Enzymatic characterization of apple (*Malus* × *Domestica*)

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Abstract. Secondary metabolites play a major role as quality factors in horticultural products by significantly contributing to plant health, optical attributes, sensory attributes and health beneficial effects for the consumer. Dihydrochalcones, a rare class of secondary metabolites, which is, however present in particularly high amounts in apple (*Malus × domestica*). In apple, phloridzin (phloretin 2′-O-glucoside) is prevalent representing more than 90% of the soluble phenolic compounds, but small amounts of 3-hydroxyphloretin and 3-hydroxyphloridzin are also constitutively present. For the systematic investigation, sufficiently comprehensive knowledge of the underlying pathway is yet lacking. Two types of enzymes could catalyze the reaction, polyphenol oxidases (PPOs) and/or cytochrome P450 dependent monooxygenases. To test a possible involvement of the F3'H of apple in the formation of constitutive 3-hydroxydihydrochalcones, cDNA clones of the two types of F3'Hs present in the apple was isolated and heterologously expressed in yeast.

Despite showing high F3'H enzyme activity with various flavonoid substrates, hydroxylation of dihydrochalcones was not observed at standard conditions, indicating that F3'H is not part of the dihydrochalcone pathway.

1. Introduction
Dihydrochalcones (DHCs) are a class of flavonoids. To date, approximately 256 DHCs have been described and are known to be formed in over 46 plant families [1]. The first known evidence of phloridzin production was in the apple trees. DHCs are of special interest because of their potentially valuable health effects, such as a wide range of biological and pharmacological activities. They are found in fruits, vegetables, tea, and wine. Apple is the most important source of DHCs [2], where it comprises up to 90% of the soluble phenolic compounds. Apple peel contains a greater variety of phenolic compounds compared to apple flesh [3]. Phloridzin and other related DHCs are present in a number of plant species, but high concentrations of phloridzin are only found in the genus *Malus*. Dihydrochalcones (DHCs) is defined as a major subclass of flavonoid. Flavonoid and biochemically related chalcones are important secondary metabolites [4]. Dihydrochalcones clearly show structural relation to chalcones, as both lack the heterocyclic C ring of flavonoids. They share, on the other hand, the saturated bond between C2 and C3, with flavanones and dihydroflavonols, which cannot be found in chalcone substrates. It was therefore also open whether common F3′H or a CH3H, which is, however, not present in apple, would be more specific for catalyzing hydroxylation in position 3. Whereas CH3H depends on specific motifs in the protein structure and was so far only described in Asteraceae species, F3′H can be found in many
plant species [5, 6]. In *Malus* sp., at least 3 genes encoding F3'Hs have been identified [7] and it could not be excluded that they are involved in the 3-hydroxyphloridzin biosynthesis besides their essential role in the flavonoid pathway.

To test a possible involvement of the F3'H of apple in the formation of constitutive 3-hydroxydihydrochalcones, cDNA clones of the two types of F3'Hs present in the apple was isolated and heterologously expressed in yeast. Two types of enzymes could be responsible for the introduction of an additional hydroxyl group in position 3 polyphenol oxidases PPOs and cytochrome P450 dependent enzymes.

2. Materials and method

Young leaves of *M. x domestica* cv. Rebella and of *M. x domestica* cv. Golden Delicious trees grafted on M9, planted in 2003, were harvested in spring 2014 in the experimental orchard of the University of Natural Resources and Life Sciences in Vienna frozen in liquid nitrogen and stored at-80 °C until use for several enzyme assays. mRNA was extracted with the µMACS mRNA isolation Kit (MiltenyiBiotec, Germany). cDNA was synthesized using the SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer. Based on specific sequence information of *F3'H* (NCBI No FJ919633 and FJ919631) [5], full size *F3'H* cDNA clones were isolated. Heterologous expression in yeast and enzyme assays were performed. The enzyme assay contained in a final volume of 100 µl:

- 0.036 nmol (14C)-naringenin (100 Bq)
- 40 µl enzyme preparation
- 5 µl NADPH (0.83 mg / 100 ml distilled water H2O)
- 55 µl 0.1 mol/l KH2PO4 + 0.4 % Sodium ascorbate, pH 7.5

Crude extract, buffer solution was transferred to the reaction tubes and the assay was started by the addition of 5 µl NADPH. With open lid reaction tubes, the enzyme assay was incubated for 30 min at 30°C. The assay was terminated by the addition of 10 µl of acetic acid and 70 µl ethyl acetate. The tubes were centrifuged at 13,000 x g and 25°C for 3 min. The organic phase was collected separately from the aqueous phase and the necessary product separation was achieved by thin-layer chromatography using cellulose TLC plates in the CAW solvent system (chloroform: acetic acid: water = 50: 45: 5). The plates were scanned on a TLC-linear analyzer. The product formed was quantified by calculating the conversion yield related to the substrate, and identified with the help of reference compounds.

3. Results and discussion

3.1. Enzyme activity as a function of pH (optimal pH)

Table 1 shows the enzyme activity of Flavanoid 3’ hydroxylase (F3’H) HII b with naringenin (Nar), Dihydrokaempferol (DHK) and Kaempferol (Km) as a substrate as a function of pH. The optimum for the reaction of F3’H, when catalyzed by the enzyme HII b and with naringenin as a substrate, was determined between pHs 6.00 and 7.00. Within these parameters, the optimum was determined to be pH 6.75. To determine the pH optimum with DHK and Km, pH values ranging from 6.0 to 7.0 were applied. The optimal pH with DHK was observed at pH 6.5, whereas with Km the highest yield was achieved at pH 6.75. Enzyme activity of F3’H HIIb with three different substrates was found to increase gradually with pH increases of 0.25, then decrease as pH rose above the optimum.
Table 1. Enzyme activity of F3’H HIIb with different pHs and substrates.

|       | Nar   |       | DHK   |       | Km    |       |
|-------|-------|-------|-------|-------|-------|-------|
| pH    | U (%) | pH    | U (%) | pH    | U (%) |
| 6     | 17.5  | 6     | 40    | 6     | 29    |
| 6.25  | 35    | 6.25  | 48    | 6.25  | 40.5  |
| 6.5   | 40.5  | 6.5   | 58    | 6.5   | 46    |
| 6.75  | 50.5  | 6.75  | 53    | 6.75  | 57.5  |
| 7     | 43    | 7     | 21    | 7     | 53    |

3.2. Enzyme activity as a function of temperature (optimal temperature)
This experiment was conducted to measure the optimal temperature of F3’H HII b with naringenin as a substrate. The reaction mixtures were incubated at different temperatures ranging from 0 to 60°C. The maximum activity was detected at 25°C, with a conversion rate of 60%. Naringenin formation gradually increases from 0 to 25°C and then decreased at higher temperatures. At 60°C however, 20 % of the optimum was still observed. Enzyme activity at different temperatures can be seen in table 2.

Table 2. Enzyme activity of F3’H HII b in different temperatures

| Temperature (°C) | U (%) |
|------------------|-------|
| 0                | 9     |
| 10               | 21    |
| 20               | 40    |
| 25               | 60    |
| 30               | 40    |
| 40               | 18    |
| 50               | 17    |
| 60               | 12    |

3.3. Enzyme activity as a function of time (time linearity)
This experiment was carried out to check the time linearity during the F3’H HII b reaction. The reaction mixtures were incubated between 0 to 60 minutes at the optimal pH 6.75 and the optimal temperature of 25°C. Under this condition, naringenin formation was linear up to 10 minutes. At higher incubation times naringenin formation was observed to level out. Enzyme activity of F3’H HII b at different incubation times can be seen in table 3.

Table 3. Enzyme activity of F3’H HII b at different time

| Time (Minute) | U (%) |
|---------------|-------|
| 0             | 8     |
| 10            | 78    |
| 20            | 92    |
| 30            | 93    |
| 40            | 94    |
| 50            | 92    |
| 60            | 92    |

3.4. Enzyme activity as a function of protein concentration (protein linearity)
Protein linearity was investigated to determine the optimal substrate concentration for maximum conversion rates with Nar, DHK and Km. With naringenin as a substrate, the reaction maintained linearity
with protein concentrations up to 3µg in 50µl. However, using DHK and Km as a substrate, protein concentration was linear up to 2 µg and 1.5 µg. The conversion rate with naringenin, dihydrokaempferol, and kaempferol were observed at 56 %, 24 %, and 50%. Enzyme activity of F3’H HII b with three different substrates in different amounts is available in table 4.

### Table 4. Protein linearity of F3’H HII b with different substrate

| Enzyme (µ) | Nar U (%) | DHK Enzyme (µ) | U (%) | Km Enzyme (µ) | U (%) |
|------------|-----------|----------------|-------|---------------|-------|
| 0          | 2         | 0              | 3     | 0             | 6     |
| 1          | 20        | 0.2            | 3     | 0.5           | 8     |
| 2          | 36        | 0.4            | 6     | 1             | 26    |
| 3          | 56        | 0.6            | 1     | 1.5           | 50    |
| 4          | 63        | 0.8            | 3     | 2             | 62    |
| 5          | 81        | 1              | 5     | 3             | 75    |
| 8          | 93        | 2              | 24    | 5             | 81    |

#### 3.5. Enzyme activity as a function of substrate concentration

Substrate concentration and conversion rates were determined, and the kinetic data can be seen in table 5. The apparent Michaelis constant (k_m), maximum reaction velocity (V_max) and the ratio (V_max/k_m) were determined for naringenin (figure 1), dihydrokaempferol (figure 2) and Kaempferol (figure 3) as substrates. The V_max/K_m of F3’H HII b with naringenin, dihydrokaempferol and kaempferol were 18 l/s*kg, 18 l/s*kg, and 32 l/s*kg, respectively.

### Table 5. Kinetic values of F3’H HIIb in different substrates concentration

| [S] nmol | 1/S | 1/V [kg Protein/kat] | [S] nmol | 1/S | 1/V[kg Protein/kat] | [S] nmol | 1/S | 1/V [kg Protein/kat] |
|----------|-----|----------------------|----------|-----|---------------------|----------|-----|---------------------|
| 0.024    | 4.17E+06 | 4.06E+05           | 0.024    | 4.17E+06 | 2.61E+05          | 0.012    | 8.33E+06 | 6.52E+05 |
| 0.036    | 2.78E+06 | 3.07E+05           | 0.036    | 2.78E+06 | 2.28E+05          | 0.024    | 4.17E+06 | 4.32E+05 |
| 0.048    | 2.08E+06 | 3.33E+05           | 0.048    | 2.08E+06 | 2.07E+05          | 0.036    | 2.78E+06 | 3.23E+05 |
| 0.06     | 1.67E+06 | 2.66E+05           | 0.06     | 1.67E+06 | 2.00E+05          | 0.048    | 2.08E+06 | 3.33E+05 |

**Figure 1.** Michaelis-Menten graph of F3’H HIIb with naringenin.
Hydroxylation in position 3 of dihydrochalcones shows high similarity with the introduction of a second hydroxyl group in the B-ring of flavonoids and chalcones, which are catalyzed by the cytochrome P450 dependent monooxygenases flavonoid 3’-hydroxylase (F3’H) and chalcone 3-hydroxylase (CH3H) [8]. In general, F3’Hs shows a broad substrate specificity, catalyzing the hydroxylation of flavanones, dihydroflavonols, and flavonols, as well as in some cases leucoanthocyanidins or flavones, as substrates [9]. Hydroxylation of the closely related chalcones, in contrast, seems to require a particular architecture of the active site and is, therefore, restricted to the closely related CH3Hs [6, 8]. Constitutively present 3-hydroxyphloridzin could increase the velocity of this defense reaction. In the case of the fire blight resistant crabapple cv. Evereste, however, a correlation between pathogen resistance and the constitutive presence of sieboldin, which also shows a 3,4-dihydroxylation pattern, was not observed [10].

Despite showing high F3’H activity with various flavonoid substrates, hydroxylation of dihydrochalcones was not observed, indicating that F3’H is not part of the dihydrochalcone pathway. The recombinant MdF3’HII was functionally active, and converted flavanone, dihydroflavonol and flavonol substrates, but not the flavone, chalcone or leucoanthocyanidin substrates. At standard conditions, *in vitro* conversion of phloretin into 3-hydroxyphloretin was not observed. The mutated MdF3’HI, which showed the same amino acid sequence as the previously published enzyme [7], confirmed that phloretin is not a substrate, or at least a much worse substrate, for the F3’H in *Malus* than the common flavonoid substrates.
The recent case of the gm-orange petunias has, however, clearly demonstrated that even if a compound is a bad substrate, that in vitro conversion at standard conditions is not observed, accumulation of its converted metabolites can be nonetheless observed in a suitable physiological background [11]. A final clarification will only be achieved by silencing of the Malus F3'Hs and testing the effect on the levels of constitutively present 3-hydroxyphloridzin.

4. Conclusion

Despite showing high F3'H activity with various flavonoid substrates, hydroxylation of dihydrochalcones was not observed, indicating that F3'H is not part of the dihydrochalcone pathway.

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