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Influence of the presence of NaY zeolite on the activity of horseradish peroxidase in the oxidation of phenol

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Abstract

In this work a new enzyme–zeolite interaction was examined. Horseradish peroxidase (HRP-C), a heme-containing enzyme, shows an activity increase for HRP-C catalysed phenol and 4-aminoantipyrine co-oxidation in aqueous medium, in the presence of NaY zeolite, although the attempts to use this enzyme immobilized on the zeolite resulted in an almost complete loss of activity.

Pseudo-homogeneous mechanical mixtures of enzyme and zeolite in solution were compared at five different temperatures (10, 20, 25, 30 and 35 °C), and have shown possible interactions which influence the enzyme activity. In order to better analyse the interactions effects, co-immobilization of the enzyme and zeolite was performed in gelatin particles; in this case the enhancement in enzymatic activity was much larger than when pseudo-homogeneous mixtures were used. HRP-C immobilized in gelatin showed activity increases as large as 100% when zeolite NaY is co-immobilized in the same particles, depending on the experimental conditions.

Although the mechanism by which the zeolite induces this effect still requires clarification, one possible explanation is that some ionization of OH groups of the enzyme occurs, due to ion exchange of Na-ions of the zeolite and protons of the enzyme, an effect which might also occur in the gelatin matrix; the zeolite may also influence the conformation of the enzyme by means of the electrical fields that are generated at the surface of these materials, which are known to influence the catalytic action of zeolites.

Keywords: Horseradish peroxidase; Zeolites; Heterogeneous catalysis; Phenol oxidation; Gelatin immobilization

1. Introduction

The use of zeolites in conjunction with enzymes has been reported in different contexts and is advantageous because of the particular properties of zeolites, such as their ability to concentrate species inside their pore system, regulate the activity of adsorbed water or supply acidity that can be used in bi-functional enzymatic-acid catalysts. Thus, zeolites, also known as molecular sieves, have been used to control the micro-environment of enzymes, such as lipases [1–11], for various reactions, or α-chymotrypsin and thermolysin for peptide synthesis [12], or in the dynamic resolution of enantiomeric species [13]. Their ability to adsorb water is particularly important when they are used in reaction mixtures which comprise enzymes in organic media; their ability to adsorb of water in condensation reactions can help to shift the reaction in the direction of the products [1–5,14–16].

However, in some cases, there are both desirable and undesirable effects. For example, in the conversion of ascorbic acid with a lipase (from *Candida antarctica* type B) addition of zeolites increased the enzyme activity, but the zeolites component also adsorbed and degraded the reactant [17]. The synthesis of butyl isobutyrate is another example of the benefit in the selective adsorption of water in the organic media [18]; however, in this case the adsorption of isobutyric acid on the zeolite also constitutes a problem.

Zeolites can also be easily changed by ion exchange, something that allows them to be tuned, both in acid/base and electrolytic properties, so as to better match them to a particular application. Acid base effects in non-aqueous media have been reported in the context of zeolite/enzyme systems [19–21].

It was also reported that zeolites could enhance protein synthesis in cell-free system [22], partially due to the removal of inhibitory substances from the reaction mixture system [23].

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Zeolites have also been used in the construction of enzyme-modified carbon paste electrodes, for instances with tyrosinases on zeolite for the detection of catechol [24].

It has also been found that zeolites increased storage and operational stability of lipases in hydrolysis and synthesis reactions, when the lipase was adsorbed on the zeolite; in these studies it was also found that NaY was the most favourable one, and that the presence of acidity in the zeolite was detrimental to the enzyme [1–6]. Apart from the advantages found for enzyme stability, the use of immobilized enzymes is also beneficial since it produces heterogenized catalysts which can be more easily used and recovered.

In this work we inspected the influence of the presence of NaY zeolite on the activity of horseradish peroxidase, for the co-oxidation of phenol and 4-aminoantipyrine with hydrogen peroxide, in aqueous medium, a reaction which is often used for analytical purposes for the detection of phenol [25] as well as for glucose determination [26]. The studies included the influence of zeolite on the free enzyme and on the enzyme entrapped in gelatin particles along with the zeolite.

2. Experimental

2.1. Materials

Horseradish peroxidase (HRP-C [E.C.1.11.1.7] MW = 40 kDa) with a specific activity of 252 U/mg (according to the pyrogallol method performed by the supplier) was obtained from Bioenzyme and stored at −18 °C. Aqueous solution of H_2O_2 (≈30%) was supplied by BDH. Gelatin, sodium phosphate and phenol were purchased from Merck and the 4-aminoantipyrine (Am-NH_2) was purchased from Sigma–Aldrich. Aqueous solutions of H_2O_2 were standardised by permanganometry using $KMnO_4$ standard solution (Merck). Distilled water was used in all the experiments and all solutions were stored at 4 °C.

The zeolite used was NaY, in powder form (LZ Y52 from Union Carbide with Si/Al = 2.5 and particle size ≈4 μm, formed by crystallites of ≈0.5 μm). For the control experiments a porous silica (EP10X from Crosfield with particle size ≈53 μm), hereafter referenced as $(SiO_2)_p$, was used. The zeolites and the silica samples were calcined, under air at 793 K, prior to use, to ensure a clean surface. After calcination they were stored in a closed vessel under a constant and high humidity atmosphere.

The Lowry method used sodium carbonate (Na_2CO_3), potassium sodium tartrate ($C_4H_4KNaO_6$) and copper sulphate ($CuSO_4$), all purchased from Merck. The Folin–Ciocalteu's phenol reagent was supplied by Fluka and albumin bovine fraction V (BSA) was obtained from BDH.

2.2. Instrumentation and equipment

Spectrophotometric measurements were performed on a HITACHI U-2000 UV/Vis spectrophotometer equipped with a thermostatically controlled cell compartment. The cell volume is 1 ml in volume and has a path length of 1 cm. The data from the spectrophotometer was collected with an external computer.

A thermostat bath model Thermomix®/Frigomix® was used to maintain temperature stability.

To homogenize the reaction mixture a miniature electronic stirrer (Model 300—Rank Brothers, UK) was used.

Thermogravimetric measurements were performed on a SDT 2960 Simultaneous DSC-TGA from TA Instruments. These measurements were only used to determine the amount of water that is adsorbed in the zeolite under storage conditions and, thus, establish a suitable dry calculation basis for the weight of the zeolites that were used in the experiments.

A B. Braun Biotech International Christ® Alpha 2–4 equipped with a Pfeiffer Balzers Duo 008 B vacuum pump was used to lyophilise the gelatin samples.

The gelatin particle images were taken with a COHU RGB camera (the software of acquisition was Matrox Inspector 2.1) mounted on an Olympus CX40 optical microscope under transmitted brightfield illumination, with a vertical and horizontal field width of 0.10 mm and NA (100× lens) = 1.30.

2.3. Methods

2.3.1. HRP-C enzyme concentration

The HRP-C concentration was determined spectrophotometrically at 404 nm using a molar absorption coefficient value of 102,000 M^{-1} cm^{-1} [25,27,28].

2.3.2. Modified Lowry method

To determine the amount of enzyme adsorbed on the zeolite, for the purpose of obtaining the adsorption isotherm, the enzyme–zeolite samples (≈10 mg) were dissolved at 100 °C with 2 ml of a 2 M NaOH solution. A dilution of the previous solution was performed (1:3) and 5 ml of the Lowry solution [29] was added with stirring. The Lowry solution was prepared with a 25:1 proportion of two solutions, the first one was a sodium carbonate solution with 5% (g/cm^3) and the second one was a potassium tartrate with 1% (g/cm^3) dissolved with a 0.5% (g/cm^3) copper sulphate solution. After 15 min, 1 ml of the Folin–Ciocalteu's solution (1:1 dilution of the original reagent) was added. The final solution absorbance was measured after 30 min at 750 nm. This procedure was also carried-out with a zeolite sample without enzyme.

The calibration curve was performed with several dilutions of a 100 μg/ml BSA solution and followed the same method.

The water content of each solid sample was measured by thermogravimetry.

All assays were performed in triplicate.

2.3.3. Gelatin–enzyme entrapment

The standard procedure for the enzyme entrapment, adapted from [30], was as follows: 0.5 g of gelatin dissolved in 4.5 ml of buffer solution and mixed with 0.5 ml of HRP-C solution (15.14 mg/ml); the final solution was divided in two parts to ensure that both had the same enzyme/gelatin ratio. In one part the adequate zeolite or silica quantity was added to reach a final proportion of 1:100, 2:100, 5:100 of m_{HRP}/m_{zeo} and 1:100 of $m_{HRP}/m_{(SiO_2)_p}$ ratio. The other part was used as reference

Table 1
Mass proportions for each gelatin sample

	Samples							
	Ref. 1	1:100	Ref. 2	2:100	Ref. 3	5:100	Ref. 4	1:100
H_2O (wt.%)	16.5	20.0	13.3	16.1	17.6	19.1	16.6	10.2
Gelatin (wt.%)	82.3	31.6	85.4	47.3	81.1	61.3	82.2	35.5
HRP-C (wt.%)	1.25	0.48	1.29	0.72	1.23	0.93	1.24	0.54
NaY (wt.%)	–	48.0	–	35.9	–	18.6	–	–
$(SiO_2)_p$ (wt.%)	–	–	–	–	–	–	–	53.8

Ref. corresponds to the sample reference, i.e., without the zeolite or the porous silica.

(hereafter these samples will be referenced as Ref. 1 to 4 (see Table 1 for composition), respectively).

With soft stirring, the solution was solidified at 4 °C and the resulting solid was washed with buffer solution.

All the samples were lyophilised and transformed into powder. These powders were sieved through a mesh with an aperture of 0.141 mm, to standardise particle dimensions.

Thermogravimetric experiments were made to confirm the protein quantity in each preparation. Table 1 shows the mass proportion for each gelatin sample.

2.3.4. Kinetic measurements

A standard reaction mixture [31] was defined as a solution containing 0.4 mM 4-aminoantipyrine (Am-NH_2), 25 mM phenol in 100 mM sodium phosphate pH 7.0 buffer. The reaction mixture was prepared shortly before use. Average enzyme concentrations in the solution were 0.43, 0.77 and 1.03 mg/l of HRP-C enzyme, and these concentrations were obtained by using an adequate quantity of gelatin powder.

Kinetic experiments were carried out in a thermostatted spectrophotometric cell that was used as a microbatch reactor, with stirring (650 rpm for homogeneous and heterogeneous reactions). The general procedure was as follows: 975 μl of the standard reaction mixture was added to the cell, followed by the addition of 25 μl of H_2O_2 of adequate concentration. The absorbance at 490 nm was then followed as a function of time.

The molar absorptivity value used for the dye product ($\varepsilon = 11.12 \pm 0.39\ mM^{-1}\ cm^{-1}$) was determined spectrophotometrically by slow and controlled addition of hydrogen peroxide to the Am-NH_2/phenol mixture until the reaction was complete [32].

A reaction scheme is next represented for better understanding and to characterise the standard kinetic measurements. This reaction scheme was adapted from reference [32] and combines the Change-George catalytic cycle, the generation of the dye product, other possible reactions due to partial inactivation of enzyme and finally a possible disproportionation reaction of hydrogen peroxide:

$$E + H_2O_2 \xrightarrow{k_1} E_I + H_2O \quad (1)$$

$$E_I + PhOH \xrightarrow{k_2} E_{II} + PhO^{\bullet} \quad (2)$$

$$E_{II} + PhOH \xrightarrow{k_3} E + PhO^{\bullet} + H_2O \quad (3)$$

$$PhO^{\bullet} + Am\text{-}NH_2 \underset{k_{-4}}{\overset{k_4}{\Leftrightarrow}} PhOH + Am\text{-}NH^{\bullet} \quad (4)$$

$$PhO^{\bullet} + Am\text{-}NH^{\bullet} \xrightarrow{k_5} Am\text{-}NH\text{-}OPh \quad (5)$$

$$Am\text{-}NH\text{-}OPh + H_2O_2 \xrightarrow{k_6} Dye + 2H_2O \quad (6)$$

$$E_{II} + H_2O_2 \xrightarrow{k_{app}} E_{III} + H_2O \quad (7)$$

$$E_{III} \xrightarrow{k_a} E + O_2{}^{-\bullet} + H^+ \quad (8)$$

$$E_{III} + PhOH \xrightarrow{k_b} E_I + PhO^{\bullet} + H_2O \quad (9)$$

$$2H_2O_2 \xrightarrow{k_8} 2H_2O + O_2 \quad (10)$$

3. Results and discussion

In the first part of this work, immobilized HRP-C on zeolite Y was used in a similar manner (adsorption) to the one used to immobilize lipase [1–5]. The zeolite adsorbed the enzyme quite well (Fig. 1), as expected according to published data [33]. Despite the dispersion of the data, adsorption measurements fit reasonably well to the following Langmuir type isotherm:

$$\frac{m_{HRP}}{m_{zeo}} = \frac{23.5[HRP]}{12.4 + [HRP]} \quad (11)$$

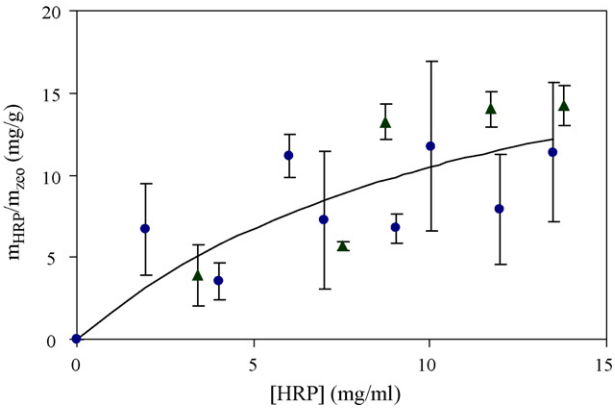

Fig. 1. Adsorption isotherm of HRP-C onto NaY zeolite at 25 °C. Standard error bars are shown for each data point. Different data symbols points correspond to two independent experiments; line corresponds to a Langmuir type isotherm (see text for details).

where the mass of HRP-C adsorbed per mass of zeolite is given in mg/g and enzyme concentration in mg/ml.

The monolayer capacity value in this equation (23.5 mg/g) is consistent with the expectable zeolite particle dimensions. In fact, the zeolite particle radius is expected to be between 250 and 450 nm, and with an HRP-C Stokes' radius (given by the empirical expression based on the protein molecular weight R_p (Å) = 0.38 (MW)$^{0.4}$ [34]) and a zeolite density of $\rho_{zeo} = 1.33$ g/cm^3, the mass of HRP-C immobilized on the outer zeolite surface should be between 12.6 mg/g (for the larger particle size) and 23.7 mg/g.

However, the immobilized enzyme was almost completely devoid of activity. Some residual activity was found after the preparations were left in solution for a while but it was observed that this residual activity was produced by enzyme that desorbed from the zeolite, once the immobilization samples were placed in solution.

Other approaches were made, such as, enzyme immobilization in the presence of a substrate (phenol, H_2O_2 and both at the same time) to protect and prevent changes in the active site and also a covalent immobilization. The last one was prepared first with the zeolite silanization by 3-aminopropyltriethoxysilane (3-APTES) and posterior cross-linking with glutaraldehyde and also glutaraldehyde directly onto zeolite. All of these methods produced inactive catalysts.

Control experiments were also carried in which only enzyme, only zeolite or a mixture of both with no prior immobilization was placed in solution.

In these experiments it was observed that the reaction was not catalysed by the zeolite alone, but it was also observed that the enzyme in the presence of zeolite seemed to have a slight increase in activity, as it will be described below.

3.1. Simple mixtures

As stated above, during the control experiments, where controlled amounts of zeolite were added to the standard reaction mixture containing free HRP-C, it was found that the addition of zeolite increased the rate of reaction by more than 10%. A set of experiments was then carried out to investigate this influence.

Fig. 2 represents the relative initial activity when different amounts of zeolite were added to the reaction solution, for five different temperatures.

Although the experimental points do not have a clear trend and are somewhat scattered, the global influence of the zeolite can be clearly seen. The scattering can be explained by the increased error involved in the measurement of the absorbance in the solutions containing zeolite in suspension, since the zeolite particles produce some scattering of the light.

As can be seen in Fig. 2 for 1 mg/l of enzyme, the increase is in many cases more than 10% and the largest increase in initial activity was observed with 0.3 mg of zeolite added at 25 °C (●).

It was observed that the decrease of the enzyme concentration clearly reduces the effect that zeolite has on the overall initial rate, probably because part of the enzyme is adsorbed on the zeolite, reducing the amount of enzyme available for the reaction.

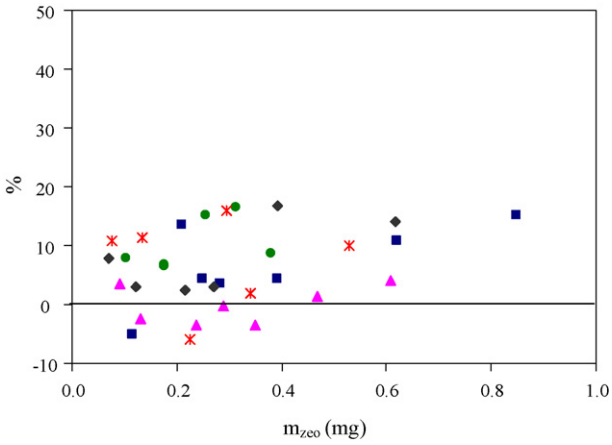

Fig. 2. Relative activity of HRP-C (with and without zeolite) after a mechanical mixture. Assays using 0.1 mM of H_2O_2 concentration and 1 mg/l of HRP-C for different temperatures (10 °C (■), 20 °C (▲), 25 °C (●), 30 °C (♦), 35 °C (✻)).

To have a better view of the increase in the initial rate of product formation, Fig. 3 represents a comparison, at 25 °C, for different hydrogen peroxide concentrations.

This effect may arise from interactions of the enzyme with the zeolite or from adsorption and concentration of reactants, by the zeolite, somewhere along the reaction mechanism. This ability to concentrate species was reported in literature [35,36] and it was confirmed that zeolite Y can adsorb phenolic compounds [37,38]. Although this effect is possible, for the reasons presented below, we do not think that this constitutes the major effect. It is true however, that the zeolite is capable of adsorbing both reactants and, thus, can influence the bimolecular reaction rates.

The possibility that the zeolite alters the conformation of the enzyme, even if it is not directly adsorbed onto it, either by influencing the micro-environment around the enzyme or by an adsorption/desorption process or by means of the electrical fields that exist even at the outer surface of the zeolites [39,40] can also occur, leaving the enzyme in a more active conformation.

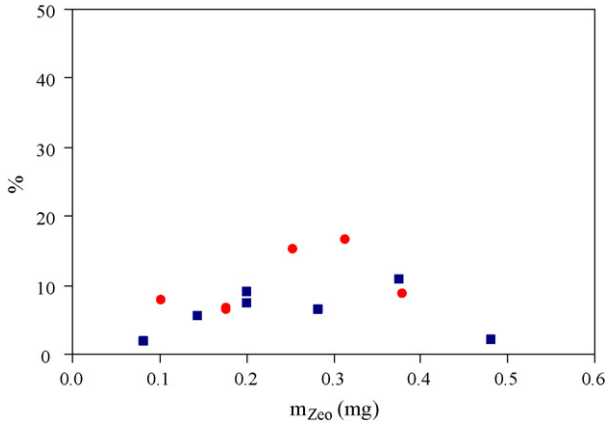

Fig. 3. Relative activity of HRP-C (with and without zeolite) after a mechanical mixture. Kinetic measurements using 0.1 mM (●) and 0.15 mM (■) of H_2O_2 concentration at 25 °C and 1.03 mg/l of HRP-C.

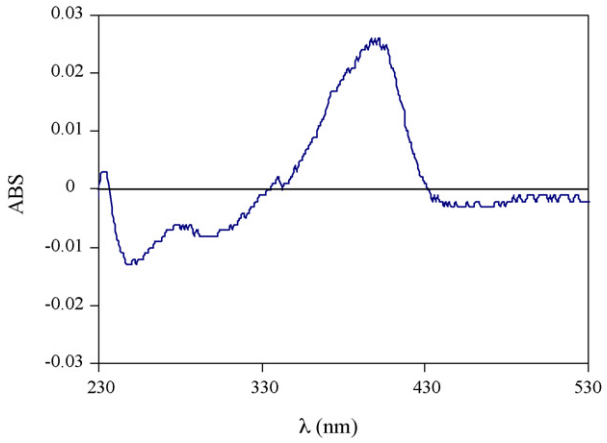

Fig. 4. Difference between the supernatants after contacting the zeolite and the initial HRP-C solution spectres. Initial HRP-C concentration of 14 mg/ml, 20 ml/g of ratio solution to zeolite mixture. Due to the high enzyme concentrations spectra were obtained after a 1:50 dilution.

However; the ionization of OH groups of the enzyme, due to ion exchange of Na-ions of the zeolite and protons of the enzyme is also a probable explanation for the observed effects; this ion exchange process can also occur with the gelatin matrix.

In order to investigate this possibility, a concentrated (14 mg/ml) solution of HRP-C was contacted with zeolite NaY. A high concentration was used, along with a low relative amount of zeolite (280 mg_{HRP}/g_{zeo}) so as to ensure that the concentration of enzyme in solution was not significantly changed by immobilization of the enzyme onto the zeolite; changes in the concentration of the enzyme due to adsorption on the zeolite are estimated as being below 0.5 mg/ml (less than 5% change).

After a certain amount of time, the zeolite was removed and the supernatant solution collected. UV/vis spectra were obtained of the original solution and after the contact with the zeolite. The difference between the two spectra is shown in Fig. 4.

Since the solution was contacted with zeolite, some decrease in enzyme concentration would be expected, due to adsorption. However, observing Fig. 4, it can be seen that although some areas have decreased in absorbance, others have increased.

HRP-C concentration is usually determined spectrophotometrically by the absorbance at 404 nm, using a molar absorption coefficient value of 102,000 $M^{-1}\,cm^{-1}$, as it was done in this work as indicated in Section 2. However, at 280 nm there is also a maximum of absorbance, with an absorbance coefficient value of 24,000 $M^{-1}\,cm^{-1}$ [41]. The heme active site is the primary responsible for the absorbance at 404 nm, while the amino acids with aromatic rings (tryptophan (Trp) and tyrosine (Tyr)) that are characteristic of many proteins are the main responsible for the absorbance at 280 nm.

As it can be seen in Fig. 4, at 280 nm a decrease in absorbance is observed, as expected due to the occurrence of some enzyme adsorption. However, at 404 nm, the absorbance after the immobilization increased instead of decreasing. Since this wavelength reveals modifications on the heme active site, it seems that, the zeolite induces a virtual increase in the horseradish peroxidase concentration.

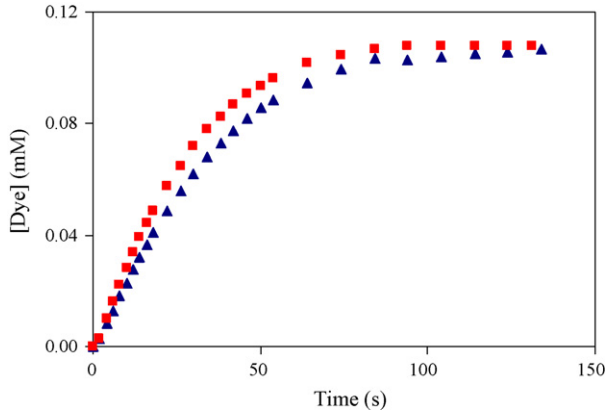

Fig. 5. Standard kinetic measurements using 1 mg/l of enzyme concentration taken from samples before (▲) and after (■) contacting with the zeolite. Reactions were carried out with 0.225 mM of H_2O_2.

It seems clear, in any case, that the contact with zeolite induced a change in the functional groups and/or conformation of the enzyme.

To check this effect, the activity of the solutions, before and after the enzyme had been contacted with the zeolite was measured and the kinetic curves can be seen in Fig. 5. The samples used to measure the activity were made so as to have 1 mg/l of enzyme, as measured at 280 nm.

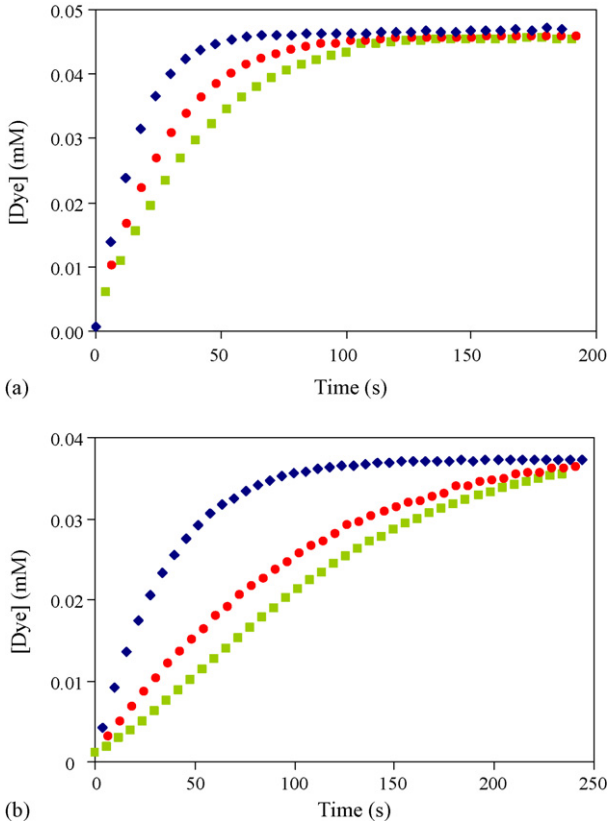

Fig. 6. Illustration examples of the kinetic reactions using free enzyme (♦), entrapped enzyme (■) and using entrapped enzyme with zeolite (●). Reaction with 2:100 of m_{HRP}/m_{zeo} ratio zeolite, using 1.03 mg/l of HRP-C and 0.09 mM of H_2O_2, at 25 °C (a) and with 1:100 of m_{HRP}/m_{zeo} ratio zeolite, using 0.43 mg/l of HRP-C and 0.085 mM of H_2O_2, at 10 °C (b).

After the enzyme has been contacted the zeolite there is an increase of 24% in the initial reaction rate.

Since the zeolite is no longer present in the solution when the activity measurement is carried out it seems likely that this change is due to a stable change in the enzyme conformation or ionization state.

3.2. Gelatin—entrapment experiments

Simply mixing the zeolite into the reaction mixture containing the enzyme presents several drawbacks, both from the experimental point of view, since the zeolite interferes with the absorbance measurements, and from the conceptual point of view, since the zeolite is likely to adsorb part of the free enzyme, thus reducing the total amount of available enzyme, and the zeolite is not kept in the vicinity of the enzyme at all times.

In order to overcome these difficulties, a co-immobilization procedure was established, where both the zeolite and the enzyme were trapped inside gelatin particles. In this way a heterogenized catalyst was produced, with varying amounts of HRP-C and zeolite.

In this way the catalyst particles contains, in close vicinity, both the zeolite and the enzyme. Control experiments were carried out with no zeolite (for comparison purposes with the zeolite containing particles) and by replacing the zeolite with a porous silica.

Fig. 6 exhibits two examples showing the difference between free enzyme and the entrapped enzyme, with or without zeolite, for two different temperatures, when using equal amounts of total enzyme in solution. As it was expected, the enzyme immobilization decreases its activity. Comparing free enzyme (♦) and entrapped enzyme (■) a decrease of 56% can be observed for example (a) and of 84% for (b) on the relative activity. However, comparing the entrapped enzyme with and without zeolite, it can be seen that in both cases there is an improvement of around 15% on the enzyme activity in the presence of the zeolite. Nevertheless, comparing the entrapped enzyme with zeolite (●) still has a lower activity than the free enzyme (♦).

The relative activities, comparing gelatin entrapment with and without zeolite, as function of temperature, enzyme and zeolite concentration, are represented in Fig. 7. As it can be seen, at 10 °C the increases are higher than for other temperatures and reach values as high as 120%. In general, the best results are obtained with 0.43 mg/l total enzyme concentration and a 1:100 proportion of m_{HRP}/m_{zeo}. This is consistent with the effect being more pronounced for smaller reaction rates and less enzyme.

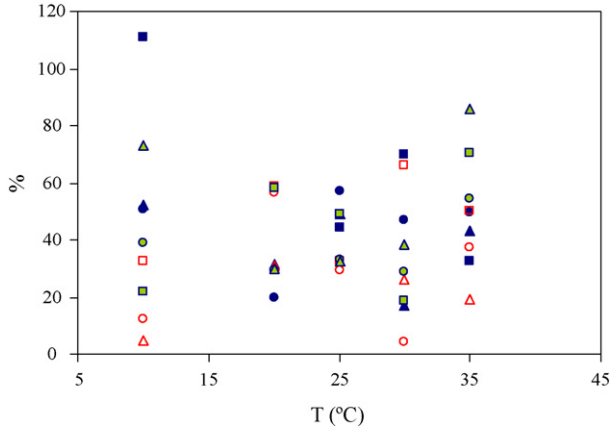

Fig. 7. Relative activity of HRP-C after immobilization by entrapment with and without zeolite, using 0.09 mM of H_2O_2 concentration, showing temperature influence. Symbols shape correspond to 0.43 mg/l (■), 0.77 mg/l (●) and 1.03 mg/l (▲) HRP-C concentrations and symbol filled type correspond to 1:100 (■), 2:100 (□) and 5:100 (▣) of m_{HRP}/m_{zeo}.

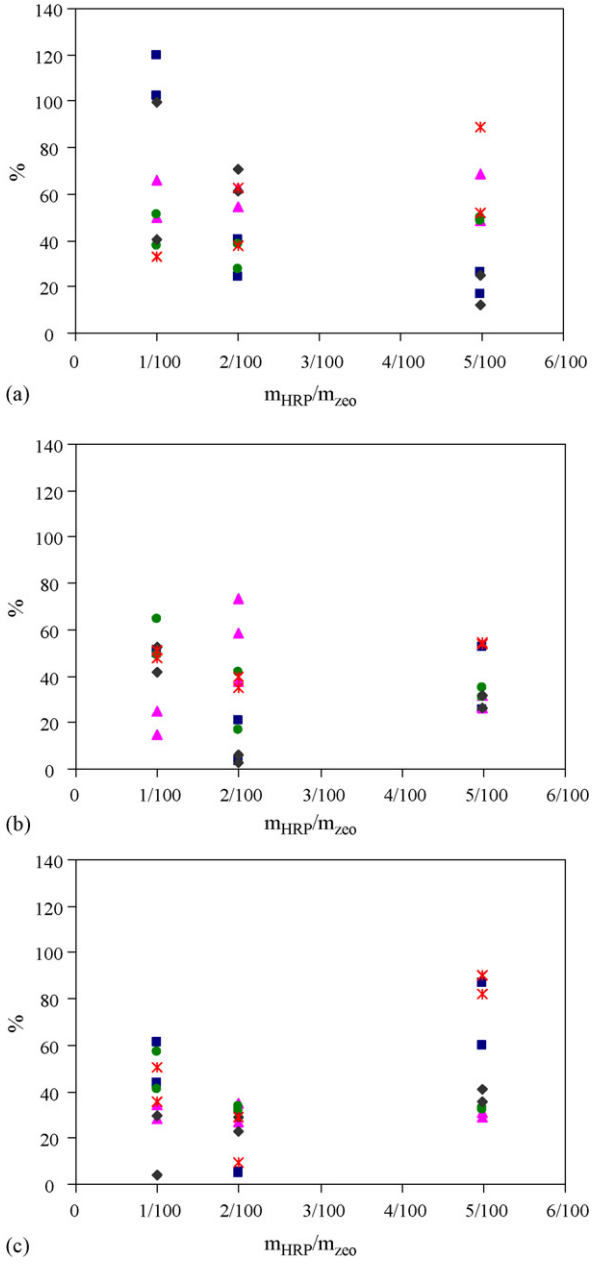

Fig. 8. Relative activity of HRP-C after immobilization by entrapment with and without zeolite, using 0.09 mM of H_2O_2 concentration, showing m_{HRP}/m_{zeo} ratio influence. Symbols correspond to different temperatures (10 °C (■), 20 °C (▲), 25 °C (●), 30 °C (♦), 35 °C (✻)) for 0.43 (a), 0.77 (b) and 1.03 (c) mg/l HRP-C concentrations.

Both 0.77 mg/l (●) and 1.03 mg/l (▲) of enzyme concentrations have comparable results with 2:100 (□) and 5:100 (■) of m_{HRP}/m_{zeo} with an average increase in activity of 40%.

In terms of usage of this catalyst, the ability to produce a catalyst that is in a heterogeneous form and where the activity of the enzyme is enhanced by the use of a zeolite component is of potential interest.

The increase is particularly interesting for processes that are operated at lower temperatures, since for lower temperatures best results are achieved. Even if the process is to be operated at higher temperatures, substantial gains in activity are still obtained.

Fig. 8 compares the effects of different zeolite to enzyme ratios in the gelatin entrapment for all five temperatures. The three groups (a, b and c) correspond to three enzyme concentrations in solution (0.43, 0.77 and 1.03 mg/l).

Although a unique pattern cannot be observed, there is a general trend for the existence of a minimum for middle range values of the ratio.

When comparing the results obtained using these composite particles, containing the enzyme, the zeolite and the gelatin as binding agent with the particles where only the enzyme is entrapped in the gelatin, it has to be taken into consideration that the zeolite particles are likely to change the porosity of the gelatin particles, by reducing the diffusion limitations, and could, thus, explain the observed increase in activity.

In order to understand the effect of the zeolite's porosity, another control experiment where a porous silica (with a porous volume of 1.77 cm^3/g) replaced the zeolite in the gelatin, was carried out. This material has a much larger pore volume than the zeolite (which only has a pore volume of 0.29 cm^3/g) and, thus, should allow for a much better access to the gelatin particles than the zeolite. The experimental conditions for this control were set similar to the ones that produced the best results with the zeolite (10 °C, 1:100 of $m_{HRP}/m_{(SiO_2)_p}$ ratio for 0.43, 0.77 and 1.03 mg/l of HRP-C concentration). The activity of the porous silica containing catalysts was only around 12% higher than the particles with no porous solid for the first two and 8% for the last one.

We can conclude that, although the porosity effect may play a role, which should be larger for the porous silica than for the zeolite, this seems to be a minor one.

In order to clarify the structure of the gelatin particles some images of these particles, with the entrapped zeolite and enzyme are shown in Fig. 9.

It is clear in Fig. 9(a) that the zeolite is constituted by very small particles. Comparing Fig. 9(b) that is a representative particle of the enzyme entrapped in gelatin and Fig. 9(c)–(e), the zeolite particles are easily recognised inside the gelatin structure. It should be noted that the enzyme concentration in the particles is always the same and, thus, in the 5:100 and 2:100 of m_{HRP}/m_{zeo} ratio samples, the gelatin is in large excess in comparison with zeolite; however, in the 1:100 sample, the zeolite

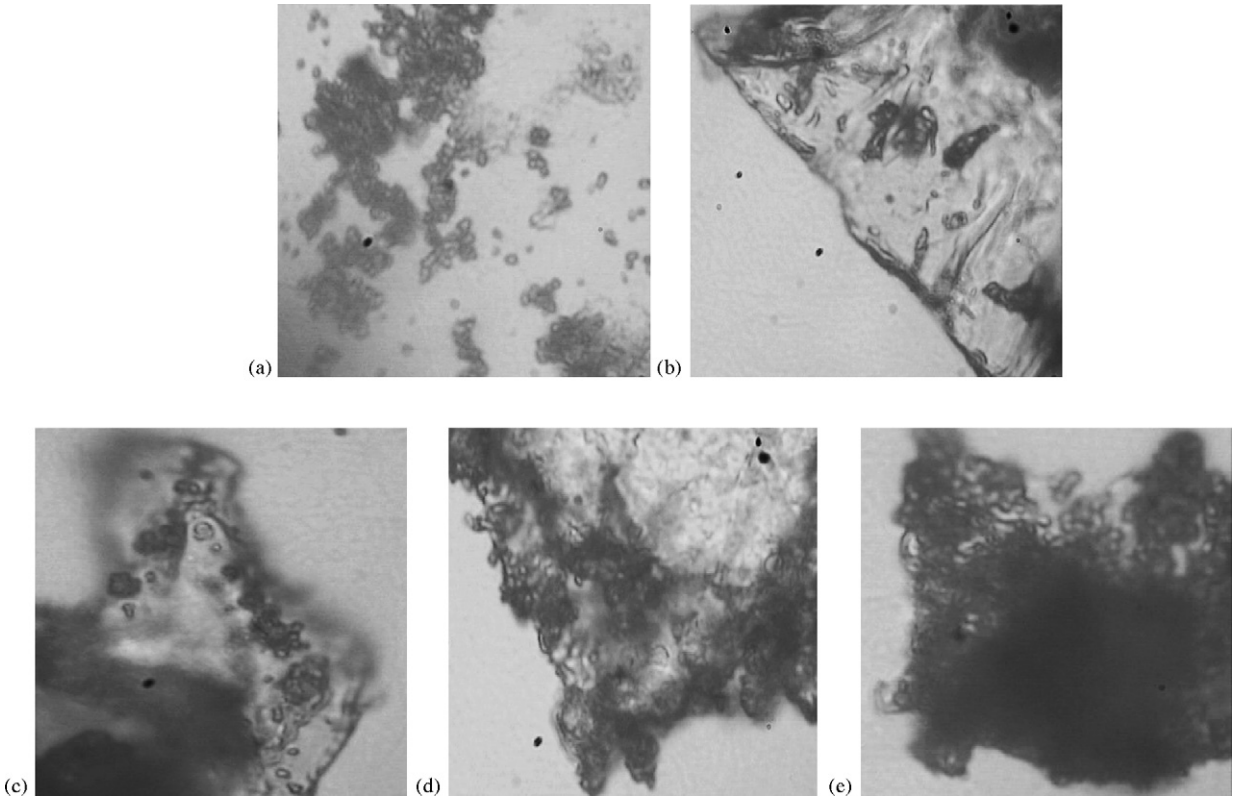

Fig. 9. Microscopic optical images (with vertical and horizontal field width of 0.10 mm) of the NaY zeolite (a), entrapment particles without zeolite (b), and with zeolite for 5:100 (c), 2:100 (d) and 1:100 (e) of m_{HRP}/m_{zeo} ratio. Note that, for enzyme containing particles, since the enzyme concentration is always the same, the lower percentages correspond to larger amounts of zeolite in the mixture.

Table 2
Amounts adsorbed on each sample, per unit mass of solid material, starting from an initial substrate concentration of 0.05 mM

	NaY	$(SiO_2)_p$	Ref. 1	1:100 $m_{HRP}/m_{(SiO_2)_p}$	1:100 m_{HRP}/m_{zeo}
Phenol (mmol/g)	0.017	0.029	0.183	0.040	0.094
Am-NH_2 (mmol/g)	0.013	0	0.063	0.036	0.021

is now the main component and the gelatin serves as a binding material containing the enzyme.

Another relevant note relates to the easiness of maceration when zeolites are present in the gelatin entrapment. Fig. 9(c) and (d) shows that zeolite is located mainly at the periphery of the particles. When the gelatin preparation is ground into small particles, for use in a reactor, it was noted that the preparations without zeolite are very difficult to grind, since they are very flexible. When zeolite is present; however, they are easier to grind, probably because they tend to separate in zeolite-rich regions, leading to the zeolite enrichment near the periphery of the particles.

In order to check for the possibility of the zeolite concentrating one or more of the reactant species, and thus influence the reaction rate of some steps in the mechanism (like steps (4), (5) or (6)) producing a positive reaction in the global reaction rate, the adsorption of phenol and Am-NH_2 were measured on the zeolite and also on the composite particles containing both zeolite and enzyme entrapped in gelatin, as well as on the control particles, either with only gelatin or with the zeolite replaced by a porous silica.

A solution with an initial concentration of 0.05 mM for each substrate (phenol and Am-NH_2 separately) was contacted with each sample presented in Table 2, for 10 min. To this solution the adequate particles quantity to reach 1 mg/l of enzyme concentration was added; for the NaY and silica, which have no enzyme, similar total amounts were used. Table 2 shows the amounts adsorbed for each sample.

As it can be seen, phenol and Am-NH_2 adsorption are very similar on NaY and also that the gelatin particles Ref. 1 adsorb more of either substrates than the zeolite or porous silica. Moreover, phenol adsorption is always higher when compared with Am-NH_2. The comparison between 1:100 m_{HRP}/m_{zeo} and the Ref. 1 samples seems to indicate that the species concentration by zeolite is not the main reason for the increase in activity that is later observed, since the former adsorbs less than the latter and, thus, the simple gelatin entrapment should provide an increase in activity when immobilizing the enzyme; the effect, however, still has to be taken into consideration, since reaction conditions inside the zeolite are significantly different from solution chemistry.

4. Conclusions

Enzyme–zeolite interactions are gaining new potential in catalytic improvements. In this work it was shown that the activity of horseradish peroxidase can increase in the presence of zeolite NaY. This increase seems to occur both when the zeolite is present, as a suspension, in a solution containing the enzyme and when both the enzyme and the zeolite are entrapped in gelatin particles.

The preparation of these composite particles has the advantage of ensuring a close proximity between the enzyme and the zeolite components, producing a heterogeneous catalyst, which is easier to re-use and retains more activity than the simple heterogenization of the enzyme. Since the enzyme adsorbed on the surface of the zeolite seems to be completely inactive, the co-immobilization procedure also has the advantage of preventing loss of enzyme by adsorption on the zeolite.

It was found that low enzyme concentration (0.43 mg/l), higher zeolite concentration (1:100 of m_{HRP}/m_{zeo} ratio) and lower temperatures (10 °C) present the best results in the composite enzyme/zeolite/gelatin particles.

From the results that were obtained it can be seen that the increase in activity is likely to be linked to some ionization of OH groups of the enzyme, due to ion exchange of Na-ions of the zeolite and protons of the HRP, rendering it more active. Moreover, a part of this increase can be attributed to the changes in the porosity of the gelatin particles by the porous particles, as it was evidenced by the control experiments with porous silica; however, this effect seems to be minor. Also to be taken into consideration are possible adsorption effects that can increase the rate of bimolecular reactions.

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