

Procedure for Obtaining Stable Protein Extracts of Cereal Flour and Whole Meal for Size-Exclusion HPLC Analysis

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ABSTRACT

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Experiments were conducted to determine the extent of instability of size-exclusion HPLC extracts prepared from flour, semolina, or whole meal. Procedures to obtain stable extracts were investigated. Whole meal extracts of durum wheat and triticale were the most unstable samples, whereas bread wheat showed smaller changes. Samples prepared from crushed maturing grains, especially during early stages, were greatly influenced by the instability process. By using protease inhibitors, evidence was obtained that endogenous proteases were the source of the instability.

Reproducible peak 1 (polymeric protein) results were obtained for a period of at least 72 hr after extract preparation if protein extracts were heated for 2 min at 80°C in a water bath immediately after filtration into the sample vials and before SE-HPLC analysis. This treatment is a viable solution to avoid sample instability in whole meal and developing grain extracts, particularly when large sample sets are prepared for automatic injection into the HPLC.

Size-exclusion HPLC is a valuable technique widely used nowadays for measuring the relative proportions of the main endosperm proteins (glutenins, gliadins, albumins, and globulins) of bread wheat and other cereals such as durum wheat and triticale. These determinations are particularly important because the relationship between protein classes (e.g., glutenin-to-gliadin ratio) and the molecular size distribution of polymeric proteins affect quality attributes such as mixing properties estimated by the mixograph (Gupta et al 1993, Gupta and MacRitchie 1994). The reliability of SE-HPLC and the small amount of sample required for the determination make it an ideal tool for the early detection of promising material in plant breeding programs.

Although as little as 10 mg of white flour is sufficient for SE-HPLC analysis, milling of grains to obtaining white flour or semolina (mill product coarser than flour, usually obtained from durum wheat) requires a larger amount of grain material, mainly defined by the mill's performance. Due to the fact that in very early generations it is not possible to have enough grains to accomplish milling, whole meal flours have been used as an alternative. Furthermore, for study during grain development, crushed whole grains or endosperm parts are used instead of flour (Khan and Bushuk 1976). In these cases, instability of samples when screening large sample sets of cereals by automated sampling SE-HPLC is observed. Huebner and Bietz (1985) and Dachkevitch and Autran (1989) suggested that proteases could be involved in the decrease of the polymeric protein peak of the chromatogram (peak 1). Previous studies had shown that proteolytic activity was present in grains of bread and durum wheat, particularly during early development stages (Bushuk et al 1971). Peak 1 is mainly made up of glutenins (Batey et al 1991, Larroque et al 1997), which are the most important endosperm proteins related to quality properties of flour. They are composed of polymers of high molecular weight and low molecular weight glutenin subunits joined by disulfide bonds. High molecular weight glutenin subunits (HMW-GS) have been widely studied in the last 20 years (Payne et al 1981, Shewry et al 1992) and according to

Gupta et al (1995) and Popineau et al (1994) are the major proteins contributing to dough properties. More recently, research conducted on the effect of insect enzyme damage to wheat flours showed proteases had a selective action on the hydrolysis of HMW-GS (Every et al 1998). In addition, the effect of fungal proteases, associated with Fusarium head blight, on wheat storage proteins has influenced the stability of extracts used for SE-HPLC analysis (Nightingale et al 1999).

Dachkevitch and Autran (1989) recommended sample extraction at 60°C for 2 hr to avoid sample deterioration. Jia et al (1996) stirred flour samples for 2 hr at room temperature, followed by a denaturation of the proteases for 5 min at 90°C before centrifugation.

The aim of this work was to identify the magnitude of extract instability in flour, semolina and whole meal samples (mature and developing grain) and to develop a procedure for obtaining stable extracts.

MATERIALS AND METHODS

Plant Material

Durum wheat (*Triticum turgidum* L. group durum (Desf.) Husnot cultivars Abadia (HMW-GS 20x+20y), Mexicali (7+8), Rugby (6+8), Wollaroi (7+8), Celta (7+8), Castigo (6+8), and Almoçave (20x+20y), bread wheat (*T. aestivum* L.) cultivar Hartog (1,17+18,5+10), and a triticale (*X Triticosecale* Wittmack) sample were used in the experiment. Extracts were prepared from flour, whole meal, or semolina. Crushed freeze-dried grain samples from bread wheat cultivar Buck Poncho (2*,7+8,5+10), harvested at weekly intervals during grain development (7, 14, 21, 28, and 40 days after anthesis) were also tested.

SE-HPLC

For total protein analysis, 10 mg of material was extracted with 1 mL of 0.5% SDS-phosphate buffer, pH 6.9, subjected to 15 sec of sonication, centrifuged at 17,000 × g for 15 min; the supernatant was filtered through a 0.45-µm filter into a glass vial. Samples were immediately heated for 2 min in a water bath at 80°C and placed in the autosampler carousel for automatic injection. Samples were kept in the carousel until the last injection. Total protein extracts (20 µL) were injected into a Phenomenex Biosep SEC-4000 column (Phenomenex, Torrance, CA) and run for 35 min on an isocratic gradient of 50% water (+0.05% TFA) and 50% acetonitrile (+0.05% TFA) using an HPLC system series 600 (Waters Corp., Milford, MA) comprising two model 510 pumps, a 712 WISP automatic sampler, and a model 481 UV-visible

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detector at 214 nm (Batey et al 1991). Relative molecular weight distribution of polymeric proteins, obtained by separately measuring the extractable (which does not include sonication treatment) and unextractable polymeric protein (where the pellet obtained from the extractable step is sonicated for 30 sec) was assessed according to Gupta et al (1993).

To prove the involvement of proteases in the instability process, protease inhibitors antipain and carboxypeptidase inhibitor (all supplied by Sigma (St. Louis, MO) were added to the standard extraction buffer at 4 µg/mL.

SDS-PAGE

Extracts were freeze-dried immediately after loading aliquots on to the SE-HPLC column, dissolved in 200 µL of 2% SDS extraction buffer containing β-mercaptoethanol and loaded onto 10% SDS gels (Payne et al 1981). Gel scanning was performed using a ScanJet 5100C scanner (Hewlett Packard Co., Palo Alto, CA) and Phoretix 1D vers. 3.01 software (Phoretix International, Newcastle upon Tyne, England).

Experimental Milling

Grains from Hartog were milled using a Bühler experimental mill (Bühler AG, Uzwil, Switzerland) to investigate the effect of the different fractions obtained with this mill on the stability of the samples. Eight different cuts were obtained as products: reduction

roll 1, reduction roll 2, reduction roll 3, break roll 1, break roll 2, break roll 3, pollard (bran containing some flour), and bran. A blended flour (R1+R2+R3+B1+B2+B3) was used as a control. Measurements were recorded at 0, 2, 4, 24, 48, and 72 hr after extract preparation.

RESULTS AND DISCUSSION

Different degrees of sample stability were found for the analyzed samples. This was related to the ratio between external coats and endosperm, particle size, and enzyme activity. Samples with smaller particle size showed more stability. Flour samples were more stable than semolina and much more stable than whole meal. Figure 1A shows typical overlapped SE-HPLC profiles of unheated samples of Rugby at 0 and 72 hr after extract preparation. A clear indication of sample instability is present in the 72-hr extract, where a decrease in the area of peak 1 along with an increase in the area of peak 3 is observed. Similar behavior was found when developing grain extracts were analyzed (Fig. 1B).

A heating treatment was used to overcome such deterioration. Heating of the extracts during 2 min at 80°C in a water bath immediately after centrifugation and filtration into the sample vials and before SE-HPLC analyses was sufficient to keep the sample stable for at least 72 hr. Figure 1C shows SE-HPLC profiles of heated extracts from Rugby at 0 and 72 hr after sample preparation. In this case, only a minor difference (middle to end part of peak 1) is found between both overlapped profiles. The SDS-PAGE fractionation (Fig. 1D) shows 1) freeze-dried extract of a fresh sample from Rugby, 2) freeze-dried extract of a heated sample after 72-hr of being prepared, and 3) freeze-dried extract obtained from an unheated sample after 72-hr of being prepared. Notably, almost complete disappearance of HMW-GS and some ω-gliadins occurred in lane 3, while the heated extract (lane 2) showed similar composition as the fresh extract (lane 1). When fresh and 72-hr semolina and whole meal extracts from Celta, Castigo, and Almoçave were compared, results showed differential behavior. In all cases, whole meal extracts were less stable than semolina, as indicated by the relative proportion of HMW-GS bands in SDS-PAGE gels. For Celta, while fresh whole meal extracts had 9.87 + 4.99% for HMW-GS 7 + 8 respectively, 72-hr extract values were 2.79 and 2.50%, respectively. On the other hand, semolina extract values were 9.5 + 5.38% for fresh samples and 6.45 + 4.19% for the 72-hr sample, respectively. Values for Almoçave (HMW-GS 20) were 16.92% (fresh whole meal), 6.69% (72-hr whole meal), 15.35% (fresh semolina) and 14.49% (72-hr semolina extract). Whole meal extracts from Castigo (HMW-GS 6+8) had values of 5.87 + 2.99% (fresh) and 2.64 + 2.59% (72-hr extract). Results for semolina

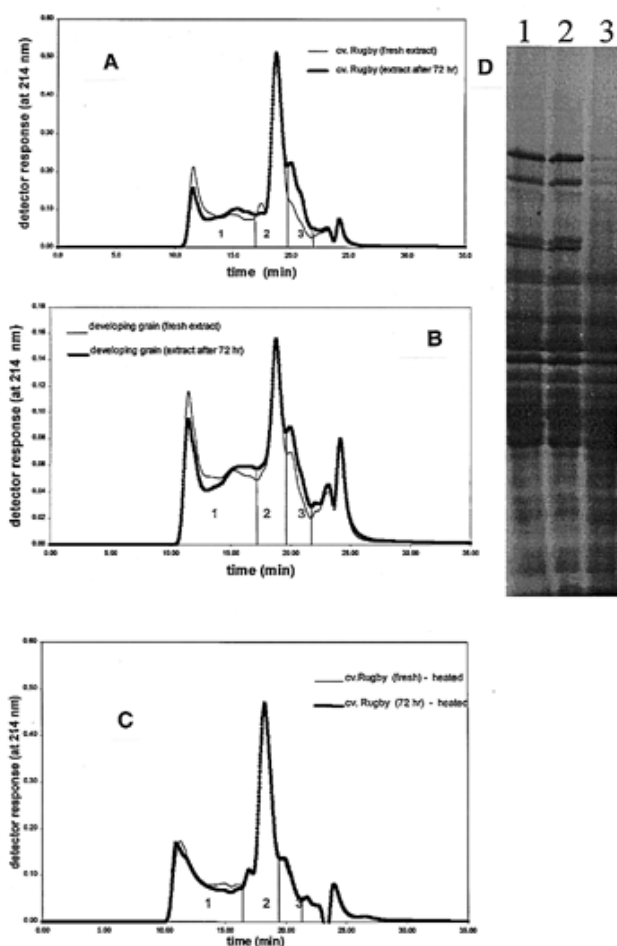


Fig. 1. A, Overlapped Size-exclusion HPLC profiles of fresh and 72-hr extracts from cv. Rugby. B, Overlapped SE-HPLC profiles of fresh and 72-hr extracts obtained from developing grain (whole meal) of cv. Buck Poncho. C, Overlapped heated samples of fresh and 72-hr extracts from cv. Rugby. D, SDS-PAGE fractionation of freeze-dried extract of a fresh sample from cv. Rugby (1); freeze-dried extract of a 72-hr heated sample (2); (3) freeze-dried extract obtained from a 72-hr unheated sample.

TABLE I
Size Exclusion HPLC Peak 1 Values (% of total area) from Unheated and Heated Samples

Cultivar	SE-HPLC % Peak 1			
	Fresh	0 hr	33 hr	72 hr
Unheated				
Ardente		35.97a	33.54b	31.19b
Abadia		35.98a	29.98a	25.56a
Rugby		38.88b	35.49bc	31.24b
Mexicali		43.02c	37.14cd	32.21b
Triticale spp.		45.49d	39.92d	35.62bc
Wollaroi		48.33e	44.36e	39.95b
Heated				
Ardente	38.03a	36.35a	36.42a	36.04a
Abadia	40.00b	37.57ab	37.85ab	37.34ab
Rugby	40.02b	38.18b	38.75b	38.86b
Mexicali	46.29c	44.68c	44.93c	44.21c
Triticale spp.	47.45c	45.97c	46.36c	46.39d
Wollaroi	49.97d	48.82d	49.86d	48.10d

^a Values within the same subset (unheated or heated) followed by the same letter are not significantly different ($P < 0.05$).

extracts from the same cultivar were 6.89 + 4.51% (fresh) and 4.36 + 3.50% (72-hr extract), respectively.

Table I shows the effect of instability of the samples on the polymeric peak of the SE-HPLC profile (%) when unheated and when heated. Results are dependent on extract stability, with statistically significant differences between treatments varying with time.

The disappearance of HMW-GS in peak 1 is associated with an increase in peak 3, corresponding to albumins and globulins in a fresh sample. We assumed that the change in HMW-GS could be explained by the action of proteases that affect particularly the HMW-GS, producing small fragments of proteins that, because of their small size, are detected in the region of peak 3 on the HPLC. Larger amounts of enzymes in the grain of wheat and cereals in general are located in the external area of the grain (bran and aleurone). The results of the experiment with inhibitors showed that samples prepared with the buffer containing antipain (a nonselective serine and cysteine protease inhibitor) had better stability than the other inhibitor and the untreated control, but less stability than the heated extract (polymeric peak at 0 hr = 100%; antipain at 72 hr = 96.32%, carboxypeptidase inhibitor = 94.59%, unheated control = 91.04%, heated control = 99.64%). When different streams from a Bühler experimental milling of Hartog were extracted for SE-HPLC analysis, results showed a different degree of stability according to the type of fraction analyzed. Bran and pollard were the samples that suffered the most changes. Pollard percentages were 87.1, 77.9, and 71.1% (polymeric peak at 0 hr = 100%) at 24, 48 and 72 hr after extract preparation. Bran had values of 90.5, 85.3, and 80.3% for the same periods. On the other hand, samples from break and reduction roll showed a more stable behavior. Polymeric peak percentages after 72 hr of extraction were 89.1, 92.2, and 89.8% for reduction rolls 1, 2, and 3; and 93.9, 94.0, and 87.8% for break rolls 1, 2, and 3, respectively. A pool of the six rolls (which could be considered as commercial flour) had a value of 94.03% of the original polymeric peak 72 hr after preparation. Because the particle size is to a certain extent associated with the ratio between external coats and endosperm, it seems that the particle size is an indirect measure of potential enzyme activity.

CONCLUSIONS

When whole meal or flour sample extracts (from mature or immature grains) are heated in a water bath at 80°C for 2 min, SE-HPLC results are not affected by changes in the proportion of peak 1 (decreasing, when not heated) and peak 3 (increasing, when not heated). The instability, which happens in samples not immediately injected into the SE-HPLC column after extraction, is particularly important in whole meal samples from durum wheat as well as from immature grains.

The treatment presented here does not introduce any appreciable change in the profile of the chromatograms, it is not toxic, as could be the case when using enzyme inhibitors of chemical origin, nor is it time consuming or expensive.

The nature of the proteolysis was enzyme-related and indirectly related to particle size and the ratio between external coats and endosperm.

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