General Fluorimetric Flow-through Sensor for the Determination of Oxidase Substrates

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A new flow-through sensor for the determination of hydrogen peroxide based on the dual immobilisation of the catalyst and the reaction product (permanent immobilisation in a reactor and transient retention in the flow-cell, respectively) is proposed. The support packed in the flow-cell is an aminopropyl-bonded silica gel non-fluorescent material. The approach provides a linear range between 0.03–17 ng ml^{-1} H_2O_2 (r^2 = 0.9954, RSD values less than 3.5%). This sensor is 80 times more sensitive than a photometric flow-through biosensor previously reported and based on similar principles.

Flow-through optical biosensors have been demonstrated to be effective and reliable systems for increasing sensitivity on the determination of substrates involved in an enzymatic reaction catalysed by oxidases.[1,2] Its application in fluorimetric detection has been limited by the native fluorescence common to a series of available supports. The use of supports either which exhibit low or residual fluorescence and those capable of retaining the product of a given enzymatic reaction have been investigated in this research. A new flow-through optical sensor for the determination of oxidase substrates based on fluorimetric detection is presented.

The method is based on the catalytic reaction between 4-hydroxyphenylacetic acid (4-HPAA) and hydrogen peroxide, in the presence of peroxidase (POD), to yield a dimer which exhibits high fluorescence, according to the following reaction:

$$\text{4-HPAA} + H_2O_2 \xrightarrow{\text{POD}} (\text{4-HPAA})_2 + H_2O$$

The product formed in the condensation reaction is retained on an aminopropyl–silica sorbent. The retention process is monitored fluorimetrically at λ_{ex} = 315 and λ_{em} = 410 nm, at which the condensation product is highly fluorescent.The product was removed from the sorbent by chloride present in the eluent.

Experimental

Instrument and Apparatus

A Kontron spectrofluorimeter furnished with an 18 µl flow-cell and equipped with a Knauer recorder was used. A Gilson Minipuls-2, four-channel peristaltic pump with a rate selector, two Reodyne 5041 injection valves (one of them modified to act as a switching valve), and Teflon tubing of 0.5 mm id were also used. A PC system equipped with a DAS-8PGA interface (Metrabyte) was employed for acquisition and processing of the relative fluorescence intensity–time data.

Reagents

The buffer used was an aqueous solution containing 10 mmol l^{-1} ammonium chloride (Merck no. 1145; Merck, Darmstadt, Germany) adjusted to pH 8.0 with 1 mmol l^{-1} ammonium hydroxide (Merck no. 5432). A solution containing 0.15 mmol l^{-1} 4-hydroxyphenylacetic acid (Sigma no. H-4377; Sigma–Aldrich, Madrid, Spain) was prepared in the buffer solution. The eluent was an aqueous solution containing 0.1 mol l^{-1} sodium chloride and 0.1 mol l^{-1} hydrochloric acid. A stock solution containing 10 000 U of peroxidase (donor : hydrogen peroxide oxidoreductase; E.C. 1.11.1.7) Type VI from horseradish (Sigma P-8375) was prepared by dissolving the contents of a vial in 2 ml of 0.1 mol l^{-1} potassium dihydrogenphosphate (Merck no. 4871) buffer adjusted to pH 7.0 with 0.1 mol l^{-1} sodium hydroxide (Merck no. 6498). The sorbent was aminopropyl–silica, 40 µm particle size, 60 Å porosity bonded silica gel (Analytichem, Harbor City, CA, USA, no. 1210-2041). All solutions were prepared using doubly distilled water of high purity obtained from a Millipore Milli-Q Plus system.

Enzyme Immobilisation

Peroxidase was immobilised on controlled-pore glass, CPG-75 200 (from Sigma) using the glutaraldehyde–bovine serum albumin method (GA–BSA) proposed for protein immobilisation such as enzymes and antibodies.[3] Teflon tubing of different lengths and 0.4 mm id was then packed with the support-peroxidase conjugated and stored in buffer solution at 4 °C. Under these conditions the enzyme activity remains constant for at least 2 months.

Sorbent Conditioning

The sorbent was washed with water and conditioned with 2 mol l^{-1} sodium chloride, 2 mmol l^{-1} hydrochloric acid and water, which converted it into the chloride form.[4]

Manifold and Procedure

The continuous approach, which includes the flow-through sensor, was built as a modification of a manifold previously proposed by the authors.[1] The main modification was the location of a POD reactor prior to the switching valve instead of in the flow-cell. This change prevented biocatalyst inactivation by the eluent (a concentrated solution of strong acid). Fig. 1 depicts the manifold used. The sample was injected *via* IV into a buffer stream, which merged at point ‘a’ with the substrate solution and passed through the POD reactor, where the enzymatic reaction took place. The reaction plug was driven to the flow-cell where the condensation product was transiently retained. A rising curve was obtained as a result of retention of the fluorescent product. A constant fluorescence intensity was reached when the whole injected plug passed through the optical path. As the selecting valve, SV, was switched, the eluent was allowed to reach the flow-cell and the product was removed, so that the support was regenerated.

Results and Discussion

The chemical, physical and flow-injection (FI) variables affecting the method were optimised using the univariate method and aqueous standard solutions of hydrogen peroxide. The range within the variables was optimised and the optimum values found are shown in Table 1.

Preliminary experiments were carried out in order to select the best sorbent, which should be endowed with two essential features: firstly, low native fluorescence, thus making the sensor highly compatible with fluorescent species retained on it; secondly, the support should be an effective and selective sorbent for retention of the fluorescent dimer in the presence of other reaction ingredients. With these features in mind, different supports were investigated. Sorbents based on divinylbenzene (DVB), such as Dowex and Amberlite, exhibited high emission under the experimental wavelengths, so they were of no use. Sorbents based on hydrocarbon structure, such as Sephadex, Sephacel, cellulose or dextran, were rejected due to their compactness, which gave rise to shortcomings owing to over-pressure in the flow-cell. An aminopropyl-bonded silica gel from commercial solid-phase extraction cartridges was chosen as being optimal. This sorbent exhibits both relatively low native fluorescence at the experimental wavelengths and strong retention of the reaction product as the NH_2-group endows the sorbent two main properties: polar interactions *via* hydrogen bonding and anion-exchanger capability (pK_a value lower than 9.8).

After selecting the sorbent material, the height of its packing in the flow-cell was optimized. A comparison of the fluorescent signal obtained by the same concentration of hydrogen peroxide in the presence and absence of the packed material in the flow-cell showed an improvement of the sensitivity of 20 times, which clearly demonstrates the *in situ* concentration step occurring at the detection point and justified the use of integrated retention–detection.

A 10 mmol l^{-1} ammonium chloride solution, adjusted to pH 8.0 with 1 mmol l^{-1} ammonium hydroxide, provided the best conditions for both development of the catalysed reaction and strong sorbent retention. A 0.15 mmol l^{-1} 4-hydroxyphenyl acetic acid solution was selected as being optimal. A higher concentration of this substrate promoted the uncatalysed reaction, which resulted in a sustained baseline rise due to slow continuous retention of the reaction product. On the other hand, lower concentration resulted in poor development of the enzymatic reaction. A 0.1 mol l^{-1} sodium chloride and 0.1 mol l^{-1} hydrochloric acid solution was appropriate for removal of the retained product from the sensor.

Temperatures in the range between 20–45 °C had no effect on the analytical signal, so room temperature was used.

The flow rate through the system dramatically influenced the performance of the continuous manifold. The enzyme–substrate contact and the retention equilibrium were strongly dependent on this dynamic variable. A flow rate of 0.8 ml min^{-1} was selected as optimal. Lower values had no significant influence on the signal, whereas higher flow rates resulted in poor development of both the reaction and retention steps. The injection of sample volumes over 2 ml did not increase the analytical signal, probably because the sorbent was already saturated with the condensation product. Time intervals between injection and the switching of valve SV for an elution longer than 250 s did not increase the analytical signal; shorter times decreased the signal, thus providing a way to expand the determination range of the analyte.

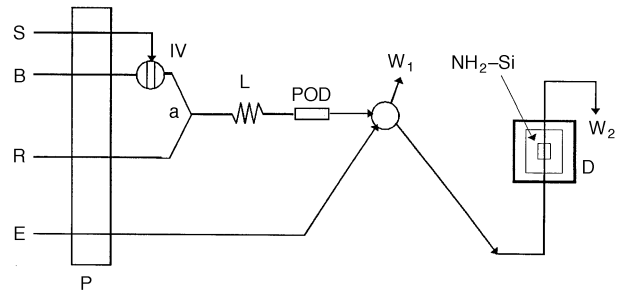

Fig. 1 Flow-through sensor integrated in a continuous manifold for the determination of hydrogen peroxide: S, denotes sample; B, buffer solution; R, substrate solution; E, eluent; P, peristaltic pump; IV, injection valve; L, reactor; POD, enzymatic reactor with immobilised peroxidase; SV, selecting valve; D, fluorimetric detector; W_1 and W_2, waste; NH_2–Si, solid support packed in the flow-cell for retention of the reaction product.

Table 1 Optimisation of variables influencing the method

Variable	Range studied	Optimum value
Physical—		
Temperature/°C	20–40	25
Chemical—		
[NH_4OH–NH_4Cl]/mmol l^{-1}	5–500	10
pH	6.0–10.0	8.0
[4HPAA]*/mmol l^{-1}	0.05–2.0	0.15
[NaCl–HCl]/mmol l^{-1}	0.1–200	100
Flow injection—		
Flow rate/ml min^{-1}	0.2–1.5	0.8
Injection volume/μl	50–3000	2000
IMER† length/cm	0.2–2	0.5
Switching time/s	60–300	250

* 4HPAA denotes 2-hydroxyphenylacetic acid. † IMER, immobilised enzymatic reactor.

Features of the Method

The calibration graph was run using the optimal values of variables stated in the previous section. Standard solutions of hydrogen peroxide (standardised by titration with potassium permanganate) at concentrations between 0.001 and 100 ng ml^{-1} were injected in triplicate into the dynamic manifold. Table 2 summarises the figures of merit. As can be seen, the method shows a linear range between 0.034 and 17 ng ml^{-1}, with a regression coefficient of 0.9954. The precision, expressed as RSD and calculated from 11 solutions of hydrogen peroxide injected in triplicate, was more than acceptable.

The relatively high value of the intercept of the calibration curve is the result of two effects: native fluorescence and light scattering of the support packed in the flow-cell.

Comparison of the Proposed Flow-through Fluorimetric Sensor With a Previous Flow-through Photometric Biosensor

The results achieved by the proposed dynamic–manifold flow-through fluorimetric sensor were compared with those obtained with a flow-through photometric biosensor previously reported by the authors.[1] With this aim in view, Table 2 includes the figures of merit of the methods for hydrogen peroxide developed by the two dynamic assemblies. As can be seen, the fluorimetric sensor is about 80 times more sensitive than the photometric biosensor. In addition, fluorimetric detection is usually more selective than photometric detection. The precision, expressed as per cent. of relative standard deviation, and the sampling frequency achieved were similar in both instances. The chemistry involved in the fluorescent reaction is simpler than that of the photometric reaction.

Table 2 Comparison of methods based on flow-through sensors: use of the proposed fluorimetric sensor *versus* a photometric biosensor

	Fluorimetric flow-through sensor	Photometric flow-through biosensor[1]
Equation*	$I = 19.284\ (\pm 1.269) + 7.547\ (\pm 0.180) \times C$	$A = 0.003\ (\pm 0.001) + 0.043\ (\pm 0.001) \times C$
r^2	0.9954	0.9945
Linear range/ng ml^{-1}	0.034–17	3–200
Sampling frequency/h^{-1}	16	16
RSD (%)†	3.6	2.5

* *I* denotes fluorescence intensity, *A*, absorbance, *C* concentration of H_2O_2 in ng ml^{-1}. † For hydrogen peroxide, 0.2 and 10 ng ml^{-1} for fluorimetric and photometric determination.

Conclusions

The most outstanding features of the proposed flow-through sensor are: its potential for the implementation of a number of methods for the determination of oxidase substrates from different areas (namely, phosphate, sulfite, glucose, cholesterol, *etc*.) based on fluorimetric detection; the enhancement in sensitivity with respect to other previous designs of the authors based on photometric detection; and the improvement in selectivity involved in fluorimetric rather than photometric detection, which will be a key aspect when the sensor is applied to natural samples. The change required in the assembly in order to develop methods for this purpose is only the inclusion of the target oxidase reactor in the dynamic manifold prior to the peroxidase reactor in order to yield the H_2O_2 on which the latter biocatalyst acts. The wide linear interval of the calibration curve enables the reduction of the number of standards and avoidance of sample dilution. Also the low determination limit achieved makes possible a dramatic dilution of the target samples, thus lowering the potential interferents.

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