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Operational stability of immobilised horseradish peroxidase in mini-packed bed bioreactors

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Abstract

Mini-packed bed bioreactors containing horseradish peroxidase (HRP) immobilised on alkylamine controlled pore glass (CPG) were assembled for monitoring and quantification of hydrogen peroxide (H_2O_2), using a flow injection analysis (FIA) system. Samples (25 μl) were injected in a carrier stream containing the HRP reducing substrates, phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP). A linear response of the flow system was obtained for concentrations of H_2O_2 lower than 11 mM.

Different immobilisation protocols [e.g., covalent binding using glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimine (EDC) hydrochloride and adsorption followed by cross-linking] were tested in order to obtain high operational stabilities.

High operational stabilities were obtained when HRP was covalently immobilised using glutaraldehyde (less than 3% of the initial conversion was lost after 24 h of continuous operation). EDC-bound HRP however showed a lower operational stability (40% of the initial conversion was already lost after 24 h of continuous operation).

HRP was also adsorbed on the surface of CPG and further cross-linked with glutaraldehyde. When a washing step was included before the cross-linking step, the bioreactors rapidly lost their initial activity. The elimination of the washing step increased the amount of protein loaded and the initial conversion of the bioreactors. Furthermore, only 10% of the initial conversion was lost after 20 h of continuous operation at 32 °C.

HRP glycans were oxidised with sodium periodate in order to introduce aldehyde groups, highly reactive towards primary amino groups. This technique allowed a direct coupling between the oxidised enzyme and the support, although EDC was also used to mediate this coupling. Both immobilised preparations showed high protein loadings (31 and 65 mg/g, respectively) and high operational stabilities (only 8% of the initial conversion loss during 24 h). This technique led to the formation of HRP homoconjugates (dimers and trimers).

Keywords: Horseradish peroxidase; Immobilised bioreactors; Flow injection analysis; Operational stability; Storage stability; Sodium periodate; Oxidised HRP

1. Introduction

Analytical methods for process monitoring and control are constantly evolving in response to the fast development of bioengineering and biotechnology. In particular, the detection of hydrogen peroxide (H_2O_2) is of great importance in many areas, including industrial processes, clinical analyses and environment.

Peroxidases have been intensively used in the construction of biosensing devices, not only for the detection of H_2O_2 and small organic peroxides, but also in combination with H_2O_2-producing oxidases for the detection of their substrates, e.g., glucose [1], alcohol [2], cholesterol [3], amino acids [4], etc.

Horseradish peroxidase (HRP; E.C. 1.11.1.7; donor: H_2O_2 oxidoreductase) is a glycoprotein, containing one heme (ferriprotoporphyrin IX) prosthetic group and two calcium ions. HRP polypeptide chain is glycosilated at eight specific asparagines. The glycans are composed of mannose, xylose, fucose and *N*-acetylglucosamine and account for ca. 20% of the total enzyme molecular weight [5].

Flow injection analysis (FIA) systems based on immobilised enzyme reactors are particularly well suited for process monitoring and for continuous on-line analysis,

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especially when sample treatment and transport needs to be automated. These systems generally offer a rapid, selective and sensitive method to monitor a biological process [6]. Nevertheless, the biological component usually fails to exhibit a long operational stability [7].

Recently, an in situ strategy has been proposed to increase the operational stability of mini-packed bed reactors containing alcohol oxidase from *Hansenula polymorpha* [2,8]. This strategy was also successfully applied to others immobilised oxidases (e.g., *Pichia pastoris* alcohol oxidase, glucose oxidase, lactate oxidase and galactose oxidase) incorporated into FIA systems. Briefly, the strategy relies on the use of HRP and reducing substrates to promote the immediate degradation of the H_2O_2 formed inside the bioreactor. In these studies, HRP was used dissolved in the stream that fed the bioreactor. However, this procedure results in a large consumption and waste of this enzyme. This problem can be tackled by using immobilised HRP bioreactors.

In this paper, the operational stability of HRP bioreactors, obtained with different immobilisation procedures, was studied in order to develop a stable analytical tool capable of performing reproducible and accurate measurements of H_2O_2 in a FIA system.

2. Materials and methods

2.1. Materials

Horseradish (*Armoracia rusticana*) peroxidase was purchased from Biozyme, UK, as a brown freeze-dried powder with a specific activity of 252 U/mg versus pyrogallol. Hydrogen peroxide was purchased from Merck and the reducing substrates, phenol-4-sulfonic acid (PSA) sodium salt and 4-aminoantipyrine (4-AAP) were purchased from Fluka and Sigma, respectively. Alkylamine controlled pore glass (CPG) with 80–120 mesh and 700 Å pore size was obtained from Sigma. Glutaraldehyde was purchased from Merck and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) hydrochloride and sodium periodate from Sigma. All solutions were prepared in distilled de-mineralised water.

2.2. Methods

2.2.1. Oxidation of HRP

HRP was oxidised for 20 min at room temperature with sodium periodate: 1 ml of 0.1 M $NaIO_4$ was added to 5 ml of 4 mg/ml HRP in 10 mM 2-(*N*-morpholino)ethanesulfonate (MES) buffer, pH 5 [9]. During this time aliquots were taken and the enzymatic activity measured. After periodation, the excess periodate was removed by gel filtration in a 20 cm^3 Econo-Pac disposable chromatography column (Bio-Rad) packed with a Sephadex G-25 coarse gel (Pharmacia). The column was equilibrated and eluted by gravity flow with 0.1 M phosphate buffer, pH 7.0. Fractions with 1.5 ml were collected and the absorbance at 404 nm was measured. The fractions with an absorbance higher than 0.1 were pooled and further concentrated by ultrafiltration using disposable concentration units from Amicon (Centricon).

2.2.2. Immobilisation procedure

HRP was covalently immobilised on aminated controlled pore glass using different cross-linkers: glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.

2.2.2.1. Glutaraldehyde coupling. Aminopropyl-CPG beads (approximately 1 ml) were suspended for 2 h with a top over bottom agitation at 25 °C, in 10 ml of a 5% (v/v) glutaraldehyde solution prepared in 0.1 M phosphate buffer, pH 7. Next, the support was thoroughly washed with phosphate buffer and an equivalent volume of 20 mg/ml HRP was added. The coupling of HRP to the support proceeded for 18 h with gentle agitation at 4 °C. In the end, the support was thoroughly washed with 0.1 M phosphate buffer, pH 7.

2.2.2.2. EDC coupling. About 50 mg of aminopropyl-CPG were suspended in 1 ml of a HRP solution (15 mg/ml native HRP or 14 mg/ml oxidised HRP) in 0.1 M EDC, 0.1 M MES buffer, pH 5.4. The coupling of HRP to the support proceeded for 18 h with top over bottom agitation at 4 °C. In the end the support was thoroughly washed with 0.1 M phosphate buffer, pH 7.

2.2.2.3. Direct coupling. About 50 mg of aminopropyl-CPG was suspended in 0.8 ml of a solution containing 27 mg/ml oxidised HRP in borate buffer, pH 8.5 and top over bottom agitated for18 h, at 4 °C.

2.2.2.4. Adsorption and cross-linking with glutaraldehyde. HRP was adsorbed on the surface of aminopropyl-CPG during 16 h, at 25 °C in 0.1 M phosphate buffer, pH 7.6. The support was washed with the same buffer and cross-linked with different concentrations of glutaraldehyde, 1–10% (v/v), prepared in the same buffer. Other immobilised preparations were obtained eliminating the washing step and adsorbing the enzyme at different pH values (0.1 M phosphate buffer pH 6.6 and 7.6 and in 0.1 M Tris/phosphate buffer pH 8.8). Lower concentrations of glutaraldehyde (0.25–1.5%, v/v) were used to cross-linking the enzyme.

2.2.3. Preparation of the packed-bed bioreactor

The immobilised preparations of HRP and oxidised HRP were packed into glass mini-columns (2 mm i.d., 25 mm length and 0.5 mm wall thickness). The CPG beads were supported in both ends by glass wool and connected to the external circuit with silicon tubing. The length of the HRP packed-beds was 6 mm, originating bioreactors with 19 mm^3. This bioreactor size was chosen since no further improvement in conversion was observed for bioreactors with higher volumes.

2.2.4. Activity determination

HRP activity was measured using phenol-4-sulfonic acid and 4-aminoantipyrine as reducing substrates according to the following equation:

$$2H_2O_2 + \text{PSA} + \text{4-AAP} \xrightarrow{\text{HRP}} \text{quinoneimine dye} + 3H_2O + NaHSO_4$$

The quinoneimine dye produced has a typical magenta colour and maximum absorption around 490 nm ($\varepsilon = 5.56\,\text{mM}^{-1}\,\text{cm}^{-1}$, determined experimentally towards H_2O_2 [10]). The activity was determined by monitoring the increase in absorbance at 490 nm associated with the consumption of H_2O_2 with a Hitachi U-2000 spectrophotometer. One unit of activity (U) was defined as the amount of enzyme that catalyses the conversion of 1 μmol of H_2O_2 per minute at 25 °C. The activity assays were performed using a standard assay reaction mixture containing 0.4 mM 4-AAP and 25 mM PSA in 0.1 M phosphate buffer, pH 7.0 [10].

2.2.4.1. Free HRP. In a typical assay, 25 μl of a HRP sample was added to 1 ml of the standard assay reaction mixture. The reaction was initiated by the addition of 25 μl of 250 mM H_2O_2 and the absorbance at 490 nm was monitored for 1 min, in a magnetically stirred quartz cell (Hellma), thermostatised at 25 °C.

2.2.4.2. Immobilised HRP. The activity of immobilised HRP was measured using a total recycle reactor. The system consisted of a jacketed glass stirred tank, a Watson Marlow peristaltic pump, a 18 mm^3 glass flow cell (Hellma) and a packed-bed bioreactor with the immobilised enzyme, connected in series with Omnifit Teflon tubing. A certain volume (10 ml) of the standard assay reaction mixture was added to stirred tank and recirculated 3.5 ml/min. The piping system was previously filled with the standard assay reaction mixture. The reaction was initiated by the addition of 250 μl of 250 mM H_2O_2 to the glass tank. The system was thermostatised at 25 °C.

2.2.5. Protein determination

Protein concentration was determined using the Folin phenol reagent. A known amount of support (approx. 10 mg) was completely hydrolysed in 2N NaOH during 20 min at 100 °C. The samples were cooled down and diluted to 0.5N NaOH. The hydrolysed protein was then determined using the Lowry method [11]. Albumin was used as a standard.

2.2.6. Operational stability

The operational stability of HRP was assessed during the continuous reduction of H_2O_2 in the standard assay reaction mixture at 32 °C. HRP bioreactors were continuously fed with 0.21 mM H_2O_2 in the standard assay reaction mixture and the production of the quinoneimine dye was monitored at 490 nm in a 18 mm^3 glass flow cell (Hellma). The concentration of H_2O_2 chosen to perform this studies correspond to the maximum amount of H_2O_2 that can be produced by H_2O_2-producing oxidases.

2.2.7. Flow system

The flow injection apparatus (Fig. 1) consisted of a Gallamp peristaltic pump connected with Accurated Tygon tubing (Elkay). Samples (25 μl) were injected using a Rheodyne rotatory PTFE valve, fitted to an Omnifit universal switching module. Absorbances were measured with a Jencons spectrophotometer, using a 18 mm^3 glass flow cell, and the output recorded on a Gallenkamp Datatrace flat-bed recorder. Teflon tubing (0.8 mm i.d.) and connectors were supplied by Omnifit.

2.2.8. Protein electrophoresis

Native and oxidised HRP were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [12]. Mini SDS-PAGE gels were prepared using a Bio-Rad Mini Gel Kit. The separating gel had a final concentration of 15% (w/v) acrylamide, 0.87% (w/v) methylenebisacrylamide, 0.1% (w/v) SDS, 0.373 M Tris–HCl, 4.4 mM *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and 1.5 mM $(NH_4)_2S_2O_8$ and was topped with water saturated with butanol until the gel set. The stacking gel had a final concentration of 5% (w/v) acrylamide, 0.14% (w/v) methylenebisacrylamide, 0.1% (w/v) SDS, 0.125 M Tris–HCl, 17 mM TEMED and 2.2 mM $(NH_4)_2S_2O_8$. Samples (20 μl) were added to 5 μl of a loading buffer (0.35 M SDS, 0.25 M Tris, 25% (v/v) glycerol and 0.05% (w/v) bromophenol) and boiled for 2–5 min. The samples were then loaded onto the gel and a constant current of 80 mA (40 mA

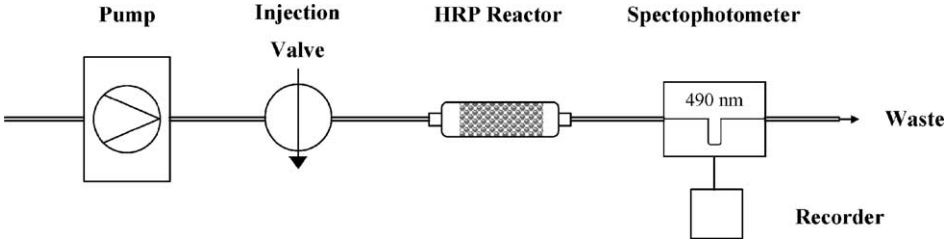

Fig. 1. FIA manifold.

per gel) was applied. Gels were developed with Commassie [13] and silver nitrate [14,15].

3. Results and discussion

HRP was immobilised onto aminopropyl-controlled pore glass supports using two distinct methodologies, adsorption followed by cross-linking and covalent binding.

3.1. Adsorption

HRP was immobilised by adsorption on CPG followed by cross-linking with glutaraldehyde using two different procedures. When a washing step between the adsorption and the cross-linking step was included, HRP packed-bed bioreactors presented a low operational stability. This was attributed to HRP desorption during washing which led to lower protein loadings. Moreover, lower operational stabilities were obtained at higher concentrations of glutaraldehyde (Fig. 2).

In view of these results, the cross-linkage with glutaraldehyde was performed immediately after the adsorption of HRP to the CPG support and low concentrations of the cross-linker (up to 1.5%) were used. Fig. 2 shows the decay on conversion by HRP immobilised preparations cross-linked with different glutaraldehyde solutions prepared in phosphate buffer, pH 7.6. Elimination of the washing step increased both the initial conversion and the stability of the bioreactors. In fact, comparing the data obtained with 1% glutaraldehyde there was an increase of 30% in the initial conversion and of 115% in the conversion after 12 h of continuous operation.

Table 1 summarises the results obtained with glutaraldehyde solutions prepared in phosphate buffer, pH 6.6 and in Tris/phosphate buffer, pH 8.8, confirming that the stability

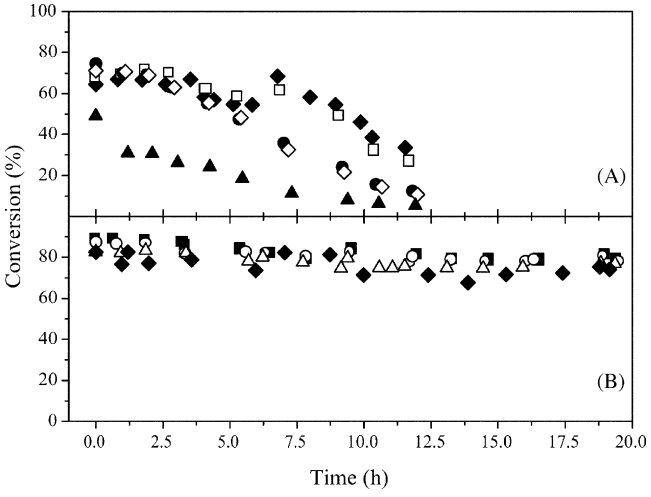

Fig. 2. 0.12 mM H_2O_2 conversion by HRP immobilised on CPG by adsorption, followed by cross-linking with different concentrations of glutaraldehyde prepared in phosphate buffer pH 7.6. (A) With a washing step: 1% (◆), 2% (□), 4% (●), 6% (◇) and 10% (▲); and (B) without a washing step: 0.25% (■), 0.50% (○), 0.75% (△) and 1% (◆).

Table 1
Effect of pH and glutaraldehyde concentration in the cross-linking step on the initial volumetric productivity (q_v), initial conversion and conversion after 12 and 20 h of continuous reduction of 0.12 mM H_2O_2

GA (%)	pH	q_v (mol/m^3 min)	Conversion (%)		
			0 h	12 h	20 h
0.25	6.6	5.61	90	79	72
	8.8	5.14	83	69	75
0.50	6.6	5.28	85	74	74
	8.8	5.71	92	78	83
0.75	6.6	5.14	83	71	71
	8.8	5.61	90	82	83
1.0	6.6	5.19	83	73	74
	8.8	5.66	91	83	86
1.5	6.6	5.12	82	75	68
	8.8	5.66	91	68	21

of the bioreactors can be enhanced by eliminating the intermediate washing step. In general, higher conversions are obtained when using glutaraldehyde solutions prepared in alkaline buffers (pH 8.8). Nevertheless, at pH 8.8 the decrease in the enzyme stability is already observed for concentrations of glutaraldehyde higher than 1.0%. The decrease in conversion that is observed when high concentrations of glutaraldehyde are used, reflects a deactivation caused by an excess of cross-linkage of the enzyme, that ultimately may lead to changes in the three-dimensional structure of the enzyme.

3.2. Covalent binding

HRP was covalently immobilised onto aminopropyl-CPG using two different linkers, glutaraldehyde and a soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimine hydrochloride. Glutaraldehyde binds proteins through their free amino groups, especially those from lysine (#6 on HRP) residues. On the other hand, EDC binds proteins to aminated supports through the carboxyl groups from aspartate (#21) and glutamate (#8) residues. Although the total number of carboxyl groups (#29) is much higher than the number of amino groups, the amount of HRP immobilised with EDC, via carboxyl groups, was much lower than with glutaraldehyde (five times lower, Table 2). Nevertheless, the activity in both bioreactors was the same. The operational stability of glutaraldehyde and EDC-bound HRP was measured in 19 mm^3 reactors as shown in Fig. 3. Both preparations exhibited similar initial H_2O_2 conversions but the glutaraldehyde coupling led to a higher long-term stability, probably due to the higher protein loading. In fact, after 10 h of continuous operation, the conversion of the bioreactor containing the EDC-bound HRP started to decrease while the glutaraldehyde-bound HRP bioreactor remained stable for more than 24 h. The half-life (time at which 50% of the initial conversion was lost) of both reactors was determined by linear regression and the value was expressed in number

Table 2
Activity, initial volumetric productivity (q_v), initial conversion and conversion after 24 and 60 h of HRP bioreactors using different immobilisation procedures and respective protein loading and respective half-life expressed in number of 25 μl injections of 0.21 mM H_2O_2

HRP	Protein (mg/g)	Activity (U/cm^3)	q_v (mol/m^3 min)	Conversion (%)			Half-life (injections)
				Initial	24 h	60 h	
Native HRP							
HRP + GA	21	17.4	7.80	72	70	19	112 800
HRP + EDC	3.9	18.6	7.90	73	29	2	48 000
Oxidised HRP							
HRPox	31	19.1	7.80	72	66	22	108 000
HRPox + EDC	65	17.4	7.33	67	62	29	110 400

of injections, which was done by converting the amount of H_2O_2 converted during this half-life time into the corresponding number of injections of 25 μl samples of 0.21 mM H_2O_2. The EDC-bound HRP bioreactor allows the injection of 48000 samples and the glutaraldehyde-bound HRP bioreactor of more than 112,000 samples in a FIA system (Table 2).

3.3. Oxidation of HRP

HRP carbohydrate chains were oxidised using sodium periodate, which acts on vicinal diols and originates two aldehyde groups. The loss of activity during the periodate oxidation in MES buffer, 10 mM, pH 5.5, was evaluated by measuring HRP activity during the course of oxidation. Although there was an abrupt loss in the initial activity down to 42%, no further decrease was observed as oxidation proceeded (Fig. 4). A significant amount of the initial activity that was lost after oxidation could be recovered (76%) after removing the periodate by gel filtration. In order to minimise the activity loss, HRP oxidation was performed in different 10 mM buffers, namely, phosphate buffer pH 6.5, acetate buffer pH 5.2 and also in water ($pH_{initial} = 4.8$; $pH_{final} = 3.8$). The lowest activity loss was observed in phosphate buffer (only 12.5%), followed by acetate buffer (21%) and water (29%). The highest activity loss was in fact observed in the initial MES buffer (58%). The activity of HRP in these buffers was very similar but higher values were obtained in phosphate buffer, which was thus adopted to perform further studies.

With oxidation, HRP carbohydrates can be converted to polyaldehydes and directly coupled to an aminated support. EDC was also used to mediate this coupling. Both immobilised preparations exhibited high protein loading (31 and 65 mg/g, respectively) and operational stability (Fig. 3), although the EDC-bound oxidised HRP presented a slightly lower initial conversion (67% against 72%, Table 2). Conversion remained stable for 10 h and after 24 h of continuous operation the decrease in conversion was less than 8%. The half-life of these bioreactors indicates that more than 100,000 samples can be analysed in a FIA system (Table 2).

Comparing both native and oxidised HRP covalently bounded bioreactors, the highest retention of the initial conversion was observed with the EDC-bound oxidised HRP, which is probably a consequence of the high protein loading obtained with this immobilisation protocol. Nevertheless, the glutaraldehyde-bound HRP bioreactor also presents a good performance, considering that this bioreactor contains only 30% of the protein of the former.

A SDS-PAGE was run in order to evaluate if periodation had produced any significant change in the protein structure. Two gels were run in duplicate, but one was developed using

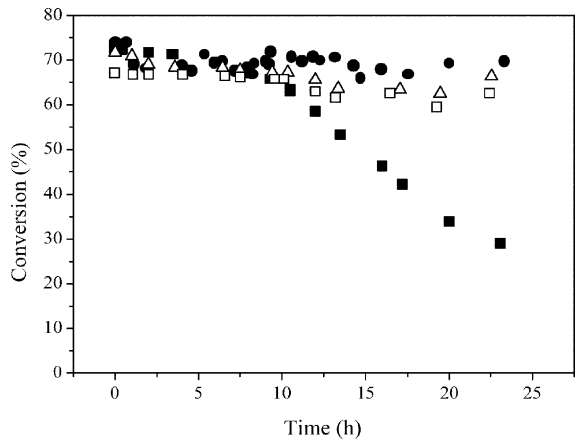

Fig. 3. 0.21 mM H_2O_2 conversion by HRP (full symbols) and oxidised HRP (open symbols) immobilised on CPG by covalent binding using glutaraldehyde (●), EDC (■, □) and direct coupling (△).

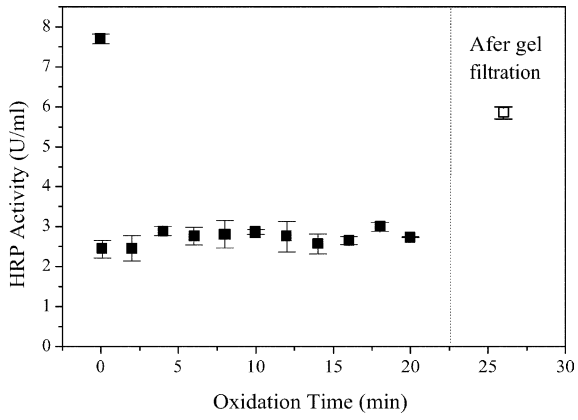

Fig. 4. HRP activity during the oxidation with 17 mM sodium periodate in 10 mM MES buffer, pH 5.5 (■) and after a gel filtration operation (□).

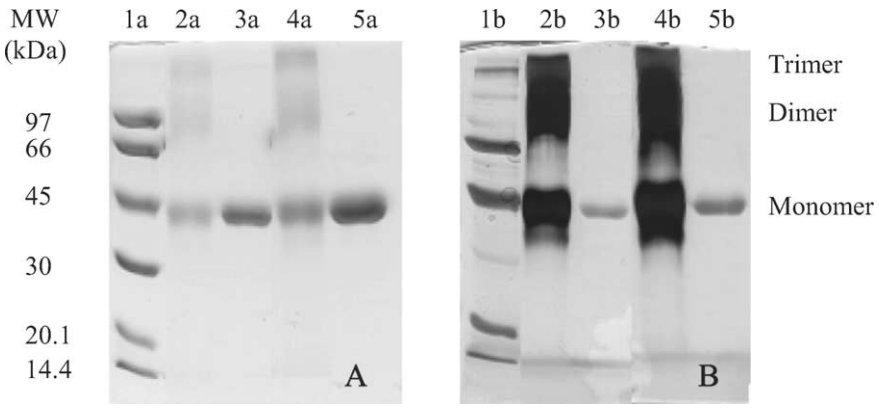

Fig. 5. SDS-PAGE gel developed with brilliant blue Commassie (A) and $AgNO_3$ (B). Lanes 1a and 1b: molecular weight markers; lanes 2a and 2b: 0.9 mg/ml oxidised HRP; lanes 3a and 3b: 1 mg/ml native HRP; lanes 4a and 4b: 1.8 mg/ml oxidised HRP; lanes 5a and 5b: 2 mg/ml native HRP.

blue Commassie and the other with silver nitrate (Fig. 5). The intensity of the bands on a gel stained with Commassie depends upon the protein concentration, and for this reason band intensity in gel A is equivalent for oxidised and native HRP. On the other hand, the intensity of the bands developed with silver nitrate is enhanced by the presence of aldehyde groups. This explains why in gel B the bands of oxidised HRP are much more intense than the bands of native HRP. The gels show that upon periodation dimers and trimers of HRP are formed probably by reaction of the aldehyde groups introduced with the amino groups from amino acid residues present in the surface of a neighbouring enzyme. The formation of these homoconjugates may explain the observed decrease in the activity of HRP since it may affect the structure of the active centre or hinder substrate access. Nevertheless, a significant amount of HRP (44 kDa) remains in the monomeric form.

3.4. H_2O_2 assay in a FIA system

The bioreactor containing HRP immobilised with glutaraldehyde was incorporated into a flow injection analysis system (Fig. 1) and its stability was tested by multiple manual injections of 25 μl H_2O_2 samples. No changes in peak heights were observed after more than 200 manual injections during several weeks. Different time intervals between successive injections were tested, namely 2.0, 1.0 and 0.5 min. The best peak resolution was observed with a 2 min interval. Nevertheless, the system can accurately analyse about 100 samples per hour.

H_2O_2 samples of different concentrations (1–18 mM) were injected through an injection valve positioned upstream of the immobilised bioreactor. The peak heights obtained are directly proportional to H_2O_2 concentration for concentrations lower than 11 mM (Fig. 6) and the following calibration curve was obtained:

$$\text{peak height (\%)} = (7.1 \pm 0.2) \times H_2O_2\,(\text{mM}) + (0.9 \pm 0.1)$$

with a correlation factor higher than 0.997.

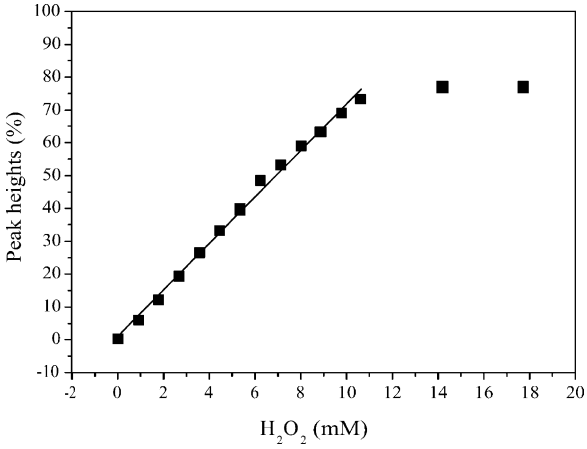

Fig. 6. Calibration curves for H_2O_2 using a HRP packed bed reactor, after 25 μl injections of H_2O_2 standards.

3.5. Storage stability

The storage stability of the glutaraldehyde-bound HRP bioreactors was studied at 4 and 25 °C. As Fig. 7 shows, there was no loss in activity after more than 1 month storage.

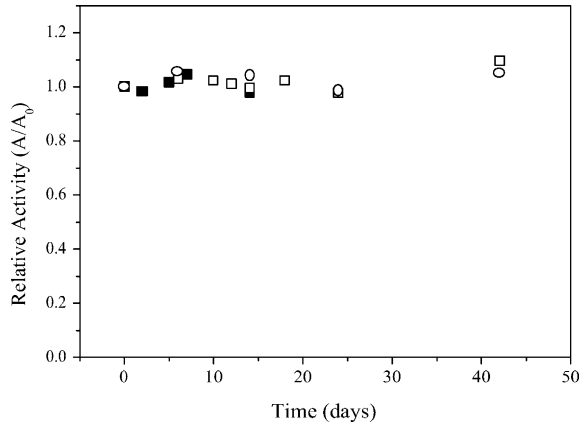

Fig. 7. Storage stability of glutaraldehyde-bound HRP bioreactors at 4 °C (○) and 25 °C (□). Effect of the standard assay reaction mixture on the stability of HRP at 25 °C (■).

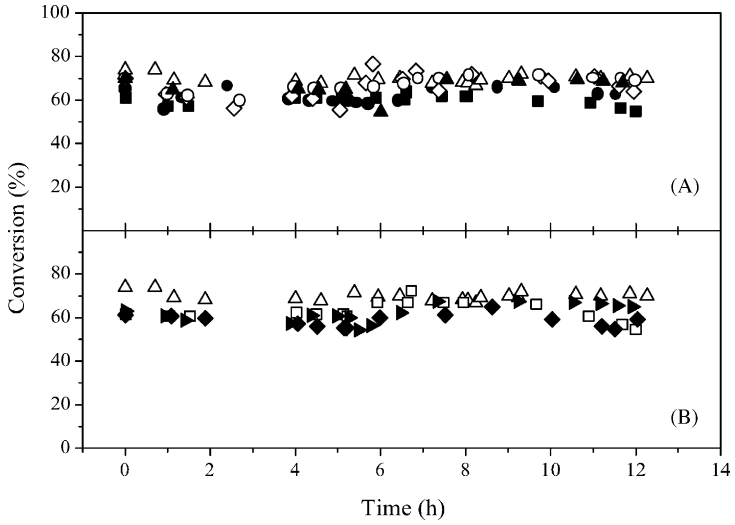

Fig. 8. Operational stability of HRP during the reduction of 0.21 mM H_2O_2 in the standard assay reaction mixture at different pH values. (A): pH 6 (■), pH 6.5 (●), pH 7 (△), pH 7.5 (◇), pH 8 (○) and pH 8.5 (▲), in the present of different complex media at pH 7. (B): none (△), fermentation media (□), brandy (▶) and beer (◆).

Moreover, these bioreactors did not loose activity after more than 1 year at 4 °C. The effect of the standard assay reaction mixture on the stability of HRP immobilised bioreactors was also evaluated (Fig. 7). A glutaraldehyde-bound HRP bioreactor was continuously fed with the standard assay reaction mixture (0.4 mM 4-AAP and 25 mM PSA in 0.1 M phosphate buffer, pH 7) and the activity was measured along the time. Again no loss in activity was observed during 2 weeks. Hence, HRP bioreactors show high stability during storage and no inhibition by the reducing substrates is observed.

3.6. Effect of pH

If HRP bioreactors are to be used for the quantitative determination of other substances such as the substrates of H_2O_2-producing oxidases (e.g., glucose oxidase, alcohol oxidase), then HRP should present a good pH stability in order to work at the oxidase optimum pH. The operational stability of glutaraldehyde-bound HRP bioreactors was then studied at different pH values from 6 to 8.5 (Fig. 8A) and a high retention of the initial conversion is observed in this range of pH values. At pH 6 and 6.5 slightly lower (less than 70%) conversions are however obtained.

3.7. Effect of complex media

The operational stability of glutaraldehyde-bound HRP immobilised reactors was studied in the present of complex media in order to simulate the analyses of real samples, such as fermentation media, beer and brandy. Substrates and metabolites which are typically present in a fermentation medium were added to the standard assay reaction mixture in order to obtain a nominal concentration of 1.7 g/l pyruvate, 0.4 g/l succinate, 4.2 g/l yeast extract, 8.3 g/l glucose, 0.4 g/l glycerol and 6.0 g/l acetate. Beer (5% ethanol) and brandy (38% ethanol) samples were diluted in the standard assay reaction mixture in order to obtain an ethanol concentration of about 6.5 mM. Although in the presence of these complex media, the initial conversion is about 15% lower, high retentions are still observed (Fig. 8B). The decrease in the initial conversion is probably a result of some inhibition of HRP by the added components, specially the yeast extract.

4. Conclusions

HRP mini-packed bioreactors were successfully constructed with HRP immobilised using different procedures: adsorption followed by cross-linking and covalent binding using glutaraldehyde and EDC. The performance of the adsorbed/cross-linked bioreactors was dependent upon the concentration of the glutaraldehyde solution used. The type of buffer used to adsorb the enzyme also affected the bioreactor initial conversion.

Regarding the covalent binding, the choice of the immobilisation reagent seems to be critical in order to achieve high operational stabilities. Glutaraldehyde-bound and EDC-bound HRP bioreactors exhibited similar initial conversions but the latter failed to retain the initial conversion after more than 10 h of continuous operation.

Upon oxidation of HRP with sodium periodate, highly reactive aldehyde groups can be introduced, which allow a direct coupling of the oxidised enzyme to the support with high yields. The highest protein loading was achieved using EDC to mediate the binding of the oxidised HRP to the support. High operational stabilities and conversion degrees were obtained with both oxidised HRP preparations.

Glutaraldehyde-bound HRP bioreactors present high operational stability at different pH values (6–8.5) and in the presence of complex media, such as beer, brandy and

fermentation media. These bioreactors also present high storage stability at 4 and 25 °C and no inhibition by the reducing substrates (4-AAP and PSA) was observed.

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