HETEROGENEITY OF WHEAT ENDOSPERM PROTEOLIPIDS (CM PROTEINS)

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Key Word Index—*Triticum aestivum*; *Triticum durum*; *Aegilops squarrosa*; Gramineae; wheat; proteolipids; CM proteins; gliadins; albumins; electrofocusing; electrophoresis.

Abstract—Proteins extracted with $CHCl_3$–MeOH from wheat endosperm have been fractionated by Sephadex G-100 and the 15000–20000 MW range fraction, designated CM protein, has been examined by combined electrofocusing (pH range 5–8) and electrophoresis (pH 3·2) and the heterogeneity of the electrophoretic components has been ascertained. It has been shown by joint mapping and by sequential extraction that CM proteins are extracted by 70% EtOH but not by H_2O, although they can be made water-soluble after dialysis against an acid buffer, pH 3·2, 3 M urea, without losing their solubility in $CHCl_3$–MeOH mixtures. It is concluded that CM proteins fit the definition of a Folch–Lees proteolipid. The *Triticum aestivum* (genomes ABD) map can be reconstructed by mixing *T. durum* (AB) and *Aegilops squarrosa* (D). The low intragenomic variability of CM protein is confirmed.

INTRODUCTION

The electrophoretic patterns (pH 3·2) of $CHCl_3$–MeOH (2:1, v/v) extracts of partially delipidated endosperm from *Triticum aestivum* L. consistently show three fast main bands, CM1, CM2 and CM3, and those of *T. durum* Desf. only two, CM2 and CM3 [1]. Garcia-Olmedo and Carbonero [2] purified and partially characterized CM1 and CM2, showing that they were controlled by chromosomes 7D and 7B respectively. The third component, CM3, has been recently purified by Redman and Ewart [3] and by Aragoncillo [4]. The latter has also purified a faster variant of CM3, designated CM3', which is present only in a few *T. durum* varieties.

There is indirect evidence that more than one protein might be present in band CM3 of *T. aestivum* (genomes ABD): (i) both *Aegilops squarrosa* (D) and *T. durum* (AB) have a CM3 band [4]; (ii) the CM3/CM2 and CM3/CM1 ratios, which are rather constant within the species, are significantly depressed in both ditelosomics and in the monosomic of chromosome 4A, but no suppression of CM3 takes place [4]; (iii) amino acid analysis of CM proteins purified by ion exchange [3] and by direct elution of trichloroacetic acid precipitated bands from an acrylamide slab show a considerable lack of agreement [4].

The electrophoretic patterns (pH 3·2) of wheat endosperm proteins extracted with 70% ethanol (EtOH) have a number of components migrating ahead of gliadins which are of great taxonomic significance, as shown by Johnson and co-workers [5–7]. Waines [8] has recently implicated homoeologous chromosome groups 4 and 7 in the control of some of these proteins. CM proteins are soluble in 70% EtOH and the three variant patterns, CM1–CM2–CM3, CM2–CM3, and CM2–CM3', can be recognized among the more complex patterns of EtOH-extracted proteins [3, 4]. It has also been observed that these proteins are at least partially soluble in water [3, 4].

We report here an investigation of the heterogeneity of CM proteins and their relationship with the 70% EtOH and H_2O-extracted proteins.

RESULTS

Gel filtration

Proteins extracted with chloroform–methanol ($CHCl_3$–MeOH) (2:1) from partially delipidated

Table 1. Distribution of map components in different protein preparations*

Protein preparation	Component number†																					Others
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Purified CM-protein (CM)	−	+	+	+	−	−	−	+	+	−	+	+	+	−	−	+	+	−	−	−	−	−
Chloroform–methanol extract (CME)	−	+	+	+	+	−	−	+	+	−	+	+	+	−	−	+	+	−	−	±	±	−
70% ethanol extract (EE)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Water extract (WE)	+	−	−	−	+	+	+	−	±	+	−	−	−	+	+	+	−	±	±	±	+	+

* +, Present; −, absent; ±, detected at the overloading level.
† See Fig. 2.

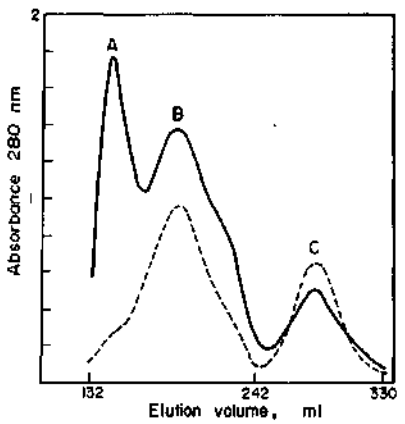

Fig. 1. Sephadex G-100 gel filtration profile of extracts. Crude $CHCl_3$–MeOH extract (——) and the same extract after dialysis, centrifugation and freeze drying of the supernatant (– – –). Peak A are glutenins, peak B gliadins and peak C CM-proteins.

wheat endosperm can be fractionated on Sephadex G-100 (Fig. 1). Three fractions are obtained: peak A, which appears with the exclusion volume, is precipitated by dialysis against water and is probably composed of glutenins; peak B elutes in the 40000–60000 MW range and yields an electrophoretic pattern identical with that of the classical gliadins extracted with 70% EtOH; and peak C, which is eluted in 15000–20000 MW range, consists of the CM proteins. Proteins extracted with 70% EtOH or with water also yield a peak in the same range as peak C, which includes the proteins that move ahead of gliadins in electrophoresis at pH 3·2 [9, 10].

Combined electrofocusing and electrophoresis

Two-dimensional mapping by combined gel electrofocusing and electrophoresis was carried out at optimal and at overloading concentrations of the following protein preparations: peak C (CM), $CHCl_3$–MeOH extract (CME), 70% EtOH extract (EE) and water extract (WE). Binary mixtures of these extracts were also run to confirm homology of spots. A composite map for *T. aestivum* var. Chinese Spring is presented in Fig. 2 and the observed distribution of components in the different preparations is summarized in Table 1. Densitometric profiles of the three latter types of preparation from *T. aestivum* var. Candeal are superposed in Fig. 3. Maps of CM and CME have

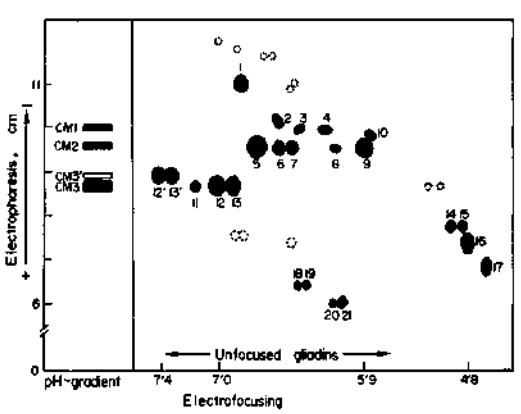

Fig. 2. Composite map obtained by combined electrofocusing and electrophoresis of protein extracts. Spots shown in broken line represent components extracted only with water (A components).

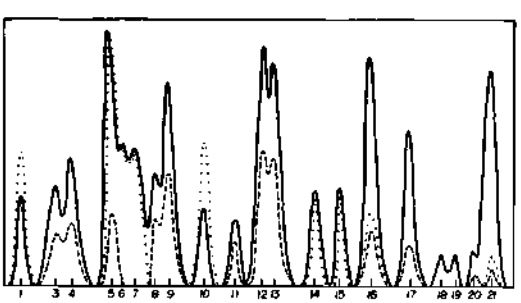

Fig. 3. Densitograms of maps obtained with different protein preparation of *Triticum aestivum* var. Candeal. Spot numbers are those given in Fig. 2. EE, 70% EtOH extract (——); WE, water extract (·····); CME, $CHCl_3$–MeOH extract (– – –). All three extracts were obtained from the same amount of endosperm (25 mg). The CM protein densitogram, at the appropriate scale, was identical with that of CME but without peaks 5, 10 and 21.

the same components in the zone of faster electrophoretic migration, with the exception of component 5 and the traces of 20 and 21 which are missing from CM.

All CME components are extracted more readily with 70% EtOH: spots 2, 3, 4, 8, 9, 11, 12 and 13 in the CME map have 50–70% of the intensity of those in the EE map while spots 5, 16, 17, 20 and 21 in the CME map have 5–20% of the intensity; components 1, 6, 7, 10, 14, 15, 18 and 19 are only present in the EE map. These results are consistent with those obtained by sequential extraction. Extraction with $CHCl_3$–MeOH after 70% EtOH yields none of the components of the CME map, while extraction with 70% EtOH after $CHCl_3$–MeOH gives a complete EE map in which spots 2, 3, 4, 8, 9, 11, 12 and 13 are much weakened.

The WE map has 9 components in common with the EE map (spots 1, 5–7, 10, 14–16 and 21). Some of these, 14–16 and 21, are extracted more effectively with 70% EtOH than with water. In addition WE has a number of components, designated A in Fig. 2, which are not detected in either CME or EE. Extraction with water after 70% EtOH yields only the A components plus spots 1 and 10. The map obtained with the 70% EtOH extract of water extracted flour closely resembles that obtained with CM. Thus, water does not extract CM proteins from flour. WE has two minor components overlapping with EE components 11–12 but different from them as demonstrated in *T. durum* var. Ledesma, whose EE map lacks 11 and has a variant of 12 but whose WE map still has the two weak components in the same position as 11 and 12.

Proteins extracted with $CHCl_3$–MeOH from EE yield the same map as CME. CM proteins are readily soluble in $CHCl_3$–MeOH, in water and in 70% EtOH after gel filtration in acid buffer, pH 3·2, 3 M urea, dialysis against water and freeze-drying. However, water does not extract CM proteins from CME or EE.

Analysis of tetraploid and hexaploid wheats

In Table 2, CME and EE phenotypes of hexaploid wheats (genomes ABD) and tetraploid wheats (AB), as well as that of *Aegilops squarrosa* (D), are recorded. Equivalence of spots was ascertained by joint mapping. All hexaploids have the phenotype of variety Chinese Spring with few exceptions: component 2 is absent in six varieties, a variant pattern is obtained for components 18–21 in two varieties, and some additional components are present in the synthetic *T. spelta* which are contributed by *T. carthlicum*.

All tetraploid phenotypes, natural as well as obtained by genome extraction, are identical, with the exception of variety Ledesma, which has components 12′–13′ of different mobility and isoelectric points than 12–13, and the already mentioned additional components of *T. carthlicum*. All components of *T. durum* maps are recognized in *T. aestivum* maps.

Table 2. Distribution of map components in *Aegilops squarrosa* and several *Triticum* genotypes*

Samples	Component number†																				Others
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
Triticum aestivum var.:																						
Chinese Spring	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Candeal	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Prelude	+	+	+	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+	+	+	+	−
Rescue	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Thatcher	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Traquejos	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	v	v	v	−
Canalejas	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	v	v	v	v	−
Chamorro	?	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Triticum spelta	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Triticum durum var.:																						
Senatore Capelli	−	−	−	−	−	+	+	+	+	?	−	+	+	+	+	+	−	+	+	+	+	−
Ledesma	−	−	−	−	−	+	+	+	+	?	−	v	v	+	+	+	−	+	+	+	+	−
Tetraprelude	−	+	−	−	−	+	+	+	+	?	−	+	+	+	+	+	−	+	+	+	+	−
Tetrarescue	−	−	−	−	−	+	+	+	+	?	−	+	+	+	+	+	−	+	+	+	+	−
Tetrathatcher	−	−	−	−	−	+	+	+	+	?	−	+	+	+	+	+	−	+	+	+	+	+
Triticum carthlicum	−	−	−	−	−	+	+	+	+	?	−	+	+	+	+	+	−	+	+	+	+	+
Aegilops squarrosa	+	−	+	+	+	−	−	−	−	−	+	+	+	−	−	−	+	+	+	+	+	+

* +, Present; −, absent; v, possible genetic variant present; ?, presence not unequivocally established.

† See Fig. 2.

All components of *Ae. squarrosa* maps are present in *T. aestivum*. Three of them, 12, 13 and 20 are also present in *T. durum*.

DISCUSSION

Heterogeneity of CM proteins

The above results indicate that not only CM3 is heterogeneous, as suspected, but also other electrophoretic bands seem to yield more than one component.

The possibility of artifacts cannot be overlooked. These could arise during electrofocusing or in the previous handling of the protein preparations. Our unpublished genetic evidence permits discarding some of the potential cases of artifact formation: the genetic control of component 2 is independent from that of 3–4; gene(s) for component 5 are located in a different chromosome than those for 6–7 and 8–9; 11 is equally independent of 12–13; component 16 is controlled by a different chromosome than 17; and the genetic variation of components 18–21 precludes their being artifacts.

These considerations narrow the possibilities to the pairs 3–4, 8–9, 12–13 and 12′–13′, that could represent one protein each, and components 6, 7, 14 and 15, that are controlled by the the same chromosome arm and could be different modifications of the same protein.

Different electrofocusing conditions have been employed to investigate if these could alter the patterns: no significant changes were observed when persulfate was substituted by riboflavin in preparation of the gel or when the sample was applied on top of the column instead of incorporated into the polymerization mixture.

It does not seem likely that the pairs are the result of incomplete delipidation because the prolonged exposure to acid during gel filtration and dialysis against water does not alter the pattern. However, they could represent *in vivo* modified forms of the same polypeptides. In this connection, it is particularly striking that the two components of CM3′, 12′–13′, differ to the same extent in their isoelectric points and electrophoretic mobility from components 12–13 (CM3). In any case, the elucidation of this matter will depend on purification of the individual components or on further genetic studies.

In conclusion, CM3 and possibly CM1 and CM2 are heterogeneous, which would explain the already mentioned differences in the amino-acid composition data [3, 4].

Classification of CM proteins as proteolipids

Proteolipids were first isolated from brain tissue by Folch and Lees [11] and defined as proteins insoluble in water and aqueous solvents but soluble in $CHCl_3$–MeOH mixtures. Although especially abundant in nervous tissue, proteolipids are also widely distributed in animal and plant tissues [12]. Zill and Harmon [13] have reported on chloroplast proteolipids and Rohrlich and Niederauer [14] described a proteolipid from wheat endosperm, a non-photosynthetic plant tissue, which was recovered from the interface of the Folch wash of endosperm lipids extracted with $CHCl_3$–MeOH. The CM proteins are readily soluble in $CHCl_3$–MeOH (2:1) and in water after gel filtration in acid buffer, dialysis against water and freeze-drying. In a similar manner, the Folch–Lees protein is still soluble in $CHCl_3$–MeOH mixtures after it has been made water soluble by dialysis against solvents of increasing polarity, but is not water-soluble in its native state or when obtained by vacuum evaporation of the $CHCl_3$–MeOH extraction mixture [12, 15]. On this basis, CM proteins should be classified as true proteolipids and not as intrinsic contaminants of gluten of the albumin type, as Meredith [16] tentatively classified a fraction, designated σ, obtained from the $CHCl_3$–MeOH extract of gluten and probably equivalent to CM protein.

This proteolipid fraction is also a part of the so-called [9, 10, 17–19] albumins plus globulins peak of gel filtration profiles of proteins extracted with 70% EtOH and probably of similar gel filtration peaks from other extracts such as those obtained with 2M urea, diluted acids and aqueous alcohols.

Low genetic variability of CM proteins

The present results confirm at much greater resolution the previous observation by one-dimensional electrophoresis of the low intragenomic variability of the non-gliadin 70% EtOH proteins [5–7, 20] and of the CM proteins [1]. The extreme usefulness of these proteins in phylogenetic studies, which has been extensively demonstrated by Johnson and coworkers, is based precisely on this pro-

perty, which makes them good genomic markers. For the same reasons, these proteins should be excellent chromosome markers at the present degree of resolution.

The low genetic variability of these proteins is in sharp contrast with the great variability of gliadins recently observed by Wrigley and Shepherd [21] using combined electrofocusing and electrophoresis.

EXPERIMENTAL

Biological material. The following wheats were used in this study: *Triticum aestivum* varieties Chinese Spring, Candeal, Chamorro, Traquejos, Canaleja, Rescue, Thatcher and Prelude; *T. durum* varieties Senatore Capelli and Ledesma; *T. carthlicum* M-1; *Aegilops squarrosa* C-1; a synthetic *T. spelta* (*T. carthlicum* × *Ae. squarrosa*) and the tetraploid versions of Rescue, Thatcher and Prelude obtained by Kaltsikes *et al.* [22] by D genome extraction.

Extracts. The crude $CHCl_3$–MeOH (2:1) extract was obtained from delipidated flour as previously described [1] and dispersed in 3 M urea, dialysed against H_2O for 48 hr, centrifuged at 3000 *g* for 15 min, and the supernatant freeze-dried.

For the mapping experiments, from 1/2 to 6 kernels were crushed between two metal plates with a hammer, defatted with light petrol (50–70°, 10 v/w) and the solvent removed *in vacuo*. The samples were extracted 3× with the appropriate solvent (8 v/w each time) and the solvent of the combined extracts eliminated *in vacuo* or by freeze-drying. When sequential extractions were performed, after the third treatment with the first solvent (100 v/w), the solvent residues in the sample were removed *in vacuo* and the second solvent then added.

Gel filtration. Both the crude and the dialysed $CHCl_3$–MeOH extracts (100–400 mg of each) were subjected to gel filtration through Sephadex G-100 (2·5 × 90 cm column) in aluminium lactate buffer 0·1 M, pH 3·2, 3 M urea. Fractions corresponding to peak C (Fig. 1) were pooled, dialysed against H_2O for 48 hr and freeze-dried.

Combined electrofocusing and electrophoresis. The method described by Wrigley [23] was used with the following modifications: polyacrylamide gels were 2 × 140 mm, ampholine pH range 5–8, voltage was brought up to 470 V never exceeding 0·5 mA/tube and then maintained for 6 hr 30 min, electrophoresis time was 6 hr at 10 V/cm. The unfixed electrofocused gel can be stored frozen for several days without affecting the two dimensional pattern. Under the above conditions, the gliadins are not focused and migrate very little into the starch gel. The pH gradient was determined according to Drysdale *et al.* [24]. Densitometry was performed in a Chromoscan (Joyce and Loebl) with the 620 nm filter. A wider sample holder was especially made which allowed scanning of half a map each time.

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REFERENCES

1. Garcia Olmedo, F. and Garcia Faure, R. (1969) *Lebensm. Wiss. U. Technol.* **2**, 94.
2. Garcia Olmedo, F. and Carbonero, P. (1970) *Phytochemistry* **9**, 1495.
3. Redman, D. G. and Ewart, J. A. D. (1973) *J. Sci. Fd Agr.* **24**, 629.
4. Aragoncillo, C. (1973) Dr. Ing. Agr. Thesis, Polytechnical University of Madrid.
5. Johnson, B. L. and Hall, O. (1965) *Am. J. Botany* **52**, 506.
6. Hall, O., Johnson, B. L. and Olered, R. (1966) *Proc. 2nd int. Wheat Genetics Symp.*, Lund 1963, *Hereditas*, Suppl. Vol. **2**, 47.
7. Johnson, B. L. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 1398.
8. Waines, J. G. (1973) *Proc. 4th Int. Wheat Genet. Symp. Columbia* [Sears, E. R. and Sears, L. M. S., Eds] (University of Missouri), pp. 873–877.
9. Huebner, F. R. and Rothfus, J. A. (1971) *Cereal Chem.* **48**, 469.
10. Charbonnier, L. (1973) *Biochimie* **55**, 1217.
11. Folch, J. and Lees, M. (1951) *J. Biol. Chem.* **191**, 807.
12. Folch-Pi, J. and Stoffyn, P. J. (1972) *Ann. N.Y. Acad. Sci.* **195**, 86.
13. Zill, L. P. and Harmon, E. A. (1961) *Biochim. Biophys. Acta* **53**, 579.
14. Rohrlich, M. and Niederauer, Th. (1967). *Fette Seifen, Anstrichmittel* **69**, 226.
15. Tennenbaum, D. and Folch-Pi, J. (1966) *Biochim. Biophys. Acta* **115**, 141.
16. Meredith, P. (1965) *Cereal Chem.* **42**, 149.
17. Jones, R. W., Babcock, G. E., Taylor, N. W. and Dimler, R. J. (1963) *Cereal Chem.* **40**, 409.
18. Meredith, O. B. and Wren, J. J. (1966) *Cereal Chem.* **43**, 169.
19. Bietz, J. A. and Wall, J. S. (1972) *Cereal Chem.* **49**, 416.
20. Waines, J. G. (1969) PhD. Thesis, University of California, Riverside, Calif.
21. Wrigley, C. W. and Shepherd, K. W. (1973) *Ann. N.Y. Acad. Sci.* **209**, 154.
22. Kaltsikes, P. J., Evans, L. E. and Bushuk, W. (1968) *Science* **159**, 211.
23. Wrigley, C. W. (1970) *Biochem. Genet.* **4**, 509.
24. Drysdale, J. W., Righetti, P. and Bunn, H. F. (1971) *Biochim. Biophys. Acta* **229**, 42.