

Characterization of Mal d 1, the 18-kD Major Apple Allergen, at the Molecular Level

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Introduction

Apple allergy is very common in areas with birch trees but has a low prevalence in other regions, for example the Mediterranean area. It belongs to the phenomenon 'oral allergy syndrome', and is mainly due to the existence of cross-reactive major allergens in both birch pollen and apple fruit. Immunoblot studies with apple extracts separated by SDS-PAGE, and skin prick test results with purified allergens have shown that between 65 and 90% of apple-allergic patients reveal IgE specific for the major apple allergen which has a molecular weight of approximately 18 kD [1–3]. In the present article, we report the current knowledge about the characteristics of this allergen which has been named Mal d 1 (allergen 1 from *Malus domestica*).

Apple Extract Quality and Source Material

Allergen preparation from apples is difficult. Special extraction procedures have to be applied to obtain immunologically active apple extracts. Extract quality is mainly affected by endogenous enzymatic activities, particularly that of polyphenoloxidases. To circumvent allergen degradation during the extraction procedure, enzyme inhibitors like diethyldithiocarbamate or ethylenediaminetetraacetic acid, or a cold solvent precipitation procedure have to be applied [4–6]. On the contrary, commercial prick test solutions from apples

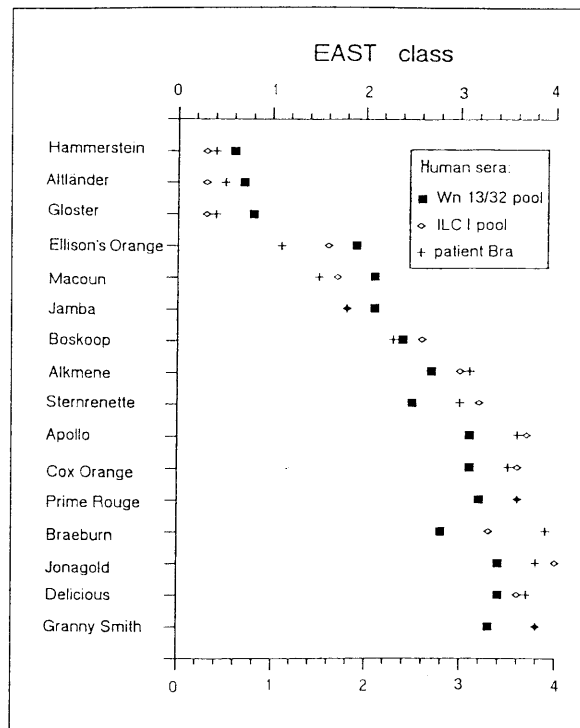


Fig. 1. Determination of specific IgE to 16 apple varieties by enzyme allergosorbent test (EAST). The three sera tested were from patients sensitized to birch pollen and apples. The pool sera were from 7 (Wn 13/32) and 4 (ILCI) patients. All apple extracts were prepared from mature fruit, and a standardized amount of protein (12 µg/ml) was used for allergen disk preparation. No IgE binding was determined with a control serum from 6 nonallergic donors [12].

have recently again been found to be unreliable for the diagnosis of this kind of fruit allergy [2, 3]. Scratch testing or the prick/prick method with fresh fruit material are therefore commonly used [7–10] but these methods cannot be standardized since, for example, they depend on the major allergen content of the fruit.

In contrast, it has recently been shown that stable prick test solutions can be prepared from high-quality crude extracts by replacing phenol, the common preservative with, for example, 0.12% cysteine and 0.7% sodium citrate in PBS [11]. Figure 1 points to the importance of the selection of source material: extracts from 16 apple strains were tested for their capability of binding specific IgE from birch pollen and apple-allergic patients. With a standardized

amount of protein, extracted from mature fruit, drastic differences of allergenic activity were detected between apple varieties not only by an enzyme allergosorbent test and immunoblotting, but also by dose-related histamine release and skin prick tests [12]. These differences were due to the major allergen content of the fruit. Furthermore, it has been shown that the Mal d 1 content of Golden Delicious apples rises considerably during maturation and storage [13].

On the basis of band intensity in SDS-PAGE, we have estimated a mean amount of 1–5 mg Mal d 1 per 100 g fresh weight in mature Golden Delicious apples. Therefore, one bite (approx. 10 g) which is able to elicit symptoms in apple-allergic patients represents 0.1–0.5 mg of the ingested major allergen [13].

Cross-Reactivity and Allergenic Potency of Mal d 1

The cross-reactive nature of birch pollen-related food allergies has been known for many years [1, 4, 5, 14, 15]. Since nearly all studies on this subject have been performed with crude extracts, the allergenic properties of pure Bet v 1 and Mal d 1 have not been investigated in a comparative study. Recently, we have isolated both Bet v 1 and Mal d 1 by preparative SDS-PAGE followed by electroelution and have investigated their immunologic properties [3]. Figure 2 shows SDS-PAGE analyses of crude extracts and the purified major allergens.

Despite the denaturing conditions which were applied for purification, both allergens were highly active. As an example, table 1 shows the results of comparative skin prick tests with whole allergenic extracts and the purified major allergens. The tests were performed in a group of 33 patients who were allergic to birch pollen and apples, and a control group of 10 patients without fruit and birch pollen hypersensitivity. High positivity rates (73–97%) were obtained even with low amounts (5 µg/ml) of the isolated allergens. In the case of Bet v 1, sensitivity of 97% was identical with that of whole birch pollen extract. Somewhat lower sensitivities were obtained with the Mal d 1 preparations when compared to whole apple extracts. In addition, the two-sided χ^2 test revealed that the positivity rates with Mal d 1 were significantly lower when compared to Bet v 1 ($p = 0.01$). Negative prick test results were obtained in the control group. However, patients suffering from birch pollinosis but without apple hypersensitivity have not been studied. One has to expect that latent sensitization to fruit among these patients would have influenced the results.

Further investigations by dose-related histamine release and specific IgE inhibition with patients' sera revealed that the allergenic activity of Bet v 1 was at least 15- and 17-fold, respectively, higher than that of Mal d 1.

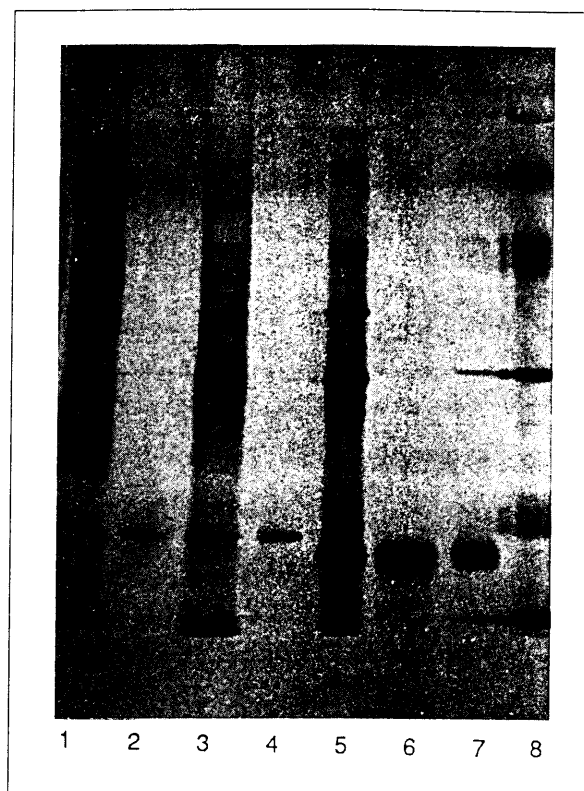


Fig. 2. SDS-PAGE analysis of purified Mal d 1 and Bet v 1. Protein bands were made visible by silver staining. Lane 1: Golden Delicious extract (GD); lane 2: Mal d 1, isolated from GD; lane 3: Granny Smith extract (GS); lane 4: Mal d 1, isolated from GS; lane 5: birch pollen extract; lane 6: Bet v 1 (EE), isolated by electroelution; lane 7: Bet v 1 (A), isolated by mAb affinity chromatography; lane 8: low molecular weight marker proteins [3].

The following conclusions may be drawn from our studies with purified major allergens: (1) Bet v 1 has all allergenic epitopes of Mal d 1, but Mal d 1 is only a weak inhibitor of the IgE reactivity with the major birch pollen allergen, probably due to the absence of some Bet v 1 epitopes on the fruit allergen. An additional explanation for the latter observation may be a lower association constant of Bet v 1-specific IgE to Mal d 1 epitopes. (2) Mal d 1 represents most of the allergenicity of apple fruit. (3) The high lability of allergens during extraction or processing of apples is probably not due to destruction of discontinuous epitopes, but to interactions with compounds from the fruit tissue, and most of these reactions are catalyzed by enzymes.

Table 1. Results of the skin prick tests in patients allergic to birch pollen and apples, using whole allergenic extracts and the major allergens isolated by electroelution [3] (for abbreviations, see fig. 2)

Prick test solution	Protein, $\mu\text{g/ml}$	Patients, n	Frequency of positive prick tests, n	% of positive prick tests
Birch pollen extract	20	33	32	97
Bet v 1 (EE)	5	33	32	97
Golden Delicious extract	42	33	30	91
Mal d 1 (GD)	5	33	24	73
Granny Smith extract	46	20	19	95
Mal d 1 (GS)	5	33	25	76

Molecular Characteristics of Mal d 1 and Other Homologous Allergens from Plant Foods

The first step in analyzing the properties of Mal d 1 at the molecular level was to apply two-dimensional immunoblotting and N-terminal amino acid microsequencing [16]. The results pointed to a molecular weight of approximately 18 kD and a pI of 5.5. Only Bet v 1-specific immunodetection systems were applied. The reactions of these with Mal d 1 again clearly showed that both allergens share common epitopes. As an example, figure 3a and b presents two-dimensional immunoblots of Golden Delicious extracts which were tested with the Bet v 1-specific mAb BIP 1 (a gift from Prof. Kraft, Vienna) and with serum of an allergic patient with specific IgE exclusively directed against Bet v 1.

N-terminal microsequencing of 26 amino acids of Mal d 1 revealed 62% sequence identity with the corresponding sequence of Bet v 1 [16, 17]. Microsequencing analysis of Mal d 1 from three different apple varieties (Golden Delicious, Granny Smith, Braeburn) led to identical N-terminal sequences [18]. One exception was position 9 of Golden Delicious, where phenylalanine and tyrosine were simultaneously detected, probably indicating the existence of two closely related isoallergens. With Bet v 1-specific immunodetection systems and inhibition experiments with purified Bet v 1, additional cross-reactive allergens from that family have been identified in cherry, pear, celery, carrot and hazelnut [18–21]. On the basis of 15 amino acid residues, the sequence identity of the allergens from cherry, celery and carrot with Bet v 1 were 67, 40 and 28%, respectively. Comparison with Mal d 1 revealed identities of 80, 33

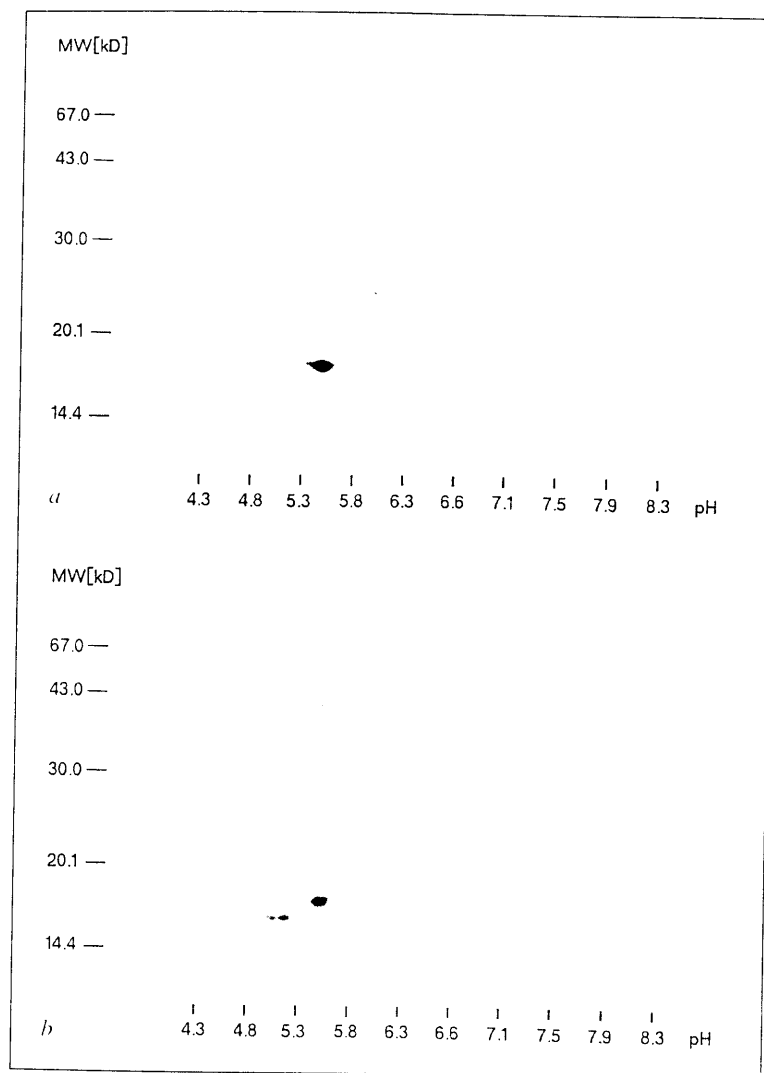


Fig. 3. Two-dimensional immunoblot analysis of Golden Delicious extract. *a* Immunodetection with mAb BIP 1, raised against Bet v 1, culture supernatant diluted 1:175. *b* Reactivity of IgE antibodies from patient Bra. On one-dimensional SDS-PAGE immunoblots, IgE of the patient reacts exclusively with Bet v 1 from birch pollen extract and Mal d 1 from Golden Delicious apple extract, serum diluted 1:25 [16].

AA		NS
	ACAATACTCTCAAGCCTCAATAAAACCATCATCCTTCCTTGCTCATTTTCCAACCTTTTTTAATCATC	-1
	ATG GGT GTC TAC ACA TTT GAG AAC GAG TTC ACC TCT GAG ATT CCA CCA TCA AGA TTG TTC	60
	<u>M G V Y T E E N E E T S E L P P S/P R L E</u>	
20	AAG GCC TTT GTC CTT GAT GCT GAC AAC CTC ATC CCC AAG ATT GCA CCC CAG GCA ATC AAG	120
	<u>K A E V L D A D N L I P K I A P Q A I K</u>	
40	CAA GCT GAA ATC CTT GAA GGA AAC GGT GGC CCC GGA ACC ATC AAG AAG ATC ACT TTT GGT	180
	<u>Q A E I L E G N G G P G T I K K I T F G</u>	
60	GAA GGC AGC CAG TAC GGC TAC GTG AAG CAC AGG ATT GAC TCG ATT GAC GAA GCA AGC TAC	240
	<u>E G S Q Y G V K H R I D S I D E A S Y</u>	
80	TCA TAC TCC TAC ACT TTG ATT GAA GGA GAT GCT TTG ACA GAC ACC ATC GAG AAA ATA TCT	300
	<u>S Y S Y T L I E G D A L T D T I E K I S</u>	
100	TAC GAG ACC AAG TTG GTG GCA TGT GGA AGT GGT TCC ACC ATC AAG AGC ATC AGT CAT TAC	360
	<u>Y E T K L V A C G S G S T I K S I S H Y</u>	
120	CAC ACC AAG GGA AAC ATT GAA ATC AAG GAA GAG CAC GTC AAG GTT GGA AAA GAG AAG GCC	420
	<u>H T K G N I E I K E E H V K V G K E K A</u>	
140	CAT GGT TTG TTC AAG CTT ATT GAG AGC TAC CTT AAG GAC CAC CCC GAC GCA TAC AAC TAA	480
	<u>H G L F K L I E S Y L K D H P D A Y N</u>	
	ATTAATCATAAGTATCTATGGTGTGTTTGGGTGTTACCGTTACTATATGGTCAGTCGAAGTTGTGTGGCTTTCTTTGTA	560
	TGTTTTTTTTCACGTCAGCCAAAAGTAATAACCATGGTTTGGGCAGTTTGGGCTTGAAAAATAAGCTTATGGTTGTGATG	640
	ATCTTCTGTGCGGATGCTTTGTTTGAAAAAAGTTGGCAGACTGAACGGAGTGATTATCTTCTATAAAATATAATGAA	720
	ATTACTTTTTTAATCAAAAAA	744

Fig. 4. Overall nucleotide sequence of the cDNA and deduced amino acid sequence of the major apple allergen Mal d 1. Numbers indicate amino acid residues (left) and nucleotide position (right) of the sequence. The single letter code for amino acids is used. Amino acids identified by N-terminal microsequencing are underlined [22].

and 21%. All the cross-reactive fruit allergens had molecular weights between 15 and 18 kD.

The N-terminal sequence information has been used to design a Mal d 1-specific nucleotide primer. The Mal d 1 cDNA was amplified from reversely transcribed mRNA by a polymerase chain reaction (PCR) [22]. The cDNA product was cloned into *Escherichia coli* and subsequently sequenced. The missing 5'-end of the apple cDNA sequence was analyzed by a 5'-RACE method.

Figure 4 shows the complete cDNA sequence which has been obtained by dideoxy cycle sequencing and the deduced amino acid sequence of the allergen. Thus, Mal d 1 is the first birch pollen-related fruit allergen with a completely known sequence. From these results it can be deduced that it is a 158 residue protein with a calculated molecular weight of 17.5 kD. Mal d 1 has no

N-glycosylation site. Since Mal d 1 binds to some lectins (e.g. ConA, unpubl. results), it may carry O-linked sugar residues. This would also explain the different migration characteristics of Mal d 1 and Bet v 1 in SDS-PAGE (fig. 2). Although the molecular weights of both allergens calculated from the deduced amino acid sequence are nearly identical (17.4/17.5 kD) [17, 22], the apparent molecular weight of Mal d 1 observed in SDS-PAGE is clearly higher (see fig. 2).

The deduced amino acid sequence of Mal d 1 is 63% identical with one isoform of Bet v 1 (BV SC3) [23], and the identity with Api g 1 from celery is 39% (see C. Ebner, this issue). Our Mal d 1 sequence differs in three amino acid residues from the sequence recently analyzed by Breiteneder et al. [24] (98% identity), pointing to a low microheterogeneity of the fruit allergen. In accordance with the observation made with Bet v 1, Api g 1 and the partial sequences of the cherry and carrot allergens, a high sequence identity of approximately 50% with some intracellular 'pathogenesis-related proteins' from food plants, for example pea or soybean, has been found [18, 22]. The exact physiological functions of these proteins are unknown.

Conclusions

The investigations of Mal d 1 and Api g 1 may serve as a model for typical birch pollen-related food hypersensitivities. Together with the studies of other fruit and vegetables, the results clearly highlight the molecular basis of the above phenomenon: the majority of these food allergies is caused by a family of low molecular weight allergens which all, like Bet v 1, may belong to a group of pathogenesis-related proteins, being involved in plant defense mechanisms. Individual sensitization patterns of different patients to various kinds of fruit may be due to the sequence variations among the Bet v 1-related food allergens. All present results of studies with Bet v 1/Mal d 1 emphasize that sensitization is due to birch pollen inhalation. When Bet v 1-specific IgE has been raised and the concentration exceeds an individual threshold level, apple allergy occurs. For patients sensitized to the major allergens, there is no evidence that apple ingestion may be the reason for sensitization. However, the situation may be different in areas without birch trees. In Spain, for example, the pan-allergen profilin has recently been identified as an important cross-reactive allergen in grass pollen, apple and peach [25], but the potency of apple profilin to orally sensitize atopic patients is also unknown. Clear answers to the questions in the context of oral sensitization against the plant food allergens can only be given at the T-cell level. Multiple T cell epitopes inducing enhanced IL-4 production have recently been found on Bet v 1 [26–28]. Three

highly cross-reactive epitopes from the major allergens of hazel and alder pollen contained between 1/12 and 3/12 amino acid changes. One of the two peptides, which differed in three amino acids from Bet v 1, induced a 10-fold lower proliferation rate with the T-cell clone from a Bet v 1-allergic patient when compared to the corresponding sequence of Bet v 1. On the contrary, a minimum of 3/12 changes occurs with 14 distinct epitopes of Bet v 1 and the corresponding Mal d 1 sequences. One epitope that has been described as being immunodominant (Bet v 1 77-92) contained 10/16 exchanges. These findings may support the view of a lower sensitization potency of the apple allergen. Since T-cell stimulation experiments with purified or recombinant Mal d 1 are being performed by several researchers, we can expect clear results on this subject in the near future. Further work is also needed to investigate the structure of the cross-reactive B-cell epitopes.

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