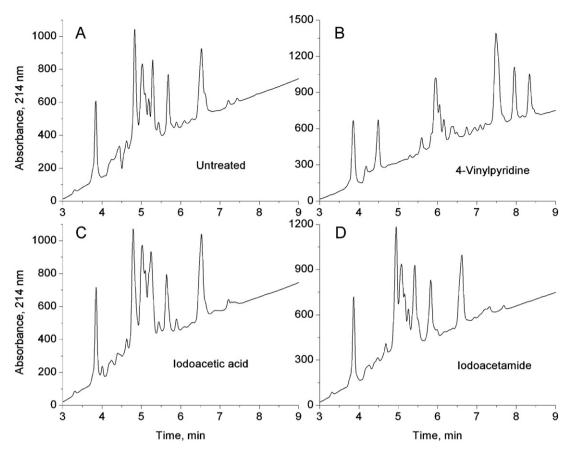
Fig. 1. Comparison of non-alkylated (A) and alkylated (B–D) acetone precipitated kafirins. IE-HPLC separations required a mobile phase containing 60% ACN/0.1% formic acid (solvent A) and 60% ACN/0.1% formic acid/667 mM Gdn-HCl (solvent B). The gradient conditions were 0 min, 0% B; 11 min, 16% B; 16 min, 18% B; 18 min, 60% B with a flow rate of 0.60 mL min^{-1} and column temperature at 40 °C. All samples were injected at 5 μL.

The first mobile phase system evaluated for separating sorghum prolamins consisted of 60% ACN containing 0.1% formic acid using a Gdn-HCl gradient (0–667 mM) to elute the proteins. As with the IE-HPLC separation of wheat and barley proteins, an acidic mobile phase was used to insure that all the proteins would be positively charged [12]. The chromatogram of kafirins separated with this mobile phase system is shown in Fig. 1A. An important consideration in the analysis of kafirins by HPLC is the alkylation of the proteins to prevent re-oxidation of disulfide bonds and loss of peaks [10]. The choice of alkylation reagents is especially important in IE-HPLC as alkylation of cys residues can alter the charge on the proteins and potentially alter binding (and elution) from the columns. For this reason, three different alkylation reagents were investigated, 4-VP which adds a positive charge to cys residues, iodoacetic acid which adds a negative charge and iodoacetamide which does not add a charge to cys residues [27]. The greatest impact on the kafirin separations was seen when 4-VP was used as the alkylation reagent (Fig. 1B). Compared to the unalkylated sample (Fig. 1A), the peaks were spread over a greater elution window and resolution was improved with the 4-VP treated sample. As 4-VP adds a positive charge to cys residues, it would be expected that this would result in greater retention on the cationic ion-exchange column. The use of 4-VP to alkylate wheat proteins prior to RP-HPLC has also been shown to improve the resolution of some proteins [28]. Little change in the overall separation was noticed when either iodoacetic acid (Fig. 1C) or iodoacetamide (Fig. 1D) were used. Because the use of 4-VP resulted in improved resolution, this alkylation reagent was chosen for the remainder of the project.

The use of alkylation reagents presents a potential problem for ion-exchange separations in that the excess alkylation reagents may bind to the column and co-elute with proteins and potentially mask protein peaks. For the analysis conducted in Fig. 1, an acetone precipitation step was used to isolate the proteins from excess reagent after being alkylated. Precipitated proteins were then re-dissolved in sample buffer and injected directly onto the cation-exchange column. If this was not done, the excess 4-VP peak eluted in the middle of the protein separation and clearly masked protein peaks (Fig. 2). In order to use the Gdn-HCl/ACN mobile phase combination, samples must be pre-treated to remove excess 4-VP. We found that the simplest method for this was to precipitate the proteins with acetone; attempts to use spin columns to remove the excess reagent did not completely remove the 4-VP peak. Dialysis could be used, but was more time consuming than a simple precipitation step.

The second mobile phase system evaluated was based on the use of a pH 3.0 TEAP buffer for both maintaining an acidic pH and for elution of the proteins from the column by using a gradient of increasing TEAP buffer content (5–300 mM). Again, an acidic pH was selected to insure that all the kafirin proteins were positively charged. Fig. 3 shows the separation of kafirins under optimized conditions using the ACN/TEAP mobile phase system. Samples were alkylated using 4-VP. A comparison was made using both precipitated kafirins (to remove excess 4-VP) and non-precipitated samples. As can be seen in Fig. 3, the 4-VP is adjacent to the solvent peak early in the chromatogram so it does not mask the kafirin peaks. Elution times were slightly different for the precipitated vs. the non-precipitated samples, most likely due to the excess 4-VP altering the protein binding to the column. Thus, the ACN/TEAP mobile phase could be used without the need to pre-treat the samples. For this reason, this mobile phase was used for the
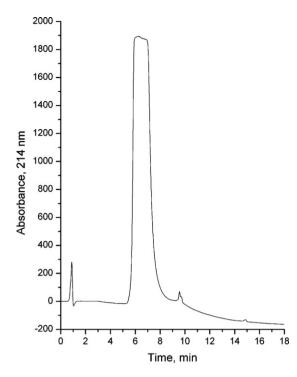
Fig. 2. IE-HPLC separation of non-precipitated kafirins alkylated with 4-VP using the same conditions as Fig. 1.
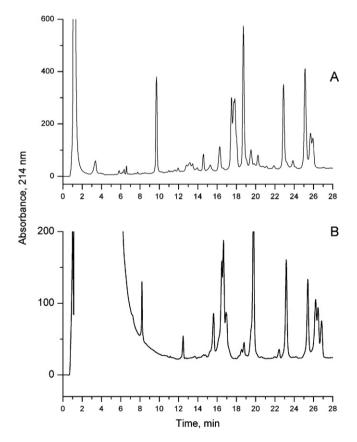

Fig. 3. Comparison of precipitated (A) and non-precipitated (B) kafirins IE-HPLC separations using a mobile phase containing 60% ACN/5 mM TEAP pH 3 (solvent A) and 60% ACN/300 mM TEAP pH 3 (solvent B). The gradient conditions A were 0 min, 0% B; 11 min, 22% B; 28 min, 49% B; 30 min, 70% B with a flow rate of 0.50 mL min^{-1} and column temperature at 60 °C. Both samples were injected at 5 µL. Samples used for this comparison were analyzed on separate days and are not consecutive injections.
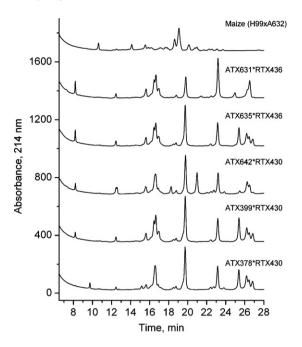

Fig. 4. IE-HPLC chromatograms of prolamins from maize and various sorghum hybrids. Flour extracts were analyzed under the conditions in Fig. 3.

remainder of the project and all further separations used the ACN/TEAP gradient method without precipitation of the samples to remove excess 4-VP. Good run-to-run repeatability was found with using the ACN/TEAP mobile phase, with RSD for the major peaks less than 0.09% over 10 consecutive injections.

3.2. Hybrid differentiation

To evaluate the resolution of the ACN/TEAP mobile phase, kafirins from related hybrids were separated to determine if IE-HPLC could be used to differentiate sorghum hybrids. Maize proteins from a commercial maize hybrid were also separated for comparison to the sorghum separations. Fig. 4 shows the separation of prolamins from five sorghum hybrids and the maize sample. Even though some of the sorghum hybrids had one of the same parent lines, differences in kafirin pattern were still readily visible. For example, two hybrids have an RTx436 parent and peaks from 6 to 18 min in these samples look very similar in both shape and absorbance. However, beyond 18 min, absorbance and the number of peaks vary. This demonstrates that "fingerprinting" of sorghum proteins was possible with this method, even with related hybrids. Prolamins from the maize sample were also resolved into a number of peaks which show the potential for this method to be translated to other cereals closely related to sorghum.

3.3. Comparison of IE- and RP-HPLC kafirin separations

To further characterize the separation of kafirins by IE-HPLC, separations were compared to RP-HPLC separations of kafirins. This comparison was conducted to gain insight into the ion-exchange separation itself and to evaluate the potential of using IE-HPLC in combination with RP-HPLC for two-dimensional separations of kafirins. To help identify the peaks in the IE-HPLC separations, individual peaks were collected from the ion-exchange separation, lyophilized, re-suspended, and then separated by RP-HPLC. The comparison between IE-HPLC and RP-HPLC is shown in Fig. 5. The separation of kafirins by RP-HPLC has been well characterized [11] and it is known that the α-kafirins elute last during RP-HPLC separations, in this case between ~9 and 13 min. Peaks from
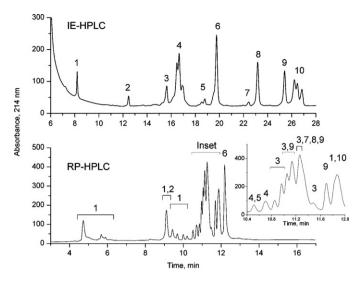
Fig. 5. Comparison of kafirins separated by IE- (top) and RP-HPLC (bottom). IE-HPLC peaks are labeled and shown where they eluted on RP-HPLC with an inset of closely eluted peaks. The ion-exchange separation for the collection was performed as Fig. 3. Reverse-phase separations required 0.1% TFA (solvent A) and ACN/0.07% TFA (solvent B) and gradient conditions of 0 min, 20% B; 5 min, 40% B; 15 min, 60% B; 17 min, 20% B with a flow rate of 0.70 mL min^{-1}, column temperature at 55 °C and injection column of 5 μL.

Fig. 6. RP-HPLC chromatograms of collected IE-HPLC peaks. RP-HPLC conditions were the same as described in Fig. 5.

virtually the entire IE-HPLC separation were found in this region of the RP-HPLC chromatogram suggesting that the α-kafirins had a high degree of charge heterogeneity. This is in agreement with earlier separations of kafirins by IEF [8] and FZCE [11].

RP-HPLC separations of individual collected IE-HPLC fractions are shown in Fig. 6 for the main α-kafirin regions. IE-HPLC peaks 1–4 contained mainly α-kafirins that eluted in the earliest region of the α-kafirins on the RP-HPLC separation (~11–11.5 min). There was then a shift to the later eluting RP-HPLC (~12–13 min) in IE-HPLC peak 6. IE-HPLC peaks 7 and 9 were also found to contain
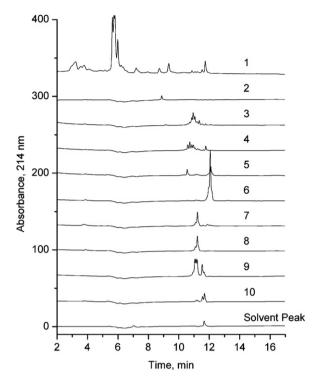
mostly earlier eluting α-kafirins on RP-HPLC (~11–12 min), while IE-HPLC peaks 9–10 contained peaks eluting around 11.5–12 min on RP-HPLC (Fig. 6).

The clustering of the α-kafirins was much easier to identify when data were plotted in a 3D chromatogram (Fig. 7A) or in a 2D contour plot (Fig. 7B). In these plots, α-kafirins formed three distinct clusters. The combination of IE-HPLC × RP-HPLC was
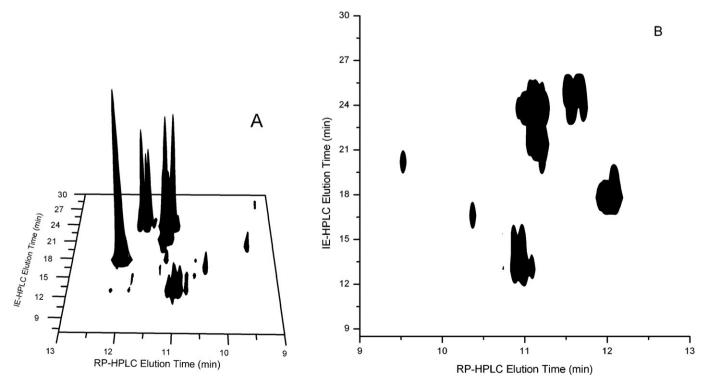

Fig. 7. 3D chromatogram (A) and 2D contour plot (B) of IE-HPLC × RP-HPLC separations.

particularly useful in this aspect as α-kafirins eluting from the RP-HPLC separation between 11 and 11.5 min had greatly different IE-HPLC elution times; roughly 10–12 min apart for these 'clusters.' Thus using IE-HPLC and RP-HPLC can be used to gain new information on the poorly characterized α-kafirins, much more than can be obtained via SDS-PAGE or any 1D chromatographic separations. Research is in progress to utilize IE-HPLC × RP-HPLC separations of kafirins to identify allelic variants at the protein level and to further characterize the biochemical differences in the α-kafirins 'clusters' identified in this research.

The 2D IE-HPLC × RP-HPLC plots also provide insight into the mode of separation of the kafirins on the cation exchange columns with the mobile phases used in this study. Separation of kafirins with the two mobiles tested could be due purely to IEC, hydrophilic interaction chromatography (HILIC), or a mixed mode IEC/HILIC separation [29]. HILIC separations have been conducted using cation exchange columns in the presence of high levels of organic solvents [e.g. 20,21]. However, from the 2D plots shown in Fig. 7, the separation of kafirins using the TEAP/ACN buffer does not appear to be a purely HILIC mechanism. If this was the case, it would be expected that peaks would show an inverse relationship between the separations on the cation exchange column and the RP-HPLC dimension, i.e. the most hydrophobic proteins would elute first from the cation exchange column in HILIC and these proteins would elute last from the RP-HPLC columns. This is clearly not the case shown for the 2D separations of α-kafirins shown in Fig. 7. For example, proteins eluting at approximately 11 min on the RP-HPLC dimension have widely different elution times on the cation exchange column (spanning 12–24 min). Elution of the γ-kafirins would also seem to indicate a mixed separation mode as these proteins elute first on both the cation exchange and RP-HPLC columns (Fig. 5), but migrate first in FZCE separations in acidic buffers [11], demonstrating that they have the highest charge density at low pH of all the kafirins and therefore should elute last from the cation exchange column in a pure ion-exchange separation. It should also be pointed out that the separation mode may differ between the TEAP/ACN and Gdn-HCl/ACN buffer systems as evidenced by the difference in the overall elution times between the two buffer systems (~30 min and 10 min, respectively).

4. Conclusions

The non-water soluble sorghum storage proteins, kafirins, were successfully separated with high resolution by IE-HPLC on a non-porous cationic column using mobile phases carefully selected to maintain the solubility of the kafirins during separation. A mobile phase consisting of 60% acetonitrile utilizing a gradient of TEAP was found to provide the best separations. The α-kafirins were resolved into numerous peaks and by combining IE-HPLC with RP-HPLC into 2D separations, the α-kafirins were found to form distinct clusters not easily identifiable in simple 1D IE-HPLC or RP-HPLC separations. This opens new possibilities for characterizing kafirins, especially α-kafirins, and determining their roles/responses in various conditions such as digestion and environmental stress during kernel development. This methodology may also be useful for other non-water soluble proteins.

References

[1] B.R. Hamaker, A.W. Kirleis, L.G. Butler, J.D. Axtell, E.T. Mertz, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 626.
[2] K.G. Duodu, A. Nunes, I. Delgadillo, M.L. Parker, E.N.C. Mills, P.S. Belton, J.R.N. Taylor, J. Cereal Sci. 35 (2008) 161.
[3] K.G. Duodu, J.R.N. Taylor, P.S. Belton, B.R. Hamaker, J. Cereal Sci. 38 (2003) 117.
[4] P.R. Shewry, J.A. Napier, A.S. Tatham, Plant Cell 7 (1995) 945.
[5] J.M. Shull, J.J. Watterson, A.W. Kirleis, J. Agric. Food Chem. 39 (1991) 83.
[6] P.S. Belton, I. Delgadillo, N.G. Halford, P.R. Shewry, J. Cereal Sci. 44 (2006) 272.
[7] J.H. Xu, J. Messing, PNAS 105 (2008) 14330.
[8] L.V.S. Sastry, J.W. Paulis, J.A. Bietz, J.S. Wall, Cereal Chem. 63 (1986) 420.
[9] J.S.C. Smith, O.S. Smith, Theor. Appl. Genet. 76 (1988) 97.
[10] S.R. Bean, B.P. Ioerger, D.L. Blackwell, J. Agric. Food Chem. 59 (2011) 85.
[11] S.R. Bean, G.L. Lookhart, J.A. Bietz, J. Agric. Food Chem. 48 (2000) 318.
[12] I.L. Batey, in: J.E. Kruger, J.A. Bietz (Eds.), High-Performance Liquid Chromatography of Cereal and Legume Proteins, AACC, St. Paul, MN, 1994, pp. 373–392.
[13] A.J. Alpert, in: E. Grushka, N. Grinberg (Eds.), Advances in Chromatography, vol. 44, CRC Press, Boca Raton, FL, 2006, pp. 317–329.
[14] J. Van Ede, J.R.J. Nijmeijer, S. Welling-Wester, J. Chromatogr. 476 (1989) 319.
[15] Dj. Josic, W. Hofmann, B. Wieland, R. Nuck, W.W. Reutter, J. Chromatogr. 359 (1986) 315.
[16] S.H. Park, S.R. Bean, J. Agric. Food Chem. 51 (2003) 7050.
[17] A.P. Schellinger, P.W. Carr, LC–GC North Am. 22 (2004) 545.
[18] S.R. Bean, B.P. Ioerger, S.H. Park, H. Singh, Cereal Chem. 83 (2006) 99.
[19] J.E. Rivier, J. Liq. Chromatogr. 1 (1978) 343.
[20] H. Lindner, W. Helliger, in: M.I. Aguilar (Ed.), HPLC of Peptides and Proteins, Methods in Molecular Biology, vol. 251, Humana Press, New York, NY, 2004, pp. 75–88.
[21] H. Lindner, B. Sarg, W. Helliger, J. Chromatogr. A 782 (1997) 55.
[22] J.E. Rivier, J. Chromatogr. 202 (1980) 211.
[23] R.B. Pepinsky, Anal. Biochem. 195 (1991) 177.
[24] F.R. Huebner, J.S. Wall, Cereal Chem. 51 (1974) 228.
[25] M. Friedman, J. Protein Chem. 20 (2001) 431.
[26] J.A. Bietz, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, CRC Press, New York, 2002, pp. 533–569.
[27] M.W. Crankshaw, G.A. Grant, Curr. Protoc. Protein Sci. (2001), 15.1.1.
[28] T. Burnouf, J.A. Bietz, J. Chromatogr. 299 (1984) 185.
[29] C.T. Mant, R.S. Hodges, J. Sep. Sci. 31 (2008) 2754.