Quadrupole time-of-flight mass spectrometry: A method to study the actual expression of allergen isoforms identified by PCR cloning

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Background: Over the past 2 decades, molecular biology has shown that most major allergens exist in multiple isoforms. Very little is known about the relevance of allergen isoforms at the level of expressed protein (ie, actual allergen exposure).
Objective: The aim of this study was to evaluate the applicability of state-of-the-art quadrupole time-of-flight mass spectrometry (Q-TOF MSMS) to the identification and quantification of allergen isoforms at the protein level.
Methods: Q-TOF MSMS is a mass spectrometric approach for sequencing peptides and proteins. In our study it was applied to recombinant (r)Mal d 1, rBet v 1a and rBet v 1d, and natural (n)Mal d 1 from fruits of *Malus domestica*, cultivar Granny Smith.
Results: Q-TOF MSMS allowed sequencing of about 70% of all amino acids in Mal d 1 and about 60% of those in Bet v 1. Mixing experiments with rBet v 1 isoforms and with rMal d 1 and nMal d 1 revealed that the technique allows identification of isoforms in mixtures down to a level of at least 5%. Recombinant Mal d 1 was identified as a Mal d 1a representative, whereas Granny Smith apples were shown to contain Mal d 1b–like allergen isoforms. In this apple cultivar hitherto unreported modifications of Mal d 1b were identified. Q-TOF MSMS allowed semiquantitative measurement of allergen at the femtomole to picomole level.
Conclusion: Q-TOF MSMS is a powerful tool to find out whether an allergen isoform, detected at the cDNA level, is really expressed in quantities relevant for the allergic patient. (J Allergy Clin Immunol 2002;110:131-8.)

Key words: *Amino acid sequencing, apple, Bet v 1, food allergy, isoforms, Mal d 1, mass spectrometry, quadrupole-time-of-flight, quantification*

Food allergy induced by fruits of the Rosaceae family, such as apple, pear, peach, and cherry, is intimately linked to sensitization to birch pollen.[1,2] Its clinical presentation is almost exclusively limited to symptoms of the oral cavity, the so-called oral allergy syndrome. The link between birch pollen allergy and fruit oral allergy syndrome is IgE against the major birch pollen allergen Bet v 1 that cross-reacts with homologous proteins in fruits (eg, Mal d 1 in apple). Both allergens share approximately 56% of their primary structure.[3,4] Bet v 1 and Mal d 1 have a molecular mass of approximately 17.5 kd and consist of 159 and 158 amino acids, respectively. For both allergens, isoforms with a limited number of amino acid exchanges were reported.[5-9]

Abbreviations used

CE:	Collision energy
PDMS:	Plasma desorption mass spectrometry
Q-TOF MSMS:	Quadrupole time-of-flight mass spectrometry

Recently, Son et al[4] analyzed 12 Mal d 1 isoforms cloned from 7 apple cultivars. They compared their sequences with the 3 primary structures available in the database, which they designated Mal d 1a, b, and c. Mal d 1a and Mal d 1b differ at 15 amino acid positions, Mal d 1a and Mal d 1c at 5 positions, and Mal d 1b and Mal d 1c at 3 positions. Six clones showed characteristic features for Mal d 1a and 6 for Mal d 1b. The analysis further revealed that only 10 of 15 amino acid differences were really distinctive between Mal d 1a and b. None of the clones analyzed was similar to Mal d 1c. All reported isoforms were deduced from cDNA-derived nucleotide sequences. Although an extremely powerful tool in the search for allergen isoforms, molecular biology does not easily give information on the actual quantitative importance of isoforms at the level of protein expression. In addition, no information is gained on posttranslational modifications that are potentially involved in allergenicity, as has been reported for plant glycosylation.[10,11]

For several allergens, significant differences in allergenicity were reported for naturally occurring isoforms. For Bet v 1, for example, isoforms were described that virtually did not bind IgE on immunoblot.[5-9] Also, for Mal d 1, differences in IgE-binding potency have been observed.[4] Strategies to develop low-allergenic apples by means of conventional breeding or genetic modification

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depend heavily on the identification and characterization of hypoallergenic isoforms and on their quantification at the protein level. Swoboda et al[12] matched cDNA-derived sequences of several isoforms of Bet v 1 with mass spectrometry–derived molecular masses of enzymatic digests to confirm the sequences at the protein level. Their results indicated that there were no posttranslational modifications and allowed a quantitative estimate of the relative importance of isoforms. In this article we explore the potential of recent developments in mass spectrometry to sequence allergen isoforms and simultaneously to quantify them. Liquid chromatography in combination with 2-dimensional mass spectrometry and, in particular, quadrupole time-of-flight MSMS (Q-TOF MSMS) have been shown to allow fast amino acid sequence determination of proteins.[13,14] Furthermore, Q-TOF MSMS has also successfully been applied for quantitative measurement of small molecules, such as drugs,[15,16] and for detection of posttranslational modifications of proteins.[17,18] The technique was primarily designed to identify proteins on the basis of amino acid sequences of a few peptides derived from the protein after tryptic digestion and not for elucidation of complete amino acid sequences. Essentially, the technique is based on 2 subsequent steps of mass spectrometry. First, a protein is digested by a protease, like trypsin, after which the exact mass of the resulting peptides is determined. Then each peptide is separately fragmented by means of sequential cleavage of the composing amino acids, which are identified in the correct sequence in a second mass spectrometric step. Ideally, this results in a complete sequence of a protein.

The aim of this study was to evaluate the potential of Q-TOF MSMS to determine the quantity of individual allergen isoforms as expressed protein. Recombinant (r)Bet v 1a and d, as pure compounds and as mixtures, and rMal d 1 were used to study whether the technique allows designation of the allergen to a defined isoform family. Subsequently, affinity-purified Mal d 1 from the highly allergenic apple cultivar Granny Smith was analyzed to determine the isoform composition and compare this with the reported PCR-based observations that both Mal d 1a and Mal d 1b isoforms are represented in this cultivar. Recombinant Mal d 1 was used to test the feasibility of Q-TOF analyses for quantitative measurement of allergenic proteins.

METHODS

Purification of natural Mal d 1

Apple fruits (*Malus domestica*, cultivar Granny Smith) were obtained from a local grocery store and extracted according to the method of Björksten et al,[19] followed by removal of pectins by addition of 5 mmol/L Ca^{2+}, Mg^{2+}, and Mn^{2+} (chloride salts in each case), centrifugation, and dialysis of supernatant against PBS. Mal d 1 was subsequently affinity purified with Sepharose-coupled mAb 5H8 directed to Bet v 1 and shown to be cross-reactive to fruit homologues from the Rosaceae family.[20] Further characterization showed that both Mal d 1a– and Mal d 1b–type allergens were recognized by this antibody. Immunoblot analysis after isoelectric focusing demonstrated that this mAb recognizes a conserved epitope in multiple Bet v 1 isoforms.[20] Purified natural (n)Mal d 1 aliquots of 50 µg were lyophilized and stored at –20°C until further analysis.

Recombinant allergens

Recombinant Bet v 1a and rBet v 1d, as well as rMal d 1, were purchased from Biomay (Linz, Austria).

SDS-PAGE

For SDS-PAGE, slab gels composed of 12% acrylamide sample gels (0.1 mol/L Tris/HCl [pH 8.8], 75-mm length) overlayed with 5% acrylamide stacking gel (0.1 mol/L Tris/HCl [pH 6.8], 13-mm length) were used. Protein samples at 1 mg/mL water, to which one fourth volume sample buffer (1 mol/L Tris/HCl [pH 6.8], 8% SDS, 40% glycerol, 20% β-mercaptoethanol, and 4% bromophenol blue) was added, were heated for 5 minutes at 95°C and cooled to room temperature. After addition of iodoacetamide (final concentration, 0.050 mol/L), 10- to 20-µL aliquots were run on SDS-PAGE. Protein bands were stained with colloidal Coomassie Brilliant Blue for 1 hour and destained overnight in water that was refreshed after the first hour.

Gel pieces containing rBet v 1a, rBet v 1d, rMal d 1, or apple-derived nMal d 1 were cut to 1 mm^2. In-gel digestion with trypsin of allergenic protein was performed according to the method of Jensen et al.[21] Digested samples were desalted with a ZipTip C_{18}-cartridge (Millipore, Bedford, Mass), according to the manufacturer's instructions. Protein was recovered in 10 µL of 50% MeOH plus 0.5% HCOOH and 10 µL of 100% MeOH.

Q-TOF Nanoflow Electrospray Ionization-MS and -MSMS analyses

All analyses were carried out on a Q-TOF 2 facility (Micromass, Manchester, United Kingdom). The Microchannel Plate Detector was set at 2700 V, capillary voltage at 900 V, cone voltage at 35 V, and low-mass and high-mass resolutions at 10. The instrument was calibrated for accurate mass at least daily before analysis of peptides with [Glu^1]-fibrinopeptide B (Sigma, St Louis, Mo). The calibration procedure was repeated until mass differences throughout the MSMS spectrum were smaller than 0.015 d. [Glu^1]-fibrinopeptide B was also used at 0.2 µmol/L as an internal standard in quantitative measurements. Data processing and amino acid sequence analysis were performed with MaxEnt3 and MassLynx/Biolynx 3.4 software (Micromass), respectively. Manual sequencing was performed when automated analyses were considered unsatisfactory.

After deioniziation, mixtures of tryptic peptides were applied to the Q-TOF Z-spray source in 8-µL aliquots through nanoflow probe tips (Type F 6028634, Micromass). In the MS mode, collision energy (CE) was maintained at 10.0 V and analysis time at 5 minutes. In the MSMS mode CE was maintained at 10.0 V for the first 30 seconds to obtain parent peak information. Then CE was increased to a voltage that resulted in a balanced fragmentation pattern for the remainder of the analysis time. Analysis time was maintained routinely at 5 minutes but was increased to 10 minutes if required for a sufficient quality MSMS spectrum.

RESULTS

Sample preparation

On SDS-PAGE, rMal d 1, nMal d 1, rBet v 1a, and rBet v 1d migrated at apparent molecular weights of 22, 19, 18, and 17 kd, respectively (data not shown). All 4 allergens were cut out and treated with trypsin for analysis on Q-TOF MSMS.

Recombinant Bet v 1 isoforms: Sequence analysis

For both commercially available recombinant isoforms of Bet v 1, rBet v 1a and rBet v 1d, 6 of 19 possible tryptic

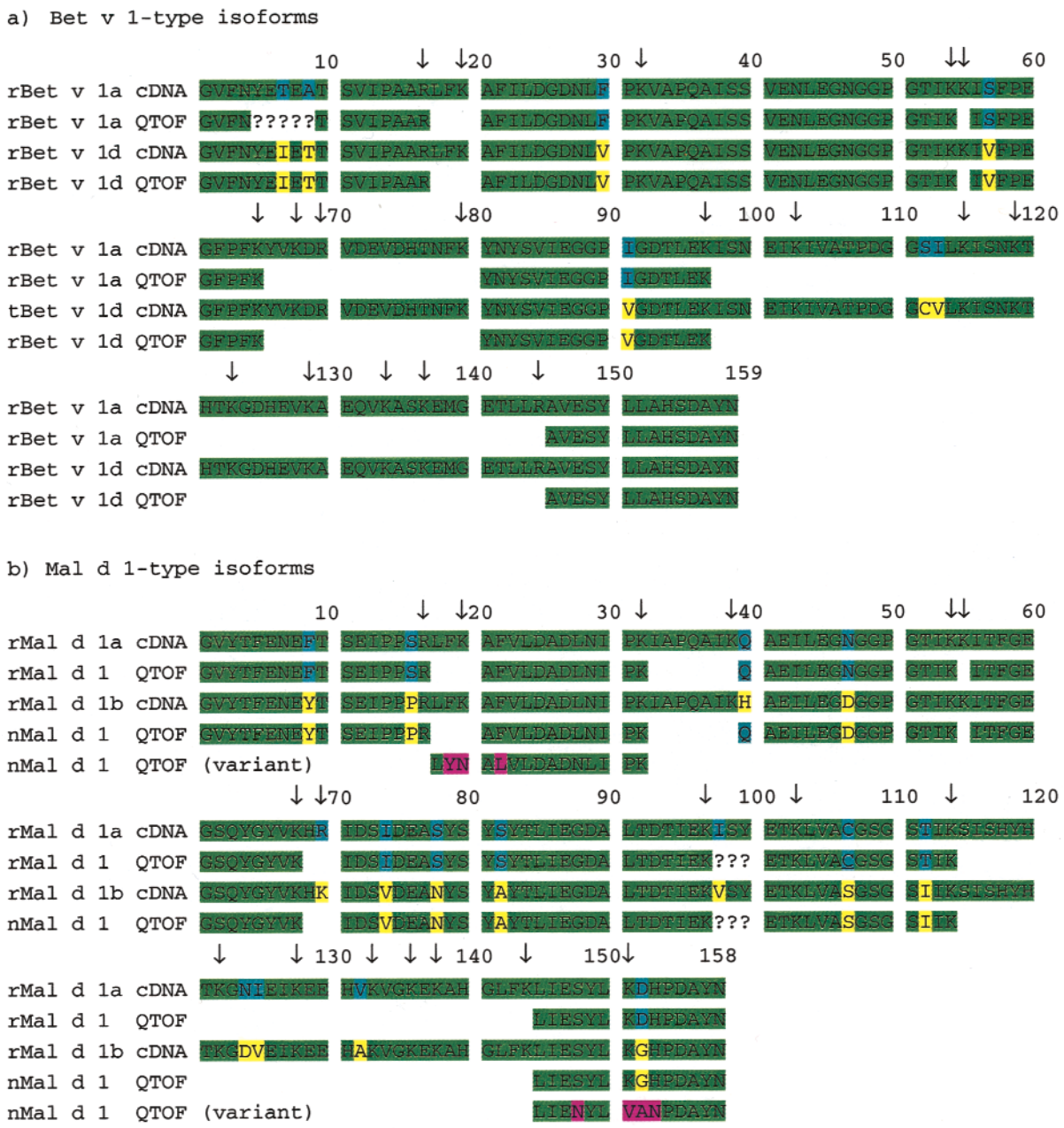

FIG 1. Amino acid sequences of rBet v 1a and rBet v 1d **(A)** and rMal d 1, rMal d 1b and nMal d 1 **(B)**, as revealed by cDNA and Q-TOF MSMS analyses. nMal d 1 was isolated from fruits of *Malus domestica* cv Granny Smith. Empty sites represent peptides not observed in Q-TOF MS and hence not sequenced by MSMS. *Question marks,* Tryptic peptides detected in Q-TOF MS, which could not be sequenced in MSMS-analyses (see text); *blue/yellow boxes,* amino acids, which differ between Bet v 1a and Bet v 1d and between Mal d 1a and Mal d 1 b; ↓, trypsin cleavage site. Numbers refer to the amino acids in the protein from N- to C-terminus. C at position 107 of rMal d 1a was detected as the carboxyamidomethyl derivative in Q-TOF MSMS. The cDNA-derived amino acid sequences of Bet v 1–type isoforms were provided by Biomay (Linz, Austria); those of Mal d 1–type isoforms were provided by Son et al.[4]

peptides corresponding to amino acids 1 to 17, 21 to 32, 33 to 54, 56 to 65, 81 to 97, and 146 to 159 were sequenced by means of Q-TOF MSMS (Fig 1, *A*). From the first tryptic peptide of rBet v 1a (amino acids 1-17), amino acids 5 to 9 could not be sequenced with Q-TOF MSMS because of a reproducible lack of subfragment information in the spectra. Hence 56.6% of all amino acids in rBet v 1a and 59.8% of those in rBet v 1d were sequenced. These included 3 of 7 amino acid differences between both isoforms (colored blue and yellow in Fig 1, *A*).

Recombinant Bet v 1a and rBet v 1d were mixed at ratios of 1:1, 1:9, and 9:1 to address the potential of Q-TOF MSMS for the detection of different isoforms in mixtures. Mixing of isoforms did not influence the Q-

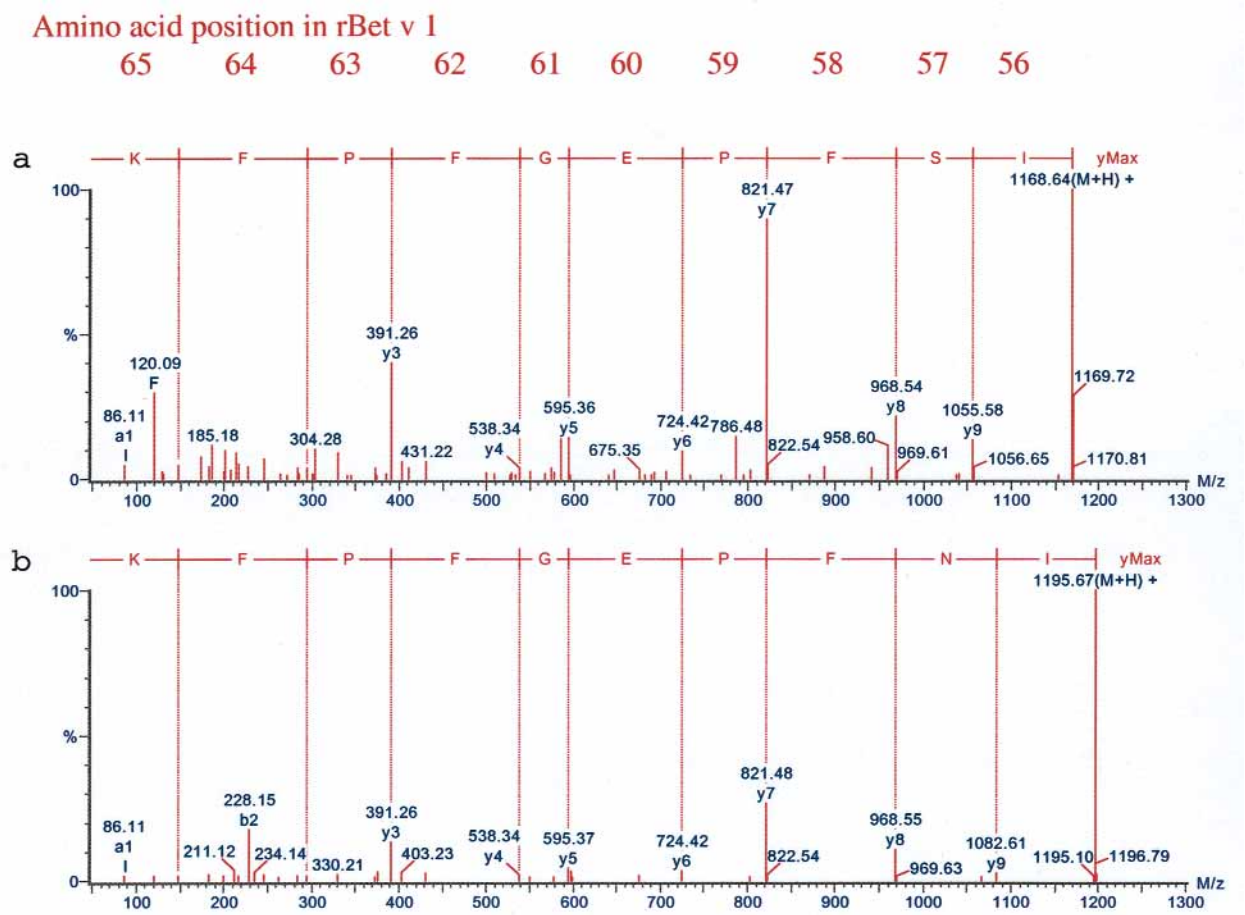

FIG 2. Q-TOF MSMS spectra of 2 tryptic peptides, both corresponding to amino acids 56-65, of rBet v 1a **(a)** and rBet v 1d **(b)**, which were analyzed from a mixture at a weight ratio of 1:1. The amino acids, given in the spectra, are arranged in reversed order (from C- to N-terminus) because Q-TOF MSMS spectra obtained in this mode are of better quality.

TOF MS or MSMS spectra, as illustrated for the MSMS spectra peptide comprising amino acids 56 to 65 of rBet v 1a and rBet v 1d, in which only amino acid 57 is different (Fig 2). The quality of the spectra is dependent on the absolute amount of peptide, also in isoform mixtures. Thus it could be calculated from the signal size that proportions of about 2% of the least abundant isoform would have been detected, whereas proportions down to 5% could also be sequenced.

Recombinant Mal d 1 and nMal d 1: Sequence analysis

Q-TOF MS analyses of tryptic digests of rMal d 1 and nMal d 1, revealed 8 of 19 of the theoretically possible tryptic peptides, as anticipated from the available sequence information for Mal d 1.[4] From 7 of these peptides, comprising amino acids 1 to 17, 21 to 32, 40 to 54, 56 to 68, 71 to 97, 104 to 114, and 145 to 151, the sequences could be elucidated completely for both recombinant and natural allergen (Fig 1, *B*). MSMS analyses of the eighth fragment from both rMal d 1 and nMal d 1 pointed in the direction of a peptide comprising amino acids 98 to 103. From this fragment, however, only the last 3 amino acids could be sequenced because of a reproducible lack of subfragment information in the MSMS spectra. Together, the 8 peptides occurring in both rMal d 1 and nMal d 1 encompassed 107 (67.7%) of 158 amino acids in this protein. On the basis of this analysis, the commercially available rMal d 1 was identified as a member of the Mal d 1a isoform group. Seven of 10 unique Mal d 1a amino acids (colored blue in Fig 1, *B*) were detected. Peptide 71 to 97 revealed a mass increment of 4.2 d compared with the expected mass for this peptide on the basis of the published sequence for Mal d 1a. This difference was explained by the MSMS spectrum as a substitution of threonin by proline at position 92. In addition, for the peptide comprising amino acids 104 to 114, it was shown that the cysteine at position 107 was modified to its carboxyamidomethyl derivative.

The MSMS spectra of both nMal d 1 bands indicated that the Granny Smith cultivar contained Mal d 1b–type allergen, with 6 of 10 unique Mal d 1b amino acids detected (Fig 1, *B*). Only at position 40 did the nMal d 1 have a Mal d 1a–like glutamine instead of a histidine. At

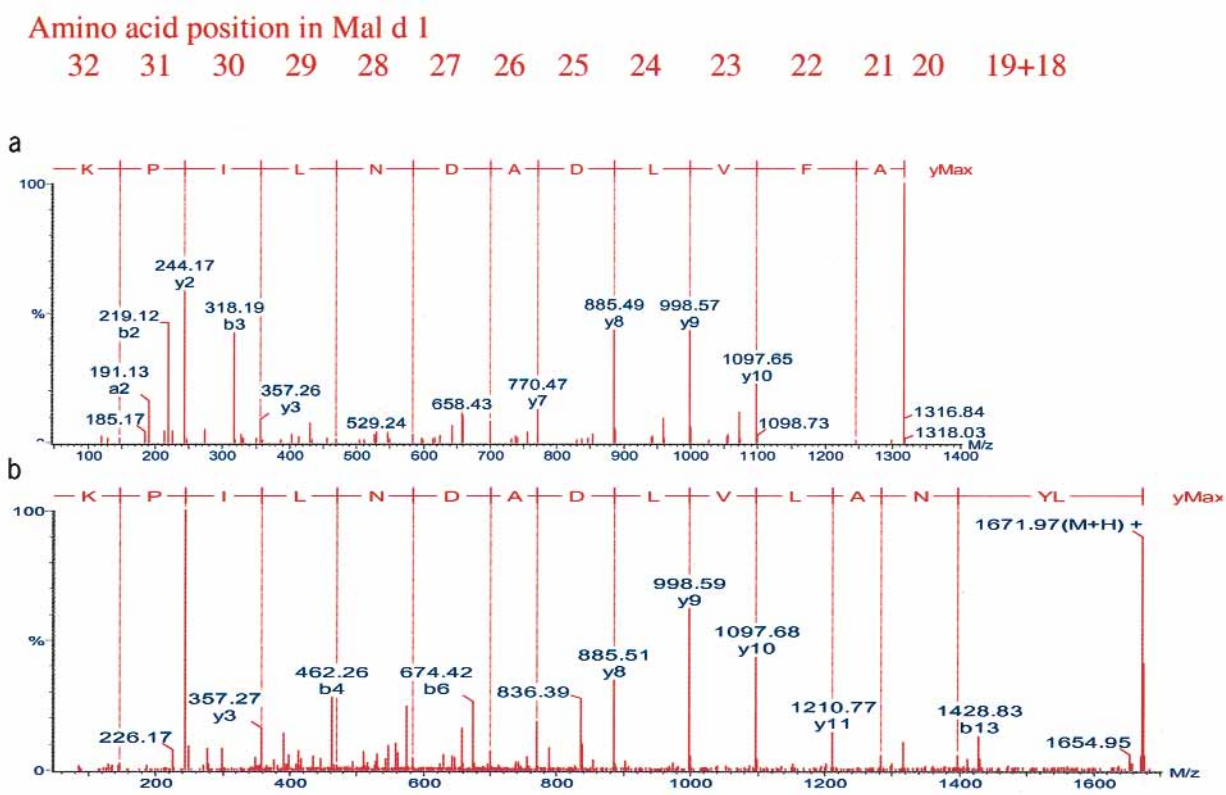

FIG 3. Q-TOF MSMS spectra of 2 of 10 tryptic peptides obtained from nMal d 1 isolated from *Malus domestica* cv Granny Smith. The peptides correspond to amino acids (N- to C-terminus): 21-32 **(a)** and 18-32 **(a)**. The peptide in **b** is an elongated form of that in **a**; the elongation is due to a lysine-to-asparagine substitution at position 20 in the Mal d 1 molecule, which makes it inaccessible to tryptic cleavage. For further details, see Fig 2.

position 50, a new polymorphism was found: a valine instead of a proline. Two additional peptides were detected in the tryptic digest of nMal d 1. MSMS spectra revealed that they were overlapping, with peptides 21 to 32 and 145 to 151 encompassing amino acids 18 to 32 and 145 to 158, respectively (Figs 1, *B*, and 3). These elongated peptides were explained by replacement of lysines at position 20 and 151 with an asparagine and a valine, respectively, thereby removing a cleavage site for trypsin. These peptides contained several additional thus-far-unreported polymorphisms (Figs 1, *B*, and 3).

As observed for rBet v 1, mixing of isoforms did not affect the Q-TOF spectra for the peptides from the individual isoforms. Hence also for Mal d 1, proportions of approximately 2% of the least abundant isoform would have been detected, whereas proportions down to 5% could also be sequenced.

Recombinant Mal d 1: Quantification and detection threshold

From the numeric data provided by Q-TOF analyses, the total ion count* values obtained from tryptic peptides in the first-dimension mass spectrometry show the best linear correlations. Regression coefficients varied from 0.91 to 0.98 for the tryptic peptides obtained from rMal d 1, which was used for these experiments (Fig 4). This indicates a high predictive value in quantification studies. Slopes of the regression lines indicate detection thresholds in the femtomole (10^{-15}) to picomole (10^{-12}) range, which makes this approach feasible for detection of Mal d 1 in much smaller quantities than obtained from a single apple fruit.

DISCUSSION

At the DNA level, it has been demonstrated that many major allergens exist in multiple isoforms. This has also been reported for the major apple allergen Mal d 1. Recently, Son et al[4] proposed to group isoforms of Mal d 1 into 3 main families according to the 3 sequences that were present in the database at that time: Mal d 1a, cloned from cultivar Granny Smith, and Mal d 1b and c, both cloned from cultivar Golden Delicious. They sequenced 12 new PCR clones from 7 different cultivars, including Granny Smith and Golden Delicious, and could classify 6 as Mal d 1a–like clones and 6 as Mal d 1b–like clones. In 3 cultivars (Golden Delicious, Gala, and Gloster) both Mal d 1a and Mal d 1b were found.

*Q-TOF equipment measures the abundance of peptide ions as electric signals. Total ion count represents the total number of ions generated by each peptide on the Q-TOF detector.

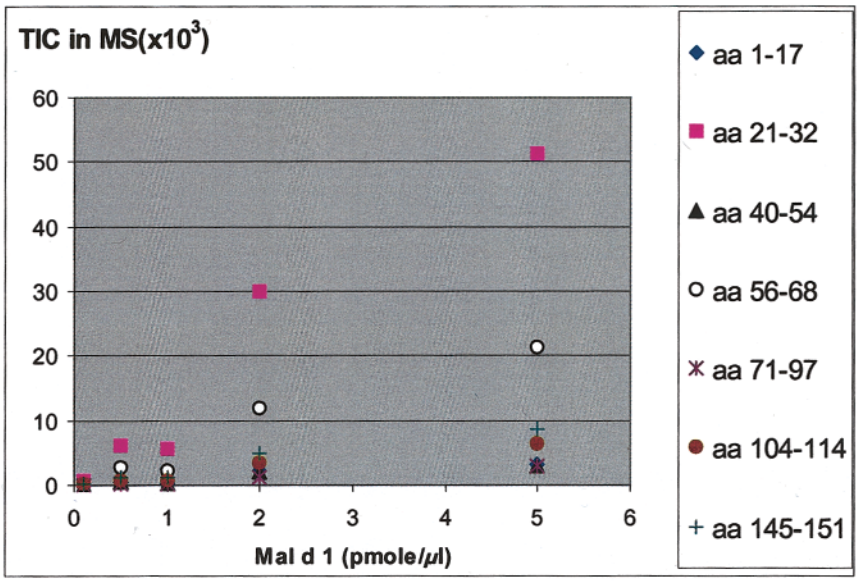

FIG 4. Quantitative relationship between the concentration of 7 rMal d 1–tryptic peptides and the signal size, obtained in Q-TOF analysis. The signal size represents the total ion count *(TIC)*, obtained in the Q-TOF MS mode of the 7 peptides that could be completely sequenced by Q-TOF MSMS (see Fig 1, *B*). The numbers in the insert refer to amino acid *(aa)* position in rMal d 1.

From Granny Smith, only Mal d 1b was picked up as protein in our study, but from this cultivar, Mal d 1a also was picked up as DNA. In other words, it is very likely that most cultivars contain DNA coding for both Mal d 1a and Mal d 1b. Mal d 1c was not found. Concerning the actual expression of Mal d 1 isoforms, the reports available until now describe that the 15 to 37 N-terminal amino acids of Mal d 1 showed approximately 65% homology to Bet v 1 and cross-react with IgE against this latter allergen.[22-24] The sequence data in these studies are consistent with our observations, in which Q-TOF MSMS allowed sequencing of approximately 70% of all amino acids in Mal d 1 and more than 55% of amino acids in Bet v 1a and Bet v 1d. In our study it was shown that affinity-purified Mal d 1 from Granny Smith contains Mal d 1b–like isoforms. Mal d 1a and Mal d 1c were not detected, indicating that these isoforms do most likely represent less than 2% of the total Mal d 1 content. Using larger amounts of total protein could have further enhanced the sensitivity for other than the most abundant isoforms.

Commercially available rMal d 1 was thought to have a sequence identical to that of Mal d 1c[L1]. In line with this, Son et al[4] used this recombinant product as a Mal d 1c reagent in their study. Sequence analysis of this recombinant reagent by means of Q-TOF MSMS has now demonstrated that this assumption is possibly not correct. With the exception of a proline instead of a threonine at position 92, rMal d 1 was identical to Mal d 1a, including positions 107 and 111, for which amino acid exchanges in Mal d 1c were reported. Polymorphisms at positions 121 and 139 could not be sequenced. The question can, however, be raised regarding whether Mal d 1c really exists or was perhaps a cloning or sequencing artifact. If a true isoform, it is most likely of minor importance. This is further supported by the absence of this isoform among the clones reported by Son et al,[4] as well as in the affinity-purified Mal d 1 from cultivar Granny Smith analyzed in this study.

Swoboda et al[12] applied plasma desorption mass spectrometry (PDMS) to study the natural occurrence of 11 cloned isoforms of the major birch pollen allergen Bet v 1. Similar to our approach, protease-digested samples of natural allergen were subjected to mass spectrometric analysis. By using trypsin and endoproteinase Glu-C, they were able to confirm 100% of the sequence of the 11 isoforms of Bet v 1 in the natural allergen source. PDMS does not allow true sequencing but only reveals the accurate molecular mass of peptides, which can then be matched to a known sequence. Unknown polymorphisms can not be determined by means of PDMS. Q-TOF MSMS not only accurately determines the mass of a peptide but also determines its exact sequence in the second-dimension MS. In this way it was shown that rMal d 1 contains a proline at position 92 instead of the threonine expected from the published sequence. Most likely, this points to a sequencing artifact in the original clone. In addition, nMal d 1 isoforms were found with polymorphisms thus far not reported in cDNA clones. Most of these polymorphisms were detected in extended versions of peptide 21 to 32 (ie, peptide 18-32 with a tyrosine, asparagine, and leucine at positions 19, 20, and 22, respectively) and of peptide 145 to 151 (ie, peptide 145-158 with an asparagine, valine, alanine, and asparagine at positions 148, 151, 152, and 153, respectively). Extended peptides were explained by the exchange of lysines at positions 20 and 151, resulting in removal of cleavage sites for trypsin. Using the calibration curves obtained with rMal d 1, the extended peptides 18 to 32 and 145 to 158 were

estimated to account for 25% to 50% of the total nMal d 1 content, respectively. PDMS could not have revealed these polymorphisms. Finally, at position 50, a valine was found instead of a proline, possibly pointing toward a sequencing artifact in the original Mal d 1b clone.

A disadvantage of Q-TOF MSMS is that not every peptide generated by means of trypsin digestion can be analyzed. The background of this will not be discussed in detail here, but it suffices to say that the technique requires tryptic peptides that do not decompose under the circumstances encountered during Q-TOF analysis. Smaller peptides tend to be unstable because of high charge densities and will therefore be selectively excluded, as was observed for the Bet v 1 and Mal d 1 isoforms. In the context of this study, it is more relevant to evaluate whether Q-TOF MSMS was able to determine those sequences that distinguish isoforms and that are of relevance to IgE binding. For the 11 isoforms of Bet v 1 reported by Swoboda et al,[12] it was shown that polymorphisms are scattered over the whole sequence. A total of 27 positions contained amino acid changes compared with Bet v 1a. Sequence data from our Q-TOF MSMS analysis covered 10 of 27 of these positions, allowing distinction among the 11 isoforms. Polymorphisms at positions 10, 30, 57, 112, 113, and 125 were postulated to be important for IgE binding,[5,9] and amino acids 42 to 52 form the major part of the 16 amino acids constituting the conformational Bet v 1 epitope.[9] The hypoallergenic isoform Bet v 1d contains 4 of these polymorphisms (30, 57, 112, and 113). Ferreira et al[5] constructed a hypoallergenic mutant with exchanges at all sites, with the exception of amino acids 42 to 52. Ideally, Q-TOF MSMS analysis should cover those parts of the molecule with relevance for allergenicity, which is the case for all the above-mentioned polymorphisms, except those at amino acids 112, 113, and 125 in Bet v 1. By analogy to Bet v 1d, Son et al[4] constructed a mutant of Mal d 1 by introducing a cysteine at position 111 instead of the serine present in both Mal d 1a and Mal d 1b. This mutation resulted in significantly decreased IgE binding. In the search for naturally occurring hypoallergenic Mal d 1 isoforms to be applied in breeding of low-allergenic apples, Q-TOF MSMS analysis of amino acids 111 and 112 therefore appears to be essential. In contrast to Bet v 1, both for rMal d 1 and nMal d 1, a peptide encompassing amino acids 104 to 114 was sequenced. All the other positions claimed to be important for Bet v 1 allergenicity, with the exception of position 125, were covered in the analysis of Mal d 1 samples.

In the last decade, amino acid sequences of allergens on the basis of deduction from nucleotide sequences have been extensively documented. By using Q-TOF MSMS, these data can now be evaluated with regard to protein quantity of allergen isoforms, which ultimately determines the allergenicity, and, where applicable, with regard to posttranslational processing. This makes Q-TOF MSMS a valuable new approach for analyses of allergens and allergenicity. Further development of basic knowledge and of hardware and software is required to further extend the potential of this technique for characterization of allergen and epitope structure analysis. In the applied field, Q-TOF MSMS can be used to detect sources with hyperallergenic or hypoallergenic activity, which will be relevant to establishing the effect of breeding programs and process technology on food safety.

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