



Effect of harvest maturity, duration of storage and shelf life of apples on the allergen Mal d 1, polyphenoloxidase activity and polyphenol content

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ABSTRACT

Freshly consumed apples can cause allergenic reactions because of the presence of allergens (in middle Europe Mal d 1) and their cross-reactivity with sensitising allergens of other species. Knowledge of endogenous factors affecting the allergenic potential of apples would provide important information to apple breeders, growers and consumers for the selection of hypoallergenic genotypes, adoption of agricultural practices for decreasing the allergenic potential and the consumption of fruits with reduced amounts of allergens.

Fruit samples were collected from three apple varieties and from fruits of two different trials, set up to assess the effect of ripeness on synthesis of Mal d 1 at harvest date, as well as on Mal d 1 after storage and shelf life. Oxidative reactions catalysed by polyphenol oxidase (PPO) and/or peroxidase (POD), present in apple, may be involved in decreasing its allergenicity. So, we focused on the classification of varieties according to the activity of polyphenol oxidase and polyphenol content. The apple allergen Mal d 1 was ripening-related; the amount increased with duration of storage. In most cases no differences were found in Mal d 1 content in regard to the stage of ripeness at harvest. Tendendiously, storage and shelf life led to higher Mal d 1 levels in unripe and overripe harvested fruit. Storage and stage of ripeness did not affect PPO activity. The allergenicity of apple fruits was affected by different internal factors, such as PPO activity, polyphenol content, especially catechin and epicatechin, and the antioxidative capacity. Whereas higher PPO activities and polyphenols contents result in less extractable Mal d 1, higher antioxidative activity can inhibit the interaction between oxidised phenols and Mal d 1, resulting in higher allergenicity (extractable Mal d 1). The extractable Mal d 1 was greatest in 'Topaz' fruit (at medium), followed by 'Golden Delicious' and 'Braeburn'. This study represents one of the first attempts to evaluate any relationship between Mal d 1 content and PPO, total phenol content and antioxidative capacity in different apple varieties.

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1. Introduction

In central Europe and North America, the major apple allergen Mal d 1 belongs to the pathogenesis-related proteins. These proteins are synthesised in response to several stress factors, including biotic and abiotic stimuli, as well as pesticide treatment or senescence processes (Breiteneder & Ebner, 2000). It was shown that storage of apple fruit led to a significant accumulation of Mal d 1 protein (Bolhaar et al., 2005; Sancho et al., 2006b; Vieths, Jankiewicz, Schöning, & Aulepp, 1994). A ripening-related occurrence of Mal d 1 was reported as well, with increasing allergen amounts during the growth period (Vieths, Schöning, & Jankiewicz, 1993). Which mechanisms led to this synthesis have yet to be analysed; a role of ethylene is suggested. As apples are available throughout the year, a significant number of consumed fruit had

been stored for several weeks or even months. These fruits contain higher amounts of the allergen.

During storage, enzymatic browning can occur, which causes unpleasant colours and flavours and a loss of nutrients (Goupy et al., 1995). Browning is dependent on polyphenol content and polyphenoloxidase (PPO) activity (Goupy et al., 1995). PPO is a copper enzyme, widely distributed in the plant kingdom (de los Angeles Serradell et al., 2000). It catalyses the oxidation of polyphenols to their corresponding quinones (Rocha & Morais, 2001; Tsurutani, Yanagida, Hagiwara, Murata, & Homma, 2002). The formed quinones take part in secondary reactions involving polyphenols, amino acids and proteins which lead to brown polymers (melanins) (Lattanzio, 2003). The enzyme is generally located in plastids of intact cells (Tsurutani et al., 2002), while substrates of the enzyme, the polyphenols, are located in the vacuoles; they come into contact when cells are broken by cutting or bruising (Rocha, Pilar Cano, Galeazzi, & Morais, 1998). Furthermore, low temperature stress might induce radical oxygen species which could react with membrane lipids. This can result in an

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intracellular decompartmentalization, thus permitting enzymes and substrates to come into contact (Lattanzio, 2003). Recent research reported a soluble PPO in many apple cultivars with a ten times lower activity than that of the plastid-bound enzyme (Tsurutani et al., 2002). Changes in membrane permeability during ripening processes lead to greater amounts of soluble PPO (Lattanzio, 2003). PPO activity was shown to be cultivar-dependent (Rocha & Morais, 2001). To reduce browning reactions during storage and astringent taste caused by phenolic compounds, apple cultivars with lower polyphenolic content have been bred.

However, the oxidised polyphenols can interact with allergenic proteins, which leads to a decrease in allergenicity (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). Hjerno et al. (2006) found that the up-regulation of oxidative enzymes was related to a lower allergenicity in strawberry fruit. The reaction between quinones and allergenic proteins leads to changes in tertiary structure, resulting in a loss of conformational epitopes and therefore lower allergenicity (Garcia, Wichers, & Wichers, 2007). So, cut products of cultivars, with high polyphenol content and high PPO activity, might have lower allergenicity.

Our aim was to determine whether harvest maturity has an impact on storability and allergenicity of apple fruit in respect to Mal d 1 content and PPO activity. To our knowledge, correlations among PPO activity, polyphenol content and allergen content in apple fruits have not so far been investigated. So, another objective of this study was to show relationships among Mal d 1 content, PPO activity and polyphenol content during storage. Additionally, shelf life at 20 °C was tested, to simulate the conditions during the handling chain or at the consumer's home. Therefore three cultivars that differ in allergen and polyphenol content were selected, harvested at different stages of ripeness, and stored for 8 and 12 weeks and an additional shelf life period.

2. Material and methods

2.1. Plant material and storage conditions

Fruit of three apple cultivars (*Malus domestica*, Borkh.), 'Braeburn', 'Topaz' and 'Golden Delicious', were cultivated at the Centre of Competence Klein-Altendorf, Germany. These varieties were selected because they differ in their polyphenol contents from low ('Braeburn') to high ('Topaz'), which was determined in previous studies (Matthes and Schmitz-Eiberger, 2009). Each variety was harvested at the unripe (10–14 days before predicted optimal harvest date), ripe and overripe (10–14 days after harvest of ripe fruits) stage. Streif-Index was used to determine maturity and, as a consequence, the optimal harvest date for each cultivar. The index was calculated as fruit firmness/[soluble solid content x starch degradation value] (Höhn, Datwyler, Gasser & Jampen, 1999). Fruit firmness was determined by a fruit pressure tester (TR, mod. FT 327, Italy). Harvest dates and Streif-indices are displayed in Table 1. Subsequent to harvest, fruit were washed and protein was extracted. To minimise variability, fruit were picked from specific positions of the tree, so that the environmental impacts (e.g. irradiation) were comparable.

For storage experiments, fruit were stored in a cold chamber (2 °C) for 8 and 12 weeks. Subsequent to this, some fruit were stored under shelf life conditions (20 °C) for two weeks to simulate the conditions at the consumer's home or during handling in the production chain.

2.2. Preparation of protein extracts

Extraction of the protein was carried out according to Bjoerksen, Halmepuro, Hannuksela, and Lahti (1980). Peel and pulp were

Table 1

Harvest dates and values for parameters of Streif-index of 'Topaz', 'Golden Delicious' and 'Braeburn' fruit harvested at different stages of ripeness.

Harvest	Cultivar	Firmness [kg/cm ²]	Soluble solids [°Brix]	Starch degradation [1–10]	Streif- Index
14.09	Topaz unripe	11.2	12.64	1.9	0.47
28.09	Topaz ripe	9.33	14.05	4.26	0.17
09.10	Topaz overripe	8.46	14.89	6.11	0.10
14.09	GD unripe	9.06	12.99	3.93	0.19
28.09	GD ripe	8.62	13.52	4.23	0.16
05.10	GD overripe	8.42	14.6	6.53	0.09
09.10	Braeburn unripe	10.61	11.59	2.63	0.37
19.10	Braeburn ripe	9.81	12.94	3.81	0.23
30.10	Braeburn overripe	9.10	13.44	7.57	0.09

homogenised with potassium-phosphate buffer (10 mM K₂HPO₄, 10 mM KH₂PO₄, pH 7.1) containing sodium diethyldithiocarbamate trihydrate (10 mM), ethylenediamine-tetraacetic acid (EDTA) (2 mM) and polyvinylpyrrolidone (PVPP, 2%) in a ratio of 1:1.5 w/v, using the knife mill Grindomix (Retsch, Haan, Germany). The extracts were prepared from the same ratio of peel and pulp for each cultivar. The core was removed before extraction. After incubation for 4 h at room temperature, the homogenates were centrifuged at 4 °C for 15 min at 3500g. The supernatants were collected and subsequently frozen in aliquots at –80 °C. For each variety, extracts were prepared fivefold.

Extraction of polyphenols was performed by following the same protocol, but the buffer did not contain PVPP, because it is able to bind to polyphenols, so that they could not be detected.

2.3. Analysis of total phenol content

Total phenols were measured using the Folin–Ciocalteu reagent (Singleton & Rossi, 1965). An aliquot of 0.5 ml of the sample was mixed with 0.5 ml of distilled water and 0.5 ml of the Folin–Ciocalteu reagent was added. After 30 s, 5 ml of aqueous sodium carbonate (20%) were added and the mixture was incubated for 30 min at room temperature in the dark. Extinction was spectrophotometrically determined at 720 nm. Total phenol content was calculated using gallic acid as standard. Results were expressed as mg gallic acid units (GAU) 100 g^{–1} FW.

2.4. Extraction of PPO

Extraction of PPO was carried out according to Rocha et al. (1998). Two grammes of fresh apples (peel and pulp) were shock-frozen in liquid nitrogen and ground in a mixer mill (Retsch, Haan, Germany). The samples were homogenised in sodium-phosphate buffer (pH 6.5) containing Triton X100 (0.25%) and soluble polyvinylpyrrolidone (1%) in a ratio of 3:2 (v:w). The homogenates were centrifuged at 4 °C for 25 min at 3500g. The supernatants were collected, filled up to 5 ml with sodium-phosphate buffer (pH 6.5) and stored at –80 °C prior to analysis. Extracts were prepared, fourfold, of a composite sample of 5 fruit.

2.5. PPO assay

Enzymatic activity of the PPO was analysed according to a method described by Rocha et al., 1998. The oxidation of the substrate was followed by monitoring the increase of absorbance at a

wavelength of 420 nm at 25 °C in a Lambda 5/15 spectrophotometer (Perkin Elmer) for 4 min. The reaction mixture contained 2.8 ml of a substrate solution of 4-methylcatechol (0.033 M) and 0.2 ml of the enzyme solution (Quiles et al., 2005; Rocha et al., 1998). The reference cuvette was filled with substrate solution. A change of absorbance value of 0.001 was defined as one unit of enzyme activity. The straight line section of the activity curve was used to express the enzyme activity (U/min/ml).

2.6. Protein determination

Total protein concentration was determined with the Roti-Nanoquant protein assay kit (Carl Roth, Germany) according to the manufacturer's guidelines. Bovine serum albumin (BSA) was used as standard protein.

2.7. Sandwich-ELISA

Quantification of Mal d 1 contents of all samples was determined in triplicate by Sandwich Enzyme-linked-Immunosorbent-Assay (ELISA). A Sandwich-ELISA was developed for quantification of Mal d 1. Microtitre plates (Nunc-Immuno Plate Maxi Sorp™ surface, Nalge Nunc International, Denmark) were coated with 1:1000 sheep anti-mouse Ig AP 302 (Chemicon, Germany) diluted in PBS-buffer (phosphate-buffered saline, pH 7) at room temperature for one hour. After every incubation step, four washes with PBS-T (v/v 0.05% Tween 20 in PBS) were conducted. A monoclonal antibody, specific for Mal d 1 (1:100), diluted in PBS-T, was incubated at 4 °C overnight. All further dilutions were performed with PBS-T buffer. In the next step the wells were incubated with dilutions of the apple extracts (1:4–1:512 in dual steps) in triplicate and recombinant Mal d 1 was used as reference (2000–2.4 ng/ml). After incubation for one hour at room temperature, a rabbit anti-Mal d 1 antibody was incubated (1:1000). Detection was performed with goat anti-rabbit IgG (Sigma, A0545, Germany) conjugated with a peroxidase (1:20000), which was incubated at room temperature for one hour. Finally, the plates were incubated for 15 min with substrate (citrate buffer, pH 3.95). To stop the reaction, 50 µl of 25% sulphuric acid were added. Photometric detection was performed at 450 nm in a microplate reader (Labsystems Multiscan RC). Recombinant Mal d 1 (Biomay, Austria) was used as standard and total Mal d 1 content was calculated by a four-parametric calibration curve. Values were expressed as ng Mal d 1/ml and converted to µg/g FW.

2.8. Statistics

The experimental data were analysed using the statistic programme SPSS 14.0 for Windows (Munich, Germany). The statistical analysis was performed by one way analysis of variance (ANOVA) at a probability level of 95%. Means were compared by Tukey's multiple range test after the data were checked for normal distribution and variance homogeneity. Any relationships among Mal d 1 content, PPO activity and polyphenol content were calculated as linear regressions from 450 values for allergens, polyphenols and PPO activity, each.

3. Results

3.1. General

An overview of harvest dates and parameters for Streif index is given in Table 1. Streif indices were calculated from the other ripeness parameters. Streif indices for unripe apple fruits were lower; indices for ripe apple fruits were equivalent to the recommended

values and, for ripe apple fruit, the values were higher (recommended values for Braeburn: 0.16–0.22, Golden Delicious 0.09–0.12, Topaz: 0.07–0.18 (Höhn et al., 1999)).

3.2. Mal d 1

At harvest, Mal d 1 contents were too low to be quantified in all apple cultivars and at all stages of maturity. An increase in Mal d 1 content was observed during storage in all cultivars (Table 2).

In most cases no differences in Mal d 1 content were detected in fruit of different harvest dates. After an additional shelf life of 12 weeks for stored 'Topaz' fruit, and 8 weeks for stored 'Braeburn' fruit, significantly higher Mal d 1 concentrations were detected in the overripe fruit in comparison to the unripe fruit. In all other storage durations no differences in Mal d 1 content between the stages of ripeness were found (Table 2).

Storage for 8 weeks led to a significant Mal d 1 synthesis in the fruit of all cultivars. With duration of storage a slight increase in allergen content was shown between 8 and 12 weeks of storage in 'Topaz' fruit, irrespective of the stage of ripeness at harvest. Storage for 12 weeks did not increase Mal d 1 content in 'Braeburn' or 'Golden Delicious' fruit in comparison to storage for 8 weeks (Table 2).

Additional shelf life, for 2 weeks, of the 8 week-stored fruit led to a marked increase in allergen amount in the unripe and overripe 'Braeburn' fruit. In all other cultivars, and harvest maturities, a slight increase could be observed. Storage, at ambient temperature, of 12 week cold-stored fruit of all cultivars led to Mal d 1 accumulation in unripe and overripe harvested fruit. In ripe fruit, Mal d 1 remained stable (Table 2).

Calculated percentage of Mal d 1/total soluble protein ranged from 0.2% for 8 week-stored unripe 'Braeburn' fruit to 3.2% for 'Topaz' fruit stored for 12 weeks + 2 weeks of shelf life (data not shown).

3.3. PPO

PPO activity in the fresh harvested fruit was lowest in the cultivar 'Topaz' (1647–2709 U/min/ml). PPO activity in 'Braeburn' fruit was from 3007–4450 U/min/ml and that of 'Golden Delicious' was from 2970 and 3649 U/min/ml (Table 3). Differences between the stages of ripeness could not be detected at any time in 'Golden Delicious' fruit. In 'Topaz' fruit that were stored for 12 weeks, a significantly higher PPO activity was found in ripe fruit than in unripe fruit. 'Braeburn' fruit that were stored for 12 weeks and an additional shelf life period, displayed significantly higher PPO activity

Table 2

Mal d 1 content of apple cultivars harvested at different stages of ripeness and after 8 and 12 weeks of cold storage and an additional shelf life (sl) period (mean, $n = 5$, n.d. = not detectable).

Cultivar	Stage of ripeness	Mal d 1 content [µg/g FW]			
		8 weeks storage	8 + 2 sl storage	12 weeks storage	12 + 2 sl storage
Braeburn	unripe	nd c B	7.6 a A	3.4 a AB	6.8 a A
	ripe	1.8 b A	4.6 a A	10.9 a A	6.7 a A
	overripe	4.8 a A	8.1 a A	4.4 a A	6.0 a A
GD	unripe	7.6 a A	17.1 a A	9.2 a A	14.6 a A
	ripe	7.3 a A	12.5 a A	10.3 a A	8.7 a A
	overripe	6.0 a B	11.4 a B	10.5 a B	18.6 a A
Topaz	unripe	1.4 a B	12.0 a A	5.1 a AB	7.3 b AB
	ripe	6.3 a A	13.3 a A	16.1 a A	12.3 ab A
	overripe	2.4 a B	11.2 a AB	11.9 a A	15.1 a A

Lower case letters indicate significant differences at $P \leq 0.05$ between stages of ripeness among fruit of one cultivar within a storage period. Upper case letters within a row indicate significant differences between the different storage durations for fruit of the same cultivar and harvest maturity.

Table 3

PPO activity of apple cultivars harvested at different stages of ripeness, and after 8 and 12 weeks of cold storage and an additional shelf life (sl) period (mean, $n = 5$).

Cultivar	Stage of ripeness	Harvest	PPO activity [U/ min/ ml]			
			8 weeks storage	8 + 2 sl storage	12 weeks storage	12 + 2 sl storage
Braeburn	unripe	3370 a A	3917 a A	4172 a A	3587 a A	3665 ab A
	ripe	3007 a A	3510 a A	3721 a A	4040 a A	4329 a A
	overripe	4450 a A	3966 a A	4182 a A	2619 a A	2574 b A
GD	unripe	2970 a A	3910 a A	3592 a A	3805 a A	3063 a A
	ripe	2978 a BC	3765 a AB	4278 a A	2675 a C	3697 a ABC
	overripe	3649 a A	4315 a A	3285 a A	3548 a A	4013 a A
Topaz	unripe	2709 a A	4289 a A	4316 a A	2299 b A	4144 a A
	ripe	1647 a B	3988 a A	4065 a A	3647 a AB	3139 a AB
	overripe	1889 a B	3798 a AB	4411 a A	3184 ab AB	3604 a AB

Lower case letters indicate significant differences at $P \leq 0.05$ between stages of ripeness among fruit of one cultivar within a storage period. Upper case letters within a row indicate significant differences between the different storage durations for fruit of the same cultivar and harvest maturity.

Table 4

Polyphenol content of apple cultivars harvested in different stages of ripeness at harvest, after 8 and 12 weeks cold storage and additional shelf life (sl) period (mean, $n = 5$).

Cultivar	Stage of ripeness	Harvest	Polyphenols [mg GAU/ 100 g FW]			
			8 weeks storage	8 + 2 sl storage	12 weeks storage	12 + 2 sl storage
Braeburn	Unripe	27.7 a B	26.6 b B	35.9 a A	31.4 a AB	31.4 a AB
	Ripe	29.0 a A	29.2 ab A	35.0 a A	31.9 a A	34.3 a A
	Overripe	26.9 a B	37.8 a A	29.3 b B	34.3 a AB	27.2 a B
GD	Unripe	30.8 a A	30.5 a A	28.4 a A	29.0 a A	31.6 a A
	Ripe	28.4 a AB	32.1 a AB	25.5 a B	34.2 a A	26.0b B
	Overripe	31.9 a AB	33.1 a AB	28.4 a B	34.7 a A	29.2 ab AB
Topaz	Unripe	31.4 a BC	37.6 a AB	33.7 a ABC	42.5 a A	31.6 a C
	Ripe	33.2 a B	37.0 a B	37.4 a B	44.0 a A	26.0 b B
	Overripe	36.6 a A	39.8 a A	38.4 a A	34.7 b A	29.2 ab A

Lower case letters indicate significant differences at $P \leq 0.05$ between stages of ripeness among fruit of one cultivar within a storage period. Upper case letters within a row indicate significant differences between the different storage durations for fruit of the same cultivar and harvest maturity.

in ripe fruit than in overripe fruit. In all other varieties, no differences were detected.

In most cases, storage did not affect PPO activity in the first 8 weeks of storage. Only in the case of ripe 'Topaz' fruit was PPO activity higher after 8 weeks of storage in comparison to harvest. During shelf life, PPO activity remained at a high level in 'Topaz' fruit, whereas, after 12 weeks, PPO activity declined markedly. Ripe harvested 'Golden Delicious' fruit showed a higher PPO activity after 8 weeks and additional shelf life period in comparison to harvest. 12 weeks of storage led to a significant decrease in PPO activity, to a value lower than the activity measured at harvest. In 'Braeburn' fruit no significant differences were detected (Table 3).

3.4. Polyphenols

In most cases, harvest maturity did not influence polyphenol content of fruit. During the storage period, polyphenol content either remained stable or increased. The stage of maturity at harvest seems to have no impact on cold storability of apple fruit in regard to polyphenol content. Shelf life, in some cases, led to significantly lower polyphenol contents (Table 4).

4. Discussion

In the present research, the actual importance of external (maturity, storage, shelf life) and internal (polyphenol content, PPO activity, antioxidative capacity) factors for the allergenic potential of apple fruit is described, with implications for common agronomical practices and postharvest management, as well as for the selection of hypoallergenic apple genotypes. The results provide first hints of the relationship between apple allergy and secondary ingredients, which may give helpful information for breeders and the selection of hypoallergic apple varieties. Among the cultivars and harvest dates the detected Mal d 1 content varies between 0.5 and 15 $\mu\text{g/g}$ of FW. Other studies have found Mal d 1 levels between 3.8 and 72.5 $\mu\text{g/g}$ of FW in the pulp (Sancho et al., 2006) or 0.71–20.17 $\mu\text{g/g}$ FW in fresh fruits and 5.95 until 200 $\mu\text{g/g}$ FW in stored fruits. Our results are at the lower end of the range. Stability of the extraction procedure and reproducibility of the sandwich ELISA were clear. Nevertheless we had a high variability in Mal d 1 content within one cultivar. In a study conducted by Asero, Marzban, Martinelli, Zaccarini, and Laimer da Camara Machado (2006), 'Jonagold' fruits showed an inter apple variability up to 20-fold and an intra-apple variability of up to 5-fold. In our study, sampling was standardised as far as possible, with the same standards at every location, as described under Materials and methods. Data on the influence of storage and cultivation methods are scarce. Mal d 1 is a pathogenesis related protein (PR-protein), so it can be concluded that stress factors can lead to protein synthesis. Further, factors affecting PR synthesis seem to be processes that occur during senescence and storage. Mal d 1 content increased in this study during storage in a cold chamber throughout 12 weeks. This is consistent with other studies in which higher Mal d 1 contents were determined over a 5 month storage period under ambient storage conditions cold storage, and storage under modified atmosphere (Sancho et al., 2006). Opposite results were reported for the apple allergen Mal d 3. During postharvest storage, Mal d 3 decreased to very low amounts after 4 and 5 months of storage (Sancho et al., 2006). However, Mal d 1 gene expression might be regulated differently from that of Mal d 3. In comparison to the 8 week-stored fruit, Mal d 1 concentration remained unchanged in the longer stored fruit. This might be due to accelerated degradation processes or reduced *de novo* synthesis.

Ripening of the fruit is associated with an upsurge in the rate of respiration and ethylene synthesis. Hsieh, Moos, and Lin (1995) observed an increase in Mal d 1 content during ripening and tried to evaluate whether this could be caused by ethylene. They did not find any correlation between Mal d 1 content and the expression of one of the enzymes of ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid oxidase, so they suggested that this allergen was not affected by senescence processes that occur during storage, but by factors that are related to tolerance to several abiotic and biotic stress factors (Matthes & Schmitz-Eiberger, 2009). In the present study, fruit were picked at 10–14 days intervals to investigate any influence of maturity. The results showed that, in most cases, no differences existed in Mal d 1 levels between the different harvest maturities. Data on Mal d 1 content, in relation to storage time and maturity at harvest, are scarce. Sancho et al.

(2006) found no differences during storage between fruit that were picked at two different harvest dates. Some studies have evaluated Mal d 1 gene expression during ripening of the fruit and found an increasing gene expression during the growth period (Vieths et al., 1993). This increase was cultivar-dependent, as synthesis of Mal d 1 was shown in the cultivar 'Golden Delicious' but not in 'Boskoop' fruit (Vieths et al., 1993). Hsieh et al. (1995) did not find a clear correlation between the stage of ripeness and the amount of Mal d 1 protein. For the apple allergen Mal d 3, a ripening-related accumulation was reported (Sancho et al., 2006b). This allergen is accumulated at the end of the growth period and time of picking had a strong effect on Mal d 3 expression.

Murata, Tsurutani, Tomita, Homma and Kaneko (1995) investigated polyphenol content and PPO activity in ripening apple fruit. Total phenol content decreased up to 3.5 months after flowering; then it remained at a constant level. PPO activity increased after flowering; then activity fluctuated; 4.5 months after flowering, the PPO activity decreased. The authors explain this by inactivation and denaturation processes that occur during the growth period. Similar results were obtained by Kim et al. (2001); PPO gene expression was reduced with ripening of apple fruit.

During storage, the PPO activity remained unchanged during the first 8 weeks of storage, whereas a slight decline was observed from the 8th to the 12th week. Literature reports are similar. Barret, Lee & Liu (1991) reported significant losses in PPO activity, combined with a lower tendency to browning, of 'Golden Delicious' fruit after 7–14 weeks of storage under controlled atmosphere. Leja, Marezek, and Ben (2001) determined PPO activity during long term storage of apple fruit. High activities at harvest decreased during storage for 120 days, irrespective of the storage conditions (controlled atmosphere and cold storage).

Enzymatic browning is seen as one of the major causes of spoilage during postharvest treatment, storage and processing (Quiles et al., 2005). In healthy plant tissues, the ripening process induces changes in cell membrane permeability, in phenolic metabolism and in PPO properties, as more enzymes become soluble (Lattanzio, 2003).

Hence, it has been hypothesised, that the allergenicity of former apple varieties were lower, most probably due to higher polyphenol content and the assumed oxidative reactions between apple phenols and the allergen (Rudeschko et al., 1995). A reduction in allergenicity could be due to the masking of IgE binding sites on the allergenic protein, through cross-linking of proteins induced by oxidative enzymes (Chung, Maleki, & Champagne, 2004). Polyphenol oxidase (PPO) is the main factor involved in these oxidative reactions in fruits and vegetables (Garcia et al., 2007). The latter author's suggest that the decrease of immunoreactivity is due to formed o-quinones, modifying the tertiary structure of the allergens and leading to the formation of cross-linked protein-polymers. So there might be reactions between polyphenols and the allergen Mal d 1 during storage, when oxidative reactions occur. The lower allergenicity of cut apple products can be explained by these reactions (Garcia et al., 2007). Antioxidants can be potent protectors of Mal d 1 allergenicity, by inhibiting the reactions between phenols and Mal d 1 (Garcia et al., 2007). A study on the cherry allergen, Pru av 1, also found a decrease in allergenic potential of the protein in the presence of polyphenols and PPO (Gruber et al., 2004).

The allergenicity of 'Braeburn' fruits was the lowest, possibly due to its high PPO activity and its high polyphenol content. Garcia et al. (2007) showed that the addition of PPO to a Golden Delicious apple extract decreased IgE-binding by Mal d 1, resulting in a decline of allergenicity. Apple peel treated with a combination of the oxidative enzyme and the polyphenol catechin showed a marked inhibition of IgE binding by Mal d 1. These experiments may explain the fact that the allergenicity in 'Braeburn' fruits

was very low in comparison to the other two varieties. Additionally, earlier studies showed that 'Braeburn' fruits had a low antioxidative capacity (Schmitz-Eiberger, Weber, Treutter, Baab, & Lorenz, 2003). Garcia et al., 2007 showed, that antioxidants, e.g. DIECA, can be potent protectors of Mal d1 allergenicity. So, only a very low inhibition of the complex reaction between oxidised phenols and Mal d 1 can occur, resulting in a decrease of IgE binding, compared to the other varieties.

For 'Golden Delicious', the measured Mal d 1 was at a medium level, while the PPO activity and antioxidative capacity (Schmitz-Eiberger et al., 2003) were also at a medium level and content of polyphenols was similar to that of the 'Braeburn' variety. The combination of PPO and polyphenols indicates medium oxidation processes, causing medium allergenicity of all apple varieties evaluated.

It should be noted, that 'Topaz' is an apple variety with high polyphenol content, relatively low PPO activity but also a high antioxidative capacity (Schmitz-Eiberger et al., 2003). High concentrations of Mal d 1 were measured, although the apple variety also has high catechin contents (Schmitz-Eiberger et al., 2003). In this variety, the IgE binding of Mal d 1 is possibly reduced a.) by low progression of oxidative processes (low PPO contents) and b.) by the inhibition of these processes by high antioxidative capacity.

5. Conclusion

Harvest maturity seems to have no impact on storability in regard to Mal d 1 content. This study represents one of the first attempts to evaluate any relationship between Mal d 1 content and PPO activity, total phenols, antioxidative capacity, catechin and epicatechin content in different apple varieties. Although our results for Mal d 1 content are conclusive, we found high variabilities between fruits, showing that environmental conditions are important for allergenicity of apple fruits. Studies in which all environmental conditions can be standardised, e.g. in a climatic chamber, and the influence of only one factor can be varied, should give valid results for the impact of single environmental conditions on allergenicity. Further long-term studies of Mal d 1, PPO, polyphenol (e.g. catechin and epicatechin) and determination of the antioxidative capacity in a wide range of cultivars will be conducted (Kiewning & Schmitz-Eiberger, in preparation) to clarify which cultivars constantly express low amounts of the allergen. This knowledge would allow breeders and growers to select hypoallergenic cultivars and agricultural practices to provide fruit with decreased allergenic potential.

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