

Interaction of allergens from house-dust mite and from cereal flours:

Dermatophagoides pteronyssinus α -amylase (Der p 4) and wheat and rye α -amylase inhibitors

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Major wheat and rye allergens associated with baker's asthma have been identified as specific inhibitors of *Dermatophagoides pteronyssinus* α -amylase (Der p 4). The most active inhibitors were the wheat tetrameric one and the rye dimeric RDAI-1. These data suggest that interaction between mite and cereal allergens (α -amylase/inhibitor complexes) might occur in house-dust-mite-infested flours. In contrast to the strong inhibition of the *D. pteronyssinus* enzyme by the wheat and rye inhibitors, the amylases from *D. farinae*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae* were much less affected. Moreover, the *D. pteronyssinus* amylase showed higher pH optimum (6.5 versus 5.0) and activity level (2–6 times/mg of protein in whole-mite extracts) than those from the three other mite species.

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House-dust mites are a common cause of asthma and other allergic diseases (1). As *Dermatophagoides pteronyssinus* and *D. farinae* are the most abundant species, many studies have been devoted to characterization of their allergenic components. Several major allergens are digestive enzymes (2), and, in the case of *D. pteronyssinus*, the α -amylase has been identified as one of them (3). No data on the allergenic properties of this enzyme from other mites have been reported so far.

On the other hand, inhibitors of insect α -amylases present in cereal flours are major allergens in baker's asthma (4–7), an allergic occupational disease caused by the inhalation of cereal flour (8). All these allergens belong to a single protein family, which has been extensively studied in wheat, barley, and rye (9–11). Three classes of active inhibitor species, corresponding to monomeric, homodimeric, and heterotetrameric (three different subunits) forms, are present in wheat and barley, while only the dimeric one has been found in rye (9–11). All three inhibitor types are differentially active against α -amylases from Coleoptera and Lepidoptera

species that are important cereal pests (12, 13). In spite of the extensive infestation found in many cereal flour samples, the effect of these allergens, or that of any other plant inhibitor, on the mite amylases has not yet been investigated.

We report here the inhibition of *D. pteronyssinus* amylase by allergens (inhibitors) from wheat and rye flour, an effect which indicates that interactions between mite allergens and flour allergens probably take place in mite-infested flour. This behavior of *D. pteronyssinus* amylase with cereal inhibitors seems to be specific, as the equivalent enzymes from other house-dust (*D. farinae*) or storage mites (*Lepidoglyphus destructor* and *Tyrophagus putrescentiae*) were much less susceptible to the same cereal inhibitors.

Material and methods

Mite extracts

The following mite species were used in this study: *D. pteronyssinus*, *D. farinae*, *L. destructor*, and *T. putrescentiae*.

Mites isolated from growth media (purity above 99%) were extracted with 20 mM NH_4HCO_3 (10% w/v) for 4 h at 4°C. The extracts were centrifuged and dialyzed against H_2O (membrane pore size 3500 Da). After addition of sucrose to a final concentration of 5% (w/v), the solutions were filtered (0.2 μm pore size) and freeze-dried.

Isolation of cereal inhibitors (allergens)

Flours from bread wheat (*Triticum aestivum* cv. Chinese Spring), pasta wheat (*T. turgidum* cv. Enano de Andujar), rye (*Secale cereale* INIA C/171M), and barley (*Hordeum vulgare* cv. Bomi) were used to obtain protein preparations enriched with α -amylase inhibitors, as well as purified allergens.

Crude inhibitor preparations were obtained by flour extraction with 0.15 M NaCl and $(\text{NH}_4)_2\text{SO}_4$ precipitation, as previously described (14). These preparations were subjected to gel filtration on Sephadex G-100 under nondissociating conditions, as in Gomez et al. (14), to obtain fractions enriched in tetrameric, dimeric, and monomeric inhibitors. Purification of individual inhibitors (allergens) from the appropriate gel-filtration fractions was performed by preparative reverse-phase high-performance liquid chromatography, under conditions previously reported (5–7, 10, 11, 15). Homogeneity of the purified proteins was checked by two-dimensional electrophoresis (isoelectrofocusing with starch-gel electrophoresis) (14). Protein concentration was quantified according to Smith et al. (16).

Amylase activity and inhibition tests

Amylase activity of mite extracts was tested essentially as in Bernfeld (17), with the modifications of Gutierrez et al. (13). For determination of the pH optima of the amylases in different mite extracts, 20 mM sodium acetate (pH 4–5.5), 100 mM NaCl, 0.1 mM CaCl_2 , and 20 mM potassium phosphate (pH 6–7.5), 100 mM NaCl, 0.1 mM CaCl_2 buffers were used.

Inhibition assays were carried out in the buffer with optimal pH for each mite extract. Briefly, 230 μl of this buffer, containing the mite amylase and appropriate amounts of inhibitor preparation, was incubated at 25°C for 30 min. After addition of 230 μl of 1% starch solution (incubation at 25°C for 30 min) and 460 μl of 3,5-dinitrosalicylic acid reagent, the final mixture was heated in a boiling water-bath (5 min), cooled on ice, and diluted with H_2O (2 ml), and its absorbance was measured at 550 nm. All tests were performed with approximately 1 unit of α -amylase, defined as the amount

of enzyme required to produce the reducing equivalents of 1 μmol maltose in our experimental conditions.

Isoelectrofocusing of *D. pteronyssinus* amylases

House-dust mite amylases were separated by isoelectrofocusing in a Multiphor II (Pharmacia Biotech) electrophoresis apparatus connected to a circulating cooling bath. Polyacrylamide gels (5%; 0.3-mm-thick; 17 cm between electrodes) containing 5.6% (v/v) ampholytes, pH 5–8, and 12.5% (v/v) glycerol were prefocused at 600 V and 22 W for 1 h, and, after sample loading, subjected to 600 V for 4 h 30 min. Electrolytes were 0.5 M acetic acid (anode) and 0.1 M NaOH (cathode). Staining was carried out with 1% (w/v) soluble starch in 20 mM potassium phosphate, pH 6.5, 100 mM NaCl, and 0.1 mM CaCl_2 , for 10 min, followed by a 5 M $\text{I}_2/10$ M KI solution in distilled H_2O .

Five micrograms of protein from the mite extract was incubated with different purified inhibitors (1.0 μg) for 30 min before electrophoretic separation.

Results

The α -amylase activities at different pHs of extracts from the four mites studied are summarized in Fig. 1. A negligible activity at alkaline pH was found in all cases (not shown). No amylase activity was detected in extracts of the corresponding mite culture media. The optimal pH was 5.0 for *L. destructor*, *T. putrescentiae*, and *D. farinae*, while that for *D. pteronyssinus* was 6.5, in agreement with previous data (18). Extreme differences in the level of amylase activity per mg of protein (at optimum pH) were also found, showing the highest value for *D. pteronyssinus* (2.3–6.2 times higher than those of *D. farinae* and *T. putrescentiae*, respectively).

This difference between *D. pteronyssinus* and the other three mites considered was further evident when the effect of cereal inhibitors on their α -amylases was tested (Table 1). Only α -amylase activity of the former species was strongly inhibited by crude inhibitor preparations of bread wheat and rye. However, no significant effect of the barley inhibitors was found for any of the four amylases tested. The differential susceptibility of the mite amylases to wheat inhibitors was further confirmed when gel-filtration fractions corresponding to tetrameric, dimeric, and monomeric forms were tested (Table 1; fractions from rye were not assayed because only inhibitors of the dimeric class are present in this cereal species (10, 11)).

The wheat tetrameric inhibitors were 2- to 6-fold more effective than the dimeric and monomeric

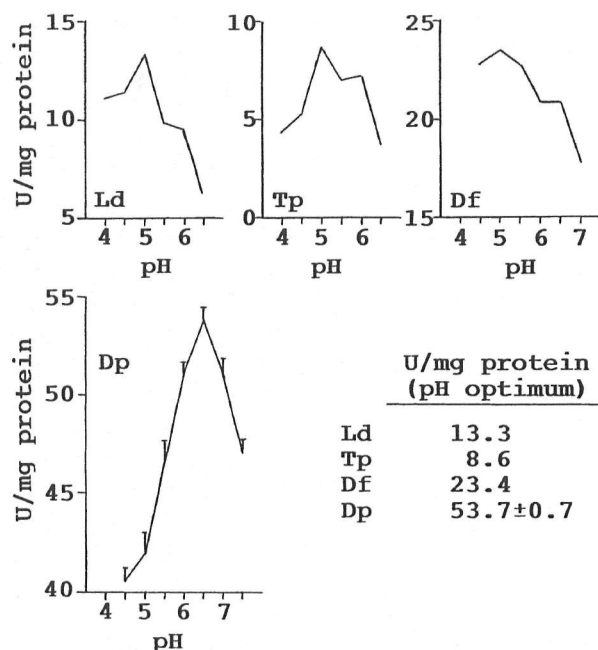


Fig. 1. Amylase activity compared with pH of whole-mite extracts from *Lepidoglyphus destructor* (Ld), *Tyrophagus putrescentiae* (Tp), *Dermatophagoides farinae* (Df), and *Dermatophagoides pteronyssinus* (Dp). Unit of α -amylase activity is defined in Material and methods section. Values are means of $n=2$ (Ld, Tp, and Df) or $n=6$ (Dp). Bars correspond to standard error (SE).

Table 1. Inhibition of α -amylase activity of *Dermatophagoides pteronyssinus* (Dp), *D. farinae* (Df), *Lepidoglyphus destructor* (Ld), and *Tyrophagus putrescentiae* (Tp) by crude inhibitor preparations from bread wheat (W), barley (B), and rye (R), as well as gel-filtration fractions corresponding to wheat tetrameric (Tw), dimeric (Dw), and monomeric (Mw) inhibitors. Tests were carried out at optimal pH values (6.5 for Dp, and 5.0 for Df, Ld, and Tp) with approximately 1 unit of enzyme activity. Five micrograms of inhibitor preparation was assayed in each case

Sample	Inhibition (%)			
	Dp ¹	Df ²	Ld ²	Tp ²
W	83	10	25	27
B	15	—	—	7
R	71	4	12	25
Tw	83	20	25	23
Dw	50	7	22	30
Mw	18	6	6	18

¹ Means of $n=4$. Standard error (SE) was under 7% in all cases.

² Means of $n=2$. —=no inhibition.

ones, respectively, toward *D. pteronyssinus* amylase (Fig. 2). In fact, only a weak inhibition was found for the monomeric fractions even at high protein levels (up to 5 μ g/assay). When purified inhibitors were assayed, the wheat tetramer reconstituted from its isolated subunits (a mixture of WTAI-CM2 plus 2 WTAI-CM3 plus WTAI-CM16 or -CM16*, where none of these subunits is inhibitory by itself) showed a strong inhibitory effect, in contrast to the lack of inhibition of its barley equivalent (Table 2).

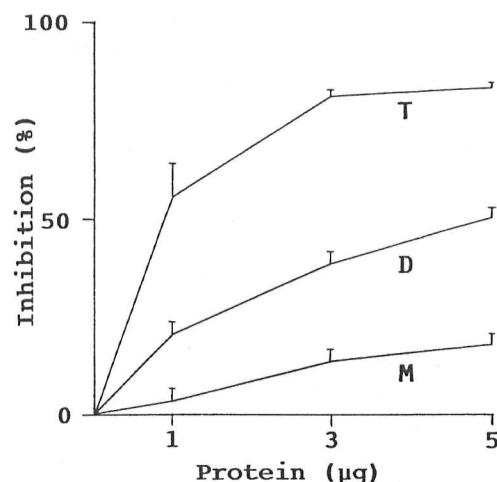


Fig. 2. Inhibition of *D. pteronyssinus* amylase activity by protein preparations enriched in tetrameric (T), dimeric (D), and monomeric (M) inhibitors from bread wheat (*T. aestivum*). Values are means of $n=4$. Bars correspond to SE. Tests were carried out with 1 U α -amylase (see Material and methods section).

Table 2. Inhibition of α -amylase activity of *D. pteronyssinus* by purified inhibitors from wheat, rye, and barley flour. Wheat tetrameric inhibitor was reconstituted from appropriate mixtures of its purified subunits: WTAI-CM2+2WTAI-CM3B+WTAI-CM16 or WTAI-CM2+2WTAI-CM3B+WTAI-CM16* (glycosylated form of WTAI-CM16). Similarly, barley tetrameric inhibitor was obtained by mixing BTAI-CMa+2BTAI-CMd+BTAI-CMb. RDAI-1 (rye dimeric α -amylase inhibitor-1) and WDAI-1 (wheat dimeric α -amylase inhibitor-1; synonymous 0.53) are equivalent components from rye and wheat, respectively. Values are means $\pm$ SE ($n=4$). Tests were carried out with approximately 1 unit of α -amylase

Purified inhibitors	Inhibition (%)			
	μ g/assay ²	1	3	5
WTAI-CM2 + 2WTAI-CM3B + WTAI-CM16 ¹		55 $\pm$ 1	65 $\pm$ 1	72 $\pm$ 1
WTAI-CM2 + 2WTAI-CM3B + WTAI-CM16* ¹		53 $\pm$ 1	65 $\pm$ 1	71 $\pm$ 1
BTAI-CMa + 2BTAI-CMd + BTAI-CMb ¹		n.d. ³	n.d.	—
	μ g/assay	0.5	1.5	2.5
RDAI-1		58 $\pm$ 2	71 $\pm$ 1	79 $\pm$ 1
WDAI-1		6 $\pm$ 2	15 $\pm$ 2	20 $\pm$ 3

¹ No subunit was inhibitory by itself up to 5 μ g/assay.

² Amount (μ g) of protein/assay was adjusted to obtain the same concentration for tetrameric and dimeric inhibitors (21.7, 43.4, and 86.9 pmoles). 1 inhibitor: 1 enzyme stoichiometry has been proposed for all inhibitor types (23).

³ n.d. = nondetermined. —=no inhibition.

Interestingly, similar effects were found when either the WTAI-CM16 subunit or its glycosylated form -CM16* (7) were included in the tetramer. WTAI-CM16* has been characterized as a major flour allergen associated with baker's asthma (4, 7).

Among the other purified members of the cereal α -amylase inhibitor family assayed, including WMAI-1 (synonymous 0.28) (9) and WDAI-1 (synonymous 0.53) (15) from *T. aestivum*; BMAI-1 (5, 7) and BDAI-1 (9) from *H. vulgare*; and RAI-1, -2, and -3 (11), as well as Sec c 1 (6) from *S. cereale*,

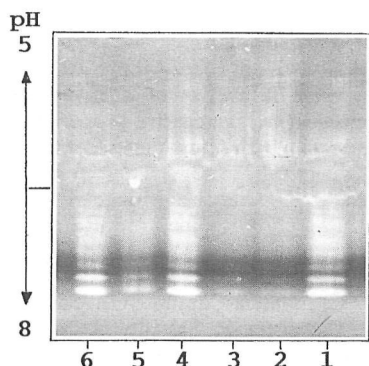


Fig. 3. Detection of *D. pteronyssinus* amylase isozymes in polyacrylamide gels after isoelectrofocusing (pH 5–8). Whole-mite extracts (5 µg) were directly electrophoresed (lane 1), or previously incubated with 1 µg of wheat tetramer including WTAI-CM16 (lane 2) or -CM16* subunit (lane 3), barley tetramer (lane 4), wheat dimeric inhibitor WDAI-1 (lane 5), and wheat monomeric inhibitor WMAI-1 (lane 6).

only the rye homodimeric inhibitor RDAI-1 (11) was strongly active toward *D. pteronyssinus* amylase (Table 2). This effect seems to be specific for the rye component, as its wheat homologue WDAI-1 (11), exerted a much weaker inhibitory effect. Like the wheat subunit WTAI-CM16*, RDAI-1 is a major flour allergen implicated in baker's asthma, an occupational allergic disease associated with flour manipulation (19).

An alternative approach to confirm the interaction between house-dust mite amylases and cereal allergens was the analysis of this enzymatic activity in native isoelectrofocusing gels (Fig. 3). Around six bands (presumably isozymes) were detected. Previous incubation with flour inhibitors effective against the mite enzymes, such as wheat tetramers, reduced drastically the intensity of all bands (Fig. 3, lanes 2 and 3), while the treatment with noneffective flour inhibitors (barley tetramer, wheat WDAI-1 or WMAI-1; Fig. 3, lanes 4–6) did not change the isozyme pattern obtained.

Discussion

The α -amylase from *D. pteronyssinus*, designated Der p 4, has been found to be a prominent allergen (3). Purified Der p 4 presented charge heterogeneity, indicating that several isoforms (isoenzymes) were included in the allergen preparation (3). No differences in allergenic reactivity have been described among the different isoforms (3). Although the α -amylase has not been detected as allergenic in other mites, amylases from other sources, such as the fungus *Aspergillus oryzae* and barley, have also been implicated in allergic reactions (20, 21).

We report here the differential behavior of *D. pteronyssinus* amylase when compared to amylases of another house-dust mite, *D. farinae*, or of

storage mites, such as *L. destructor* and *T. putrescentiae* (Fig. 1 and Table 1). Besides showing higher activity levels per mg of protein in whole-mite extracts, the enzyme from *D. pteronyssinus* has a different pH optimum (6.5 versus 5.0, although amylases of the other three mites seem to have a secondary peak of activity at pH ~6). Moreover, it is the only amylase strongly inhibited by proteinaceous α -amylase inhibitors present in wheat and rye flour.

In the case of wheat, the tetrameric inhibitors show the strongest effect on *D. pteronyssinus* amylase, followed by the dimeric and the monomeric ones in decreasing order (Fig. 2). This result, as well as the lack of effect of barley inhibitor preparations, is in line with previous results on lepidopteran pest amylases (12). To our knowledge, this is the first report on proteinaceous inhibitors of a mite amylase, a finding of potential interest not only for plant protection but also clinically.

A screening of 12 purified inhibitors from wheat, rye, and barley flours showed that the wheat tetramer (reconstituted from its purified subunits) and the rye dimeric RDAI-1 are the truly effective ones. Comparison with the inhibitory levels obtained for crude protein preparations suggests that inhibitor subunits might be partially inactivated during the isolation procedure and/or that only a partial reconstitution of the dimeric and tetrameric forms is achieved, a methodologic limitation previously pointed out for this inhibitor family (12, 13). Interestingly, the homologues of the wheat tetrameric inhibitor (barley) and of RDAI-1 (wheat dimeric WDAI-1) show very little or no inhibitory activity, indicating a specific effect on *D. pteronyssinus* amylase. In this context, the comparison of RDAI-1 and WDAI-1 seems particularly significant, because both components have very similar N-terminal amino acid sequences and inhibitory effects on insect and mammalian α -amylases (11), but clearly different allergenic properties (19). The effect of the wheat tetramer and RDAI-1 is not restricted to particular amylase isozymes, but extends to all the bands detected by isoelectrofocusing (Fig. 3).

The wheat tetrameric inhibitor subunits and RDAI-1 are abundant proteins in the corresponding flours (11, 14). More interestingly, both WTAI-CM16* (4, 7) (a glycosylated subunit of the wheat tetramer) and RDAI-1 (19) have been identified as major allergens associated with baker's asthma. Mite infestation of flour is quite common, *D. pteronyssinus* being very abundant in some samples (22). Therefore, mite-allergen (amylase)/cereal-allergen (inhibitor) complexes are likely to accumulate in infested flours and in dust samples from bakeries. Furthermore, the expected molecular

size of the complex in the case of mite amylase/wheat tetrameric inhibitor (around 100 kDa) could facilitate the cross-linking of IgE molecules and, thus, the release of inflammation mediators. From a clinical point of view, this enzyme-inhibitor interaction may be of interest in immunotherapy. Combination of mite and flour extracts for treatment of baker's asthma patients could be advisable when cross-sensitization has been detected.

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