LWT - Food Science and Technology 73 (2016) 320–325

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Inhibitory effect of ethanol, sulphur dioxide and proanthocyanidinic tannins on lysozyme antimicrobial activity in model wine

Ilaria Benucci, Elena Cappannella, Katia Liburdi*, Marco Esti

Dept. for Innovation in Biological, Agro-food and Forest Systems, University of Tuscia, Via S. Camillo de Lellis, 01100 Viterbo, Italy

ARTICLE INFO

Article history:
Received 3 March 2016
Received in revised form
26 May 2016
Accepted 13 June 2016
Available online 15 June 2016

Keywords:
Hen egg white lysozyme
Oenococcus oeni
Ethanol
Sulphur dioxide
Proanthocyanidinic tannins

ABSTRACT

The antimicrobial activity of lysozyme from hen egg white (HEWL) was tested against *Oenococcus Oeni* cell as substrate in wine-like acidic medium, composed of tartaric acid buffer (pH 3.2) fortified with ethanol (EtOH), free sulphur dioxide (SO_2), grape skin and seed tannins, within the average range of their concentration in wine in order to identify, for each compound, the nature, the mechanism and the extent of inhibition. All of the tested wine constituents were reversible inhibitors for HEWL activity, with a limited inhibiting effect observed for both EtOH and free SO_2, which proved to be competitive and mixed-type inhibitors, respectively. Contrarily, proanthocyanidinic tannins had the strongest inhibiting effect, which affected muramidase activity in two different ways: grape skin tannins proved to be uncompetitive while seed tannins were mixed-type inhibitors.

1. Introduction

Lysozyme from hen egg white (HEWL) is a natural protein with bactericidal activity, extensively used in food industry against Gram-positive bacteria (Cappannella et al., 2016), whom application is permitted by the European food legislation (Liburdi, Benucci, & Esti, 2014). HEWL catalyzes the hydrolysis of the β (1,4) glycosidic bond between the N-acetylmuramic acid and the N-acetyl-D-glucosamine of peptidoglycan (muramidase activity), leading to the degradation of peptidoglycan in the cell wall of Gram-positive bacteria, resulting in cells lysis (Lasanta, Roldán, Caro, Pérez, & Palacios, 2010).

In winemaking, HEWL can be used to limit the spontaneous growth of lactic acid bacteria, thus controlling malolactic fermentation (Azzolini, Tosi, Veneri, & Zapparoli, 2010; Bartowsky, 2009; Tirelli & De Noni, 2007), with the aim of reducing the sulphur dioxide (SO_2) dosage (Bartowsky, Costello, Villa, & Henschke, 2004; Liburdi et al., 2014; Wu & Daeschel, 2007). Among lactic acid bacteria, *Oenococcus oeni* is the most important involved in accomplishing the malolactic fermentation, since many strains of this species are well adapted to survive and grow in wine (Bartowsky, 2005; Cappello, Stefani, Grieco, Logrieco, & Zapparoli, 2008).

Several studies have described the effects exerted on HEWL antimicrobial activity by some wine constituents such as SO_2, which is usually added to wine due to its anti-microbial and anti-oxidant properties (Palenzuela, Simonet, Rìos, & Valcàrcel, 2005) and grape polyphenolic compounds (Lasanta et al., 2010; Tirelli & De Noni, 2007). A significant depletion of muramidase activity was observed in presence of free SO_2 (Green & Daeschel, 1994; Lasanta et al., 2010; Tirelli & De Noni, 2007), due to its ability to inactivate numerous enzymes by splitting their disulphide linkages. Moreover, both free SO_2 and H_2SO_3 were able to convert disulphide bonds of enzymes or proteins into thiosulphonates and thiols (Esti, Benucci, Liburdi, & Garzillo, 2011; Malhotra & Hocking, 1976).

The biological activity of phenolic compounds as enzyme inhibitors has been extensively reported (Haslam, 1996). Most of these molecules in wine derive from grape berries and are anthocyanins and condensed tannins, also known as proanthocyanidins (Gonçalves, Soares, Mateus, & de Freitas, 2007). Several studies have proved that tannins act as protein-complexing agents (Prigent et al., 2009; Rawel, Meidtner, & Kroll, 2005), resulting in the denaturation of some enzymes as well as inhibiting proteases (Gonçalves et al., 2007; Liang, Huang, & Kwok, 1999). Furthermore, the interaction between proanthocyanidins and HEWL was investigated, demonstrating the influence of these compounds on muramidase activity (Guzzo, Cappello, Azzolini, Tosi, & Zapparoli, 2011; Tirelli & De Noni, 2007).

Although various authors have investigated the effect of SO_2 and

* Corresponding author.
E-mail address: k.liburdi@unitus.it (K. Liburdi).

http://dx.doi.org/10.1016/j.lwt.2016.06.035
0023-6438/

phenolic compounds on HEWL antimicrobial activity (Lasanta et al., 2010; Palenzuela et al., 2005; Tirelli & De Noni, 2007), to our knowledge an inhibition study of the main wine constituents has yet to be carried out. An inhibitor can be defined as any substance that reduces the velocity of a reaction catalysed by an enzyme (Esti et al., 2011) and inhibition studies are useful for determining the nature of the interaction between E and I (reversible or irreversible inhibition), as well as the mechanisms that cause this phenomenon (competitive, uncompetitive or mixed-type inhibition) and the extent of inhibition (inhibition constant value, K_i and K_i'). As stated by other authors, graphical methods represent the common tool in the diagnosis of enzyme inhibition (Antunes, Marinho, Barreto, Pavão, & Pinto, 2003) and they are often used to estimate the inhibition constants (Cornish-Bowden, 1974; Cortes, Cascante, Cardenas, & Cornish-Bowden, 2001).

With the aim of gaining greater insight into the above-mentioned issue and optimizing the application of HEWL for controlling the spontaneous *O. oeni* growth in wine, this paper was aimed to carry out an inhibition study, in model wine, of the potential inhibitors naturally present in wine, such as EtOH, free SO_2, grape skin and seed tannins, by identifying the nature, the mechanism and the extent of inhibition for each compound.

2. Materials and method

2.1. Materials

HEWL (EC 3.2.1.17; systematic name: peptidoglycan n-acetylmuramic hydrolase) and *O. oeni* lyophilised cells (lot no. E6003/3014/8032) are oenological preparation that were kindly supplied by Lallemand Inc. (Italy). The protein content of HEWL preparation was 8% (Bradford, 1976).

Grape skin and seed tannins, as preparations intended for oenological use, were supplied by EVERINTEC (Venice, Italy). EtOH and all the other chemicals were of analytical grade which were purchased from Sigma-Aldrich (Milano, Italy).

2.2. Muramidase activity

The activity of HEWL toward a microbial substrate (*O. oeni*) was investigated at 20 °C both in tartaric acid buffer (TB, a solution of tartaric acid/sodium tartrate at 0.03 mol, pH 3.2) and in model wine mimicking wine conditions (TB-EtOH, consisting of TB with ethanol 12%v/v).

The cell lysis was detected by measuring the decrease in OD_{600nm} *vs* time (Deckers, Vanlint, Callewaer, Aertsen, & Michiels, 2008; Esti, Liburdi, Palumbo, Benucci, & Garzillo, 2014) in 4-ml quartz cuvettes (1 cm light path), where the following reagents were mixed to reach a final volume of 3.5 ml: 0.5 ml of sucrose (0.27 M), 0.5 ml of saline solution (NaCl 0.9%), 0.1 ml of HEWL (1 g/l), tartaric acid buffer, an increasing amount of substrate (0–5.7 g/l) and the following potential inhibitors at various concentrations: EtOH (0, 6, 9, 12, 15% v/v), free SO_2 (0, 0.005, 0.01, 0.018, 0.025 g/l), grape skin (0, 0.03, 0.05, 0.08, 0.1 g/l gallic acid eq) and seed (0, 0.03, 0.05, 0.08, 0.1 g/l gallic acid eq) tannin preparations. The lytic reaction was monitored in continuous mode at 20 °C using a spectrophotometer (Shimadzu UV-2450) equipped with a thermostat cell (MPM Instruments Type M 900-TI) with magnetic stirring. One unit of HEWL activity (U) was defined as a decrease of 0.001 OD_{600} 1/min. Moreover, HEWL specific activity was expressed, considering HEWL protein content.

2.3. Inhibition study

In this paper graphical methods have been applied in order to achieve the diagnosis of enzyme inhibition and the estimation of inhibition constants, as suggested by Segel (1975). Irreversible and reversible inhibition have been distinguished by plotting ΔA/min *vs* $[E]_t$, where $[E]_t$ represents the amount (μl) of enzyme added to the assay. If an irreversible inhibitor is present, the "plus inhibitor" curve has the same slope as the control curve, but it intersects the horizontal axis at a position equivalent to the amount of enzyme that is irreversibly inactivated. For a reversible inhibitor, the curve has a smaller slope than the control curve and goes through the origin (Esti et al., 2011).

Assuming that only a single substrate is involved in the reaction and that only one type of inhibitor is present at any time, the interaction between a reversible I and E, or ES, can be described by various inhibition models: competitive, uncompetitive and mixed-type inhibition. The equilibria (eq. (1) and eq. (2)):

$$E + I \underset{K_i}{\rightleftharpoons} EI \tag{1}$$

$$ES + I \underset{K_i'}{\rightleftharpoons} ESI \tag{2}$$

are defined by the thermodynamic constants, Ki or Ki', respectively:

$$K_i = \frac{[E][I]}{[EI]} \tag{3}$$

$$K_i' = \frac{[ES][I]}{[ESI]} \tag{4}$$

The inhibition constant value reflects the concentration of an inhibitor, which reduces the rate of an enzyme-catalysed reaction by 50%. These definitions and equilibria describe the various types of enzyme inhibition. For each inhibition model (competitive, uncompetitive or mixed-type inhibition), the kinetic equation used is a modification of Michaelis-Menten, in which the parameters K_M and V_{max} are replaced by the corresponding apparent kinetic parameters $K_{M(app)}$ and $V_{max(app)}$ (Segel, 1975).

2.4. Determination of kinetic parameters

The kinetic parameters (V_{max} and K_M) of HEWL were determined according to the Michaelis–Menten equation, fitting experimental data by a non-linear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.). The goodness-of-fit of each data set to its best-fit theoretical kinetic curve was assessed as the square of the correlation coefficient (r^2). K_M (Michaelis–Menten constant) is equal to the substrate concentration when the initial velocity is one-half of the maximum velocity (V_{max}), thus indicating catalytic efficiency.

3. Results and discussion

3.1. Inhibition nature

In winemaking, the presence of various compounds (i.e. EtOH, free SO_2, grape skin and seed tannins) could affect the application of HEWL by reducing its antimicrobial activity (Green, 1995; Guzzo et al., 2011; Lasanta et al., 2010; Tirelli & De Noni, 2007). For each potential inhibitor, a preliminary trial was carried out in order to identify the nature of the interaction between E and I (reversible or irreversible inhibition),using a graphical method, which consists in plotting ΔA/min *vs* the lysozyme volume in the assay.

The "inhibitor added" curves had a smaller slope than the control curve, and passed through the origin (Fig. 1). Therefore, all the inhibitors tested (EtOH, free SO_2, skin and seed tannins) proved