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Cinnamic carbohydrate esters: new polymeric supports for the immobilization of horseradish peroxidase

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Abstract

Several crosslinking photopolymers were used to cover 3 mm diameter glass beads for immobilising HRP. These immobilization supports were prepared from the totally cinnamoylated derivatives of 4-hydroxybenzaldehyde, glycerine (GLICN), D-sorbitol (SOTCN), D-manitol (MNTCN), 1,2-*O*-isopropylidene-α-D-glucofuranose (IPGSCN), D-glucuronic acid (GLUCNA), D-gulonic acid (GULCNA), sucrose (SSCN), D-glucosone (GSOCN), D-arabinose (ASCN), D-fructose (FSCN), D-glucose (GSCN), ethyl-D-glucopyranoside (EGSCN), maltose (MSCN), inuline (INCN), dextrine (DXICN), dextrane (DXACN) and polyvinylic alcohol (PVCN). The following partially cinnamoylated derivatives were also used: 2,3,4,6-tetracinnamoyl-D-glucopyranose (GPSTCN), obtained by partial hydrolysis of EGSCN in acid medium, and 3,5,6-tricinnamoyl-D-glucofuranose (GFSTCN), which was obtained by the acid hydrolysis of IPGSCN. The derivatives obtained were cross-linked by irradiation in the ultraviolet region, where these prepolymers show maximum sensitivity. The enzyme was immobilized by adsorption on to the support. The immobilized enzymatic activity varied with the length of incubation (2–21 h) and depended on the chemical nature of the support used. The effect of irradiation time on initial enzymatic activity and on that remaining after storage of the samples with immobilized enzyme was studied; both were seen to be related with the cross-linking density of the final polymer. The immobilized enzyme was more resistant than the soluble enzyme to inactivation by H_2O_2 at neutral pH, and provided good yields after thermal treatment at this pH value. The results show that the cinnamic esters described are suitable supports for immobilising peroxidase and can be used for different applications of the enzyme on an industrial scale.

Keywords: Horseradish peroxidase; Immobilization; Cinnamic carbohydrate esters; Enzyme kinetics; Peroxidase stability

1. Introduction

The insolubilization of photosensitive polymers is one of the oldest of photochemical reactions (Delzenne, 1972, 1974). The first synthetic photopolymer used, which solved the problems associated with natural photopolymers (Delzenne, 1974; Egerton, Hyde, Trigg, Payne, Beynon, Mijovic, & Reiser, 1981; Minsk, Smith, Van Deusen, & Wright, 1959; Tsuda, 1964), was polyvinyl cinnamate (PVCN) (Minsk et al., 1959), which, in turn, was replaced by a broad class of photopolymers based on cycloadditions (Delzenne, 1972; Williams, Laakso, & Borden, 1971). The polymer (olefins) is insolubilized by cyclodimerization of two cinnamoyl groups (excited chromophores) belonging to two different chains by means of a 'four-centre' polymerization step (Wendell, 1983), by absorbing light energy (Ranby, 1998). This photoreaction takes place in crystalline state, providing polyesters that contain units of cyclobutane. The carbonyl group provides the double reactive bond with the necessary polarization and the phenyl group increases the polarisability, thus increasing the chromophore's power to absorb light (Williams, 1974).

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The presence of truxylic and not truxinic acid in the crosslinked PVCN requires stereoregularity between chains (Reiser, 1980).

From a chemical point of view, the applications differ according to the properties of the material, which change through the action of light (Delzenne, 1974; Trout, Schmieg, Gambogi, & Weber, 1998). For example, there are applications based on the modulation of solubility (Plambeck, 1956), solubility and adhesion (Buehler, 1993; Celeste, 1969), permeability (Gervay & Walker, 1973, 1974, 1975), the physical state (Cohen & Fan, 1979), and the refraction index (Chandross & Tomlinson, 1979; Colburn, 1997). The materials can also be used for immobilising different types of molecules (additives, reagents) and biomolecules (enzymes, proteins), which is the subject of this report. After irradiation, the synthesised polymers that act as carriers of reactive groups (Fréchet, 1981) are insoluble in aqueous medium, which is the enzyme reaction and/or storage medium.

Both partially and totally cinnamoylated derivatives have been prepared on glass beads capable of binding the HRP enzyme rapidly and simply by physical adsorption interactions (Rojas-Melgarejo, Rodríguez-López, García-Cánovas, & García-Ruiz, 2004). The structural differences between them permitted a study of their effect on the catalytic activity they retained (Chibata, 1978; Sturgeon, 1988; Guilbault, 1984) and of their stability in the face of different inactivating factors, such as temperature and $[H_2O_2]$ (Grzywnowicz, Brzyska, Lobarzewski, & Greppin, 1992), comparing these factors with each other and with respect to the soluble enzyme. The HRP enzyme was chosen for this study because of its interest for industry, where it is used in many oxidative processes (Hiner, Hernández-Ruíz, Arnao, García-Cánovas, & Acosta, 1996; Tatsumi, Wada, & Ichikawa, 1996).

2. Materials and methods

2.1. Materials

HRP isoenzyme C was purchased from Sigma (type VI; RZ = 3.2) and used without further purification The concentration of HRP was determined spectrophotometrically using a Soret extinction coefficient of 102 $mM^{-1}cm^{-1}$ (Schonbaum & Lo, 1972). Reagent-grade H_2O_2 (33%, v/v) was obtained from Scharlau (Spain). ABTS in the crystallized diammonium salt form was purchased from Sigma (Spain). All other chemicals were of analytical grade. Ultrapure water from Milli-Q system (Millipore Corp.) was used throughout this research.

2.2. Spectrophotometric assays

Spectrophotometric measurements were made with a UNICAM 8625 UV/Visible spectrophotometer controlled by a PC running Unicam Software Scan 8620/25 after adjusting to the desired wavelength. A variable flow peristaltic pump (DINKO D25V) was used to pump the reaction medium through the spectrophotometer, which contained a quartz cuvette (1 cm).

2.3. Preparation of D-glucosone

Twelve grams of copper (II) acetate and 7.21 g of D-fructose were dissolved in 120 ml methanol/acetic acid/water (12:1:2) and heated until reflux temperature was attained. This temperature was maintained for 2 h, after which the reaction medium was filtered to eliminate the copper (II) oxide formed. The solution was then passed through an Amberlite IR-120 column to remove any remaining Cu^{+} and Cu^{2+}. The resulting solution was poured into a flask and the solvent was eliminated in a rotary evaporator. The 6.77 g of D-glucosone thus obtained was used as starting material to obtain the corresponding cinnamate.

2.4. Preparation of ethyl-α-D-glucopyranoside

Fifteen grams of D-glucose, 160 ml of absolute ethanol and a few drops of sulphuric acid were placed in a 250 ml flask and adjusted to a pH of 3–4. The flask was placed in a water bath and heated to reflux temperature, which was maintained for 2 h. When the reaction had finished, the flask was allowed to cool and the contents were neutralized with lead carbonate. The reaction medium was then filtered and the solvent was eliminated from the resulting solution in a rotary evaporator. The resulting solid was dissolved in methanol and brought to dryness again. The final product obtained was used for preparing the cinnamic acid ester.

2.5. Preparation of the totally cinnamoylated derivatives

*Carbohydrate derivatives.*In all cases preparation followed a modified version of the method proposed by Van Cleve (Van Cleve, 1963), in which 0.02 mol of carbohydrate were dissolved in 100 ml of pyridine. A mixture of pyridine and DMF (2:1) was used to prepare the derivatives of ethyl-α-D-glucopyranose, D-arabinose and dextrine, in a ratio of 1:2 to prepare the dextrane derivative and a ratio of 1:1 for D-glucose. The mixture was heated at 60 °C for one hour to ensure complete dissolution. After cooling to room temperature, 0.03 mol of cinnamic acid chloride was added for 4-hydroxybenzaldehyde cinnamate and PVCN, 0.07 mol for GLICN, INCN, DXICN, DXACN, IPGSCN and GFSTCN, 0.10 mol for GSOCN, ASCN, EGSCN, GLUCNA, GULCNA and GPSTCN, 0.12 mol for FSCN and GSCN, 0.15 mol for SOTCN and MNTCN, and 0.19 mol for SSCN and MSCN. The reaction was allowed to proceed at room temperature for 4 h, after which the resulting mixture was poured into vigorously stirred water. The precipitate obtained, after decanting and filtering this

mixture, was dissolved in chloroform and purified by adding, one drop at a time, to vigorously shaken hexane. The solid obtained was redissolved and reprecipitated before being dried on P_2O_5 at reduced pressure.

Polyvinylic alcohol derivatives. In this case, 0.045 mol of polyvinylic alcohol were completely dissolved in 100 ml of *N*-methyl-2-pyrrolidone by heating to 80 °C. The mixture was then cooled and thermostatted at 25 °C before 0.078 mol of cinnamoyl chloride and 6.36 ml of pyridine were added. The reaction was carried out at 25 °C in an inert N_2 atmosphere for 24 h, after which the reaction mixture was poured into strongly stirred acidulated water. The precipitate, which appeared was dissolved in ethylmethylketone and once again precipitated in water. The solid obtained was dried in a reduced pressure oven at 30 °C and then kept in a P_2O_5 reduced pressure drier.

4-*Hydroxybenzaldehyde derivatives.*The method followed to prepare this cinnamate was similar to that described for synthetising totally cinnamoylated carbohydrate derivatives, although, because there is only one free hydroxyl to esterify, the quantity of cinnamoyl chloride added was equimolar with respect to the quantity of the 4-hydroxybenzaldehyde used plus a 20% excess to ensure complete cinnamoylization.

2.6. Hydrolysis of the derivatives

Preparation of 2,3,4,6-tetracinnamoyl-D-glucopyranoside. This support was prepared by hydrolysis in an acid medium of totally cinnamoylated derivative of ethyl-D-glucopyranoside. For this, an aqueous solution of HCl (pH 3.0) was added to the glass beads covered with the immobilization support. This was maintained for 30 min, after which the hydrolysis medium was withdrawn and a new aqueous acid solution was added and maintained for 30 min. The last hydrolysis medium was withdrawn again and the final support was thoroughly washed with abundant distilled water. Lastly, the glass beads were rinsed with 0.1 M aqueous potassium phosphate solution (pH 9.1) for approximately 10 min to adjust the pH to the value previously used for the immobilization. Finally, the glass beads were dried by reduced pressure suction.

Preparation of 3,5,6-tricinnamoyl-D-glucofuranose. This support was prepared by hydrolysis of 1,2-*O*-isopropylidene-α-D-glucofuranose cinnamate. In an acid medium the procedure used was similar to that mentioned for 2,3,4,6-tetracinnamoyl-D-glucopyranoside.

2.7. HRP immobilization

Glass beads of 3.0 ± 0.2 mm diameter with a contact surface of 7995 $cm^2\,kg^{-1}$ were purchased from Labor (Barcelona, Spain) and covered with the different immobilization supports prepared as indicated in previous works (Rojas-Melgarejo et al., 2004). A chloroform solution of the immobilization supports synthesized at 15 $g\,l^{-1}$ was prepared, in which the glass beads were immersed. A film of prepolymer was formed on the beads and the solvent was eliminated by evaporation and suction at reduced pressure. After drying, the prepolymer film was polymerized by irradiation in the ultraviolet zone for 15 min using an Osram HOL-125W mercury vapor lamp. To immobilize HRP, a 5 ml solution of this enzyme (0.11 μM) in 0.1 M aqueous phosphate solution (pH 9.1) was added to a syringe containing 15 g of glass beads covered with 1.5 mg of the corresponding immobilization support. The reaction was allowed to proceed for 21 h at 4 °C, ensuring that the enzyme solution retained its initial enzymatic activity during this time. After immobilization the reaction medium was withdrawn and the immobilized enzyme was thoroughly rinsed in distilled water, checking that no HRP molecules were lost during this process by measuring the enzymatic activity present in the rinsing fractions at different times.

2.8. Peroxidase activity assay

Soluble and immobilized HRP enzymatic activity was determined using H_2O_2 as oxidizing substrate and ABTS as reducing substrate The assay medium contained 1 mM ABTS and 1 mM H_2O_2 in phosphate–citrate buffer, pH 4.5. To assay the immobilized enzyme, syringes containing 15 g of glass beads with HRP bound were used as small packed bed continuous reactors with recirculation and descending flow. The substrate solution was pumped at 50–55 ml min^{-1} using a peristaltic pump. The initial rate was measured between the first 30 and 90 s of the reaction as the increase in absorbance at 414 nm ($\varepsilon_{414\ nm} = 31.1\ mM^{-1}cm^{-1}$) caused by the ABTS oxidation product. One unit of enzymatic activity (U) was defined as the quantity of enzyme forming 1 μmol of product in 1 min at the specified pH at 25 °C. For immobilized enzyme the activity is expressed as peroxidase units per gram of dry support ($U\,g^{-1}$).

2.9. Thermal stability of immobilized and soluble HRP

The thermal stability of immobilized HRP was studied at neutral pH (0.1 M sodium phosphate buffer pH 7.0) by incubation at 60 °C For the immobilized enzyme, 5-ml of preheated buffer were added to a series of syringes each containing 15 g of glass beads covered with HRP immobilized on the different supports. The syringes were then introduced in a water bath at the desired temperature. At each incubation time indicated in the results and Table 5 (0.5 or 10 min), a syringe was removed from the bath and rapidly cooled to room temperature using abundant distilled water and the A_r was determined. The A_r (%) was calculated taking the initial activity (100%) to be the activity present in the samples with immobilized enzyme after immobilization and rinsing.

2.10. Stability during storage

The stability of immobilized HRP enzyme during storage at −18 °C was evaluated by storing samples immobilized in 5 ml of distilled water for 33 days.

2.11. Enzyme inactivation by hydrogen peroxide

The effect of H_2O_2 on immobilized HRP was stsudied at both pH 4.5 and 7.0, including in the reaction medium 10 mM H_2O_2 and 0.75 mM ABTS in sodium citrate–phosphate buffer (pH 4.5) or 0.1 M sodium phosphate (pH 7.0) at 25 °C The assay medium was continuously recirculated as indicated above and the enzymatic reaction was followed at 414 nm for 30 min. The data obtained were compared with those published for the soluble enzyme (Rojas-Melgarejo et al., 2004).

3. Results and discussion

3.1. Cinnamic carbohydrate esters as immobilized supports

For this research 4-hydroxybenzaldehyde cinnamate, GLICN, SOTCN, MNTCN, IPGSCN, GLUCNA, GULCNA, SSCN, GSOCN, ASCN, FSCN, GSCN, EGSCN, MSCN, INCN, DXICN, DXACN and PVCN (Fig. 1) were synthesized and used as supports for HRP immobilization. Elemental analysis of MSCN (as representative of a polymer of middle to low molecular weight), and INCN (high molecular weight) provided C/H values very similar to their corresponding theoretical values. In addition, the visible–UV spectra in the 240–340 nm range agreed with those described by other authors (Wojciechowski, Okrasa, & Kozanecki, 2002), with absorption maxima at 278–280 nm.

One of the advantages of these supports is that all the hydroxyl groups are esterified with cinnamoyl groups, as can be deduced using various experimental analyses. As regards the ^{1}H-NMR analyses, signals were observed at $\delta = 4.4$–4.9 and 5.3–6.1 due to the carbohydrates present, at $\delta = 6.4$; 6.6 and 7.8 due to the vinylic protons and $\delta = 7.4$ due to the aromatic protons. The integration signals corresponded to totally esterified derivatives. The absence of hydroxyl groups was confirmed by analysing samples to which deuterated water had been added. The ^{13}C-NMR and 'DEPT' (Distortionless Enhancement by Polarization Transfer) spectra and the results of the different two dimensional experiments (COSY and C/H ratio), agreed with the structure proposed for these compounds. The assignment of the infrared spectrum bands agreed with those of the synthetised products. Thus, the bands at 1720, 1312 and 1160 cm^{-1} are typical of α, β-unsaturated esters, those at 1637, 1285, 980 and 865 cm^{-1} are due to the (trans) conjugated carbon–carbon double bond, those at 1600, 1500 and 1450 cm^{-1} correspond to the aromatic nucleus and those at 770 and 685 cm^{-1} are due to monosubstitute benzene. Also appearing were bands (for example, those at 1331, 1100, 1072 and 960 cm^{-1}) corresponding to polysaccharide (Wojciechowski et al., 2002).

Differential thermal analysis (DST) of the totally cinnamoylated derivatives of polysaccharides, oligosaccharides and polyvinylic alcohol suggested that all these immobilization supports are very stable at the working temperatures common in the industrial processes where they might be used, since no fusion or vitreous transition points were observed between 0 and 300 °C.

In the case of the cinnamoylated derivatives of hexitoles and monosaccharides (FSCN, GSCN, GSOCN and EGSCN), sharp heat absorption peaks were observed, (probably as a result of melting), although they were not detected in the second ramp since the samples had oligomerized. Within the group of cinnamoylated monosaccharide derivatives, those of ASCN and IPGSCN, showed clear melting points at relatively low temperatures so that even in the second heating ramp heat absorption peaks were still visible due to the presence of molecules that had not oligomerized after initial heating at 300 °C. These compounds need to be deeply crosslinked to obtain supports unalterable by heat. The derivatives were therefore crosslinked by irradiation in the ultraviolet region, where the cinnamic carbohydrate esters showed maximum sensitivity.

The cinnamoyl derivatives (immobilization support) were precipitated on glass beads (inert matrix) by solvent evaporation (Wójcik, Lobarzewski, Blaszczynska, & Fiedurek, 1987). The best precipitation results were obtained at support concentrations close to 15 g l^{-1}. Under these experimental conditions, the support retained was approximately 0.1 mg per gram of glass beads.

3.2. HRP immobilization

Several aspects, including HRP concentration, support concentration, immobilization time, and pH, were studied to ascertain the best immobilization conditions The representation of the enzymatic activity retained versus HRP concentration showed a sigmoidal behaviour, which tended to a constant value as a result of the saturation of the immobilization support (Rojas-Melgarejo et al., 2004). Immobilization times varied from 2 to 21 h and the behaviour observed depended on the immobilization support used (Fig. 2). Although the immobilization time was to a certain extent independent of the enzymatic activity retained by HRP, the activity did show a slight tendency to increase as immobilization time increased in the case of some supports. The most obvious examples are the ASCN and SSCN derivatives, whose values almost doubled as the immobilization time increased from 2 to 21 h. Whatever the case, the exact immobilization time will obviously depend on the industrial application for which the enzyme is to be used. In our experiments, an immobilization time of 21 h

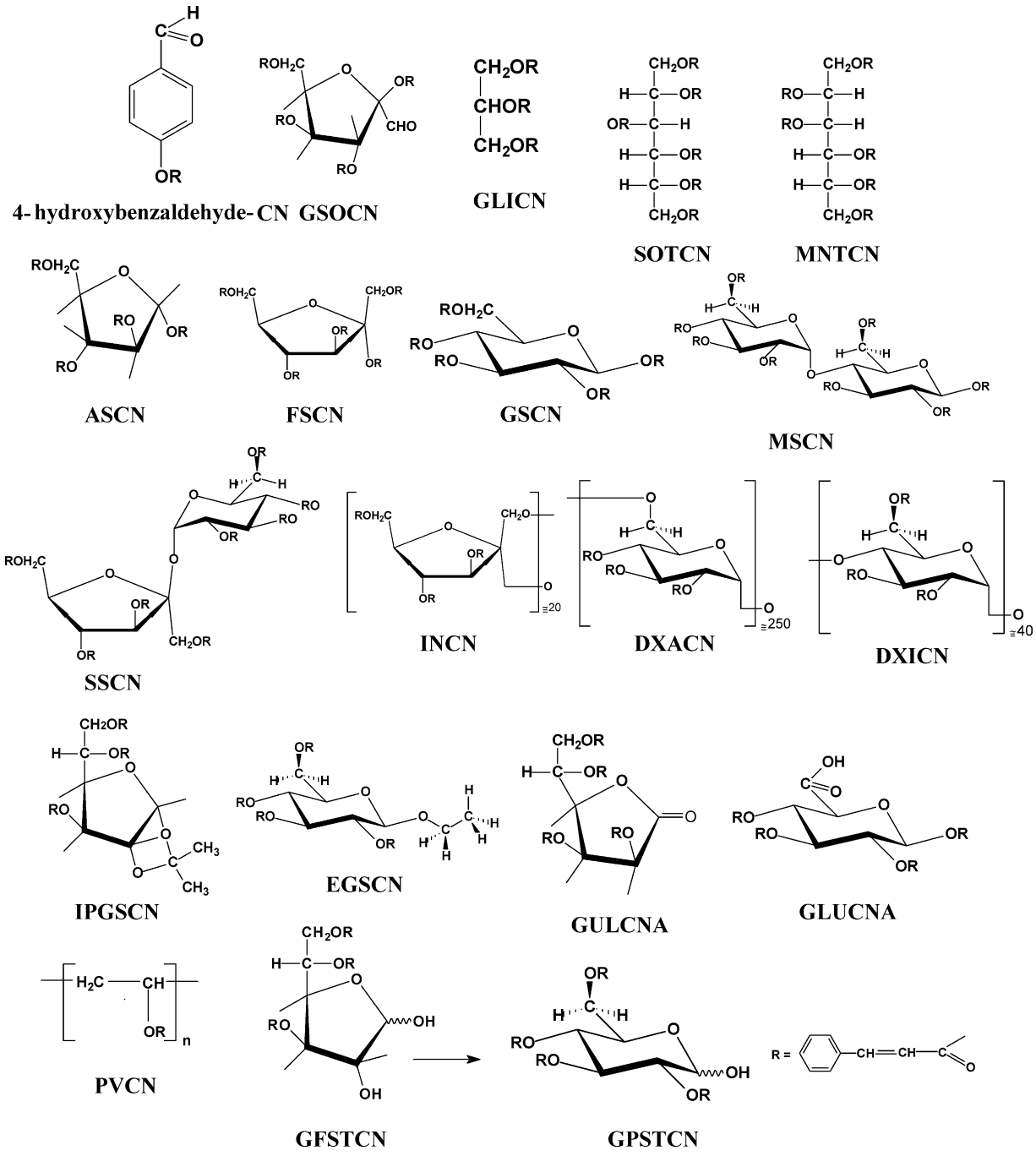

Fig. 1. Schematic representation of the structure of the supports used for HRP immobilization.

was used to enable the retained enzyme to take on a stable structure following the corresponding conformational changes.

3.3. Behaviour of immobilized HRP in cinnamic carbohydrate esters

In order to study the properties of immobilized HRP on cinnamic carbohydrate esters and to check the applicability of these supports for peroxidase immobilization, several aspects were investigated:

*Chemical nature.*The effect of the nature of the totally cinnamoylated derivatives and of the partially cinnamoylated derivatives 2,3,4,6-tetracinnamoyl-D-glucopyranose (GPSTCN) and 3,5,6-tricinnamoyl-D-glucofuranose (GFSTCN) on the retained enzymatic activity was studied (Table 1). The findings enabled us to classify the immobilization supports according to the value of

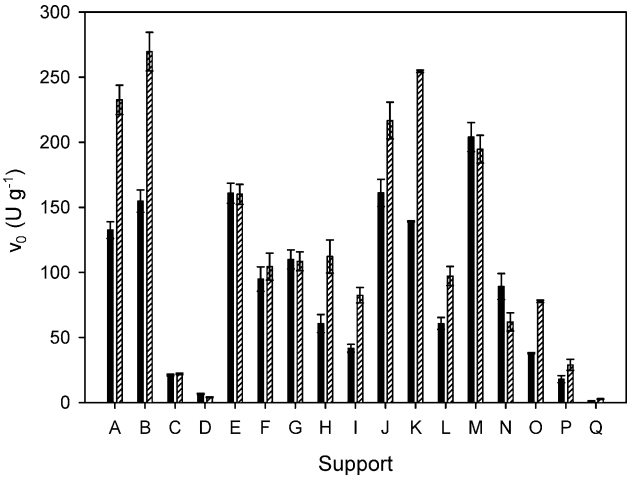

Fig. 2. Effect of immobilization time on enzymatic activity initially retained by immobilized HRP. (A) ASCN, (B) SOTCN, (C) MNTCN, (D) IPGSCN, (E) EGSCN, (F) GSCN, (G) MSCN, (H) DXICN, (I) DXACN, (J) FSCN, (K) SSCN, (L) INCN, (M) GSOCN, (N) GULCNA, (O) GLUCNA, (P) GPSTCN and (Q) GFSTCN. ■ 2 h and ▨ 21 h.

the enzymatic activity shown by the protein immobilized on them. SOTCN, SSCN, ASCN, FSCN, GSOCN and EGSCN exhibited higher levels of immobilized enzyme activity whereas on MNTCN, IPGSCN, GPSTCN and GFSTCN the activity was lower.

The presence of free polar groups (–OH) or the use of high molecular weight monomers to prepare the support lowered the retained activity, while the use of lineal and flexible monomers (SOTCN) led to maximum values of enzymatic activity being retained. Smaller rings (ASCN, FSCN and GSOCN), with a furanose structure, led to higher levels of retained enzymatic activity than those obtained with their analogues of six members with a pyranose structure (GSCN or EGSCN) (Fig. 1).

Table 1
Effect of chemical nature of the immobilization support on initial enzymatic activity of immobilized HRP

Support	v_0 (U g^{-1})
SOTCN	262.36±14.46
SSCN	254.55±0.87
ASCN	226.49±10.99
FSCN	210.00±13.60
GSOCN	196.41±10.70
EGSCN	174.42±8.39
DXICN	117.15±13.31
MSCN	112.52±7.52
GSCN	99.22±9.83
INCN	96.03±7.23
DXACN	84.95±6.07
GLUCNA	77.52±0.87
GULCNA	63.06±7.07
GPSTCN	27.48±4.12
MNTCN	21.69±0.58
IPGSCN	4.34±0.31
GFSTCN	2.31±0.26

Irradiation time.The effect of irradiation time was studied on (i) initial activity retained (Table 2), (ii) its stability during storage (Table 3) and (iii) crosslinking density (Table 4), using the derivatives GLICN, ASCN, GSOCN, SSCN and INCN (11.8 g l^{-1} in all cases) irradiated between 0 and 10 min as immobilization supports. After recording the initial enzymatic activity, the samples were stored to study the effect of irradiation time on the level of activity retained during storage. The results of this assay are presented as a percentage of the initial enzymatic activity remaining after storage. Steps were taken to ensure that no enzyme was released to the medium during storage. In the case of INCN, SSCN and ASCN, after a low irradiation time, a slight release of active enzyme to the storage medium was observed, although such release was much more pronounced in the case of SSCN and ASCN. This did not occur for irradiation times of 10 min, since cross-linking is complete and the support has already reached its maximum posible molecular weight.

To study the effect of irradiation time on crosslinking density, an emulsion of each of the supports was prepared from a chloroformic solution on a transparent quartz cuvette, and the ultraviolet spectra were recorded in the 240–340 nm interval at different irradiation times (0–10 min). The value of maximum absorption ($\lambda = 280$ nm), was used to calculate the crosslinking density (ρ), or the fraction of crosslinked monomers (Table 4). Note the greater variation in the ρ values calculated for the shorter irradiation times, while any variation was much less at longer times. Fitting the data for ρ by linear regression to the Neparian logarithm of irradiation time (expressed in minutes) and by reference to the ordinate on the origin and slope, respectively, it was possible to obtain the threshold sensitivity (46.73×, 45.03×, 51.72, 47.56× and 36.53×10^{-2} for GLICN, ASCN, GSOCN, SSCN and INCN, respectively) and the overall reaction rate (11.82×, 13.97×, 13.08×, 11.43× and 10.29×10^{-2} for GLICN, ASCN, GSOCN, SSCN and INCN, respectively), which that determine the quality of our immobilization supports as photopolymers. It can be seen that the values of both parameters increase as molecule size decreases, pointing to the greater ease of polymerization since the structural rigidity and esteric impedimentes of the molecules decrease, thus facilitating the dimerization of cinnamoyl groups. This pattern is only broken by GLICN, possibly due to the low degree of cinnamoyl group substitution, which would limit dimerization and polymerization.

We also studied the effect of crosslinking density on the initial enzymatic activity retained (Tables 4 and 2) and stability during storage (Tables 4 and 3). Except for INCN, all the supports showed a maximum at a ρ value of 0.4–0.6. In the case of SSCN, this maximum is displaced towards ρ values of 0.4, probably due to the greater molecular size of the initial monomer, while in the case of ASCN the maximum is displaced towards 0.6 for the opposite reason. This suggests that there is an optimum crosslinking density for obtaining the best enzymatic activity results

Table 2
Effect of irradiation time on initial retained activity, v_0 (U g^{-1}), of HRP immobilized on totally cinnamoylated derivatives of glycerine (GLICN), D-arabinose (ASCN), D-glucosone (GSOCN), sucrose (SSCN) or inuline (INCN)

$t_{irrad.}$ (s)	v_0 (U g^{-1})				
	GLICN	ASCN	GSOCN	SSCN	INCN
0	43.68±2.12	583.72±28.31	330.91±18.03	921.57±3.13	263.22±19.82
10	54.38±2.64	719.67±34.90	389.92±21.25	585.17±1.99	257.73±19.41
16	63.06±3.06	755.54±36.64	301.41±16.43	503.02±1.71	242.69±18.27
40	61.61±2.99	535.99±26.00	391.08±21.31	577.07±1.96	236.32±17.79
80	75.21±3.65	511.12±24.79	377.48±20.57	431.86±1.47	242.69±18.27
100	88.80±4.31	417.69±20.26	–	373.14±1.27	–
140	83.31±4.04	559.42±27.13	238.35±12.99	362.73±1.23	222.44±16.75
300	70.58±3.42	361.28±17.52	231.98±12.64	315.00±1.07	202.48±15.25
600	65.95±3.20	210.00±10.19	135.08±7.36	190.33±0.65	163.43±12.31

(Jayakumari & Rajasekharan-Pillai, 1991). This maximum is not observed for INCN possibly due to the high degree of crosslinking obtained at low radiation times.

Thermal stability. The thermal stability of immobilized enzymes is one of the most important criteria to be borne in mind for their subsequent application. Several industrial applications of peroxidases involve high temperatures, which increase the yields and profitability of the processes. In general, the activity of immobilized enzyme preparations are more resistant to heat and denaturing agents than their soluble counterparts (Ulbrich, Schellenberger, & Damerau, 1986). The effect of temperature on the stability of HRP immobilized on the immobilization supports prepared was studied at 60 °C in 0.1 M sodium phosphate buffer (pH 7.0) (Table 5).

No great differences were seen between the values shown by the different samples of immobilized enzyme after heating for 30 s at 60 °C (heat shock) and all showed values of around 24.4%. Of note was the excellent behaviour shown by GSOCN, EGSCN, MNTCN, SOTCN and the derivative GLUCNA (more than 30%). The characteristics of these supports (low molecular weight and structural flexibility) favour their UV-light dependent cross-linking as described in the previous section. This gives rise three-dimensional networks that could affect the stability of the immobilized enzyme. This assay permitted us to classify the supports according to their capacity to maintain the enzyme in good condition in the face of high temperatures, pH and inactivators, and, presumably, to maintain its accessibility to the substrate and the easy diffusion of the reaction product to the reaction medium. After 10 min at 60 °C (prolonged heat treatment) all the samples with immobilized enzyme showed activity of about 7.6%, once again the activity retained by GSOCN, SOTCN, EGSCN, MNTCN and GLUCNA being of note, together with GPSTCN (retention in excess of the mean). When the combined effect of heat shock and prolonged heat treatment (Table 5, R_3 (%)) were taken into account, the activity initially retained after the heat shock decreased to around 31% through the effect of the prolonged heat treatment. GSOCN once again behaved well (≅40%), as did the above-mentioned derivatives and MSCN, with values of above the mean.

Inactivation of immobilized HRP by hydrogen peroxide. Peroxidases can be inactivated by several mechanisms: (i) reactions between hydrogen peroxide or associated radical species and intermediates of the enzyme catalytic cycle (Arnao, Acosta, Del Río, Varón, & García-Cánovas, 1990; Hiner, Hernández-Ruíz, García-Cánovas, Smith, Arnao, & Acosta, 1995; Baynton, Bewtra, Biswas, & Taylor, 1993); (ii) irreversible reactions between the enzyme and phenyl or phenoxy radicals formed by one-electron oxidation of phenol substrates during the catalytic cycle (Baynton et al., 1993; Aitken & Heck, 1998); (iii) adsorption of polymerized phenols on peroxidase, which

Table 3
Effect of irradiation time on retained activity after one month of storage, R (%), of HRP immobilized on totally cinnamoylated derivatives of glycerine (GLICN), D-arabinose (ASCN), D-glucosone (GSOCN), sucrose (SSCN) or inuline (INCN)

$t_{irrad.}$ (s)	R (%)				
	GLICN	ASCN	GSOCN	SSCN	INCN
0	10.60±0.51	15.98±0.78	32.93±1.79	31.08±0.11	12.31±0.93
10	11.70±0.57	28.78±1.40	51.59±2.81	29.36±0.10	14.59±1.10
16	11.01±0.53	28.67±1.39	35.56±1.94	25.84±0.09	14.54±1.09
40	11.74±0.57	22.19±1.08	47.67±2.60	33.72±0.11	14.20±1.07
80	16.54±0.80	20.83±1.01	42.06±2.29	29.76±0.10	14.78±1.11
100	15.96±0.77	19.51±0.95	–	25.98±0.09	–
140	14.24±0.69	22.44±1.09	31.35±1.71	27.13±0.09	15.09±1.14
300	13.12±0.64	16.87±0.82	27.97±1.52	23.54±0.08	15.43±1.16
600	11.40±0.55	14.69±0.71	22.88±1.25	18.53±0.06	12.21±0.92

Table 4
Effect of irradiation time on crosslinking density, ρ, of the immobilization supports: totally cinnamoylated derivatives of glycerine (GLICN), D-arabinose (ASCN), D-glucosone (GSOCN), sucrose (SSCN) or inuline (INCN)

$t_{irrad.}$ (s)	$\rho \times 10^2$				
	GLICN	ASCN	GSOCN	SSCN	INCN
0	0	0	0	0	0
10	25.35±2.44	21.86±0.70	28.69±2.63	27.32±0.91	19.93±1.99
16	30.59±2.95	26.24±0.84	33.74±3.09	31.94±1.06	22.91±2.29
40	41.58±4.01	36.85±1.18	45.42±4.17	42.33±1.41	30.82±3.08
80	50.55±4.87	48.03±1.53	56.08±5.14	51.26±1.70	38.27±3.83
100	53.32±5.14	52.19±1.66	–	53.84±1.79	–
140	57.80±5.57	57.71±1.84	64.00±5.87	57.74±1.92	44.60±4.46
300	66.16±6.38	69.32±2.21	73.70±6.76	66.20±2.20	53.26±5.33
600	72.58±7.00	76.56±2.44	80.40±7.37	73.15±2.43	61.64±6.16

$\rho = (1 - A_i/A_0)$. A_i: Value of absorbance at 280 nm for each irradiation time. A_0: Value of absorbance at 280 nm in the non-irradiated sample.

would hinder the access of a substrate to the active site of the enzyme (Nakamoto & Machida, 1992). One of the characteristic aspects of the action mechanism of peroxidases is their suicide inactivation by peroxides (Arnao et al., 1990; Rodríguez-López, Hernández-Ruíz, García-Cánovas, Thorneley, Acosta, & Arnao, 1997). This inactivating phenomenon is a problem when the enzymes are to be used for industrial purposes, whether in enzymatic reactors or/as biosensors. It is for this reason that immobilization supports that increase enzyme stability in the face of hydrogen peroxide are of great academic and economic interest. The time-course of immobilized HRP inactivation by hydrogen peroxide (10 mM) at both pH 7.0 and 4.5 was similar to that described in previous studies (Rojas-Melgarejo et al., 2004). Apparent inactivation constants were determined at both pH values (Table 6).

One of the most surprising results is the extent to which HRP is protected when immobilized on supports at pH 7.0. In all cases the value of k_i observed was lower than that determined for soluble HRP. Although it is difficult to find a molecular explanation for this, it could be surmised that the fixation of HRP to a support might inhibit its reaction with possible inhibiting agents generated during the reaction. Reactive oxygen species, such as √OH, superoxide, which are generated during catalysis, may be absorbed by the support instead of attacking essential residues of the enzyme. Furthermore, the low inactivation constant values obtained for GSOCN and EGSCN should be pointed out.

A diametrically opposite effect was observed when the assays were carried out at pH 4.5. In this case, greater inactivation was observed than at pH 7.0, especially on those supports containing carbonyl groups (Smith & March, 2001) oxidisable at acid pH (e.g. GSOCN), or which possess carboxyl groups (e.g. GULCNA and GLUCNA) which, in the presence of H_2O_2, give rise to strongly inactivating peracids. Such an effect is more marked in monosaccharide groups than in other polyhydroxylic compounds due to the glycosidic bonds that they present. In the presence of ABTS, this substrate protects the enzyme against suicide inactivation, more so at pH 4.5 than at 7.0, since at the lower value it is a better substrate ($Km_{pH\ 4.5} = 92.8$ μM; $Km_{pH\ 7.0} = 640$ μM (Rodríguez-López, Gilabert, Tudela, Thorneley, & García-Cánovas, 2000)). Therefore, the greater inactivation observed for immobilized HRP than for the soluble enzyme at pH 4.5 may be due to the reaction of aldehyde groups released from the support with the reactive groups present on the aminoacid side chains of the enzyme, which provokes its inactivation.

As regards the GSOCN polymer, the decrease in the inactivation constant as the pH of the medium changed from 4.5 to 7.0 may, as has already been mentioned, be due to the effect of the assay medium on the aldehyde groups released on the immobilization support and responsible for the inactivation of the adsorbed enzyme. The supports, which did not contain an easily oxidisable group that may be affected by the pH of the medium (ASCN, SOTCN and MNTCN) showed the lowest inactivation constant at pH 4.5. It is interesting to note that the EGSCN derivative, whose C_1 is blocked by the formation of a glycosidic bond with an ethyl group, showed an inactivation constant at acidic pH which was substantially lower than that shown by

Table 5
Thermal stability after heat shock, $R_{1(30\ s;\ 60\ °C)}$ (%), prolonged heat treatment, $R_{2(10\ min;\ 60\ °C)}$ (%) or after the two combined effects, R_3 (%), of HRP immobilized on each of the cinnamoylated supports studied

Support	$R_{1(30\ s;\ 60\ °C)}$ (%)	$R_{2(10\ min;\ 60\ °C)}$ (%)	R_3 (%)
SOTCN	28.74±1.58	11.24±0.62	39.10±2.15
SCN	19.61±0.07	5.55±0.02	28.32±0.10
ASCN	24.88±1.21	7.16±0.35	28.79±1.40
FSCN	22.75±1.47	5.28±0.34	23.16±1.50
GSOCN	32.91±1.79	13.00±0.71	39.57±2.16
EGSCN	32.61±1.57	6.87±0.33	21.11±1.02
DXICN	18.98±2.16	5.20±0.59	27.50±3.12
MSCN	16.80±1.12	6.25±0.42	37.10±2.48
GSCN	23.63±2.34	7.73±0.77	32.58±3.23
INCN	24.25±1.83	5.20±0.39	21.43±1.61
DXACN	21.00±1.50	6.30±0.45	29.83±2.13
GLUCNA	30.08±0.34	9.59±0.11	31.71±0.36
GULCNA	15.23±1.71	3.90±0.44	25.71±2.88
GPSTCN	23.68±3.55	9.30±1.40	40.00±6.00
MNTCN	30.26±0.81	11.84±0.32	39.13±1.04

Table 6
Inactivation constants by effect of H_2O_2 at pH 4.5 ($k_{i;\ pH\ 4.5}$) and 7.0 ($k_{i;\ pH\ 7.0}$) of soluble (SB) and immobilized HRP

Support	$k_{i;\ pH\ 4.5}\times 10^2$ (min^{-1})	$k_{i;\ pH\ 7.0}\times 10^2$ (min^{-1})
ASCN	8.30±0.03	–
SOTCN	8.18±0.02	10.58±0.32
MNTCN	8.28±0.32	–
IPGSCN	10.84±1.18	–
GFSTCN	7.43±0.29	–
EGSCN	8.18±0.35	7.60±0.35
GPSTCN	10.01±0.16	–
GSCN	10.20±0.20	7.30±0.27
MSCN	10.73±0.17	–
DXICN	10.99±0.14	9.28±0.26
DXACN	11.30±0.10	–
FSCN	12.71±0.22	10.57±0.34
SCN	9.13±0.11	–
INCN	10.09±0.11	8.04±0.23
GSOCN	12.37±0.09	6.63±0.35
GULCNA	12.25±0.09	–
GLUCNA	12.87±0.14	–
SB	5.29±0.06	14.98±0.76

the support obtained from hydrolysis and subsequent release of the C_1 (GPSTCN), which runs the risk of oxidation. Furthermore, the EGSCN derivative showed a similar inactivation constant at both pH values assayed, perhaps because the blocked C_1 means that it is insensitive to changes in the pH of the medium. For SOTCN, when the pH changes to neutral, the immobilized enzyme shows a similar tendency to that shown by the soluble form, that is, the inactivation constant becomes greater than at acidic pH, although it does not reach the value shown by this soluble form.

4. Conclusions

The HRP enzyme immobilized on the above mentioned supports permitted good enzymatic activity values to be obtained, the exact values depending on the chemical nature of the support used. The supports containing small molecules (preferably lineal or of furanose structure) with no free hydroxyl groups (GSOCN, SOTCN and EGSCN) provided the best results probably due to the greater flexibility of the monomer chain used and the availability of cinnamoyl groups for crosslinking.

The results justify the use of long irradiation times (15 min) to prepare the immobilization support since, (i) although maximum activity is reached after short irradiation times, longer times ensure that this activity level is repeated in subsequent experiments even though there may be slight variations in the length of the irradiation time, (ii) the retained enzyme is not released to the storage medium and (iii) a good layer of solid is formed around the glass beads because of the cosslinking of the initial monomer chains around such an inert matrix. The deterioration, breakage or release of non-crosslinked monomers to the assay or storage medium is thus avoided.

The results obtained after the initial heat shock and after more prolonged thermal treatment show that GSOCN is the best support for immobilising HRP enzyme and for subsequent heat treatment. Furthermore, it possesses an aldehyde group (C_1) and a neighbouring ketonic group (C_2) responsible for the 2,5-furanose ring, which may be responsible for the covalent immobilization of the enzyme through the initial formation of a Schiff base that subsequently may be reduced or undergo an Amadori rearrangement to form an irreversible covalent bond. Other supports providing good results are SOTCN and EGSCN since they initially retain a substantial amount of enzymatic activity and behave well in the face of heat treatment. They would therefore be useful for use in processes that involved extreme temperatures. Although MNTCN retained a low amount of enzymatic activity initially, its performance after heat treatment was good. It can also be crosslinked with another support that has a reactive group of special interest, for example the GLUCNA derivative, which also gives good yields in the face of heat treatment. The base layer of MNTCN provides a bed where the enzyme remains stable and where enzyme immobilization occurs by means of covalent bonds through the carboxyl group of the support and a suitable reactive group present on the side chains of the enzyme amino acids.

Inactivation by H_2O_2 at pH 7.0 is slower in the case of the immobilized enzyme than the soluble enzyme, as can be seen from the lower inactivation constants.

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