Biotransformation of quercetin, kaempferol and apigenin to monoglycosylated derivatives by in vitro suspension cultures of *Astragalus vesicarius* ssp. *carniolicus*

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Abstract

Biotransformation of exogenous substrates quercetin, kaempferol and apigenin by suspension cultures of *Astragalus vesicarius* ssp. *carniolicus* to their monoglycosylated derivatives was performed. The maximal enzymatic potential of cells of *A. vesicarius* ssp. *carniolicus* was evaluated by different concentrations of substrate exposure. According to quantitative ultra-high performance liquid chromatography-high resolution electrospray ionization mass spectrometry (UHPLC-HR-ESI-MS) analysis, the highest concentration of kaempferol O-glycoside (14.88 nmol/g dry weight, DW), apigenin O-glycoside (10.55 nmol/g DW) and quercetin O-glycoside (150.83 nmol/g DW) was achieved, when suspension cultures were treated with 4 mg/mL kaempferol, 4 mg/mL apigenin and 3 mg/mL quercetin, respectively. The glycosidic products of biotransformation were not detected in the untreated control.

Keywords

*Astragalus vesicarius* ssp. *carniolicus*, in vitro suspension cultures, biotransformation

Introduction

*Astragalus vesicarius* ssp. *carniolicus* is a perennial stemmed herb 30 cm high with violet petals. The plant is mainly distributed in Italy, Switzerland, Croatia and Slovenia (Zippel and Whilhalm 2009). Flavonoid production of *A. vesicarius* ssp. *carniolicus* is one of the targets in biotechnological process optimization (Krasteva et al. 2016). Quercetin is the main aglycone identified of in vitro cell suspension cultures from *Astragalus missouriensis* and *Astragalus angustifolius* in both free and bound forms (as glycosides). Flavonoid glycosides such as isoquercitrin, quercitrin, rutin, scopoletin, hyperoside, and phenolic acids – chlorogenic and p-coumaric acid have been detected. After optimization of production medium, flavonoid production for in vitro cultures of *A. angustifolius* (1.78%) and for *A. missouriensis* (1.34%) was achieved (Ionkova 2009a). Quercetin is one of the main flavonoids in our daily diet and may be used for prevention of cancer, cardiovascular diseases and neurodegenerative diseases (Shimoda et al. 2007). Many biological activities of kaempferol and its glycosides have been reported, such as anticancer, cardiovascular, antioxidant, antiinflammatory, antidiabetic, hepatoprotective and neuroprotective effects.
Materials and methods

Plant material and in vitro cultivation

*A. vesicarius ssp. carniolicus* seeds were obtained from University of Zurich Botanical Garden. The seeds were sterilized using standard procedure (Iionkova et al. 2010) and cultured in Petri dishes (150 mm × 25 mm) containing 20 mL of modified Murashige and Skoog (MS) medium (Murashige et al. 1962), supplemented with casein 1 g/L and sucrose 20 g/L (DoH). After 30 days of cultivation, the seedlings were transferred on MS medium supplemented with 1% agar agar under light regimen. Callus cultures were initiated when shoot explants were transferred on modified MS medium, supplemented with casein 1 g/L, kinetin 2 mg/L, 2,4-dichlorophenoxyacetic acid (2,4-D) 0.1 mg/L and indoleacetic acid (IAA) 0.2 mg/L (G48) (Iionkova et al. 2010) under light regimen of cultivation. Suspension cultures of *A. vesicarius ssp. carniolicus* were obtained after transferring actively growing calli to liquid G48 medium. Every two weeks the suspensions were transferred in fresh medium and growth rates were determined (Iionkova 2009b).

Biotransformation of quercetin, kaemferol and apigenin

Flavonoid substrates were dissolved in sterile H₂O and applied to the suspension cultures under aseptic conditions at the first day of cultivation at different concentrations as follows: quercetin (1, 2 and 3 mg/mL), kaemferol (2 and 4 mg/mL) and apigenin (2 and 4 mg/mL). Each concentration level consisted of three randomised samples and the results were the average of the three replicates. In addition, three controls were cultivated without addition of flavonoids.

Extraction, analytical procedure and products identification

The air-dried powdered plant material (200 mg) was extracted with 80% methanol under reflux for 30 min (3 × 50 mL). The extracts were concentrated under reduced pressure and residue was dissolved in 50 mL water and extracted with EtOAc (3 × 50 mL). EtOAc fractions were dried and concentrated under reduced pressure. For the purpose of the analysis, those samples were diluted in 10 mL volumetric flask with 80% methanol. Aliquot of each sample was analyzed by UHPLC-HR-ESI-MS, which was performed by Q-Exactive Plus Orbitrap mass spectrometer with a ESI ion source (Thermo Fisher Scientific, Bremen, Germany) with ultra-high resolution mode (70 000, at m/z 200). An UHPLC system (Dionex Ultimate 3000 RSRLC, Thermo Fisher Scientific, Bremen, Germany) was coupled to the mass spectrometer. The operating conditions of the ESI ionization device in the negative ionization mode were 3.5 kV voltage and 320 °C capillary temperature, 25 units of carrier gas flow and 5 units of dry gas flow. All other detector parameters were set in such a way as to obtain the most intense signal from [M-H]. UHPLC separations were performed on a Hypersil Gold C18 column (1.9 μm, 2.1 × 50 mm, Thermo Fisher, Scientific, USA) at 30 °C. A mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) at a flow rate of 0.3 mL/min was used. Gradient elution was performed as follows: 0 to 3 min 83% (A), 3 to 7 min 80% (A), 7 to 10 min 70% (A), 10 to 20 min 50% (A), 20 to 25 min 40% (A), 25 to 28 min 30% (A), 30 to 35 min 0% (A). Rutin dihydrate CRS (Sigma-Aldrich, Germany) used as an external standard. Five concentration levels ranging from 0.01 to 0.00001 mg/mL were injected individually in triplicate to the UHPLC-HR-ESI-MS system. Rutin was observed at tᵣᵣ = 4.88 min as a deprotonated molecular ion [M-H] with m/z 609.1454 (calc. 609.1456) and corresponding formula C₂₀H₁₇O₁₆. The flavonoid amount was determined based on the equation \( y = 2e^{1.1x} + 387440 \) and correlation coefficient \( r² = 0.9999 \).

Statistical processing

Each experiment was done in triplicate. Results were expressed as mean ± SD. MedCalc 12.3 (MedCalc Software 2012) was used. The one-way analysis of variance was performed to define the statistical significance of the amount found.

Results and discussion

After 14 days of cultivation the suspension cultures of *A. vesicarius ssp. carniolicus*, treated with 2 mg/mL quercetin, kaemferol and apigenin, reached the highest growth rates of 118.20 g/L, 170.00 g/L and 158.30 g/L. The higher concentration of the added flavonoids led to lower plant cell yield. Glycosylation of quercetin, kaemferol and apigenin was observed in all samples of suspension.
cultures, while respective O-glycosylated derivatives were not detected within the control group.

Quercetin O-glycoside was detected in the negative ion mode as a deprotonated ion [M-H]- with m/z 463.0896 at tR 5.10 min which corresponded to a molecular formula C21H19O10 (calc. 463.0871) (Suppl. material 1: Figure S1). By comparing its MS/MS spectra with a reported data (Zhou et al. 2011), the observed metabolite was tentatively identified as isoorquercitrin. The highest concentration of isoorquercitrin (150.83 nmol/g DW) was observed when suspension cultures of A. vescarius ssp. carniolicus were treated with 3 mg/mL quercetin. Isoorquercitrin was not detected in the untreated control (Table 1, Suppl. material 2: Figure S2).

Kaempferol O-glycoside was detected as a deprotonated molecular ion [M-H]- with m/z 447.0937 at tR 5.81 min which corresponded to a molecular formula C20H17O10 (calc. 447.0922), (Suppl. material 3: Figure S3). The structure of the observed metabolite was tentatively identified as kaempferol O-glycoside based on the comparison of its MS/MS spectra with fragmentation pathway reported before (Chen et al. 2015). The highest concentration of kaempferol O-glycoside (14.88 nmol/g DW) was observed when suspension cultures were treated with 4 mg/mL kaempferol. It was not detected in the control (Table 2, Suppl. material 4: Figure S4).

Apigenin O-glycoside was detected as a deprotonated molecular ion [M-H]- with m/z 431.0983 at tR 5.51 min which corresponded to a molecular formula C20H17O9 (calc. 431.0973) (Suppl. material: Figure S5). As described above the metabolite was tentatively identified as apigenin O-glycoside (Wang et al. 2018). The highest concentration of apigenin O-glycoside (10.55 nmol/g DW) was observed in suspension cultures of A. vescarius ssp. carniolicus treated with 4 mg/mL apigenin. Apigenin O-glycoside was not detected in the control (Table 3, Suppl. material 6: Figure S6).

### Table 1. Concentration of quercetin O-glycosides (nmol/g DW) in suspension cultures of A. vescarius ssp. carniolicus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Quercetin O-glycoside nmol/g DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>64.60 ± 0.08</td>
</tr>
<tr>
<td>C</td>
<td>107.66 ± 0.04</td>
</tr>
<tr>
<td>D</td>
<td>150.83 ± 0.02</td>
</tr>
</tbody>
</table>

### Table 2. Concentration of kaempferol O-glycoside (nmol/g DW) in suspension cultures of A. vescarius ssp. carniolicus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kaempferol O-glycoside nmol/g DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>14.5 ± 0.02</td>
</tr>
<tr>
<td>C</td>
<td>14.88 ± 0.05</td>
</tr>
</tbody>
</table>

### Table 3. Concentration of apigenin O-glycoside (nmol/g DW) in suspension cultures of A. vescarius ssp. carniolicus

<table>
<thead>
<tr>
<th>Sample</th>
<th>Apigenin O-glycoside nmol/g DW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>4.42 ± 0.04</td>
</tr>
<tr>
<td>C</td>
<td>10.55 ± 0.06</td>
</tr>
</tbody>
</table>

### Conclusion

This study demonstrates that suspension cultures of A. vescarius ssp. carniolicus are able to convert quercetin, kaempferol and apigenin to their 3-O-glycosidic forms. Higher concentration of externally added aglycone resulted in higher production of the corresponding glycoside. Thus, biotransformation by plant suspension cultures is considered as an important tool for glycoside production.

### Acknowledgements

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### References


Supplementary material 1

Figure S1

Authors: Pavlinka Popova, Yancho Zarev, Iliana Ionkova
Data type: image
Explanation note: UHPLC-HRESIMS spectrum of quercetin O-glycoside.
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Link: https://doi.org/10.3897/PlantSociology.68.64289.suppl1

Supplementary material 2

Figure S2

Authors: Pavlinka Popova, Yancho Zarev, Iliana Ionkova
Data type: image
Explanation note: Concentration of quercetin O-glycoside (nmol/g DW) in suspension cultures of A. vesicarius ssp. carniolicus; A – plant cells grown on G48 medium, non-treated with quercetin (control); B – plant cells grown on G48 medium, supplemented with 1 mg/mL quercetin; C – G48 medium, supplemented with 2 mg/mL quercetin; D – G48 medium, supplemented with 3 mg/mL quercetin.
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Supplementary material 3

Figure S3

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Data type: image
Explanation note: UHPLC-HRESIMS spectrum of kaempferol O-glycoside.
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Supplementary material 4

Figure S4

Authors: Pavlinka Popova, Yancho Zarev, Iliana Ionkova
Data type: image
Explanation note: Concentration of kaempferol O-glycoside (nmol/g DW) in suspension cultures of A. vesicarius ssp. carniolicus; A – plant cells grown on G48 medium, non-treated with kaempferol (control); B – plant cells grown on G48 medium, supplemented with 2 mg/mL kaempferol; C – G48 medium, supplemented with 4 mg/mL kaempferol.
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Supplementary material 5

Figure S5
Authors: Pavlinka Popova, Yancho Zarev, Iliana Ionkova
Data type: image
Explanation note: UHPLC-HRESIMS spectrum of apigenin O-glycoside.
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Supplementary material 6

Figure S6
Authors: Pavlinka Popova, Yancho Zarev, Iliana Ionkova
Data type: image
Explanation note: Concentration of apigenin O-glycoside (nmol/g DW) in suspension cultures of A. vesicarius ssp. carniolicus; A – plant cells grown on G48 medium, non-treated with apigenin (control); B – plant cells grown on G48 medium, supplemented with 2 mg/mL apigenin; C – G48 medium, supplemented with 4 mg/mL apigenin.
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