ELSEVIER
papers

Immobilization of the glycosidases: α-L-arabinofuranosidase and β-D-glucopyranosidase from *Aspergillus niger* on a chitosan derivative to increase the aroma of wine. Part II

Giovanni Spagna,*,† Franco Andreani,† Elisabetta Salatelli,† Denis Romagnoli,† Daniele Casarini, and Pier Giorgio Pifferi*,†**

**School of Specialization in Food Chemistry & Technology †Interdepartmental Centre of Biotechnological Research, Department of Industrial Chemistry & Materials **Department of Organic Chemistry "A. Mangini", University of Bologna, Bologna, Italy*

The purpose of this paper was to study the immobilization of two glycosidases, α-L-arabinofuranosidase (EC 3.2.1.55) and β-D-glucopyranosidase (EC 3.2.1.21), contained in a commercial preparation and purified as reported in Part I. The procedure which proved to be the best is simple and inexpensive to perform, employing the chitosan derivative, glyceryl-chitosan, especially synthesized and characterized, as a support. The glycosidases were adsorbed on this support and cross-linked with glutaraldehyde to prevent them from being released into the wine. Subsequent reduction of the biocatalyst with sodium borohydride allowed for improved stability. Finally, the immobilized glycosidases were compared with free ones in terms of optimum pH and temperature, stability over time, and kinetics parameters (K_m and V_{max}) after which they were employed for aromatizing a model wine solution containing aromatic precursors. © *1998 Elsevier Science Inc.*

Keywords: α-L-arabinofuranosidase; β-D-glucopyranosidase; enzyme immobilization; wine aromatization

Introduction

Glycosidases including α-L-arabinofuranosidase (Ara, EC 3.2.1.55) and β-D-glucopyranosidase (βG, EC 3.2.1.21) are enzymes of considerable importance in food technology[1–3] with potential applications in the aromatization of musts, wines, other alcoholic beverages (brandy, bitters, etc.), and fruit juices (apple, apricot, peach, papaya, passion fruit, and so on).[4–15] They contain aromatic compounds bonded to β-D-glucopyranose and α-L-arabifuranose. In particular, the more abundant aromatic precursors are found in wine in the form of diglycosidates so that the action of the glycosidases is sequential, Ara acting first, followed by βG.[16–18] In the previous paper, a simple method for the purification of Ara and βG from the *Aspergillus niger* present in a commercial enzyme preparation was described.[19] In the present study, the purified glycosidases were coimmobilized on chitosan by means of simple adsorption and subsequently on glyceryl chitosan (GCh),[20] a derivative especially prepared by the reaction of chitosan with glycidol. The glycosidases adsorbed on this latter support were then cross-linked with glutaraldehyde to prevent them from being released into the

Address reprint requests to Dr. G. Spagna, Department of Industrial Chemistry & Materials, University of Bologna, Viale Risorgimento 4, 40136 Bologna Italy
Received 2 July 1997; revised 30 October 1997; accepted 11 November 1997

655 Avenue of the Americas, New York, NY 10010
0141-0229/98/$19.00
PII S0141-0229(97)00249-4

wine. Finally, the biocatalyst was reduced with sodium borohydride in order to increase its stability over time. Chitosans offer a number of advantages for the immobilization of enzymes in the food processing industry.[21–24] In fact, they are non-toxic (foodgrade), easily available, inexpensive, moderately biodegradable, available in different forms (powder, gel, fibers, and membranes), and easy to functionalize; moreover, they exhibit a high affinity toward proteins and have already been successfully used for the immobilization of the βG contained in another type of enzyme preparation.[25–26]

Materials and methods

Materials

A commercial preparation, AR 2000 (Gist Brocades, France), purified according to the method described in a previous paper[19] was the source of the glycosidases Ara and βG. Synthetic substrates, namely the *p*-nitrophenyl of α-L-arabinofuranose (*p*-NPA) and β-D-glucopyranose (*p*-NPG) (Sigma Chemical Co., St. Louis, MO) were used for activity assays while the aromatic precursors extracted from the skins of *Moscato* grapes from Alessandria (Italy) were employed as a natural substrate.[19] Protein determination was made using Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) as a colorimetric agent and bovine serum albumin (BSA; Sigma) as a standard. The following supports were used for the immobilization tests: carboxymethyl-cellulose (CM-cell; CM23), diethylaminoethyl-cellulose (DE-cell; DE11) supplied by Whatman (London, England) chitosan supplied by Protan (Redmond, Washington, USA) and glyceryl-chitosan (GCh) synthesized by reaction of chitosan with glycidol (2-3-epoxy-1-propanol) at 96% supplied by Sigma. Glutaraldehyde (GA; 50%, Fluka, Buchs, Switzerland) and $NaBH_4$ (Sigma) were used as a cross-linking agent for the glycosidases adsorbed on the GCh and as a reducing agent, respectively. All other reagents not specified here were high purity and supplied by Sigma.

Synthesis of GCh

GCh was synthesized according to the method for agarose functionalization reported by Guisan *et al*.[27] A sample of chitosan taken from the same lot used for the tests of immobilization by simple adsorption was ground and sieved to obtain particle sizes between 75–125 μm. Powdered chitosan was suspended in 0.1 M NaOH, recovered by filtration, and repeatedly washed and filtered using (in sequence) water (four times) and ethanol (three times), and finally dried at 60°C under dynamic vacuum pumping until a constant weight was retained. The degree of N-acetylation of chitosan was determined by infrared (IR) spectroscopy[28] as well as by acidimetry[29] and colorimetry[30] of the amino group. The N-acetylation percent values determined with the three aforementioned techniques showed excellent agreement. Dry chitosan (0.9 g) was suspended in 0.17 M NaOH (12 ml) and treated under slow stirring with $NaBH_4$ (0.342 g). The mixture was cooled (3–4°C) and glycidol (6 ml) was added dropwise at a temperature below 18°C. Upon addition being completed, the temperature was held constant at 20°C for about 18 h. The resulting GCh was filtered, washed thoroughly with distilled water and then with methanol, and finally dried at 60°C under dynamic vacuum pumping until a constant weight was retained (about 1.2 g). The quantitative determination of unreacted amino groups was performed according to the colorimetric method of Ngo.[30] Microanalytical results (C, H, N, O) were provided by Redox Laboratories (Milan, Italy). IR spectra of chitosan and GCh were obtained using a Perkin-Elmer 1,750 Fourier Transform infrared (FTIR) spectrophotometer. The samples were prepared by mixing the powders with KBr (about 0.3% w/w) and pressing the dilute mixture to form a clear semitransparent pellet. ^{13}C-NMR solid state CP-MAS spectra of chitosan and GCh were obtained using a Bruker CXP 300 spectrometer operating at 75.45 MHz. The solid samples were packed into a 7 mm zirconia rotor and spun at the magic angle with a spinning rate in the order of 3–4 KHz. The ^{1}H pulse duration for a 90 flip angle was 4 μs which was also the value for ^{13}C as set by Hartmann-Hahn conditions.[31] A contact time of 2 ms was adopted for cross-polarization while the recycle delay for CP experiments was generally 5 s. The number of transients, in the order of several thousands, was varied to achieve good signal-to-noise ratios. The chemical shifts were measured with respect to the lower frequency signal for adamantane at 29.4 ppm and with respect to TMS (by replacement).

Adsorption

A sample of 10 mg chitosan or GCh in a 10-ml test tube was equilibrated at room temperature for at least 15 min by means of 5 ml 0.025 M citrate-phosphate (C-P) buffer at pH values between 3.4–7.0. Five ml of the purified enzyme solution[19] containing about 15–20 U of βG and 60–120 U of Ara were brought to the same pH of the equilibration with HCl or NaOH (0.1 M) and then added to the support. Finally, the tube was plugged and stirred in a carousel (75 rpm) at 3–4°C overnight. The same procedure was adopted for CM-cell and DE-cell except for the fact that in this case, the supports were pretreated with HCl (0.1 M) and NaOH (0.1 M) according to the method recommended by the manufacturer (Whatman).

Cross-linking

A sample of 5 ml glutaraldehyde (GA) at different concentrations (0.1–2%) dissolved in a 0.025 M C-P buffer at pH values between 3–7 was added to the glycosidases adsorbed at pH 5.0 on 10 mg of CGh followed by stirring in a carousel at 4°C for 7 h and at 25°C for 30 or 60 min. After centrifugation at 2,000 *g* for 10 min, the biocatalyst was washed twice at room temperature with 5 ml of a 1 M NaCl solution.

Reduction of Schiff bases

Before reducing the biocatalyst with $NaBH_4$ at an alkaline pH, the stability of the free glycosidases contained in the purified enzyme preparation[19] was tested in a 0.1 M carbonate-bicarbonate buffer at pH 9.0 and 25°C, and subsequently in the same buffer containing 10 mg ml^{-1} $NaBH_4$ at the storage temperatures of 4 and 25°C.

The glycosidases were adsorbed on GCh at pH 5.0 and cross-linked with GA at pH 5.0 and 25°C for 60 min. The biocatalyst thus obtained was reduced under light stirring at 4 and 25°C for 1 h with 5 ml of a solution of 1 or 10 mg ml^{-1} of $NaBH_4$ dissolved in a 0.1 M buffer at pH 8 (phosphate-phosphate), 9 and 10 (carbonate-bicarbonate). The biocatalyst was then filtered and thoroughly washed with 0.1 M C-P buffer at pH 7 until the pH of the wash water was neutral. After centrifugation at 2,000 *g* for 10 min, the biocatalyst was washed twice at room temperature with 5 ml of a 1 M NaCl solution.

Enzyme assays

Depending on whether the enzyme was free or immobilized, 50 μl of a solution or a suspension of enzyme were added with stirring at 25°C to 450 μl of 2.0 mM *p*-NPA or 5.5 mM *p*-NPG dissolved in 0.1 M C-P buffer at pH 3.5. The assay was performed for 0.5–5 min after which 1 ml of 1 M Na_2CO_3 was added to stop the reaction and allow the yellow color of the *p*-nitrophenolate ion to develop. This color was read against the blank at a λ of 400 nm and

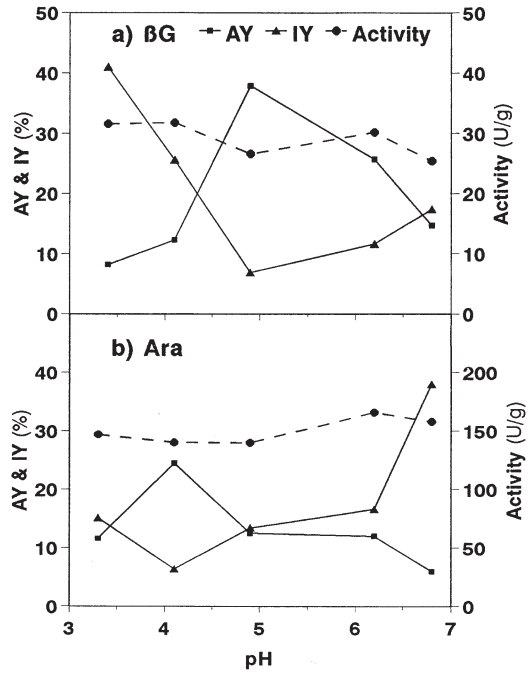

Figure 1 Immobilization of the glycosidases, βG (a) and Ara (b), by adsorption onto chitosan as a function of pH

determined by using a ϵ of 18,300 M^{-1} cm^{-1}. The order of the reagents was reversed in the blank test, i.e., the Na_2CO_3 solution was added to the substrate before the enzyme. In determining immobilized enzyme activity, the insoluble material was removed by centrifugation at 2,000 *g* for 15 min before measuring absorbance. One unit of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenolate min^{-1} under assay conditions.[25] Finally, protein determination was made by colorimetric reaction with Coomassie Blue G250 according to Bradford.[32]

Adsorption yield (*AY*) and immobilization yield (*IY*) are defined as follows: AY = Uads/Ueq × 100; IY = Uact/Uads × 100 where *Ueq* are the enzyme units which are equilibrated with the support, *Uads* are those which remain bound to the support after separation of the supernatant, and *Uact* are those which are active in the immobilized enzyme.

The activity of the free or immobilized glycosidases was also tested on natural substrates according to the method described in Part I.[19] Samples of 1–2 ml of the free enzyme or the immobilized enzyme suspension were added to 30 ml of a model wine solution in a test tube which contained 9 g l^{-1} tartaric acid in 10% ethanol adjusted to pH 3.3 with potassium carbonate and added with the aromatic precursors extracted and isolated from 100 g of *Moscato* skin grapes. The tube was sealed with a screw cap and incubated at 25°C for 18 h. The analysis was made using a Carlo Erba 5300 gas chromatograph fitted with a Durabon Wax column.[33]

Characterization

The following parameters were determined for the free and the immobilized enzymes: optimum pH (between 3.0–7.0 in 0.1 M C-P buffer) at 25°C; optimum temperature (between 20–90°C) at pH 3.5; stability over time at 57°C (pH 3.5 in 0.1 M C-P buffer) and at 25°C in the wine (*Trebbiano*, Emilia Romagna, Italy); and finally, the kinetic parameters K_m and V_{max} (at pH 3.5 and 25°C) calculated according to Lineweaver Burk.[34]

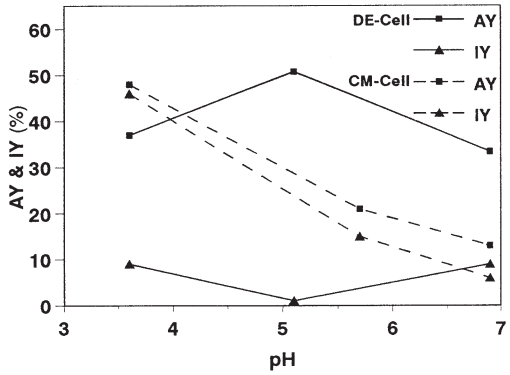

Figure 2 Immobilization of βG by adsorption on diethylaminoethyl-cellulose (DE-cell) and carboxymethyl-cellulose (CM-cell) as a function of pH

Results and discussion

Immobilization by adsorption onto chitosan

Upon pH values being varied, the glycosidases, βG and Ara, adsorbed on chitosan exhibited similar trends in terms of yields and activity (*Figures 1a* and *1b*). βG and Ara AY followed a bell-shaped pattern with maximum adsorptions at pH values close to 5 and 4, respectively. IY demonstrated an opposite trend, with minimum values being recorded at maximum AY values. Activity was therefore fairly constant at rather low mean values of 30 U g^{-1} for βG and 150 U g^{-1} for Ara.

The main interactions between the enzymes and chitosan are of an electrostatic nature, even if other weak interactions such as hydrogen bonding and Van der Waals forces are also present. The enzyme capture (AY) peak recorded in the central pH zone is presumably ascribable to the electrostatic interaction between the negatively charged enzymes and the positively charged chitosan (pKa of about 6.4). The difference in the maximum values of AY between the two enzymes is probably related to their different isoelectric points (pI of about 4.0–4.5 for βG and 3.3–3.7 for Ara).[35–38] A possible explanation of the IY trend is that the catalysis of the glycosidases involves an acid/base-type behavior of the carboxyl groups present at the active site.[39–40] At pH values for which the electrostatic interaction between the dissociated carboxyl groups of the active sites of the enzymes and the protonate amino groups of the chitosan is at a maximum, the enzyme molecules may preferentially orientate themselves so that their active sites come to be facing the surface of the support. If this is the case, the carboxyl groups would be less available for catalysis and the accessibility of the substrate to the active sites would be reduced (steric hindrance). Conversely, as can be seen in *Figures 1a–1b*, at extreme pH values, reduced adsorption is associated with an increase in IY.

To test these hypotheses, adsorption of βG and Ara was performed on two differently derived cellulose supports. One is the DE-cell, which is basic and contains tertiary amino groups which may simulate the structure of chitosan, and the other is the CM-cell which is acid and contains carboxyl groups. As can be seen in *Figure 2*, the βG

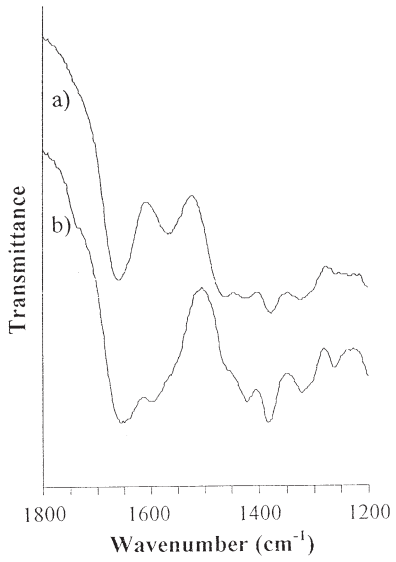

Figure 3 IR spectra of GCh (a) and chitosan (b)

adsorbed on the DE-cell exhibits a behavior similar to that on chitosan, that is with opposite trends in AY and IY. The electrostatic interaction of the positively charged support with the negatively charged enzymes once again causes a sensible inactivation. On the contrary, AY and IY for the βG adsorbed on the CM-cell follow parallel trends, diminishing as pH increases, and with IY values always greater than those reported for the DE-cell. Unlike on the DE-cell, the electrostatic interaction occurring during glycosidase immobilization on the CM-cell involves the protonate amino groups of the enzyme and the dissociated carboxyl ones of the support. This means that the enzyme molecules are capable of orientating themselves and that the carboxyl groups, including those present at the active site, are less involved. On both the DE- and CM-cell, Ara yield trends were similar to those found for βG (data not shown). The results obtained in these immobilization tests on cellulose derivatives confirm that the carboxyl group rather than the amino one is involved in the catalysis. It may therefore be concluded that chitosan is not the ideal support for the immobilization of βG or Ara by simple adsorption.

Characterization of GCh

The % N-deacetylation of the chitosan used to synthesize GCh was 62%.[28–30] This means that the starting chitosan was the copolymer possessing 62% of β-(1 → 4)-2-amino-2-deoxy-D-glucopyranose units and 38% of the corresponding 2-acetamido units. After reaction with glycidol, an average number of 95 glyceryl groups per 100 copolymeric units of GCh was calculated on the basis of the carbon-to-nitrogen ratio as determined by elemental analysis (46.7% C, 7.2% H, 5.7% N, 40.5% O). Notwithstanding, an average number of 30 unreacted primary amino groups per 100 copolymeric units of GCh was still found to be present.[30] Overall, these results may reflect the fact that, in the alkaline medium used for the reaction between chitosan and glycidol, a random distribution of N-mono-, N-bis-, and O-alkylation occurred. This influence of the pH is similar to that observed by Loubaki *et al*.[20]

Figure 3 shows the infrared spectra of chitosan and GCh between 1,800 and 1,200 cm^{-1} with the amide I band at 1,655 cm^{-1} having been assumed as an internal standard to compensate for differences in sample concentration. As can be seen, at the frequency of about 1,550 cm^{-1}, typical of the amide II band in chitin and in highly acetylated chitosans,[41] the chitosan does not exhibit any clear absorption, but only a very slight upward swelling at about 1,562 cm^{-1}, caused by the rather intense absorption at 1,600 cm^{-1} which can be ascribed to the NH_2 deformation band.[42] On the other hand, in the spectrum of GCh, the amide II band can be clearly observed at 1,562 cm^{-1}, despite the same extent of N-acetylation of chitosan. This is due to the marked decrease in the intensity of the 1,600 cm^{-1} absorption band, indicative of the reaction of glycidol with the amino groups of chitosan; moreover, the intensities of both the CH stretching vibration of the methylene group in the region of 2,850–2,900 cm^{-1} and the CH bending band at 1,461 cm^{-1} (not shown) increase for GCh, thus confirming the introduction of glyceryl groups in the structure of chitosan.

A comparative analysis of the ^{13}C-NMR solid state CP-MAS spectra of GCh and chitosan also provided structural information on GCh. The signals in the 50–110 ppm range observed for the unsubstituted chitosan were assigned on the base of the solution data.[43–45] The two peaks at 57.4 and 60.6 ppm were assigned to C2 and C6 of the glycosidic rings, the most intense peak at 75.4 ppm was attributed to C3, C5, while the signals at 83 and 105 ppm were assigned to C4 and C1, respectively. Considerable differences were observed between the relative intensity signals of chitosan and GCh, suggesting the introduction of a glyceryl group in the structure. Although in GCh the signal broadness prevented discrimination between N- and O-alkylation in the chitosan unit, several considerations could be drawn by comparing experimental and calculated shifts from empirical equations,[46] for both O- and N-alkylation. The observed reduction of the broad peak at 57.4 ppm was attributed to the CH_2 of the glyceryl group N-substituted and to the C2 of unreacted chitosan; moreover, the increased area of the signal at 61.8 ppm was attributed to C2 N-substituted and to C6 of unreacted chitosan. The emerged signal at 71.8 ppm, however, attributed to the OCH_2 of the glyceryl group and the increased area of the peak at 75.0 ppm suggest that O-alkylation also occurred. This reinforces the interpretation of the FTIR data and confirms that a mixture of N- and O-alkylation has occurred in the substitution of chitosan with glycidol.

Immobilization by adsorption on GCh

Figure 4 shows the yields and activities of the glycosidases βG and Ara adsorbed onto GCh as a function of pH. A comparative analysis of the data shown in *Figure 4* with the corresponding data shown in *Figure 1* clearly indicates higher catalytic responses for GCh as compared to chitosan with increases in activity being up to about 12 times for βG (350 U g^{-1}) and 29 times for Ara (4,400 U g^{-1}).

The improvement in yields may be ascribable to the presence on the GCh of hydroxylate side chains which make

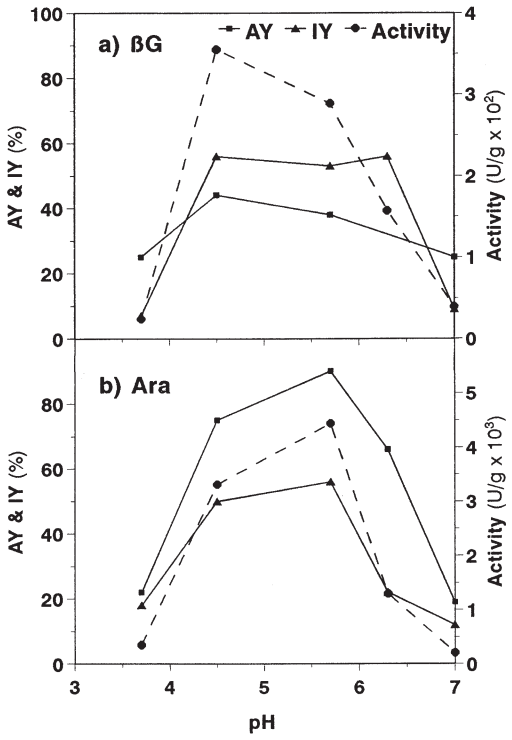

Figure 4 Immobilization of the glycosidases, βG (a) and Ara (b), by adsorption onto GCh as a function of pH

it even more hydrophilic than chitosan, therefore endowing it with the possibility of forming a greater number of hydrogen bridges with the enzyme molecules, in particular with the carbohydrate moiety of the enzyme;[36–37] moreover, there may be a reduction in enzyme-support interaction owing to the reduction in the amino groups occurring during the functionalization reaction and the presence of the glyceryl groups in the GCh which may distance the enzyme from the support surface (space-arm effect). This latter effect may play a crucial role in increasing the accessibility of the substrate toward the active site.

Cross-linking and reduction

Despite the excellent results obtained for immobilization on GCh, in solutions with a high ionic force such as wine, electrostatic interactions caused the glycosidases to be released. The subsequent cross-linking of the adsorbed enzyme with a bifunctional agent was performed in order to prevent the enzyme from being released in the wine. GA was employed as the cross-linking agent since it is inexpensive, easy to use, and does not react with the carboxyl groups which, as already mentioned, are probably present in the active site of the glycosidases.[39–40]

GA is capable of reacting via the aldehyde groups with the nucleophile groups such as the amino ones present on the surface of the enzyme and chitosan derivative, thus forming Schiff bases. Monsan *et al.*[47] have discussed the possibility of GA reaction also via the α,β-unsaturated double bonds originating from its polymerization (aldolic condensation) which is favored by the increase in pH so that

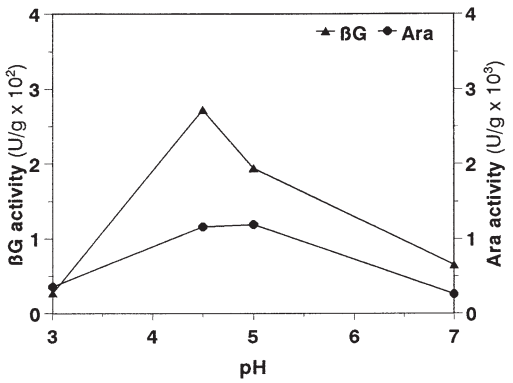

Figure 5 Immobilization of the glycosidases, βG and Ara, by cross-linking on GCh as a function of the activation pH (GA 2.0% at 25°C)

as pH varies, the reactivity of GA changes; therefore, GA would be able to form both enzyme-enzyme and enzyme-support bonds, thus enhancing the adsorption interactions.

Before proceeding with immobilization, the stability of the free enzymes was tested in a glutaraldehyde solution. The activity of the enzymes in the GA solution (2% at pH 5.0 and 25°C) only slightly decreased with such decrease occurring during the first hour of contact and being greater for Ara (about 20%) than for βG (about 10%).

The enzymes adsorbed onto GCh were cross-linked with GA at different pHs and concentrations. As the activation pH varied, the activity trends of both enzymes exhibited a bell-shaped pattern with maximum values around pH 4.5–5.0, i.e., in the optimum adsorption zone (*Figure 5*). This finding suggests that at the above pH values, GA is capable of "blocking" the enzyme molecules in the optimal conformation and orientation corresponding to the highest catalytic activity. Notwithstanding, while at the peak of the curve the activity of βG is essentially retained (about 280 U g^{-1}), that of Ara drastically drops (about 1,200 U g^{-1}). As for the free enzymes, therefore, Ara was found to be more sensitive to the inactivating action of GA than βG even after immobilization on GCh.

The activity of both glycosidases did not change appreciably when GA concentration (at pH 4.5) was increased from 0.1–2%; nevertheless, a partial release of the immobilized glycosidases (about 20% after 3 days at pH 3.5) was reported at low GA concentrations (0.1–1%), an effect which was not observed at GA concentrations greater than 1%. This finding is in line with that of Blanco[48] according to whom adsorption becomes irreversible only in the case of a minimum bond threshold being formed between the enzyme and the support.

Even though no glutaraldehyde was seen to have been released, the biocatalyst was treated with sodium borohydride in order to improve biocatalyst stability by reducing the C=H and CH=O groups.[48] Sodium borohydride was used as the reducing agent since it is nontoxic, inexpensive, and highly reactive and selective toward the Schiff bases and the aldehyde groups; moreover, the small dimensions of its molecules permit it to diffuse more easily inside the matrix. This reducing agent may, however, exert an inacti-

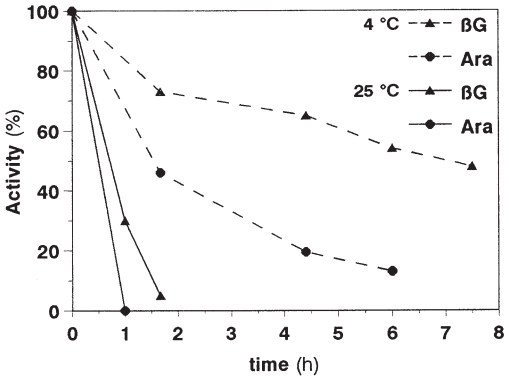

Figure 6 Stability of the glycosidases, βG and Ara, free in a solution of $NaBH_4$ (10 mg ml^{-1}, pH 9.0) at two different temperatures (4 and 25°C)

vating effect on the enzymes. In fact, sodium borohydride must be employed at an alkaline pH (8–10) at which value the protein structure of the enzyme can be denatured and the serine and threonine residues reduced to alanine and α-amino butyric acid, respectively. Finally, sodium borohydride can break up the disulfide bridges, although this effect can be ruled out in the case of the enzymes examined since their amino acid composition does not contain any cysteine.[36–37]

Before testing the operative conditions for the reduction of the glycosidases, the stability of the purified free enzymes was tested at alkaline pHs in the absence and presence of the reducing agent. βG proved to be fairly stable at pH 9 and 25°C with a half-life of about 80–100 h while under the same conditions, Ara was seen to inactivate much more easily with its half-life being about 5–6 h. The addition of sodium borohydride to the purified enzyme solution at pH 9.0 and 25°C caused a sharp drop in activity in particular for Ara while at 4°C the decrease in activity was acceptable at least during the first hour of reaction (about 20 and 30% for βG and Ara, respectively) (*Figure 6*). In view of these results, the cross-linked glycosidase reduction tests were conducted at 4°C for 1 h.

The drop in activity at basic pHs can be ascribed to the conformational deformation undergone by the enzyme molecules at pH values which are very far from their pI. Given that the pI of both glycosidases is similar, it may be assumed that the protein structure of Ara is less compact, i.e., more "soft" than that of βG;[49–50] moreover, the fact that Ara exhibits a higher sensitivity also to sodium borohydride may instead be partially ascribable to the presence, in its primary structure, of a greater amount of the hydroxylate amino acids, serine and threonine, as compared to βG.[36–37]

The activity trend for the cross-linked glycosidases as a function of the increase in the reduction pH between 8–10 was similar for both enzymes. In particular, while a decrease in activity (from about 280 to 100 U g^{-1} for βG and from about 1,200 to 510 U g^{-1} for Ara) was observed at 1.0 mg ml^{-1} of the reducing agent a fairly constant activity was observed at 10 mg ml^{-1} (about 175 U g^{-1} for βG and 770 U g^{-1} for Ara). A possible explanation is that at all pH values, the reduction of Schiff bases was incomplete at the

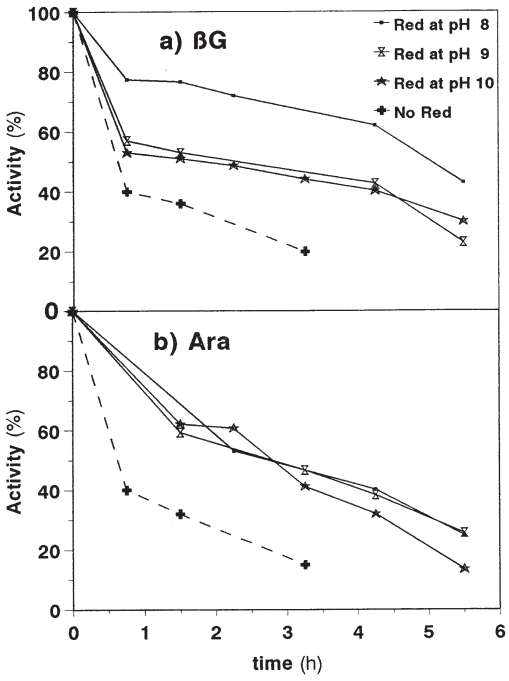

Figure 7 Stability (at pH 3.5 and 57°C) of the glycosidases, βG (a) and Ara (b), immobilized by cross-linking on GCh, nonreduced and reduced with $NaBH_4$ (10 mg ml^{-1}) at different pH values (8, 9, and 10)

lower concentration of the reducing agent (in fact, the typical yellow color of the matrix was maintained) so that the inactivating effect of the alkaline environment was able to prevail over the stabilizing effect of the reduction reaction. Conversely, at the higher concentration of the reduction agent, reduction was complete as demonstrated by the complete decoloring of the biocatalyst. The enzymes were thus "frozen" in their original cross-linked conformation and protected against the inactivating effect of the reduction pH.

Before establishing the optimum reduction pH, an accelerated stability test was conducted on the nonreduced and reduced biocatalyst. Biocatalyst stability at the lower concentration of the reducing agent was practically the same as that of the non-reduced one at all reduction pHs while at the higher concentration, the stability of the reduced glycosidases was considerably higher than that of the nonreduced ones (*Figures 7a–7b*). The increase in stability was essentially independent of the reduction pH value except for βG reduced at pH 8 which maintained its activity more than at other pHs. The following reduction conditions were therefore adopted: $NaBH_4$, 10 mg ml^{-1}; pH 8, and temperature at 4°C.

Characterization of free and immobilized enzymes

The enzymes immobilized on GCh were characterized and compared with the free ones.

The optimum pH of the immobilized βG was found to be shifted toward more acid values (from about 4.5 to 4.0) (*Figure 8a*) while no shift was observed in the optimum pH

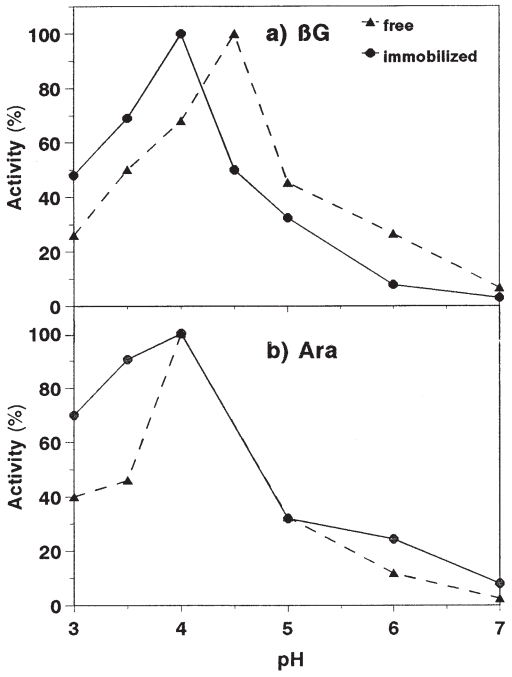

Figure 8 Activity of the glycosidases, βG (a) and Ara (b), immobilized by cross-linking on GCh and subsequently reduced, as a function of pH

for Ara which remained around 4 (*Figure 8b*). Notwithstanding for both immobilized glycosidases and in particular for Ara, greater percent activities were recorded at acid

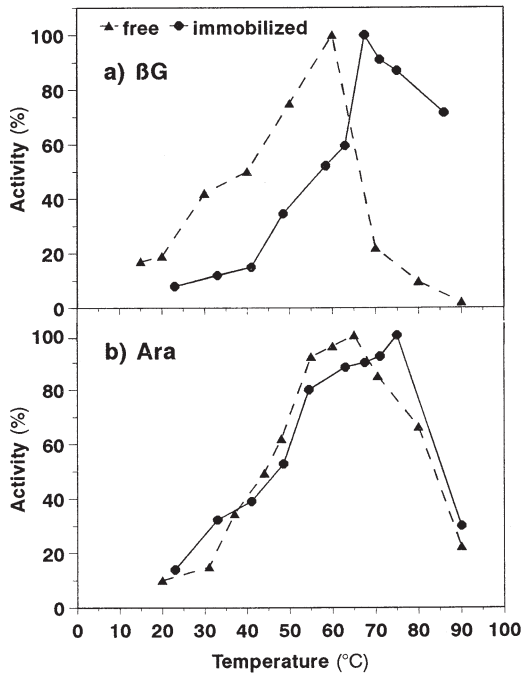

Figure 9 Activity of the glycosidases, βG (a) and Ara (b), immobilized by cross-linking on GCh and subsequently reduced, as a function of temperature

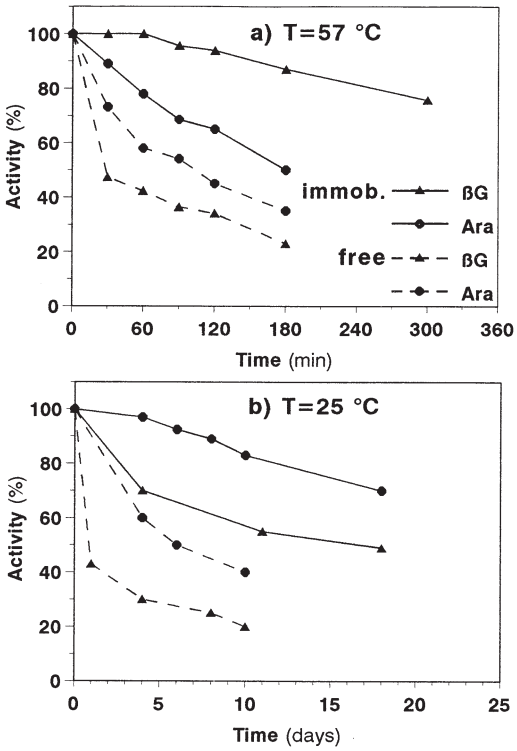

Figure 10 Stability of the glycosidases, βG and Ara, immobilized by cross-linking on GCh and subsequently reduced, at different storage temperatures: 57°C (a) and 25°C (b)

pH values between 3–3.5, a fact which is advantageous for oenological applications. The behaviors observed are typical of polycationic supports such as chitosan and GCh which cause an unequal distribution of the H^+ and OH^- ions in solution with a greater concentration of OH^- ions in proximity to the microenvironment of the immobilized enzyme. The latter is therefore affected by a pH value higher than that of the bulk of the solution.[51]

The optimum temperature of the immobilized βG (*Figure 9a*) was increased from 60 to 67°C, its stability decreasing more slowly at higher temperatures. The behavior of immobilized Ara is similar (*Figure 9b*), with the optimum temperature having been increased from 65 to 75°C; moreover, the stability of the glycosidases also increased with immobilization (*Figure 10*). At 57°C after 2 h, activity retention rose from about 30 to 90% for βG and from about 40 to 65% for Ara. As expected at 25°C, absolute values were higher and after 10 days, activity retention rose from about 20 to 55% for βG and from about 40 to 80% for Ara.

The presence in the immobilized glycosidases of intra- and intermolecular covalent bonds and of bonds with the support confers rigidity to the secondary and tertiary protein structure of the enzymes which are therefore less affected by the denaturing effect of the temperature and acquire greater stability; moreover, the presence on GCh of side chains containing oxydryl groups may favor the solvation of the enzyme and the retention of its globular structure.[52–53]

Regarding the K_m of the immobilized enzymes, that of

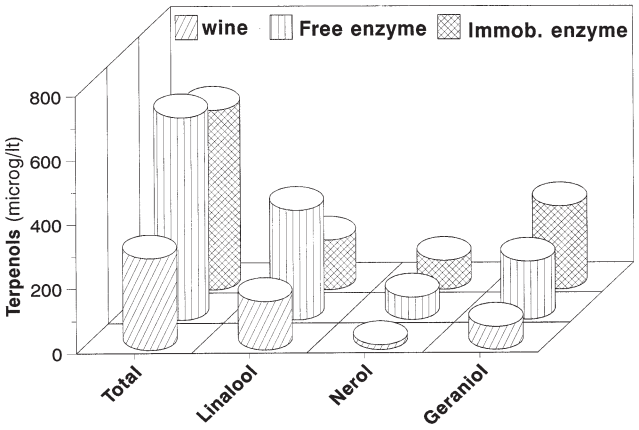

Figure 11 Free terpenols present in a model wine as such or treated with glycosidases, free and immobilized by cross-linking on GCh and subsequently reduced

βG did not vary (being about 0.60 mM) while that of Ara increased (from about 0.63 to 1.3 mM). The increase in K_m in immobilized enzymes is mainly ascribable to the conformational changes which the protein undergoes following immobilization on the support and/or to the difficulty of the substrate to diffuse toward the catalytic site.[51] The V_{max} of the immobilized enzymes diminished with βG from 4.0 to 1.2 U mg^{-1} protein and Ara from 37 to 20 U mg^{-1} protein. The reduction of V_{max} in the immobilized as compared to the free enzymes is essentially ascribable to the partial inactivation of the former and to the fact that, when immobilized, the enzyme is not in the best condition for catalysis.[51]

Both the free and immobilized enzymes were capable of releasing a high amount of total terpenols from the model wine solution (*Figure 11*) even though the action of the immobilized ones was slightly less (by about 10%); moreover, the immobilized enzymes were seen to be less selective when compared to the free ones toward the glycosidate precursors of linalool, a tertiary alcohol, than toward those of nerol and geraniol, both primary alcohols. This behavior may be explained by presuming that the glycoside of the tertiary alcohol is less accessible to the catalytic site of an immobilized enzyme than to that of a free one.

Conclusions

Chitosan was suitable for achieving high adsorption of the glycosidases βG and Ara which, however, were sensibly inactivated. On the other hand, the chitosan derivative, GCh, further increased the adsorption of the glycosidases (in particular of Ara) while allowing significant increases in activity. Cross-linking of the enzymes adsorbed onto GCh with glutaraldehyde prevented their release into the wine without the activity of βG being adversely affected even though that of Ara was reduced. The subsequent reduction in the Schiff's bases formed by the reaction between glutaraldehyde and the biocatalyst, despite causing a further decrease in activity, nevertheless led to a considerable increase in stability.

As compared to the free glycosidases, the ones immobilized on GCh exhibited an increase in relative activity and stability at acid pH values (that is, at values suitable for the potential application of the biocatalyst in oenology) as well as a higher optimum temperature. Finally, the immobilized glycosidases proved to be capable of increasing the aroma of a model wine solution even though, compared to the free ones, they exhibited a lower selectivity toward linalool.

Acknowledgments

We are very grateful to G. Versini and A. Della Serra for their help in the gas chromatographic analysis of aroma. Special thanks are also given to the Italian CNR-I. Co.C.E.A. (Bologna) for making available the Solid State NMR facilities. This research was supported by the Italian CNR, special project RAISA, subproject no. 4.

References

1. Wrostald, R. E., Wightman, J. D., and Durst, R. W. Glycosidase activity of enzyme preparations used in fruit juice processing. *Food Technol*., 1994, **11,** 90–98
2. Gueguen, Y., Chemardin, P., Janbon, G., Arnaud, A., and Galzy, P. A very efficient β-glucosidase catalyst for the hydrolysis of flavor precursors of wines and fruit juices. *J. Agric. Food Chem*. 1996, **44,** 2336–2340
3. Aryon, A. P., Wilson, B., Strauss, C. R., and Williams, P. J. The properties of glycosidases of *Vitis vinifera* and a comparison of their β-glucosidase activity with that of exogenous enzymes. An assessment of possible applications in enology. *Am. J. Enol. Viticult*. 1987, **38,** 182–188
4. Stahl Biskup, E., Intert, F., Holthnujzen, J. H., Stengele M., and Schultz, G. Glycosically bound volatiles-A review 1986–1991. *Flav. Fragr. J*. 1993, **8,** 61–80
5. Williams, P. J., Sefton, M. A., and Francis, I. L. Glycosidic precursors of varietal grape and wine flavor. In: *Flavor Precursors. ACS Symp. Ser*. 1992, **490,** 74–86
6. Nicolini, G., Mattivi, F., and Versini, G. Uso di glicosidasi esogene in mosti ed attività collaterali. *Riv. Vitic. Enol*. 1994, **1,** 45–60
7. Gunata, Z. Y., Bayonove, C. T., Tapiero, R. E., and Cordonnier, R. Hydrolysis of grape monoterpenyl β-D-glucosides by various β-glucosidases. *J. Agric. Food Chem*. 1990, **38,** 1232–1236
8. Winterhalter, P. Bound terpenoids in the juice of the purple passion fruit (*Passiflora edulis* Sims). *J. Agric. Food Chem*. 1990, **38,** 452–455
9. Schawab, W. and Schreier, P. Glycosidic conjugates of aliphatic alcohols from apple fruit (*Malus sylvestris* Mill cult. Jonathan). *J. Agric. Food Chem*. 1990, **38,** 757–763
10. Krammer, G., Winterhalter, P., Schawb, M., and Schreier, P. Glycosidically bound aroma compounds in the fruit of prunus species: Apricot (*P. armeniaca*), Peach (*P. persica*), Yellow Plum (*P. domestica*, L. ssp. *Syriaca*). *J. Agric. Food Chem*. 1991, **39,** 778–781
11. Krammer, G., Winterhalter, P., Schawb, M., and Schreier, P. Glycosidically bound aroma compounds in the fruit of prunus species: Apricot (*P. armeniaca*), Peach (*P. persica*), Yellow Plum (*P. domestica*, L. ssp. *Syriaca*). *J. Agric. Food Chem*. 1991, **39,** 778–781
12. Chassagne, D., Bayonove, C., Crouzet, J., and Baumes, R. Formation of aroma by enzymic hydrolysis of glycosidically bound components of passion fruit. *Bioflavour 95, Djon (France)* Les Colloques, n. 75 (INRA, Paris, Eds.) 1995, 217–222
13. Marlatt, C., Ho, C.-T., and Chien, M. Studies of aroma constituents bound as glycosides in tomato. *J. Agric. Food Chem*. 1992, **40,** 249–252
14. Wu, P., Kuo, M.-C. Hartman, T. G., Rosen, R. T., and Ho, C.-T. Free and glycosidically bound aroma compounds in pineapple (*Ananas comosus* L. Merr.). *J. Agric. Food Chem*. 1991, **39,** 170–172

15. Schwab, W., Scheller, G., and Schreier, P. Glycosidically bound aroma components from sour cherry. *Phytochemistry*, 1990, **29,** 607–612
16. Gunata, Z., Bitteur, S., Brillouet, J. M., Bayonove, C., and Cordonnier, R. Sequential enzymic hydrolysis of potentially aromatic glycosides from grape. *Carbohydr. Res*. 1988, **184,** 139–149
17. Gunata, Z., Dugelay, I., and Bayonove, C. Actions des glycosidases exogenes au cours de la vinification: Libération de l'arome à partir de precurseurs glycosidiques. *J. Int. Sci. Vigne Vin*. 1990, **24,** 133–144
18. Vorin, S. G., Baumes, R. L., Bitteur, S. M., Gunata, Z. Y., and Bayonove, C. L. Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes. *J. Agric. Food* 1990, **38,** 1373–1378
19. Spagna, G., Romagnoli, D., Bianchi, G., Martino, A., and Pifferi, P. G. A simple method for purifying glycosidases: α-L arabinofuranosidase and β-glucopyranosidase from *Aspergillus niger* to increase the aroma of wine. Part I. *Enzyme Microb. Technol*. 1998, **22,** 298–304
20. Loubaki, E., Sicsic, S., and Le Goffic, F. Modification chimique du chitosane avec la δ-gluconolactone, la β-propiolactone et le glycidol. *Eur. Polym. J*. 1989, **25,** 379–384
21. No, H. A. and Meyers, S. P. Preparation and characterization of chitin and chitosan, a review. *J. Aqua. Food Prod. Technol*. 1995, **4,** 27–52
22. Weiner, M. L. An overview of the regulatory status and of safety of chitin and chitosan as food and pharmaceutical ingredients. In: *Advances in Chitin and Chitosan* (Brine, C. J. Sanford, P. A., and Zikakis, J. P., Eds.). Elsevier Science Publ. LTD, New York, 663
23. Li, Q., Dunn, E. T., Grandmaison, E. W., and Goosen, M. F. A. Applications and properties of chitosan. *J. Bioact. Compat. Polym*. 1992, **7,** 370–397
24. Widmer, F. Chitosan: A promising support for enzyme immobilization. Preliminary-system design and assessment with *Aspergillus niger* β-Galactosidase (lactase) *Chemische Rundschau* 1979, **32,** 2–3
25. Martino, A., Durante, M. J., Pifferi, P. G., Spagna, G., and Bianchi, G. Immobilization of β-glucosidase from a commercial preparation. Part 1. A comparative study of natural supports. *Process Biochem*. 1996, **3,** 281–285
26. Martino, A., Pifferi, P. G., and Spagna, G. Immobilization of β-glucosidase from a commercial preparation. Part 2. Optimization of the immobilization process on chitosan. *Process Biochem*. 1996, **3,** 287–293
27. Guisan, J. M. Aldehyde-agarose gels as activated supports for immobilization-stabilization of enzymes. *Enzyme Microb. Technol*. 1988, **10,** 375–382
28. Baxter, A., Dillon, M., Talyor, A. K. D., and Roberts, G. A. F. Improved method for IR determination of the degree of N-acetylation of chitosan. *Int. J. Biol. Macromol*. 1982, **14,** 166–169
29. Sannan, T., Kurita, K., and Inakura, Y. Studies on chitin. 2. Effect of deacetylation on solubility. *Makromol. Chem*. 1976, **177,** 3589–3592
30. Ngo, T. T. Colorimetric determination of reactive amino groups of a solid support using Traut's and Ellman's reagents. *Appl. Biochem. Biotechnol*. 1986, **13,** 213–219
31. Yannoni, C. S. High resolution NMR in solids: The CPMAS experiment. *Acc. Chem. Res*. 1982, **15,** 201–208
32. Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. 1976, **72,** 248–254
33. Versini, G., Rapp, A., Scienza, A., Dalla Serra, A., and Dell'Eva, M. New bound terpenic and nor-isoprenic components identified in grapes. *Proc. Int. Symp. Aromatic Sub. Grapes Wines* (Scienza, A. and Versini, G., Eds.) 1989, 71–92
34. Lineweaver, H. and Burk, D. The determination of enzyme dissociation constants. *J. Am. Chem. Soc*. 1934, **56,** 658–666
35. Gunata, Z., Brillouet, J. M., Voirin, S., Baumes, R., and Cordonnier, R. Purification and some properties of an α-L-arabinofuranosidase from *Aspergillus niger*. Action on grape monoterpenyl arabinofuranosylglucosides. *J. Agric. Food Chem*. 1990, **38,** 772–776
36. Van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J., and Visser, J. Induction, purification, and characterization of arabinases produced by *Aspergillus niger*. *Arch. Microbiol*. 1991, **15,** 723–728
37. Himmel, M. E., Adney, W. S., Fox, J. W., Mitchell, D. J., and Baker, J. O. Isolation and characterization of two forms of β-D-glucosidase from *Aspergillus niger*. *Appl. Biochem. Biotechnol*. 1993, **39/40,** 213–225
38. Watanabe, T., Sato, T., and Yoshioka, S. Purification and properties of *Aspergillus niger* β glucosidase. *Eur. J. Biochem*. 1992, **209,** 651–659
39. Legler, G. Glycoside hydrolases: Mechanistic information from studies with reversible and irreversible inhibitors. *Adv. Carbohy. Chem. Biochem*. 1990, **48,** 319–383
40. Whithers, G. S. Enzymatic cleavage of glycosides: How does it happen? *Pure Appl. Chem*. 1995, **67,** 1673–1682
41. Domszy, J. C. and Roberts, G. A. F. Evaluation of infrared spectroscopic techniques for analysing chitosan. *Makromol. Chem*. 1985, **186,** 1671–1677
42. Wei, W., Shuiquin, B., Shuquing, L., and Wen, Q. Determination of the Mark-Houwink equation for chitosans with different degrees of deacetylation. *Int. J. Biol. Macromol*. 1991, **13,** 281–285
43. Domard, A., Gey, C., Rinaudo, M., and Terrasin, C. ^{13}C and ^{1}H-NMR spectroscopy of chitosan and N-trimethyl chloride derivatives. *Int. J. Biol. Macromol*. 1987, **9,** 233–237
44. Loubaki, E., Ourevitch, M., and Sicsic, S. Chemical modification of chitosan by glycidyl trimethylammonium chloride. Characterization of modified chitosan by ^{13}C and ^{1}H-NMR spectroscopy. *Eur. Polym. J*. 1991, **27,** 311–317
45. Kim, J. H. and Lee, Y. M. Synthesis and properties of diethylaminoethyl chitosan. *Polymer* 1993, **34,** 1952–1957
46. Kalinowski, O. H., Berger, S., and Braun, S. *The Chemical Shift in Carbon-13 NMR* spectroscopy. John Wiley and Sons, New York, 1988, 92-449
47. Monsan, P. Optimization of glutaraldehyde activation of a support for enzyme immobilization. *J. Mol. Catal*. 1977/78, **3,** 371–384
48. Blanco, R. M. and Guisan, J. M. Stabilization of enzymes by multipoint covalent attachment to agarose aldehyde gels. Borohydride reduction of trypsin agarose derivatives. *Enzyme Microb. Technol*. 1989, **11,** 360–366
49. Haynes, C. A. and Norde, W. Globular proteins at solid/liquid interfaces. *Coll. Surfaces B: Biointerfaces* 1994, **2,** 517–566
50. Norde, W. Adsorption of proteins from solution at the solid liquid interface. *Adv. Coll. Interface Sci*. 1986, **25,** 267–340
51. Chibata, I. Properties of immobilized enzymes and microbial cells. III. In: *Immobilized Enzymes* (Chibata, I., Ed.). Halsted Press, New York, 1978, 108–147
52. Mozhaev, V. V. and Martinek, K. Structure stability relationship in protein: A guide to approaches to stabilizing enzymes. *Adv. Drug Deliv. Rev*. 1990, **4,** 387–419
53. Mozhaev, V. V. Mechanism based strategies for protein thermostabilization. *Trend Biotechnol*. 1993, **11,** 88–95