Differential effects of high-lysine mutations on the accumulation of individual members of a group of proteins encoded by a disperse multigene family in the endosperm of barley (*Hordeum vulgare* L.)

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The CM proteins are a group of major salt-soluble endosperm proteins encoded by a disperse multigene family. The effects of high-lysine mutations on the net accumulation in barley endosperm of three members of this group (CMa, CMb, and CMe) have been investigated. Genes *CMa*, *CMb* and *CMe* are located in chromosomes 1, 4, and 3 respectively. Protein CMe has been found to be identical with a previously described trypsin inhibitor. The three proteins have been quantified in the different genetic stocks by HPLC. The different high-lysine mutations have different effects on the expression patterns of the three genes: CMe is markedly decreased and CMa and CMb are increased in mutant Risø 1508, whereas all three proteins are decreased in Risø 527 and increased in Risø 7 with respect to the wild-type Bomi; CMa and CMb are increased and CMe is unaffected in mutant Risø 56 with respect to the wild-type Carlsberg II; and protein CMe is markedly decreased in Hiproly barley as compared with its sister line CI4362. The implications of these results in connection with the evolution of CM proteins and with the characterization of high-lysine mutations are discussed.

Cereal grains are the main world-wide source of energy and protein in food and feed. Their storage tissue, the endosperm, can be considered the main edible plant product. Endosperm protein has a low nutritional value because of the low proportion of certain essential amino acids in the prolamin fraction, which in most species represents over half of the total protein. Extensive biochemical and genetic studies have been carried out on this tissue, not only because of its economic importance but also as a model developmental system in plants.

In the case of barley the storage prolamins (hordeins) have a low lysine content and, consequently, this amino acid is the most deficient in the total kernel protein. Most of the lysine in barley endosperm is present in the salt-soluble, glutelin, and residue fractions, especially in the first one, which represents up to 25% of the total protein and contributes about 50% of the total lysine. The overall amino acid composition of the endosperm can be altered through appropriate changes in the proportions of major proteins by genetic manipulation [1]. A number of high-lysine mutants with an improved nutritional value of the grain protein have been obtained [2–4]. In each case the mutation affects a single locus and has marked effects on endosperm development, the most prominent of which are an impaired synthesis of specific hordeins and of starch [4, 5]. The decrease of the hordein fraction and the concomitant increase in other proteins brings about an increase in the overall lysine proportion [4, 5]. Although the effects of some of these mutations on the expression of genes encoding hordeins and some salt-soluble proteins, such as β-amylase, protein Z and the chymotrypsin inhibitors, have been well documented [6–10], no detailed quantitative study has been carried out for other major endosperm proteins. We report here such a study for three homologous proteins, CMa, CMb, and CMe. These are major salt-soluble proteins, which can be also extracted with chloroform/methanol mixtures [11–14]. It has been previously shown that proteins CMa and CMb are members of a group of homologous proteins, the CM proteins, which are encoded by a disperse multigene family [15], whereas the inclusion of protein CMe in this group was only tentative, as it was solely based on its solubility properties [15]. We now demonstrate, as a preliminary investigation, that this protein is a true CM protein and is identical to a previously reported trypsin inhibitor from barley endosperm [16, 17].

MATERIALS AND METHODS

Biological material

The following barley genetic stocks were used in the present study: *Hordeum vulgare* L. cv. Bomi and its mutants Risø 7, 527 and 1508 [2, 4]; cv. Carlsberg II and its mutant Risø 56 [2, 4]; line CI4362 and its high-lysine sister line Hiproly [18]; and line HL82/3, carrying gene *lys* from Hiproly [4]. All these were the kind gift of H. Doll (Risø National Laboratory, Denmark).

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Abbreviations. SDS-PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; RCD index, relative compositional difference index; BzArgNan, benzoyl-1-arginyl-*p*-nitroanilide

Reagents

Acetonitrile was from Scharlau and Carlo Erba. High-performance liquid chromatography (HPLC) ultrapure water, generated by a Milli-RO4 coupled to a Milli-Q water purification system (Millipore, Bedford, MA), was used in the preparation of all buffers. All other reagents were analytical grade.

Protein extraction

Barley endosperm was obtained by hand-dissection, for small samples, and by milling, as previously described [13–15], for preparative purposes. The ground endosperm was delipidated with 10 volumes (1 h, room temperature) of light petroleum (b.p. 40–60° C). Salt-soluble proteins were extracted with 0.5 M NaCl (3 × 10 volumes, 1 h, 4° C) and precipitated by adding trichloroacetic acid to a final concentration of 15%. The precipitate was repeatedly washed with cold acetone and the residual solvent eliminated *in vacuo*. CM proteins were extracted with chloroform/methanol (2:1, v/v; 3 × 10 volumes, 1 h, room temperature) and the solvent evaporated *in vacuo*.

Electrophoresis

Starch gel electrophoresis was carried out in 0.1 M aluminium lactate buffer (pH 3.2), 3 M urea, for 4 h at 20 V/cm. Gels were stained with 0.05% Nigrosine in methanol/H_2O/acetic acid (5:5:1, by vol.). Two-dimensional electrophoresis was as follows: the first dimension was a pH-gradient electrophoresis on preformed pH gradients (ampholines pH 4–9 or 3–10; 5% polyacrylamide; 2-mm × 12-mm columns; 2 h 45 min at 470 V; samples inserted at acid end) and the second dimension was a starch gel electrophoresis as described above. Separations by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) and staining of gels was carried out by published procedures [19, 20].

Protein purification

Salt-extracted proteins, obtained as described, were treated with chloroform/methanol (2:1, v/v; 2 × 10 vol., 1 h, room temperature) and the insoluble material eliminated by centrifugation. The solvents from the supernatant were evaporated *in vacuo*, and the resulting residue was redissolved in 0.1 M acetic acid and freeze-dried. The extract was then fractionated by preparative pH-gradient electrophoresis (ampholines pH 4–9; 5% polyacrylamide; 8-mm × 160-mm column; 4 h 30 min, at 470 V and 4° C). Protein bands were visualized by placing the extruded gels in 50% $(NH_4)_2SO_4$ for 20 min at 4° C, excised with a razor blade, and homogeneized with 0.1 M acetic acid (2 × 10 vol, 12 h, room temperature). Gel residues were eliminated by centrifugation and the supernatants freeze-dried. The freeze-dried material was redissolved in a small volume of 0.1 M acetic acid and the protein precipitated with trichloroacetic acid (final concentration 15%). The precipitate was washed repeatedly with acetone and the residual solvent eliminated *in vacuo*. Protein CMe, which corresponded to the fastest migrating band, was obtained by this procedure together with proteins CMa and CMb, which were are also obtained as previously described [13].

Trypsin inhibition test

Purified proteins were tested for trypsin inhibitor activity following the procedure of Erlanger et al. [21] as modified by Boisen and Djurtoft [22].

Amino acid analysis and protein sequencing

Amino acid analyses were performed by standard procedures. Half-cystine was determined as in [23].

CMe native protein (1.0 mg) in 200 µl 1.0 M Tris/HCl buffer, pH 8.5, containing 0.002 M EDTA and 6.0 M guanidinium chloride, was reduced with 0.1 M dithiothreitol at 37 °C for 100 min. Radioalkylation was achieved by addition of 10 µl iodo[^{14}C]acetic acid (1 µCi/µl) to the mixture and incubation for 15 min at room temperature in the absence of light. Unlabelled iodoacetic acid was then added to a final concentration of 80 mM and excess reagents were removed by gel filtration on a column (1.5 × 20 cm) on Sephadex G-25.

Automatic Edman degradations were performed by previously published methods [24, 25]. The phenylthiohydantoin derivatives were identified by back-hydrolysis [26] and by thin-layer chromatography [27]. Aliquots were also taken for ^{14}C measurements for identification of carboxymethylcysteine.

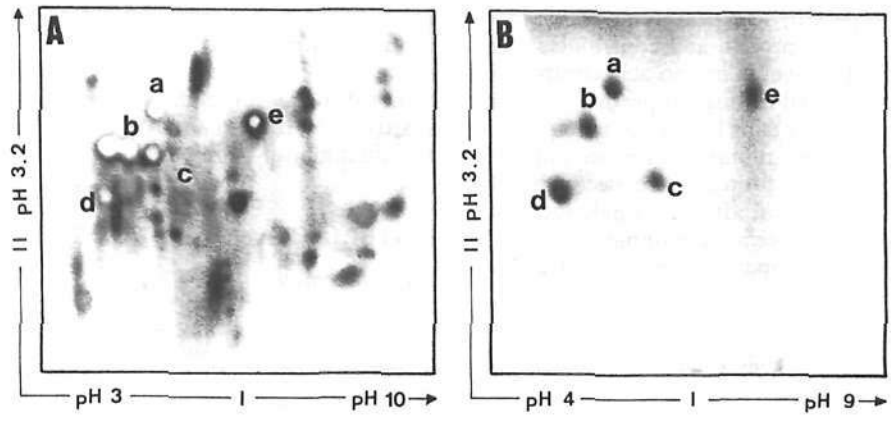

Fig. 1. *Fractionation by combined pH-gradient electrophoresis (pH range 3–10 or 4–9, as indicated; 1st dimension) × electrophoresis (2nd dimension) of A) salt extract from mature barley endosperm (cv. Bomi) and B) CM proteins extracted with chloroform/methanol (2:1, v/v). Positions of CM proteins are indicated (a–e)*

High-performance liquid chromatography

Chromatography with reversed-phase columns was carried out essentially as described for ribosomal proteins [28, 29] with a high-performance liquid chromatograph (Waters, Milford, MA, USA) equipped with a variable ultraviolet detector, model 480 Lambda-Max, as well as an automated gradient controller, model 680 and two solvent-delivery systems, models 680 and 45, respectively. Reversed-phase separations were performed on Vydac TP-RP (C18, particle size 10 µm, pore size 300 nm, column size 250 × 4.6 mm; support was from Chrompack, Mullheim and the steel column from Knauer, Berlin, FRG). Proteins were eluted at room temperature, using gradients made from buffer A (0.1% trifluoroacetic acid in water) and buffer B (0.1% trifluoroacetic acid in acetonitrile). The aqueous and organic buffers were degassed by vacuum and sonication respectively. Measure-

Table 1. *Antitrypsin activity of proteins CMe, CMa and CMb*
The tests were carried out by a modification [22] of the procedure of Erlanger et al. [21]

Protein tested	Amount	A_{410}	Inhibition
	µg		%
–	–	0.22	–
Soybean trypsin inhibitor	5	0.00	100
	1	0.05	77
Bovine serum albumin	20	0.22	0
CMe	20	0.00	100
	10	0.00	100
	5	0.01	95
	2	0.05	77
	1	0.07	68
CMa	20	0.22	0
CMb	20	0.22	0

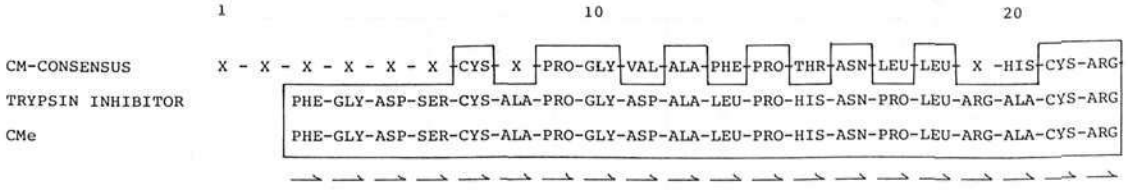

Fig. 2. *N-terminal amino acid sequence of CMe aligned with the CM-consensus sequence [30] and that of the trypsin inhibitor [17].* The number of residues to the left of the first Cys is variable from 3 to 6 in the sequenced CM proteins from cereals [30]. → Sequencer. Homologies are boxed

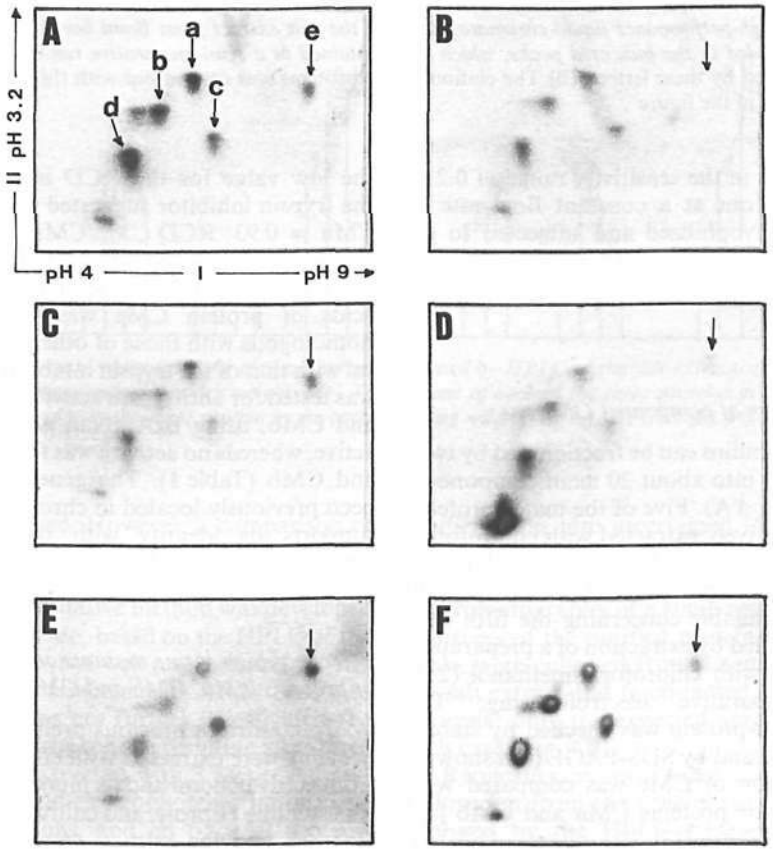

Fig. 3. *Two-dimensional electrophoresis of CM proteins from the following stocks: (A) Bomi, (B) Risø 1508, (C) CI4362, (D) Hiproly, (E) Carlsberg II, and (F) Risø 56. (A, C, E) wild type; (B, D, F) high-lysine.* Protein corresponding to the same endosperm dry weight was inserted in each gel. Positions of CM proteins are indicated (a – e)

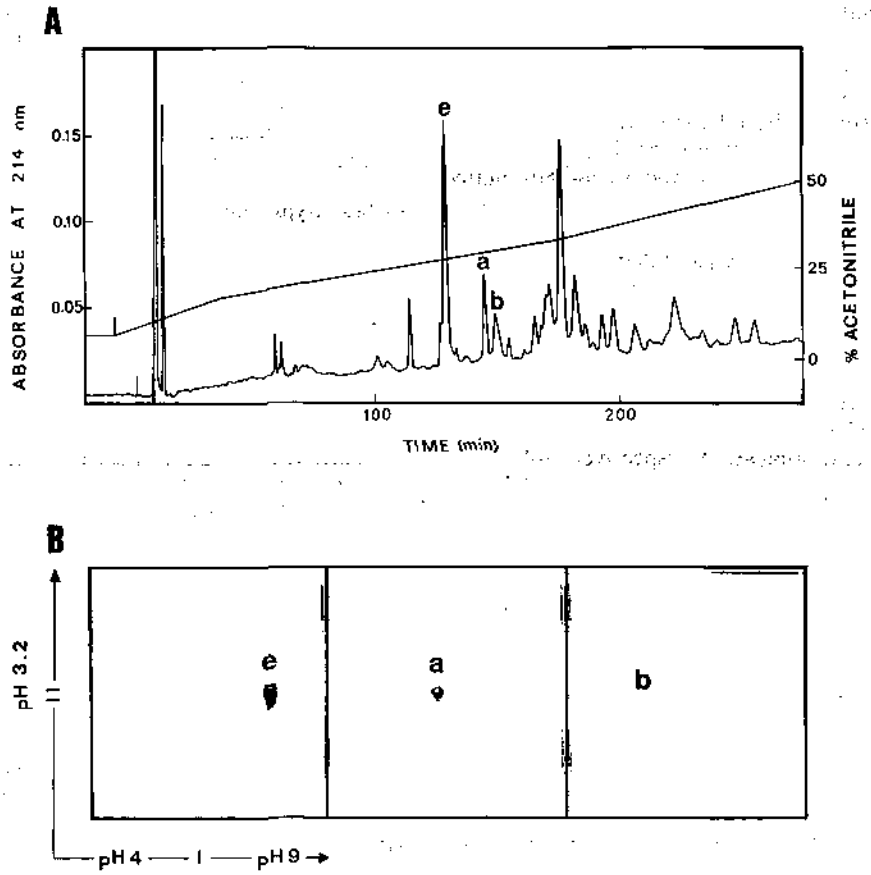

Fig. 4. *(A) Fractionation by high-performance liquid chromatography of the salt extract from Bomi barley. (B) Two-dimensional starch gel electrophoresis of proteins included in the indicated peaks, which were obtained in a semi-preparative run.* (A) Peaks corresponding to CM proteins, e, a and b are indicated by these letters. (B) The elution of the proteins was carried out with the gradients of acetonitrile in 0.1% trifluoroacetic acid as indicated in the figure

ments were made at 214 nm in the sensitivity range of 0.2 to 2.0. All runs were carried out at a constant flow rate of 0.5 ml/min. Aliquots were lyophilized and subjected to gel electrophoresis and amino acid analysis when required.

RESULTS

Purification and identification of component CMe

Barley albumins and globulins can be fractionated by two-dimensional electrophoresis into about 20 main components and several minor ones (Fig. 1 A). Five of the major proteins (CMa to CMe) can be selectively extracted with chloroform/methanol mixtures (Fig. 1 B). We had presented evidence of sequence homology among the first four CM proteins [14, 30] but no information was available concerning the fifth one, CMe. This protein was purified by extraction of a preparation of albumins and globulins with chloroform/methanol (2:1, v/v), followed by preparative electrofocusing. The homogeneity of the purified protein was checked by starch-gel electrophoresis at pH 3.2 and by SDS-PAGE (not shown). The amino acid composition of CMe was compared with those previously reported for proteins CMa and CMb [13] and for the barley trypsin inhibitor [17]. The indexes of relative compositional differences (RCD) [14, 31] for the binary comparisons of protein CMe with each of the two CM proteins indicated homology with a confidence of 95%, whereas the low value for the RCD index in the comparison with the trypsin inhibitor suggested possible identity (RCD CMe/CMa = 0.93; RCD CMe/CMb = 1.00; RCD CMe/trypsin inhibitor = 0.19; RCD <1 indicates homology with 95% confidence; see [14]). The sequence of the 19 N-terminal amino acids of protein CMe was obtained and found to be homologous with those of other CM proteins [30] and identical with that of the trypsin inhibitor [17] (Fig. 2). Protein CMe was tested for antitrypsin activity together with proteins CMa and CMb, using BzArgNan as substrate, and found to be active, whereas no activity was found for its homologues CMa and CMb (Table 1). The gene encoding protein CMe had been previously located in chromosome 3 [15], which further supports its identity with the trypsin inhibitor, whose structural gene had been assigned to the same chromosome [32].

Effects of high-lysine mutants on the net accumulation of proteins CMa, CMb and CMe

To confirm a previous preliminary observation [15], CM proteins were extracted with chloroform/methanol (2:1, v/v) from cultivar Bomi and its mutant Risø 1508, line CI4362 and its sister line Hiproly, and cultivar Carlsberg II and its mutant Risø 56, and the extracts were fractionated by two-dimensional electrophoresis as shown in Fig. 3. The yields and relative proportions of the individual CM proteins were altered in all the high-lysine mutants, and especially protein CMe was

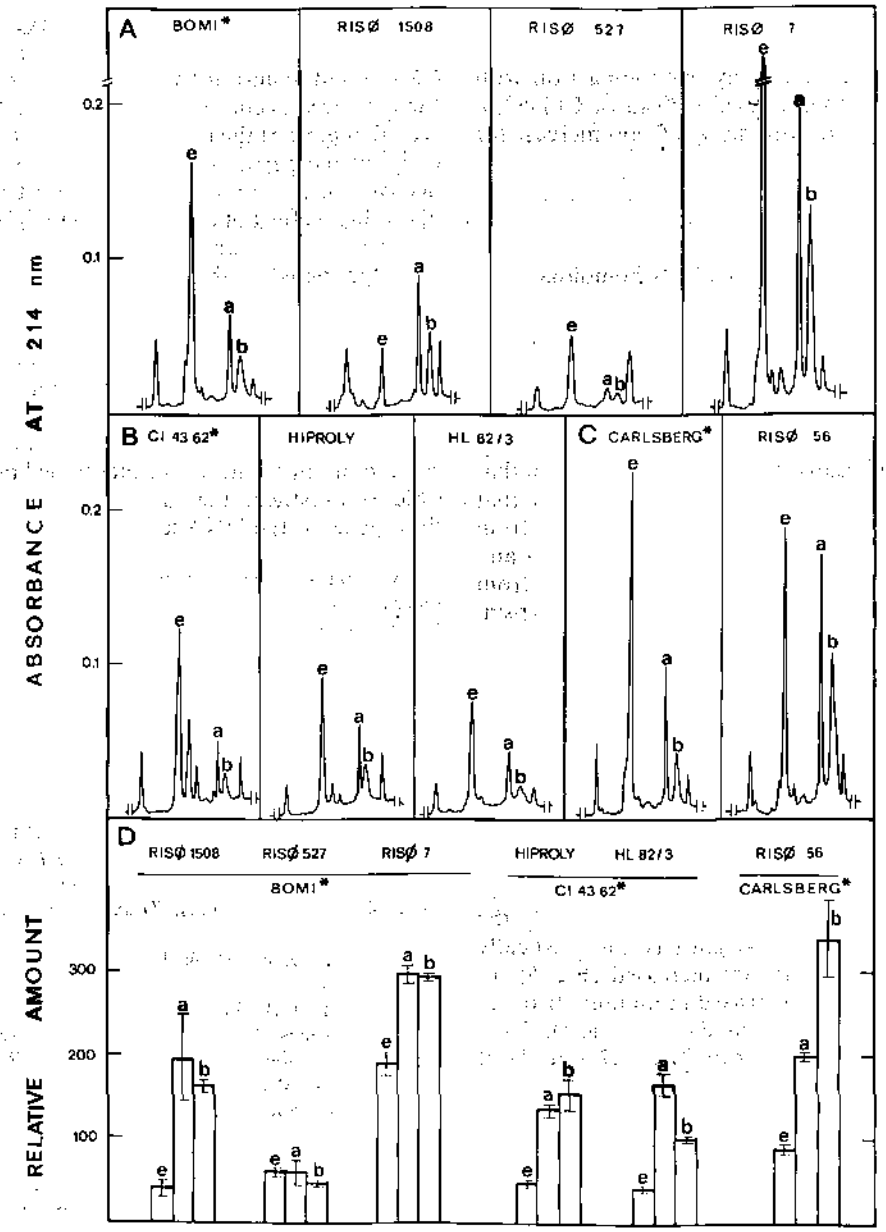

Fig. 5. *(A–C) Tracings of appropriate zones from the chromatograms obtained by HPLC of the salt extracts from the indicated genetic stocks. All chromatograms represent the same endosperm dry weight. (D) The amount of each of the three proteins in each of the stocks is represented as a percentage of the absolute amount of the same protein in the corresponding wild-type (*).* HPLC runs were carried out as in Fig. 5

absent or significantly decreased. However, a comparison of the two-dimensional electrophoretic gels in Fig. 1A, B indicated that extraction with chloroform/methanol mixtures was not quantitative, so a quantitative method was developed for proteins CMa, CMb and CMe, based on the HPLC of the salt extracts. Protein CMd was not well resolved and protein CMc was not quantitatively extracted with 0.5 M NaCl, so their quantitative variation was not further investigated. To check quantitative extraction, the residue resulting after three salt extractions was further extracted with 3 M urea/aluminium lactate buffer, pH 3.2, and electrophoresed in the same buffer at overloading conditions, and no trace of proteins CMa, CMb and CMe was found (data not shown). Solubility of the three proteins in the solvents used in the HPLC gradient, as well as in mixtures representing intermediate points of the gradient, was also ascertained. A chromatogram of the salt extract from Bomi barley is presented in Fig. 4A. Peaks corresponding to each of the three proteins were identified by cochromatography of a small amount of the salt extract with an excess of the purified protein investigated. The identified peaks were collected from a semi-preparative HPLC run of the salt extract and fractionated by two-dimensional electrophoresis; only the expected single protein was observed in each case (Fig. 4B).

Replicate samples of salt extracts, corresponding to 15 mg endosperm from the genetic stocks included in Fig. 4, were analysed by the HPLC method together with additional mutants (Risø 527, Risø 7, HL 82/3). Representative tracings of appropriate zones of the chromatograms are presented in Fig. 5A. The amount of each of the three proteins in each of

the mutants is represented as a percentage of the absolute amount of the same protein in the corresponding wild type in Fig. 5B. In the case of HL 82/3, only the comparison with CI4362 is represented; if Bomi is used instead of CI4362, a similar pattern is obtained but with no significant increase for either CMa or CMb.

DISCUSSION

The demonstration that protein CMe is homologous to other CM proteins further extends this group in barley and implicates one more chromosome as carrier of members of the dispersed CM gene family. Thus, proteins CMa, CMb, and CMe are encoded by genes located in barley chromosomes 1, 4, and 3 respectively [15]. The identity of CMe and the trypsin inhibitor was suspected because of the location of its corresponding gene and because we have recently shown that the CM proteins are included in a protein superfamily that also includes inhibitors of α-amylase and of trypsin from cereals, and certain storage globulins from castor bean and rape seed [30]. Proteins CMa and CMb have diverged from CMe to the extent of not sharing its ability to inhibit trypsin. This divergence seems to have also involved the 'regulatory' properties of these genes, as they are differentially affected by the different high-lysine mutations. A common feature of all mutant lines, except Risø 527, is that the amounts of CMe relative to those of CMa and CMb are significantly reduced as compared to those in their respective wild types (Fig. 5). Apart from this, each mutation seems to alter the expression pattern of the three genes in a specific way, which will be briefly discussed.

In mutant Risø 1508, which has slightly higher protein content and lower seed size than the wild-type Bomi [2], the amount of protein CMe, on a dry-weight basis, is drastically reduced whereas CMa and CMb are increased (Fig. 5). In contrast, all three proteins are decreased in mutant Risø 527 and significantly increased in mutant Risø 7 (Fig. 5). Total protein content is about 10% higher in Risø 527 and about equal in Risø 7, as compared with the parent variety, whereas seed size is about 25% lower in Risø 527 and about equal in Risø 7 [2]. The gene in Risø 1508, designated *lys3a*, has been located in chromosome 7 [33], so the observed effects on genes located in chromosomes 1, 3, and 4, are necessarily *trans*. A profound alteration of the expression pattern of genes encoding B, C, and D hordeins, located in the short and long arms of chromosome 5, has been also assigned to gene *lys3a* [6]. To our knowledge the chromosomal locations of the high-lysine genes in mutants Risø 7 and Risø 527 have not been established.

The high lysine content of Hiproly barley is associated with gene *lys*, also located in chromosome 7 [34]. The variations of proteins CMe, CMa, and CMb in Hiproly barley, with respect to its sister variety CI4362, are similar to those observed for the pair Risø 1508/Bomi. A line carrying gene *lys* from Hiproly, designated HL 82/3, has been selected at Risø from a cross (Hiproly × Bomi) × Bomi [4]. The decrease of CMe is again observed, using either CI4362 or Bomi as terms of comparison, but no increase is observed for proteins CMa and CMb in comparison with Bomi. Previous reports have indicated that Hiproly and high-lysine lines derived from it have a reduced prolamin content and a higher one of four salt-soluble proteins, namely protein Z, β-amylase and chymotrypsin inhibitors CI-1 and CI-2 [8, 35, 36].

The gene in mutant Risø 56, designated *Hor2ca*, is actually a deletion-mutation at the *Hor2* locus in chromosome 5, which codes for B hordein [7]. In this case, accumulation of proteins CMa and CMb is markedly enhanced, while the amount of CMe is not significantly affected. This result indicates that the enhanced accumulation of proteins CMa and CMb, observed in most of the high-lysine mutants, could be an indirect effect of the partial block of hordein synthesis, whereas the decrease of protein CMe and of Hordeins would be a more likely direct effect of mutant 'regulatory' genes acting in *trans*.

The *in vivo* function of these proteins is unknown, although, based on the *in vitro* inhibitory activities of some of them, it has been speculated that they may have a protection role during endosperm development and germination. They are synthesized as precursors by membrane-bound polysomes, but are recovered as soluble components in subcellular fractionation experiments [37]. When more information becomes available concerning the detailed structures of genes corresponding to the above-mentioned proteins it will be possible to investigate the basis of the differential expression of these genes at the DNA level.

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DISCUSSION

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