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Purification of rosmarinic acid extracts from *Lavandula vera* MM cell biomass

Milen Georgiev [a], Elena Kovacheva [b,*], Nadejda Marcheva [b], Mladenka Ilieva [a]

[a] *Department of Microbial Biosynthesis and Biotechnologies-Laboratory in Plovdiv, Institute of Microbiology, Bulgarian Academy of Sciences, 26 "Maritza" Blvd., 4002 Plovdiv, Bulgaria*

[b] *Department of Analytical Chemistry, University of Food Technologies, 26 "Maritza" Blvd., 4002 Plovdiv, Bulgaria*

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Abstract

Two approaches for purification of rosmarinic acid extracts, obtained from *Lavandula vera* MM cell biomass were investigated: adsorption on different Amberlite XAD resins and extraction with ethylacetate. Results showed that when ethylacetate extraction was used, 18.7 times higher level of purity compared to initial material was achieved. Similarly the obtained preparations from adsorption on Amberlite XAD-7 resin and elution with 80% ethanol contained 29.8% rosmarinic acid, which is 7.7 times higher purity compared to initial material. The precipitation of rosmarinic acid as Ca-rosmarinate was investigated as well. The obtained rosmarinic acid preparations were with low yield (34% of rosmarinic acid fell into sediment).

Keywords: Cell biomass; *Lavandula vera* MM; Purification; Rosmarinic acid

1. Introduction

Rosmarinic acid (RA) possesses various biological activities, such as antibacterial, antiviral, anti-inflammatory and it is also an antioxidant that shows a very low toxicity (Parnham & Kesselring, 1985). The process for obtaining RA from plant cell cultures has been developed successfully and transferred in bioreactors with different volumes and the achieved yields are higher, compared to those reported for intact plants (Georgiev, Pavlov, & Ilieva, 2004; Petersen, 1999; Su, Lei, & Kao, 1995; Ulbrich, Wiesner, & Arens, 1985; Zhong, Chen, & Chen, 2001). However, its isolation from cell biomass and further purification are important steps for the creation of economically efficient biotechnological process for production of RA.

In our previous work (Georgiev, Kovacheva, Pavlov, & Ilieva, 2003) the optimal extraction conditions for RA from cell biomass of the *Lavandula vera* MM cell suspension were established. The aim of the present work was to study further purification of these extracts for obtaining RA preparations with different purity.

2. Materials and methods

2.1. Obtaining the crude extract of rosmarinic acid from biomass of L. vera MM

As an initial material for purification crude extract of rosmarinic acid was used. It was obtained according to Georgiev et al. (2004) as follows: after the cultivation process was completed the wet biomass was extracted with 50% (v/v) ethanol (for 1 h) at 70 °C. The extract

* Corresponding author. Tel.: +359 32 603 683; fax: +359 32 644 102.

E-mail addresses: lbpmbas@plov.omega.bg, elkovap@yahoo.com (E. Kovacheva).

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was evaporated to dryness, the dry residue was dissolved in 70% (v/v) ethanol and stored for 24 h at −10 °C. The precipitate was filtered off and the filtrate was used for further experiments. Its volume was reduced by evaporation (rotary evaporator Laborota 4002, Heidolph) and then it was lyophilized (lyophilizer Alpha 1-2, Christ). The lyophilized extract contained 38.80 mg RA per g dry weight.

2.2. Chemicals

Rosmarinic acid standard was purchased from Extrasynthese (Genay, France). Amberlite XAD-2, XAD-4, XAD-7 resins and ethylacetate were supplied by Sigma–Aldrich (St. Louis, MO, USA). All other chemicals used in this work were of analytical grade.

2.3. Rosmarinic acid determination

RA was determined spectrophotometrically according to Georgiev et al. (2004) at 327 nm using spectrophotometer Shimadzu UV–Vis 1240. The presence of RA in the different fractions was checked by TLC-chromatography on silicagel G_{254} using the following mobile system: chloroform:methanol:acetic acid:water – 59:29:8.4:3.6. The bands were inspected under UV light at 356 nm.

2.4. Purification approaches

2.4.1. Adsorption on different Amberlite XAD resins

Samples of 0.25 g from the crude lyophilized extract were dissolved in water (20 ml, pH 3) and the solution were poured on columns with Amberlite XAD-2, XAD-4 and XAD-7 (diameter 12 mm, height 9–10 mm, weight of resin 10 g). The columns were washed with water (15 ml, pH 3) and the elution was performed in two variants using aqueous ethanol solutions (40% and 80%). RA in each fraction (5 ml) was determined.

2.4.2. Extraction with ethylacetate

Samples of 0.5 g lyophilized extract of RA were dissolved in water (10 ml, pH 3) and 0.5 g NaCl was added. Extraction was performed with ethylacetate (3×20 ml) and combined ethylacetate extracts (after the water was removed by anhydrous Na_2SO_4) were evaporated to dryness. The dry residues were dissolved in ethanol and determination of RA was performed.

2.5. Precipitation with calcium hydroxide

Samples of 0.5 g lyophilized crude extract of RA were dissolved in 10 ml water. Calcium hydroxide (saturated solution) was added until reaching a level of pH 10. The obtained precipitate, containing Ca-rosmarinate was centrifuged at 5000*g* for 20 min, and the supernatant was used for determination of the residual RA. The second precipitation of RA in supernatant was performed under the above described conditions with new amounts of calcium hydroxide. The procedure was repeated once more.

2.6. Statistical analyses

All experiments were carried out in triplicate, the reported results are the averages of at least three measurements, and the coefficients of variations, expressed as the percentage ratio between standard deviations (SD) and the mean values, were found to be <10 in all cases.

3. Results and discussion

3.1. Purification approaches

The downstream processes for isolation and further purification of the metabolites from plant cell cultures had sufficient impact on the common economical effectiveness of the plant cell biotechnologies. In this research two different approaches for purification of crude extracts of rosmarinic acid from cell biomass of *L. vera* MM and obtaining of enriched RA preparations were investigated: by adsorption on Amberlite XAD resins and by extraction with ethylacetate. Such preparations could be used in the food and cosmetic industries.

3.1.1. Adsorption on different Amberlite XAD resins

Water solutions with concentrations 0.5–1.0 mg RA per ml were used for the experiments, which correspond to its concentration in the crude extract from cell biomass. Purification by adsorption on non-ionic polymeric Amberlite XAD resins (XAD-2, XAD-4 and XAD-7) was investigated. It is known that XAD resins have greater adsorption capacity and are easier to elute than other adsorbents as alumina, silica gel and etc. (Aiken, Thurman, Malcolm, & Walton, 1979). The investigated Amberlite XAD-2 and XAD-4 are styrene divinylbenzene copolymers with hydrophobic nature, while Amberlite XAD-7 is an acrylic ester polymer of intermediate polarity with hydrophilic nature. It was established that all resins under study were good adsorbents of RA, but the elution profiles were different (Fig. 1). Ulbrich et al. (1985) established that elution of RA from Amberlite XAD-2 could be performed with good yield using 40% methanol. Since the aim of our investigation was to obtain enriched preparation of RA for food industry aqueous ethanol solutions were used instead of methanol. The obtained results showed that 40% ethanol was not appropriate as eluent, concerning all three resins under study, because the full elution of RA from Amberlite XAD-2 and XAD-4 was completed after 16–20 fractions

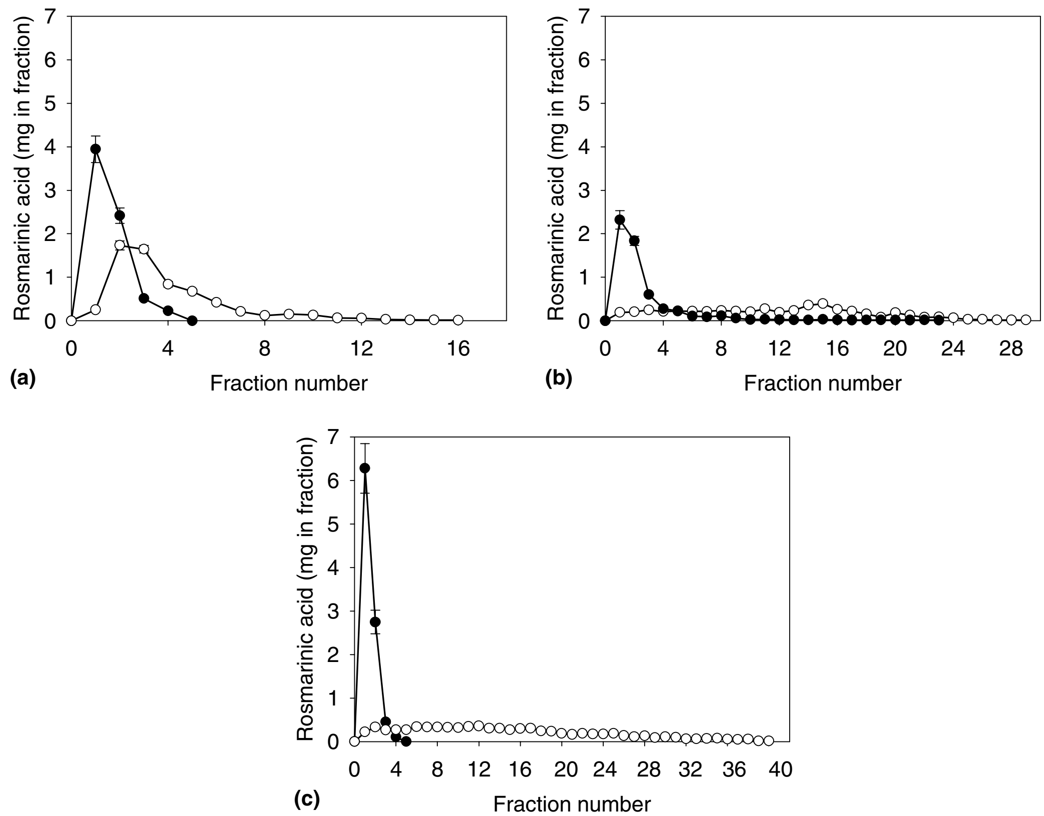

Fig. 1. Elution profile of rosmarinic acid on Amberlite resins eluted with 40% (○) and 80% (•) aqueous ethanol solutions: (a) XAD-2; (b) XAD-4; (c) XAD-7. Each fraction is 5 ml. Bars represent standard deviation.

(Fig. 1(a) and (b)). Its amounts in them were low, which corresponded to the low yield of eluted RA and required large amount of ethanol. No sufficient elution of RA with 40% ethanol from Amberlite XAD-7 was established (Fig. 1(c)). When the ethanol concentration in eluent was increased up to 80% better elution of RA for all resins under study was observed (Fig. 1) and the main part of adsorbed amounts of RA flowed out with the first five fractions. The best results were obtained when Amberlite XAD-7 was used and elution was performed with 80% ethanol. In this case about 98.8% of adsorbed RA was recovered in the combined eluents and this yield is 28.9% and 38.2% higher than those obtained with Amberlite XAD-2 and XAD-4, respectively (Table 1). The different elution profiles can be connected with differences of chemical nature of Amberlite XAD-2, 4 and 7 resins and especially with their physical properties (specific surface, specific pore volume and solvent uptake) (Aiken et al., 1979; Parrish, 1977). The RA preparations, obtained by adsorption on Amberlite XAD-7 and elution with 80% ethanol (Table 1) contained 29.8% RA, which is 7.68 times higher purity compared to initial extract.

Table 1
Purification of rosmarinic acid extracts from *L. vera* MM cell biomass

	Yield (%)	RA amount (%) in preparation
Crude extract[a]	100	3.88
Adsorption on Ambelite XAD		
XAD-2 elute[b]	73.20 ± 4.80	27.94 ± 1.59
XAD-4 elute[b]	61.04 ± 3.59	18.93 ± 1.48
XAD-7 elute[b]	98.81 ± 0.34	29.80 ± 1.59
Extraction with ethylacetate	74.87 ± 5.16	72.56 ± 2.03

[a] Crude extract contained 38.80 mg rosmarinic acid per g dry weight.

[b] Elution was performed with 80% (v/v) aqueous ethanol solution.

3.1.2. Extraction with ethylacetate

It is known that phenolic acids, respectively, RA can be extracted with the esters of aliphatic carboxylic acid such as ethylacetate (Kovatcheva, Pavlov, Koleva, Ilieva, & Mihneva, 1996; Psotova et al., 2003). Based on our preliminary experiments the appropriate ratio 1:2 (aqueous solution of RA, saturated with NaCl:ethylacetate) was chosen and extraction was performed three times. Results showed that RA amount in the ethylacetate extract was 72.56% (Table 1), which is 18.7 times higher than in crude extract. Achieved RA content in preparation was 2.43 times higher in comparison with that obtained using Amberlite XAD-7 resin and 80% ethanol as eluent.

Table 2
Separation of rosmarinic acid with calcium hydroxide

Step	RA (mg in solution)	Precipitated RA (mg)	Precipitation (%)
Crude extract	19.40	0	0
First precipitation	17.49 ± 0.18	1.91 ± 0.18	9.85
Second precipitation	13.38 ± 0.29	4.11 ± 0.29	31.05
Third precipitation	12.82 ± 0.05	0.56 ± 0.05	33.90

3.2. Precipitation of rosmarinic acid with calcium hydroxide

In some early investigations (Ellis & Towers, 1970; Razzaque & Ellis, 1977; Scarpati & Oriente, 1958), concerning isolation of RA, it was precipitated as a lead salt. This procedure is not appropriate taking into consideration the toxicity of lead. Recently, Tanaka, Kiyomatsu, Nonaka, and Ishimaru (2001) used calcium hydroxide for precipitation of RA. The similar scheme was used in this investigation and the precipitation was performed in three steps (Table 2). According to the final result it was found that the precipitation of RA with $Ca(OH)_2$ was not an appropriate method for obtaining enriched RA preparation, because only 34% of RA was precipitated as Ca-rosmarinate. The reason for this unsatisfactory yield is low specificity of $Ca(OH)_2$, which precipitated not only RA from crude extract.

4. Conclusions

This study represents the results from different approaches for obtaining enriched preparations of RA. By means of ethylacetate extraction the yield of 74.87% was achieved and the obtained preparations contained 72.56% RA. By adsorption on Amberlite XAD resins the best results, concerning yield and RA content in preparations (98.8% and 29.8%, respectively), were achieved, when Amberlite XAD-7 was used and subsequent elution was performed with 80% ethanol. Likewise, calcium hydroxide offers another possibility for obtaining RA-containing preparations, though the yield was characterized as low.

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