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Inhibitory activities against heterologous α-amylases and in vitro allergenic reactivity of Einkorn wheats

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Abstract Salt extracts from seeds of 36 lines of Einkorn wheats were analyzed for their inhibitory activity towards two insect (*Tenebrio molitor*, Coleoptera, and *Ephestia kuehniella*, Lepidoptera) and one mammalian (human salivary) α-amylases. Whereas all ten *T. monococcum* accessions tested were active towards the lepidopteran enzyme, they had no effect on the coleopteran or the mammalian ones. More variability was found among the 21 lines of *T. boeticum* analyzed, although none of them inhibited human α-amylase. The five accessions of *T. urartu* showed even greater diversity. Among all Einkorn accessions tested, only two *urartu* lines affected the three α-amylases. These lines displayed inhibition patterns similar to those of *T. aestivum* and *T. turgidum* cultivars. Since several bread-wheat α-amylase inhibitors are major allergens associated with baker's asthma, we also studied the in vitro allergenic activity of salt extracts from the Einkorn wheats under study. No significant differences in IgE-binding were found between these accessions and the *T. aestivum* or *T. turgidum* cultivars. Furthermore, putative allergens with molecular sizes in the range of 20–60 kDa were detected in these Einkorn wheats.

Key words α-Amylase inhibitors · Allergens · *Triticum monococcum* · *Triticum boeticum* · *Triticum urartu*

Introduction

Einkorn wheat includes cultivated and wild diploid species (2n = 2x = 14; genomes AA) of the genus *Triticum*. The assignation of these diploid wheats to specific taxa is still controversial and, as a consequence, their classification differs in germ plasm collections. Three Einkorn species have been proposed: *Triticum monococcum* L., *T. boeticum* Boiss. and *T. urartu* Thum.; although some authors consider *T. boeticum* as a subspecies of *T. monococcum* (Bell 1987). Despite the fact that low genetic diversity has been found in these three species using isozyme and RFLP markers (Smith-Huerta et al. 1989; Le Corre and Bernard 1995), a number of accessions with unique RFLP patterns have been reported (Castagna et al. 1994).

Einkorn wheats have been analyzed to study the phylogeny of polyploid wheats. Recent evidence suggests that *T. urartu* is the donor species of the A genome present in *T. turgidum* L. (pasta wheat, genomes AABB) and *T. aestivum* (bread wheat, genomes AABBDD) (Kerby and Kuspira 1987; Dvorak et al. 1993; Takumi et al. 1993). On the other hand, several quality traits of the Einkorn wheats (dough characteristics, high protein content, tolerance to biotic and abiotic stress, etc.) have led to their use in bread and durum wheat-breeding programs, as well as to develop new lines of *T. monococcum*.

In *T. aestivum* and *T. turgidum* the major fraction of salt-soluble endosperm proteins is represented by 13–16 kDa polypeptides belonging to the cereal α-amylase inhibitor family (García-Olmedo et al. 1987). These inhibitors are encoded by a multigene family dispersed over chromosomes 3, 4, 6 and 7 of genomes A and B (Carbonero et al. 1993). Interestingly, no members of this family have been detected at the protein level which are encoded by the A genome. However, homologous DNA sequences have been found in chromosomes 4A and 7A by Southern analysis, suggesting that these A genes are either silent or else expressed at only a very low level (García-Maroto et al. 1990).

No inhibitory activity against insect or mammalian α-amylases has been previously reported in *T. monococcum* or in the majority of *T. boeticum* accessions analyzed (Bedetti et al. 1974; Vitozzi and Silano 1976; Gomez et al. 1989; Konarev 1994). By contrast, water

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extracts from *T. urartu*, like those from all other hexa-, tetra- and di-ploid *Triticum* species tested, inhibit heterologous α-amylases (Vitozzi and Silano 1976; Konarev 1994). The activity of the inhibitors towards pest enzymes has led us to propose their use in plant protection (García-Olmedo et al. 1987).

A further relevant aspect of this cereal inhibitor family comes from the finding that several members are prominent allergens in allergic diseases (baker's asthma) provoked by the inhalation of cereal flour (Sanchez-Monge et al. 1992; Armentia et al. 1993).

In the present report we have analyzed the activity against insect and mammalian α-amylases of NaCl extracts from ten accessions of *T. monococcum*, 21 of *T. boeticum*, and five of *T. urartu*. New inhibitory properties have been uncovered in most samples tested. In addition, the in vitro IgE-binding capacity of these salt extracts has been studied using sera of allergic patients and their reactivities have been compared with those of *T. aestivum* and *T. turgidum* cultivars.

Materials and methods

Plant material

Thirty six genotypes of Einkorn wheat (ten of *T. monococcum*, 21 of *T. boeticum* and five of *T. urartu*) were analyzed (Table 1). All of them, except *T. monococcum* UP-1, were kindly supplied by Drs. F. Salamini and M. Heun (Max Planck Institut, Köln, Germany). Code numbers (Table 1) correspond to those of the Max Planck collection. Three cultivars of *T. aestivum*, three of *T. turgidum* and one accession of *T. tauschii* (diploid, genomes DD) were used as controls.

Protein extraction

De-hulled kernels were mortar-ground, and 500 mg of the resulting flour were extracted with 0.5 M NaCl (2 × 1:10 w/v; 1 h; 25°C). The salt extracts were dialyzed against 0.1 M ammonium acetate, and then heated at 60°C during 1 h (to inactivate the endogenous amylases) and centrifuged. The final supernatants were used in the inhibition tests. Protein concentration was quantitated by the method of Smith et al. (1985).

Inhibition tests

Inhibition of insect and mammalian α-amylases was tested according to Bernfeld (1955), with the modifications introduced by Gutierrez et al. (1993). Tests were carried out at the pH optimum of each α-amylase assayed, using the following buffers and temperatures: 20 mM sodium acetate, 100 mM NaCl, 0.1 mM $CaCl_2$, pH 5.4 (25°C) for *Tenebrio molitor* (Coleoptera) α-amylase; 25 mM glycine, 100 mM NaCl, 0.1 mM $CaCl_2$, pH 9.5 (25°C) for the enzyme from *Ephestia kuehniella* (Lepidoptera); and 20 mM potassium phosphate, 67 mM NaCl, 0.1 mM $CaCl_2$, pH 6.9 (37°C) for human salivary α-amylase. Briefly, the salt extracts (5 and 50 μg of protein) were dissolved in 230 μl of buffer containing the amylase, incubated for 30 min, and then 230 μl of 1% starch solution was added. After incubation for 30 min, addition of 460 μl of 3,5-dinitrosalicylic acid reagent, heating at 100°C for 5 min, cooling on ice, and diluting with 2 ml of water, the absorbance of the final mixture was measured at 550 nm. All tests were performed with approximately 1 unit of α-amylase, defined as the enzyme activity required to produce the reducing equivalents of 1 μmol of maltose in our experimental conditions.

Table 1 Einkorn wheat lines analyzed in this study. Code numbers (ID) refer to those of the Einkorn wheat collection of the Plant Breeding and Yield Physiology Department, Max-Planck Institut, Köln, Germany, except for *T. monococcum* accession UP-1

Sample	Number	ID	Geographic origin
T. monococcum spp	1	UP-1	Spain
T. monococcum sinskajae	2	69	Daghestan
T. monococcum vulgare	3	103	Balkan region
T. monococcum sinskajae	4	120	Daghestan
T. monococcum vulgare	5	237	Austria
T. monococcum flavescens	6	303	France
T. monococcum vulgare	7	330	Spain
T. monococcum spp.	8	397	Yugoslavia
T. monococcum spp.	9	495	Turkey
T. monococcum spp.	10	571	Macedonia
T. boeticum	11	49	Iraq
T. boeticum	12	581	Iran
T. boeticum	13	607	Turkey
T. boeticum	14	697	Turkey
T. boeticum	15	776	Iraq
T. boeticum	16	801	Iraq
T. boeticum	17	881	Iraq
T. boeticum	18	900	Iraq
T. boeticum	19	913	Iraq
T. boeticum	20	933	Iran
T. boeticum	21	938	Iran
T. boeticum	22	1108	Turkey
T. boeticum	23	1109	Turkey
T. boeticum	24	1201	Iraq
T. boeticum	25	1208	Iraq
T. boeticum	26	1230	Iraq
T. boeticum	27	1237	Iraq
T. boeticum	28	1285	Turkey
T. boeticum	29	1297	Turkey
T. boeticum	30	1303	Turkey
T. boeticum	31	1305	Turkey
T. urartu	32	122	Unknown
T. urartu	33	126	Unknown
T. urartu	34	386	Turkey
T. urartu	35	388	Lebanon
T. urartu	36	394	Russia

Immunodetection

The IgE-binding capacity of the salt extracts was determined by dot-blot assays, using a pool of sera from five patients with baker's asthma (kindly provided by Dr. A. Armentia, Hospital Rio Ortega, Valladolid, Spain). This pool was RAST (radioallergosorbent test) class 4 (the highest class of RAST indicating a high level of specific IgE in the sera) when tested by the Phadebas-RAST Kit (Pharmacia).

Samples (1 μg of protein) were solubilized in NaCl/Tris (20 mM Tris/HCl, 150 mM NaCl, pH 8.3), 0.001% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, and heated at 100°C during 5 min. After adsorption to poly (vinylidene difluoride) (PVDF) membranes, immunodetection was carried out by treatment with 1:3 sera dilutions and ^{125}I-labelled anti-human IgE, as described by Lughtenberg et al. (1975). Negative (BSA) and positive (wheat and barley purified allergens of the α-amylase/trypsin inhibitor family) controls were included. To quantify the responses obtained, the radioactivity of each spot was determined directly in the PVDF membranes using a Phosphor Analyst (BioRad). Samples (10 μg of protein) seperated by SDS-PAGE were electrotransferred to PVDF membrances, as in Sanchez-Monge et al. (1992), and immunoblotted by the procedure described above.

Cluster analysis

For the analysis of the relationships between wheat samples. the arcsine transforms (Sokal and Rohlf 1981) of the inhibition values (50 μg of protein/assay) were used as characters, and their distances were estimated as Euclidean distances. The methods employed were Maximum Likelihood, Neighbor Joining and UPGMA, and the programs were those included in the PHYLIP package of J. Felsenstein. Significance was assessed by the bootstrapping method (Efron 1979).

Table 2 Inhibitory activity of salt extracts from Einkorn wheats against *T. molitor*, *E. kuehniella* and human salivary α-amylases. Several *T. aestivum* and *T. turgidum* cultivars, as well as a *T. tauschii* accession, were used as reference samples. Values are means ($n = 2$). Mean and standard deviation for each species are indicated.

Wheat lines	Inhibition[a] (%)						
		T. molitor		*E. kuehniella*		Human saliva	
	μg/assay	5	50	5	50	5	50
T. monococcum	1	–	8	4	54	–	–
	2	–	11	7	57	–	3
	3	–	–	21	61	1	4
	4	–	–	–	48	–	–
	5	1	6	9	58	–	–
	6	1	10	21	64	–	–
	7	–	–	1	54	–	–
	8	–	9	–	49	–	–
	9	4	11	–	54	–	–
	10	3	6	24	49	–	–
Mean ± σn		0.9 ± 1.3	6.1 ± 4.3	8.7 ± 9.2	54.8 ± 5.0	0.1 ± 0.3	0.7 ± 1.4
T. boeticum	11	79	81	67	75	–	–
	12	5	27	1	72	1	–
	13	42	77	34	75	4	–
	14	75	85	61	73	–	–
	15	74	80	58	70	–	–
	16	85	92	63	75	–	–
	17	88	100	59	65	1	–
	18	70	100	57	66	–	3
	19	76	100	49	67	4	3
	20	2	65	1	63	5	1
	21	–	78	9	64	–	–
	22	68	85	60	72	–	–
	23	83	100	46	62	5	–
	24	75	100	40	70	1	–
	25	–	26	–	64	–	–
	26	–	63	1	57	–	–
	27	72	95	48	69	2	–
	28	2	9	–	55	1	1
	29	71	78	56	71	–	–
	30	1	35	–	64	–	–
	31	2	10	–	41	–	–
Mean ± σn		46.1 ± 36.0	70.7 ± 29.9	33.8 ± 26.4	66.1 ± 7.8	1.1 ± 1.7	0.3 ± 0.8
T. urartu	32	–	4	–	49	–	–
	33	–	2	2	60	–	–
	34	84	97	44	66	–	–
	35	11	46	30	68	10	29
	36	10	49	23	75	10	24
Mean ± σn		21.0 ± 31.8	39.6 ± 34.9	19.8 ± 16.7	63.6 ± 8.7	4.0 ± 4.8	10.6 ± 13.0
T. aestivum	cv Chinese Sp.	73	95	48	65	55	63
	cv Anza	77	95	56	67	48	61
	cv Cajeme	76	95	51	70	15	31
Mean ± σn		75.3 ± 1.6	95.0 ± 0.0	51.6 ± 3.3	67.3 ± 2.0	39.3 ± 17.4	51.6 ± 14.6
T. turgidum	cv Senatore C.	51	80	34	79	43	54
	cv Enano And.	33	65	29	78	44	57
	cv Peñafiel	67	96	63	63	57	64
Mean ± σn		50.3 ± 13.8	80.3 ± 12.6	42.0 ± 14.9	73.3 ± 7.3	48.0 ± 6.3	58.3 ± 4.1
T. tauschii	acc UP-2	47	86	32	78	1	17

[a] Tests were carried out using approximately 1 unit of α-amylase. – = no inhibition

Results and discussion

Inhibition of heterologous α-amylases by salt extracts from Einkorn wheats

The inhibitory properties against α-amylases from three different origins, *T. molitor* (Coleoptera, pH optimum 5.4), *E. kuehniella* (Lepidoptera, pH optimum 9.5) and human saliva (pH optimum 6.9), of salt extracts from Einkorn wheat seeds were tested. Both insects are important cereal pests. The results obtained are summarized in Table 2.

All the *T. monococcum* accessions analyzed showed very similar inhibitory properties. None of them was active towards the coleopteran or the human salivary enzymes, in agreement with previous data (Bedetti et al. 1974; Vitozzi and Silano 1976; Gomez et al. 1989; Konarev 1994). However, inhibition of the *E. kuehniella* α-amylase was observed in all samples, specially when high amounts of protein/assay were used. In contrast with most *T. aestivum* and *T. turgidum* cultivars, the *T. monococcum* lines were poorly effective at low protein levels. These results indicate that components active towards lepidopteran α-amylase are present in the seeds of *T. monococcum*. Although such components remain to be characterized, preliminary data indicate that a major fraction of the inhibitory activity is associated with low-molecular-weight non-protein fractions of the salt extracts (R. Sánchez-Monge and G. Salcedo, unpublished). Thus, the major *T. monococcum* inhibitors do not appear to be equivalent to those described in *T. aestivum* and *T. turgidum* and most likely do not belong to the cereal α-amylase/trypsin inhibitor family.

By contrast with previous data (Vitozzi and Silano 1976; Konarev 1994), most *T. boeticum* lines were active against both insect α-amylases and showed inhibition patterns very similar to those found in *T. aestivum* cultivars. However, while the major monomeric and homodimeric inhibitors of hexaploid wheat also inhibit the human salivary enzyme (Sanchez-Monge et al. 1989; Gomez et al. 1991; see also Table 2), this α-amylase was not affected by any of the *T. boeticum* lines tested here. The variability in inhibitory properties among the *T. boeticum* samples was higher than in *T. monococcum*. Furthermore, some *boeticum* accessions (i.e. nos. 28 and 31) showed inhibition profiles like those observed in the latter species.

This variability was even higher among the *T. urartu* lines analyzed, which showed three different inhibitory patterns. Two of them were represented by *T. monococcum*-like (nos. 32 and 33) and the most abundant *T. boeticum*-like (no. 34) samples. The third one (nos. 35 and 36) displayed an inhibitory specificity similar to that of *T. aestivum* and *T. turgidum*, although its effects against *T. molitor* and human salivary enzymes was weaker. Inhibitiors active towards both α-amylases have been described in several *T. urartu* lines (Vitozzi and Silano 1976; Konarev 1994).

The relationships between the wheat samples included in Table 2 were estimated by the Neighbor Joining method using the inhibition levels exerted towards the three α-amylases when the higher amount of protein (50 μg) was assayed. Their clustering, shown in Fig. 1, must be considered only as indicative, since it does not appear significant by the bootstrapping method, probably due to the low number of characters involved in the analysis. However, the agreement between the different methods used (Neighbor Joining, Maximum Likelihood and UPGMA) suggests that the clustering shown in Fig. 1 is not a by-product of the structure of the set of data.

Based on the analysis three groups of samples can be considered. Group *a* comprises all the *T. aestivum* and *T. turgidum* cultivars, the *T. tauschii* accession and *T. urartu* lines nos. 35 and 36. Group *b* includes all the *T. monococcum* lines, five *T. boeticum* accessions (nos. 12, 25, 28, 30 and 31) and two *T. urartu* lines (nos. 32 and 33). Most *T. boeticum* samples and *T. urartu* no. 34 belong to group *c*.

This clutering suggests that the level of similarity within *T. monococcum* is higher than in *boeticum* or *urartu*. Futhermore, it reveals that only some *T. urartu* lines are closely related to the tetra- and hexa-ploid cultivated wheats.

Fig. 1 Clustering of the wheat lines included in Table 2 The relationship between samples was estimated from the inhibition values by the Neighbor Joining method. (*1–36*) Einkorn wheat lines according to the numbering in Table 1: *T. aestivum* cultivars Chinese Spring (*Ch*), Anza (*An*) and Cajeme (*Ca*): *T. turgidum* cultivars Senatore Capelli (*Se*), Enano de Andujar (*En*) and Peñafiel (*Pe*); *T. tauschii* accession UP-2 (*Tt*)

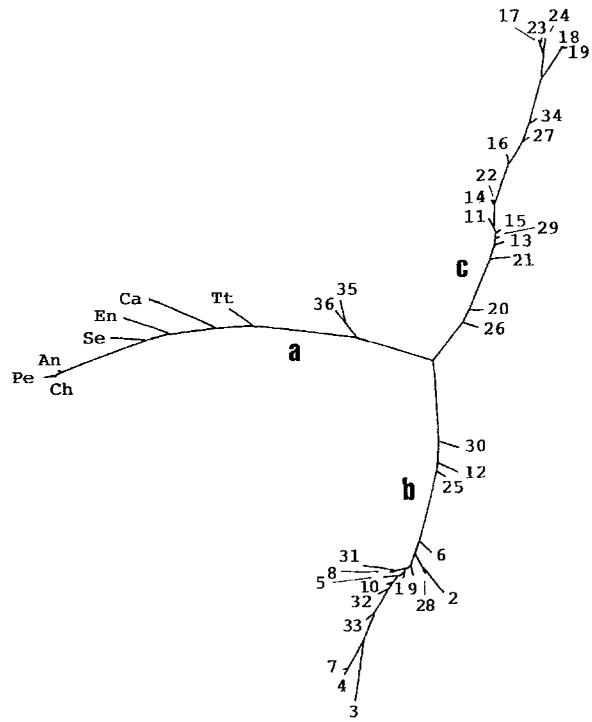


Table 3 In vitro IgE-binding capacities of Einkorn wheats compared to those of *T. aestivum* and *T turgidum* cultivars. The radioactivity associated with the spots of Fig. 2 was quantified in the PVDF membranes using a Phosphor Analyst

Wheat lines		IgE-binding (PDU units)[a]	Wheat line		IgE-binding (PDU units)
T monococcum	1	14.8	*T. boeticum*	28	14 6
	2	9.3		29	18.5
	3	13.0		30	20.8
	4	26.1		31	12.4
	5	17.9	Mean ± σn		16.6 ± 3.7
	6	16.0	*T. urartu*	32	16.8
	7	18 3		33	15.8
	8	14.3		34	20.9
	9	11.8		35	21.0
	10	9.5		36	18.1
Mean ± σn		15.1 ± 4.7	Mean ± σn		18.5 ± 2.1
T. boeticum	11	15.1			
	12	15.5	*T. aestivum*	cv Chinese S.	12.9
	13	16.4		cv Anza	20.6
	14	15.1		cv Cajeme	15.5
	15	26.9	Mean ± σn		16.3 ± 3.1
	16	18.8			
	17	20.4	*T. turgidum*	cv Senatore Cap.	8.6
	18	10 5		cv Enano And	13.7
	19	13.7		cv Peñafiel	7.9
	20	11.1	Mean ± σn		12.6 ± 2.9
	21	14.0			
	22	19.9	*T tauschii*	acc UP-2	14.6
	23	19.3			
	24	20 4	*Controls*[b]		
	25	14.4	BSA		0.7
	26	16.8	WTAI-CM16		2.1
	27	14.7	CM16*		288 3
			BMAI-1		317.7
			BDAI-1		21 0

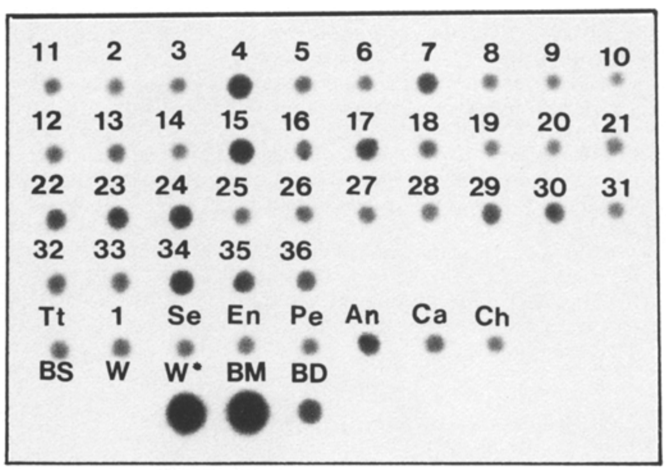
[a] Phosphor densitometric units × 10^5
[b] See legend of Fig. 2

In vitro allergenic reactivity of Einkorn wheats

The putative absence of members of the α-amylase/trypsin inhibitor family, some of which are major allergens associated with baker's asthma (Sánchez-Monge et al. 1992; Armentia et al. 1993), in all lines of *T. monococcum* (and probably in some *T. boeticum* and *T. urartu* accessions), prompted us to compare their allergenic reactivities with those of tetra- and hexa-ploid wheats. Thus, the IgE-binding capacity of salt extracts from Einkorn wheats was assayed using sera from patients allergic to wheat flour. The results obtained are summarized in Fig. 2 and Table 3. No significant differences in IgE-binding capacity were found between *T. aestivum* (or *T. turgidum*) and Einkorn wheats.

The presence of allergens with molecular masses above 20 kDa and not related to the α-amylase inhibitors has been previously described in bread-wheat flour (Posch et al. 1995; Sandiford et al. 1995). To investigate the presence of such allergens in Einkorn wheat, we immunoblotted salt-soluble proteins fractionated by SDS-PAGE with sera of allergic patients. A representative example of the results obtained is shown in Fig. 3. Reactive (IgE-binding) bands of molecular masses between 50 and 20 kDa were detected in all Einkorn

Fig. 2 IgE immunodetection of salt-soluble proteins from Einkorn wheats. Samples were adsorbed onto PVDF membranes and treated with a pool of sera from baker's asthma patients and ^{125}I-labelled anti-human IgE antibody (*1–36*) Einkorn wheat lines according to the numbering of Table 1: *T. aestivum* cultivars Chinese Spring (*Ch*), Anza (*An*) and Cajeme (*Ca*); *T. turgidum* cultivars Senatore Capelli (*Se*), Enano de Andujar (*En*) and Peñafiel (*Pe*). *T tauschii* accession UP-2 (*Tt*) Positive (wheat allergen CM16*, W*; barley allergen BMAI-1, BM: and barley allergen BDAI-1, BD) and negative (bovine serum albumin, BS; and wheat protein WTAI-CM16, W) responses were also included



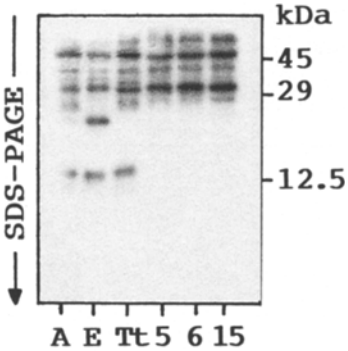


Fig. 3 IgE immunodetection of SDS-PAGE fractionated salt-soluble proteins from the following samples: *T. aestivum* cv Anza (*A*), *T. turgidum* cv Enano de Andujar (*E*), *T. tauschii* acc UP-2 (*Tt*), *T. monococcum* acc nos. 5 and 6 (*5*, *6*) and *T. boeticum* acc no. 15 (*15*). Immunodetection was as in Fig. 2

wheats, as well as in *T. aestivum*, *T. turgidum*, and *T. tauschii*. Such allergens probably account for the in vitro reactivities observed in most *T. monococcum*, *T. boeticum*, and *T. urartu* lines.

Interestingly, no bands of 12–16 kDa (corresponding to α-amylase inhibitors) were detected in the Einkorn wheats analyzed (see Fig. 3, lanes 5, 6, and 15), except in the case of *T. urartu* lines nos. 35 and 36. This supports the proximity of both accessions to the tetra- and hexaploid wheats suggested by our cluster analysis (Fig. 1).

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