Theoretical and Applied Genetics 48, 279-284 (1976)
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The Phylogenesis of Protein α-Amylase Inhibitors from Wheat Seed and the Speciation of Polyploid Wheats

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Summary. Protein α-amylase inhibitors extracted with water from seeds of a number of *Triticum* and *Aegilops* species were characterized according to their molecular weights and action specificities towards human salivary and *Tenebrio molitor* L. α-amylases. Four inhibitor peaks, with molecular weights 60000, 44000, 22000 and 11000, active towards the two amylases have been detected. Another inhibitor peak with molecular weight 11000, only active towards the insect α-amylase, has been found in several species tested. *Triticum urartu* showed only the 22000 inhibitor peak, while other diploid *Triticum* species did not exhibit any inhibitory activity. All the diploid *Aegilops* species tested contained α-amylase inhibitors and the inhibitor patterns differed greatly even for closely related species. In general, tetraploid *Triticum* species (*turgidum* and *timopheevi*) exhibited amylase inhibitor patterns of higher complexity than diploid *Triticum* and *Aegilops* species.

The relationships existing among the amylase inhibitor patterns of the *Triticinae* species tested are consistent with the hypothesis of the polyphyletic origin of tetraploid wheats by Sarkar and Stebbins (1956) and suggest that the amylase inhibitors from diploid species all derive from common ancestral genes.

Introduction

The nature of the donor of the B genome to polyploid wheats is still controversial, in spite of the number of investigations available on several diploid species showing some properties related to those of the B genome. Kimber's (1973) recent hypothesis, that the species donor of the B genome might be extinct, clearly highlights the difficulties encountered in the recognition of the B genome donor. Although *Agropyron triticum* and *Aegilops bicornis* have been indicated (McFadden and Sears 1944; Sears 1956) as involved in the phylogenesis of emmer wheats, most of the investigations carried out up to now have centred on the hypothesis that *Aegilops speltoides*, or a closely related species, is the donor of the B genome. The evidence supporting the role of *Ae. speltoides* in the phylogenesis of emmer wheats is based upon spikelet and chromosome morphology, meiotic chromosome pairing, nuclear DNA content, acidic phosphatase electrophoretic patterns and geographical distribution patterns (Jenkins 1929; Pathak 1940; Riley *et al.* 1958; Rees 1963; Giorgi and Bozzini 1969; Jaaska 1971). However, the value of some of this data has been questioned by Sears (1969), and cytogenetical, biochemical and morphological evidence against the role of *Ae. speltoides* has been reported (Kihara and Yamashita 1956; Sears 1956; Kimber 1966; Riley and Chapman 1966; Johnson and Kimber 1967; Bozzini *et al.* 1970; Athwal and Kimber 1972; Dvorak 1972; Kimber and Athwal 1972). Recently, Shands and Kimber (1973) suggested that *Ae. speltoides* could instead be the donor of the G genome to *Triticum timopheevi*. Because of the difficulties encountered in identifying an *Aegilops* species able to fully account for the features of the B genome, other possibilities have been exploited. According to Sarkar and Stebbins (1956), as well as other authors (Zohary and Feldman 1962; Giorgi and Bozzini 1969; Kimber and Athwal 1972), tetraploid wheats may be polyphyletic in origin, that is, they might have arisen from intercrossing of two or more amphiploids, each containing the A genome but with a different *Aegilops* second genome. On the basis of the differences found among gel-electrophoretic patterns of albumins extracted from tetraploid wheats and a number of *Aegilops* species, Johnson (1972) revived for consideration Camara's (1935) old hypothesis, according to which tetraploid wheats have arisen by autoploidy, B as well as A genomes being derived from diploid wheats. Other biochemical investigation from this Laboratory on albumin α-amylase inhibitors indicated however that unidentified *Aegilops* species had been involved in the speciation of polyploid wheats (Bedetti *et al.* 1974). In this study, in fact, no α-amylase inhibitory activity was detected in *Triticum boeoticum* and its domesticated derivative *Triticum monococcum*, whereas all the *Aegilops* species tested contained albumin amylase inhibitors. Subse-

quently, Johnson (1975) identified *Triticum urartu* as the species which contributed the B genome to emmer wheats, although Konarev *et al.* (1974) had concluded from their immunochemical studies on gliadins that *T. urartu* contributed the A genome to emmer wheats.

As Johnson's investigations were mainly based on the analysis of albumin fractions, and wheat amylase inhibitors make up as much as two-thirds of wheat albumins (Petrucci *et al.* 1974), we have considered it worthwhile to carry out further investigations on albumin amylase inhibitors to clarify the nature of the B genome donor species. We compared the amylase inhibitor composition of a number of *Aegilops* and *Triticum* species including *T. urartu*. We also intended to gather further information on these naturally occurring inhibitors which, up to now, have been studied at molecular and structural levels mainly in *Triticum aestivum* (Feillet and Nimmo 1970; Shainkin and Birk 1970; Sodini *et al.* 1970; Cantagalli *et al.* 1971; Saunders and Lang 1973; Silano *et al.* 1973; Petrucci *et al.* 1974; Buonocore *et al.* 1975; Redman 1975; Silano *et al.* 1975; Deponte *et al.* 1976; Petrucci *et al.* 1976; Buonocore *et al.* 1976).

Materials and Methods

Extraction of α-amylase inhibitors was carried out with distilled water on 13-20 seeds of each species tested after embryo removal, according to Bedetti *et al.* (1974). Water extracts were submitted to gel filtration on a Sephadex G-100 column as previously described (Bedetti *et al.* 1974) and apparent molecular weights of the eluted inhibitor fractions were calculated from the corresponding retention volumes according to Andrews (1964). Inhibitory activity of the column eluate towards human salivary and *Tenebrio molitor* L. (yellow mealworm) α-amylases was determined continuously as previously described (Vittozzi *et al.* 1976). Amylase inhibition was expressed as arbitrary inhibition units. One inhibition unit represents the inhibitor amount that gives 30% inhibition of the amount of amylase producing 0.3 μeq maltose under described experimental conditions (Vittozzi *et al.* 1976).

Results

Water extracts obtained according to Bedetti *et al.* (1974) from seeds of a number of *Triticum* and *Aegilops* species were submitted to gel filtration on a Sephadex G-100 column and the eluates were assayed for inhibitory activity towards human salivary and *T. molitor* α-amylases. Retention volumes and apparent molecular weights of the inhibitor peaks obtained are shown in Figs. 1 and 2.

One inhibitor peak (molecular weight 22000) active towards both the mammalian and insect amylases was present in the elution profile of *T. urartu*, whereas no amylase inhibitory activity was detected in *T. monococcum*, *T. boeoticum* or *T. thaoudar*. This result compares well with those previously reported by Johnson (1975), who showed that *T. urartu* contains albumin fractions absent from *T. boeoticum* and *monococcum*.

The amylase inhibitor pattern of *T. urartu* was identical to that of *Aegilops mutica*, but the two tetraploid wheats tested (*Triticum turgidum* and *Triticum timopheevi*) contained a much higher number of inhibitor peaks. In addition to the three inhibitor peaks found in *T. turgidum*, *T. timopheevi* showed an inhibitor peak with molecular weight 44000, active towards the two amylases, which was present in *Ae. speltoides* and absent from all the other *Aegilops* species tested. As already established with the manual amylase assay (Bedetti *et al.* 1974), *T. turgidum* showed an inhibitor composition qualitatively identical to that of *T. aestivum*.

Regardless of the similarity of their genomes, the seven diploid *Aegilops* species tested showed very different inhibitor compositions (Figs. 1 and 2). From a qualitative standpoint, only *Aegilops longissima* showed an inhibitor pattern identical to that of *T. turgidum*. This result is in agreement with those of Johnson (1972), who found that the albumin electrophoretic pattern of *Ae. longissima* was substantially represented in the profiles of tetraploid wheats. Moreover, the amylase inhibitor pattern of *T. turgidum* can be obtained by adding that of *T. urartu* to that of *Ae. umbellulata*, or by combining more than two inhibitor patterns of Figs. 1 or 2.

As appears from Figs. 1 and 2, the ratios of inhibitory activities towards human salivary and *T. molitor* α-amylases of inhibitor peaks with identical molecular weights vary to a large extent with the species tested. A possible explanation for this result might be that these inhibitor peaks contain different amounts of inhibitor components with different activities, towards the two amylases. Such a heterogeneity has already been shown for inhibitor peaks from *T. turgidum* and *T. aestivum* (Silano *et al.* 1973; Bedetti *et al.* 1974;

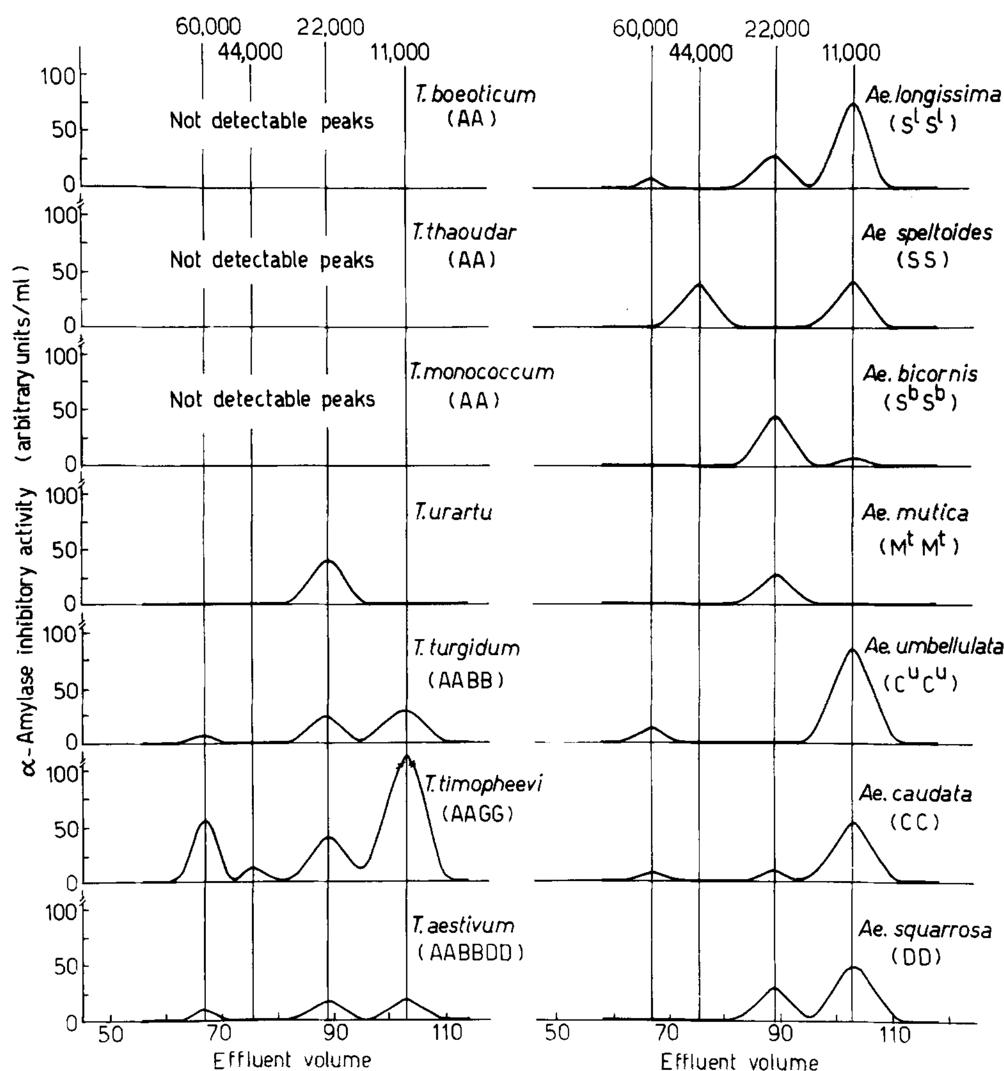

Fig. 1. Gel filtration patterns on Sephadex G-100 of *Tenebrio molitor* L. α-amylase inhibitors from *Triticum* and *Aegilops* species. About 2 ml of water extract were applied on a Sephadex G-100 column (2 cm × 100 cm), eluted with phosphate buffer (pH 7.4) and assayed for inhibitory activity as previously described (Vittozzi *et al.* 1976)

Petrucci *et al.* 1974; Deponte *et al.* 1976), as well as for those from *Ae. speltoides* and *Ae. squarrosa* (Bedetti *et al.* 1974).

Discussion

As shown in Table 1, which summarizes the results of Figs. 1 and 2, with this study on seven *Aegilops* and seven *Triticum* species, we have detected five inhibitor types differing in their molecular weights or in their specificities towards human salivary and *T. molitor* α-amylases. The presence of different inhibitor types is a property common to both the *Aegilops* and *Triticum* genus, but no species was found to contain all the five inhibitor types.

When diploid *Aegilops* and *Triticum* species are compared, it appears that the frequency of α-amylase inhibitor peaks is much higher in the *Aegilops* genus than in *Triticum*. In fact, among *Triticum* diploid species only *T. urartu* contained α-amylase inhibitors. Moreover, *T. urartu* only exhibited the 22000 inhibitor peak that was also present in the *Aegilops* species tested, except for *Ae. umbellulata* and *Ae. speltoides*. The presence of this peak in *T. urartu* might indicate that some transfer of α-amylase inhibitor coding genes has occurred from *Aegilops* species to diploid *Triticum*

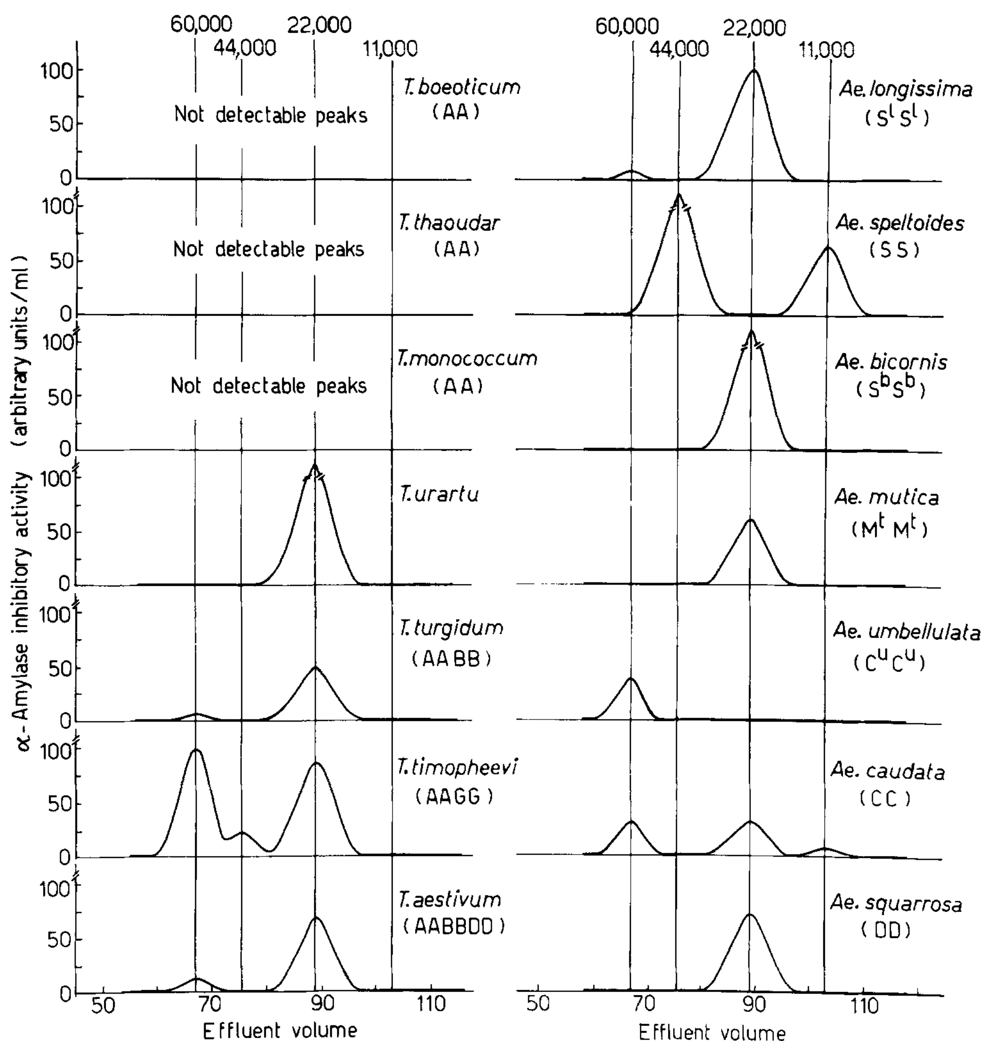

Fig. 2. Gel filtration patterns on Sephadex G-100 of human salivary α-amylase inhibitors from *Triticum* and *Aegilops* species. About 2 ml of water extract were applied on a Sephadex G-100 column (2 cm × 100 cm), eluted with phosphate buffer (pH 7.4) and assayed for inhibitory activity as previously described (Vittozzi *et al.* 1976)

species. The correspondence of the molecular weights of the inhibitor peaks obtained from different diploid *Aegilops* species suggests that these peaks all derive from common ancestral genes. Further investigations are needed to show whether, as observed in *T. aestivum* (Deponte *et al.* 1976), the inhibitor peaks with higher molecular weights in the *Aegilops* species are composed of subunits with molecular weight 11000. If this is the case, the appearance or disappearance of an inhibitor peak might even be the result of a relatively simple mutation capable of affecting polymerization properties of the basic peptide units of the amylase inhibitors. Therefore, the five inhibitor types of Table 1 might derive from a very limited number of ancestral genes coding for 11000 peptide units and the observed distributions of the inhibitor peaks in diploid *Aegilops* species might indicate a duplication of these ancestral genes followed by divergence of duplicated genes through mutation.

Although the classification of species belonging to related genera has so far been mainly based upon cytological and morphological methods, there is increasing evidence as to the value of biochemical criteria, such as the additiveness of parental protein patterns, to establish evolutionary relationships among *Triticinae* species (Johnson and Hall 1965; Johnson 1967; Waines 1969; Williams 1971; Bedetti *et al.* 1974). Therefore, from our results, some conclusions

Table 1. Properties and presence frequency of α-amylase inhibitors from *Triticum* and *Aegilops* genera

Molecular weight of inhibitor fraction	Activity towards		Range of ratios of inhibitory activity towards human salivary amylase to *T. molitor* amylase	Presence frequency *	
	human salivary amylase	*T. molitor* amylase		in *Aegilops* genus	in *Triticum* genus
12,000	-	+	0.0	4/7	3/7
12,000	+	+	0.2 - 2.0	2/7	0/7
24,000	+	+	2.0 - 5.5	6/7	4/7
44,000	+	+	2.0 - 4.0	1/7	1/7
60.000	+	+	0.9 - 6.0	3/7	3/7

* The seven species of each genus tested are those indicated in Fig. 1
+ indicates inhibition;
- indicates no inhibition.

about the phylogenesis of tetraploid wheats may be drawn. In general, tetraploid *Triticum* species (*turgidum* and *timopheevi*) exhibited amylase inhibitor patterns of higher complexity than diploid *Triticum* and *Aegilops* species, suggesting that different *Aegilops* diploid species could have contributed inhibitor coding genes to tetraploid wheats. Such a mixed contribution might have occurred through repeated hybridization among different *Triticum* × *Aegilops* amphiploids in agreement with the polyphyletic origin of polyploid wheats suggested by Sarkar and Stebbins (1956). The conclusion of Giorgi and Bozzini (1969) is of interest here, according to which an exchange of chromosome segments between homoeologous chromosomes in hybrids or amphiploids, before the occurrence of the mutation restricting the pairing to homologous chromosomes, probably played an essential role in wheat speciation. Therefore, in our opinion, the hypothesis of polyphyletic origin of polyploid wheats (Sarkar and Stebbins 1956) should be thoroughly investigated with other methods. In particular, the presence in *T. timopheevi* of the 44000 inhibitor peak, peculiar to *Ae. speltoides*, points to the importance of this *Aegilops* species in the speciation of *T. timopheevi*, thus confirming the hypothesis of Shands and Kimber (1973). On the other hand, the presence in this tetraploid wheat of other inhibitor peaks absent in *Ae. speltoides* clearly indicates that other *Aegilops* species, possibly closely related to those involved in the phylogenesis of emmer wheats, may have contributed to the speciation of *T. timopheevi*. Moreover, our results are not in agreement with Johnson's (1975) identification of *T. urartu* as the B genome donor species to *T. turgidum*, and instead indicate a possible role of *Ae. longissima* in the speciation of emmer wheats.

Acknowledgements

We are indebted to Dr. A. Bozzini, Chief of Crop and Grassland Production Service, Division AGP, FAO, for supplying the *Triticum* and *Aegilops* samples studied in this paper. Moreover, we would like to thank Dr. A. Bozzini for his stimulating advice and support.

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Received May 4, 1976
Communicated by H. Stubbe

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