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Analysis of wheat flour proteins related to grain hardness using capillary electrophoresis

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Abstract

A simple and fast capillary electrophoresis method was used for the analysis of wheat flour proteins related to grain hardness. The study has shown that the ratio of the peak areas of two proteins, puroindolines *a* and *b*, can be used to distinguish hard and soft varieties of breadwheat. In addition, the results indicate that hard breadwheats can be further separated into two different sub-types. This novel finding suggests that hard breadwheat may have evolved from the original soft breadwheat by two different routes. The method has the potential to provide a new diagnostic tool for wheat breeders and the milling industry.

Keywords: Wheat flour; Proteins; Puroindolines; Friabilins

1. Introduction

The milling quality of breadwheat (*Triticum aestivum*) is usually described as being 'hard' or 'soft'. Durum wheat (*Triticum durum*), preferred for pasta making, is described as 'very hard'. Operationally, milling quality depends mainly on the physical hardness of the starchy endosperm of the grain, the 'endosperm texture'. Studies to understand milling quality [1,2] have established that hard and soft allelic forms of a single gene, the *Hardness* gene, are responsible for the bimodal distribution of endosperm texture amongst *T. aestivum* cultivars.

A protein named 'friabilin' because of its association with soft, i.e. friable, endosperm [3–5], and other related lipid-binding hydrophobic proteins extracted from wheat flour by means of the non-ionic detergent Triton X-114 (TX114) (puroindolines *a* and *b*) [6,7], are intimately involved in the phenomenon of endosperm texture in a way that is still not clearly defined, though both friabilin and one puroindoline have been closely linked to the *Hardness* gene [8].

Capillary electrophoresis (CE) is a highly efficient separation technique that is easily automated, has the advantage of on-line detection and uses little sample and reagents. Recently, the CE technique was used to differentiate cultivars of wheat, oats and rice [9,10]. A low pH phosphate buffer with the addition of cellulose was used to separate wheat proteins (gliadins), oat proteins (avenins) and rice proteins (prolamins). A similar approach was also used for the analysis of genetic variants of milk proteins [11,12]. Simultaneous separation of whey proteins and casein fractions provided the fingerprints corresponding to the genetic variants in milk from different species. At low pH, the charge of the silanol groups on the capillary wall is reduced to almost zero. This reduces electroosmotic flow (EOF) and

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the attraction of positively charged proteins to the capillary wall. The adsorption of proteins is prevented even further by adding modified celluloses [9,10] and by the use of coated capillaries [11,12]. Thus, CE is an obvious choice for the present work, which aims to identify biochemical markers for grain hardness, from the fine detail of the puroindoline/friabilin populations in wheat varieties.

2. Experimental

2.1. Chemical

Tris [tris(hydroxymethyl)aminomethane], EDTA (ethylenediamineteraacetic acid, disodium salt: dihydrate) and Triton X-114 (*tert*.-octylphenoxypolyethoxyethanol) were from Sigma (Poole, UK). Potassium chloride, sodium dihydrogenorthophosphate monohydrate and other chemicals were all AnalaR regrade from BDH (Poole, UK). Diethyl ether and ethanol were from Rathburn (Walkerburn, UK). MHPC (methylhydroxypropylcellulose, viscosity of a 2% aqueous solution 40 000–60 000 cP) was from Hercules (Salford, UK). The water used was from the Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. Wheat flours

White flours were taken from the 1995, 1996 and 1997 UK wheat harvests, from samples of pure varieties grown at various sites under the Recommended List Growing Trials conducted by the National Institute of Agricultural Botany. The wheats were milled on a laboratory Buhler mill at the Campden & Chorleywood Food Research Association under a standard milling protocol. The particle size index (PSI) of the Buhler-milled flours was measured as the percentage mass of particles able to pass through a 20-cm metal sieve with 75-μm mesh. Endosperm texture of a grain sample was assigned according to the rule that Hard<50%<Soft for the Buhler-milled flours.

Owing to changes of the milling process which started with the 1997 harvest, only the PSI values from wheat harvest of years 95/96 were available for the purpose of this work.

2.3. Extraction of proteins from wheat flours

The extraction method used was a modification of a procedure described by Blochet [6]. Soluble proteins were first extracted by mixing 0.5 g wheat flour with 5 ml of Tris–KCl buffer (pH 7.8, 100 m*M* Tris–HCl, 5 m*M* EDTA+0.1 *M* KCl) at 4°C for 1 h. After centrifugation at 2 000 *g* the residue was mixed with 5 ml of Tris–KCl buffer containing 4% (v/v) TX114 at 4°C for 1 h. After centrifugation as before, the supernatant was collected and heated at 30°C (above the 'cloud point' of this detergent) for 1 h, and the resultant emulsion was centrifuged. Tris–KCl buffer (5 ml) containing 1% (v/v) TX114 was then added to the separated lower TX114-rich layer. The solution was mixed at 4°C for 30 min, heated at 30°C for 1 h and centrifuged. To obtain a crude puroindoline extract and remove TX114, the lower TX114-rich layer was mixed with 5 ml of diethyl ether–ethanol (1:3, v/v) and left overnight (−20°C). The precipitated proteins were collected by centrifugation, and washed with diethyl ether–ethanol (1:3, v/v) to remove all traces of the TX114 detergent. The protein pellet was air-dried briefly and resuspended into 0.5 ml 50 m*M* acetic acid. The protein concentration of this solution was determined using the Bradford dye-binding assay [13]. The protein extracts were then standardised to 0.5 mg/ml total protein for CE analysis.

2.4. The analysis of protein extracts by CE

Analyses were carried out using a Beckman P/ACE MDQ system (Beckman Instruments, Fullerton, CA, USA) controlled by an IBM PC 350 computer with PACE system MDQ software, version 1.2. The separation was performed using a fused-silica capillary (Composite Metal Service, Hallow, UK), 40 cm (30 cm to detector)×50 μm I.D. The sample was introduced at the anode with pressure (0.01 MPa, 20 s). The electrophoresis buffer was 100 m*M* phosphate buffer containing 0.05% MHPC, pH 2.5 [9]. The protein separation was carried out with a constant voltage of +15 kV at 25°C and monitored by UV detection at 214 nm. Before each run, the capillary was rinsed with 0.1 *M* NaOH (0.14 MPa, 1 min), followed by the water (0.14 MPa, 1 min) and the electrophoresis buffer (0.14 MPa, 3 min).

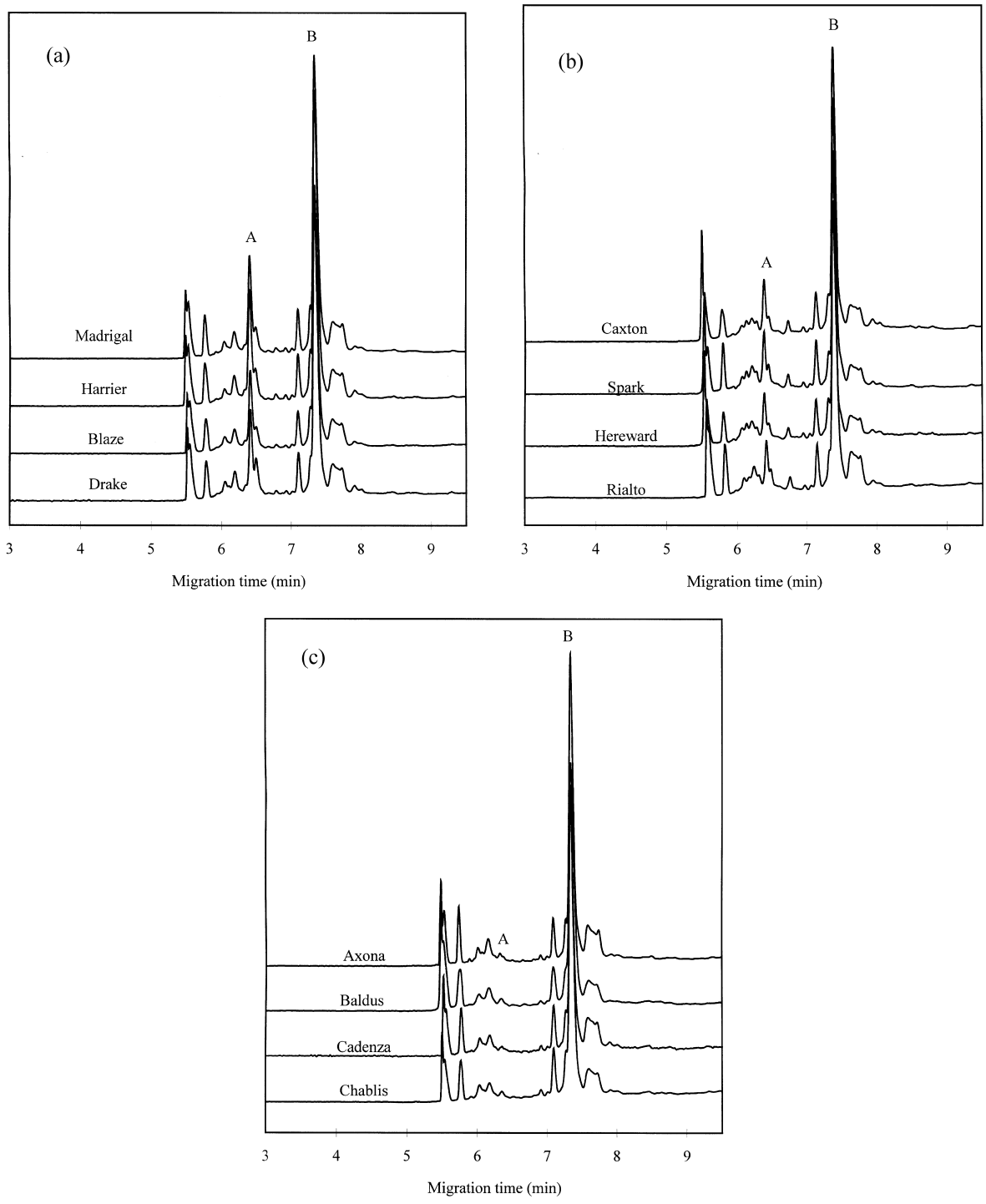

Fig. 1. CE patterns of breadwheat: (a) soft, (b) hard-type 1, (c) hard-type 2. Conditions: capillary, 40 cm (30 cm to detector)×50 μm I.D. fused silica; buffer, 100 m*M* phosphate containing 0.05% MHPC, pH 2.5; injection, 1.5 p.s.i., 20 s (1 p.s.i.=6894.76 Pa); separation, +15 kV, 25°C; absorbance, 214 nm. Peaks: (A) puroindoline *a*; (B) puroindoline *b*.

Identification of the peaks corresponding to puroindolines *a* and *b* was carried out by spiking with authentic pure lyophilised proteins kindly supplied by Dr D. Marion, INRA-Nantes, France.

3. Results and discussion

For each wheat variety, duplicate flour samples from different harvest years at various sites were used to represent the variation of sampling within a single variety. Duplicate extraction was carried out for each wheat flour and duplicate CE runs for each protein extract.

Fig. 1 shows the CE patterns of soft and hard breadwheat. Peak A was identified as puroindoline *a* and peak B as puroindoline *b* by spiking with the pure lyophilised proteins. Using a peak area ratio of puroindoline *b*/puroindoline *a*, it was found that all soft varieties (PSI>50%) tested had a ratio of less than 4, whilst all hard varieties had a ratio of greater than 4 (Table 1). Hard varieties were also found to consist of two distinct groups, i.e. those with a ratio of 4–10 (type 1; Fig. 1b) and those with a ratio greater than 30 (type 2; Fig. 1c).

Table 1
Protein peak area ratio (peak B/peak A) and the particle size index (PSI) for different varieties of wheat

Variety	Ratio of peak B/peak A in CE profiles		PSI (%), year 95/96
	Year 95/96	Year 97	
Blaze	2.9	3.3	57
Consort	3.3	3.5	56
Crofter	2.6	3.8	49
Drake	3.1	3.5	53
Harrier	3.5	3.0	52
Madrigal	3.5	3.4	61
Riband	3.3	3.0	56
Abbot	5.9	6.9	41
Caxton	4.8	6.7	40
Charger	5.7	7.4	41
Hereward	7.7	7.3	38
Rialto	5.5	6.8	42
Spark	5.9	5.1	40
Axona	40	41	38
Baldus	40	41	36
Caxenza	51	62	38
Chablis	53	52	36
Soissons	30	34	39

The relative peak area ratios of B/A from the same extract were shown to be highly reproducible, 0.98% R.S.D. for 10 analyses within 1 day, and 1.64% R.S.D. for 20 analyses over a period of 1 week.

Statistical analysis of the CE peak area ratio data indicated that the major source of variation in the data was the flour type (soft, hard-type 1 or hard-type 2) and that this was considerably greater than other factors, such as the year in which the wheat was grown, and experimental variability associated with extraction and CE analysis (Table 2a).

Differences between the peak area ratios for the three flour types were statistically significant, the 95% confidence intervals indicating no overlap between the soft, hard-type 1 and hard-type 2 CE peak area ratios of peak B/A (Table 2b).

The results clearly suggest that the CE protein profiling of wheat flour protein extracts was not only able to distinguish soft and hard varieties of breadwheat, but also the division of two types of hard varieties.

The biochemical distinction, in terms of puroindoline *b*/puroindoline *a* ratios, between two different subtypes of hard *T. aestivum* varieties is apparently a novel finding. However, during the course of the present work, two papers [14,15] on the molecular genetics of North American wheat varieties showed that there are two subtypes of the hard allele of the

Table 2
Results of statistical analysis (analysis of variance, ANOVA) to estimate the variability due to sampling, flour type, extraction and CE analysis, and to show the significance of the identification of different flour types (soft, hard-type 1 and hard-type 2) using the peak area ratio

(a) Variation contributed by each factor

Factor	Variance estimate
Sampling (year 95/96 or 97)	0.00
Type of flour (soft, hard-type 1 or hard-type 2)	1.80
Extraction within a variety	0.12
CE replicate analysis of each extract	0.02

(b) 95% Confidence intervals (C.I.)

Wheat flour type	Mean ratio (peak area B/A)	C.I. (95%)
Soft	3.28	2.55–4.20
Hard-type 1	6.40	4.99–8.21
Hard-type 2	43.76	34.13–56.12

Hardness gene. The authors interpreted their DNA sequence data in terms of two different mutations in an original form of the *Hardness* gene, corresponding to the original hexaploid *T. aestivum* as it evolved by addition of the DD genome to the AABB genome of a tetraploid wheat. Since the DD diploid species is very soft, this original *T. aestivum* was assumed to have the soft allele of the *Hardness* gene, and the two hard forms were taken as having subsequently evolved from this.

One of the mutations was identified as conversion of the puroindoline *a* gene to a null form that is not expressed in the wheat. It is reasonable to conjecture that this type of hard breadwheat corresponds to the type identified in the present work as having a puroindoline *b*/puroindoline *a* CE ratio greater than 30; it may be that this represents an infinite ratio, i.e. the absence of puroindoline *a*, which has been lowered by the presence of traces of other proteins at the migration point of puroindoline *a* in the CE profiles.

The second mutation identified by Giroux and Morris [14,15] was found to be a point mutation which converted a single glycine residue in puroindoline *b* into a serine residue. It is not clear whether this second type of hard North American variety is the same as the second type of hard European variety that was found in the present work as having a B/A ratio in the range 5.0–7.5. Work on both pedigrees of wheat by both experimental approaches would have to be done to establish such an identity. There is currently no evidence that the identity is not true, but it cannot be ruled out that more than two subtypes of the hard allele of the *Hardness* gene have evolved around the world, during the millenia that have passed by since the emergence of hexaploid breadwheat.

Although only one set of PSI data was available, the close correspondence (Fig. 2) between operational endosperm texture (PSI) and the precise biochemical and genetic nature of the puroindolines expressed in the endosperm, is good evidence upon which to draw the conclusion that puroindolines are in fact the product of the *Hardness* gene (considered to be a

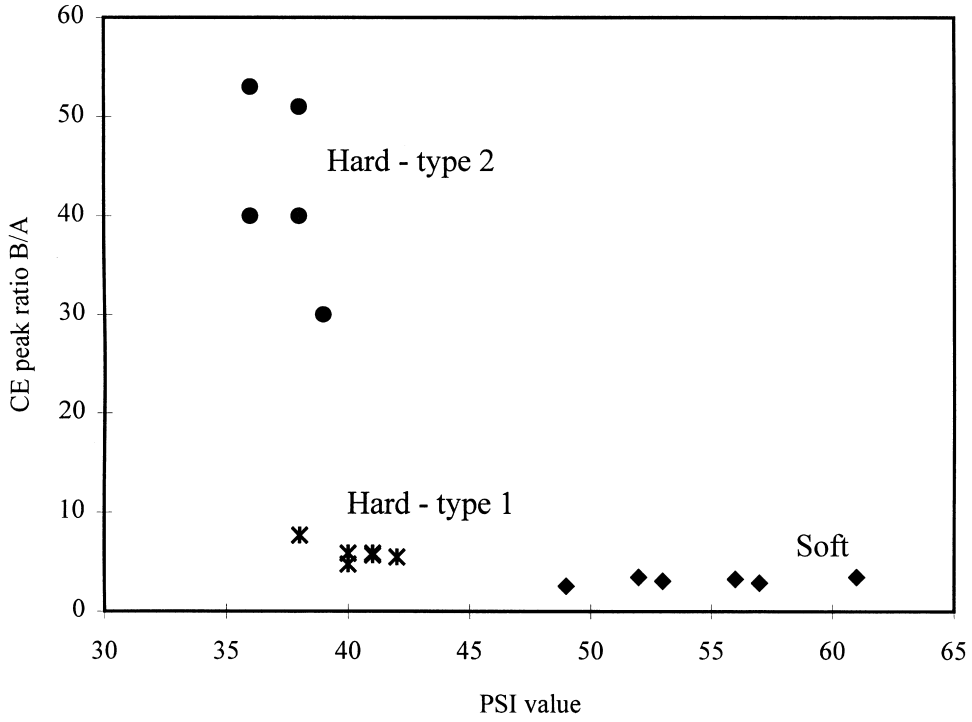

Fig. 2. Ratios for peak areas B/A of wheat flours plotted against endosperm texture of the wheats as measured by particle size index (PSI) of the flours (hard<50%<soft).

complex locus). Further, it is also reasonable to conclude that these puroindolines are the major causative factor for the physical differences seen between hard and soft endosperms of breadwheat, which exhibit, respectively, strong and weak adhesion between the starch granule and the surrounding protein matrix.

4. Conclusions

CE was used for the first time to provide identification of biochemical markers for grain hardness. Using the ratio of the peak areas of the two proteins—puroindolines *a* and *b* (the products of the *Hardness* gene)—soft and hard varieties of breadwheat were distinguished; in addition, two sub-types of hard breadwheat were discovered.

The main advantage of CE compared with sodium dodecyl sulfate–polyacrylamide gel electrophoresis is the on-line quantitative detection of proteins, and compared with HPLC, the small sample volumes that can be used (nl–μl). The present extraction method uses 0.5 g of flour. Theoretically it is possible to miniaturise it further, such that it could be used on half a single grain (10–20 mg of flour). This would provide a new diagnostic tool for wheat breeders and the milling industry.

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