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Subunits of tetrameric α-amylase inhibitors of *Hordeum chilense* are encoded by genes located in chromosomes 4H^{ch} and 7H^{ch}

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Summary. Three proteins (components 1, 2, and 4) of the non-prolamin, 70% ethanol soluble fraction from the endosperm of *Hordeum chilense* have been identified as putative subunits of the tetrameric inhibitors active against insect α-amylases. In experiments carried out with the synthetic alloploid Tritordeum (*H. chilense* × *Triticum turgidum* conv. *durum*), previously described proteins from *T. turgidum,* designated CM2, CM3 and CM16, have been also identified as subunits of α-amylase inhibitors. Genes for components 1 and 4 of *H. chilense* have been located in chromosomes 4H^{ch} and 7H^{ch}, based on the analysis of *H. chilense-T. turgidum* addition lines. Subunits of the inhibitors from wheat and from cultivated barley had been previously assigned to chromosomes of the same homoeology groups.

Key words: *Hordeum chilense* – Chromosomal location – α-Amylase inhibitors

Introduction

The aqueous-ethanol extracts of many cereal endosperms-including wheat, barley and rye-contain a group of low molecular weight polypeptides (12–16 kdalton), besides the more abundant prolamins (see Garcia-Olmedo et al. 1982 and Shewry and Miflin 1985 for reviews). These proteins are also soluble in salt solutions and are members of a family of α-amylase/trypsin inhibitors which are encoded in wheat and barley by disperse multi-gene families (Fra-Mon et al. 1984; Barber et al. 1986a, b; Sanchez-Monge et al. 1986a).

Some components of the family are selectively extracted by chloroform/methanol mixtures and are designated CM-proteins (see García-Olmedo et al. 1982). We have recently shown that in barley (*Hordeum vulgare* L.), three of the CM-proteins, namely CMa, CMb and CMd, are the subunits of a tetrameric inhibitor which is specific for the α-amylases from the larvae of the insect *Tenebrio molitor* (Sanchez-Monge et al. 1986b). The genes for these three subunits had been previously located in chromosomes 1 and 4 of *H. vulgare* (Salcedo et al. 1984), and those for the putative equivalent proteins from wheat in chromosomes of groups 4 and 7 (Aragoncillo et al. 1975).

The wild South American barley *H. chilense* has been extensively studied in recent years because of its good crossability with other wild and cultivated *Triticeae,* which makes it an interesting potential source of agronomic traits. Among the interspecific hybrids obtained, those with *Triticum turgidum* have received special attention (Martin and Sanchez-Monge Laguna 1982; Padilla and Martin 1983). The synthetic amphiploid (*H. chilense* × *T. turgidum*), designated Tritordeum, shows a good agronomic performance, and the addition lines derived from it have been used to locate structural genes for different isozymes from *H. chilense* (Fernandez and Jouve 1987a, b).

The present report deals with the identification of three proteins from *H. chilense* as subunits of the tetrameric α-amylase inhibitors and with the chromosomal location of the genes for two of them. In the course of this study previously described proteins from *T. turgidum* have been also identified as components of the same type of inhibitor.

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Materials and methods

Biological materials

The following plant material was used throughout this study: *Hordeum chilense* Brongn var. *muticum* (Presl) Hauman (genomes $H^{ch}H^{ch}$); *Triticum turgidum* conv. *durum* (genomes AABB) cv 'Mexican 248 × Andalucia 344' (MA) and cv 'Cocorit'; *Hordeum vulgare* (genomes HH) cv 'Bomi'. Tritordeum (genomes $AABBH^{ch}H^{ch}$) line 'CHMA' (Martin and Sanchez-Monge Laguna 1982) and the monosomic addition lines of *H. chilense* chromosomes in *durum* wheat (Fernandez and Jouve 1987c). The identification of *H. chilense* chromosomes in the addition lines was carried out according to Fernandez and Jouve (1984).

Protein extraction and gel filtration

Kernels (30 mg) were extracted with 70% ethanol (v/v) according to Rodriguez-Loperena et al. (1975). The fraction under 25 kdalton of the crude 70% ethanol extract from Tritordeum endosperm was prepared following Salcedo et al. (1980).

The crude inhbitor preparation from Tritordeum endosperm, its fractionation by gel filtration under non-dissotiating conditions on Sephadex G-100 and the assay of the eluted fractions for their inhibitory activities against the α-amylases from *Tenebrio molitor* larvae was carried out essentially as previously described (Sanchez-Monge et al. 1986b). Gel fractionation under dissociating conditions of the 60 kdalton molecular weight fraction of the inhibitor preparation was performed using the same elution buffer plus 4 *M* guanidinium chloride.

Two-dimensional gel electrophoresis

The 70% ethanol extract from individual kernels and the different gel filtration fractions were subjected to two-dimensional gel electrophoresis, isoelectrofocusing (IEF, pH 4–9) and starch-gel electrophoresis (SGE, pH 3.2), as described by Fra-Mon et al. (1984). Non-dissociating conditions in the first dimension (IEF) were achieved omitting 6 *M* urea.

Results

Non-prolamin ethanol-soluble proteins from H. chilense

The four main components (1–4) of the non-prolamin fraction from the 70% ethanol extract of *H. chilense* can be separated by combined electrofocusing and electrophoresis, as shown in Fig. 1 A. These four components can be also identified after the two-dimensional fractionation of the 0.5 *M* NaCl extract (not shown). A comparison with the equivalent fraction of *H. vulgare* cv 'Bomi' (Fig. 1 B, C) indicates that the *H. chilense* proteins have similar isoelectric points and electrophoretic mobilities as the subunits (CMa, CMb, CMd) of the tetrameric inhibitor of insect α-amylases, previously described in the cultivated barley, although there is no overlapping of spots, except for component 2, in the two-dimensional map of a mixture of extracts from the two species (Fig. 1 C).

Association of components 1, 2, and 4 from H. chilense

A crude inhibitor preparation from Tritordeum endosperm, obtained by precipitation of the salt extract with 50% saturated $(NH_4)_2SO_4$, was subjected to gel filtration as indicated in Fig. 2 A. The peak with an apparent molecular weight of 60 kdalton was active against the α-amylase from *T. molitor*. This fraction was subjected to SDS-electrophoresis, yielding three prominent bands with apparent molecular weights in the 12–15 kdalton range and bands in the 50–60 kdalton range (not shown). Gel filtration under dissociating conditions of the same fraction allowed the separation of a main peak with an apparent Mr under 25 kdalton, which showed inhibitory activity (90% inhibition using 10 µg of protein/assay), and a minor peak of higher molecular weight, which was not inhibitory (Fig. 2 B). Components 1, 2 and 4 of the non-prolamin ethanol-soluble proteins were identified by two-dimensional electrophoresis in the crude inhibitor preparation (Fig. 3 A), in the 60 kdalton fraction obtained by gel filtration under non-dissociating conditions (Fig. 3 B) and in the peak under 25 kdalton obtained from the previous 60 kdalton fraction by gel filtration under dissociating conditions (same pattern as in Fig. 3 B). These components were found to be absent from any other gel filtration fraction analysed. The identity of these *H. chilense*

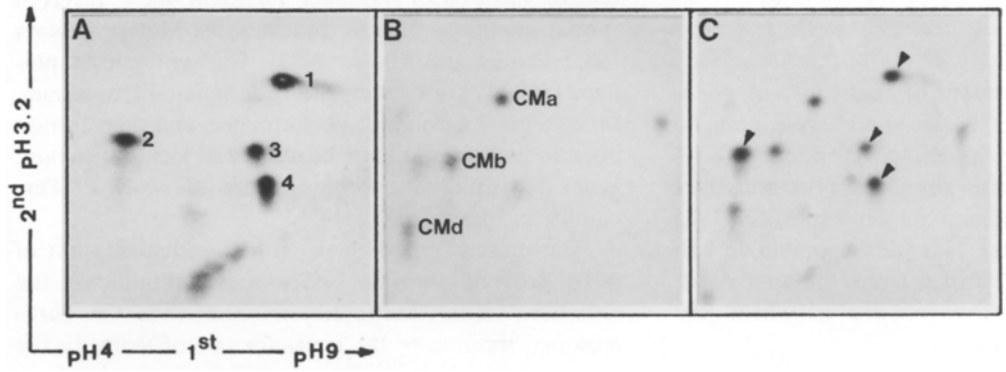


Fig. 1 A–C. Fractionation by combined electrofocusing (pH 4–9) and starch-gel electrophoresis (pH 3.2) of 70% ethanol extracts from: **A** *Hordeum chilense;* **B** *H. vulgare;* **C** a mixture of *H. chilense* and *H. vulgare* extracts. Only pertinent zones of the two-dimensional maps are shown. *Arrows* point to the positions of *H. chilense* components in the mixture

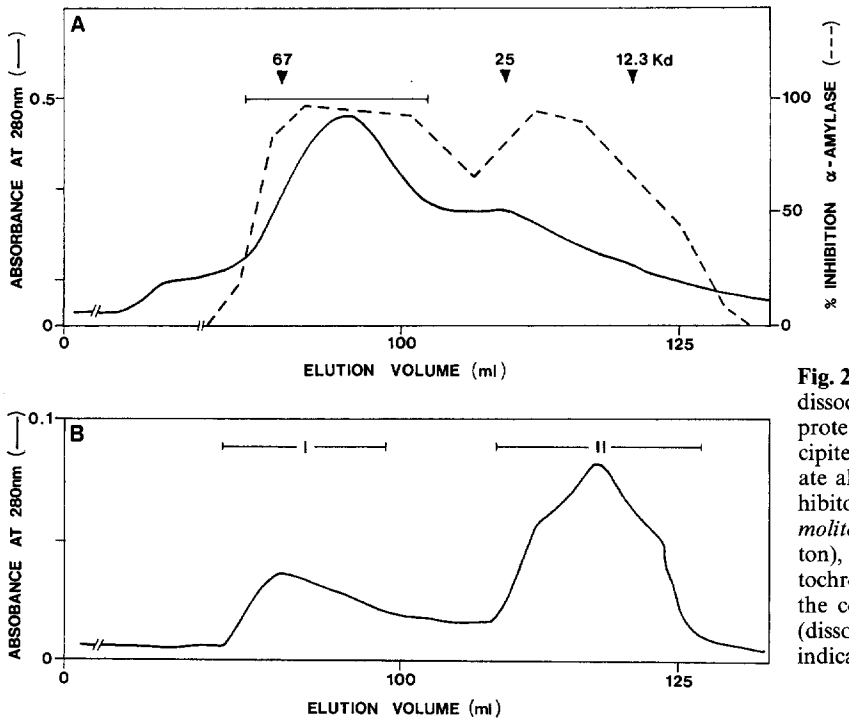
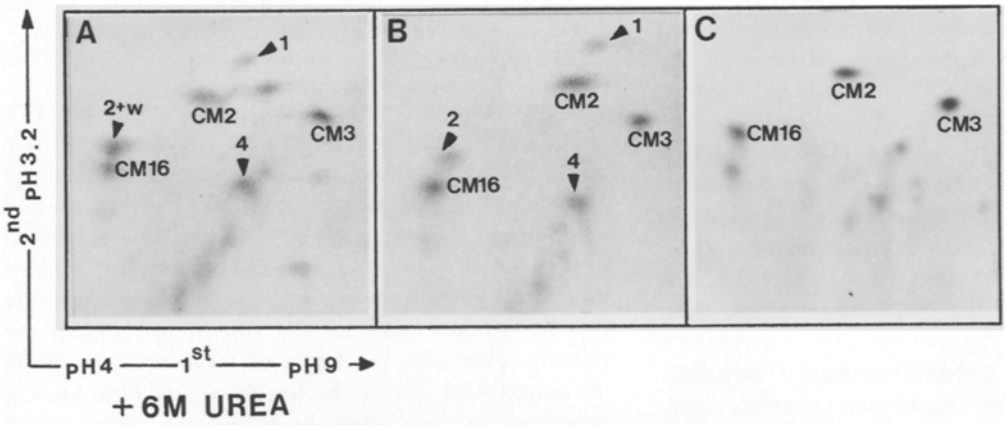


Fig. 2. **A** Gel filtration on Sephadex G-100 (non-dissociating conditions) of Tritordeum endosperm proteins extracted with 150 mM NaCl and precipited with 50% saturated $(NH_4)_2SO_4$. Appropriate aliquots of eluted fractions were tested for inhibitory activity againsts α-amylases of *Tenebrio molitor* larvae. Bovine serum albumin (67 kdalton), chymotrypsinogen (25 kdalton) and cytochrome c (12.3 kdalton) were used to calibrate the column. **B** Gel filtration on Sephadex G-100 (dissociating conditions) of the 60 kdalton fraction indicated in Fig. 2 A



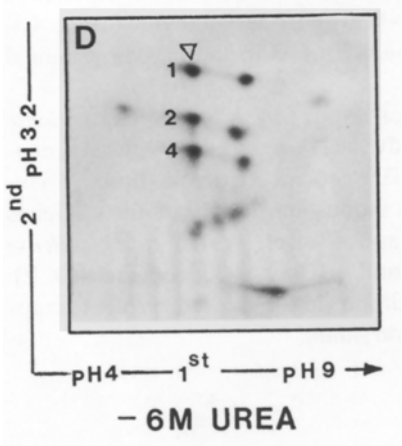


Fig. 3 A–D. Two dimensional maps (IEF pH 4–9×SGE pH 3.2) of the following samples: **A** Crude inhibitor preparation from Tritordeum endosperm; **B, C** 60 kdalton gel filtration fraction of the crude inhibitor preparations from Tritordeum and *Triticum turgidum*, respectively. **D** 70% ethanol extract from *H. chilense* (using non-dissociating conditions in the 1st dimension; association is indicated by a *vertical arrow head*, ▽). *Numbers* and *arrows* (▼) indicate the positions of *H. chilense* components in the two-dimensional maps. Only pertinent zones of the protein maps are shown. w: wheat spot that overlaps with component 2

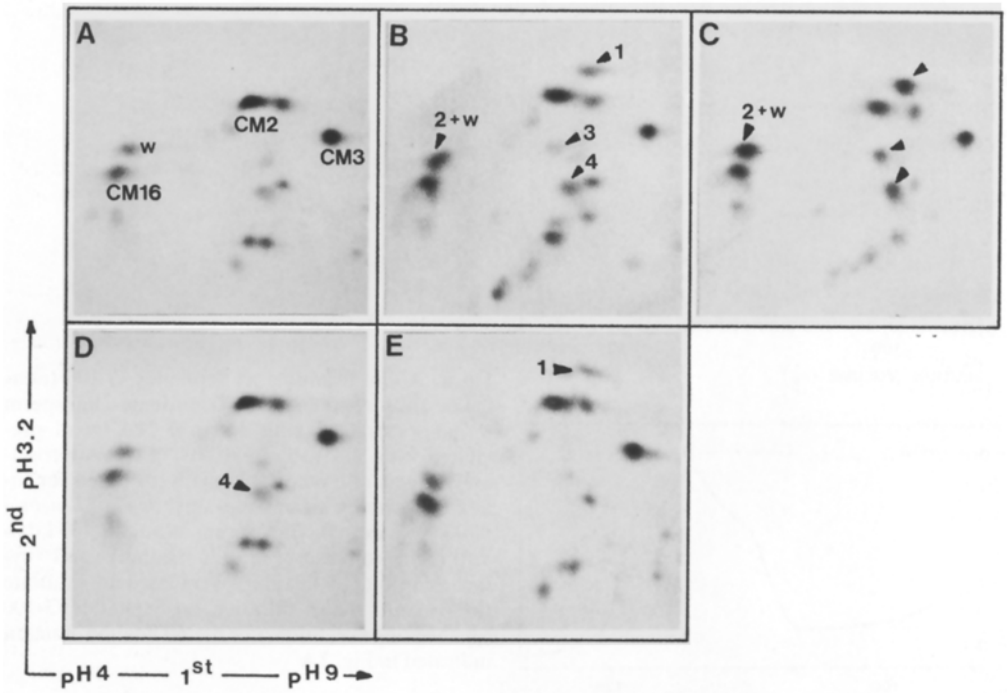


Fig. 4 A–E. Two-dimensional maps (IEF pH 4–9 × SGE pH 3.2) of 70% ethanol extracts from : **A** *Triticum turgidum* conv. *durum;* **B** Tritordeum; **C** a mixture of *H. chilense* and *T. turgidum* extracts; **D, E** monosomic addition lines *H. chilense/durum* wheat $4H^{ch}$ and $7H^{ch}$, respectively. *Arrows* and *numbers* indicate the positions of *H. chilense* components in the different protein maps. Only pertinent zones of the two-dimensional maps are shown. w: wheat spot that overlaps with component 2

proteins in the two-dimensional maps was ascertained by: (1) analysis of the appropriate mixtures of the samples mentioned above with 70% ethanol extracts from *T. turgidum* and *H. chilense;* (2) comparison of the 60 kdalton fraction from Tritordeum (Fig. 3 B) with that obtained using similar methods from *T. turgidum* endosperm (Fig. 3 C). This comparison made it possible to discriminate between *H. chilense* component 2 and an overlapping wheat spot (designated w in Figs. 3 A, 4A) that was not present in the 60 kdalton fraction from *T. turgidum.*

Previously described CM-proteins from *T. turgidum,* CM2, CM3 and CM16 (Rodriguez-Loperena et al. 1975; Barber et al. 1986 b) were identified in the two-dimensional maps from Tritordeum by co-electrophoresis of the 60 kdalton fraction of Fig. 2 A with authentic samples of the proteins. All the main components that appeared in the 60 kdalton fraction of the crude inhibitor preparation from the amphiploid (Tritordeum) could also be identified among the polypeptides with a molecular weight under 25 kdalton of the ethanol extract from Tritordeum endosperm (not shown). The specific association of components 1, 2 and 4 from *H. chilense* was direcly demonstrated in this species by using non-dissociating conditions in the first dimension of the two-dimensional electrophoretic separation (comparison of Figs. 1 A, 3 D).

Chromosomal location of genes encoding components 1 and 4 of H. chilense

The separation of non-prolamin proteins of the 70% ethanol extracts from *T. turgidum* conv. *durum* (cv 'MA' and cv 'Cocorit' showed identical protein maps), the amphiploid *H. chilense* × *T. turgidum* (Tritordeum) and a mixture of the extracts from both species is presented in Fig. 4 A-C. Three of the *H. chilense* components mentioned above (see Fig. 1 A) can be identified in the two-dimensional maps of the amphiploid and of the mixture. However, the levels of these components in the amphiploid seem to be low (compare Figs. 4 B, C), especially in component 3. *H. chilense* component 2 overlapped with a wheat spot (designated *w* in Fig. 4 A, C) and could not be identified in the two-dimensional maps.

Chromosomes carrying genes for the *H. chilense* proteins under study were identified by two-dimensional analysis of 70% ethanol extracts from *H. chilense/durum* wheat monosomic addition lines. Genes for components 1 and 4 were located in *H. chilense* chromosomes $7H^{ch}$ and $4H^{ch}$, respectively (Fig. 4 D, E). The remaining addition lines showed protein maps similar to that of *T. turgidum.*

Discussion

The 70% ethanol extract from the endosperm of *H. chilense* contains four main salt-soluble components of low molecular weight, three of which have been identified as putative subunits of the tetrameric α-amylase inhibitors of this species, based on the known properties of these subunits in *H. vulgare* (Salcedo et al. 1982; Sanchez-Monge et al. 1986 b). Apart from similar solubility properties, components 1, 2 and 4 had similar apparent molecular weights, isoelectric points and, electrophoretic mobilities at acid pH to those of proteins CMa, CMb and CMd, which are subunits of the inhibitor from the cultivated species. Furthermore, the three components co-eluted with a peak of inhibitory activity of about 60 kdalton after gel-filtration under non-dissociating conditions. When this 60 kdalton fraction was subjected to gel filtration under dissociating conditions, both the inhibitory activity and the three components co-eluted as a peak of about 12–15 kdalton. In the course of these experiments, which were carried out with the synthetic alloploid Tritordeum, the previously described proteins CM2, CM3 and CM16 from *T. turgidum* were similarly identified as possible components of the tetrameric inhibitor of this species. A wheat tetrameric inhibitor had been previously described by Buonocore et al. (1985) but its subunits had not been characterized. A more complete characterization of both the *H. chilense* and *T. turgidum* inhibitors is presently under way.

Genes encoding the alluded wheat proteins have been located in chromosomes 4A (CM3, CM16) and 7B (CM2), while those corresponding to the cultivated barley have been assigned to chromosomes 4H (CMb, CMd) and 1H (CMa), chromosome 1H being homoeologous to group 7 of wheat (Aragoncillo et al. 1975; Salcedo et al. 1984). In line with these observations, genes for components 1 and 4 have been respectively assigned to chromosomes $4H^{ch}$ and $7H^{ch}$ by two-dimensional electrophoretic analysis of the *H. chilense*/*T. turgidum* monosomic addition lines. Components 1 and 4 are probably the same as those previously reported as low molecular weight "storage" proteins by Fernandez (1986). It has been recently established, using different isozyme markers, that chromosomes $4H^{ch}$ and $7H^{ch}$ are respectively homoeologous to groups 4 and 7 of wheat (Miller et al. 1985; Fernandez and Jouve 1987 b). The present results further support the proposed homoeologies and indicate that the genes for the inhibitor subunits were in their present linkage groups prior to wheat/barley evolutionary branching.

The trypsin/α-amylase inhibitor family has been extensively used in phylogenetic studies because of its low intra-specific variability (Rodriguez-Loperena et al. 1975; Salcedo et al. 1984). In a previous survey of *H. vulgare* cultivars and *H. spontaneum* accessions virtually no divergence was found for the inhibitor subunits (Molina-Cano et al. 1987). The lack of coincidence in the two-dimensional electrophoretic map of the subunits of *H. chilense* and *H. vulgare* reported here reflects a considerable evolutionary divergence between the two species (Love 1982 and others).

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