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Lysozyme immobilized on chitosan beads: Kinetic characterization and antimicrobial activity in white wines

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ABSTRACT

In order to be able to use lysozyme as an anti-microbial agent during the winemaking process, hen egg-white lysozyme (HEWL) was covalently immobilized on chitosan beads. A cell suspension of *Oenococcus oeni*, an enological strain involved in the winemaking process, was utilized as enzyme substrate. Both a kinetic study and evaluation of antibacterial activity, of the free and immobilized HEWL, were performed in model and real white wines. The catalytic parameters V_{max} and k_{cat} turned out to be higher for free-HEWL in model and white wines, demonstrating that covalent immobilization affected cell lysis velocity. However the K_m values were similar for the free and immobilized enzyme in wine model and only slightly lower in white wines for the immobilized biocatalyst. Moreover, the covalent immobilization also reduced the lysozyme antimicrobial efficiency, although it is worth noting that, in white wine, the antimicrobial activity of immobilized HEWL is not affected by the concentrations of free SO_2 and total phenols. Even though the proposed covalent immobilization strongly affected the lysozyme catalytic and antimicrobial efficiency, it improved the stability of immobilized HEWL in white wine, thus demonstrating its potential for use as an efficient antimicrobial agent in wine-making applications.

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1. Introduction

In winemaking processes the hen egg-white lysozyme (HEWL), an enzyme with muramidase activity, is used to control the growth of *Oenococcus oeni* (Cejudo-Bastante et al., 2010; Delfini et al., 2004; Gerbaux et al., 1999; Gerbaux, Villa, Monamy, & Bertrand, 1997; Lasanta, Roldán, Caro, Pérez, & Palacios, 2010). HEWL is a 129-amino acid enzyme and as a muramidase it has the ability to hydrolyze the beta (1,4) glycosidic bond between the N-acetylmuramic acid and the N-acetyl-D-glucosamine of peptidoglycan, the major component of the cell walls of Gram-positive bacteria (Hashemi, Aminlari, & Moosavinasab, 2014; Losso, Nakai, & Charter, 2000). Use of lysozyme in winemaking was approved by OIV in 1997 (resolution OENO 10/97), while the successful control of lactic acid bacteria (LAB) growth using HEWL has been well demonstrated in both white and red wines (Bartowsky, 2009; Gao et al., 2002; López et al., 2009). However, over the last decade several case studies have revealed severe allergic reactions due to the presence of lysozyme in wines (Weber, Steinhart, & Paschke, 2007; Weber et al., 2009). Thus, wines treated with lysozyme must be subject to specific labeling in accordance with the European Commission Regulation (EU) No. 1266/2010 of 22 December 2010, which amends directive 2007/68/EC as to the labeling requirements for wines. In light of HEWL's usefulness and its simultaneous allergenic properties, lysozyme immobilization could represent an interesting compromise for its use in wine. Having the lysozyme covalently immobilized on solid supports could allow for its removal from the wine so as to exclude the need for its presence in the label.

As reported by Liburdi, Benucci, and Esti (2014), HEWL has been successfully immobilized on various materials, including organic and inorganic polymers, using physical and chemical mechanisms. Generally chemical immobilization, such as covalent binding method, is mainly used when a reaction process does not require enzyme in the final product (Nisha, Arun Karthick, & Gobi, 2012). To date, few studies have addressed the development of covalent immobilized HEWL for winemaking applications (Liburdi, Straniero, Benucci, Garzillo, & Esti, 2012; Zacchigna et al., 1999) and no study have been conducted in real wine measuring the kinetic and antimicrobial properties of insoluble lysozyme. In the last decades, many studies have been conducted relative to HEWL immobilized on different chitosan-based materials, especially for

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food packaging applications (Duan, Park, Daeschel, & Zhao, 2007; Park, Daeschel, & Zhao, 2004; Wen et al., 2016; Yuceer & Caner, 2014). Chitosan and its derivatives, have proven to be excellent and safe candidates for food enzyme immobilization (Ge, Zhao, Mo, Li, & Li, 2012; Krajewska, 2004; Spagna, Barbagallo, Greco, Manenti, & Pifferi, 2002), mainly due to their non-toxic, biocompatible and biodegradable properties.

Moreover chitosan is considered as an antimicrobial agent when the high density of free amino group are present in the polymer structure (Liu, Du, Yang, & Zhu, 2004).

In this work, lysozyme was covalently immobilized on chitosan, the kinetic parameters and the antibacterial activity of both the free and immobilized HEWL were studied in model and real white wines using *O. oeni* as substrate.

2. Materials and methods

2.1. Materials

The lysozyme-based (E.C.3.2.1.17) enological preparation from hen egg white (HEWL) and *O. oeni* lyophilized cells, were kindly donated by Lallemand Œnologie (Blagnac Cedex, France). The chitosan beads, named Chitopearl BCW-3010 (BCW), were obtained from Wako Chemicals GmbH (Neuss, Germany).

All other chemicals were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Chemical characterization of white wines

3 white wines, from different grape cultivars (Chardonnay, Moscato and Sauvignon *blanc*), were collected in an Italian market and used for the kinetic and antimicrobial study of free and immobilized lysozyme. The wine chemical parameters, reported in Table 1, were determined with officially endorsed tools such as the European Official Methods (Regulations (EU) No 2676/90). All samples were analyzed in triplicate, and the mean and standard deviation (±SD) were reported in Table 1.

2.3. Cell substrate characterization

O. oeni cells (0.2 mg ml^{-1}), were rehydrated, washed in saline solution (NaCl 0.9%), and grown in 50 ml of modified ATB (Acidic Tomato Broth): casein peptone tryptic digest (10 g l^{-1}); yeast extract (5 g l^{-1}); glucose (10 g l^{-1}); MgSO4·7H2O (0.2 g l^{-1}); MnSO4·H2O (0.05 g l^{-1}); cysteine-HCl (0.5 g l^{-1}), and filtered tomato juice (25% v/v; Oxoid LTD., England). The cell concentration was spectrophotometrically monitored by measuring the optical density at 600 nm (OD600 nm; Shimadzu 2450 UV/VIS) to describe the *O. oeni* growth curve until the steady state was achieved (28 h at 37 °C and shaking at 170 rpm). At regular time intervals (1 h), cell culture aliquots were collected and diluted to obtain measurable OD600nm values (below 2 Unit Absorbance, UA). At the same time, 0.1 ml of conveniently diluted culture was plated on agarised ATB medium (agar 15 g l^{-1}), and bacterial cell viability was measured as the number of colony forming units (CFU) per ml. The equation of the straight line (y = 2*108x, where y: CFU/mL, x: OD600 and R2: 0.99) was then used to calculate CFU/mL values corresponding to the different absorbance values and cell substrate concentrations used in the enzymatic assay. Moreover, cell stability was tested by monitoring OD600nm for 300 s in the absence of lysozyme. The experiment was performed three times with triplicates during the spectrophotometric measuring.

2.4. Immobilization procedure

The HEWL immobilization on BCW was performed using glutaraldehyde as crosslinker (Oliveira et al., 2001). These biocatalysts (GDA-HEWL) were incubated, with slow stirring, for 2 h at room temperature. At the end of the incubation, samples were washed three times with immobilization phosphate buffer (Na_2HPO_4/NaH_2PO_4 0.1 M, pH 7) and then let stand for 20 min in a 0.1-M glycine solution.

At the end of immobilization time, supports were washed three times with 2 M NaCl solution to remove all non-covalently bound proteins. Immobilization yield (IY, %) was determined by Bradford's method (Bradford, 1976), using *Coomassie* brilliant blue reagent and measuring absorbance at 595 nm. Bovine serum albumin (BSA) was used as standard.

2.5. Assay of HEWL catalytic activity

Kinetic characterization of free or immobilized lysozyme was carried out at 25 °C, both in model wine (tartaric buffer, pH 3.2, ethanol 12% v/v) and in 3 different white wines (Chardonnay, Moscato and Sauvignon blanc) using *O. oeni* as substrate. The cell lysis was detected by measuring the decrease in optical density at 600 nm (OD_{600}) using a Perkin-Elmer Lambda 25 UV/VIS (Beaconsfield Buks, B).

The assay mixture contained increasing amounts of cell substrate (1×10^8–4×10^8 CFU mL^{-1}), 0.5 mL of sucrose (0.27 M) for cells membrane stabilization, 0.5 mL of saline solution (NaCl 0.9%), 0.1 mL of lysozyme (2 mg ml^{-1}) and tartaric buffer (pH 3.2) or real wine to reach a final volume of 3.5 mL.

The lytic reaction was carried out in 4-mL cuvettes with a 1 cm light path for 300 s at 600 nm with stirring at 25 °C in a SHIMADZU UV 2450 spectro-photometer with a thermostated cell (MPM Instruments Type M 900-TI). One unit of lysozyme activity (U.) was defined as a decrease of 0.001 OD_{600} units/min. For each trial a blank correction was made, using a sample not containing enzyme.

The activity of immobilized lysozyme (GDA-HEWL) was determined by adding 1 g of lysozyme immobilized chitosan beads to the cell suspension (final volume 3.5 ml, as described previously) and agitating at 50 rpm with end-over-end rotation. After 30 min, the cell suspension was separated from the biocatalyst. The OD_{600} was measured before and after the treatment to calculate the activity. In order to consider the chitosan antimicrobial activity, a blank assay with the carrier beads without LYZ, was done to account the OD_{600} loss due to non-enzymatic reactions.

Table 1
Chemical parameters measured in the three white wines: Chardonnay, Sauvignon b. and Moscato.

Wine	pH	Titratable acidity (tartaric acid, g*L^{-1})	Alcoholic degree (ethanol, % v/v)	Total SO_2 (mg*L^{-1})	Free SO_2 (mg*L^{-1})	Malic acid (g*L^{-1})	Polyphenols (mg*L^{-1})
Chardonnay	3.4 (±0.02)	5.6 (±0.4)	13.5 (±0.7)	90 (±3.2)	18 (±0.9)	1.3 (±0.2)	191 (±7.2)
Moscato	3.5 (±0.01)	5.4 (±0.6)	12.9 (±0.6)	109 (±4.5)	24 (±1.5)	0.5 (±0.03)	423 (±12.3)
Sauvignon blanc	3.3 (±0.01)	5.9 (±0.3)	12.7 (±0.8)	83 (±2.1)	13 (±1.1)	0.9 (±0.06)	140 (±11.8)

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