JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Dimeric Inhibitors of Human Salivary α-Amylase from Emmer (*Triticum dicoccon* Schrank) Seeds

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The proteins belonging to the cereal trypsin/α-amylase inhibitor family are abundant water/salt-soluble flour proteins active against α-amylases from several seed parasites and pests and inactive against endogenous α-amylases. Three α-amylase inhibitor families have been described in cereals that vary in size and are differently expressed among Triticeae seeds. The present work investigates the presence of human salivary α-amylase inhibitors in emmer (*Triticum dicoccon* Schrank) flour. The isolation was obtained by a series of chromatography steps, and the purification progress was monitored through the inhibition of human salivary α-amylase activity. The purified fraction was subjected to protein sequencing by tandem mass spectrometry (MSMS) of the tryptic digests obtained after the sample separation on 2-DE. MSMS data indicated that the emmer α-amylase inhibitory fraction was composed of two newly identified proteins [emmer dimeric inhibitor 1 (EDI-1) and emmer dimeric inhibitor 2 (EDI-2)] sharing very high identity levels with related proteins from *Triticum aestivum*.

KEYWORDS: Human salivary α-amylase; dimeric inhibitor; emmer; tandem mass spectrometry

INTRODUCTION

Protein inhibitors of α-amylases were discovered in wheat endosperm over 60 years ago (*1*). It was known that the bulk (about two-thirds) of wheat albumins consisted of a few amylase inhibitor families phylogenetically related and coded by a small number of parental genes (*2*). In the absence of dissociating agents, wheat α-amylase inhibitors could be fractionated by gel filtration into three inhibitor families, with apparent molecular masses close to 60, 24, and 12 kDa (*3*). Under denaturing conditions the three families revealed various degrees of polypeptide aggregation into heterotetrameric inhibitors (60 kDa) constituted by three different subunits, one of which was present in two copies; homodimeric inhibitors (24 kDa) having two identical subunits of about 12 kDa each; and monomeric inhibitors having a molecular mass of about 12 kDa. These inhibitors, belonging to the cereal trypsin/α-amylase inhibitor family (*4*), are active against insect and mammalian α-amylases, but not against the cereal enzymes.

In wheat, the monomeric inhibitors are strongly active against insect α-amylases but weakly, or not at all, active against mammalian salivary or pancreatic α-amylase; on the other hand, the homodimeric inhibitors are generally more effective against the mammalian enzymes (*5*). Several monomeric and homodimeric α-amylase inhibitors have been characterized (*2, 6*), and the complete amino acid sequences of one monomeric and two homodimeric inhibitors have been determined (*7–9*). The heterotetrameric inhibitor family is characterized by a much greater inhibitory activity against *Tenebrio molitor* α-amylase than against the salivary enzyme (*10*). García-Maroto et al. (*11*) characterized three cDNA clones corresponding to the three types of subunits of the wheat tetrameric inhibitor. The deduced amino acid sequences of the mature polypeptides are homologous to those of the dimeric and monomeric α-amylase inhibitors.

The majority of the heterologous α-amylase inhibitors in plants seem to play a protective role against the attacks of animal predators, insects, and bacteria. The inhibition of these organisms' hydrolytic enzymes may be toxic or even lethal or could induce the evolution of different feeding habits.

A link between plant defense proteins and plant food allergens has been established during the past decade. Constitutive and inducible proteinaceous inhibitors of heterologous hydrolytic enzymes are included in such plant defense proteins. Some of these proteins (i.e., cereal trypsin/α-amylase inhibitor family) are major allergens in the allergic diseases provoked by inhalation (bakers' asthma) or ingestion of cereal flour (*4*).

Cereals are the staple food consumed by the majority of the world population; the oldest cultivated cereals were hulled wheats, characterized by grains covered by the spikelet glumes after threshing. Their production has been replaced by that of dehulled and higher yielding wheats. However, recently we have

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10.1021/jf071739w CCC: $37.00
Published on Web 11/20/2007

witnessed a renewed interest in the seeds of hulled wheats for human nutrition due to their increasing popularity as environmentally friendly cereal crops for the production of niche products. Despite this, knowledge of the biochemical characteristics of these cereal seeds is quite scarce and often conflicting (*12*). Their antimetabolic compound content is probably partially responsible for the suitability of these species for growth in harsh ecological conditions without the need for pesticides and fertilizers.

For some time we have focused our research interest on the antinutritional and potentially allergenic factors in caryopses of one of the hulled wheats most commonly found in Italian markets, the tetraploid *Triticum dicoccon* Schrank, also called emmer (*12*, *13*). In the present work we have searched for protein inhibitors active against human salivary α-amylase and isolated some of them in the effort to further contribute to the knowledge of the biochemical and nutritional characteristics of this ancient species in comparison to highly domesticated cereal crops.

MATERIALS AND METHODS

Plant Material. Emmer seeds were purchased from the Consorzio Produttori Farro della Garfagnana (Piazza al Serchio, Lucca, Italy). The seeds were ground for 2.5 min in a steel ball mill (Retsch GmbH & Co. KG, Haan, Germany) cooled with dry ice. The flour was used immediately.

Isolation of α-Amylase Inhibitors. Twenty grams of emmer flour was extracted twice, by continuous stirring with 100 mL of 0.15 M NaCl for 1 h at 5 °C. The slurry was centrifuged at 17000*g* for 15 min at 5 °C. The supernatant was brought to 50% saturation with solid ammonium sulfate and stirred for 30 min at 5 °C. After centrifugation at 17000*g* for 15 min at 5 °C, the precipitate was suspended in 100 mM ammonium acetate buffer, pH 6.9, and dialyzed overnight at 5 °C (3.5 kDa MWCO dialysis tubing) against the same buffer. The dialyzed extract was centrifuged at 27000*g* for 10 min at 5 °C, and the supernatant was chromatographed on a Sephadex G-100 (1.4 × 83.5 cm; Sigma-Aldrich, Milan, Italy) calibrated with gel filtration standards (Bio-Rad Laboratories, Milan, Italy) and equilibrated with 100 mM ammonium acetate buffer, pH 6.9, at the flow rate of 0.6 mL/min. The fractions eluted with an approximate M_r of 24 kDa were collected and dialyzed overnight at 5 °C against 20 mM sodium acetate buffer, pH 5.2. The dialyzed extract was centrifuged as above, and the supernatant was loaded on a CM-cellulose column (2.5 × 20 cm; Sigma-Aldrich, Milan, Italy) equilibrated with 20 mM sodium acetate buffer, pH 5.2. After the nonbound material had been washed with equilibration buffer, the column was eluted at the flow rate of 2 mL/min, with a three-step gradient of the sodium acetate buffer (20–200, 200, and 200–300 mM). The eluted fractions were assayed for human salivary α-amylase (HSA) inhibitory activity, and those showing α-amylase inhibition were pooled and lyophilized.

Purification of the HSA inhibitors from the CM pool was achieved by reversed phase (RP)-HPLC on semipreparative and analytical C-18 columns (Nucleosil, 10 mm × 250 mm, 300 Å, 7 μm; and Nucleosil, 4 mm × 250 mm, 300 Å, 5 μm; Macherey-Nagel GmbH & Co. KG, Düren, Germany).

For the semipreparative separation the lyophilized CM pool was dissolved in 80% solvent A [H_2O/0.05% trifluoroacetic acid (TFA)] and 20% solvent B [acetonitrile (CH_3CN)/0.05% TFA] and injected on the column equilibrated with 80% solvent A and 20% solvent B. The separation was achieved with a two-step linear gradient,, from 20 to 30% of solvent B in 10 min and from 30 to 50% of solvent B in 80 min, at 50 °C and at a flow rate of 3 mL/min. The individual fractions eluted were collected and assayed for HSA inhibitory activity. The fractions showing inhibition were pooled and freeze-dried.

The lyophilized pool was dissolved and injected onto the analytical column equilibrated with 80% solvent A and 20% solvent B. The elution was performed at a flow rate of 1 mL/min, with a two-step linear gradient, from 20 to 30% solvent B in 10 min and from 30 to 50% solvent B in 160 min, at 50 °C. The fraction exhibiting HSA inhibitory activity was collected, analyzed by 15% SDS-PAGE (*14*), lyophilized, and utilized for in-solution and in-gel α-amylase inhibition assays, 2-DE separation, and molecular weight and sequence determination by MS techniques.

Protein Quantification. Protein content was measured with the Bradford method (*15*).

α-Amylase Assays. The activity assay of α-amylases (EC 3.2.1.1) from human saliva and from *Bacillus subtilis* (type II-A) was performed according to the method of Bernfeld (*16*) with slight modifications (*17*). All assays were performed in triplicate. The amount (milligrams) of maltose produced was calculated by using a standard curve. One unit of α-amylase activity was defined as the amount of enzyme required to produce 1 mg of maltose in 3 min at pH 6.9 and 37 °C.

α-Amylase Inhibition Assays. The HSA and *B. subtilis* α-amylase inhibition assays were performed as described for the α-amylase assay, with appropriate amounts of inhibitor preparation added into the preincubation mixture. The inhibitor preparation from early purification stages (crude extract, G-100, and CM chromatography fractions) was treated for 5 min at 100 °C to denature the endogenous amylase activities; purified samples were not heat-treated before being assayed for inhibition. Control assays were run as described in Fontanini et al. (*17*). All inhibition assays were performed in triplicate.

7.5% Non-denaturing Starch−PAGE and In-Gel Inhibition Assay of the Isolated α-Amylase Inhibitors. Non-denaturing 7.5% PAGE minigels of the isolated inhibitors were performed according to the method of Fontanini et al. (*17*) with the addition of 2% soluble potato starch to the separating gel mixture. The gels were stained with 0.02% PhastGel Blue R-350 (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

In-gel inhibition assay of the isolated α-amylase inhibitors was performed on 7.5% starch−PAGE minigels; after the electrophoretic run, the minigels were washed with distilled water and incubated for 2 h at 37 °C in 20 mL of sodium phosphate buffer, pH 6.9, to which had been added 6.7 mM NaCl and containing 5 units/mL of HSA. At the end of the incubation time the minigels were fixed in 10% TCA for 10 min and washed with distilled water. The HSA inhibitory activity appeared as a dark band on a pale yellow background, after staining of the gels with Lugol's reagent (6.7 mg/mL KI and 3.3 mg/mL I_2).

2-DE of the α-Amylase Inhibitors. Fifteen microliters of α-amylase inhibitors was added to 110 μL of 25 mM Tris-HCl, pH 9.0, containing 2% CHAPS, 7 M urea, 2 M thiourea, 0.5% IPG buffer (pH 3–10), and traces of bromophenol blue (BBP). The sample was reduced with 43 mM DTT for 2 h, alkylated for 1 h with 60 mM acrylamide, and applied on an Immobiline DryStrip gel (7 cm, pH 3–10, Amersham Biosciences, Milan, Italy) by overnight rehydration. Isoelectric focusing was performed at 20 °C with a Multiphor II apparatus (Amersham Biosciences) for 1 min at 200 V, followed by a linear voltage ramp to 3500 V in 90 min and 1 h of constant voltage (3500 V). The strip was equilibrated for the second dimension separation in 50 mM Tris-HCl, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, and traces of BBP (equilibration buffer) added with 25 mM DTT. After 20 min, the strip was alkylated in equilibration buffer containing 360 mM acrylamide for 20 min. The second dimension separation was performed on a 15% SDS-PAGE minislab gel. The minigel was stained with 0.02% PhastGel Blue R-350 and destained with methanol/acetic acid/water (3:1:6, v/v/v).

MALDI-TOF MS of Isolated Human Salivary α-Amylase Inhibitors. The MALDI mass spectrum of the isolated inhibitors was acquired in the mass range of 4000–19000 Da, using a Voyager DE-PRO time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a UV nitrogen laser (337 nm). Spectra were obtained in positive linear ion-delayed extraction mode and were averaged from 200 laser shots to improve the signal-to-noise ratio. Sample preparation was performed according to the dried droplet method (*18*) using sinapinic acid (SA) as matrix. The matrix solution was prepared by dissolving SA in CH_3CN/0.1% TFA (60:40, v/v) at a concentration of 10 μg/μL. Mass assignments were made using insulin (5734 Da), cytochrome *c* (12360 Da), and myoglobin (16951 Da) as external standards.

In-Gel Digestion of Protein Spots and Mass Spectrometric Analyses. Selected protein spots from the 2-DE gel were excised,

washed, and subjected to in-gel trypsin digestion according to the method of Shevchenko et al. (*19*). After trypsin (modified porcine trypsin, Promega, Madison, WI) had been soaked into the gel pieces, the supernatant containing excess trypsin was removed, and the gel pieces were covered with 50 μL of 50 mM NH_4HCO_3 and incubated at 37 °C overnight. The enzymatic reaction was stopped by cooling the gel pieces and the supernatant solution at −24 °C.

For MALDI-MS analysis, 10 μL of the supernatant solutions containing tryptic peptides was subjected to micropurification (desalting/concentration) with a homemade 5 mm nanocolumn packed with C18 resin (POROS R2, Applied Biosystems) in a constricted GELoader tip (Eppendorf Scientific, Westbury, NY), according to the method of Gobom et al. (*20*). The tryptic mixture was then eluted with 0.8 μL of matrix and deposited directly onto the MALDI target. The matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid (CHCA) in CH_3CN/0.1% TFA (70:30, v/v) at a concentration of 10 μg/μL. MALDI mass spectra were acquired in the *m*/*z* 600–4000 mass range in a positive ion reflectron-delayed extraction mode and were averaged from 250 laser shots to improve the signal-to-noise ratio.

MALDI-TOF spectra were calibrated using bovine β-lactoglobulin (Sigma-Aldrich, Milan, Italy) tryptic peptides as external standards.

Bioinformatic Search. MALDI-TOF peptide mass data were used to perform protein identifications by searching the NCBInr database with Mascot (www.matrixscience.com) server used in the PMF mode. The following parameters were used for database searches: taxonomy, *viridiplantae* (green plants); monoisotopic mass accuracy, 100 ppm; missed cleavages, 2; allowed modifications, propionamide Cys (fixed), oxidation of Met (variable), transformation of N-terminal Gln and N-terminal Glu residue in the pyroglutamic acid form (variable).

Capillary RP-HPLC/nESI-MSMS. HPLC grade water and CH_3CN were provided by Carlo Erba (Milan, Italy). Capillary RP-HPLC/nESI-MSMS was performed using an Ultimate 3000 system (LC Packings, Dionex, Sunnydale, CA) coupled with a linear ion trap nanoelectrospray mass spectrometer (LTQ, Thermo Electron, San Jose, CA). Initially, 5 μL of the tryptic peptides solution from spots 1 and 2 was directly loaded onto a C18 μ-precolumn (5 × 0.3 mm, 100 Å, 5 μm, PepMap, LC Packings, Dionex) with 0.1% aqueous formic acid (FA) at a flow rate of 20 μL/min for 4 min. Subsequently, peptides were applied onto a C18 capillary column (150 × 0.18 mm, 300 Å, 5 μm, Thermo Electron) and eluted at room temperature with a linear gradient of CH_3CN/0.1% FA/H_2O/0.1% FA from 5 to 50% in 50 min at a flow rate of 2 μL /min. The samples originated from the very faint spots 3 and 4, instead, were subjected to a second extraction with CH_3CN, concentrated to 15 μL, and then injected into the precolumn.

Repetitive mass spectra were acquired over the *m*/*z* 150−2000 mass range in positive ion mode using the following electrospray ion source parameters: capillary temperature, 220 °C; spray voltage, 2.3 kV.

The amino acid sequences of the peptides detected during RP-HPLC/nESI-MS were determined by MSMS. The operating conditions for MSMS analysis were as follows: isolation width, 3 Da; normalized collision energy, 23 au; and activation *Q*, 0.250.

Data analysis and sequence data handling were performed using the General Protein/Mass Analysis for Windows (GPMAW) software.

RESULTS

Semipreparative RP-HPLC of the CM pool resolved several peaks eluting between 35.5 and 47.75% acetonitrile (data not shown). The in-solution inhibition assays of these fractions showed the prevalence of HSA inhibitory activity in a 37% acetonitrile-eluting peak. This fraction was further purified by three rounds of analytical RP-HPLC. The last analytical separation resolved two peaks, a minor one eluting at 31.6% acetonitrile and a major one eluting at 31.75% acetonitrile, that were carefully recovered to avoid cross-contamination (**Figure 1**). The minor peak showed no HSA inhibition, whereas the major peak inhibited 93.1% of the HSA activity.

The inhibitory fraction analyzed by 15% SDS-PAGE resolved into two close-migrating protein bands (**Figure 1**, inset).

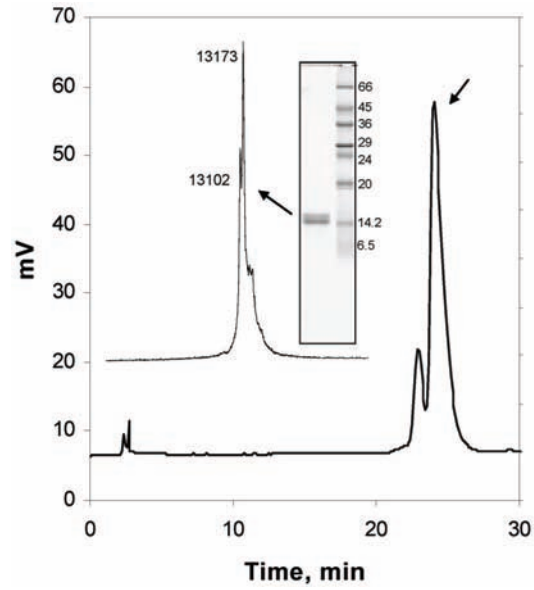

Figure 1. Isolation of the emmer seed human salivary α-amylase inhibitory fraction (arrow) by analytical RP-HPLC (C18). (Inset) 15% SDS-PAGE and MALDI mass spectrum acquired in the *m*/*z* range 4000–19000 of the collected peak. The MALDI spectrum was obtained operating in linear ion mode and using SA as matrix. The spectrum was calibrated using a mixture of insulin, cytochrome *c*, and myoglobin as external standards.

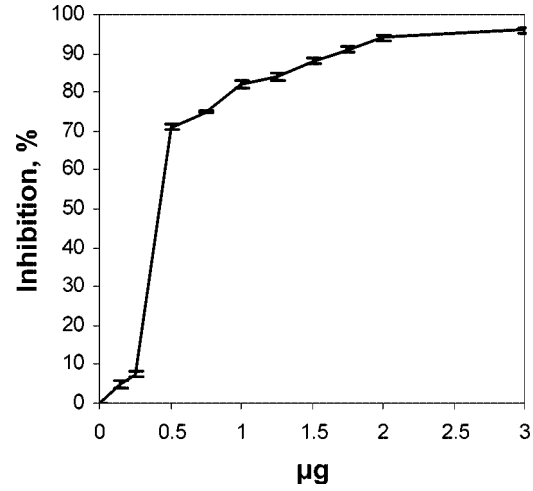

Figure 2. Effect of various concentrations (micrograms) of emmer inhibitory fraction on 0.5 unit of human salivary α-amylase. Vertical bars indicate standard deviation.

MALDI-TOF analysis of the HSA inhibiting fraction showed two peaks at *m*/*z* 13102 and 13173 (**Figure 1**, inset).

The in-solution inhibition tests carried out using increasing concentrations of this fraction showed a dramatic decrease of the HSA activity already with 0.5 μg of inhibitor (71% inhibition); the inhibition rose to 96% inhibition when 3 μg of inhibitor was used (**Figure 2**).

No inhibition was detected against *B. subtilis* α-amylase, even with significantly high protein levels (4 μg, data not shown).

Non-denaturing starch−PAGE analysis of the inhibitory fraction resolved one protein band on the Coomassie stained gel (**Figure 3A**); a duplicate of this gel gave a strong positive reaction when subjected to the in-gel inhibition assay (**Figure 3B**).

When the sample was analyzed by 2-DE, it resolved in a major spot (spot 1), a minor one (spot 2), and two additional faint spots (spots 3 and 4, **Figure 4**).

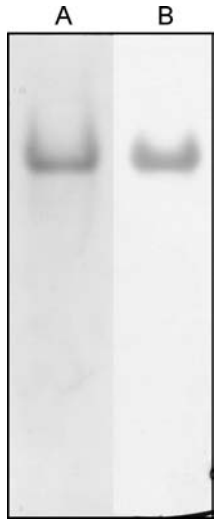

Figure 3. Non-denaturing starch−PAGE (7.5%) of the inhibitory fraction (27 μg) from emmer seeds.: (**A**) Coomassie staining; (**B**) HSA in-gel inhibition assay.

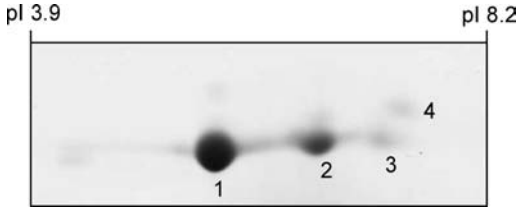

Figure 4. 2-DE analysis of the emmer seeds inhibitory fraction (15 μg). The first dimension separation was performed in the pH range of 3–10 on a 7 cm Immobiline DryStrip gel. The second dimension separation was on 15% SDS-PAGE; the spots revealed after protein staining with CBB R-350 were numbered 1–4.

To verify the protein sequence of the inhibitory fraction components, the four spots resolved on 2-DE gels were excised and subjected to in-gel trypsin digestion, mass spectrometric analyses, and bioinformatic search.

The MALDI-TOF mass spectrum of the tryptic peptides originated from spot 1, acquired in the *m/z* 600–4000 mass range (**Figure 5A**), showed intense signals, which allowed its identification with a *Triticum aestivum* dimeric α-amylase inhibitor (NCBInr General Index |65993807) by bioinformatic search. The peaks at *m/z* 1148.7, 1320.8, 1552.7, 1577.7, 1584.8, 1691.9, and 1901.8 corresponded to the expected fragments T9, T2, T5, T1, T3, T10, and T6, respectively (**Table 1**). The signals at *m/z* 2057.9, 2821.4, 3435.6, and 3591.6 were due to fragments T6+T7, T9+T10, T5+T6, and T5+T6+T7, originating from partial cleavage of the $Arg^{84}-Arg^{85}$, $Lys^{100}-Leu^{101}$, $Lys^{66}-Glu^{67}$, and $Lys^{66}-Glu^{67}/Arg^{84}-Arg^{85}$ bonds, respectively. Furthermore, in the MALDI spectrum two peaks were present at *m/z* 1585.8 and 1882.8, which could not be assigned to any of the expected tryptic fragments, whereas signals corresponding to the C-terminal peptides T11 (MH^+ 763.4) and T4 (MH^+ 1896.8) were absent. The T8 peptide was not expected to be revealed because its MH^+ (*m/z* 474.3) fell out of the mass range covered.

The reconstructed total ion current (TIC) chromatogram of the tryptic digest of spot 1 is shown in **Figure 5B**. The data obtained confirmed the identification of all the fragments already detected by MALDI-TOF MS. In addition, the fragment $Val^{14}-Arg^{21}$, originating from the cleavage of the $Arg^{21}-Pro^{22}$ bond, was detected in the fraction eluting at 18.5 min; the TIC chromatogram also showed a peak at 24.4 min, which gave a MH^+ at *m/z* 1882.8, having a difference of 14 Da from the theoretical fragment T4 (MH^+ 1896.8). Further analysis by capillary RP-HPLC/nESI-MSMS (see Supporting Information **Figure 1A**) demonstrated that this mass difference was attributable to the point substitution $Glu^{40}\rightarrow Asp^{40}$; the modified peptide is indicated as T4* in **Table 1**. The absence of the theoretical fragment T4 in the RP-HPLC/nESI-MSMS analysis confirmed the result already obtained by MALDI-MS. Consistent with the MALDI mass spectrum, the TIC chromatogram showed that the theoretical fragment T3 (MH^+ 1584.8) and a peptide with *m/z* 1585.8 coeluted at 21.5 min. Due to the isolation window of 3 Da in the MSMS experiment, separation of the two peptides' molecular ions was not achieved, and therefore the tandem mass spectrum was constituted by a superimposition of the fragment ions generated. Careful analysis of the tandem mass spectrum revealed that the sequence difference between the two peptides was due to the substitution $Asn^{29}\rightarrow Asp^{29}$, consistent with the difference of 1 Da between the observed MH^+ of the two fragments (see Supporting Information **Figure 1B**).

Moreover, the C-terminal peptide T11, previously undetected, was identified in the peak eluting at 17.5 min (**Figure 5B**). Unambiguous attribution of this signal to fragment T11 was obtained from the MSMS spectrum (see Supporting Information **Figure 2A**). Also, the TIC chromatogram (**Figure 5B**) showed that the peak eluting at 16.8 min was due to a peptide with MH^+ at *m/z* 692.2, a mass value that did not correspond to any of the expected tryptic fragments. Interpretation of the MSMS spectrum (see Supporting Information **Figure 2B**) revealed that the sequence of this fragment was GVAAYPD. This sequence corresponded to the C-terminal peptide T11 (**Table 1**), minus the C-terminal Ala residue. Because the $Asp^{123}-Ala^{124}$ cleavage could not be explained by the enzymatic activity of trypsin, we could assume the presence in spot 1 of a second protein truncated at the Asp^{123} residue. This explanation was in agreement with the difference of 71 Da observed for the two proteins detected in the MALDI mass spectrum (**Figure 1**, inset).

In conclusion, the combined MALDI-TOF MS and RP-HPLC/nESI-MSMS data indicated that more than one protein was present in spot 1; the protein sequences were completely covered, with the exception of the T8 tetrapeptide (97% sequence coverage). The proteins were characterized by the point substitution $Glu^{40}\rightarrow Asp^{40}$ with respect to the NCBInr sequence gi|65993807. Moreover, one protein was truncated at the level of the Asp^{123} residue, and sequences containing both Asn^{29} and Asp^{29} were present.

In-gel digestion and subsequent MALDI-TOF MS and capillary RP-HPLC/nESI-MSMS of spots 2 and 3 gave the same results obtained for spot 1 (data not reported).

The MALDI-TOF mass spectrum of the tryptic peptides originating from spot 4 and acquired in the *m/z* 600–4000 mass range showed intense signals (**Figure 6A**) that allowed identification, by bioinformatic search, of a *T. aestivum* tetrameric α-amylase inhibitor subunit (NCBInr gi|21705). The signals at *m/z* 1023.5, 1175.6, 1182.5, 1813.9, 1903.9, 2476.3, and 3312.8 corresponded to the peptide fragments T8, T3, T2, T7, T4, T1, and T9, respectively (**Table 2**). The spectrum also individuated a signal at *m/z* 2192.0, corresponding to the sequence Glu^4-Arg^{21} derived by the aspecific cleavage of the Asn^3-Glu^4 bond; the peptides T5 (MH^+ 661.4) and T6 (MH^+ 742.4) were not detected. Peptides containing Met residues gave additional peaks at $MH^+ + 16$ originating from the oxidation of this amino acid to methionine sulfoxide (*m/z* 1829.9, 2208.0, and 2492.2).

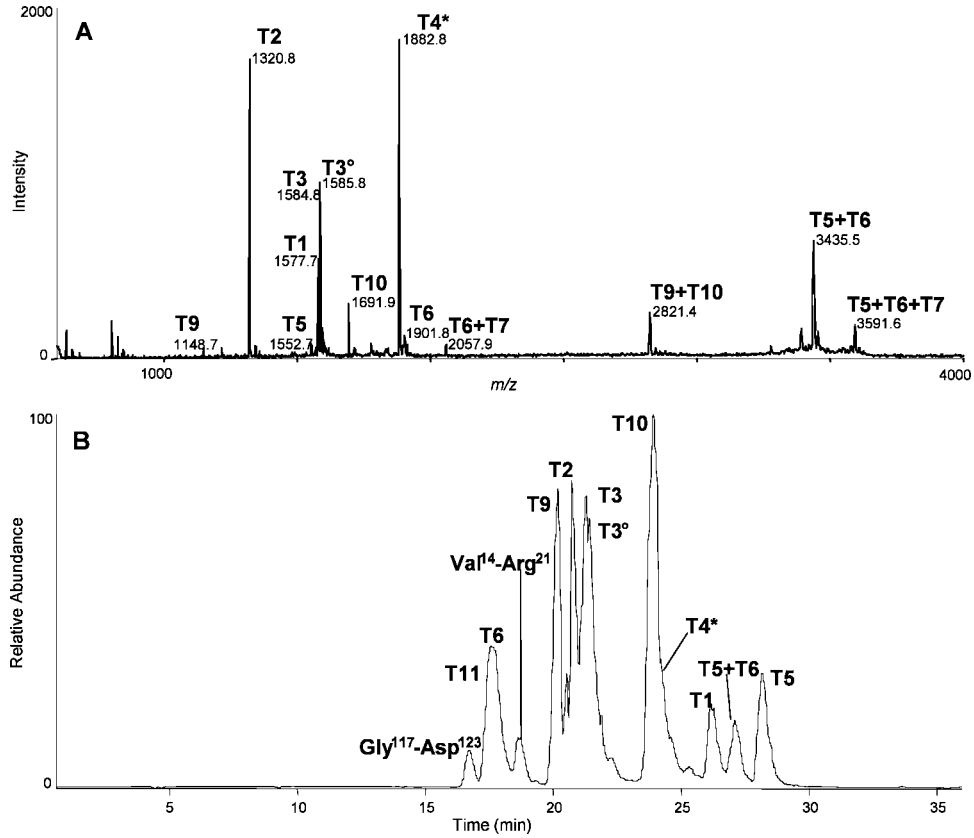

Figure 5. Analyses of tryptic peptides obtained by the in-gel digestion of spot 1: (**A**) MALDI-TOF mass spectrum acquired in the *m*/*z* range of 600–4000 obtained by operating in reflectron ion mode and using a CHCA as matrix (the spectrum was calibrated using a tryptic digest of bovine β-lactoglobulin as external calibrant); (**B**) total ion current (TIC) chromatogram of the tryptic peptides performed by RP-HPLC/nESI-MS.

Table 1. Sequence Position, Calculated Monoisotopic MH^+, and Experimentally Measured MH^+ of Spot 1 Tryptic Fragments

fragment	position	MH^+ monoisotopic	MALDI-MS measured MH^+	ESI-MS measured MH^+	sequence
T1	1–13	1577.7	1577.7	1577.3	SGPWMCYPGYAFK
T2	14–25	1320.8	1320.8	1320.5	VPALPGCRPVLK
	14–21	883.5		883.4	VPALPGCR
T3	26–39	1584.8	1584.8	1584.8	LQCNGSQVPEAVLR
T3°	26–39	1585.8	1585.8	1585.5	LQCDGSQVPEAVLR
T4	40–53	1896.8			ECCQQLADISEWCR
T4*	40–53	1882.8	1882.8	1882.4	DCCQQLADISEWCR
T5	54–66	1552.7	1552.7	1552.3	CGALYSMLDSMYK
T6	67–84	1901.7	1901.8	1901.5	EHGVQEGQAGTGAFPSCR
T5+T6	54–84	3435.5	3435.6	3436.0	CGALYSMLDSMYKEHGVQEGQAGTGAFPSCR
T7	85	175.1			R
T5+T6+T7	54–85	3591.6	3591.6		CGALYSMLDSMYKEHGVQEGQAGTGAFPSCRR
T6+T7	67–85	2057.9	2057.9		EHGVQEGQAGTGAFPSCRR
T8	86–89	474.3			EVVK
T9	90–100	1148.6	1148.7	1148.4	LTAASITAVCK
T10	101–116	1691.9	1691.9	1691.4	LPIVIDASGDGAYVCK
T9+T10	90–116	2821.5	2821.4		LTAASITAVCKLPIVIDASGDGAYVCK
T11	117–124	763.4		763.2	GVAAYPDA
	117–123	692.3		692.2	GVAAYPD

The tryptic mixture was further analyzed by capillary RP-HPLC/nESI-MSMS (**Figure 6B**). This analysis confirmed all of the assignments included the peptide Glu^4-Arg^{21} sequence and allowed the detection of the peptide T6 (retention time of 17.5 min). The results obtained allowed 96% coverage of the sequence of the *T. aestivum* tetrameric α-amylase inhibitor subunit (NCBInr gi|21705), T5 being the only undetected fragment.

DISCUSSION

The genes coding for the three amylase isoinhibitor families (mono-, di-, and tetrameric) may be derived from a very limited number of ancestral genes coding for polypeptide sequences with molecular mass close to 12 kDa. Replication of these ancestral genes and divergence by various mechanisms have probably given rise to the three main families of genes coding for the many subunits of the different inhibitors (*21*). One of the most common forms of sequence variation is single nucleotide polymorphisms (SNPs), that is, single base-pair substitutions which can be found in both the coding and noncoding regions of the genome. The majority of coding regions SNPs are single-base substitutions that may or may not result in amino acid substitutions. Genetic variations have been

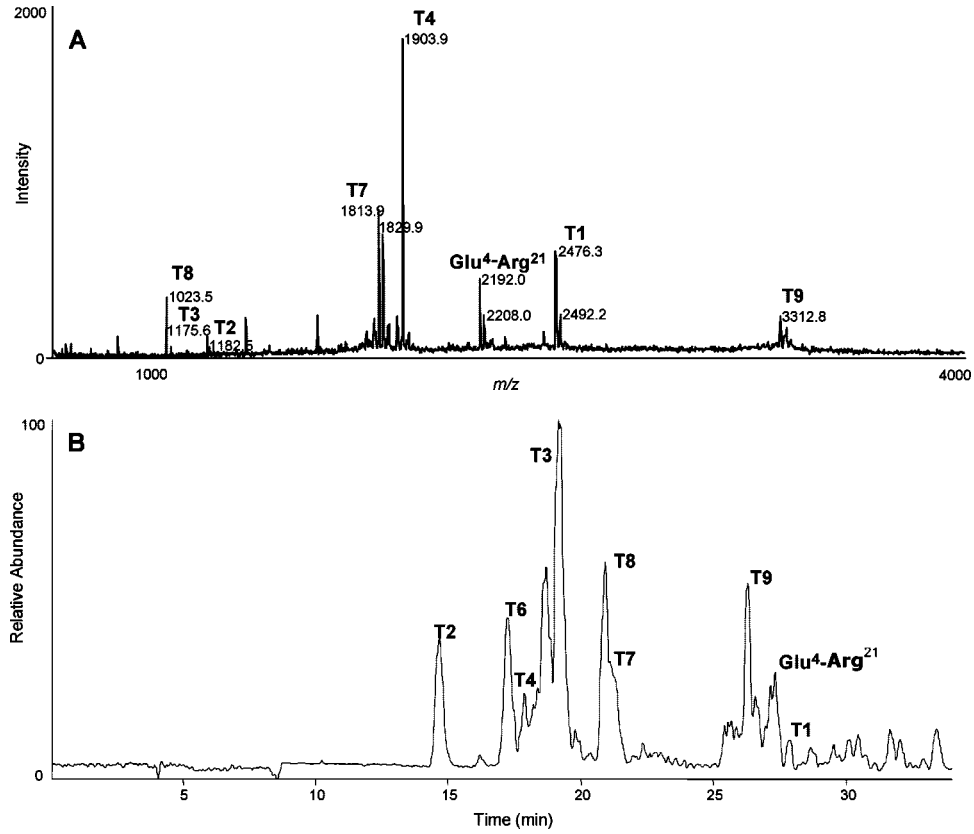

Figure 6. Analyses of tryptic peptides obtained by the in-gel digestion of spot 4: (**A**) MALDI-TOF mass spectrum acquired in the *m*/*z* range of 600–4000 obtained operating in reflectron ion mode and using a CHCA as matrix (the spectrum was calibrated using a tryptic digest of bovine β-lactoglobulin as external calibrant); (**B**) total ion current (TIC) chromatogram of the tryptic peptides performed by RP-HPLC/nESI-MS.

Table 2. Sequence Position, Calculated Monoisotopic MH^+, and Experimentally Measured MH^+ of Spot 4 Tryptic Fragments

fragment	position	MH^+ monoisotopic	MALDI MS measured MH^+	ESI MS measured MH^+	sequence
T1	1–21	2476.2	2476.3	2476.9	IGNEDCTPWMSTLITPLPSCR
	4–21	2192.0	2192.0	2192.3	EDCTPWMSTLITPLPSCR
T2	22–30	1182.6	1182.5	1182.2	DYVEQQACR
T3	31–41	1175.6	1175.6	1175.2	IETPGSPYLAK
T4	42–56	1903.9	1903.9	1903.3	QQCCGELANIPQQCR
T5	57–61	661.4			CQALR
T6	62–67	742.4		742.2	YFMGPK
T7	68–83	1813.9	1813.9	1814.3	SRPDQSGLMELPGCPR
T8	84–91	1023.5	1023.5	1023.2	EVQMDFVR
T9	92–119	3312.5	3312.8	3313.3	ILVTPGYCNLTTVHNTPYCLAMEESQWS

detected in dimeric α-amylase inhibitors from cultivated wheat and its putative diploid progenitors (*22*). Wang et al. (*23*) identified 11 dimeric α-amylase inhibitor genes from *T. aestivum* 'Chinese Spring' that encoded proteins with highly coherent deduced amino acid sequences (95.1%) and all containing 10 cysteine residues.

Mass spectrometry analyses of spot 1 indicated that the inhibitory fraction isolated from emmer seeds was indeed a mixture containing more than one inhibitor protein.

This fact was not surprising because the mass spectrometric data themselves demonstrated that these proteins had very similar amino acid sequences (*24*); consequently, the electrophoretic and chromatographic techniques that we have used and that have been routinely used to isolate and purify these proteins in the past could not efficiently discriminate between proteins having few amino acid substitutions often involving residues with very similar charges and molecular weights.

Combinations of the structural features deduced from the mass spectrometric data for spot 1 generated four hypothetical proteins: two proteins with 124 amino acids, one with the amino acid substitution $Asn^{29}\rightarrow Asp^{29}$ (13172 and 13173 Da, respectively), and two proteins with 123 amino acids, one with the same amino acid substitution (13101 and 13102 Da, respectively). All four of these hypothetical proteins present the point substitution $Glu^{40}\rightarrow Asp^{40}$. The accuracy of mass determination by MALDI mass spectrometry in the 14000 Da mass range is not sufficient to discriminate 1 mass unit; thus, from the present data, the occurrence of the substitution $Asn^{29}\rightarrow Asp^{29}$ in the complete sequence or in the truncated form remains undetermined. With this structural uncertainty, the 124 amino acid protein is named "emmer dimeric inhibitor 1" (EDI-1, 13173 or 13172 Da) and the 123 amino acid protein is named "emmer dimeric inhibitor 2" (EDI-2, 13101 or 13102 Da).

The inhibitors EDI-1 and EDI-2 identified in spot 1 share high identities (from 89 to 98% of sequence identity) with the deduced sequences of dimeric α-amylase inhibitors of *T. aestivum* (*23*) and with the sequences of the so-called 0.19 and 0.53 inhibitors (*8, 9*); however, the amino acid substitution in

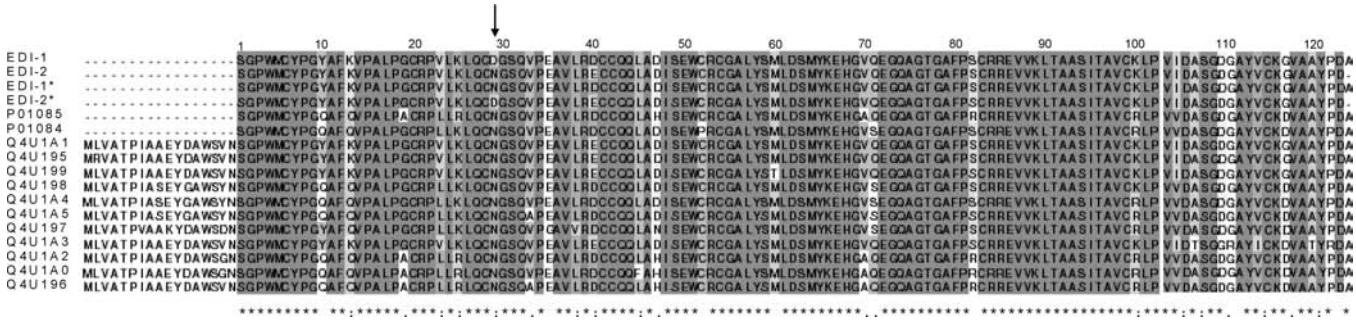

Figure 7. Alignment of the amino acid sequences of the isolated dimeric α-amylase inhibitors from emmer (EDI-1 and EDI-2) with the mature proteins of inhibitors 0.19 and 0.53 and the deduced sequences of dimeric α-amylase inhibitors from *T. aestivum*. The alignments were obtained through the MUSCLE web server at http://www.drive5.com/muscle/ (*32*). EDI-1* and EDI-2* indicate the possible alternative inhibitor forms suggested by the RP-HPLC/nESI-MS analyses. The arrow points to the amino acid in position 29, which cannot be assigned inequivocably to the complete (EDI-1/EDI-1*) or truncated inhibitor protein (EDI-2/EDI-2*).

position 29 (Asn→Asp) of EDI-1 or EDI-2 and the Ala deletion in position 124 of EDI-2 are not present in any of the sequences reported in **Figure 7**. The positions of this amino acid substitution and deletion do not influence the HSA inhibitory activity of EDI-1 and EDI-2, because they are located outside the three regions of interest postulated by Franco et al. (*25*) for the interaction between α-amylase inhibitors and HSA. Using the modeled complex of HSA-0.19, proposed by Franco et al. (*25*), three zones were revealed in the 0.19 inhibitor sequence: the His^{47} residue, the Ser^{49} residue, and the $V^{104}VDA^{107}$ sequence that is involved in the inhibitor protruding long loop (residues 100–113). It was suggested that this loop region was to play a pivotal role in conferring inhibition specificity for α-amylases of mammalian origin. In the wheat inhibitors that weakly inhibit the α-amylases from human saliva and pancreas (such as the inhibitor 0.28) this segment is replaced by the shorter sequence $P^{104}NP^{106}$ (*26*). EDI-1 and EDI-2 sequences presented two substitutions in positions 47 (His→Asp) and 105 (Val→Ile) (**Figure 7**), which are comprised within the three regions of interest proposed for the HSA-0.19 model (*25*). Wang et al. (*22*) stated that only α-amylase inhibitors closely related to inhibitor 0.19 from the D genome of *Aegilops tauschii* and common wheat (*T. aestivum*) presented the His^{47}; this residue was replaced by Asp or Asn in all other inhibitor sequences from *Triticum urartu* (A genome), *Aegilops speltoides* (B genome), and *Triticum durum* (AB genome). Hence, this substitution in position 47 of EDI-1 and EDI-2 sequences is in agreement with the emmer wheat genomic constitution (AB genome).

Wang et al. (*22*) have also hypothesized that the lack of amylase inhibitory activity in diploid wheats (*Triticum monococcum* and *Triticum urartu*) might be due to a number of possible reasons, among which was the substitution Ile^{105}→Val^{105} in the sequence $V^{104}VDA^{107}$. Conversely, this substitution is present in both emmer dimeric inhibitors, which are highly active against HSA (**Figure 2**). This substitution does not appear to alter the validity of the model proposed, on the basis of the similar biochemical characteristics of the amino acids involved.

Due to the Ala^{124} deletion, the EDI-2 sequence was made up of 123 amino acids (**Figure 7**). A protein, named WPR26, with a similar deletion has been described by Feng et al. (*27*) in wheat seeds. On the basis of the location of Ala^{124} in a probable flexible region of the protein, it is unlikely to form strong interactions with HSA (*26*). Hence, this deletion should not impair the binding between EDI-2 and HSA. WPR26 was found to be active against insect α-amylases and totally inactive against HSA; however, the inhibitor specificity was attributed to the replacement of the sequence $V^{104}VDA^{107}$ by the shorter $P^{104}NP^{106}$, which is typical of α-amylase inhibitors lacking activity against HSA.

The effect of increasing concentrations of emmer inhibitors on HSA activity showed that to obtain 50% inhibition of 1 unit of HSA required about 1 μg of the emmer inhibitors (**Figure 2**). Despite the different methodologies used for the inhibition measurement, these data are in agreement with the value obtained by Maeda et al. (*9*) for the 50% inhibition of 1 unit of HSA by the 0.19 inhibitor.

Some conflicting data concern the ability of dimeric inhibitors to inhibit α-amylase of bacterial origin. Takase (*28*) demonstrated that the α-amylase from *B. subtilis* was strongly inhibited by the wheat α-amylase inhibitors 0.53 and 0.19, whereas Buonocore et al. (*2*) reported a complete inactivity of 0.19 against the bacterial enzyme. Our data are in agreement with Buonocore et al. (*2*), confirming the lack of specificity of EDI-1 and EDI-2 for the α-amylase of bacterial origin.

Wheat dimeric α-amylase inhibitors are always described as homodimers, constituted by two identical polypeptides that are able to self-associate in vitro (*29*). However, in *Setaria italica* a 24 kDa heterodimer has been described, made up of non-covalently linked subunits of 12 and 16 kDa (*30*). Recently, Zoccatelli et al. (*31*) have demonstrated that dimeric inhibitors of *T. aestivum* can exist in vitro as heterodimers without losing the inhibitory capacity; it is possible that the HSA dimeric inhibitors identified in spot 1 aggregate both in homodimeric and in heterodimeric forms (based on the combination of the hypothetical monomers identified).

The finding in spots 2 and 3 of polypeptides identical to those found in spot 1 can be explained by an artifactual horizontal streaking; this could be due to a reduced solubility of the sample in rehydration buffer and consequently to the repeated cycles of lyophilization/solubilization it underwent during the purification process.

The mass spectrometric data identified the protein present in spot 4 as a subunit of the wheat heterotetrameric inhibitor of heterologous α-amylases (NCBInr gi|21705). Heterotetrameric inhibitors are composed by different subunits whether isolated from tetraploid (CM2, CM16, and CM3) or hexaploid (CM1, CM2, CM3, CM16, and CM17) wheats. The proteins are soluble both in NaCl solutions and in chloroform/methanol mixtures, from which the acronym CM-proteins is taken. The sequence of the protein found in spot 4 was identified with the CM16 subunit of the heterotetrameric inhibitor of *T. aestivum* (*11*). This protein component was not visible in the MALDI mass spectrum due to its lower amount with respect to the dimeric inhibitors. Possibly, of the two protein bands revealed on the

SDS-PAGE analysis of the inhibitory fraction (**Figure 1**, inset), the faster migrating protein corresponds to a mixture of the dimeric inhibitor subunits and the slower migrating protein to the CM16 polypeptide. The heterotetrameric inhibitors are active against both insect and human α-amylases but are not active against the enzymes from bacteria (*B. subtilis*) or fungi (*Aspergillus oryzae*). Reconstitution experiments of the tetrameric inhibitors showed that three types of subunits, having little or no inhibitory activity by themselves or in binary combinations, are required to obtain fully active inhibitors (*10*). On these accounts, the CM16 subunit from spot 4 should be present in our sample as a minor protein contaminant but would not contribute at all to the inhibitory activity we measured against HSA.

The present study was carried out as part of a project aimed to investigate the presence of antinutritional factors in rustic cereal species such as emmer (*T. dicoccon* Schrank). From this perspective we have focused our research on protein inhibitors able to severely suppress the activity of human salivary α-amylase. Our findings have indicated the presence in emmer mature seeds of two isoinhibitors (EDI-1 and EDI-2) with minimal polypeptide variability and identical specificity against HSA. These results confirm the existence in tetraploid wheats of a family of highly similar α-amylase inhibitors coded by a multigene family, as already discussed by Wang et al. (*22*, *23*).

Further investigation of these inhibitor classes in emmer seeds will examine their ability to act against pest amylases, thereby investigating the possibility of their playing a role in making emmer a very rustic species.

Supporting Information Available: Figures 1 and **2** representing the MSMS spectra of selected peptides from the in-gel tryptic digestion of spot 1. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Kneen, E.; Richardson, M. An amylase inhibitor from certain cereals. *J. Am. Chem. Soc.* **1943**, *65*, 1247.

(2) Buonocore, V.; Petrucci, T.; Silano, V. Wheat protein inhibitors of α-amylase. *Phytochemistry* **1977**, *16*, 811–820.

(3) Deponte, R.; Parlamenti, R.; Petrucci, T.; Silano, V.; Tomasi, M. Albumin α-amylase inhibitor families from wheat flour. *Cereal Chem.* **1976**, *53*, 805–820.

(4) Salcedo, G.; Sánchez-Monge, R.; García-Casado, G.; Amentia, A.; Gomez, L.; Barber, D. The cereal α-amylase/trypsin inhibitor family associated with bakers' asthma and food allergy. In *Plant Food Allergens*; Mills, E. N. C., Shewry, P. R., Eds.; Blackwell Publishing: Oxford, U.K., 2004; pp 70–86.

(5) Silano, V.; Furia, M.; Gianfreda, L.; Macri, A.; Palescandolo, R.; Rab, A.; Scardi, V.; Stella, E.; Valfre, F. Inhibition of amylases from different origins by albumins from the wheat kernel. *Biochim. Biophys. Acta* **1975**, *391*, 170–178.

(6) García-Olmedo, F.; Salcedo, G.; Sánchez-Monge, R.; Gomez, L.; Royo, J.; Carbonero, P. Plant proteinaceous inhibitors of proteinases and α-amylases. *Oxford Surv. Plant Mol. Cell Biol.* **1987**, *4*, 275–334.

(7) Kashlan, N.; Richardson, M. The complete amino acid sequence of a major wheat protein inhibitor of α-amylase. *Phytochemistry* **1981**, *20*, 1781–1784.

(8) Maeda, K.; Hase, T.; Matsubara, H. Complete amino acid sequence of an α-amylase inhibitor in wheat kernel. *Biochim. Biophys. Acta* **1983**, *743*, 52–57.

(9) Maeda, K.; Kakabayashi, S.; Matsubara, H. Complete amino acid sequence of an α-amylase inhibitor in wheat kernel (0.19-inhibitor). *Biochim. Biophys. Acta* **1985**, *828*, 213–221.

(10) Gomez, L.; Sánchez-Monge, R.; García-Olmedo, F.; Salcedo, G. Wheat tetrameric inhibitors of insect α-amylases: alloploid heterosis at the molecular level. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3242–3246.

(11) García-Maroto, F.; Maraña, C.; Mena, M.; García-Olmedo, F.; Carbonero, P. Cloning of cDNA and chromosomal location of genes encoding the three types of subunits of the wheat tetrameric inhibitor of insect α-amylase. *Plant Mol. Biol.* **1990**, *3*, 845–848.

(12) Capocchi, A.; Fontanini, D.; Muccilli, V.; Cunsolo, V.; Saviozzi, F.; Saletti, R.; Lorenzi, R.; Foti, S.; Galleschi, L. NsLTP1 and nsLTP2 isoform in soft wheat (*Triticum aestivum* cv. Centauro) and farro (*Triticum dicoccon*) Schrank bran. *J. Agric. Food Chem.* **2005**, *53*, 7976–7984.

(13) Capocchi, A.; Fontanini, D.; Muccilli, V.; Cunsolo, V.; Saviozzi, F.; Saletti, R.; Foti, S.; Galleschi, L. Tetraploid and hexaploid wheats express identical isoforms of nsLTP1. *J. Agric. Food Chem.* **2006**, *54*, 2372–2377.

(14) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(15) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(16) Bernfeld, P. Amylases, a and b. *Methods Enzymol.* **1955**, *1*, 149–158.

(17) Fontanini, D.; Capocchi, A.; Saviozzi, F.; Galleschi, L. Simplified electrophoretic assay for human salivar α-amylase inhibitor detection in cereal seed flours. *J. Agric. Food Chem.* **2007**, *55*, 4334–4339.

(18) Kussmann, M.; Nordhoff, E.; Rahbek-Nielsen, E.; Haebel, S.; Larsen, M. R.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kristensen, A. K.; Palm, L.; Roepstorff, P. Matrix-assisted laser desorption/ionization mass spectrometry sample preparation techniques designed for various peptide and protein analytes. *J. Mass Spectrom.* **1997**, *32*, 593–601.

(19) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850–858.

(20) Gobom, J.; Nordhoff, E.; Mirgorodskaya, E.; Ekman, R.; Roepstorff, P. Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **1999**, *34*, 105–116.

(21) Buonocore, V.; De Biasi, M.-G.; Giardina, P.; Poerio, E.; Silano, V. Purification and properties of an α-amylase tetrameric inhibitor from wheat kernel. *Biochim. Biophys. Acta* **1985**, *831*, 40–48.

(22) Wang, J.-R.; Wei, Y.-M.; Yan, Z.-H.; Zheng, Y.-L. Detection of single nucleotide polymorphisms in 24 kDa dimeric α-amylase inhibitors from cultivated wheat and its diploide putative progenitors. *Biochym. Biophys. Acta* **2005**, *1723*, 309–320.

(23) Wang, J.-R.; Yan, Z.-H.; Wei, Y.-M.; Nevo, E.; Baum, B. R.; Zheng, Y.-L. Molecular characterization of dimeric α-amylase inhibitor genes in wheat and development of genome allele-specific primers for the genes located on chromosome 3BS and 3DS. *J. Cereal Sci.* **2006**, *43*, 360–368.

(24) Wang, J.-R.; Zhang, L.; Wei, Y.-M.; Yan, Z.-H.; Baum, B. R.; Nevo, E.; Zheng, Y.-L. Sequence polymorphisms and relationships of dimeric α-amylase inhibitor genes in the B genomes of *Triticum* and S genomes of *Aegilops*. *Plant Sci.* **2007**, *173*, 1–11.

(25) Franco, O. L.; Rigden, D. J.; Melo, F. R.; Bloch, C., Jr.; Silva, C. P.; Grossi-de-Sá, M. F. Activity of wheat α-amylase inhibitors towards bruchid α-amylases and structural explanation of observed specificities. *Eur. J. Biochem.* **2000**, *267*, 2166–2173.

(26) Payan, F. Structural basis for the inhibition of mammalian and insect α-amylases by plant protein inhibitors. *Biochim. Biophys. Acta* **2004**, *1696*, 171–180.

(27) Feng, G. H.; Richardson, M.; Chen, M. S.; Kramer, K. J.; Morgan, T. D.; Reeck, G. R. α-Amylase inhibitors from wheat: amino acid sequences and patterns of inhibition of insect and human α-amylases. *Insect Biochem. Mol. Biol.* **1996**, *26*, 419–426.

(28) Takase, K. Site-directed mutagenesis reveals critical importance of the catalytic site in the binding of α-amylase by wheat proteinaceous inhibitor. *Biochemistry* **1994**, *33*, 7925–7930.

(29) Sánchez-Monge, R.; Gomez, L.; García-Olmedo, F.; Salcedo, G. New dimeric inhibitor of heterologous α-amylases encoded by a duplicated gene in the short arm of chromosome 3B of wheat (*Triticum aestivum* L.). *Eur. J. Biochem.* **1989**, *183*, 37–40.

(30) Nagaraj, R. H.; Pattabiraman, T. N. Isolation of an amylase inhibitor from *Setaria italica* grains by affinity chromatography on Blue-sepharose and its characterization. *J. Agric. Food Chem.* **1985**, *33*, 646–650.

(31) Zoccatelli, G.; Dalla Pellegrina, C.; Mosconi, S.; Consolini, M.; Veneri, G.; Chignola, R.; Peruffo, A.; Rizzi, C. Full-fledged proteomic analysis of bioactive wheat amylase inhibitors by a 3-D analytical technique: identification of new heterodimeric aggregation states. *Electrophoresis* **2007**, *28*, 460–466.

(32) Edgar, R. C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797.

Received for review June 13, 2007. Revised manuscript received October 1, 2007. Accepted October 18, 2007. The research was funded by the Italian Ministry of University and Research (MiUR) within the program "Incentivazione alla mobilità di studiosi stranieri e italiani residenti all'estero".

JF071739W