



Study of Two Different Immobilized Acid Proteases for Wine Application

Katia Liburdi, Ilaria Benucci, and Marco Esti

Food Science and Technology Department, University of Tuscia, Viterbo, Italy

This study compares the hydrolytic efficiency in model wine (pH 3.2 tartaric acid buffer with 12% v/v ethanol) of two immobilized enzymes. Food-grade acid proteases (from *Aspergillus saitoi* and stem bromelain) were covalently immobilized on two solid supports, aminopropyl controlled pore glass beads and Eupergit®C. The effects of pore diameter, immobilization procedure, and protein properties on the activity of biocatalysts were evaluated to determine the feasibility of immobilized proteases in wine. The results expressed in terms of yield of immobilized enzyme, enzyme activity, and kinetic parameters ($V_{\max}$ and K_m) demonstrated that the best performance of both proteases was when immobilized in Eupergit®C.

Key Words: acid proteases; aminopropyl porous glass (APG) beads; covalent immobilization; Eupergit®C; model wine

INTRODUCTION

The presence of residual unstable proteins in wine is a concern for winemakers because these molecules can precipitate during storage, causing the appearance of sediments and hazes (Bakalinsky and Boulton, 1985). Such wine is considered defective and unmarketable unless properly treated and rebottled. Generally, the fine bentonite is the preferred and most commonly used method of inducing protein wine stability, as it absorbs proteins by cation exchange and preferentially removes those proteins most positively charged at wine pH. However, this treatment has disadvantages; in particular, bentonite is considered as responsible for the simplification of the aromatic wine profile and for the loss of color and compounds useful for the wine structure (Høj et al., 2000).

An alternative approach is the enzymatic hydrolysis of proteins into smaller, more soluble polypeptide molecules, thereby reducing the incidence of precipitation. This requires proteolytic enzymes that are active in a mildly acid medium and such enzymes are often referred to as acid proteases. As proteolytic enzyme mixtures, the protease could be added to the wine in the free form, but they can degrade themselves and cannot be recycled. Moreover, the soluble enzymes may be less efficient and may remain in the product. Therefore, they may catalyze further undesirable reactions. In order to improve the efficient use of enzyme and decrease the cost of biocatalysis application, enzyme immobilization technology could be employed. It is an effective means that leads to enzyme reuse and improved stability. In particular, proteases have been successfully immobilized on many different types of carriers (Tang et al., 2006; Ding et al., 2001; Ganesh Kumar et al., 2009).

Protein immobilization is generally carried out by adsorption or entrapment and encapsulation in polymeric substances, as well as by covalent bonds to solid matrices. The covalent coupling of enzymes to insoluble matrices has been by far the most commonly used technique, and covalently bonded enzymes, usually obtained using mild reaction conditions, show higher protein stability. Moreover, proteinases have been successfully immobilized on many different type of carriers with a variety of chemical and physical characteristics, such as controlled amine terminal magnetic particle (Ding et al., 2001), chitosan beads (Leuba and Widmer, 1979), extrusion soybean fiber (Cheng, 2002), and magnetic polymer microsphere containing styrene, hydroxyethyl methacrylate, divinyl benzene, and Fe_3O_4 (Li et al., 1994).

The porous aminopropyl glass (APG) are widely use as support for chemical immobilization. The several advantages of APG are well known, namely the variety of available controlled shapes, sizes and characteristics, developed methodologies for covalent coupling of enzymes, stability against chemical substances, and physical resistance to mechanical effects (Gómez et al., 2006). Nevertheless, considering that APG is rather expensive support, a cheaper material such as the Eupergit[®]C was also investigated. EupergitC are microporous, epoxy-activated acrylic beads; the immobilization on these resins is rapid and easy, both at laboratory scale and industrial scale and does not require additional reagents or special equipments (Boller et al., 2002).

In this study, the development and the application to a model wine of acid proteases immobilized on APG and EupergitC was investigated. The catalytic activities, which are routinely evaluated to indicate the effectiveness of a proposed immobilization procedure, were determined. Moreover, taking into account that the chemical modification and the properties of support may influence diffusion related restrictions associated with the immobilized enzyme, the kinetic parameters were determined.

Specifically, the aims of this study were a) to evaluate the feasibility of immobilization of two acid proteases from microbial and plant sources for

use in acid environments of wine; and b) to assess the effect of immobilization method using two different supports, APG and Eupergit C, on enzyme activity.

MATERIALS AND METHODS

Reagents

The two acid proteases were commercial solid preparations: an aspartic protease from *Aspergillus saitoi* (As) (EC 3.4.23.18, 1 U/mg of solid with 9.1 U/mg of protein using haemoglobin as substrate), and a cysteine proteinase, bromelain from pineapple stem (Br), (EC 3.4.22.32, 2.86 U/mg of solid and 2 U/mg of protein using N α -Z-L-lysine p-nitrophenyl ester as substrate), both purchased from Sigma-Aldrich (Milan, Italy). Disodium phosphate, trichloroacetic acid (TCA), and all other chemicals were obtained from Merck AG (Darmstadt, Germany). Eupergit C (Eup), epoxy-activated acrylic beads with a diameter of 100–250 μ m, were a gift from Degussa Rohm GmbH&Co. (KG, Pharma Polymers, Darmstadt, Germany); aminopropyl controlled pore glass beads (APG) (500Å pore size, 200–400 mesh) were from Sigma-Aldrich (Milan, Italy). Analytical grade reagents, without any additional purification, were used to prepare all solutions in distilled and deionised water.

Enzyme Immobilization

Immobilization on APG

For this enzyme immobilization, a procedure described in the literature was followed (Walt and Agayn, 1994). The quantity of 0.1 g of APG was activated with 1 mL of a glutaraldehyde (GA) solution (2.5%; v/v) in 0.1 M of pH 7.0 phosphate buffer solution (Guisàn et al., 1997). The suspension was maintained under constant agitation for 3 h and then washed profusely with distilled water. Thereafter, 2 mL of the enzymatic solution (5 mg mL⁻¹) was added, slowly stirred for 1 h at room temperature and then for 4 h at 4°C. The glass beads were finally separated by filtration, washed 3 times with phosphate buffer, once with 1 M NaCl and left in repose for about 20 min in a 75 mM glycine solution at 4°C.

Immobilization on Eupergit C

This immobilization was carried out in 1 M sodium phosphate pH 7 at room temperature for 20 h, using 0.1 g of Eupergit C and 1 mL of enzyme suspension (5 mg mL⁻¹). The mixture was maintained at room temperature during 24 h. Finally, the derivatives were washed with an excess of distilled water and stored at 4°C.

Determination of Bound Protein

The yield of immobilized protein (Y%), expressed as percentage of removed protein, was determined by the ratio of the difference of its amount present in the solution before (P_i) and after coupling to supports (P_f) respect the initial value:

$$Y (\%) = \frac{P_i - P_f}{P_i} \times 100 \quad (1)$$

The protein concentration was spectrophotometrically (Abs 595 nm) determined using bovine serum albumin as reference standard (Bradford, 1976). The values reported are means of triplicate $\pm$ standard deviation ($\pm$ SD) measurements.

Protease Activity

Protease activity was assayed using casein as substrate, following the method described by Anson (1938). Briefly, the enzymatic solution used for the immobilization or the immobilized enzyme, were incubated in model wine (pH 3.2 tartaric acid buffer with 12% v/v ethanol) using saturated casein solution (12 mg mL⁻¹) as substrate. Tyrosine released from casein hydrolysis was detected after reaction with Folin Ciocalteu reagent at 660 nm with a spectrophotometer (Perkin-Elmer Lambda 25 UV/VIS; Beaconsfield-Buys, B), using 10-mm silica cuvette. One unit of protease activity (U) is defined as the amount of enzyme able to produce 1 μ mol of tyrosine per min at pH 3.2 (20°C), using saturated casein solution. The reaction rate (rt) was calculated as μ mol mL⁻¹ min⁻¹ of tyrosine produced at pH 3.2 (20°C), by the following expression:

$$rt (\text{mmol}^{-1} \text{min}^{-1}) = \frac{\Delta A/\text{min}}{0.6896} \quad (2)$$

where 0.6896 (mM⁻¹ cm⁻¹) is the molar extinction coefficient (ϵ) of tyrosine released from casein.

Specific activity of free (U_f) and immobilized proteases (U_i) was estimated by dividing the reaction rate (rt) by the amount of protein in the enzymatic solution (Y_f) and by the amount of bound protein (Y_b), respectively, as:

$$U_f \text{ mg}^{-1} \text{ protein} = rt/Y_f \quad (3)$$

$$U_i \text{ mg}^{-1} \text{ bound protein} = rt/Y_b \quad (4)$$

To study the enzymatic stability, 100 mg of immobilized biocatalysts were incubated in 50-mL flask, containing 25 mL of model wine maintained on plate-rotating agitation at 400 rpm for 6 d. The U_i was determined measuring, for each assay (every 24 h), the total tyrosine content of the same reaction mixture at 20°C, after a daily casein addition. Values reported are means of triplicate $\pm$ standard deviation measurements.

Retained Enzyme Activity Ratio

This parameter represents the activity retained from immobilized proteases as a consequence of the enzyme immobilization as well as its inactivation. The retained activity ratio (Ra) was calculated by:

$$Ra (\%) = \frac{U_i}{U_f} \times 100 \quad (5)$$

where U_f and U_i are the total units of proteases activity before and after immobilization process, respectively, measured as described in previous paragraph. Values reported are means of triplicate $\pm$ standard deviation ($\pm$ SD) measurements.

Kinetic Study of Immobilized Enzyme

Protease activity measurements of immobilized biocatalysts were checked by varying casein concentration at constant temperature (20°C) and pH (3.2) according to the Anson (1938) method as previously described.

Kinetic parameters, maximum reaction rate ($V_{\max}$), and Michaelis–Menten constant (K_m) were determined for both immobilized enzymes using the Michaelis–Menten equation:

$$V = \frac{[S] V_{\max}}{[S] + K_m} \quad (6)$$

where $[S]$ is the casein concentration (0–0.35 mg mL^{−1}) and V is the initial reaction rate of the enzyme (mmol mL^{−1} min^{−1}), calculated as mmol of tyrosine produced mL^{−1} min^{−1} at pH 3.2 (20°C), using its molar extinction coefficient (ϵ) of 0.6896 mM^{−1} cm^{−1}. K_m and $V_{\max}$ were estimated using a nonlinear regression by GraphPad Prism 5.0 (GraphPad software, Inc.), the values reported are means of triplicate $\pm$ standard deviation ($\pm$ SD) measurements.

RESULTS AND DISCUSSION

Influence of Immobilization Procedure on Protease Performance

The results of the immobilization process, expressed as yield of immobilized protein (Y %), specific activity of free (U_f) and immobilized proteases (U_i) and retained enzyme activity (Ra%) are reported in Table 1.

Both supports showed a good protein-binding capacity for the two enzymatic extracts (>55%), indicating that the chosen immobilization reactions are all effective. The Y (%) measured for Br-catalysts on APG and Eupergit C were 78% and 85%, respectively. These values resulted in significantly higher activity than that measured for *A. saitoi* protease (56% for APG and 70% for Eupergit C). These results should be explained in terms of nucleophilic property of cysteine (7.4 residues/mol) (Husain and Lowe, 1968) that can be considered an exceptional ligand due to the chemical reactivity of its thiol group (Giles et al., 2003).

The Ra (%), calculated comparing the specific activity of free (U_f) and immobilized (U_i) proteases, is one of the most important aspects in the enzyme immobilization process. The low Ra values measured for immobilized biocatalysts indicate that immobilization procedure had profound effect on the activity of immobilized proteases. However, the As-Eup and Br-Eup showed higher retained activity (14% and 13%, respectively) with respect to As-APG and Br-APG (6% and 2%, respectively). It can be explained by enzyme partial denaturation resulting from glutaraldehyde action (Benkhelifa et al., 2005) that causes the “rigidification” of enzyme and/or by the steric hindrance which prevents the substrate from reaching the active site.

The differences between two supports were determined. The best performances obtained for proteases immobilization on Eupergit C were probably due to the higher specific reactive surface of the support, and to the incubation conditions such as, longer time (24 h) and higher reaction temperature (25°C), that are recognized to substantially increase the formation of covalent linkages between many proteins and different supports (Guisàn et al., 1991).

Kinetic Parameters

Kinetic parameters were estimated by the nonlinear regression of initial reaction rate versus substrate concentration [Casein] plots (Fig. 1). Different kinetic parameters of immobilized proteases were reported and compared in Table 2.

The K_m values indicate evidence to the extent to which the immobilized enzyme has access to the substrate. The lower value of K_m represents higher affinity between enzyme and substrate; *A. saitoi* and bromelain immobilized on Eupergit C (As-Eup, Br-Eup) had lower K_m values than other biocatalysts, indicating higher affinity of the two enzymes on the acrylic carrier towards

Table 1: Parameters obtained from proteases immobilization process on APG and Eupergit®C. Yield of immobilized protein (Y %) were determined by the ratio of the difference of protein amount present in the solution before and after coupling to supports. Specific activity of free (U_f) and immobilized proteases (U_i) was estimated by dividing the reaction rate (r_t) by the amount of protein in the enzymatic solution and by the amount of bound protein. Retained activity ratio (Ra) was calculated dividing the total units of proteases activity before (U_f) and after immobilization process (U_i).

	Immobilized enzyme				Free enzyme	
	As-APG	As-Eup	Br-APG	Br-Eup	As	Br
Yield of immobilized protein (Y) (%)	56 (± 3)	70 (± 2)	78 (± 1)	85 (± 2)	—	—
Specific activity (mmol min ⁻¹ mg ⁻¹ of protein)	(U _f)	—	—	—	0.29 (± 0.00)	0.34 (± 0.00)
Retained activity (Ra) (%)	(U _i)	0.02 (± 0.00) 6 (± 1)	0.04 (± 0.00) 14 (± 1)	0.01 (± 0.00) 2 (± 0.5)	0.045 (± 0.02) 13 (± 1)	— — —

Values are mean (± SD) of three replications.

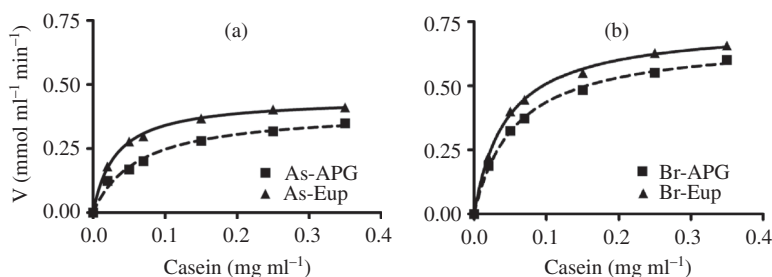


Figure 1: Michaelis-Menten plots of: a) *A. saitoi* proteases (As-APG, As-Eup) and b) Bromelain (Br-APG, Br-Eup) immobilized on APG and Eupergit®C, respectively. Kinetic curves were defined using a nonlinear regression by GraphPad Prism 5.0.

Table 2: K_m and $V_{\max}$ of *A. saitoi* protease (As-APG, As-Eup) and bromelain (Br-APG, Br-Eup) immobilized on APG and Eupergit®C, respectively, measured varying casein concentration (0–0.35 mg mL^{-1}) at constant temperature (20°C) and pH (3.2).

Kinetic parameters	Biocatalysts			
	As-APG	As-Eup	Br-APG	Br-Eup
$V_{\max}$ ($\text{mmol mL}^{-1} \text{min}^{-1}$)	0.40 (± 0.02)	0.45 (± 0.01)	0.68 (± 0.01)	0.73 (± 0.01)
K_m (mg mL^{-1})	0.06 (± 0.01)	0.03 (± 0.002)	0.06 (± 0.003)	0.04 (± 0.002)

Kinetic parameters were estimated using a nonlinear regression by GraphPad Prism 5.0. Values are mean ($\pm$ SD) of three replications.

casein compared to APG biocatalysts. The lower values of $V_{\max}$ for both enzymes immobilized on APG explained by corresponding lower values of Y and R_a could be attributable to conformational changes in the tertiary structure of the enzyme that occurred in the course of immobilization (Ayhan et al., 2002).

Enzyme Stability

The activity of two immobilized microbial proteases during time (Fig. 2) shows that the hydrolyzing action of both biocatalysts decrease during the 6 d of evaluation and the lower value was measured for As-APG at the end of experiment (1.3 U g^{-1} of bound protein). After multiple use, the best operational stability was exhibited by As-Eup (loss of 57% of its initial value in 6 d), whereas the As-APG activity decreased more than 70% in the first 2 d. Finally, *A. saitoi* protease on Eupergit C revealed the highest and lasting catalytic activity in model wine with added casein.

The response of bromelain on APG and Eupergit C was similar to As-catalysts (Fig. 3). Analogous to the data observed for As-Eup, Br-Eup revealed higher specific activity in comparison with Br-APG at the start and the end of evaluation. The lower hydrolytic efficacy is probably explained by the enzyme

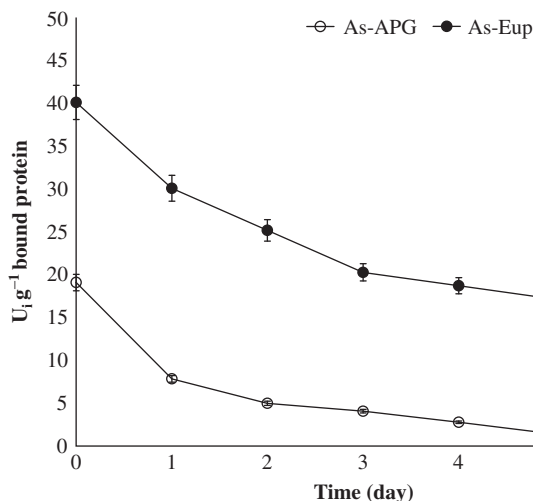


Figure 2: Activity (U_i) of *A. saitoi* protease immobilized on APG (As-APG) and Eupergit®C (As-Eup) measured for 6 d at 20°C in batch reactor, containing model wine (pH 3.2 tartaric acid buffer with 12% v/v ethanol) and casein as substrate (12 mg mL⁻¹). U_i was daily determined spectrophotometrically at 660 nm measuring the tyrosine released from casein hydrolysis.

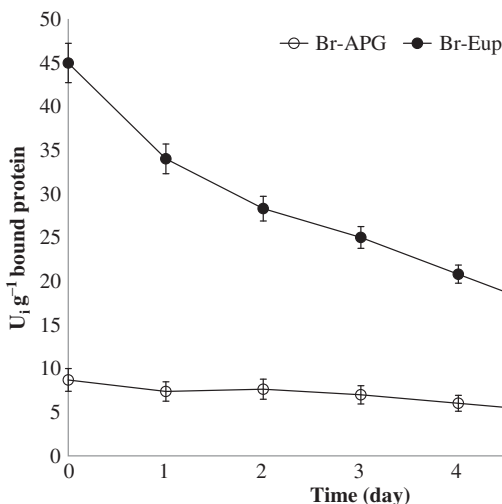


Figure 3: Activity (U_i) of bromelain immobilized on APG (Br-APG) and Eupergit®C (Br-Eup) measured for 6 d at 20°C in batch reactor, containing model wine (pH 3.2 tartaric acid buffer with 12% v/v ethanol) and casein as substrate (12 mg mL⁻¹). U_i was daily determined spectrophotometrically at 660 nm measuring the tyrosine released from casein hydrolysis.

conformational changes induced by the cross linker. Finally, the best catalytic activity observed for both proteases coupled on Eupergit C should be ascribed to both the higher content of oxirane groups on the acrylic support surface, which resulted in a stronger enzyme-binding and the larger pore diameter

that prevented the leaching of protein and favored the molecular diffusion of substrate (Yiu et al., 2001).

CONCLUSION

Eupergit C and APG reticulated with glutaraldehyde supports, evaluated for immobilizing acid proteases (aspartic and cysteinic) showed a good protein-binding capacity (> 55%) for both the 2 enzymes from *A. saitoi* and pineapple stem. The best coupling capacity of Eupergit C should be ascribed to both higher specific reactive surface of support, and mild incubation conditions. Moreover, kinetic parameters for immobilized proteases on Eupergit C showed higher affinity towards casein substrate. Both enzymes, tested for 6 d in model wine added with casein, revealed higher specific activity when immobilized on Eupergit C when compared to APG. A big pore diameter support is, in fact, favourable for the catalytic performance of enzyme. Otherwise, the reticulation with glutaraldehyde of APG immobilization procedure could strongly reduce the activity of bound proteases.

NOMENCLATURE

Y	yield of immobilized protein (%)
P _i	protein amount present in the solution before coupling to support (mg BSA equiv.)
P _f	protein amount present in the solution after coupling to support (mg BSA equiv.)
rt	tyrosine reaction rate (mmol ⁻¹ min ⁻¹)
U _f	specific activity of free protease (mmol min ⁻¹ mg ⁻¹ of protein)
U _i	specific activity of immobilized protease (mmol min ⁻¹ mg ⁻¹ of protein)
Y _f	amount of protein in the enzymatic solution (mg ⁻¹)
Y _b	amount of bound protein (mg ⁻¹)
Ra	retained activity (%)
V _{max}	maximum tyrosine formation rate (mmol mL ⁻¹ min ⁻¹)
K _m	Michaelis–Menten constant (mg mL ⁻¹)

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