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The impact of catechin and epicatechin, total phenols and PPO activity on the Mal d 1 content in apple fruit

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ABSTRACT

The most important apple allergen in Central Europe and North America is Mal d 1. Apples are a very important source of secondary plant metabolites like polyphenols in human nutrition. It is known that oxidised phenols can bind proteins. These irreversible bindings can lead to a reduced allergenicity. The most important phenols in apple are epicatechin, catechin and their polymeric structures, which have been identified as substrates of the polyphenoloxidase (PPO). The aim of this study was to analyse the influence of naturally occurring catechin and epicatechin contents in apple on the allergenicity of apple fruits. Fruits of the cultivars 'Elstar', 'Diwa' and 'Boskoop' were harvested and stored for 8 and 12 weeks in a cold-chamber at 2 °C. Mal d 1-, catechin-, epicatechin- and total phenol content as well as the activity of PPO were determined. Correlation analysis showed that naturally occurring catechin as well as epicatechin has no impact on the Mal d 1 content of the tested cultivars: correlation coefficient ranged from −0.203 to 0.501 for the correlation between Mal d 1 and catechin. The results further indicated that the activity of PPO is more important than the content of total phenols to reduce the Mal d 1 level. If there is a high PPO activity, Mal d 1 could be reduced even if the total phenol concentration is low.

1. Introduction

Allergic reactions after eating an apple are not rare. Between 0.4–6.6% of adults and 2.2–11.5% of children aged from 0 to 6 years are reporting perceived fruit allergy. In the last group 8.5% perceived reactions against apple (Zuidmeer et al., 2008). Up to now four main classes of apple allergens are identified: Mal d 1, Mal d 2, Mal d 3 and Mal d 4 (Botton et al., 2008). In Central Europe and North America, the most important apple allergen is Mal d 1, a protein which belongs to the pathogenesis-related proteins (PR-proteins), subgroup 10 (Breiteneder & Ebner, 2000; Pühringer et al., 2000). It is a labile protein, which is sensitive to heat, pepsin digestion and oxidation (Asero, Marzban, Martinelli, Zaccarini, & Laimer da Câmara Machado, 2006; Hsieh, Moos, & Lin, 1995). Due to these properties the allergic reactions are normally mild oral symptoms such as itching in the oral cavity or swelling of the lips. These symptoms are called the oral allergy syndrome (OAS) (Mari, Ballmer-Weber, & Vieths, 2005). The allergic reaction against Mal d 1 is based on an immunologic cross-reactivity between the major birch-pollen allergen Bet v 1 and the major apple allergen Mal d 1 (Björkstén, Halmepuro, Hannuksela, & Lahti, 1980; Ebner et al., 1991). The amino acid sequences of Bet v 1 and Mal d 1 show high homologies (Schöning, Ziegler, Vieths, & Baltes, 1996; Vanek-Krebitz et al., 1995). Hence, both proteins share common IgE epitopes, which are the basis for an immunological reaction (Vanek-Krebitz et al., 1995). Due to this, up to 70% of birch-pollen-pollinosis patients develop food allergies, most frequently to apples (Eriksson, Formgren, & Svenonius, 1982; Geroldinger-Simic et al., 2011).

Apples are available throughout the whole year. Because of this they are a very important source of secondary plant metabolites like polyphenols in human nutrition. A high intake of secondary plant metabolites is reported to be beneficial to human health and to play a role in the prevention of cardiovascular diseases, cancer, stroke and osteoporosis (Boyer & Liu, 2004; Scalbert, Manach, Morand, Remesy, & Jimenez, 2005). Furthermore, polyphenols have additional effects. It is difficult to isolate Mal d 1 in the native state because of the phenolic compounds. It is known that oxidised phenols can bind proteins. These irreversible bindings can induce changes of the tertiary structure of the proteins. In the case of allergens, they can lose the epitopes, the binding site for antibodies (Chung, Maleki, & Champagne, 2004; Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). This in turn can be the reason for a reduced allergenicity. There are two possibilities for the reaction of phenols with proteins. On the one hand the phenols react reversibly with proteins by hydrogen binding and on the other the phenols react irreversibly by oxidation followed by covalent condensations (Loomis & Battaile, 1966). This irreversible

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oxidation is induced by the activity of the polyphenoloxidase (PPO). The oxidation of phenols results in products which are highly unstable and afterwards undergo many secondary reactions, for example with proteins (Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994). To avoid these reactions between phenols and proteins, PVPP, EDTA and DIECA (in the further text mentioned as phenol-inhibitors) are useful inhibitors for the protein–phenol reaction (Björkstén et al., 1980).

In a previous study Schmitz-Eiberger and Matthes (2011) pointed out the relationship between the Mal d 1 content and total phenols as well as PPO activity. The experiments of this study continued the former experiments of Schmitz-Eiberger and Matthes (2011).

The total phenol contents and pattern of phenol compounds varies significantly among the cultivars (Napolitano et al., 2004; Tsao, Yang, Young, & Zhu, 2003). The most important phenols in apple are epicatechin, catechin and their polymeric structures, as well as chlorogenic acid (Napolitano et al., 2004; Podsedek, Wilska-Jeszka, Anders, & Markowski, 2000). Epicatechin and catechin further have been identified as substrates of PPO (Rocha & Morais, 2001). There are a few studies which reported that addition of certain phenols can reduce the allergenicity of different fruits (García, Wichers, & Wichers, 2004; Gruber et al., 2004).

Therefore, the aim of this study was to investigate the influence of naturally occurring catechin and epicatechin contents in apple on the allergenicity of apple fruits. For this, extraction of proteins was performed on the one hand with the phenol inhibitors PVPP, DIECA and EDTA and on the other hand without these inhibitors. To identify the influence of catechin, epicatechin and total phenols by correlation analysis, the concentrations of catechin, epicatechin as well as the total phenols were determined. The activity of polyphenoloxidase was also measured. For this we chose three different apple cultivars which are known from our previous studies to have a high ('Elstar'), moderate ('Boskoop') and low ('Diwa') Mal d 1 content.

2. Material and methods

2.1. Material

Fruits of the three apple cultivars 'Boskoop', 'Diwa', and 'Elstar' were cultivated at the Research-Station Klein-Altendorf. Fruits were harvested at the optimal harvest date, according to the Streif-Index (DeLong, Prange, Harrison, Schofield, & DeEll, 1999). They were picked from definite position for excluding high variability between individual apple fruits. After harvest, fruits were stored for 8 and 12 weeks in a cold chamber at 2 °C.

2.2. Preparation of protein extracts

Extraction of the proteins was carried out according to the method of Björkstén et al. (1980). For the extracts of every cultivar a mixed sample of five fruits, including peel and pulp, were homogenised with potassium phosphate buffer (10 mM K_2HPO_4, 10 mM KH_2PO_4, pH 7), containing 10 mM sodium diethyldithiocarbameate trihydrate (DIECA), 2 mM ethylendiaminetetraacetic acid (EDTA) and 2% polyvinylpolypyrolidone (PVPP) in a relation of 1:1.5 w/v. To include the effect of polyphenols on Mal d 1 a second variant of extracts was produced in the same way, but excluded the polyphenol-inhibitors DIECA, EDTA and PVPP. Afterwards the homogenates were incubated on a shaker for 4 h at room temperature and were finally centrifuged at 4 °C for 15 min at 4000×*g*. The supernatants were collected and frozen at −80 °C. For each cultivar extracts were prepared 3-fold.

2.3. Quantification of Mal d 1

Quantification of Mal d 1 was done by a sandwich enzyme-linked immunosorbent assay (ELISA). Microtiter plates (F96 Maxisorp Nunc-Immuno-Plate, Nunc A/S, Roskilde, Denmark) were coated with 1:1000 sheep anti-mouse Ig AP 302 (Chemicon, Millipore) diluted in a phosphate-buffered saline buffer, pH 7 (PBS). After incubation for 1 h at room temperature, the plates were washed (TECAN Columbus Washer) four times with PBS-T (v/v 0.05% Tween 20 in PBS). This washing step was conducted after every incubation step. Afterwards a monoclonal antibody specific to Mal d 1 diluted in PBS-T (1:100) was incubated at 4 °C overnight. On the next day the plates were incubated with dilutions of the extracts (1:4–1:512 in PBS-T in dual steps) in triplicate and with dilutions of recombinant Mal d 1 (Biomay AG, Vienna, Austria) (2000–2.4 ng ml^{-1}) as a reference. After incubation for 1 h at room temperature the plates were coated with a polyclonal rabbit serum with specifity for Bet v 1 diluted in PBS-T (1:1000) for another hour at room temperature. For detection, the wells were incubated for 1 h at room temperature with goat anti-rabbit IgG (Sigma, A0545, Germany) labelled with a peroxidase (diluted 1:20 000 in PBS-T). Finally the plates were incubated for 15 min with the substrate 3,3′,5,5′-tetramethylbenzinidine (citrate buffer, pH 3.95). The reaction was stopped with sulfuric acid (25%). Photometric detection was performed at 450 nm in a microplate reader (Labsystems Multiskan®). The recombinant Mal d 1 was used as a standard. Total Mal d 1 content was calculated by a four-parametric calibration curve (SigmaPlot 11).

2.4. Extraction of catechin and epicatechin

Pressurized liquid extraction was carried out using an Accelerated Solvent Extractor (ASE 200, Dionex, Idstein, Germany). Briefly, 0.1 g of freeze-dried and powdered apple was mixed with Hydromatrix HM-N (Separtis, Grenzach-Wyhlen, Germany) and afterwards packed into 11 ml stainless steel extraction cells after insertion of two cellulose filters (Schleicher and Schüll, Dasseln, Germany). For extraction, water and acetone in a ratio of 1:1 (v:v) were used. Afterwards ASE-extracts were filled up to 60 ml with distilled water and used for solid-phase extraction (SPE). SPE was performed using an ASPEC Xli (Gilson, Middleton, USA). Chromabond® PA SPE cartridges (Macherey–Nagel, Düren, Germany) were conditioned with 3 ml dimethyl sulfoxide (DMSO) containing 1% formic acid and 0.3% trifluoroacetic acid and washed with 5 ml of water. ASE-extracts were loaded onto the cartridge in 3 steps. The cartridge was washed after every application of extract. The phenolic compounds were eluted with 1.25 ml DMSO containing 1% formic acid and 0.3% trifluoroacetic acid.

2.5. Identification of catechin and epicatechin by HPLC-UV-DAD

The HPLC-UV-DAD analysis were carried out using a Dionex summit system (Idstein, Germany) with a Phenomenex Aqua column (150 × 2 mm, 3 µm particle size; Phenomenex, Aschaffenburg, Germany) operated at 35 °C. The mobile phase consisted of 1% acetic acid in water (mobile phase A) and 1% acetic acid in acetonitrile (mobile phase B). A gradient elution program was used as follows: Starting at 0% B with a linear gradient to 40% B after 80 min, from 40% to 100% B after 81 min. The column was washed with 100% B for 10 min and reequlibrated for 10 min with the initial conditions. The flow rate was 0.3 ml min^{-1} and the injection volume for all samples was 5 µl. The chromatogram monitored at 200–595 nm with a wavelength of 280 nm for quantification.

2.6. Extraction of PPO

Extraction of PPO was carried out according to Rocha, Cano, Galeazzi, and Morais (1998). Samples of freeze-dried and powdered apples (peel and pulp) where homogenised in sodiumphopshate buffer (pH 6.5) containing Triton X100 (0.25%) and