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# BIOCONVERSION OF APIGENIN-7-O-β-GLUCOSIDE IN AQUEOUS TWO-PHASE SYSTEM

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*The study is concerned with the conversion of apigenin-7-O-glucoside into apigenin in polyethylene glycol 6000 / dextran 20000 aqueous two-phase system by β-glucosidase. Apigenin was separated from apigenin-7-O-glucoside and β-glucosidase by their partition into opposite phases. In 14% PEG / 22.5% DEX aqueous two-phase system obtained yield of apigenin in top phase was 108%.*

KEYWORDS: Apigenin; apigenin-7-O-glucoside; camomile; extractive bioconversion; aqueous two-phase system

## INTRODUCTION

Apigenin, 5,7,4'-trihydroxyflavone, belongs to flavones, one of the major subclasses of plant flavonoids found in camomile. Apigenin exhibits a distinct anxiolytic activity without evidencing sedation or muscle relaxant effects at doses similar to those used for classical benzodiazepines without the anticonvulsant action (1). It also shows inhibitory effect on TPA-mediated tumour promotion and antimutagenicity (2,3). Moreover, preliminary studies also showed the topical use of apigenin reduced UV-induced skin tumorogenesis (3).

Like the most flavones, apigenin is usually found in plants bound to sugars as glycosides or in ester form with tannin acids. The pharmacological activity of apigenin-glucoside form is much lower than that of aglycone – apigenin. The conversion of apigenin-7-O-glucoside was possible by autofermentation (4) and by the action of β-glucosidase (4-7). When bioconversion is conducted, reuse of biocatalyst, either the whole cell or the

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enzyme, is always beneficial. Aqueous two-phase system (ATPS) opens the possibility for performing the extractive bioconversions, in which integration of bioconversion and product removal is achieved with the posibility of repeated use of the biocatalyst. Such systems have proven to be a convenient medium for bioconversion of pharmacologically active compounds (8, 9).

The aim of this study was to investigate the possibility of bioconversion of apigenin-glucosides by β-glucosidase in the aqueous two-phase system composed of polyethylene glycol and dextran. Conditions concerning phase system composition, where highest possible separation of apigenin and apigenin-glucoside into opposite phases is achieved, were previously established in model systems.

## EXPERIMENTAL

### *Plant material*

Camomile ligulate flower: tetraploid camomile produced by the Institute for Hops, Broomcorn and Medicinal Herbs, Bački Petrovac (1997).

### *Chemicals*

Standard samples of apigenin used in the present work were purchased from Sigma-Aldrich Chemie GmbH. Dextran 20 000 ($\overline{M}_w$ 20000) (DEX) from Pharmacia (Uppsala, Sweden) and polyethylene glycol 6000 ($\overline{M}_w$ 6000) (PEG) from Merck (Germany) were used. β-Glucosidase (~6 U/mg) from Fluka AG (Switzerland) was applied. All other chemicals were of analytical reagent grade.

### *Sample preparation*

Autofermentation of Camomile ligulate flowers: Camomile ligulate flowers (10 g) were moistened with 0.1 mol/dm$^3$ Na-acetate buffer pH 5.5 (50 cm$^3$) and autofermented at 37°C for 72 hours. After that, the sample was dried at 40°C and extracted by 70% ethanol (5 g of sample and 250 cm$^3$ of solvent) using ultrasound (40 min). After filtration, the dry extract was obtained by solvent evaporation under vacuum. The dry extract was dissolved in 2 cm$^3$ 70% ethanol (Extract A).

Camomile ligulate flowers extract: Camomile ligulate flowers (2 g) were extracted by 70% ethanol (100 cm$^3$) using ultrasound (40 min). After filtration, the dry extract was obtained by solvent evaporation under vacuum. The dry extract was dissolved in 2 cm$^3$ 70% ethanol (Extract B).

### *Aqueous two-phase system preparation*

Phase systems for model system experiments were prepared by weighting PEG and DEX masses and dissolving in acetate buffer (0.1 mol/dm$^3$ Na-acetate buffer pH 5.5), and then extract A (0.1cm$^3$) was added and mixed thoroughly (10). The total mass of the two-phase system was 2 g. Model ATPS was left to equilibrated at room temperature for 12 hours, then the upper phase was carefully collected by syringe.

*Extractive bioconversion*

The enzyme dissolved in acetate buffer (3 mg β-glucosidase in 100 g 0.1 mol/dm$^3$ Na-acetate buffer pH 5.5) or phosphate buffer (3 mg β-glucosidase in 100 g 0.1 mol/dm$^3$ phosphate buffer pH 5.5) was added to the model ATP systems. The bioconversion was performed at 55°C for 24 hours with constant mixing**.** The two phases were allowed to separate before sampling, then the upper phase was carefully collected using syringe.

*Phase sample preparation for TLC and HPLC*

Samples of the top phases (0.4 cm$^3$) were added to activated Amberlite and adjusted to a final volume of 1 cm$^3$ with acetate buffer, then vortexed for 15 minutes and centrifuged (3000 rpm) for 10 minutes. After that samples were extracted from Amberlite with 70% ethanol (2 cm$^3$) and analyzed by TLC and HPLC methods.

*Qualitative and quantitative analyses*

TLC: Glass plates (20 x 20 cm), coated with silica gel $GF_{254}$ (Fluka AG, Switzerland) thickness 0.5 mm, were used. Previously prepared samples extracted from Amberlite with 70% ethanol were then applied: 5 μL of each sample solution was spotted on the plate, and, simultaneously, 15 μL of each solution of dry extract of Camomile ligulate flowers and dry extract of autofermented Camomile ligulate flowers in 70% ethanol (Extracts A and B) were spotted. Development was at room temperature (approximately 20°C) in a glass chamber. The mobile phase was benzol : ethylmethylketon : methanol (5.5 : 3 : 1.5; v/v/v). The elution time was about 60 min. Detection was under UV light (254 nm). After that, by spraying the plate with 96% sulphuric acid and heating, yellow spots of separated flavonoids appeared in daylight.

HPLC: Agilent Technologies 1100 HPLC system equipped with a binary pump, UV-Diode Array detector, autosampler, Chem station software was used. Chromatographic conditions were: temperature 25°C termostated, column Zorbax, SB-C18 (4.6 x 150 mm, 5 μm), guard column Zorbax, SB-C18 (4.6 x 12.5 mm, 5 μm). Elution profile was isocratic: acetonitrile/$H_2O$ (3/7, v/v) with addition of acetic acid (1% of total volume). Injection volume was 10 μl, flow rate 1.0 cm$^3$/min, and stop time 40 min. Detection and quantification were conducted at UV 340 nm. Peak purity and identity were checked by comparison of the UV spectra (DAD-detector) and co-chromatography (TLC, HPLC) with reference substance (apigenin and apigenin-7-O-β-glucoside) and literature data (6). Quantification of apigenin and apigenin glycoside was performed by HPLC method using a single point calibration with apigenin as an external standard (6).

*Partition parameters*

The partition coefficient of apigenin in the aqueous two-phase systems was defined as

$$K = \frac{C_T}{C_B} \qquad [1]$$

where $C_T$ and $C_B$ are the concentrations of apigenin in top and bottom phase, respectively.

The yield in top phase was defined as

$$Y_T\ (\%) = \frac{C_T V_T}{C_o V_o} 100 \qquad [2]$$

where:

$C_T$ and $C_o$ are concentration of apigenin in the top phase and in the extract loaded to ATPS, respectively,

$V_T$ and $V_o$ are volumes of the top phase and the extract loaded to ATPS, respectively.

## RESULTS AND DISCUSSION

Preliminary investigations in model aqueous two-phase system were conducted to find out if apigenin and apigenin-7-O-β-glucoside show affinity to the top and the bottom phase, respectively, i.e. if there is a possibility of their separation in ATPS. For that, partition experiments were performed in the systems with dextran 20 000 (DEX) and polyethylene glycol 6000 (PEG), whose concentrations varied in the range from 14 to 48% DEX and from 4 to 22.5% PEG (compositions are given in Table 1), and qualitative analysis of the top phases by TLC was carried out.

The observation of flavonoids' spots under UV light and after staining (data not shown) revealed the presence of apigenin and absence of apigenin-7-O-β-glucoside in the top phase, showing that their separation in DEX / PEG ATPS is possible. This opened next step of our investigation - selection of a system giving the highest possible partition parameters for apigenin. In order to find out appropriate conditions, partition studies were conducted in ATPS with different polymer concentrations. The concentration of apigenin in the top phase was determined by HPLC. In order to determine partition coefficient, concentration of apigenin in the bottom phase was calculated from mass balance.

**Table 1.** Effect of ATPS composition on the partition coefficient and the top phase yield of apigenin

| ATPS | PEG (%, w/w)/ DEX (%, w/w) | $V_T/V_B$ | K | $Y_T$ (%) |
|---|---|---|---|---|
| A | 4.0/ 14.5 | 0.67 | 0.161 | 10.00 |
| B | 7.5/ 15.5 | 1.67 | 0.027 | 4.31 |
| C | 14.5/ 32.5 | 1.50 | 0.154 | 18.76 |
| D | 22.5/ 14.0 | 3.75 | 0.060 | 18.37 |
| E | 8.0/ 48.0 | 0.64 | 2.940 | 65.00 |
| F | 11.5/ 40.0 | 0.87 | 0.390 | 25.35 |

The highest obtained partition coefficient for apigenin (2.94) was achieved in the system E, whose composition was 8.0% PEG and 48.0% DEX, followed by the highest top phase yield 65% (Table 1). Although content of apigenin in top phase in systems E and F was much higher then in other systems, we decided not to use them for further experiments because of their high density due to high polymer concentration, which can cause

technical problems (separation, mixing). Using the systems C and D resulted in almost equal yield of apigenin in top phase, but our choice was system D, because it contains higher percentage of PEG, which is used as an auxiliary ingredient in pharmaceutical industry.

The next step was the investigation of 14.0% PEG/ 22.5% DEX ATPS for extractive biotransformation of apigenin-7-O-β-glucoside extracted from Camomile ligulate flowers into apigenin by commercial β-glucosidase. In order to increase values for partition coefficient, enzymatic hydrolysis was also performed in phosphate buffer. Yield of apigenin in the top phase was calculated considering amounts of both apigenin and apigenin-glucoside present in the extract, determined by HPLC.

The bioconversion of Camomile ligulate flowers extract by β-glucosidase under these conditions was successful and it resulted in the top phase apigenin yield of 108% (Table 2). At the first sight surprising yield value over 100% can be explained by the hydrolysis of not only apigenin-7-O-β-glucoside, but also of other apigenin glucosides - apigenin-7-O-β-monoacetyl-glucoside and apigenin-7-O-β-diacetylglucoside present in camomile (7). Results showed that phase volume ratio increased but content of apigenin in the top phase decreased when phosphate buffer was used, which resulted in lower yield of apigenin in the top phase. In both systems, β-glucosidase was present only in the bottom phase, which opens the possibility of its recirculation.

**Table 2**. Concentration and top phase yield of apigenin after extractive bioconversion by β-glycosidase in ATPS

| **Enzymatic Conversion** | **DEX/PEG (%, w/w)** | **$V_T/V_B$** | **$C_T$ (mg/ml)** | **$Y_T$ (%)** |
|---|---|---|---|---|
| Acetate buffer | 14.0 / 22.5 | 2.91 | 0.28 | 108 |
| Phosphate buffer | 14.0 / 22.5 | 3.03 | 0.17 | 65 |

## CONCLUSION

PEG / DEX ATPS is a convenient medium for the extractive bioconversion of apigenin-7-O-β-glucoside contained in camomile flower extract into apigenin. After this process, apigenin is separated in the top phase, and, on the other hand, apigenin-7-O-β-glucoside, as well as the used enzyme β-glucosidase, in bottom phase. This experiment opens a lot of possibilities such as production of apigenin from camomile flower extract using ATPS, recirculation of the biocatalyst and obtaining the product containing apigenin and PEG, which could be used in pharmaceutical industry for production of tablets and creams.

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## БИОКОНВЕРЗИЈА АПИГЕНИН-7-О-β-ГЛУКОЗИДА У ВОДЕНОМ ДВОФАЗНОМ СИСТЕМУ

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Испитивана је конверзија апигенин-7-О-β-глукозида у апигенин помоћу β-глукозидазе у воденом двофазном систему полиетилен гликол 6000 / декстран 20000. Апигенин је био раздвојен од апигенин-7-О-β-глукозида и β-глукозидазе расподелом у различите фазе система. У воденом двофазном систему 14% PEG / 22,5% DEX постигнут принос апигенина у горњој фази је био 108%.

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