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A novel chitosan derivative to immobilize α-L-rhamnopyranosidase from *Aspergillus niger* for application in beverage technologies

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Abstract

α-L-rhamnopyranosidase (Rha, EC 3.2.1.40) is an enzyme of considerable importance to food technology in increasing the aroma of wines, musts, fruit juices and other beverages. The aim of this research is the immobilization of the Rha contained in a commercial preparation already used in the winemaking industry and purified in the manner described in a previous study [1]. The immobilization supports tested were chitin, chitosan and derivatized chitosan, diethylaminoethyl chitosan (DE-chitosan) never previously used for this type of application. Particularly, on DE-chitosan, the Rha was adsorbed and cross-linked with various bifunctional agents (glutaraldehyde, diepoxyoctane, suberimidate and carbodiimide), whose best results (immobilization yields and activity) were obtained with carbodiimide (EDC) that allowed a reduction in the involvement of the enzyme amine groups that are probably important in catalytic mechanism. In addition, the use of rhamnose and a succinimide (NHS) during cross-linking enhanced the action of the EDC and so increased the immobilization yield and activity. The immobilized Rha retained the kinetic parameters (K_m and V_{max}) of the free enzyme and increased stability. Moreover, this biocatalyst allowed an increase in the aroma in a model wine solution containing glicosidic precursors with a marked reduction in specificity toward tertiary monoterpenols as compared to the free enzyme.

Keywords: α-L-rhamnopyranosidase (Rha); Enzyme immobilization; Chitosan derivative; Wine aromatization

1. Introduction

Chitosan is a good support for enzyme immobilization in the food industry since it is non-toxic (food grade), user-friendly, available in different forms (powder, gel, fibers and membranes), has high protein affinity and allows easy derivatization; moreover, it is easily available and inexpensive since derived from chitin, a by-product of the fishing industry and one of the most common polysaccharides [2–8].

The aim of the research is the immobilization of α-L-rhamnopyranosidase (Rha, EC 3.2.1.40) on chitosaneous materials for use in beverage technologies [9,10]. It is being used increasingly to heighten the aroma of fruit juices (cherry, passion fruit, pineapple, apricot, cherry, strawberry, apple, pear, papaya, banana, tomato, etc.) [1], musts and wines [1,11–19].

Rha is an enzyme that catalyses the breakage of the glycosidic linkages of rhamnose with other compounds, including precursors of the aromatic components present in glycosidic form [20]. In wines, the rhamnose is indirectly bound by the glucose to several volatile aglycons, e.g. monoterpenes [21–24]. The hydrolytic action of the Rha along with those of other glycosidases releases fragrant compounds. The aromatic precursors also undergo slow chemical hydrolysis during aging of wine, however some of them (linalool, nerol and geraniol) may be rearranged into less fragrant compounds (α-terpineol, diols and triols, oxides) so that after aging, an aromatic wine can actually have a less intense aroma. Given its high specificity, enzymatic hydrolysis releases the terpenols bound to the glycosidic residues more rapidly and in a selective manner without

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bringing about structural alterations in the monoterpenes, thus developing a more natural aroma. Despite the applicative importance of such enzyme, few studies exist regarding its immobilization [9,25] and those found regard Rha from *Penicillium decumbens* [26]. *Aspergillus niger* produces less Rha compared to *Penicillium,* but has the advantage of being a GRAS microorganism and producing cheap enzymes that are widely employed in the food industry.

The immobilization supports tested were chitin, chitosan and a specially derivatized chitosan (diethylaminoethyl chitosan, DE-chitosan), never previously used to immobilize enzymes. The method provides for adsorption onto said support and subsequent cross-linking, as well as aromatization testing of a model solution containing glycosidic precursors.

2. Materials and methods

2.1. Materials

The α-L-rhamnopyranosidase (Rha, EC 3.2.1.40) was purified according to the method described in a previous paper [1] from a commercial preparation, AR 2000 (Gist Brocades, Seclin, France). The synthetic substrates, *p*-nitrophenyl α-L-rhamnopyranoside (pNPR) were supplied by Sigma (Milan, Italy). The aromatic precursors, extracted from the skins of *Moscato* grapes from Alessandria (Italy), were employed as a natural substrate. Protein concentration was determined after precipitation in 7% (w/v) trichloroacetic acid by means of Coomassie Blue G250 (Serva, Heidelberg, Germany) and employing bovine serum albumin as a standard (BSA; Sigma) according to Bradford (1976; 1977). The following supports were used for the immobilization tests: carboxymethyl cellulose (CM-cell; CM23), diethylaminoethyl cellulose (DE-cell; DE11) supplied by Whatman (USA), chitin and chitosan supplied by Protan (USA). Activators, functionalizing agents, cross-linkers: Glutaraldehyde (GA 50%, Fulka, Buchs, Switzerland); 1,2–7,8 Diepoxyoctane (DEP) and Dimethylsuberimidate dihydrochloride (SUB) (Aldrich-chemie, Milan, Italy); 1-ethyl-3-(3-dimethylamino propyl)carbodiimide (EDC), 2-(Diethylamino)chloroethane hydrochloride (DE-HCl) and N-hydroxysulfosuccinimide (sulfo-NHS) (Sigma). Glucose, Fructose, Rhamnose, Catechin were supplied by Sigma; the buffers used were citrate-phosphate (C-P), phosphate-phosphate (P-P), carbonate-bicarbonate (C-B). The other reagents not specified here were high purity and supplied by Carlo Erba (Rodano, Italy).

2.2. Synthesis of DE-chitosan

A sample of chitin and chitosan taken from the same lot used for the tests of immobilization by simple adsorption was ground and sieved so as to obtain particle sizes of between 75 and 125 mm. Powdered chitin and chitosan were suspended in 0.1 M NaOH, recovered by filtration and repeatedly washed and filtered in sequence using water (4 times) and ethanol (3 times), and finally dried at 60°C under dynamic vacuum pumping until a constant weight was retained. The degree of the N-deacetylation of chitosan was determined by Infrared (I.R.) spectroscopy [27,28] as well as by acidimetry [29] and colorimetry [30] of the amino group. The N-deacetylation percentage values determined with the three aforementioned techniques showed excellent agreement. DE-chitosan was synthesized according to the method described by Kim and Lee [31].

5 g of chitosan (75–125 μm) are weighed and dissolved in 200 ml of methanol solution at 10% v/v of acetic acid. The methanol avoids gelation of the aminated support and increases solubility of the aldehyde. Next, 26.35 g of benzaldehyde were slowly added in ca 5 min and left to react for ca. 24 h at 60°C. In order to obtain protection for the amino group of the support, a gel is formed of the reaction intermediate: N-benzylidenechitosan. After several washes with methanol in order to remove the unreacted benzaldehyde (spectrophotometric determination at 280 nm), the alcohol is removed by vacuum evaporation at around 30°C. The powder obtained was swollen in a solution of Chloroform/Pyridine 1:1 and continuously stirred at 60°C for 24 h. 5.73 g of DE.HCl were added and left to react at 50°C for 24 h, thus obtaining O-diethylaminoethylbenzylidenechitosan. The product obtained is suspended in an ethanolic solution of HCl 0.25 M. With the deprotection of the amino group, acid hydrolysis is completed in 24 h at 25°C. The solid residue is filtered and washed several times with ethanol, in order to remove the unreacted DE.HCl, acetone and dyethyl ether (removal of benzaldehyde). Finally, it is treated with 1 M NaOH. The synthesized DE-chitosan support is vacuum anhydrized at 60°C for 48 h, in a dryer containing P_2O_5.

Microanalytical results (C, H, N, O) were provided by Redox Laboratories (Milan, Italy). ^{13}C-NMR solid-state CP-MAS spectra of DE-chitosan were obtained using a Bruker CXP 300 spectrometer operating at 75.45 MHz. The solid samples were mixed with salt and finely ground, then were packed into a 7 mm zirconium rotor and spun at the magic angle with a spinning rate of 4 kHz. The spectra were acquired with a standard CP sequence, the ^{1}H pulse duration was 4 μs which was also the value for the ^{13}C as set by Hartmann-Hahn conditions. A contact time of 1.5 ms was adopted for the cross-polarization while the recycle delay was 5 s [32–35].

The number of transients was of the order of several thousand in order to achieve a good signal to noise ratio. The chemical shifts were calibrated by replacement with respect to the lower frequency signal for the adamantine at 29.4 ppm.

The IR spectra were obtained using a Perkin-Elmer 1,750 Fourier Transform Infrared (FTIR) spectrophotometer. The samples were prepared by mixing the compounds with KBr

(about 0.3% w/w) and pressing the diluted mixture to form a clear semi-transparent pellet.

2.3. Immobilization

A sample of 10 mg of chitin, chitosan and DE-chitosan in a 10 ml test tube was equilibrated at room temperature for at least 20 min by means of 5 ml of 0.025 M citrate-phosphate (C-P) buffer at pH values between 3.2 and 8.0, whilst 10 ml of the purified enzyme solution containing about 9–11 U of Rha (about 1U/mg of support), brought to the same pH as the equilibration with HCl or NaOH 0.1 M, were 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 0.1 M HCl and 0.1 M NaOH according to the method recommended by the manufacturer (Whatman, USA).

For the activation of the chitin and chitosan, 5 ml of GA solution of concentration varying from 0.01 to 5% (w/v) in 25 mM C-P buffer at pH 6.0 were added to 10 mg of the supports. After about 2.5 h at 25°C, the suspensions were centrifuged at ca 2,500 g for 15 min at 25°C. The activated supports were then washed at least 2–3 times with 9 ml of distilled water to remove the GA, determined at 245 nm. In conclusion, following centrifugation, 9–11 U of Rha at pH 4.0, 6.0 and 8.0 were added to the activated supports in the manner previously described. The immobilization tests giving the best results were repeated using 10 ml of ca 25 U of Rha obtained by concentrating about 3 times the enzyme solution using lyophilization.

2.4. Cross-linking

The Rha adsorbed on 10 mg of DE-chitosan at pH 8 was cross-linked with various bifunctional agents.

With the GA, 5 ml of 0.5% GA at pH 4.0, 6.0 (10 mM C-P buffer) and 8.0 (10 mM P-P buffer) were added to the Rha adsorbed on 10 mg of DE-chitosan at temperatures of 4 and 25°C for 2.5 h. Subsequently, centrifugation took place at 2500 g for 15 min at the same temperature and the support was washed with 5 ml of 0.5 M NaCl and then with water, separating the surnatant by centrifugation.

For the SUB, 5 ml of a SUB solution with concentration varying from 0.005 to 0.5% (w/v) were added to the Rha adsorbed on DE-chitosan at pH 8.0 (10 mM of P-P buffer) for ca 2 h at 25°C. The biocatalysts obtained were separated and washed as above; the DEP was treated in the same manner except as regards the different cross-linking conditions: 4°C for around 2–3 days.

For the EDC, 5 ml of 0.02 M EDC dissolved in 10 mM C-P buffer were added to the adsorbed Rha at pH between 3.0 and 9.0. The cross-linking conditions are 4°C and 2.5 h in a carousel at 75 rpm. The support is once again separated by centrifugation. In the test at pH 8.0, increasing concentrations of solid NHS up to 0.5 M were also added with the EDC.

Finally, in several tests, 5 mg of rhamnose every 10 U of Rha were also added to the enzyme solution and left to equilibrate for at least 1h at 25°C before being added to the support.

2.5. Enzyme assays

Depending on whether the enzyme was free or immobilized, 50 μl of a solution or suspension of enzyme were added by stirring at 25°C to 450 μl of 2.0 mM pNPR (α-L-rhamnopyranoside and β-D-glucopyranoside, respectively) dissolved in 0.1 M C-P buffer at pH 3.5. The assay was carried out for 1–15 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. In determining immobilized enzyme activity, the residue material was removed by centrifugation at 2,500 g for 15 min before measuring absorbance. The order of the reagents was reversed in the blank test, i.e. Na_2CO_3 solution was added to the substrate before the enzyme. The color was read against the blank at a λ of 400 nm and determined by using a ϵ° of 18,300 M^{-1} cm^{-1}. On the basis of the results of a calibration curve, the spectrophotometric readings were considered valid up to maximum absorbance of about 0.9 U.

One unit of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenolate per minute under assay conditions [36,37]. Finally, protein determination was carried out by colorimetric reaction with Coomassie Blue G250 according to Bradford [38,39].

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 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. All the assays were done in triplicate.

2.6. Aromatization

Free and immobilized glycosidases were tested on natural substrates according to the method described in a previous work [1]. Samples of 1–2 ml of the free enzyme or the immobilized enzyme suspension (about 10 U of Rha) were added to 30 ml of a model wine solution in a test tube which contained 9 g/liter of tartaric acid in 10% ethanol, adjusted to pH 3.3 with potassium carbonate and with the addition of the aromatic precursors extracted and isolated from 100 g of *Moscato* skin grapes according to Gunata et al. [40].

The tube was sealed with a screw cap and incubated at 25°C for 18 h. At least five extractions were made from the solution using 10 ml of a pentane-dichloromethane mixture (2:1 v/v). The emulsion thus formed was stirred for 5 min, allowed to stand, and finally centrifuged at 2,000 g for 5 min. A known quantity of an n-heptanol hydroalcoholic

solution (200 μg/liter) was added, as an internal standard, to the fractions of pentane-dichloromethane. The sample was then dried in a cylinder using anhydrous sodium sulphate. The liquid fraction was concentrated to a few milliliters by means of distillation in a Vigreaux column. The analysis was carried out using a gas chromatograph HRGC 5300 (Carlo Erba, Rodano, Italy) comprising a Durabon Wax column according to Versini et al. [41].

2.7. Characterization

The following were characterized: free Rha, immobilized on chitosan activated with GA (C-GA), immobilized on DE-chitosan after adsorption at pH 8.0 and cross-linked with GA (D-GA) or EDC/NHS (D-EN). The purified enzyme concentrated 3 times by lyophilization was used to equilibrate the selected supports (about 2.5 U/mg of support). The following parameters were determined: optimum pH, between 3.0 and 7.5 in 0.1 M C-P buffer at 25°C; optimum temperature, between 25 and 85°C, at pH 3.5; stability over time at 70°C, in 0.1 M C-P buffer at pH 3.5 after 3 h, and at 25°C in wine (*Moscato*, Alessandria, Italy); the kinetic parameters K_m and V_{max} (at pH 3.5 and 25°C), calculated according to Lineaweaver and Burk [42] and, finally, the effect on the activity of the ethanol (up to 33% v/v), certain monosaccharides such as rhamnose, glucose and fructose (up to 10% w/v) and catechin (up to 80 mg/liter). All the analyses were done in triplicate.

3. Results and discussion

3.1. Immobilization on chitin and chitosan

Between pH 3 and 8, the adsorption of Rha on chitosan and chitin was almost virtually zero (AY = 1–3%). The enzymes are mainly adsorbed on the chitosan by way of electrostatic interactions and other weak interactions such as those of hydrogen, Van der Waals, London, etc. [43,44]. The electrostatic interactions between enzyme and chitosan are affected by the adsorption pH that, in order to be effective, needs to be between the isoelectric point (IP) of the enzyme and the pK_a of the chitosan, about 6.3 [4]. The lack of adsorption of Rha on the chitosan could be due to the fact that the enzyme IP, about 6.0, [1] is similar to chitosan pK_a, whilst for chitin, the high degree of acetylation (about 93%) almost entirely rules out the establishment of electrostatic interactions.

In order to allow Rha adsorption, the chitin and chitosan were activated with glutaraldehyde (C-GA) to increase their reactivity toward proteins [45–47]. Starting from concentrations of glutaraldehyde (GA) ≥ 0.5%, the chitosan activated with GA (Fig. 1) shows total adsorption of Rha (AY = 100%) with trends unrelated to the support activation pH. Nevertheless, the immobilized Rha was almost entirely inactivated, with low IY values (less than 1%),

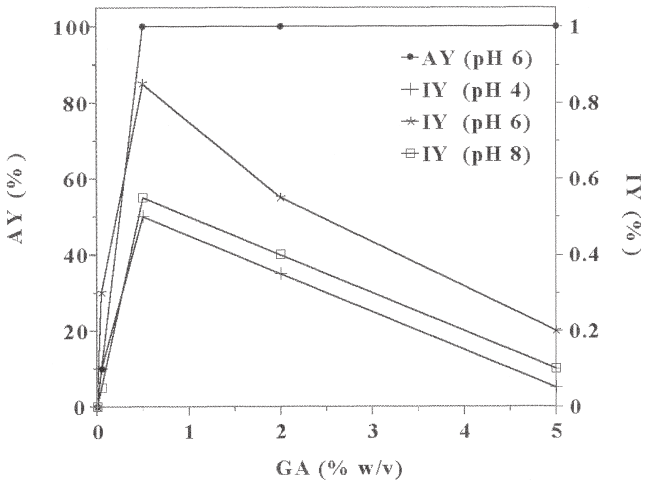

Fig. 1. Adsorption yield (AY) and immobilization yield (IY) of Rha immobilized (at pH 4.0, 6.0 and 8.0) on chitosan activated with GA (C-GA), as a function of the cross-linker concentration. Activation step at pH 6 and 25°C for 2.5 h.

reaching maximum values at 0.5% GA concentration and at pH 6.0 (9 U g^{-1} of support)

GA is a bifunctional reactive agent mainly capable of reacting with the surface amine groups of the enzyme and chitosan, through the formation of Schiff bases and Michael adducts [48]. The nucleophilic property of the amino groups required for the reaction is also assured at lower pH values by microenvironmental effects of the chitosan surface [49]. The protonated amine groups of the chitosan have a repulsive effect on the solvated hydrogen ions, so the pH close to the surface becomes higher than in the bulk of the solution, thus favoring the reaction. At low concentrations of GA, it is probable that the aldehyde groups present on the support cross-link the amine groups of the chitosan, and the few reversible bonds (Schiff bases) involving the enzyme molecules are not sufficient to avoid their leakage. By raising the concentration of GA, the increase in the aldehyde groups reaches a point of saturation that allows complete adsorption of the equilibrated enzyme. The considerable inactivation of the free and immobilized Rha induced by the GA could be due to the involvement of GA with one or more amine groups essential to catalysis in cross-linking. The maximum IY value is seen at the Rha IP (≅6.0) since at such pH the intra- and inter-molecular interactions of the immobilized enzyme molecules are at their height. It is probably here that the enzyme is to be found in its optimum globular conformation and protects itself from unfolding phenomena. On the other hand, at pH immobilization values far removed from its IP (4.0 and 8.0), the enzyme assumes a net charge different from zero, so that the repulsive phenomena eliminate the self-protection effect. At an intermediate concentration of GA (0,5%) the lower density of bond per enzyme molecules and its high amount adsorbed, probably give a less distorted conformation; this effect is favored by secondary interactions of “self-protection” between the enzyme molecules.

Unlike chitosan, in chitin, adsorption undergoes only a very slight increase (YA = 2–5%); the situation is basically identical to that of the non-activated matrix [50,51]. In such case, it is probably the chitin's high degree of acetylation that determines only reduced activation of the support. Such an hypothesis is confirmed by the fact that no staining of the chitin (typical of the imino bond formed) is noted during the activation phase.

3.2. Immobilization on derivatized chitosan

The poor results obtained from the previous methods of Rha immobilization on chitosan and chitin by adsorption of the enzyme, with or without activation of the support with GA, have forced us to consider the possibility of its derivatization. Adsorption of Rha on DE-cell and CM-cell was carried out, between pH 3.0 and 8.0, in order to assess the more efficient derivatization of the chitosan.

The Rha adsorbed on CM-cell shows elevated AY at pH levels below 6.0, while IY values rise only slightly. Such trends can be explained by assuming the establishment of enzyme-support electrostatic interactions. At acid pH, the negatively charged CM-cell is capable of absorbing Rha, got as long as it is positively charged, i.e. close to its IP, after which the increase in pH induces an electrostatic repulsion between enzyme and support. At acid pH, the slight increase in the IY could depend on the larger amount of adsorbed enzyme that minimizes its secondary interactions with the support.

The DE-cell shows contraring yield trends compared to those of the CM-cell with zero yields at pH below 5.0 and increasing at higher pH. The DE-cell is an anion exchange resin positively charged (pKa 9.0) at all pH values tested. At pH below 6.0, a situation of repulsion between Rha (IP $\cong$ 6.0) and matrix could prevail, as they are both positively charged, therefore the AY is zero. On increasing pH, electrostatic attraction rises progressively since the Rha assumes a negative charge and is captured by the positively charged DE-cell (tertiary protonated amino group).

If we compare the behavior of the two cellulosic supports, it can be noted that yields, and in particular IY, are higher for DE-cell than for CM-cell. The different activity retention could be correlated with the different amino acid residues involved in Rha adsorption and different orientation assumed by the enzyme molecules on the surface of such matrixes. Rha adsorption on the positively charged DE-cell should mainly take place through the enzyme's dissociated carboxyl groups, whilst that on the negatively charged CM-cell through the protonated amine groups (e.g. lysine). These results seem to indicate that the amino groups are essential in Rha catalysis. In addition, a "space arm" effect could occur for the DE-cell, caused by the diethylaminoethyl groups that facilitate the access of the substrate to the active site and minimize secondary deforming enzyme-support interactions.

In view of the favorable results obtained for DE-cell, in order to improve the use of the chitosan, the DE-chitosan was synthesized, as already described, and characterized.

Table 1
Experimental and theoretical C, H, N, O analysis for the samples of the unsubstituted chitosan (62% of deacetylation) and the DE-chitosan

Sample	Experimental Analysis (%)				Ratio		
	C	H	N	O	C/N	C/O	O/N
Chitosan	44.4	7.3	7.9	40.4	5.6	1.1	5.1
DE-chitosan	50.0	12.3	9.1	28.6	5.5	1.8	3.1
	Theoretical Analysis (%)						
	C	H	N	O	C/N	C/O	O/N
Chitosan	45.8	6.6	7.9	39.7	5.8	1.2	5.0
DE-chitosan	51.0	11.8	9.2	28.0	5.5	1.8	3.0

The characterization of the structural changes occurred in the derivatization from the initial chitosan up to the o-diethylaminoethyl derivative (DE-chitosan) was provided by the elementary analysis and spectroscopic (NMR and IR) data.

The elementary analysis data for the DE-chitosan, reported in Table 1, were compared with those of the unsubstituted material, and showed that the ratio C/N resulted unchanged whereas the ratio O/N decreased and the ratio C/O increased as forecast in theoretical analysis.

A further confirmation of the satisfactory derivatization of the chitosan came from the ^{13}C-NMR solid-state CP-MAS spectrum that, when compared with the initial chitosan, showed significant differences in the intensity and in the number of signals. The peak at 23.7 ppm, not observed in the chitosan, was assigned to the methyl groups of the N, N-diethylaminoethyl group. Instead the NCH_2 carbons of the same group were attributed to a part of the increased signal between 58–65 ppm where also the C_2 (NCH) and C_6 (OCH_2) carbons of the glycoside and the C_7 (OCH_2) of the diethylaminoethyl group are included. The most intense peak at 75 ppm was assigned to the carbons C_5 and C_3 (OCH) whereas the carbons C_4 and C_1 of the glycosidic ring were assigned to the broad peak at 83 and 105.5 ppm respectively.

A comparative analysis of the IR spectra of the initial chitosan and the DE-chitosan does not show substantial differences; however the DE-chitosan spectrum showed a more marked absorption band in the region of 2,850–2,900 cm^{-1}, CH stretching vibration, due to the increased number of the methylene groups. In addition to that, the disappearance, after the last reaction, of the bands observed at 760 and 720 cm^{-1} in the benzilidene intermediate suggests the substitution of the diethylamino-ethyl group on the CH_2OH of the chitosan.

DE-chitosan shows similar behavior to that of the DE-cell (Fig. 2), allowing at pH 8.0 high yields and activity of about 80 U g^{-1}; this appears to confirm the validity of the derivatization and the previous hypothesis on the DE-cell. Nevertheless, the enzyme adsorbed on DE-chitosan was

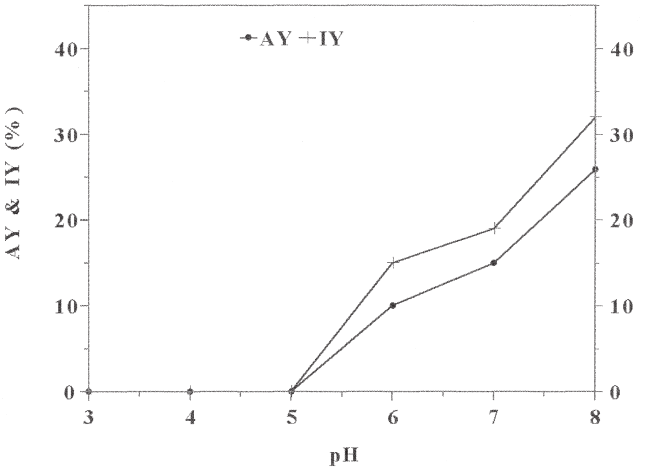

Fig. 2. Yields (AY and IY) of Rha adsorbed on DE-chitosan as a function of pH.

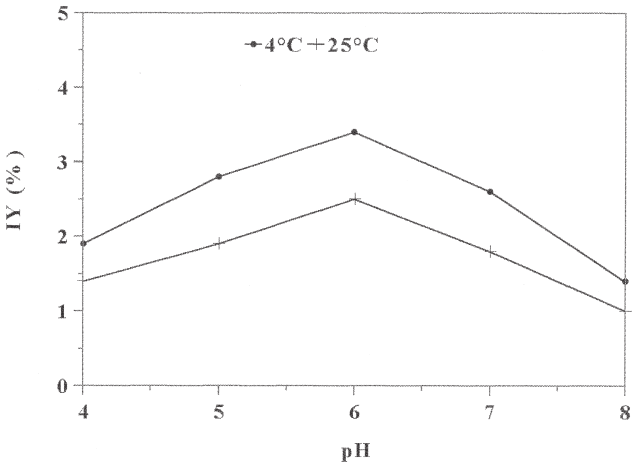

Fig. 3. IY of Rha adsorbed (at pH 8) on DE-chitosan and cross-linked with GA (at 4 and 25°C) (D-GA), as a function of pH.

released completely in solutions with high ionic strength, such as saline (2 M NaCl) and wine, or in buffer solutions at pH $\leq$ 5.0, but in the latter case the leakage took place very slowly (release of 10% after about 2.5 h). The enzyme leakage in solution at acid pH can be easily explained by considering the enzyme-matrix electrostatic repulsion due to the previously mentioned difference in IP values of the Rha and pKa of the support.

3.3. Immobilization by cross-linking on DE-chitosan

The high enzyme activity retention achieved with the Rha adsorption on DE-chitosan (at pH 8.0) and the difficulty in leakaging the Rha probably adsorbed in its optimum molecular orientation, have allowed us to carry out at a subsequent stage cross-linking with bifunctional agents at high pH and time ranges. In fact, this procedure could avoid the leakage of the enzyme and, at the same time, increase its stability.

Depending on the pH (Fig. 3), the enzyme adsorbed on DE-chitosan cross-linked with GA (D-GA) shows similar IY trends at the two temperatures tested, with a maximum at pH 6.0 and at 4°C. Nevertheless, IY values for D-GA are still low, even though higher than those of the Rha immobilized on chitosan activated with GA (C-GA). An interpretation based on enzyme IP seems plausible. At pH close to the enzyme IP, the Rha self-protection restricts the deforming action of GA. The best values for cross-linking at 4°C are mainly attributable to the lower reactivity of the GA, thus resulting in a lower density of bond per enzyme molecule with reduced conformational distortion.

Two other bifunctional reagents were tested, a diepoxide (DEP) and an iminoester (SUB), both with chain length C_8. In both cases, the cross-linking reaction is carried out at alkaline pH, in order to facilitate the S_N2 mechanism between the enzyme-support nucleophilic (aminic) groups and the electrophilic centers of the cross-linkers [52] while maintaining the optimum orientation of the enzyme adsorbed. These cross-linkers show different IY trends (Fig. 4), with maximum IY values (about 4.0%) almost coincident but slightly higher than those of cross-linking with GA. On varying the concentration of DEP, the IY show a steep rising trend up to 0.05%, then reach a plateau. This can be explained considering that on the basis of the high reaction time (2.5 days at 4°C), only at the highest concentrations of DEP, the enzyme will not be released from the support. The slight increase in IY, with respect to GA, could be due to its lower reactivity and to the higher selectivity of cross-linked reactions. In addition, its chain length (C_8) could have a positive effect on the IY, reducing the phenomenon of steric hindrance, caused by the closeness of the enzyme to the support, and making the enzyme more flexible and efficient in its interaction with the substrate. Finally, by using this cross-linker, the product formed by immobilization is an amino alcohol derivative, stable at the acid pH characteristic of wine solution.

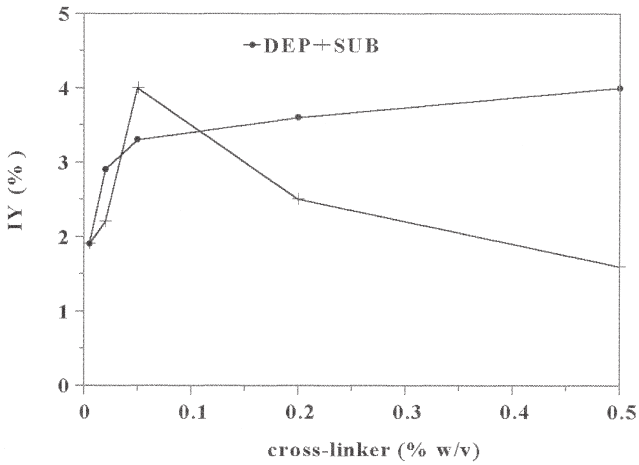

Fig. 4. IY of Rha adsorbed on DE-chitosan and cross-linked with DEP and SUB as a function of cross-linker concentration.

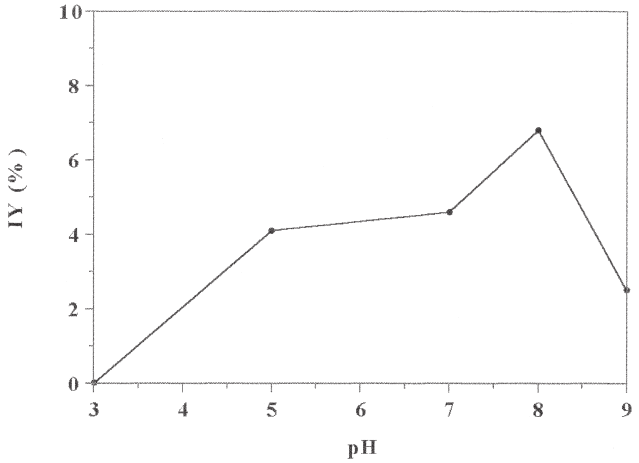

Fig. 5. IY of Rha adsorbed on DE-chitosan and cross-linked with EDC as a function of pH.

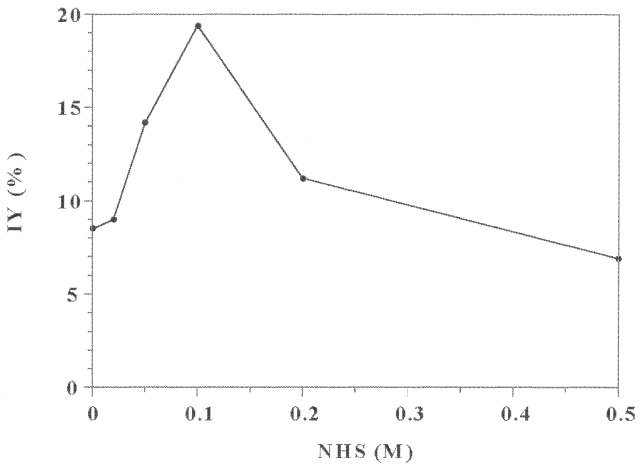

Fig. 6. IY of Rha adsorbed on DE-chitosan and cross-linked with EDC (0.02 M) and NHS (D-EN), in the presence of rhamnose, as a function of NHS concentration.

SUB is an iminoester salt with a center of unsaturation to which the nucleophilic group of enzyme and chitosan can be added under alkaline conditions, with the release of a methoxyl group and re-establishment of the imino bond [53]. Like DEP, SUB has fewer side reactions than GA, but is more reactive. The SUB IY values (Fig. 4) are always low and similar to those obtained with DEP, but in this case, their trend does show a maximum. The better results when compared to GA could be due to the same reasons that regard DEP: few side reactions and greater chain length (C_8). In addition, with SUB, protonable nitrogen is preserved at acid pH (imino group). The drastic fall in activity at higher cross-linker concentrations is probably due to the high density of the enzyme-support bonds that caused marked distortion in the enzyme structure.

In conclusion the low IY value obtained in the cross-linking with GA, DEP and SUB, all reactive toward the amino group, seems to confirm the involvement of that group in the biocatalytic mechanism. It would appear essential to minimize its involvement in the cross-linking of the adsorbed Rha using different cross-linkers capable of involving other functional groups of the enzyme.

EDC is a cross-linker of zero length that, besides having a good specificity action and moderate reactivity, can form an amide bond between carboxyl groups of the enzyme and amine groups of the support.

Formation of the amide bond basically occurs in two stages (activation and coupling): first, the activation of the carboxyl group, leading to the formation of the activated derivative O-acyl-isourea; second, the formation of an amide bond with elimination of urea that acts as a thermodynamic drive force [54].

Higher yields for EDC at pH 8 (Fig. 5) are compared to the previous cross-linkers, with IY values of about 7% and activity of about 18 U g^{-1}. The zero IY noted at pH 3.0 are probably caused by the low reactivity of EDC, in such condition, toward the carboxyl groups and thus by the leakage of the enzyme molecules during cross-linking. The possible side reaction of aqueous hydrolysis at acid pH could also reduce the efficiency of the main cross-linking one. At pH 9.0, extraneous proteins could also compete in the cross-linking reactions. Moreover, other enzyme amino acid residues containing hydroxyl (e.g. tyrosine) and carboxyl (e.g. aspartic and glutamic acids) groups could react with the electrophilic center of the EDC unsaturated carbon and with the activated enzyme carboxyl [55]. This favors the capture of Rha, but probably increases the density of bonds per enzyme molecule, thus causing conformational distortions and steric hindrance of the substrate toward the active site, amplified by the zero length of the cross-linker. Altogether, IY values are, as a whole, higher than other previous cross-linking methods thus confirming the essential biocatalytic role of the amino group [56].

The addition of rhamnose during adsorption and cross-linking with EDC has produced a slight increase in IY (up to 8%) and thus in activity (about 21 U g^{-1}). As a competitive inhibitor, rhamnose is capable of binding to the Rha active site, protecting the residual amino acids present and blocking the enzyme in its optimum conformation. This increases the resistance of the protein to deactivating distortion phenomena that may occur during immobilization.

To optimize this methodology as suggested by Klibanov et al. [57], NHS was added to EDC (Fig. 6). NHS increases EDC selectivity, reducing its competitive reactions and facilitating the main immobilization reaction, i.e. formation of the amide bond, as well as avoiding those of probable deactivation. The immobilization yield of Rha increases at intermediate NHS concentration, with a maximum IY of 19%, a slight fall compared to the simple adsorption and an activity of 50 U g^{-1}.

With NHS, the formation of the enzyme-support amide bond occurs in different stages. Firstly there is the activation of the carboxyl groups with EDC, then the formation of the N-hydroxysulfosuccinimide ester derivative that subsequently reacts with the aminic groups. Compared to the

Table 2
Physical-chemical characterisation of Rha free and immobilized on chitosan activated with GA (C-GA), DE-chitosan cross-linked with GA (D-GA) and EDC/NHS (D-EN)

Enzyme	Support	Cross-linker	AY (%)	IY (%)	Activity (U g^{-1} of support)	pH Optimum	Temperature optimum (°C)	Relative stabilities		Vmax U (mg protein)$^{-1}$ min^{-1}	Km mM
								70°C	25°C		
Free	none	none				4	70	1.0	1.0	1.30	1.35
Immobilised	chitosan	C-GA	100	0.85	21	3.5–4	75	1.9	2.2	0.0	2.70
	DE-chitosan	D-GA	25.9	3.4	22	3.5	75	1.6	2.0	0.65	2.70
		D-EN	25.9	19.4	126	3.5	70	1.3	1.6	1.20	1.35

Note: Relative stabilities at 70°C in the buffer at pH 3.5 (after 3.5 h) and at 25°C in wine (after 20 d) referred at residual activity of free enzyme (25% at 70°C and 36.5% at 25°C).

activated intermediate obtained with only EDC, the intermediate activated with NHS is affected to a lesser extent by the side reactions of aqueous hydrolysis and cross-linking between the functional groups (hydroxyl and carboxyl) of the enzyme residues (tyrosine, aspartic and glutamic acids).

The low concentrations of NHS result in its poor efficiency, as the enzyme carboxyl groups are present to a greater extent under the form of the derivative with EDC. On the other hand, by increasing the concentration of NHS, there is a rise in the forms relative to the activated carboxyl groups that make the action of the EDC more effective. Finally, at the highest concentrations of NHS, cross-linking efficiency presumably increases, but the Rha conformation, bound at several points (many reactive carboxyls), is more distorted.

3.4. Characterization of Rha

For chemical-physical characterization, the Rha immobilized on chitosan activated with GA (C-GA), on DE-chitosan and cross-linked with GA (DE-GA), and on DE-chitosan and cross-linked with EDC/NHS (D-EN), were compared to the free enzyme. The biocatalysts chosen also allow the effects of the different matrixes and cross-linking procedures to be assessed. In order to increase the activity of the immobilized Rha, all matrices were equilibrated with a more concentrated enzyme solution (about 3 times) with respect to previous tests, without the yields (AY and IY) suffering any variation. Table 2 summarizes the main results obtained regarding the characterization trials.

Fig. 7 shows relative activity trends as a function of pH. Compared to the free enzyme, the Rha immobilized on chitosan activated with GA (C-GA) has a broader peak activity at more acid pH. The enzymes immobilized on DE-chitosan (D-GA and D-EN) follow a similar pattern, with a shift of optimum pH toward the acid zone (from 4.0 to 3.5) and a more pronounced trend of activity compared to the free enzyme. In the pH range of wine (3.0–3.5), the relative activity of various immobilized enzymes and the free enzyme has the following decreasing order: D-EN, D-GA, C-GA, and free enzyme. The shift of the optimum pH of the biocatalysts, advantageous for use in wine making, is characteristic of enzymes immobilized on polycationic supports such as chitosan and DE-chitosan. These have amine groups that are easily protonable at acid pH, which determine an increase in the concentration of hydroxyls, in the microenvironment of the immobilized enzyme, that in turn raises pH with respect to the bulk of the solution [52]. This hypothesis could also explain how the type of cross-linking does not affect the behavior of the Rha immobilized on DE-chitosan (D-GA and D-EN).

Fig. 8 shows the pattern of relative activity as a function of temperature. The free enzyme and the immobilized D-EN have similar relative activity trends with coinciding values for optimum temperature (70°C). Despite the difference in the supports, the two immobilized with GA, C-GA and D-GA, have similar relative activity trends and a slightly higher optimum temperature value (75°C) compared to the previous cases. These trends could depend on the high density of bonds per enzyme molecule formed by the GA. The cross-linkers [58,59] are capable of increasing the conformational rigidity of the enzyme and thus raising the activation energy of the thermal denaturation reaction. GA

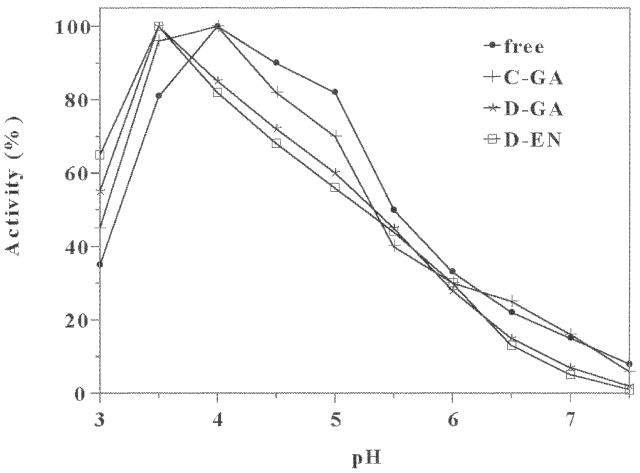

Fig. 7. Relative activity of Rha free and immobilized on chitosan activated with GA (C-GA), DE-chitosan cross-linked with GA (D-GA) and EDC/NHS (D-EN), as a function of pH. The data are the mean values of three analyses. The variability in activity was ±2% (with a 95% of confidence interval).

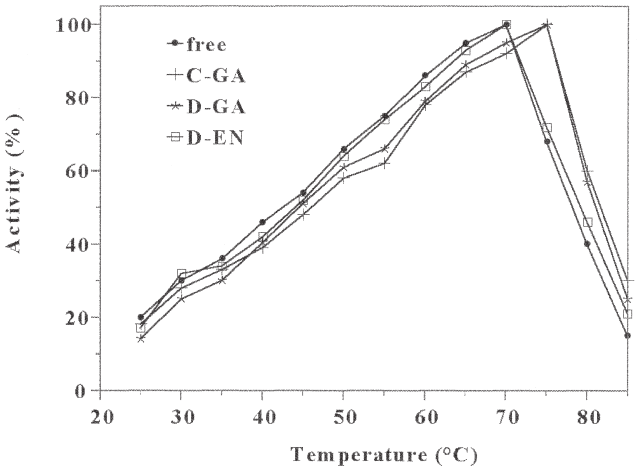

Fig. 8. Relative activity of Rha free and immobilized on chitosan activated with GA (C-GA), DE-chitosan cross-linked with GA (D-GA) and EDC/NHS (D-EN), as a function of temperature. The data are the mean values of three analyses. The variability in activity was ±2% at temperature <60°C and ±6% at temperature ≥60°C (with a 95% of confidence interval).

is a more powerful cross-linker than the others utilized, therefore the immobilized enzyme molecules are probably more cross-linked, i.e. less mobile and more distorted; this would explain the lower activity, the higher thermal resistance of Rha cross-linked with GA and the moderate influence of the type of support used (chitosan or DE-chitosan). Differently, in the case of D-EN the enzyme probably finds itself in a more similar state to that of the free one.

Stability tests for the free and immobilized state are of considerable technological interest. In accelerated stability tests at 70°C in an acid buffer (pH 3.5) it is possible to observe the following increasing order of stability: free enzyme, D-EN, D-GA, C-GA (Table 1). In tests effected in wine at ambient temperature, stability kept to the same order (Fig. 9).

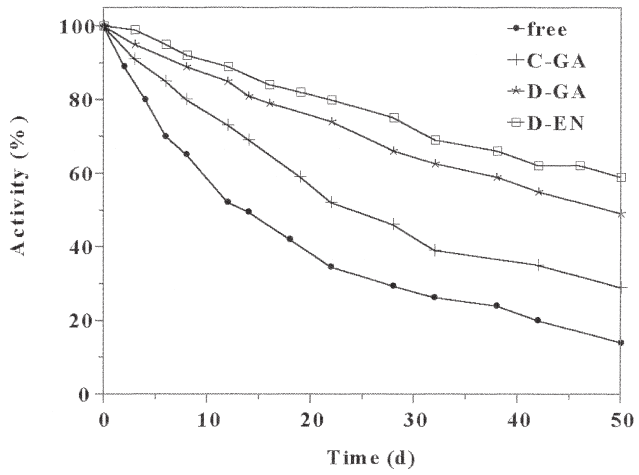

Fig. 9. Stability over time of Rha free and immobilized on chitosan activated with GA (C-GA), DE-chitosan cross-linked with GA (D-GA) and EDC/NHS (D-EN), in wine at 25°C.

These results could be explained on the basis of theories enunciated by various authors [57–59] who, as regards enzyme stability, generally assume unfolding of the protein structure one of the main phenomena involving the irreversible mechanism in enzyme inactivation. In such case, it may be supposed that the stability results are closely connected to variations in the conformational structure of Rha and particularly in the rigidity of the secondary and tertiary structure of enzyme that reduces the unfolding rate. These last data could be correlated with previous results, in particular with those of activity and optimum temperature (Table 2).

In the supports (chitosan and DE-chitosan) cross-linked with GA (C-GA and D-GA), the high reactivity of the cross-linker causes a high density of enzyme-support bonds that increases rigidity and conformational distortion of the immobilized Rha molecules. Consequently, stability and optimum temperatures are higher, whilst activity decreases due to the lesser adaptability of the enzyme's active site toward the substrate. On the contrary, the behavior of D-EN could also be explained in terms of the high structural mobility of Rha that determines greater activity and facilitates the unfolding phenomenon of its structure.

The values of kinetic parameters K_m and V_{max} are the same for the free Rha and immobilized D-EN, as well as for Rha cross-linked with GA (C-GA and D-GA). On the whole, the behavior noted in this test also confirms that the method used for immobilization of Rha on DE-chitosan cross-linked with EDC/NHS (D-EN) alters the characteristics of the enzyme to a lesser extent.

On the other hand, for the enzymes immobilized with GA (C-GA and D-GA), the reduction of V_{max} and increase in K_m are typical of the passage from a free enzyme to an immobilized one [52] and may be attributed to at least two phenomena that can occur following immobilization of the enzymes on solid supports. The first regards problems of substrate diffusion caused by the microenvironment of the immobilized enzyme. This phenomenon would result in a lower concentration value of the substrate surrounding the immobilized enzyme than in the "bulk" of the solution, thus altering the value of its K_m that would be higher than the effective one (apparent K_m). The second phenomenon, "steric effects," arises from the structural rigidity of the enzyme conformation, distorted following covalent immobilization, that could reduce the affinity toward the substrate of the active site of the immobilized enzyme, thus increasing K_m and lowering V_{max} as a result of reduced flexibility of the entire enzyme structure. Therefore, the two phenomena described above would not appear to be so important for the biocatalyst D-EN. As already suggested, its probable state of greater mobility and lesser distortion compared to the enzymes immobilized with GA (C-GA and D-GA) makes its kinetic parameters similar to those of the free Rha.

Finally, an assessment was made of the possible inhibiting action of the rhamnose and several compounds present in the must and/or wine, such as monosaccharides (glucose,

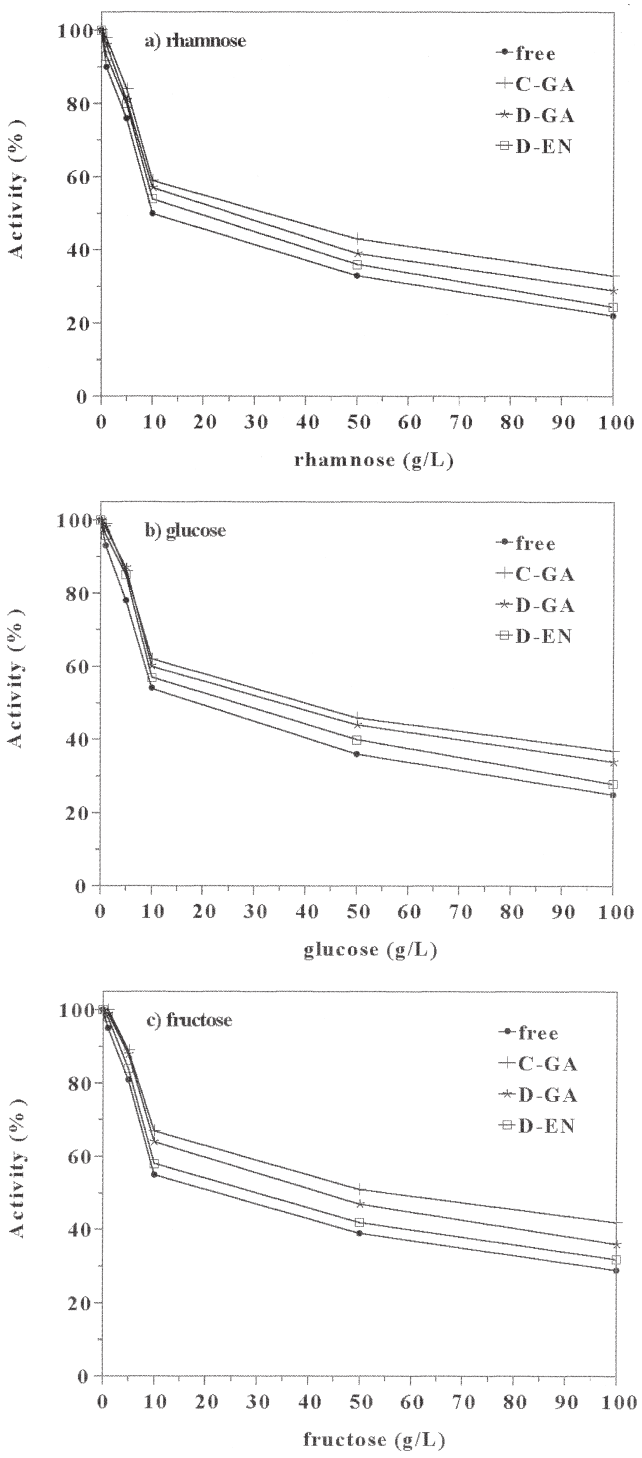

Fig. 10. Relative activity of Rha free and immobilized on chitosan activated with GA (C-GA), DE-chitosan cross-linked with GA (D-GA) and EDC/NHS (D-EN), as a function of rhamnose (a), glucose (b) and fructose (c) concentration. The data are the mean values of three analyses. The variability in activity was ±2% (with a 95% of confidence interval).

fructose), ethanol and catechins. The inhibiting action of the three monosaccharides on the free Rha and immobilized enzymes shows a similar pattern (Fig. 10) with extensive falls in activity at low concentrations (up to ca 10 g/liter) which lessen slightly upon increasing the concentration. For all monosaccharides inhibition followed the decreasing order: free enzyme, D-EN, D-GA and C-GA; whilst, using the same biocatalyst, inhibition decreased in the order, rhamnose, glucose and fructose.

In order to act, the monosaccharides must bind to specific sites of the enzyme structure; if this has become more rigid and distorted due to the immobilization, the action of these compounds is probably reduced. This could explain the high inhibiting action suffered by the free enzyme and the decreasing order of efficiency on the various immobilized Rha.

Technologically it is interesting to verify the action of ethanol on free and immobilized rhamnosidase. For free and immobilized Rha, the reduction in relative activity as a function of the rising concentration of ethanol is quite marked. At concentrations of about 15% this has already fallen to almost half the initial value. The activity reductions follow the decreasing order: C-GA, D-GA, D-EN and free enzyme, with less marked differences at the various rising concentrations of ethanol.

Ethanol acts, in an aspecific way, on the overall state of the enzyme conformation, mainly through weak interactions, in particular hydrogen bonds. These can cause its partial destabilization or more marked denaturation. Following immobilization, that presumably stiffens the enzyme structure, the action of ethanol is reduced. Nevertheless, the protective effect to be seen following immobilization is less marked for ethanol compared to monosaccharides, perhaps due to the reduced molecular size of this compound that determines easy diffusion.

Finally, no type of inhibition was noted for the free or immobilized rhamnosidase as regards phenols such as catechins (up to concentrations of 80 mg/liter).

3.5. Aromatization

Both the free enzyme and the immobilized D-EN show β-glucosidase (βG) activity; the ratio with Rha is ca 1 and 0.5 respectively, therefore the aromatization tests were carried out without adding more βG. If we consider the terpenols that offer the greatest contribution to the aroma of a *Moscato* wine (Table 3), the enzyme in both free and immobilized form (D-EN) causes a net increase in them (doubled). Nevertheless, both enzymes act with greater selectivity toward primary alcohols (citronellol, nerol, geraniol) compared to tertiary ones (linalool), especially the immobilized form. This behavior could depend on the lesser selectivity of glycosidases toward tertiary alcohol caused by phenomena of steric hindrance of the substrate toward the enzyme's catalytic site. This is even more apparent in the case of immobilized enzyme whose active sites are probably even less accessible to the substrates.

Table 3
Aromatic compounds in *Moscato* wine following treatment with or without enzyme (free and immobilized)

Aromatic compounds	Without enzyme ($\mu g\ L^{-1}$)	Free enzyme ($\mu g\ L^{-1}$)	Immobilized enzyme (D-EN) ($\mu g\ L^{-1}$)
Linalool	250	358	271
α-terpineol	60	62	63
Citronellol	45	398	418
Nerol	32	139	129
Geraniol	122	241	258
Total terpenols	509	1198	1139
Selectivity	0.8	2.1	3.0

4. Conclusions

Chitosan only adsorbs the Rha if activated with GA; nevertheless, the enzyme immobilized in this manner has almost no activities. The functionalization of chitosan with 2-diethylaminochloroethane hydrochloride (DE.HCl), decided after a preliminary test with DE-cell, allows good adsorption and activity retention. The results obtained seem to indicate that the amino group plays an essential role in the catalysis of Rha from *Aspergillus niger*.

Under the pH conditions of wine, the enzyme is almost entirely released, albeit slowly. The problem was solved by cross-linking the immobilized enzyme with bifunctional agents (GA, DEP, SUB, EDC). Amongst these, EDC in combination with NHS is the one best suited to cross-linking (D-EN), since with a view to its potential use in wine making, it combines high activity (about 130 U g^{-1}) with good stability, sufficient for aromatization, even though less than that of the Rha immobilized on chitosan activated with GA (C-GA) and DE-chitosan cross-linked with GA (D-GA). In addition, the inhibiting action on the activity of the immobilized Rha of some compounds present in the must and/or wine, such as certain monosaccharides and ethanol, was lower than that of the free enzyme and absent as regards catechins. Nevertheless, with respect to the free enzyme, the biocatalyst D-EN acts with even more marked selectivity toward primary glycosidic terpenols compared to tertiary ones and this could also alter the natural aroma profile of the wine. Thus an improvement to the immobilization method would be desirable, to make the wine aroma similar to that obtained with the free enzyme.

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