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Immobilized pineapple stem bromelain activity in a wine-like medium: Effect of inhibitors

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A B S T R A C T

The catalytic activity of stem bromelain, covalently immobilized on chitosan beads, was characterized towards a synthetic substrate (Bz-Phe-Val-Arg-pNA), in a wine-like acidic medium containing wine inhibitors (ethanol, sulphur dioxide, grape skin and seed tannins), at their average concentration range.

For all tested substances, the inhibition constant (K_i) values of immobilized bromelain were significantly higher than the corresponding values obtained in a previous work for free enzyme, thus indicating that direct covalent immobilization on chitosan beads makes protease more resistant to the inhibition effect.

Immobilized protease was affected by ethanol inhibition (competitive type) only if present at abnormal concentration for real wine. Grape skin and seed tannins exerted uncompetitive inhibition to the same extent, with a K_i value close to 1 g $l^{-1}_{\text{gallic acid eq}}$. The effect of free sulphur dioxide on immobilized bromelain changed respect to free enzyme, both becoming uncompetitive inhibitor and dramatically lowering its strength.

These results show that immobilized stem bromelain could have productive biotechnological applications in wine-making, even though further studies will be necessary to test its proteolytic activity towards wine proteins in real matrices.

Keywords: Pineapple stem bromelain; Covalent immobilization; Wine inhibitors; Wine-like medium

1. Introduction

The addition of adsorbent materials, such as bentonite, is a non-specific common practice, applied in wine industry for the prevention of protein haze. Despite its effectiveness, bentonite fining adversely affect the quality of treated wine, through the removal of colour, flavour and texture compounds (Waters et al., 2005).

The possibility of stabilizing white wine by acid proteases, able to hydrolyze specifically proteins and inert respect to other wine compounds, appears of great interest. Nevertheless, currently, enzyme application, in free form, is limited by the increased request of additive-free food, prevailing in some European countries (Feijoo-Siota and Villa, 2011). For this reason, growing attention has been given to the application of proteases immobilized on solid support, which could be apply for wine protein stabilization, by a continuous treatment, in different bioreactor configurations with no enzyme residual contamination. Stem bromelain, a cysteine proteinase extracted from the stem of pineapple plant, has been immobilized on various supports (organic and inorganic) and by different methods (Seo et al., 1998; Tan et al., 2008; Shi et al., 2007; Krajewska, 2004; Mahmood and Saleemuddin, 2007; Gupta et al., 2007).

Enzyme immobilization, achieved by fixing a biocatalyst to or within solid supports, is of great interest for applications in the food industry. Compared with their free disposable forms, immobilized enzymes avoids product contamination in the food reaction mixture, allowing their reusability (Yodoya et al., 2003).

Moreover, immobilization by covalent linkage could induce enzyme structure modification to overcome the inhibitory

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effect of different chemical compounds as well as to limit protease self-digestion phenomena (Kim, 2005). Recently, stem bromelain has been covalently and directly linked to chitosan beads without glutaraldehyde, and this food-safe biocatalyst appeared to be suitable for application in a wine-like medium (Benucci et al., 2012). Furthermore, the influence of expected inhibitors on free bromelain activity that is usually present in wine has been investigated, showing that all tested substances (ethanol, sulphur dioxide, grape skin, grape seed, ellagic and gallic tannins) were reversible inhibitors for the protease in free form (Esti et al., 2011). The strongest mixed-type inhibitor was found to be free sulphur dioxide, which is added to wine, especially white wine, during the winemaking process to prevent undesirable microbial growth and oxidation processes (Segundo and Rangel, 2001; Toniolo et al., 2010).

The present work, together with the above cited manuscripts, completes our preliminary evaluation phase, carried out using a wine-like acidic medium, of protease feasibility as a sustainable practice for white wine selective protein stabilization, and an alternative to bentonite fining. The aim of the study was to characterize the effect of inhibitors, which are usually present in wine (over their average range of concentration), on the activity of stem bromelain, covalently immobilized on chitosan beads.

Considering that protein insolubilization and wine turbidity phenomena remain unclear and the triggering event is not caused solely by the protein molecules themselves (Hsu and Heatherbell, 1987; Vincenzi et al., 2011; Batista et al., 2009), bromelain activity has been evaluated towards a synthetic substrate.

2. Materials and methods

2.1. The enzyme and chemicals

Stem bromelain (EC 3.4.22.32) from Sigma–Aldrich (Milan, Italy) was immobilized on chitosan beads (Chitopearl BCW-3010, Wako Chemicals GmbH, Neuss, Germany). The synthetic peptide substrate Bz-Phe-Val-Arg-p-nitroaniline (pNA) was purchased from Bachem, Germany. Grape skin and seed tannins, as preparations intended for enological use, were kindly supplied by EVERINTEC (Venice, Italy). All other reagents were obtained from Sigma–Aldrich (Milan, Italy).

2.2. Immobilization procedure

Stem bromelain was covalently immobilized on Chitopearl BCW-3010 by a direct mechanism, which probably involves the protease carboxyl groups of Asp or Glu residues and the amino groups of the support.

One ml of enzyme preparation (5 mg ml^{-1}), previously solubilized in tartaric buffer (tartaric acid/sodium tartrate 0.03 M, pH 3.2) was added to 100 mg of chitosan beads, and then incubated at room temperature for 12 h (Benucci et al., 2012).

2.3. Determination of immobilization yield

At the end of the immobilization time, the supports were washed three times with 2 M NaCl solution in order to remove all non-covalently bound proteins. The biocatalysts were recovered by centrifugation. After collection of the supernatant, the beads were resuspended in tartaric buffer and centrifuged. All supernatants were collected and diluted with the above buffer solution to a constant final volume in order to determine the bound protein.

IY (%) was determined by Bradford's method (Bradford, 1976), using Coomassie brilliant blue reagent and measuring absorbance at 595 nm. BSA was used as standard. The amount of bound protein was then determined indirectly from the difference between the amount of protein in solution before and after immobilization. All measurements were made in triplicate and the standard deviations were reported.

2.4. Enzymatic activity assay

Immobilized bromelain activity was assayed using Bz-Phe-Val-Arg-pNA as a substrate, at 25 °C in a wine-like acidic medium (tartaric acid/sodium tartrate 0.03 M, pH 3.2). Several concentrations of Bz-Phe-Val-ArgpNA, ranging from 0 to 200 μM, were tested in the presence of immobilized biocatalyst (100 mg).

Cleavage of the substrate results in release of free pNA, which was detected colorimetrically at 410 nm. Immobilized bromelain activity was determined measuring the change in absorbance *vs.* time for 30 min using a Shimadzu UV 2450 (Milan, Italy). The specific activity of immobilized bromelain, calculated in I.U. of pNA produced ($\varepsilon_{mM} = 8.480\ mM^{-1}\ cm^{-1}$ for pNA), was expressed as I.U. mg^{-1} of immobilized protein (Hale et al., 2005; Benucci et al., 2011). A blank correction was made using a sample that did not contain enzymes. All measurements were made in triplicate, and the standard deviations were reported.

2.5. Kinetic study and determination of kinetic parameters

A kinetic study was carried out by varying the substrate concentration (0–200 μM) at 25 °C in the presence of immobilized stem bromelain in a wine-like acidic medium, which also contained one of the following potential inhibitors (I) at different concentrations: ethanol (0, 12, 18%, v/v), free SO_2 (0, 10, 25 mg l^{-1}), skin tannin preparation (0, 0.5, 2 g l^{-1} corresponding to 0, 0.25, 0.99 $g\,l^{-1}_{gallic\ acid\ eq}$) and seed tannin preparation (0, 0.5, 2 g l^{-1} corresponding to 0, 0.30, 1.20 $g\,l^{-1}_{gallic\ acid\ eq}$).

Kinetic curves were obtained measuring proteolytic activity at different substrate concentrations (7–8 points), making three turns of measurements for each concentration. The kinetic parameters (k_{cat}, K_m, K_a) of stem bromelain were determined according to the Michaelis–Menten equation using a nonlinear regression procedure (GraphPad Prism 5.0, GraphPad software, Inc.).

The K_M value reflects the enzyme substrate complex formation, whereas k_{cat} measures the number of substrate molecules turned over per enzyme per minute. Moreover, k_{cat} is indicative of the product release velocity, and represents the maximum number of moles of substrate converted to the product per number of moles of catalyst per unit time. This parameter can be obtained from the equation $k_{cat} = V_{max}/[E]_{tot}$, where $[E]_{tot}$ is the enzyme molar concentration. In addition, the K_a, being the ratio k_{cat}/K_M, indicates the affinity of the enzyme towards the substrate. It is indicative of both reaction steps and expresses the overall catalytic efficiency.

2.6. Inhibition study

For each tested compound, the effect on immobilized bromelain activity was evaluated, identifying the inhibition type