

## Improved Resolution of Nonsilica-Based Size-Exclusion HPLC Column for Wheat Flour Protein Analyses

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The use of size-exclusion high-performance liquid chromatography (SE-HPLC) has become an important method adopted for cereal protein quality characterization within different breeding programs. Several types of SE-HPLC columns have been used for the study of wheat flour proteins.

Different attributes have been stated for available SE-columns. They include solubility of the proteins and elution conditions, minimal interaction with the column support, resolution of the peaks, and column life in a routine procedure (Huebner and Bietz 1985; Singh et al 1990; Batey et al 1991; Ciaffi et al 1996). These considerations must be taken into account before starting new analysis approaches.

Denaturing agents such SDS cause negative effects on silica-based support. Therefore, attempts to remove the SDS from the sample preparation and elution buffers have been made (Batey et al 1991). Since this work was published, many other researchers have adopted hydrophobic elution conditions for SE-HPLC wheat protein characterization to improve peak resolution (Gupta et al 1993; Larroque et al 1997; Huebner and Bietz 1999). Other research groups use hydrophilic conditions (Dachkevitch and Autran 1989; Ciaffi et al 1996; Carceller and Aussenac 1999; Tronsmo et al 2002). All of them agree on the convenience of using SDS for protein extraction. Moreover, Batey et al (1991) recommended the use of >0.3% SDS in the extracting buffer to avoid problems later in the chromatographic performance.

The aim of this note is to present the advantages of using a novel size-exclusion column, Superdex 200 (Amersham Pharmacia Biotech, Uppsala, Sweden). This column allows the use of the same SDS level in the extracting and elution buffer, improving resolution. The main peaks were characterized by SDS-PAGE confirming good resolution of the separation process.

### MATERIALS AND METHODS

#### Plant Material

Flour samples of Uruguayan bread wheat (*Triticum aestivum* L.) genotypes were used for chromatographic analyses.

#### Protein Extraction

The endosperm protein extraction was conducted as described by Larroque et al (2000), with modifications. Proteins from flour (100 mg) were extracted with 10 mL of 0.5% SDS and 0.05M

sodium phosphate buffer, pH 6.9, subjected to 15 sec of sonication (output 20W) using an Ohtake Works sonicator (Tokyo, Japan) with a 9-mm diameter probe. Several sonication times were tested to determine best conditions for protein extraction. The supernatant obtained by centrifugation of the samples at  $16,000 \times g$  for 10 min was filtered through a 0.45- $\mu$ m polyvinylidene difluoride filter membrane (Durapore, Millipore Corp., Ireland) and immediately heated for 2 min at 80°C in a water bath. For SE-HPLC analyses, 20  $\mu$ L of the protein extracts was used.

#### SE-HPLC

The column used was a Superdex 200 HR 10/30 size-exclusion analytical column (10  $\times$  300 mm) that has dextran covalent bonding to highly cross-linked porous agarose beads. This column allows the separation of proteins throughout a large molecular weight range (10,000–600,000).

The column was equilibrated with 0.05M sodium phosphate buffer (pH 6.9). Different concentrations of SDS (0.05–0.5%) and NaCl (0.05–0.2M) were evaluated to get the best resolution of the main protein classes. These results were compared with those from the column equilibrated with 15% acetonitrile containing 0.1% of trifluoroacetic acid (TFA). It was concluded that the best resolution was obtained when the same buffer, 0.5% SDS and 0.05M sodium phosphate, pH 6.9, was used both for extraction and elution.

Column elution was isocratic for 35 min with a flow rate of 0.5 mL/min at room temperature using an HPLC Äkta Purifier System (Amersham Pharmacia Biotech, Uppsala, Sweden). Absorbance was measured at 214 nm. All HPLC measurements were run in duplicate and mean values were used. Reproducibility was tested for the elution times and the quantitation of the peaks for each cultivar employed as described by Batey et al (1991).

The apparent molecular weight range of wheat proteins was estimated by calibrating the column with standards including ferritin (440,000), catalase (232,000), chymotrypsinogen A (25,000), albumin (67,000), ovalbumin (43,000), and ribonuclease A (13,700).

#### Characterization of Peaks by SDS-PAGE

HPLC fractions corresponding to the three main peaks were collected. Each fraction was precipitated with four volumes of cold acetone ( $-20^{\circ}\text{C}$ ), left overnight at  $-20^{\circ}\text{C}$ , and centrifuged 30 min at  $15,000 \times g$ . Pellets were washed with distilled water, dissolved in 0.1M acetic acid and freeze-dried (Hurkman and Tanaka 1986). Freeze-dried fractions were suspended in 40  $\mu$ L of sample buffer for electrophoresis containing 5%  $\beta$ -mercaptoethanol and centrifuged 5 min at  $16,000 \times g$ . Supernatants were heated 5 min at  $95^{\circ}\text{C}$  before electrophoresis and were loaded on a 10% SDS-PAGE gel. Electrophoresis was done using a Mini-protean II apparatus (Bio-Rad, Richmond, CA) at a constant current of 5 mA overnight. The bands were first stained with Coomassie Brilliant Blue G (CBB-G) (Neuhoff et al 1988) and restained with protein silver stain (Blum et al 1987) to increase the sensitivity.

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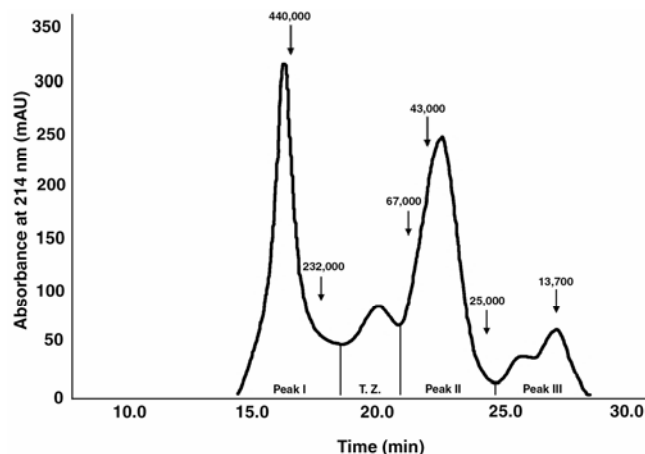
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**TABLE I**  
Evaluation of Sonication Time Effects at Fixed Power (20W) on Flour Protein SE-HPLC Fractions

Peaks	15 sec		30 sec		60 sec	
	Retention Time (mL)	% Area	Retention Time (mL)	% Area	Retention time (mL)	% Area
Glutenins (I)	8.1	39.4	8.1	40.8	8.1	38.6
Transition zone (II)	10.1	12.6	10.0	9.3	10.0	9.7
Gliadins (II)	11.3	38.6	11.3	40.7	11.3	42.6
Nongluten proteins (III)	13.0	9.4	13.0	9.2	13.0	9.0



**Fig. 1.** Characteristic SE-HPLC profile of wheat, extracted with 0.05M phosphate buffer, pH 6.9, containing 0.5% SDS. Arrows show the elution times of molecular weight markers.

## RESULTS AND DISCUSSION

Former papers stated the necessity of finding alternative column packing for a routine procedure. The convenience of using the same buffer for extracting proteins and elution in SE-HPLC has been previously highlighted. Efforts have been made to adapt the protein sample to the elution buffer (Batey et al 1991). It has been reported that high concentrations of SDS have detrimental effects on protein peak resolution when silica-based support columns are used (Batey et al 1991). Until now, all SE-HPLC published research has been based on low level concentration of SDS ( $\leq 0.1\%$ ) or the use of acetonitrile and TFA.

The matrix composition of the Superdex 200 is produced by the covalent bonding of dextran to highly cross-linked porous agarose beads. Separation properties of the composite medium are predominantly determined by the dextran component, and no contraindications are observed for detergents used at concentrations normally reported in chromatography. In addition, the use of  $\leq 1\%$  SDS is suggested by manufacturers without causing problems.

There is no extraction system that solubilizes total flour protein, but the use of SDS in the extraction buffer improved the efficiency (Singh et al 1990). Among all the buffers assayed, the best resolution of main protein classes of wheat was obtained using 0.5% SDS and phosphate, pH 6.9. The choice of this buffer helps to avoid low protein solubility caused by the use of other solvents (Fig. 1). Other buffers gave lower resolution of the three main fractions, including acetonitrile and TFA.

Sonication time was optimized to maximize glutenin extraction without degradation. The aim of sonication is to use a time long enough to give a maximum in the chromatogram area but not change the profile (F. MacRitchie, *personal communication*). As sonication breaks down the largest glutenin molecules, too long sonication time will reduce the height of the first peak and cause an increase in the areas to appear at later elution times.

The SE-HPLC profile did not change in any treatment (data not shown). Extending sonication time did not increase the size of the polymeric proteins peak (Table I). The conclusion that total protein extraction was close to complete after 15 sec of sonication.

Good resolution of each of the main peaks was observed in Fig. 1. The column thus provides a viable alternative to other columns that have been used (Dachkevitch and Autran 1989; Batey et al 1991; Ciaffi et al 1996; Larroque et al 2000). In the second peak, generally a shoulder (transition zone) is observed and contains low molecular weight glutenins and larger size gliadins (such as  $\omega$ -gliadins). This observation shows the quality of the resolution of the system in this range.

Through the analysis by SDS-PAGE of the collected fractions, the good resolution of the SE-HPLC peaks was confirmed (data not shown). The main peaks showed almost no overlap of their polypeptide composition and represented the glutenin (peak I), gliadin (peak II), and albumin and globulin (peak III) proteins.

Extracted samples were tested for stability by reinjection after 24, 48, and 72 hr, observing the same behavior previously reported by Larroque et al (2000). The reproducibility of the absolute size of peaks I and II was  $\pm 1.5\%$  between injections of the same extract and  $\pm 1.5\%$  for different extractions of the same sample run consecutively. These results are in agreement with the values reported by Batey et al (1991).

Thus, the recommended procedure using 0.5% SDS and 0.05M sodium phosphate buffer, pH 6.9, to extract proteins from flour can be used as elution buffer for SE-HPLC analysis with this hydrophilic matrix. In addition, a good resolution of wheat flour protein can be obtained for a large number of samples. Polymeric proteins were clearly grouped and separated from monomeric proteins without losing resolution throughout more than 450 sample injections. Our results suggest a possible alternative to be included for the routine analysis procedure for a large number of samples.

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