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Selective and eco-friendly synthesis of lipoaminoacid-based surfactants for food, using immobilized lipase and protease biocatalysts

Claudia Bernal [a,b,*], Fanny Guzman [c], Andres Illanes [d], Lorena Wilson [d]

[a] Instituto de Investigación Multidisciplinario en Ciencia y Tecnología, Universidad de La Serena, Raúl Bitran 1305, La Serena, Chile
[b] Tecnología Enzimática para Bioprocesos, Departamento de Ingeniería de Alimentos, Universidad de La Serena, Raúl Bitran 1305, La Serena, Chile
[c] Laboratorio de Síntesis de Péptidos, Núcleo de Biotecnología Curauma, Pontificia Universidad Católica de Valparaíso, Chile
[d] Escuela de Ingeniería Bioquímica, Pontificia Universidad Católica de Valparaíso, P.O. Box 4059, Valparaíso, Chile

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ABSTRACT

Lipoaminoacids, as surfactants, are an excellent option for food industry due to the currently trends in consumption of functional and natural ingredients. Synthesis of lauroyl glycine lipoaminoacid was carried out with a lipase from *Pseudomonas stutzeri* and a protease from *Bacillus subtilis*, which were immobilized in octyl-glyoxyl silica and glyoxyl-silica supports, respectively, comparing their catalytic performance. The enzymatic selectivity towards the lipoaminoacid instead of the dipeptide glycylglycine and synthesis yield were evaluated with respect to the characteristics of the immobilized biocatalysts and synthesis conditions. Three solvents were tested as reaction media for evaluating the expressed activity, stability and catalytic behavior during synthesis. Results indicate that both enzymes favor the lauroyl glycine synthesis over the peptide synthesis, but the immobilized protease has the best balance between selectivity and yield: 40% yield for lauroyl glycine and less than 5% for dipeptide after 96 h of synthesis, at 45 °C and acetone as solvent.

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1. Introduction

Lipoamino acids (LAA), also referred to as *N*-acyl amino acids, are a special kind of compounds based on amino acids and fatty acids. They are an interesting class of biocompatible compounds since they contain an amino acid as the hydrophilic part and a carbonated chain as the hydrophobic part, which confer surfactant behavior. LAA have attracted much attention as candidate amino acid based surfactants because of their low-toxicity and biodegradability (Chen et al., 2013). They are applicable as medical and cosmetic preservatives, and as additives for food, mineral flotation and pesticides (Shi & Wang, 2014) due to their emulsifying properties (Lu, Yuan, Fang, Wang, & Guo, 2015; Pérez et al., 2009).

The methodologies used for LAA synthesis include reactions of acylation, esterification, amidation, and alkylation of amino acids with a fatty alkyl acid binding the carboxyl group of the fatty acid at the amino, carboxyl or R group of the amino acid (Takehara, 1989). Most of these reactions have been conducted by chemical catalysis: fatty nitrile hydrolysis, amide carbonylation and Schotten-Bauman condensation (Azmi et al., 2016; Mohini, Prasad, Karuna, Poornachandra, & Ganesh Kumar, 2016). These processes are environmentally offensive requiring the use of toxic solvents, and protection and deprotection steps of chemical groups are necessary making the process cumbersome and expensive (Chen et al., 2013; Colomer et al., 2011; Kawase, Nishioka, & Oida, 2010). On the other hand, LAA enzymatic synthesis is a mild, eco-friendly and highly selective strategy when compared with traditional methodologies. However, enzymatic synthesis requires a careful enzyme selection, its stabilization under reaction conditions and an improved synthesis yield with respect to conventional methodologies (Wada et al., 2002; Xia et al., 2004; Morán et al., 2004; Zhang, Adachi, Watanabe, & Matsuno, 2005).

Proteases and lipases catalyze the hydrolysis of amides and esters, respectively; however, under suitable conditions, both enzymes can catalyze peptide synthesis or fatty acid amidation by acyl-transfer reactions (Haseena Banu, Prasad, & Bharathi, 2014; Liu, Liu, & Chang, 2007; Nuijens et al., 2011; Rodrigues Borgesa & de Carvalho Balaban, 2014; Zheng et al., 2011). Activated substrates have been mostly used in kinetically controlled reactions that are usually faster and not limited by the equilibrium. Enzyme regioselectivity is particularly important in LAA synthesis, because several parasite reactions may occur leading to different

* Corresponding author at: Instituto de Investigación Multidisciplinario en Ciencia y Tecnología, Universidad de La Serena, Raúl Bitran 1305, La Serena, Chile.
E-mail addresses: claberz@gmail.com, cbernal@userena.cl (C. Bernal).

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by-products, for example, the formation of oligopeptides that decrease the yield of LAA. It is expected that lipases and proteases will differ in this respect when catalyzing LAA synthesis.

Two strategies are used for promoting the acyl-transfer reactions with lipases or proteases that may influence the enzyme selectivity: to decrease the water activity in the reaction medium, and to increase the substrate concentration (Gumel, Annuar, Heidelberg, & Chisti, 2011; Vossenberg, Beeftink, Cohen Stuart, & Tramper, 2013; Bernal, Illanes, and Wilson, 2014). Both strategies can be approached by increasing the organic solvent concentration in the reaction medium. However, this represents a challenge for the enzymatic synthesis of LAA, since enzymes are in many cases labile in organic solvents (Fernandez-Lopez et al., 2017). This problem can be solved using immobilized enzymes, which are usually much more stable than their soluble counterparts (Vossenberg et al., 2013; Bernal, Illanes, and Wilson; 2014; Yazawa & Numata, 2014), opening a low explored research area with new topics, like the use of enzyme immobilization to modified the regiospecificity and promiscuity for non-natural reactions, (Guajardo, Bernal, Wilson, & Cabrera, 2015).

In this work, the performance of two immobilized enzymes in the synthesis of the LAA lauroyl glycine is compared in terms of selectivity and yield when these enzymes are immobilized on porous silica modified with different functional groups, emphasizing the effect of enzyme immobilization on the enzyme regioselectivity and synthesis yield. The selected enzymes are a protease from *Bacillus circulans* and a lipase from *Pseudomonas stutzeri,* which are low-cost and commercially available enzymes that have been tested in other reactions of sugar fatty acid ester synthesis with excellent results (Bernal, Illanes, & Wilson, 2014; Walsh, Bombyk, Wagh, Bingham, & Berreau, 2009; Zheng et al., 2011). The effect of the immobilized biocatalysts characteristics and the type of solvent used as reaction medium were evaluated, choosing selectivity (synthesis preference toward one product), lauric acid conversion and synthesis yield as response parameters.

2. Experimental section

2.1. Materials

The following analytical grade reagents were used without further modification. Sodium silicate (25–29% SiO_2 and 7.5–9.5% Na_2O), ethylacetate (EtAc), sulfuric acid (98%), and $NaIO_4$ were purchased from Merck (Darmstadt, Germany); cetyltrimethylammonium bromide (CTAB), $NaBH_4$, glycerin, glycidyloxypropyltrimethoxysilane (GPTMS 99.7%), trimethoxy(octyl)silane (OTMS; 96%), *N-t*-BOC-l-alanine p-nitrophenyl ester (BOC-Ala-p-NP), p-nitrophenyl butyrate (pNPB), acetone, propylene carbonate, acetonitrile, glycine, lauric acid, 9-fluorenylmethoxycarbonyl (Fmoc), trifluoroacetic acid (TFA) and triisopropylsilane were purchased from Sigma–Aldrich (St. Louis, MO, USA). Commercial lipase from *P. stutzeri* (60,000 IU per gram of powder) was kindly donated by Meito-Sangyio Ltd. (Fuchu, Japan). Commercial protease from *Bacillus subtilis* (Alcalase 2.5L) was kindly donated by Novozymes (Spain). All other reagents and solvents were of the highest available purity and used as purchased.

2.2. Synthesis, chemical modification and characterization of siliceous supports

The synthesis of silica was carried out as previously reported by Bernal et al. (2014) using the following molar composition: 1 SiO_2: 0.30; Na_2O: 0.24; cetyl trimethylammonium bromide: 7.2; ethyl acetate: 193 H_2O. This mixture was heated at 80 °C for 48 h under quiescent conditions. The solid obtained was calcined at 540 °C during 3 h.

For the functionalization of the support with glyoxyl or octyl-glyoxyl groups, 1.0 g silica (activated under vacuum at 200 °C) was contacted with 30 mL of 5% of suitable silane: 3-glycidoxypropyl)methyldiethoxysilane (GPTMS, for glyoxyl groups) or mixture 1:1 of GPTMS or trimethoxy(octyl)silane (TOS, for octyl groups) in toluene solution. The epoxy groups of both supports were hydrolyzed with 0.1 M H_2SO_4 during 2 h at 85 °C. After filtration, washing with water/acetone and drying, oxidation with 0.1 M $NaIO_4$ solution proceeded for 2 h at room temperature (Bernal et al., 2014).

2.3. Enzyme biocatalysts preparation

Assay of enzymatic activities: The enzymatic activity was measured by the increase in the absorbance at 348 nm produced by the released p-nitrophenol in the hydrolysis of 0.4 mM pNPB for lipase, or 2.5 mM BOC-Ala-p-NP for protease, both in 25 mM sodium phosphate buffer at pH 7.0 and 25 °C. To start the reaction, 0.05 mL of enzyme solution or suspension was added to 2.0 mL of substrate solution. One international unit of activity (IU) was defined as the amount of enzyme that hydrolyzes 1 μmol of pNPB or BOC-Ala-p-NP (lipase or protease, respectively) per minute under the conditions described above.

Protease immobilization: Considering the hydrophilic nature of the *B. subtilis* protease and the quantity of lysine residues on the tridimensional surface of the enzyme (Fig. S1, Supplementary Material) (Bernal, Sierra, & Mesa, 2012), this enzyme was immobilized in glyoxyl-silica (Gx). Briefly, 1 g of Gx was contacted with 5 mL of 100 mM potassium bicarbonate pH 10 containing the *B. subtilis* enzyme at different concentration (5–30 mg protein per gram of support). The suspension was incubated at 25 °C for 3 h until the activity in the supernatant was constant. The derivative was then reduced with 4 mg of sodium borohydride, for 30 min at 4 °C (Bernal, Illanes, & Wilson, 2015). Finally, the solid obtained was thoroughly washed with water and stored at 5 °C for further use.

Lipase immobilization: Considering the interfacial activation of lipases (Manoel, dos Santos, Freired, Rueda, & Fernandez-Lafuente, 2015) and the advantages of their immobilization in heterofunctional hydrophilic-hydrophobic support (Bernal et al. 2014), lipase from *P. stutzeri* was immobilized in octyl-glyoxyl-silica (OGx). Briefly, 1 g of support was contacted with 5 mL of 25 mM potassium phosphate pH 7 containing the enzyme (15 mg protein per gram of support). The suspension was incubated at 25 °C for 6 h until the activity in the supernatant remained constant; after that, the suspension was incubated at pH 10 and the process was finished as already described for the immobilization in glyoxyl-silica. Finally, the solid obtained was thoroughly washed with water and stored at 5 °C for further use.

2.4. Biocatalysts characterization

Immobilization yield in terms of protein (IYp) and activity (IYa) were calculated according to Eqs. (1) and (2), respectively

$$IYp = \frac{P_L}{P_O} \times 100 \quad (1)$$

$$IYa = \frac{A}{A_O} \times 100 \quad (2)$$

where P_O is the offered protein, P_L is the loaded protein in the biocatalyst, A_O is the offered activity and A is the expressed activity in the immobilized enzyme.

Protein was measured by Bradford methodology (Bradford, 1976) and the expressed activity determined as described above.

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