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Genes encoding α-amylase inhibitors are located in the short arms of chromosomes 3B, 3D and 6D of wheat (*Triticum aestivum* L.)

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Summary. Three α-amylase inhibitors, designated Inh. I, II and III have been purified from the 70% ethanol extract of hexaploid wheat (*Triticum aestivum* L.) and characterized by amino acid analysis, N-terminal amino acid sequencing and enzyme inhibition tests. Inhibitors I and III have identical N-terminal sequences and inhibitory properties to those of the previously described 0.19/0.53 group of dimeric inhibitors. Inhibitor II has an N-terminal sequence which is identical to that of the previously described 0.28 monomeric inhibitor, but differs from it in that in addition to being active against α-amylase from *Tenebrio molitor,* it is also active against mammalian salivary and pancreatic α-amylases. Compensating nulli-tetrasomic and ditelosomic lines of wheat cv. 'Chinese Spring' have been analysed by two-dimensional electrophoresis, under conditions in which there is no overlap of the inhibitors with other proteins, and the chromosomal locations of the genes encoding these inhibitors have been established: genes for Inh. I and Inh. III are in the short arms of chromosomes 3B and 3D, respectively, and that for Inh. II in the short arm of chromosome 6D.

Key words: Wheat endosperm – α-amylase inhibitors – Gene mapping

Introduction

Protein inhibitors of α-amylase were discovered in wheat endosperm over 40 years ago (Kneen and Sandstedt 1943). These inhibitors, which are active against insect and mammalian α-amylases, but not against the cereal enzymes, have been thought to be relevant in the protection of this tissue and in relation to its nutritional properties (for reviews see Marshall 1975; Buonocore et al. 1977; García-Olmedo et al. 1982).

The inhibitors have been classified into three families: a 12 Kd family of monomeric inhibitors, a 24 Kd family of dimers and a high molecular weight (60 Kd) family, formed by aggregates of 12 Kd subunits (Deponte et al. 1976; Buonocore et al. 1977). The chemical and inhibitory properties of the first two types have been extensively studied (Silano et al. 1973, 1975, 1977, 1978; Petrucci et al. 1974, 1976; O'Connor and McGeeney 1981 and others) and, recently, the complete amino acid sequences of one inhibitor of the first type and two of the second type have been established (Kashlan and Richardson 1981; Maeda et al. 1983, 1985). Genetic studies concerning the chromosomal locations of genes encoding the different inhibitors have not been conclusive. Pace et al. (1978) compared the gel filtration patterns of α-amylase inhibitors extracted from kernels of 11 of the 40 available compensating nulli-tetrasomic stocks of 'Chinese Spring' wheat. The analytical procedure used separated the three classes but could not distinguish between different inhibitors within each class. On the other hand, genetic studies carried out with crude albumin and globulin fractions that presumably included α-amylase inhibitors did not include purification of individual components and tests of inhibitory activity (Noda and Tsunewaki 1972; Cubadda 1975; Aragoncillo et al. 1975; Fra-Mon et al. 1984).

We report here the purification and characterization of three α-amylase inhibitors, and the chromosomal assignment of their corresponding genes.

Materials and methods

Biological materials

Milled endosperms from *Triticum aestivum* cv. 'Candeal' were used for the isolation of α-amylase inhibitors. The complete series of nulli-tetrasomic from *Triticum aestivum* cv. 'Chinese Spring' (except those nullisomic for chromosome 2A and 4A)

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and ditelosomic 3BL, 3DS and 6DS were the gift of E.R. Sears from the University of Missouri (Sears 1954, 1966).

Sources of enzymes

Trypsin, and α-amylases from the mammalian pancreas, *Aspergillus oryzae* and barley were obtained from Sigma. *Tenebrio molitor* α-amylase was prepared from larvae and the salivary α-amylase from lyophilized crude saliva.

Purification of α-amylase inhibitors

Milled endosperm was delipidated with petroleum ether (bp 50–70 °C; 10 v/w; 1 h) and extracted twice with 70% (v/v) ethanol (10 v/w; 1 h). The solvent was rotoevaporated and the residue redisolved in 3M urea, dialyzed against H_2O and freeze-dried. The ethanol extract was fractionated by gel filtration through Sephadex G-100 as previously described (Rodriguez-Loperena et al. 1975). The fraction corresponding to the peak under 25 Kd was further fractionated by ion-exchange chromatography on carboxymethyl cellulose (CM-cellulose; 50×2.6 cm column), in 0.02 M Na acetate buffer (pH 5.2), with a linear gradient of 0.03 to 0.14 M NaCl. α-Amylase inhibitor I was purified in a final step of high performance liquid chromatography (HPLC) on Vydac TP-RP (C18, particle size 10 μm, pore size 300 nm, column size 250×4.6 mm) using a 20%–35% linear acetonitrile gradient.

Two-dimensional gel electrophoresis

Individual kernels of different genetic stocks were extracted with 70% ethanol according to Rodriguez-Loperena et al. (1975). The presence of the inhibitors under study in the different extracts was investigated by two dimensional electrophoresis: first dimension, isoelectrofocusing on preformed pH gradients (pH 6–8; 5% polyacrylamide; 140×2 mm column; 67 V cm^{-1}; 7 h; insertion at acid end); second dimension, starch-gel electrophoresis (0.1 M aluminium lactate buffer, pH 3.2, 3 M urea; 28×18×0.1 cm slabs; 20 V cm^{-1}; 4 h). As a preliminary investigation, different pH gradients (4–9, 5–8, 6–8) and times (2–7 h) were tested for the 1st dimension, in order to obtain optimal separation of the three inhibitors.

Amino acid analysis and protein sequencing

Amino acid analysis and sequencing by automatic Edman degradation were performed essentially as previously reported (Lazaro et al. 1985).

Inhibition tests

Purified proteins were tested for α-amylase-inhibitory activity by the method of Benfeld (1955), except that 0.02 M Na acetate, 0.1 M NaCl, pH 5.4 buffer was used for *Tenebrio molitor* α-amylase and 0.02 M K phosphate, 0.067 M NaCl, pH 7.6 buffer for pancreatic, *Aspergillus oryzae* and barley α-amylases. All tests were carried out with approximately 1 unit of α-amylase (U.A.), defined as the amount of enzyme required to produce the reducing equivalents of 1 mg of maltose in 10 min. Inhibition of trypsin was tested following the procedure of Erlanger et al. (1961) as modified by Boisen and Djurtoft (1981).

Results

Purification and identification of three α-amylase inhibitors

A 70% ethanol extract of the endosperm of the wheat cv. 'Candeal' was subjected to gel filtration under dissociating conditions and the peak under 25 Kd was chromatographed in a column of CM-cellulose. Three fractions (I–III) with anti-α-amylase activity were obtained (Fig. 1 A). Fractions II and III were found to correspond to homogeneous proteins (here after Inh. II and Inh. III), both by two-dimensional electrophoresis and by HPLC (data not shown). Fraction I was a mixture of several components and was further fractionated by semi-preparative HPLC (Fig. 1 B). The homogeneous protein designated Inh. I was the active component of the mixture. Tests of the three protein against α-amylases from human saliva and *Tenebrio molitor* are summarized in Table 1. None of the three proteins showed inhibitory activity against, *Aspergillus oryzae* or barley α-amylases, however Inh. II showed 27% inhibition of pancreatic α-amylase at 20 μg/U.A. No antitrypsin activity was found for any of the proteins, using a 10 μg/assay.

In order to further characterize the three inhibitors, amino acid compositions and N-terminal amino acid sequences were determined. Composition data are presented in Table 2 and the compositional divergence indexes for the binary comparisons of the three proteins with the three previously characterized inhibitors are presented in Table 3. N-terminal sequences are shown in Fig. 2. No heterogeneity was found at any of the sequenced positions.

Chromosomal locations of genes encoding Inh. I, Inh. II and Inh. III

The two-dimensional electrophoretic procedure was optimized to achieve a minimum of overcrowding for the inhibitors. Best results were obtained for the pH 6–8 range in the first dimension. The chromosomes carrying genes for these proteins were identified by two-dimensional analysis of 70% ethanol extracts from compensating nulli-tetrasomics of wheat cv. 'Chinese Spring', and the chromosome arm was then investigated in the corresponding ditelosomics. The positions of the three inhibitors in the two-dimensional protein maps were ascertained by co-electrophoresis of the purified proteins with appropriate 70% ethanol extracts. The two-dimensional map of the wheat cv. 'Chinese Spring' euploid is presented together with representative parts of maps corresponding to the critical genetic stocks in Fig. 3. The gene for Inh. I was found to be located in the short arm of chromosome 3B, that for Inh. III in the homoeologous arm of chromosome 3D, and that for Inh. II in the short arm of chromosome 6D.

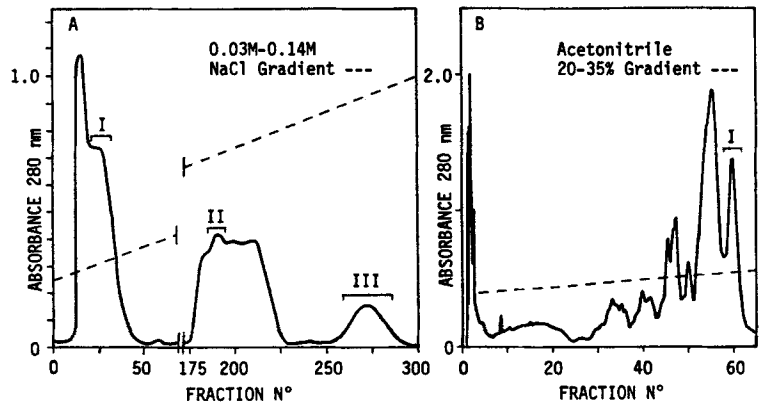

Fig. 1. **A** Fractionation of those protein components with a molecular weight under 25 Kd in the 70% (v/v) ethanol extract from wheat endosperm by ion-exchange chromatography on carboxymethyl cellulose with a linear gradient of 0.03–0.14 M NaCl in 0.02 M Na acetate buffer (pH 5.2). Fractions (I–III) with anti-α-amylase activity are indicated. **B** Fraction I from **A** was subjected to high performance liquid chromatography on a Vydac TP-RP column using a 20%–35% linear acetonitrile gradient in a 0.1% trifluoroacetic buffer. The peak corresponding to homogeneous Inh. I is indicated

Inh. I S G P W M - C Y P G Q A F

Inh. III S G P W M - C Y P G Q A F Q V P A L P A C R P L L R L Q C N

Inh. II S G P W S W C D P A T G Y K V S A L T G

Fig. 2. N-terminal amino acid sequences of Inh. I, Inh. II and Inh. III determined by automatic Edman degradation. The single-letter designations used are: Ala = A; Cys = C; Asp = D; Phe = F; Gly = G; His = H; Ile = I; Lys = K; Leu = L; Met = M; Asn = N; Pro = P; Gln = Q; Arg = R; Ser = S; Thr = T; Val = V; Trp = W; Tyr = Y

Table 1. Inhibitory activities against salivary and *Tenebrio molitor* α-amylases of Inh. I, Inh. II and Inh. III

Inhibitor	μg/U.A.[a]	% inhibition	
		Salivary α-amylase	Tenebrio α-amylase
I	1	50	–
	5	100	85
	10	–	100
II	1	–	100
	10	50	–
	30	87	–
III	1	100	97
	5	–	100

[a] U.A. ≡ 1 unit of α-amylase (see "Materials and methods")

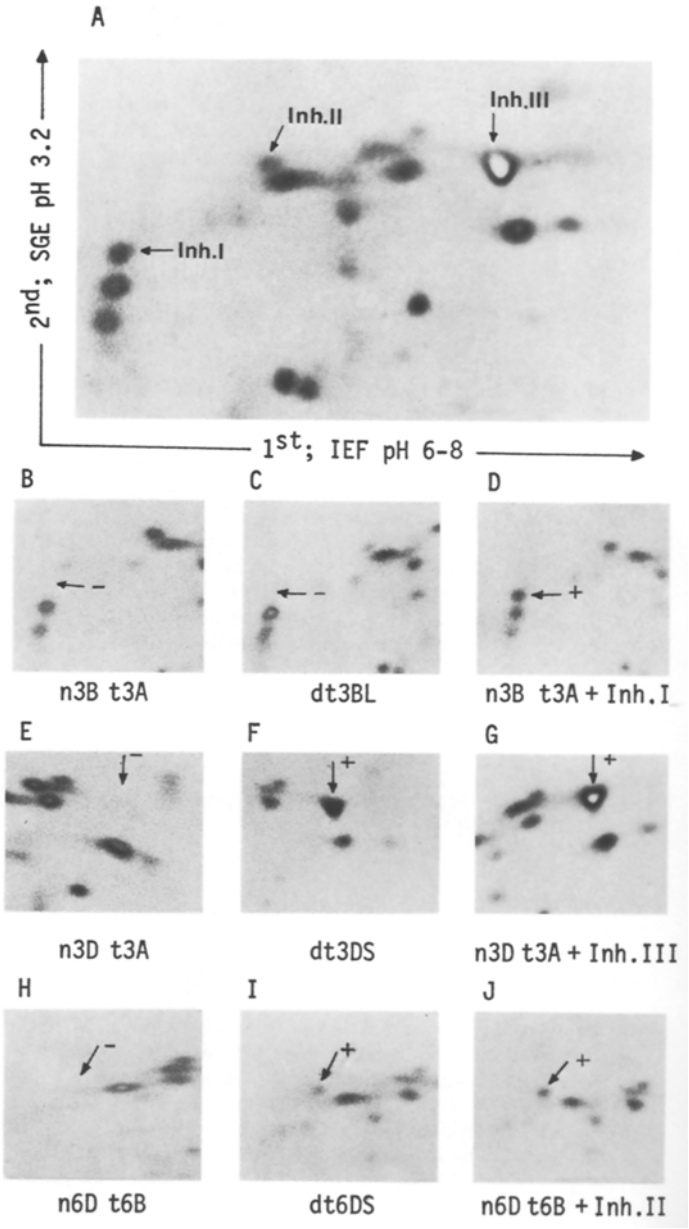

Fig. 3 A–J. Aneuploid analysis of genes encoding α-amylase inhibitors, Inh. I, Inh. II and Inh. III of wheat. **A** Fractionation by combined electrofocusing (pH 6–8) × starch-gel electrophoresis (pH 3.2) of a 70% ethanol extract from *T. aestivum* cv. 'Chinese Spring'. **B–J** Pertinent zones of the two-dimensional maps of the indicated aneuploids. n = nulli; t = tetra; dt = ditelosomic. Maps **D, G** and **J** correspond to a mixture of the indicated nulli-tetrasomics and 40 μg of purified Inh. I, Inh. III and Inh. II, respectively. *Arrows* point to the positions of Inh. I–III; – = absence and + = presence of the corresponding inhibitor

Table 2. Amino acid composition of inhibitors I, II and III and their comparison with α-amylase inhibitor 0.53, 0.28 and 0.19. The amino acid composition data of α-Amy inhibitor 0.53 and 0.19 have been deduced from the sequences in Maeda et al. (1985) and that of α-Amy inhibitor 0.28 from its sequence as reported by Kashland and Richardson (1981)

Amino acid[a]	Inh. I[b]	Inh. 0.53	Inh. II	Inh. 0.28	Inh. III	Inh. 0.19
Lys	5	4	5	6	3	3
His	2	1	1	0	2	2
Arg	6	6	7	7	8	8
Asx	8	8	8	10	8	7
Thr	4	3	3	3	4	3
Ser	9	9	7	8	7	7
Glx	14	12	16	12	14	13
Pro	8	10	10	9	10	9
Gly	12	11	11	11	10	10
Ala	13	15	10	10	15	17
Val	9	11	12	14	10	10
1/2 Cys	9	9	9	11	11	10
Met	2	3	3	3	2	3
Ile	3	3	3	2	3	3
Leu	10	10	9	9	10	10
Tyr	5	5	4	4	4	5
Phe	3	2	1	0	1	2
No. of residues	122	122	119	119	122	122

[a] Tryptophan was not considered
[b] The experimental data has been adjusted to 122, 119 and 122 for Inh. I, II and III, respectively

Table 3. Values of the compositional difference index for the binary comparisons of Inh. I, II and III with α-Amy inhibitors 0.53, 0.28 and 0.19[a]

Inhibitors	I	II	III
0.53	0.21	0.53	0.23
0.28	0.71	0.34	0.73
0.19	0.39	0.77	0.11

[a] The indexes has been calculated according to Cornish-Bowden (1980), as modified by Paz-Ares et al. (1983), using the data in Table 2

Discussion

We have obtained three different α-amylase inhibitors in a homogeneous form as judged by two-dimensional electrophoresis and by N-terminal sequencing. The N-terminal sequences of Inh. I and Inh. III are identical and correspond to those previously published for the dimeric (24 Kd) inhibitors, designated 0.19 and 0.53 (Maeda et al. 1985), whereas that of Inh. II is identical with that of the previously reported monomeric (12 Kd) inhibitor, designated 0.28 (Kashland and Richardson 1981). The compositional divergence indexes indicate that Inh. I is more closely related to 0.53 and Inh. III to 0.19; the low values of these indexes are compatible with complete identity but do not exclude differences at a few sequence positions. In the case of Inh. II, the only significant discrepancy with respect to 0.28 seems to be the possible presence in its sequence of 1 His and 1 Phe, which suggests that Inh. II is a different member of the same family.

Both Inh. I and Inh. III were active against the α-amylases of *Tenebrio molitor* and mammalian saliva, as expected from inhibitors of the dimeric type (Bounocore et al. 1977). Neither inhibitor was active against pancreatic α-amylase at the concentration tested (up to 40 μg/U.A.), which means that, although the inhibitors may be active at higher concentrations, the salivary/pancreatic activity ratio is greater than 10^3. Maeda et al. (1985) have recently reported ratios of 90–500 for this type of inhibitors. Inh. III is 4–5 times more active than Inh. I against both the insect and the salivary enzyme, which is in line with the observations of Maeda et al. (1985) for 0.19 and 0.53, and lends further support to the structural identification.

The data concerning Inh. II is more puzzling because although the structural information clearly indicates that this protein is either identical with or very closely related to the monomeric 0.28 inhibitor sequenced by Kashland and Richardson (1981), the inhibition specificity differs from that reported for that inhibitor: 0.28 is thought to inhibit only the insect enzyme, whereas Inh. II inhibits both the mammalian and the insect enzymes. The most plausible explanation

is that we are dealing with a different member of the 0.28 family, with a different specificity. Indeed, a 12 Kd inhibitor (size of the 0.28 type) with the same specificity of Inh. II has been detected in *Aegilops speltoides* by Bedetti et al. (1974). Furthermore, we have recently shown that among five related proteins of barley, there was a much closer relationship between a trypsin and an α-amylase inhibitor than between two trypsin inhibitors (Barber et al. 1986).

The analysis of nulli-tetrasomic and ditelosomic stocks by two-dimensional electrophoresis has allowed the unambiguous assignment of genes encoding the closely related inhibitors of the dimeric type, Inh. I and Inh. III to homoeologous locations, in the short arms of chromosomes 3B and 3D, respectively, which further stresses their recent, common evolutionary origin. The gene for Inh. II has been similarly located in the short arm of chromosome 6D, again in line with its inclusion in a separate structural family of inhibitors. Genes encoding proteins homologous to those reported here, active against α-amylase or trypsin, or even inactive, have been located in chromosomes of homoeology groups 3, 4 and 7 of wheat or barley (Aragoncillo et al. 1975; Salcedo et al. 1984; Barber et al. 1986). The present finding indicates that this gene superfamily also extends to group 6. Several previous reports had assigned genes for albumins and globulins to chromosome groups 3 and 6 (for a review see García-Olmedo et al. 1982), but no purification of individual components and functional or structural identification was carried out. According to their positions in the two-dimensional map and the chromosomal location of their structural genes. Inh. I, Inh. II and Inh. III correspond to component numbers 12, 8 and 11 in the two-dimensional map recently reported by us (Fra-Mon et al. 1984).

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