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Short communication

Production of combi-CLEAs of glycosidases utilized for aroma enhancement in wine

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ABSTRACT

Glycosidases are frequently used in winemaking to liberate glycosidically bound aroma compounds. Since most of the glycosidases used for diglycoside hydrolysis act sequentially, their co-immobilization is an attractive alternative from a technical and economical perspective. The enzymes α-L-arabinosidase (ARA) and β-D-glucosidase (βG) from the preparation Rapidase®AR2000 were co-immobilized in CLEAs (combi-CLEAs), evaluating the effect of bovine serum albumin (BSA) addition and the concentration of glutaraldehyde (Glu) on enzyme immobilization yield and expressed activity. Combi-CLEAs prepared with a Glu to Rapidase protein mass ratio of 0.053 and BSA to Rapidase protein mass ratios of 1, 0.33, and 0.2 were selected, evaluating their stability at simulated winemaking conditions: 25 °C, pH 3.5, and 10% (v/v) of ethanol. All combi-CLEAs were more stable than the soluble enzymes, the best result being obtained at a BSA to Rapidase protein mass ratio of 0.33. Half-lives of βG and ARA in combi-CLEAs were 43.9 and 54.9 days, respectively, whereas in the case of the soluble enzymes were only 1.3 and 6.2 days, respectively. Immobilization yields were 79.1 and 47.1% in terms of βG and ARA activity, respectively. Combi-CLEAs of glycosidases are technologically relevant robust biocatalysts for their application as aroma enhancers in winemaking.

Keywords: Glycosidases; Immobilization; Cross-linked enzyme aggregates; Stabilization; Wine; Aroma

1. Introduction

Monoterpenes are present in grapes in free and glycosidic conjugates forms, playing an important role in wine aroma. The latter are non-volatile and flavorless compounds that represent a significant reservoir of glycosidic aroma precursors; therefore, the enzymatic hydrolysis of such glycosidic conjugates is an interesting strategy for enhancing wine aroma by the release of the free aromatic compounds (Mateo and Jimenez, 2000). Sugar moieties of glycosidically conjugated forms of monoterpenes have been identified as rutinose (6-O-α-L-rhamnopyranosyl-β-D-glucopyranoside), 6-O-α-L-arabinofuranosyl-β-D-glucopyranoside, 6-O-α-L-apiofuranosyl-β-D-glucopyranoside or β-D-glucopyranoside. Most of the enzymes used for diglycoside hydrolysis act sequentially. Firstly, the corresponding sugar and glucoside are liberated by α-L-rhamnosidase (EC 3.2.1.40), α-L-arabinosidase (EC 3.2.1.55) or apiosidase and then the glucoside is hydrolyzed by β-D-glucosidase (EC 3.2.1.21) liberating the aromatic compound (Günata et al., 1988; Maicas and Mateo, 2005). The β-D-glycosidases may be plant-derived (Arayan et al., 1987) or may be produced by yeasts and filamentous fungi (Longo and Sanromán, 2006), being the latter stable at wine pH contrary to enzymes from other sources (Günata et al., 1993, 1997). Soluble enzyme preparations from *Aspergillus niger* rich in relevant glycosidases have been used in winemaking in order to liberate glycosidically bound aromatic compounds. One example is the commercial preparation Rapidase®AR2000, where the main glycosidase activities are α-L-arabinosidase (ARA) and β-D-glucosidase

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(βG) (Cabaroglu et al., 2003). βG from *A. niger* is a trimeric enzyme with molecular mass of 330 kDa, each subunit having a molecular mass of 110 kDa (Rashid and Siddiqui, 1997), while the *A. niger* ARA is a monomeric enzyme with a molecular mass of 67 kDa (Kaneko et al., 1993).

Glycosidases immobilization offers the advantage of allowing a higher control of the hydrolysis reaction time and, consequently, the wine characteristics. Additionally, enzyme immobilization allows biocatalyst reutilization and may improve its stability, extending its lifespan and obtaining a product free of enzyme, which is a critical issue in order to avoid undesired alterations of wine organoleptic characteristics. Carrier-free immobilized enzymes obtained by aggregation and crosslinking (CLEAs) have the advantages associated to an insoluble biocatalyst, but without requiring an inert support that adds in complexity and costs. The crosslinking of enzyme aggregates is a simple methodology for enzyme immobilization based in the non-denaturing precipitation of the enzyme protein and its further crosslinking with bifunctional reagents, not requiring the previous purification of the enzyme preparation (Sheldon and van Pelt, 2013; Wilson et al., 2006; Caballero Valdés et al., 2011). Since the biocatalysts are crosslinked, this immobilization methodology is especially suited for the immobilization of multimeric enzymes (Wilson et al., 2004) and the co-immobilization of several enzymes (combi-CLEAs) to be applied in sequential biocatalytic processes (Sheldon, 2007; Talekar et al., 2013; Dalal et al., 2007).

The objective of this work was to establish a methodology for the production of a combi-CLEA of glycosidases from the commercial enzymatic preparation Rapidase® AR2000, in order to obtain a stable and robust biocatalyst under winemaking conditions (low pH and presence of ethanol). The development of such a biocatalyst by this simple strategy of co-immobilization offers an attractive alternative to easily control the enhancement of wine aroma and extend biocatalysts lifespan. Handling of the catalyst is compatible with winemaking practice and is easily removed from the wine not altering its quality.

2. Materials and methods

2.1. Materials

The commercial enzyme preparation Rapidase® AR2000 was from DSM (The Netherlands). Bovine serum albumin (BSA) was from Loba Chemie (India). *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA), *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), and glutaraldehyde grade II (25%, v/v) were from Sigma–Aldrich (USA). Polyethylene glycol (PEG 400), ammonium sulfate, and tert-butanol were from Merck (Germany). All other reagents were of the highest available purity and used as purchased.

2.2. Analysis

The enzymatic activity of ARA and βG was determined using *p*NPA and *p*NPG as substrates, respectively, measuring the *p*-nitrophenol (*p*NP) release spectrophotometrically at 405 nm using a temperature-controlled cell with constant magnetic stirring. To initiate the reaction, 200 μL of the enzymatic solution or suspension was added to 1.8 mL of a solution containing 0.42 mM of *p*NPA or 0.61 mM of *p*NPG for measuring ARA or βG activity, respectively. One international unit of ARA and βG activity (IU) was defined as the amount of enzyme producing 1 μmole of *p*NP per minute at 40 °C and pH 7 (phosphate buffer 100 mM) from the corresponding *p*-nitrophenyl derivative. The molar extinction coefficient of *p*NP was 8.462 mM^{-1}.

Protein concentration was determined according to Bradford (1976).

2.3. Combi-CLEA preparation

2.3.1. Selection of the precipitating agent

PEG 400, acetone, ammonium sulfate, methanol, and tert-butanol were evaluated as precipitating agents, being different volumes of each slowly added to a set of Eppendorf tubes with 500 μL of the enzyme solution prepared in 100 mM phosphate buffer, pH 7 (0.13 $g_{Rapidase}$/mL corresponding to 2 $mg_{protein}$/mL). In order to avoid enzyme inactivation, precipitation was carried out at 4 °C. After precipitate formation, the samples were centrifuged at 10,000 × *g* for 20 min at 3 °C and the protein concentration in the supernatant was measured. The enzymatic activity recovered in the precipitate was assessed after dissolution and the calculated enzyme recovery was used as parameter for the selection of the precipitating agent.

2.3.2. Evaluation of BSA and glutaraldehyde concentration in combi-CLEAs preparation

The use of BSA as protein feeder in combi-CLEAs preparation was evaluated. Different quantities of BSA were added to 10 mL of the enzyme solution prepared in 100 mM phosphate buffer, pH 7 (0.13 $g_{Rapidase}$/mL, 2 $mg_{protein}$/mL), mixing the samples gently in order to avoid foam formation. BSA to Rapidase® AR2000 protein (RP) mass ratios and glutaraldehyde (Glu) to RP mass ratios utilized for combi-CLEAs preparation were assessed in a factorial design experiment (5 × 3). BSA/RP mass ratios of 0, 0.1, 0.2, 0.33, and 1 were evaluated, in compliance with the range of values considered by other authors (Kim et al., 2013; Galvis et al., 2012), while Glu/RP mass ratios of 0.027, 0.053, and 0.080 were evaluated. The response parameters were the immobilization yield (IY) and specific activity (IU/$g_{catalyst}$) of each enzyme immobilized in the combi-CLEAs. IY was defined as the ratio of the units of activity expressed in the combi-CLEAs (A_I) and the units of activity subjected to immobilization (A_C), as presented in Eq. (1):

$$IY = \frac{A_I}{A_C} \cdot 100 \quad (1)$$

The complete process of combi-CLEAs preparation was carried out as follows: 40 mL of the selected precipitating agent was added slowly to 10 mL of the enzyme solution (with and without BSA) under stirring at 300 rpm and at 4 °C. After adding the precipitating agent, the suspension was maintained under stirring at 300 rpm for 30 min and afterwards was centrifuged at 10,000 × *g* per 20 min at 3 °C. A volume of 25 mL of the supernatant was discarded and the precipitate was suspended in the remaining volume. Glutaraldehyde was added slowly at 4 °C under stirring at 300 rpm and after adding the crosslinking agent the suspension was maintained under stirring for 1 h at the same conditions. The suspension containing the combi-CLEAs was centrifuged at 10,000 × *g* for 20 min at 3 °C, discarding the supernatant. The combi-CLEAs were suspended in 100 mM phosphate buffer pH 7 and again centrifuged at 10,000 × *g* for 20 min at 3 °C. This washing procedure was repeated three times in order to remove the