Original scientific article- Originalni naučni rad

EFFECT OF DIFFERENT REACTION PARAMETERS ON LIPASE-CATALYZED ESTERIFICATION OF NARINGIN AND ESCULIN

Ana Milivojević[1], Marija Ćorović[2,*], Milica Carević[2], Katarina Banjanac[1], Dejan Bezbradica[2]

[1]Innovation Center, Faculty of Technology and Metallurgy, University of Belgrade, 11000 Belgrade, Serbia
[2]Department of Biochemical Engineering and Biotechnology, Faculty of Technology and Metallurgy, University of Belgrade, 11000 Belgrade, Serbia
e-mail: mstojanovic@tmf.bg.ac.rs

Abstract

Fatty acid flavonoid esters are antioxidants with high potential for food, cosmetic and pharmaceutic industry. However, their application is still not wide-spread due to low efficiency of traditional chemical esterifications, as well as the necessity for comprehensive optimization of parameters for enzymatic synthesis to increase the economic viability of process. Hereby, esterification of two important flavonoid glycosides, naringin and esculin, with oleic acid as acyl donor using Novozym® 435 was optimized in order to broaden their commercial application. The effect of solvents, flavonoid concentrations, as well as substrate molar ratio on molar conversion and product yield, were investigated. The highest molar conversions of both flavonoids were reached in acetonitrile. Also, optimal flavonoid concentrations for synthesis of naringin and esculin esters were 30 mM and 70 mM, respectively achieving concentrations of 21.2 mg/ml naringin oleate and 27.5 mg/ml esculin oleate after 72h. Further investigation has shown that substrate molar ratio significantly influences esterification of both flavonoids and it was concluded that fivefold excess of oleic acid was optimal for both reactions. Maximum conversions of 70 and 86% were achieved with esculin and naringin, respectively, under optimized reaction conditions, making obtained results promising for further investigations, including product purification and process scale-up.

Keywords: esterification, flavonoids, lipase, antioxidants.

INTRODUCTION

Flavonoids, natural polyhydroxylated phenolic compounds may be found in the nature as active components of fruits, vegetables and other plants. They can exist as aglycons but more often they form glycosides. Many works attributed their beneficial health effects to their ability to act as antioxidants, so they have high potential for food, cosmetic and pharmaceutic industry (Chebil et al., 2006; Viskupicova et al., 2012). Besides antioxidant activity, many studies have reported that flavonoids possess a wide spectrum of other biological activities, including antiallergenic, antiviral, antiinflammatory and vasodilatory actions (Danihelová et al., 2012). However, low stability and solubility of flavonoids in liphophilic environments limit their commercial applications. In order to improve their functional properties, natural flavonoids have been subjected to many structural modification, so different moieties such as aromatic acids, fatty acids and their vinyl esters, could be introduced in parent molecules (Tapas et al., 2008). Enzymatic esterification of flavonoids using lipase as biocatalyst seems to be the promising way to enhance stability and solubility of these biomolecules in lipophilic environments, since enzymatic approach is more regioselective than

chemical acylation and can be conducted under milder reaction conditions as well (Bezbradica et al., 2017; Mellou et al., 2005). Acylation of flavonoids using different aliphatic or aromatic acids shows promising industrial applications. Special attention has been paid to the production of flavonoid fatty acid esters, since fatty acids can improve physiological properties of flavonoids. Moreover the best molar conversions and initial reaction rates were achieved when fatty acids were used as acyl donors for esterification of flavonoids (Ardhaoui et al., 2004; Salem et al., 2010). In the present study we performed the one-step regioselective acylation of esculin and naringin with free monounsaturated fatty acids (oleic acid) by using the immobilized lipase B from *C. antarctica* (Novozym® 435). These two flavonoids were chosen since it had been confirmed that they possess good antioxidant and anti-inflamatory activities (El-Desoky et al., 2018; Niu et al., 2015; Pizzorno et al., 2016), rendering them as good constituents of food and cosmetics. Nevertheless, such a choice of flavonoids (different aglycon structure as well as carbohydrate moieties) for reaction of esterification, gives an insight in effect of flavonoid structure on reaction performances. Furthermore, the effect of the nature of the solvent, concentration of flavonoids and substrate molar ratio on reactions performance was investigated in order to achieve maximum yields of synthesized products.

MATERIALS AND METHODS

Enzyme and Chemicals

Lipase B from *Candida antarctica* (CAL B) immobilized on acrylic resin, Novozym® 435, was purchased from Novozymes (Bagsvaerd, Denmark). Naringin (>90%) were purchased from TCI Europe N.V., Zwijndrecht, Belgium while esculin (>97 %) was purchased from Acros Organics, New Jersey, USA. Oleic acid (85% pure) from Alfa Aesar GmbH & Co, KG, Karlsruhe, Germany was used as acyl donor. In reactions the following solvents were used: acetonitrile (HPLC grade, Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), acetone (99.5%, Zorka Pharma, Šabac, Serbia), isooctane (99.5% pure, Centrohem, Stara Pazova, Serbia) and *tert*-butyl alcohol (99%, Sigma-Aldrich Chemie GmbH, Steinheim, Germany), while methanol (HPLC grade, J.T. Baker, Center Valley, PA, USA) was used as mobile phase in HPLC analyses.

Enzymatic esterification of flavonoids

Acylation of flavonoids was performed in a sealed orbital shaken flask at 65 °C in a thermostated shaker (IKA KS 4000i Control, Staufen, Germany) at 150 rpm. Reaction mixtures consisted of flavonoid and oleic acid (concentrations specified for each experiment individually). Concentration of flavonoids was varied in range from 10 to 90 mM, while substrate molar ratio acyl acceptor: acyl donor was varied from 1:1 to 1:20. Solvents were added to reaction mixtures to reach 5 ml and reactions were initiated by adding 1 % (w/v) of enzyme. Samples for HPLC analyses (50 µl) were taken at predefined times. Control samples (without enzyme) were prepared in a same way, subjected to the same temperature treatment and product was not detected in them.

HPLC analyses

Quantitative analysis of samples was done by Dionex Ultimate 3000 Thermo Scientific (Waltham, USA) HPLC system and a reverse phase column (Hypersil GOLD C18, 150 mm × 4.6 mm, 5 µm). As a mobile phase MeOH:HCOOH=100:0.1 % was used. It was conducted by isocratic elution, eluent flow rate was of 1 ml/min and column was thermostated at 30 ˚C. Reaction mixtures were twenty to fifty times diluted and injection volumes were in range of 10 to 40 µl. Detection of products was carried out by a UV detector at 280 nm for naringin and its ester, and at 345 nm for

esculin and its derivate. Standard curves for two flavonoids were constructed. Concentrations of products were calculated using following equation:

$$C_{product}(mM) = \frac{A_{product} * V_{standard}}{slope * V_{product}} * D \quad (1)$$

Where A (mAU*min) is area of product in HPLC chromatograms, $V_{product}$ and $V_{standard}$ are injection volumes of synthesized ester and standard (naringin, esculin) respectively and D is sample dilution. Slopes were calculated for all flavonoids using standard curves.

RESULTS AND DISCUSSION

Effect of solvents on esterification of naringin and esculin

Reaction medium is important parameter influencing the reaction performance, since the chosen solvent must provide good solubility of polar flavonoids and non-polar acyl donor, as well as to ensure high activity of biocatalyst. Therefore, in this paper the influence of solvents on reactions of synthesis of naringin and esculin oleate catalyzed by immobilized lipase B from *Candida antarctica* (Novozym® 435) was investigated using various organic solvents (acetonitrile, acetone, *tert*-butanol and isooctane) with wide range of log P values from -0.33 to 4.37. Obtained results have shown that the highest conversion degrees were achieved in acetonitrile (Figure 1). Also, it was observed that synthesis of esculin oleate could be successfully conducted in *tert*-butanol as solvent, since conversion of limiting substrate was slightly lower than that obtained in acetonitrile. These results are in accordance with results obtained in majority of studies dealing with esterification of flavonoids (Milisavljević et al., 2014). However, given the literature data (De Araújo et al., 2017), low activity of CAL B in synthesis of naringin and esculin esters in acetone was unexpected. Furthermore, in isooctane, only traces of products were detected, which could be explained by poor solubility of hydrophilic esculin and naringin in such hydrophobic medium. Considering the obtained results, further esterifications of naringin and esculin were performed in acetonitrile.

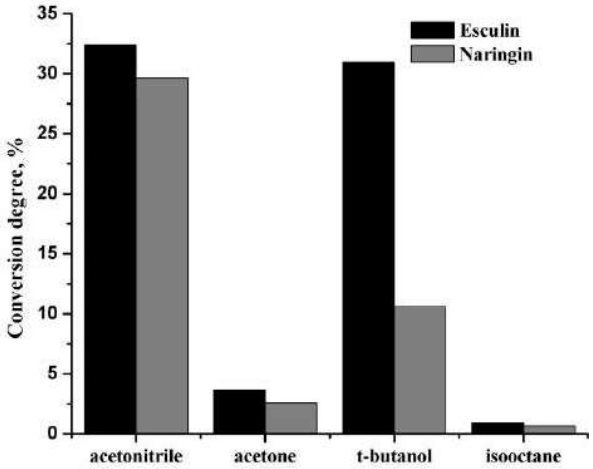

Figure 1. Effect of reaction medium on synthesis of esculin oleate and naringin oleate. The reactions were carried out at 65 °C for 30 h, with 50 mM of flavonoids, concentration of enzyme 1% (w/v), and substrate molar ratio 1:18. The volume was adjusted to 5 ml by the addition of different solvents.

Effect of flavonoid concentration on esterification of flavonoids

The other important parameter influencing the esterification of flavonoids is concentration of acyl acceptor since amount of flavonoid is limited by its solubility in reaction medium. In our study, we investigated the effect of concentration of flavonoids (naringin and esculin) on conversion degrees of limiting substrates as well as product yields. Considering the results obtained in our previous study dealing with optimization of phloridzin esterification (Milisavljević et al., 2014), concentrations of flavonoids were varied in the range of 10 to 90 mM, while substrate molar ratio acyl acceptor/donor was set at 1:13. Time courses of naringin esterifcation using different concentration of limiting substrate are shown in Figure 2a. It could be noticed that after 72 h of reaction, maximum concentration of naringin oleate, about 23 mg/ml, was accomplished in two experiments, when initial concentrations of naringin were 30 and 50 mM, while significantly lower product yields were achieved in other three experiments. Additionaly, it was observed that with increasing the concentration of acyl acceptor conversion degree of limiting substrate decrease, obtaining almost 90 % of conversion degree when initial concentration of naringin was 10 mM and 15 % using 90 mM of naringin (Figure 2b). Given the results achieved, it can be concluded that optimal concentration of naringin for esterification with oleic acid is 30 mM, so further experiments were carried out with this amount of naringin.

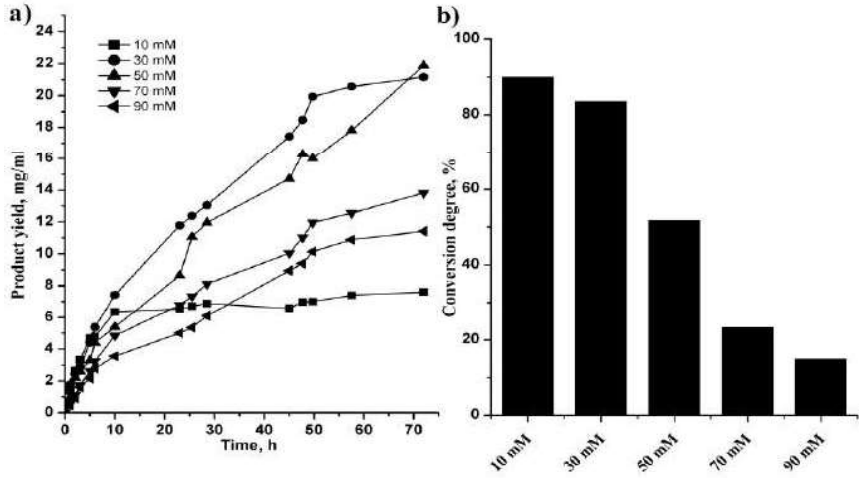

Figure 2. The effect of initial concentration of naringin on a) product yield and b) conversion degree of limiting substrate. The reactions were performed in acetonitrile at 65 °C for 72 h, with concentration of enzyme 1% (w/v), and substrate molar ratio 1:13.

On the other hand, when effect of initial concentration of esculin on conversion degree and esculin oleate yield was examined, it was observed that increasing of initial concentration of esculin from 10 to 70 mM led to increase of product yield, achieving the maximum concentration of esculin oleate (27.5 mg/ml) after 72h of reaction (Figure 3a). However, with further increase of initial esculin concentration, significantly lower concentration of product was achieved. Additionally, it was observed that with increasing of esculin concentration from 10 to 70 mM, obtained conversion degree was almost constant, around 60 %, while drastically lower conversion (11.2 %) was achieved with 90 mM of esculin (Figure 3b). Therefore, it was concluded that for esterification of esculin, 70 mM was optimal initial concentration and it was applied in further experiments.

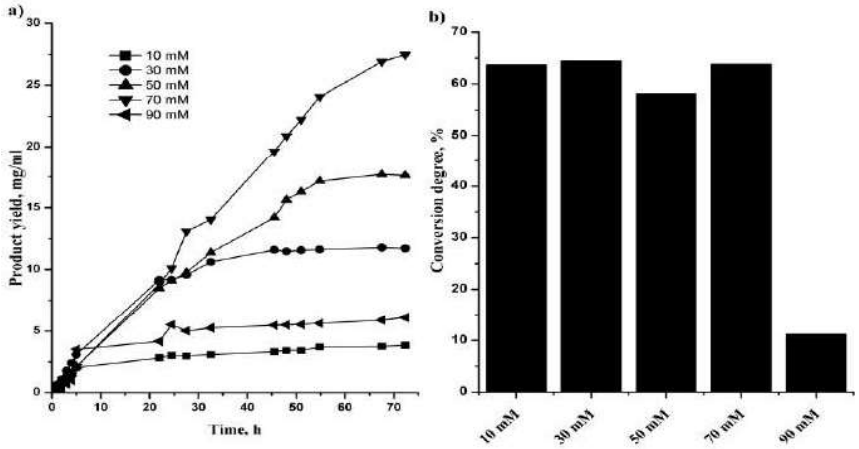

Figure 3. The effect of initial concentration of esculin on a) product yield and b) conversion degree of limiting substrate. The reactions were performed in acetonitrile at 65 °C for 72 h, with concentration of enzyme 1% (w/v), and substrate molar ratio 1:13.

Effect of substrate molar ratio on esterification of flavonoids

Typically, esterifications of flavonoids are conducted in the excess of acyl donor, since that is the facile way to shift reaction equilibrium to synthesis of the product. In order to determine the optimal molar ratio flavonoid/acyl donor for achieving the highest conversion degrees of limiting substrates, in further experiments substrate molar ratio was varied in range from 1:1 to 1:20 and its effect on conversion degrees of naringin and esculin is shown in Figure 4. Similar trend was observed in experiments with both flavonoids, since increase of substrate molar ratio from 1:1 to 1:5 led to increase of conversion degree of naringin from 17 to 86 %, as well as of esculin from 30 to 70 %, achieving maximum concentration of 26 and 48 mM of naringin oleate and esculin oleate, respectively after 72 h of reaction. Further increasing led to slightly decreased conversion of flavonoids, indicating that fivefold excess of oleic acid is optimal for acylation of both flavonoids. These results are in accordance with literature data (Gayot et al., 2003).

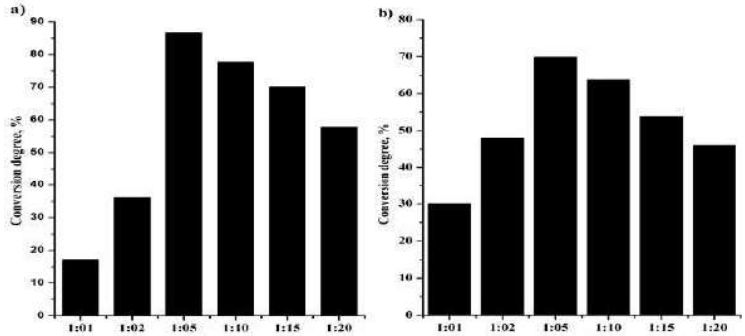

Figure 4. The influence of substrate molar ratio on conversion degree of a) naringin and b) esculin. The reactions were performed in acetonitrile at 65 °C for 72 h, with concentration of enzyme 1% (w/v), and optimal concentrations of flavonoids.

CONCLUSION

Esterification of two structurally different flavonoids, naringin and esculin, with oleic acid as acyl donor catalyzed by lipase B from *C. antarctica* (Novozym® 435) was optimized in order to achieve

the highest product yields. Optimal conditions for synthesis of naringin and esculin oleate were determined, and it was concluded that the most appropriate solvent for both reactions was acetonitrile. Additionally, maximum concentration of naringin oleate could be achieved using 30 mM of naringin and molar ratio flavonoid/acyl donor 1:5. On the other hand, the highest yield of esculin oleate was obtained using 70 mM of esculin and also fivefold excess of oleic acids. Obtained results of optimization of process parameters present an important impetus for further investigation of synthesis of esters of various flavonoids, including purification and scale-up.

ACKNOWLEDGEMENTS

Authors are grateful to the Serbian Ministry of Education, Science and Technological Development [project numbers III46010 and TR31035] for the financial support.

LITERATURE:

1. Ardhaoui, M., Falcimaigne, A., Ognier, S., Engasser, J., Moussou, P., Pauly, G., and Ghoul, M. (2004). Effect of acyl donor chain length and substitutions pattern on the enzymatic acylation of flavonoids. *Journal of biotechnology, 110*, 265-272. doi: 10.1016/j.jbiotec.2004.03.003
2. Bezbradica, D., Ćorović, M., Jakovetić Tanasković, S., Luković, N., Carević, M., Milivojević, A., and Knezevic-Jugovic, Z. (2017). Enzymatic Syntheses of Esters - Green Chemistry for Valuable Food, Fuel and Fine Chemicals. *Current Organic Chemistry, 21*, 104-138. doi: 10.2174/1385272821666161108123326
3. Chebil, L., Humeau, C., Falcimaigne, A., Engasser, J.-M., and Ghoul, M. (2006). Enzymatic acylation of flavonoids. *Process Biochemistry, 41*, 2237-2251. doi: https://doi.org/10.1016/j.procbio.2006.05.027
4. Danihelová, M., Viskupičová, J., and Šturdík, E. (2012). Lipophilization of flavonoids for their food, therapeutic and cosmetic applications. *Acta Chimica Slovaca, 5*, 59-69. doi: 10.2478/v10188-012-0010-6
5. De Araújo, M. E. M., Franco, Y. E., Messias, M. C., Longato, G. B., Pamphile, J. A., and Carvalho, P. d. O. (2017). Biocatalytic synthesis of flavonoid esters by lipases and their biological benefits. *Planta medica, 83*, 7-22. doi: dx.doi.org/10.1055/s-0042-118883
6. El-Desoky, A. H., Abdel-Rahman, R. F., Ahmed, O. K., El-Beltagi, H. S., and Hattori, M. (2018). Anti-inflammatory and antioxidant activities of naringin isolated from Carissa carandas L.: In vitro and in vivo evidence. *Phytomedicine, 42*, 126-134. doi: 10.1016/j.phymed.2018.03.051
7. Gayot, S., Santarelli, X., and Coulon, D. (2003). Modification of flavonoid using lipase in non-conventional media: effect of the water content. *Journal of biotechnology, 101*, 29-36. doi: 10.1016/S0168-1656(02)00286-9
8. Mellou, F., Lazari, D., Skaltsa, H., Tselepis, A., Kolisis, F., and Stamatis, H. (2005). Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *Journal of biotechnology, 116*, 295-304. doi: 10.1016/j.jbiotec.2004.12.002
9. Milisavljević, A., Stojanović, M., Carević, M., Mihailović, M., Veličković, D. a., Milosavić, N., and Bezbradica, D. (2014). Lipase-catalyzed esterification of phloridzin: acyl donor effect on enzymatic affinity and antioxidant properties of esters. *Industrial & Engineering Chemistry Research, 53*, 16644-16651. doi: 10.1021/ie5027259
10. Niu, X., Wang, Y., Li, W., Zhang, H., Wang, X., Mu, Q., . . . Yao, H. (2015). Esculin exhibited anti-inflammatory activities in vivo and regulated TNF-α and IL-6 production in

LPS-stimulated mouse peritoneal macrophages in vitro through MAPK pathway. *International immunopharmacology, 29*, 779-786. doi: 10.1016/j.intimp.2015.08.041

11. Pizzorno, J. E., Murray, M. T., and Joiner-Bey, H. (2016). *The Clinician's Handbook of Natural Medicine E-Book*. London: third ed. Elsevier Health Sciences.
12. Salem, J. H., Humeau, C., Chevalot, I., Harscoat-Schiavo, C., Vanderesse, R., Blanchard, F., and Fick, M. (2010). Effect of acyl donor chain length on isoquercitrin acylation and biological activities of corresponding esters. *Process Biochemistry, 45*, 382-389. doi: 10.1016/j.procbio.2009.10.012
13. Tapas, A. R., Sakarkar, D., and Kakde, R. (2008). Flavonoids as nutraceuticals: a review. *Tropical Journal of Pharmaceutical Research, 7*, 1089-1099. doi: 10.4314/tjpr.v7i3.14693
14. Viskupicova, J., Ondrejovic, M., and Maliar, T. (2012). Enzyme-mediated preparation of flavonoid esters and their applications. In D. Ekinci (Ed.), *Biochemistry* (pp. 263-286). Rijeka: InTech.