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Journal of Molecular Catalysis B: Enzymatic 28 (2004) 129–135
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
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Assay of H_2O_2 by HRP catalysed co-oxidation of phenol-4-sulphonic acid and 4-aminoantipyrine: characterisation and optimisation

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Received 26 February 2003; received in revised form 11 November 2003; accepted 2 February 2004

Abstract

A colorimetric system involving horseradish peroxidase (HRP), phenol-4-sulfonic acid (PSA) and 4-aminoantipyrine (4-AAP) for the determination of hydrogen peroxide concentration and peroxidase activity, and possible coupling with oxidases for other analytical purposes, was developed and optimised. Proposed reagents concentrations are 25 mM PSA, 0.4 mM 4-AAP, and 100 mM phosphate buffer pH 7. The absorptivity of the product calculated to the added hydrogen peroxide concentration is close to value measured for the colorimetric system that instead of PSA contained phenol. Influence of some common fermentation broth components (D-galactose, D-glucose, succinate, glycerol, acetate, yeast extract) on the colorimetric system was tested and no significant interference was found, except for yeast extract for concentrations above 5 g l^{-1}. Some advantages of the proposed colorimetric system is that PSA has better solubility, does not produce precipitates in reaction with hydrogen peroxide, and is not as toxic as phenol. The optimised colorimetric system was used in soluble and immobilised HRP kinetics experiments. Optimised colorimetric system was also successfully employed in a flow injection analysis system for hydrogen peroxide measurements and good linear range, sensitivity, reproducibility and system stability were confirmed.

Keywords: Phenol-4-sulphonic acid; 4-Aminoantipyrine; Colorimetric system; Horseradish peroxidase; Activity; Flow injection analysis

1. Introduction

Dynamic development of bioengineering and biotechnology gave boost to increasing interest for reliable and fast methods for monitoring concentrations of substrates, metabolites and inhibitors in culture media and control of bioreactors. A high specificity of enzyme-based assays combined with the advantages of flow injection systems could meet the requirements for fast and accurate process monitoring [1].

Horseradish peroxidase (HRP) (E.C.1.11.1.7) has been broadly used and investigated for analytical purposes [2]. It catalyses reduction of hydrogen peroxide in the presence of a reducing compound. Applications that involve peroxidases described in literature are based on colorimetry, chemiluminescence, fluorescence and amperometric measurements [2–4].

If the reducing agent is selected so to produce coloured product or to further react with an appropriate chromogen to produce a coloured product, then hydrogen peroxide (or an oxidase substrate if HRP is coupled to a hydrogen peroxide producing oxidase) can be determined spectrophotometrically. A wide range of reducing HRP substrates (like phenol, aminophenols, indophenols, diamines and a number of other compounds) has been used in spectrophotometric determination of H_2O_2 [2,3]. Most of these substrates, including 2,2′-azino-bis(3-ethylbenzthiazoline-6) sulphonic acid (ABTS), phenol, pyrogallol, *o*-tolidine, *o*-phenylendiamine cathecol, resorcinol, *o*-dianisidine are mutagenic, carcinogenic or extremely toxic compounds [3]. Furthermore, reaction with guaiacol gives an undefined mixture of oxidation products and absolute molar absorptivity cannot be determined [4]. Trinder et al. [5,6] developed a method for H_2O_2 determination using HRP catalysed oxidation of phenol in the presence of 4-aminoantipyrine. Trinder's coupled

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doi:10.1016/j.molcatb.2004.02.003

HRP/Phenol/4-AAP system for H_2O_2 determination could be improved by replacing toxic phenol by phenol-4-sulfonic acid (PSA) that is less hazardous to human health and environment and has much better water solubility [7]. This colorimetric system has been used to determine ethanol in beer and wine [8], and to measure peroxidase and glucose oxidase activities in soluble and immobilised enzyme preparation [9,10].

The purpose of this work was to optimise the coupled PSA/4-AAP/HRP system in respect to the concentrations of PSA, 4-AAP, buffer type and pH value. This was done by studying the apparent HRP activity and stability, and intensity of the colour obtained in catalytic oxidation of PSA in the presence of 4-AAP. In addition, effect of some common compounds of fermentation broths on the colorimetric system was investigated as this colorimetric system has been coupled with oxidases for monitoring nutrients consumption (e.g., glucose, galactose, lactate, others) and metabolites formation (e.g., ethanol) in fermentation broths. The kinetics of enzymatic redox reaction for four different reducing HRP substrates (phenol, *p*-chloro-phenol, *p*-cresol, PSA) was investigated and compared. The kinetics of immobilised HRP was also studied. Optimised system was applied in a flow-injection set-up for monitoring of hydrogen peroxide concentration.

2. Materials and methods

2.1. Materials

HRP with a specific activity of 252 U mg^{-1} (according to pyrogallol method performed by the supplier) was obtained from Biozyme (UK). Phenol-4-sulfonic acid sodium salt, potassium acetate, aqueous solution of H_2O_2 (≈30%), D-glucose, ethanol, and D-galactose were supplied by BDH (UK). Guaiacol, 4-aminoantipyrine, *p*-cresol, 4-chloro-phenol, pyrogallol, glycerol and succinic acid were purchased from Sigma (Germany) and phenol from Merck (Germany). Aqueous H_2O_2 solutions were standardised by permanganometry using $KMnO_4$ solution standard (Merck). Yeast extract was obtained from Difco (USA). All other chemicals were obtained from BDH, and were either laboratory or analytical grade. Deionised and distilled water was used in all the experiments. HRP immobilisation technique was as described in [9,12], using gluteraldehyde, and aminated CPG beads obtained from Sigma.

2.2. Methods

2.2.1. HRP enzyme concentration

Concentration of HRP in solution was determined spectrophotometrically at 404 nm using the molar absorptivity value 102,000 mol^{-1} l cm^{-1} [11]. Quantity of immobilised enzyme per mass of CPG support was determined by dissolving the CPG beads with immobilised enzyme in a 2 M NaOH solution and then following the standard Lowry procedure for the protein determination [9].

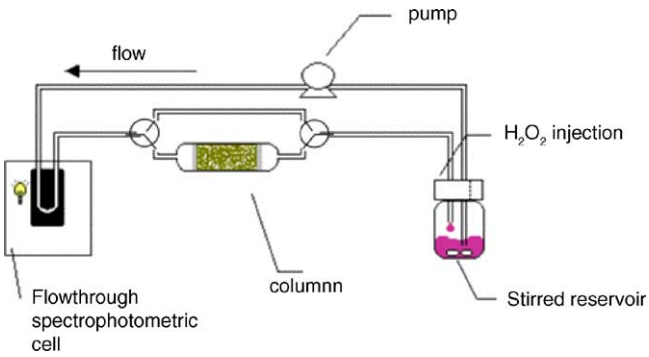

Fig. 1. Experimental set-up for HRP kinetics experiments.

2.2.2. Colorimetric measuring system

Standard assay reaction mixture was defined as a solution containing 0.4 mM 4-AAP, 25 mM phenolic compound (PSA if not explicitly stated otherwise) in 100 mM sodium phosphate buffer pH 7.0, and it was prepared shortly before use. Reaction mixture for the experiments with soluble HRP contained 0.4 mg l^{-1} of the enzyme if not stated otherwise.

2.2.3. H_2O_2 assay and HRP kinetics experiments

The experiments with dissolved HRP were carried out in a spectrophotometric cell agitated by a magnetic stirrer (Electronic Stirrer Model 300, Rank Brothers, UK) in the following way: 975 μl of the standard reaction mixture was added to the cuvette, followed by the addition of 25 μl of H_2O_2 sample or standard solution. As soon as the H_2O_2 solution was added spectrophotometer was started and absorbance at 490 nm was recorded until a stable value is reached.

Immobilised HRP kinetics experiments were performed in an external reactor system shown on Fig. 1. The same set-up was used in the experiments with soluble HRP for comparison with the immobilised enzyme activity. In the experiments with soluble HRP and analytical column, tubing and the column were rinsed with the reactional mixture prior to the kinetics experiment. After rinsing the system, connecting tubes were attached to the external reactor filled with 4.875 ml of the reactional mixture. Flow rate in both cases was 3.5 ml min^{-1}. After allowing some time for the solution in the system to homogenise, zero absorbance of the spectrophotometer was set, 125 μl of the H_2O_2 standard solution was added, and absorbances were measured and recorded.

Molar absorptivity of the reaction product at 490 nm (ε) is defined as the absorbance of the reactional mixture per mole of added hydrogen peroxide and centimetre of the light path (l) after the reaction is completed. Chemical structure of the quinone–imine dye produced in the reaction is presented in Fig. 2 [6].

Initial absorbance development rate at 490 nm is proportional to the initial reaction velocity defined as H_2O_2 con-

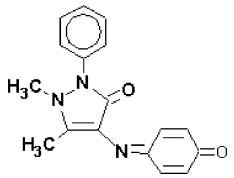

Fig. 2. Quinone–imine product.

sumption per unit of time. The reaction rate is calculated as:

$$-\frac{dc_{H_2O_2}}{dt} = \frac{1}{\varepsilon l}\frac{dA}{dt} \quad (1)$$

2.2.4. HRP buffer and pH dependence

Pyrogallol activity assay was performed according to the method described by the producer [13]. Guaiacol activity assay was performed according to [12].

2.2.5. FIA system set-up

The flow injection analysis (FIA) system was set-up with two Gilson (Middleton, USA) Minipuls 3 peristaltic pumps connected with Chemfluor FEP tubing from Tygon (Charny, France) operating with same flow rate (1 ml min^{-1}). The diluent composition was 100 mM potassium phosphate buffer pH 7.0, and the composition of the colour reagent was 0.8 mM 4-AAP, 50 mM PSA (doubled concentrations of the standard assay reaction mixture) dissolved in 100 mM phosphate buffer pH 7.0. Analytical column of 19 μl volume filled with immobilised HRP onto CPG beads was placed between the junction of the two FIA lines and reaction coil (Fig. 3). Sample injection (25 μl) was performed with an Anachem (Luton, UK) valve switching module fitted with a low pressure Rheodine injection valve, controlled by an Omnifit (Cambridge, UK) universal-switching module. Absorbance was measured with a Jencons (Leighton Buzzard, UK) spectrophotometer with a 490 nm light filter, using an 18 μl glass flow cell with a 10 mm path length from Hellma (Müllheim, Germany), and the output was recorded on a RDS-200-020K Gallenkamp (Loughborough, UK) data trace flat-bed recorder. Omnifit supplied Teflon tubing, with an i.d. of 0.8 mm. Connectors were also obtained from Omnifit and the connections were made using 1/4 and 28 thread low-pressure HPLC unions, nuts and ferules from Upchurch (Oak Harbour, USA).

3. Results and discussion

3.1. PSA/4-AAP colorimetric system optimisation

3.1.1. Influence of the reagents (PSA/4-AAP) concentration

Reactional mixture consisting of 0.4 mM 4-AAP, 0.22 mM H_2O_2 and varying concentrations of PSA in the presence of 4 mg l^{-1} HRP attains maximum absorbance 1–2 min after preparation (not shown). The dependence of the attained colour intensity and reaction rate on the PSA concentration was examined for PSA concentrations between 5.0 and 85.0 mM (Fig. 4). Two different regions of the absorbance curve can be distinguished: for low PSA concentrations the slope is much steeper than in the higher concentrations range. In the steeper region coloured product formation could be limited by quantity of available PSA and therefore directly dependent on the PSA concentration. Once the critical PSA concentration is reached it should cease to be the limiting factor for the coloured product formation and absorbance would be constant. However, there is a slight positive effect on the colour attenuation. This could be explained by the protective effect that reducing agent (PSA) has on

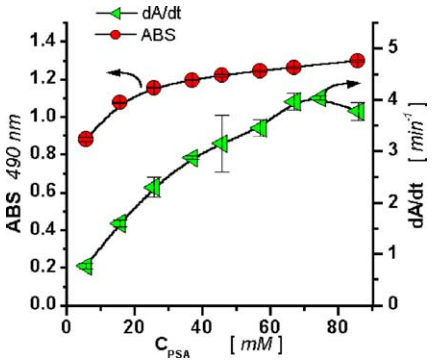

Fig. 4. PSA concentration dependence of the initial reaction rates and attained colour for the system containing 4.0 mg l^{-1} HRP, 0.4 mM 4-AAP and 0.22 mM H_2O_2.

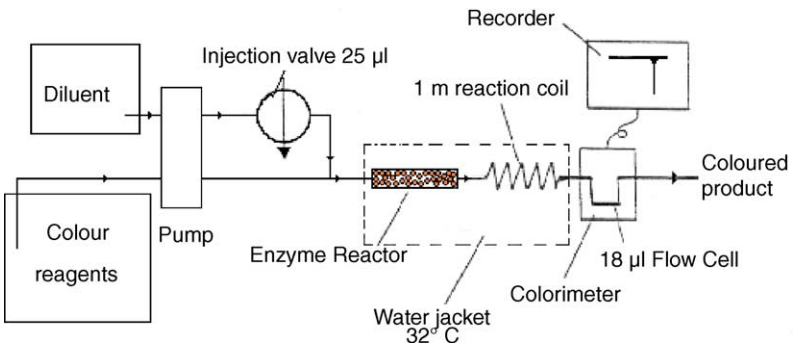

Fig. 3. FIA set-up.

HRP [14,16] and by competition between 4-AAP and PSA in reaction with HRP compound I [11,15]. The higher the concentration of phenolic reducing compound the slower the terminal inactivation of HRP and more of the hydrogen peroxide is involved in dye production. On the other hand, increasing PSA/4-AAP ratio in reactional mixture reduces the probability of direct oxidation of 4-AAP by oxidised forms of HRP (compounds I and II), which in turn reduces the concentration of aminyl radicals and the probability of their dimerisation. The predominant radical species in the mixture, tend to undergo radical transfer with amine compound due to high 4-AAP concentration relatively to phenoxy radicals [11]. Formed aminyl radicals then react with phenoxy radical rather than with another aminyl radical due to the relatively high phenoxy radical concentration. Therefore, increasing the concentration of phenolic compound enhances the dye production reactional pathway.

Between 0 and 40 mM PSA the reaction rate is linearly dependent on PSA concentration. It was also verified that the presence of PSA does not affect the stability of HRP activity for more than 90 h, even for the highest concentrations tested (data not shown). Concentration of PSA that was selected as optimal for future experiments was the smallest concentration that still provides satisfactory final colour development and good colour formation rate.

A similar study of the influence of 4-AAP concentration on colour formation was performed. The reactional mixture consisted of 25 mM PSA, 0.22 mM H_2O_2, and 0.4 and 4.0 mg l^{-1} HRP (Fig. 5a and b, respectively). It is shown that the presence of 4-AAP does not affect HRP activity for the examined concentrations of 4-AAP and for both concentrations of the enzyme. The low HRP concentration experiment (Fig. 5a) shows that after a steep region of dependence 4-AAP ceases to be the limiting factor for intensity of attained colour and reaches plateaux of maxima between 0.3 and 0.5 mM of 4-AAP. For the high 4-AAP concentrations there is a notable decay in the intensity of the attained colour. For high enzyme concentration (Fig. 5b), the decay occurs for lower 4-AAP concentrations and the maximum is far narrower. The decay in attained colour for higher 4-AAP concentrations could be due to competition between PSA and 4-AAP in reaction with the oxidised enzyme.

Concentrations of 0.4 mM 4-AAP and 25 mM PSA have been selected as standard reaction mixture. As both reaction rate and attained colour show low dependence on concentration for this particular reaction mixture composition, small changes in concentration of PSA and 4-AAP between experiments will produce small error of measurement. Absorbance at 490 nm of this standard reaction mixture when kept for 24 h in a dark bottle increases for a value of only 0.025 absorbance units.

3.1.2. Influence of the buffer solutions and pH

Variation in colour attenuation of the colorimetric system was very small between pH 6.0 and 8.5 (0.27 mM H_2O_2). The pH dependence of HRP activity in PSA/4-AAP colorimetric system is bell-shaped with maximum that is much wider than maxima for the pyrogallol and guaiacol methods (Fig. 6). This fact makes the 4-AAP/PSA system interesting for investigation of combined multi-enzyme assays with oxidases that require a narrower pH range for their optimum activity, as the PSA/4-AAP colorimetric system is functional in a wider range of pH values.

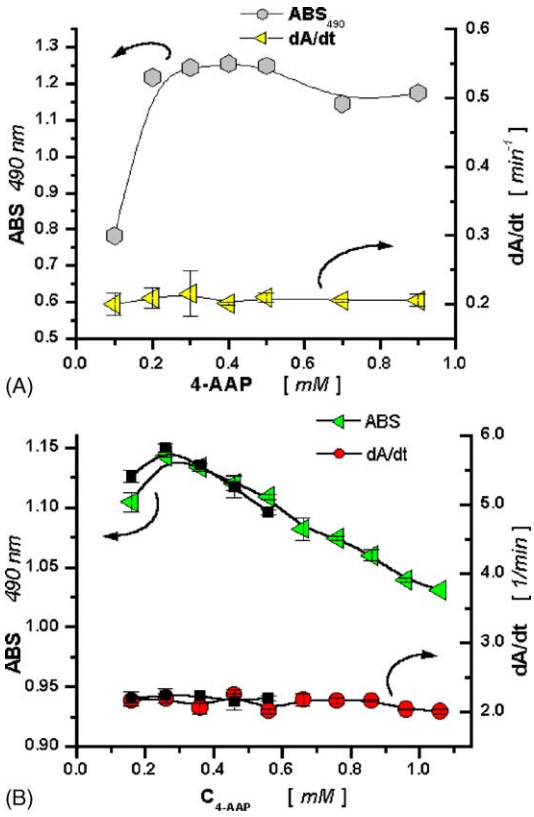

Fig. 5. Dependence of attained colour intensity and rate of colour production for different concentrations of 4-AAP for peroxidase concentration of (a) 0.4 mg l^{-1} and (b) 4.0 mg l^{-1} in 100 mM Na phosphate buffer pH 7.0, in the presence of 25 mM PSA and 0.22 mM H_2O_2.

3.1.3. Influence of the additives

Some compounds of the fermentation media may affect detection of H_2O_2. This is important for application of the

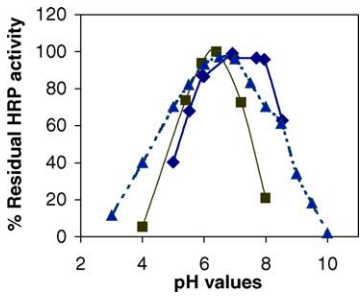

Fig. 6. Comparison of pH dependence of the HRP activity in systems with different reducing agents: (■) pyrogallol, (◆) 4-AAP, (▲) guaiacol. Buffer solutions were: acetate buffer (pH 5.0, 6.0), phosphate buffer (pH 5.5, 5.9, 6.9 and 7.0), Tris–phosphate buffer (7.7, 8.0 and 8.5).

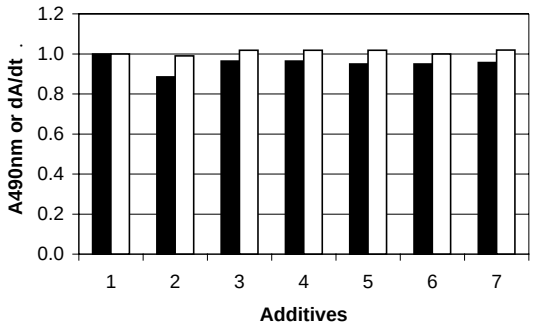

Fig. 7. Interference of some additives to HRP activity (□) and colour attenuation (■) determined by PSA/4-AAP method. (1) no additives, (2) yeast extract 25 g l^{-1}, (3) D-galactose 10.2 g l^{-1}, (4) glucose 10.0 g l^{-1}, (5) succinate 0.5 g l^{-1}, (6) glycerol 0.5 g l^{-1}, (7) acetate 7.2 g l^{-1}.

method for fermentation broth analyses. These compounds may inhibit the enzyme activity, consume H_2O_2, react with oxidised forms of HRP or react with intermediary free radicals. Interference of some of the compounds commonly found in fermentation broths was quantified by comparing colour attenuation and reaction rates for 0.18 mM H_2O_2 solutions in pure water and in solutions of additives.

Succinate, glycerol, potassium acetate, glucose and galactose (0.5, 0.5, 7.2, 10.0, 10.2 g l^{-1}, respectively) did not interfere significantly with the hydrogen peroxide determination (difference less than 5%). When present in high concentrations yeast extract interferes with the colour attenuation: in the presence of 25, 10 and 5 g l^{-1} YE final absorbance difference was 14, 9 and 4%, respectively. No significant interference with the reaction rate (i.e., HRP activity) was observed for any of the compounds (Fig. 7).

3.1.4. H_2O_2 assay in a spectrophotometric cell

Hydrogen peroxide assay in a spectrophotometric cell was performed as described in Section 2. The calibration curve was obtained after measuring absorbances at 490 nm of solutions of the standard reactional mixture in the presence of 0.4 mg l^{-1} HRP after 15 min of incubation with hydrogen peroxide solution. It had a very good linearity ($R^2 = 0.999$) up to 0.21 mM H_2O_2, with lower detection limit of 6 μM.

Table 1
Calculated apparent molar absorptivities relative to H_2O_2 concentration for different reactional systems

Compound	ε (mol^{-1} dm^3 cm^{-1})	R^2
Phenol	5,540 ± 20	0.995
p-Cl-phenol	10,600 ± 60	1.000
p-Cresol	–	–
PSA	5,560 ± 70	0.999

3.2. Comparison of different reducing agents

Reaction rates in colorimetric systems containing phenol, *p*-Cl-phenol, *p*-cresol and PSA were compared. Reactional mixtures contained 0.4 mM 4-AAP, 0.4 mg l^{-1} HRP and 25 mM phenolic compound. All assays were performed as described previously.

Molar absorptivity of the co-oxidation reaction product was determined from linear fits of the attained absorbances at 490 nm (ε) against H_2O_2 concentration plots as it was assumed that all the added hydrogen peroxide stochiometrically reacts to produce the dye. A very good linearity was obtained for up to 0.24, 0.14 and 0.21 mM of hydrogen peroxide added to the standard assay reaction mixtures containing phenol, *p*-Cl-phenol and PSA, respectively. Results are presented in Table 1. *p*-Cresol does not react with 4-AAP to produce the coloured compound. Adding hydrogen peroxide to the reactional mixture with *p*-cresol results in development of turbidity and the solution turns yellow. The different ε values for *p*-Cl-phenol and phenol and PSA

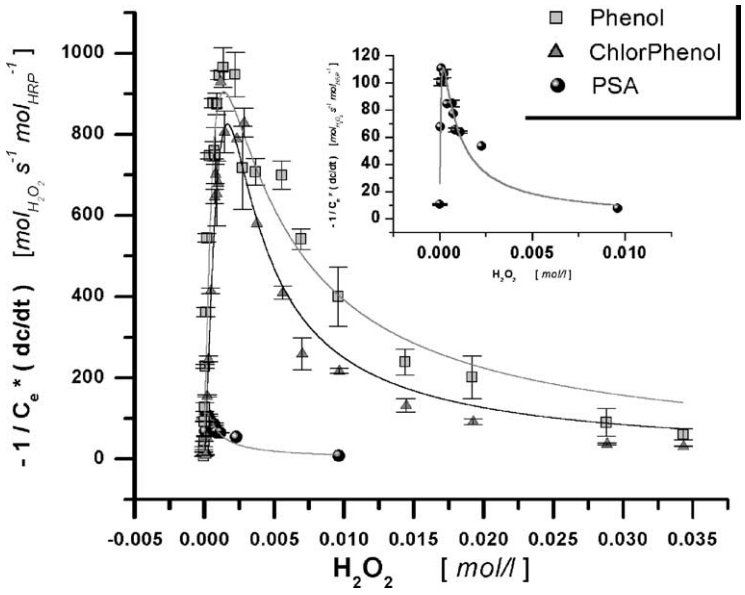

Fig. 8. HRP kinetics for different reducing agents: (■) phenol, (▲) *p*-Cl-phenol, (●) PSA.

imply different reaction mechanisms. It appears that the hydrogen peroxide consumption per mole of produced dye for *p*-Cl-phenol is half of that for PSA and phenol, as reported by Trinder and Webster [6].

Reactional mixtures containing phenol, *p*-cresol and *p*-Cl-phenol produce precipitate for hydrogen peroxide concentrations higher than a certain value. It is estimated that turbidity can be detected (at 640 nm) for the H_2O_2 concentrations higher than 0.6 mM for *p*-Cl-phenol, and 1.1 mM for phenol which are close to the concentrations stochiometrically equivalent to 4-AAP (0.4 mM in the *p*-Cl-phenol system, 0.8 mM in the phenol system), considering that a certain amount of hydrogen peroxide could be spent in side reactions with other compounds present in the solution. This could mean that as the 4-AAP is consumed, phenolic compounds start to be oxidised to produce oligo- and polymeric species with limited water solubility.

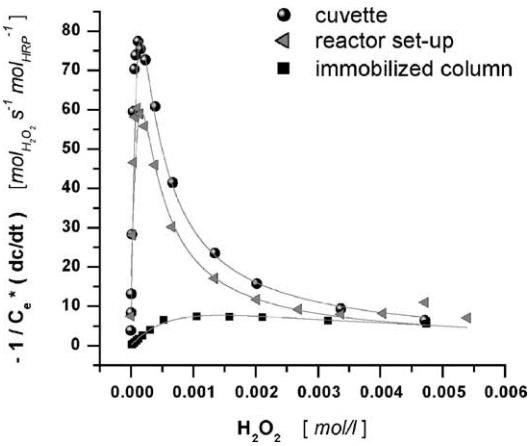

Fig. 9. HRP kinetics for different experimental set-ups in a PSA system: (■) immobilised HRP, (◀) reactor set-up, (■) cuvette set-up. Total enzyme concentration in the experiments with dissolved HRP was 0.4 mg l^{-1} and in the case of immobilised HRP it was 13.2 mg l^{-1}.

3.3. Kinetics of soluble and immobilised HRP

Kinetics experiments results for colorimetric systems with different reducing agents are presented in Fig. 8. The reaction rates for the phenol and *p*-Cl-phenol systems are about 8–10 times higher than that of PSA system. This difference cannot be explained by the systematic error caused by occurrence of turbidity in phenol and *p*-Cl-phenol systems, as the absorbance increase due to the turbidity is delayed to the initial reaction phase in which quinone–imine dye is produced. Apart from that a difference in calculated reaction rates exists even for the small peroxide concentrations for which no turbidity is detected.

Kinetics of the co-oxidation in the PSA/4-AAP system catalysed by HRP immobilised on CPG beads was determined in an experiment with an external reactor system depicted on Fig. 1. Results were compared to the kinetics of the PSA/4-AAP system in solution measured in the same external reactor set-up (in which case the column is omitted) and in a spectrophotometric cell (Fig. 9).

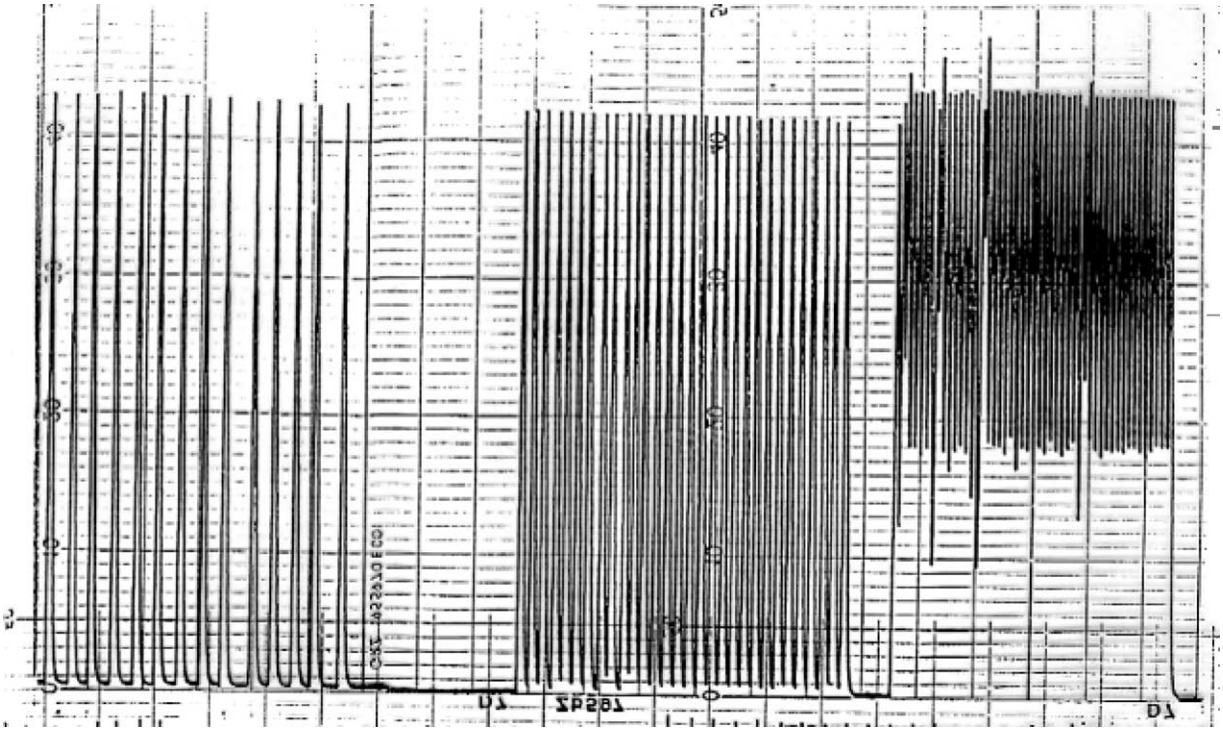

Fig. 10. FIA system response to H_2O_2 injections of 25 μl and injection intervals of 2, 1 and 0.5 min.

Fig. 9 shows that there is a certain difference in reaction rates measured in cuvette and batch reactor system for dissolved enzyme probably due to the time delay in batch set-up. There is a notable difference in reaction rates for dissolved and immobilised HRP due to the loss of activity during enzyme immobilisation that has been previously quantified to about 93% loss.

3.4. H_2O_2 assay in FIA system

The calibration of the FIA system was performed by injecting 25 μl hydrogen peroxide standards. Enzymatic column was submersed in a water bath at 32 °C. The paper charter scale was defined by 0.5 absorbance units per 100% paper chart scale and the peak height was measured. The measuring system gave linear response to H_2O_2 concentrations up to 8.8 mM H_2O_2. Lower detection limit was 0.3 mM. The loading of the standards from the injection pool into the FIA system occurred during 15 s, and the best peak definition was obtained for 2 min injection intervals. However, system can accurately analyse about 100 samples per hour (Fig. 10).

The stability of the base line was confirmed during continuous absorbance reading for 24 h and simultaneously by measuring the flow rates of diluent, colour reagent and joint line.

4. Conclusion

The optimised colorimetric system based on the HRP catalysis co-oxidation of PSA and 4-AAP for the determination of hydrogen peroxide concentration, HRP activity, and for employment in FIA systems consists of 25 mM PSA and 0.4 mM 4-AAP in 100 mM phosphate buffer pH 7.

Presence of succinate, glycerol, potassium acetate and galactose, which are all commonly present in fermentation broths, does not interfere neither with the colour production rate nor the attained colour for the tested concentrations, which allows for the colorimetric system to be used in complex fermentation media. Yeast extract interferes significantly with the colour production of the colorimetric system when present in concentrations higher than $5\,g\,l^{-1}$.

The colorimetric system that contains *p*-chloro-phenol is about twice as sensitive to hydrogen peroxide concentration as colorimetric system containing phenol or PSA (ε for *p*-Cl-phenol is twice higher). Phenol and *p*-chloro-phenol reaction mixtures produce precipitates in reaction with H_2O_2.

Reaction rates for the soluble HRP measured in an external reactor set-up are somewhat smaller than the rates measured directly in a spectrophotometric cell. Immobilised reactor peroxidatic activity per unit of concentration of present enzyme is about 10% of the activity of dissolved enzyme.

The optimised analytical colorimetric system containing PSA has been successfully employed in a flow injection system, with linear response up to 8.8 mM H_2O_2. The FIA response delay was about 15 s, allowing up to 100 sample injections per hour.

Acknowledgements

V. Vojinović and A.M. Azevedo gratefully acknowledge the Ph.D. fellowships (Praxis XXI SFRH/BD/5495/2001 and Praxis XXI BD/18216/98, respectively), and L.P. Fonseca acknowledges post-doctoral fellowship (Praxis XXI BPD/16347/98), of the Ministério da Ciência e Tecnologia, Portugal. L.P. Fonseca also wishes to thank the Enzyme Biotechnology Group of the School of Biochemistry and Molecular Biology, University of Leeds, UK, for the support during his sabbatical. This work has been performed within EU project BIO4CT972199.

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