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Enzymatic synthesis of 1,3-dihydroxyphenylacetoyl-*sn*-glycerol: Optimization by response surface methodology and evaluation of its antioxidant and antibacterial activities

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ABSTRACT

In this study, the enzymatic synthesis of phenylacetoyl glycerol ester was carried out as a response to the increasing consumer demand for natural compounds. 1,3-dihydroxyphenylacetoyl-*sn*-Glycerol **(1,3-di-HPA-Gly),** labeled as "natural" compound with interesting biological properties, has been successfully synthesized for the first time in good yield by a direct esterification of glycerol **(Gly)** with *p*-hydroxyphenylacetic acid **(*p*-HPA)** using immobilized *Candida antarctica* lipase as a biocatalyst. Spectroscopic analyses of purified esters showed that the glycerol was mono- or di-esterified on the primary hydroxyl group. These compounds were evaluated for their antioxidant activity using two different tests. The glycerol di-esters **(1,3-di-HPA-Gly)** showed a higher antiradical capacity than that of the butyl hydroxytoluene. Furthermore, compared to the *p*-HPA, synthesized ester **(1,3-di-HPA-Gly)** exhibited the most antibacterial effect mainly against Gram + bacteria. Among synthesized esters the **1,3-di-HPA-Gly** was most effective as antioxidant and antibacterial compound. These findings could be the basis for a further exploitation of the new compound, **1,3-di-HPA-Gly**, as antioxidant and antibacterial active ingredient in the cosmetic and pharmaceutical fields.

1. Introduction

Over the last few years, a commercial relevance for glycerol has been marked by a significant increase because of its rising inevitable formation as a by-product of biodiesel production (10%, w/w referred to biodiesel) [1–3]. The use of glycerol as feedstock has awakened the interest in the development of new green synthesis processes. Several studies have reported the chemistry of glycerol, starting from the classical esters to generate new products like glycerol carbonate, propanediols telomers and epoxides *via* chemical and biocatalytic reactions [3]. The phenolic compounds derived from by-product plants are highly regarded for their antioxidant capacity [4]. Recently, growing evidence has shown that phenolic compounds in olive oil could play a part in protection against cancer and more attention is focalized to its phenolic compounds [5–7]. Olive oil extraction generates solid and liquid wastes (olive leaf and waste olive oil), which represents a big environmental pollution problem [8,9]. Nevertheless, these wastes are also promising sources of phenolic compounds that can be recovered and converted to useful products by synthesis prcesses. These by-products include especially 4-hydroxy phenylethanol (tyrosol), 3,4-dihydroxyphenylethanol (hydroxytyrosol), caffeic acid and 4-hydroxyphenylacetic acid (***p*-HPA**) used for the preparation of non-steroidal anti-inflammatory drugs, *p*-coumaric acid, protocatechuic acid and many others [10–12]. These bioactive phenolic acid compounds exhibit powerful biological activities such as anti-oxidation [13,14], inhibition of human immune deficiency virus (HIV) [15], anti-inflammatory [16] and joint-degenerative effects [17].

Interestingly, two molecules, 1-feruloyl-*sn*-glycerol (FG) and 1,3-diferuloyl-*sn*-glycerol (F_2G), have been isolated from natural sources, from *Solanum tuberosum* (potato) [18]. These ferulic acid derivatives have been motivated by the feruloyl moiety which exhibited antioxidant properties and ultraviolet (UV) light absorbing [19,20]. In fact, these ferulic acid derivatives may also be an active ingredient attractive to the food and cosmetic industries.

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Besides, numerous studies have shown that F_2G has been synthesized using chemical catalysts which make the processes industrially and economically infeasible [18]. Such antioxidants have been used as natural substitution for their synthetic counterparts, such as butylated hydroxytoluene [21]. However, it is well known that chemical synthesis generates secondary compounds which must be eliminated by purification processes. In addition, chemical catalysis does not meet the requirements for food applications. For these reasons, enzymatic catalysis seems to be the ideal method which offers various advantages like specificity, milder reaction conditions and without producing secondary compounds. The enzymatic esterification of glycerol and soybean oil mono- and diacylglycerols with ethyl ferulate using *Candida antarctica*, generated the production of 1-feruloyl-*sn*-glycerol and 1,3-diferuloyl-*sn*-glycerol compounds, respectively [21]. In addition, the esterification of hydroxycinnamic acids to glycerol using feruloyl esterases, has been previously reported [22]. Vafiadi et al. [23] reported the esterification of sinapic acid to one of the primary hydroxyl groups of glycerol. The authors showed that the synthesized compound (glycerol sinapate) retained, after esterification, 89.5 ± 1.1% of its antioxidant activity against low-density lipoprotein oxidation.

In this context, the main focus of this study is to improve the biological activity of a phenolic compound, by fixing two or three molecules of ***p*-HPA** onto a hydroxyl group of glycerol **(Gly)** using Novozym 435 as a biocatalyst. Then, the antioxidant and antibacterial activities of the newly synthesized esters will be investigated.

2. Materials and methods

2.1. Chemicals and reagents

Chloroform and methanol were purchased from Scharlau (Spain); acetonitrile and acetic acid from Pharmacia (Uppsala, Sweden); 2-methyl-2-propanol from Fluka (Germany); The ***p*-HPA**; 2,2-azino-bis-3ethylbenzothiazoline-6-sulfonic acid **(ABTS)**; 4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide **(MTT)** and 2,2-diphenyl-1-picrylhydrazyl **(DPPH)** were purchased from Fluka (Switzerland); butylated hydroxytoluene **(BHT)** (purity ≥ 99%) from Sigma. *Candida antarctica* lipase (Novozym 435) from Novo Nordisk.

2.2. Experimental designs and esterification reactions

2.2.1. Box-Behnken designs and response surface analysis

Response surface methodology (RSM) is an empirical optimization technique for evaluating the relationship between experimental responses and coded factors called X_1, X_2, X_3. This method is usually used in combination with factorial design methods such as central-composite designs and Box–Behnken designs. Adopting Box–Behnken designs allowed reducing the number of experimental sets without decreasing the optimization accuracy compared with traditional factorial design methods which are based on a large number of experimental assays. Three factors (enzyme amount, *p*-HPA/glycerol molar ratio and temperature) have been selected according to preliminary assays, for enhancing the conversion yield of the synthesized product. These factors were investigated at three different levels (−1, 0, +1) in terms of coded and uncoded symbols as shown in Table 1.

An empirical model related to the factors has been obtained using a second-order polynomial equation and a multiple regression of data [24]. The general form of the second-order polynomial equation is:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j$$

Table 1
Levels of the factors tested in the Box-Behnken design.

Factors	Symbol	Coded levels		
		−1	0	+1
p-HPA/Gly molar ratio	X_1	2	5	8
Temperature (°C)	X_2	35	45	55
Enzyme amount (mg)	X_3	10	30	50

where Y is the predicted response, X_i and X_j are independent factors, β_0 is the intercept, β_i is the linear coefficient, β_{ii} is the quadratic coefficient and β_{ij} is the interaction coefficient.

2.2.2. Data analysis and software

Design-expert, version 7.0 (STAT-EASE Inc., Minneapolis, USA) was used for the experimental designs and the statistical analysis of the experimental data. The analysis of variance (ANOVA) was used to estimate the statistical parameters.

2.2.3. Esterification reactions

Glycerol contains three hydroxyl groups in its structure, and therefore three esterified derivatives with *p*-HPA were expected. HPLC analysis of the reaction mixture after 48 h showed the production of two new compounds by the enzymatic reaction. Each new compound was purified and identified by NMR analysis as described below. The purified compounds are used as external standard.

RSM was performed to optimize the enzymatic synthesis of hydroxyphenylacetoyl glycerol derivatives. Several conditions with a total of 17 runs were tested including different combinations of three factors (*p*-HPA/glycerol molar ratio, temperatures and enzyme amounts) (Table 2). In fact, the production of hydroxyphenylacetoyl glycerol derivatives was performed by the direct esterification of glycerol with *p*-HPA in screw-capped flasks. Different molar ratios of *p*-HPA to glycerol were dissolved in 3 mL of *tert*-butanol/acetonitrile volume ratio 1:1 (v/v). Since, we have shown that the adequate solvent to solubilize the two substrates is a mixture of solvent ratio *ter*-butanol/acetonitrile 1:1 (v/v). We have shown also that the addition of a more hydrophobic solvent in the reaction mixture, like *tert*-butanol (Log P = 0.8), trigger the enzyme activity.

The mixture was stirred at different temperatures in an orbital shaker at 220 rpm and in the presence of different amounts of Novozym 435 (*Candida antarctica* lipase). Controls were run in parallel, under the same conditions, without enzyme addition. After 48 h of reaction incubation, an aliquot of the mixture reaction was withdrawn and filtered to be used for HPLC analysis. The conversion yield of synthesized compounds was calculated as the ratio of the number of moles of synthesized compound (determined by an external standard range previously established for each compound) per the total number of moles of *p*-HPA. The esterification study has been carried out using four microorganism lipases (*Rhizopus oryzae, Staphylococcus aureus, and Staphylococcus xylosus*) produced in our laboratory and immobilized on $CaCO_3$ and a commercial one (*Candida antarctica* lipase immobilized on macroporous acrylic resin). Novozym 435 (*Candida antarctica* lipase) showed the highest yield conversion (data not shown) and was selected for the rest of our work.

2.3. Purification and identification of synthesized compounds: 1-hydroxyphenylacetoyl-sn-glycerol and 1,3-dihydroxyphenylacetoyl-sn-glycerol

The purification of the newly synthesized compounds was achieved by chromatography on a silica gel60 column (Merck)