

Use of Size-Exclusion High-Performance Liquid Chromatography in the Study of Wheat Flour Proteins: An Improved Chromatographic Procedure

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A procedure for using sonication and size-exclusion high-performance liquid chromatography (SE-HPLC) for measuring the relative proportions of the main size classes of wheat flour proteins was recently described by Singh et al (1990a). Although this technique is useful in indicating flour quality, we have found that a relatively rapid deterioration of column performance occurs under the suggested operating conditions. After fewer than 300 injections, a Waters Protein Pak 300 column (Millipore, Milford MA) showed a loss of resolution of the three main protein fractions—aggregated proteins, gliadins, and albumins and globulins. When the HPLC procedure was used on a TSK 4000G column (Toyo Soda, Tokyo), a similar loss of performance occurred after even fewer injections. Thus, the method as it stands has limitations as a routine procedure, particularly as these types of size-exclusion columns are very expensive compared with most other HPLC columns. To achieve acceptance as a routine procedure, either an alternative column packing must be found, or the sample preparation and running conditions must be changed to ensure that column life is extended.

These SE-HPLC columns have a silica-based support with a surface coating of a hydrophilic bonded-phase to reduce interactions of the packing material with proteins (Regnier and Gooding 1980). Column manufacturers advise that denaturing agents such as sodium dodecyl sulfate (SDS) may be used in the column but state that "there is a tendency to display shorter column life." Why this should be so is not clear. The most likely explanation is that SDS binds to the support, thus affecting the apparent pore size and the separations achieved. However, it appeared to be the amount of SDS passing through the column, not just the number, size, and/or content of the injections, that caused the loss of resolution. Removal of SDS from the sample preparation and elution buffers, therefore, seemed to be the easiest way to extend column life. This article describes the use of alternative conditions that extend column life to at least 2,000 injections.

MATERIALS AND METHODS

Flour samples with a wide range of bread-making qualities were obtained from single varieties and from a number of sources. The flours were prepared for SE-HPLC using the sonication procedure of Singh et al (1990a). However, SDS concentrations in the extractant (0.05M phosphate, pH 6.9) varied from 0.1 to 2%. In separate experiments, ethanol and water (7:3, v/v), isopropanol and water (1:1, v/v), and acetonitrile and water (1:1, v/v), each containing 0.05 or 0.1% (v/v) trifluoroacetic acid (TFA), were also used as the extractant. For HPLC, 20 μ l of each extract was injected onto a Waters Protein Pak 300 column, using a Waters HPLC system comprising two model 510 pumps, a WISP 712 automatic sampler, and a model 481 UV-visible detector at 214 nm. Pump control and data acquisition were achieved with a model 840 chromatography station.

Elution solvents used were ethanol and water (70:30 v/v),

isopropanol and water (50:50) and acetonitrile and water (50:50), the latter both with and without trifluoroacetic acid (0.1%). The flow rate with all solvents was 0.5 ml/min.

Solubility of the protein in the elution solvent was tested by adding a 100- μ l aliquot to 1 ml of the elution solvent. The UV absorbance was measured before and after centrifugation at 11,600 \times g. Initially, the near-quantitative extraction of protein reported by Singh et al (1990a) was also confirmed. Each flour sample was examined by the method of Smith et al (1985) to test how much of the flour protein was extracted. In each case, at least 95% of the protein was extracted. Individual extracts applied to the column were not examined in this way; it was assumed that the amount of protein extracted was similar on each occasion.

RESULTS AND DISCUSSION

In a chromatographic run using the method described by Singh et al (1990a), most of the SDS that comes into contact with the column is present in the elution solvent. In a 40-min run with 0.1% SDS in the elution buffer, 20 mg of SDS passes through

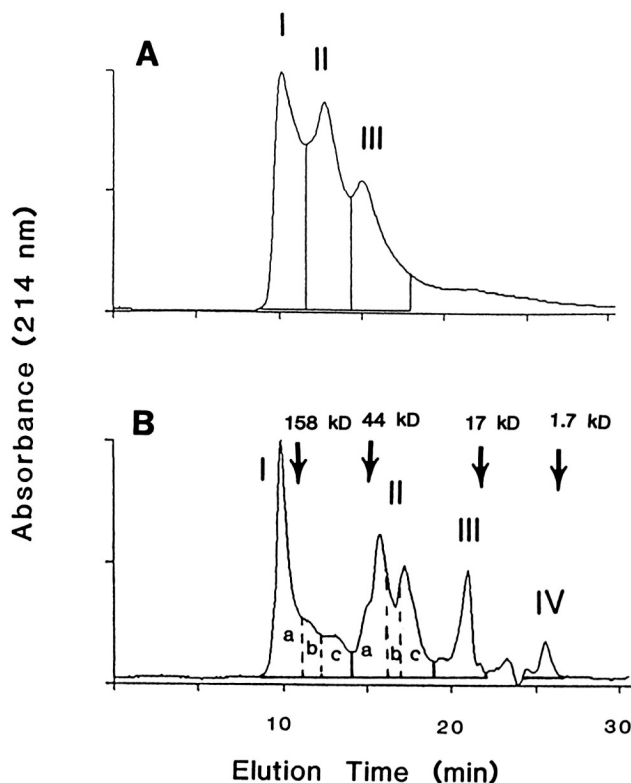


Fig. 1. Size-exclusion high-performance liquid chromatographic profiles of the wheat cultivar Cook, extracted with 0.05M phosphate buffer, pH 6.9, containing 2% (w/v) sodium dodecyl sulfate (SDS). **A**, the old elution procedure (0.05M phosphate with 0.1% SDS); **B**, the presently recommended elution procedure (50% acetonitrile and water containing 0.1%, v/v, TFA). Chromatography was performed on a Protein Pak 300 column with a flow rate of 0.5 ml/min. The elution times of molecular weight markers are shown by arrows; peak I was at the void volume.

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the column, whereas with 2% SDS in a protein extract, there is only 0.4 mg in 20 μ l. Thus, removing SDS from the elution solvent was the first approach attempted. When the elution solvent was either 70% aqueous ethanol or 50% aqueous isopropanol, very high back pressures were necessary to maintain the flow rate (0.5 ml/min.). Because high back pressure is particularly detrimental to size-exclusion columns, the use of these solvents was abandoned.

Acetonitrile, a commonly used solvent in the HPLC of proteins (Regnier and Gooding 1980), is compatible with the column packing. In addition, it has a much lower viscosity than ethanol or isopropanol and does not give as high back pressures on the column. An elution solvent of 50% aqueous acetonitrile gave partial resolution of the three main fractions of wheat flour proteins. Adding 0.1% trifluoroacetic acid to the solvent gave greatly improved resolution, significantly better than that achieved using the original SDS-phosphate buffer as the elution solvent (Fig. 1). No indication of protein precipitation was noted when the SDS extracts came into contact with the acetonitrile-water mixture. When added to acetonitrile-water mixture, aliquots of the extract gave the same UV absorbance at 280 nm before and after centrifugation. Thus, the remainder of the results presented

TABLE I
Ratio of Peak I to Peak II (Aggregated Proteins: Gliadin) for Replicate Injections of Extracts of Cook and Halberd Flour Varieties as Measured by Size-Exclusion High-Performance Liquid Chromatography^a

Variety	Extract Number	Number of Injections	Peak I: Peak II	Standard Deviation
Cook	1	5	1.21	0.013
	2	5	1.24	0.013
	3	5	1.23	0.011
	4	5	1.23	0.015
Halberd	1	4	0.99	0.005
	2	4	0.98	0.008
	3	4	1.00	0.008

^a In each case, more than 90% of the protein was extracted from the flour.

here were obtained using 50% aqueous acetonitrile containing 0.1% TFA as the elution solvent.

There was almost baseline resolution of each of the main peaks. In addition, partial resolution of the second peak into two main subpeaks occurred (Fig. 1B, IIa and b). Also, partially resolved shoulders were observed on peak I with all samples and on peak II with some samples. With SDS-phosphate as the elution solvent, much inferior resolution of peaks was noted, with no resolution of the gliadin peak (peak II) into subfractions. The improved resolution of the peaks permitted more accurate and reproducible integration of each peak. Good resolution of the peaks was also reflected in the results obtained when the peak fractions were collected and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2). The three main peaks showed almost no overlap in their peptide composition and clearly represented polymeric proteins, gliadins, and albumins and globulins, respectively. The banding patterns of reduced fractions revealed that peak I comprised polymeric proteins, mostly the low and high molecular weight subunits of glutenin (Fig. 2, lanes Ia–Ic). Fractions IIa–IIc of peak II showed some overlap with each other, with IIa containing all classes of gliadin; fractions IIb and IIc contained most of the same components, but the ω -gliadins were missing. Peak III comprised albumin and globulins (lane III), but peak IV (not shown) contained no proteinlike material large enough to be retained in the gel. This peak has been found to vary considerably in size and may contain nonprotein material that is detected at 214 nm.

To test the reproducibility of the elution times and the quantitation of the peaks, multiple extracts were made for each of two varieties (Cook and Halberd), and multiple injections of each extract were applied to the column (Table I). The reproducibility of the absolute size of peaks I and II was $\pm 2\%$ between injections of the same extract and $\pm 3\%$ for different extractions of the same sample run consecutively.

The extracted samples were tested for stability by reinjection after 12, 24, 48, and 72 hr. These samples were left in the sample injector between injections, simulating conditions that would exist for a batch of samples extracted at the same time. Differences in absolute peak size between these reinjections were approximately the same as for consecutive injections ($\pm 2\%$). The differ-

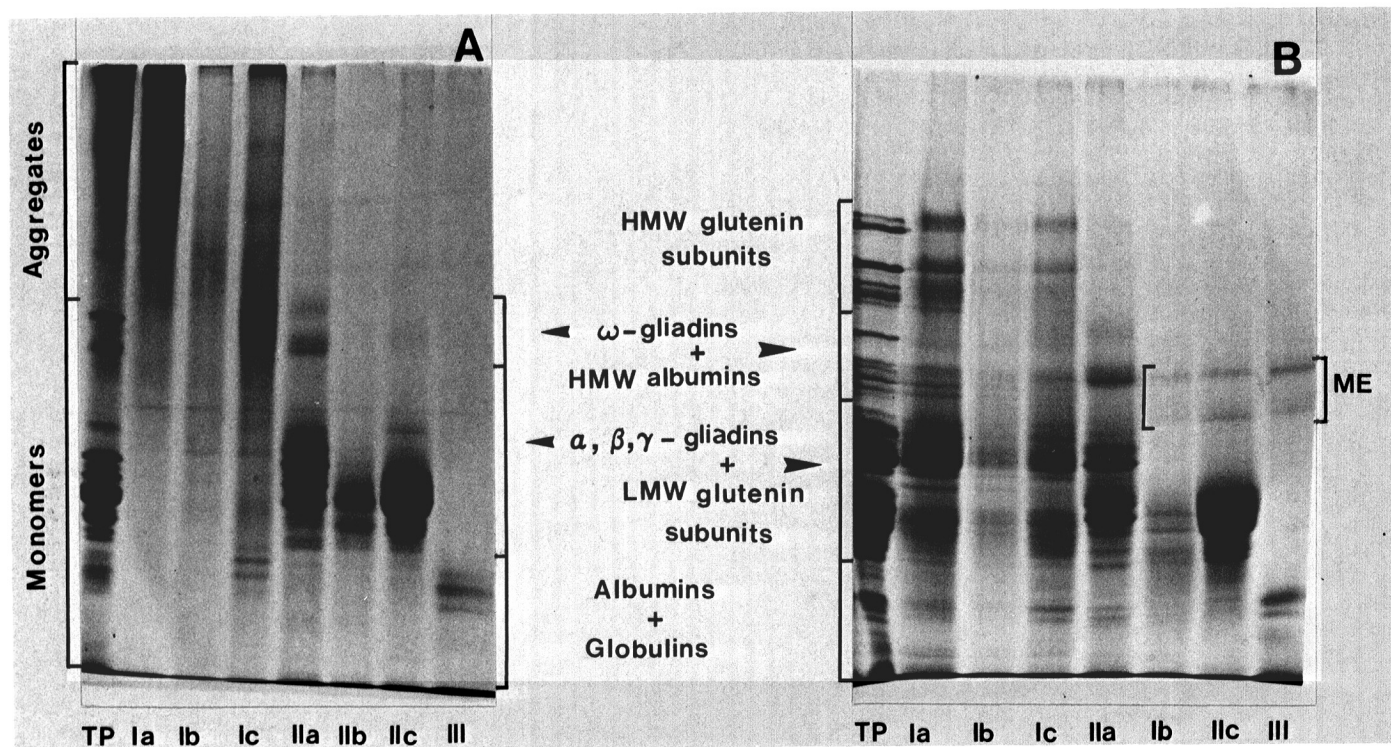


Fig. 2. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns (12% acrylamide) of flour proteins and fractions shown in Fig. 1B under unreduced (A) and reduced (B) conditions of proteins from the cultivar Cook. TP = total proteins, Ia–Ic = peak I proteins, IIa–IIc = peak II proteins, III = peak III proteins.

TABLE II
Effect of Different Extractants on the Percentage of Protein Extracted and on the Proportions of Aggregated Protein (Peak I), Gliadin (Peak II), and Albumin and Globulin (Peak III) in Extracts of the Cultivar Israel M68 as Observed by Size-Exclusion High-Performance Liquid Chromatography^a

Extractant	SDS ^b (%)	Peak (%)			Ratio of Peak I to Peak II	Protein Extracted (%)
		I	II	III		
Phosphate buffer (0.05M)	0.0	22.2	27.0	50.8	0.82	15
	0.1	46.2	42.9	10.9	1.08	90
	0.3	47.2	42.1	10.7	1.12	96
	0.5	46.2	41.7	12.1	1.11	94
	1.0	46.0	42.7	11.3	1.08	95
	1.5	46.1	42.5	11.4	1.08	99
	2.0	46.4	42.3	11.3	1.10	95
Acetonitrile and water (1:1)						
Containing 0.1% TFA ^c	...	40.7	46.8	12.5	0.87	82
Containing 0.05% TFA	...	43.1	45.3	11.6	0.95	87

^a The elution solvent was acetonitrile and water (1:1) containing 0.1% trifluoroacetic acid.

^b SDS = sodium dodecyl sulfate.

^c TFA = trifluoroacetic acid.

ences relative to each other were even less, indicating that some of the variance was due to the injector. Extracts of many different varieties showed this consistency, which indicated that the protein extracts were stable, both with respect to aggregation (and concomitant precipitation) and to enzymatic breakdown by endogenous enzymes extracted from the flour (Huebner and Bietz 1985). This stability means that a batch of samples extracted on one day may be validly compared over succeeding days using an automated sample injector. Over longer periods, a slow change occurred in the results from a column. For example, a sample extracted and subjected to chromatography showed that the ratio of peak I to peak II was about 8% lower than the same sample examined six months earlier. This difference was consistent for a set of 15 samples. Thus, it would appear that a change occurs in the column over a long period, a well-recognized phenomenon on other types of HPLC columns. However, samples may be compared with others run at different times, provided that standards are run with each batch.

The complete removal of SDS from the sample preparation buffer was not possible. Alternative solvents, with SDS replaced by sodium stearate (Kobrehel and Bushuk 1977) were outside the recommended pH range of the column. Nevertheless, ways of reducing the amount of SDS in the extractant were investigated. Table II shows the effects of decreasing the amount of SDS from 2 to 0.1%. No change occurred in the absolute or relative size of each peak until the SDS concentration was reduced to 0.3%. Below this level, some of the samples became cloudy on standing, and the relative amount of peak I was reduced. In absolute terms, peaks I and II were smaller, while peak III remained almost unchanged. It is clear that the amount of SDS in the extracting buffer must be greater than 0.3%, and a concentration of 0.5% should be used for safety.

Aqueous organic solvents, including ethanol and water (7:3), isopropanol and water (1:1), and acetonitrile and water (1:1), each containing 0.05 or 0.1% TFA (but no SDS), were not as effective in extracting the protein. Of these, acetonitrile and water with 0.05% TFA gave the best results, but not as good as those obtained with phosphate buffer containing SDS (0.5% or more) (Table II). At 0.5% SDS concentration, the amount of SDS to

which the column is exposed in this modified procedure is reduced by a factor of 200 per injection compared with the amount using the original extracting and buffer conditions described by Singh et al (1990a).

Thus, the recommended procedure uses 0.5% SDS in 0.05M phosphate buffer, pH 6.9, to extract the protein from flour, and 50% (v/v) aqueous acetonitrile containing 0.1% TFA as the elution solvent. These modifications to the method should not only ensure greatly increased column life but also provide much improved resolution of the different protein classes of wheat flours. The modified procedure has been used to measure the proportions of the three main classes of proteins for a large number of samples. As reported previously for the old method (Singh et al 1990b), highly significant statistical correlations exist between certain of these values and wheat quality parameters.

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