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Electrochemical detection of transketolase activity using a tyrosinase biosensor

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ABSTRACT

This paper proposes a new concept of transketolase (TK) activity profiling. A tyrosinase (PPO) biosensor, based on the immobilization of this enzyme in a Mg_2Al–Cl layered double hydroxide, was developed for the amperometric detection of N-acetyl-L-tyrosine ethyl ester monohydrate (N-Ac-Tyr-OEt) at −0.2 V. This compound was released during an enzymatic reaction catalyzed by TK with N-acetyl-O-(2*R*, 3*S*, 5-trihydroxy-4-oxopentyl)-L-tyrosine ethyl ester used as donor substrate. This tyrosinase biosensor was optimized for the detection of TK activity, including PPO optimum substrate concentration, electrolyte nature, pH, and influence of bovine serum albumin (BSA). It was found that N-Ac-Tyr-OEt release is dependent on TK concentration (U/mL) in the electrolyte medium. These results demonstrate the sensitivity and specificity of the tyrosinase biosensor designed for *in vitro* detection of TK activity, which is known to be involved in several diseases.

1. Introduction

Transketolase (TK, EC 2.2.1.1.) is an intracellular enzyme that is essential in the metabolism of all living cells. It is implicated in the pentose phosphate pathway. TK reversibly transfers the C1–C2 ketol unit from a donor ketose phosphate (D-xylulose-5-phosphate) to an acceptor aldose phosphate (D-erythrose-4-phosphate or D-ribose-5-phosphate) to generate a ketose phosphate (D-fructose-6-phosphate or D-sedoheptulose-7-phosphate) with a new asymmetric center in the (*S*) configuration. The mechanism of reaction of TK is mediated by its cofactor thiamine pyrophosphate (TPP) which is coordinated to a divalent cation (Mg^{2+}).

A large body of research has focused on the substrate specificity and structure of TK from yeast (*Saccharomyces cerevisiae*) (Lindqvist et al., 1992; Nikkola et al., 1994; Wikner et al., 1997) and *Escherichia coli* (Hibbert et al., 2007). These enzymes appear highly specific for ketol donor substrates but accept a wide range of acceptor aldehydes showing enantioselective properties towards hydroxyaldehyde substrates with the (*R*) configuration. The fact that TK specifically catalyzes the irreversible transfer of the ketol unit, when α-hydroxypyruvic acid is used as donor substrate makes it an ideal tool for generating D-*threo*(3*S*, 4*R*)ketoses (Charmantray et al., 2006; Hecquet et al., 1994; Kobori et al., 1991; Zimmermann et al., 1999).

In cell metabolism, TK is the most critical enzyme of the non-oxidative branch of the pentose phosphate pathway for nucleic acid synthesis. Numerous studies have proposed TK as an investigation, prevention and therapy tool for cancer, neurodegenerative diseases and diabetes (Zhao and Zhong, 2009). Since activation of the pentose phosphate pathway for D-ribose-5-phosphate synthesis has been demonstrated in tumor cells, TK has been suggested as a target for inhibition in the treatment of cancer (Comin-Anduix et al., 2001; Du et al., 2004). The crystal structure of human TK is unknown but homology modelling of human TK has been performed using the crystal structure of yeast as template in order to conduct research into structure-based drug design (Obiol-Pardo and Rubio-Martinez, 2009), leading to a new class of thiamine analogs (Le Huerou et al., 2008; Thomas et al., 2008a,b). TK enzyme variants and reduced TK activities are present in patients with the neurodegenerative disease Wernicke–Korsakoff syndrome (McCool et al., 1993). In Alzheimer's disease patients, TK variants with different isoelectric points or proteolytic cleavage lead to small TK protein isoforms (Zhao and Zhong, 2009). Reduced TK activities have been reported in diabetes mellitus patients (Thornalley et al., 2001).

In this context, simple and cost-effective methods can enable TK activity profiling for diagnostic applications. The conventional method for measuring TK activity uses D-xylulose-5-phosphate as donor substrate and D-ribose-5-phosphate as acceptor substrate. However, there is a need for new TK assays since D-xylulose-5-

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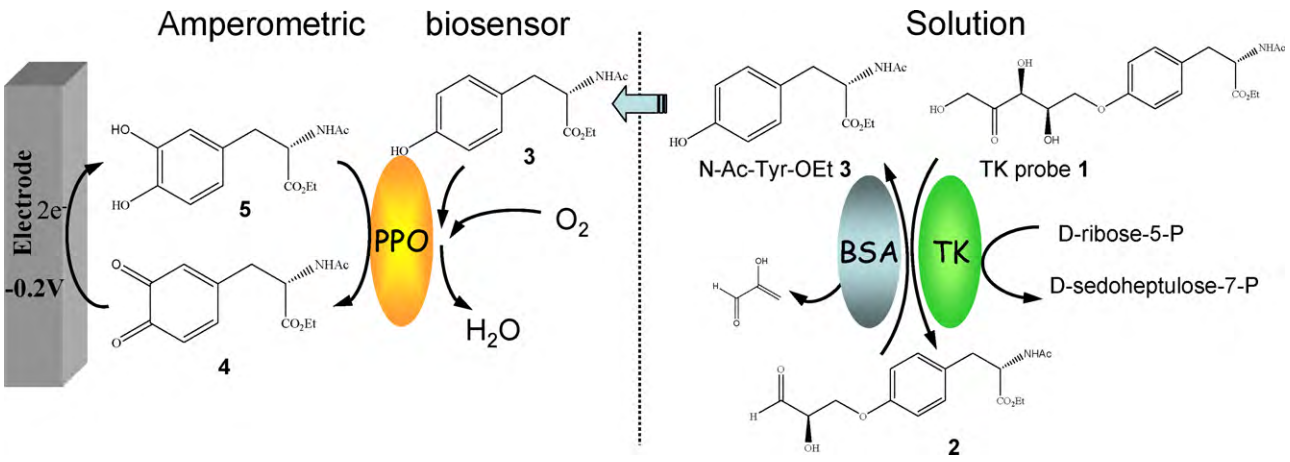


Scheme 1. Biosensing principle of TK activity.

phosphate is no longer commercially available and is difficult to chemically synthesize. There are effective methods for assaying TK using non-natural donor substrates based on spectrophotometric (Hecquet et al., 1993; Lee et al., 2008; Naula et al., 2008), colorimetric (Smith et al., 2006), and fluorimetric detection (Sevestre et al., 2006).

In this work, a versatile electrochemical biosensor is developed for monitoring TK activity in buffered solution (Scheme 1). We recently showed (Charmantray et al., 2009) that TK is able to cleave probe **1** releasing compound **2** which underwent a β-elimination reaction leading to N-acetyl-L-tyrosine ethyl ester monohydrate (N-Ac-Tyr-OEt) **3**. In the presence of bovine serum albumin (BSA) the velocity of the leaving group release was increased. Indeed, BSA was able (through lysine residues of this protein) to promote β-elimination of phenol derivatives leaving groups such as umbelliferone from appropriated compounds (Klein and Reymond, 1998; Sevestre et al., 2006).

When directly carried out in an electrolyte medium, compound **3** can be amperometrically detected using a tyrosinase (PPO, EC 1.14.18.1., polyphenol oxidase) biosensor (Scheme 1). Compound **3** is oxidized by PPO to compound **4** which undergoes a cathodic reduction to compound **5**, thus linking TK activity to an electric current.

This biosensor is based on the immobilization of PPO within layered double hydroxides (LDH) which are known to be efficient immobilization matrices for PPO (Shan et al., 2003). Layered double hydroxides (LDH) are synthetic solids with positively charged brucite-like layers of mixed metal hydroxides separated by interlayered hydrated anions, defined by the general formula $[M_{1-x}^{2+}M_x^{3+}(OH)_2]^{x+}$ $[(A^{n-})_{x/n}, yH_2O]$ (abbreviated as $M^{2+}_{(1-x/x)}M^{3+}$-A). These materials have recently been used in biosensor development as immobilization matrices for enzymes (Mousty, 2010). In the present work, we used Mg_2Al–Cl LDH. The metal cations in the LDH layers are known to have a buffer effect. The basic strength of MgAl LDH could contribute to the alkaline catalysis of β-elimination on compound **2** which is usually performed by BSA (Geraud et al., 2008). We optimized this tyrosinase biosensor for the detection of TK activity by varying several parameters, including PPO substrate quantity, electrolyte nature, pH, and study of BSA influence.

2. Materials and methods

2.1. Chemicals and reagents

Polyphenol oxidase (PPO, EC 1.14.18.1., from mushroom 5370 U mg^{-1}), 3,4-dihydroxyphenylalanine (L-DOPA), L-tyrosine, N-Acetyl-L-tyrosine ethyl ester monohydrate (N-Ac-Tyr-OEt, **3**.), bovine serum albumin (BSA), D-ribose-5-phosphate (D-ribose-5-P), glutaraldehyde solution 25% in water, 3-(N-morpholino)propanesulfonic acid (MOPS), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium phosphate monobasic and dibasic were purchased from Sigma. Thiamine pyrophosphate (TPP) was obtained from Fluka and $MgCl_2$ was obtained from Across.

2.1.1. Synthesis and stability of TK probe 1

The chemo-enzymatic synthesis of N-acetyl-O-(2*R*, 3*S*, 5-trihydroxy-4-oxopentyl)-L-tyrosine ethyl ester **1** was previously described by our group (Sevestre et al., 2006). Stock solution of TK probe **1** was prepared in ethanol (0.01 M). Sample purity and stability in time were tested in cyclic voltammetry, and no signal corresponding to N-Ac-Tyr-OEt **3** was detected. TK probe **1** is not electroactive in the potential range 1 to −0.5 V/Ag–AgCl and there is no spontaneous decomposition of this sample.

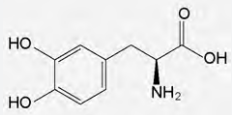
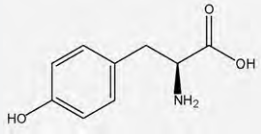
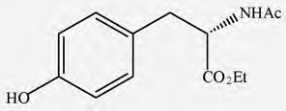
2.1.2. Production of yeast TK

TK from *S. cerevisiae* was produced from *S. cerevisiae* strain H402 × pTKL1 (donated by Pr. Gunter Schneider, Sweden). Yeast culture was prepared according to Wikner et al. (1997). TK yeast extraction was conducted from the packed cells. 10 g of cells was resuspended in 100 mL of Tris buffer (0.1 M) at pH 7.4 and placed in a one shot cell disrupter at 2 kBar. The solution was centrifuged at 15,000 rpm for 20 min. The crude TK extract has an activity of 18.7 U/mL and a specific activity of 2.6 U mg^{-1}. For analytical purposes, the crude TK extract was filtered on Microcon® centrifugal filter devices (Molecular weight cut-off 30 kDa), so as to recover the pass-through devoid of TK (see Section 3.2: detection of TK activity).

2.1.3. Synthesis of Mg_2Al–Cl LDH

$Mg_2Al(OH)_6Cl{\cdot}nH_2O$ (Mg_2Al–Cl) LDH was prepared by the coprecipitation method following the procedure published by Rives (2001). Typically, a mixed aqueous solution of $MgCl_2$ and $AlCl_3$, with a Mg^{2+}/Al^{3+} molar ratio $r = 2$, and a total 1 M concentration of metallic cations, was introduced with a constant flow into a reactor. pH was maintained constant at 9.0 throughout the coprecipitation by the simultaneous addition of a 2 M NaOH solution. The suspension was aged at room temperature with stirring for 24 h. The final product was centrifuged and washed several times with decarbonated water, and finally dried in air at room temperature. All experiments were carried out under a stream of N_2, to avoid or at least minimize the contamination by atmospheric CO_2. Formation of the LDH phase was confirmed by powder X-ray diffraction

Table 1
Analytical performance of PPO/Mg_2Al–Cl biosensor (0.05 M PBS pH 6.0, $E_{app} = -0.2$ V).

Substrate	Sensitivity (mA M^{-1} cm^{-2})	J_{max} ((A cm^{-2})	Linear range (M)	R (n)	K_M^{app} (mM)
L-DOPA 	762	135	8.0×10^{-8}–7.0×10^{-6}	0.999 (20)	0.186
L-Tyrosine 	180	9	3.0×10^{-7}–6.0×10^{-6}	0.999 (10)	0.055
N-Ac-Tyr-OEt 	969	72	8.0×10^{-8}–1.5×10^{-6}	0.998 (13)	0.085

showing a basal spacing of 0.75 nm and by FTIR (ν_{MO} at 761 and 653 cm^{-1}; ν_{OMO} at 438 cm^{-1}).

2.2. Determination of TK activity

TK activity was determined by spectrophotometric assay at 340 nm. Starting with 0.5 mL of Tris buffer (50 mM) pH 7.6, we added 100 μL of L-erythrulose (100 mM, 120 mg mL^{-1}), 25 μL of D-ribose-5-phosphate (180 mM, 50 mg mL^{-1}), 5 μL of TPP (200 mM, 10 mg mL^{-1}), 10 μL of $MgCl_2$ (100 mM, 10 mg mL^{-1}), 10 μL of NADH (14 mM, 10 mg mL^{-1}), alcohol dehydrogenase (25 units). Protein concentration was determined using the Bradford method at 595 nm. 100 μL of the sample was introduced into a tube containing 5 mL of Bradford solution (100 mg of Coomassie blue, 50 mL of ethanol and 100 mL of phosphoric acid 85% were introduced in 850 mL of H_2O). The solutions were incubated 5 min at room temperature. Absorbance was measured at 595 nm. The concentration of protein in the sample was determined using a standard curve plotted with serum albumin (1 mg/mL).

2.3. Preparation of biosensors

Glassy carbon electrodes ($\Phi = 0.5$ cm, $A = 0.196$ cm^2) were polished with an alumina slurry paste (0.05 μm) and rinsed with acetone, ethanol and water. An LDH (Mg_2Al–Cl) colloidal suspension was prepared by dispersion in deionized and decarbonated water (2.5 mg mL^{-1}) under stirring overnight at room temperature. This suspension was mixed with a PPO solution (2.5 mg mL^{-1}) in 1:1 ratio. A defined amount of the mixture (25 μg of LDH and 25 μg of PPO) was spread on the glassy carbon electrode surface, and the coating was dried at 4 °C overnight. The modified electrode was placed for 15 min in saturated glutaraldehyde vapor to cross-link the biomembrane, and the resulting electrode was incubated in buffer solution for 15 min to rehydrate the biofilm and remove any traces of glutaraldehyde.

2.4. Electrochemical instrumentation and procedure

Chronoamperometry experiments were performed with a potentiostat EA161 (EDAQ). The measurements were carried out in a conventional thermostated three-electrode cell, including an Ag/AgCl reference electrode, a platinum wire as auxiliary electrode, and a rotating glassy carbon disk (RDE, Radiometer) modified with PPO/LDH films as working electrode. The biosensors were immersed in a set volume of buffer solution (20 mL for PPO-based biosensor optimization and 5 mL for experiments with TK). The biosensor was equilibrated under applied potential for 30 min or 4 h before adding analyte.

3. Results and discussion

3.1. N-Ac-Tyr-OEt biosensor optimisation

Polyphenol oxidase (PPO) contains a coupled dinuclear copper active site that catalyzes the metabolic conversion of monophenols and diphenols to *o*-quinones. Enzymatically generated *o*-quinones can be detected *via* their electrochemical reduction at −0.2 V (Scheme 1). Substrate concentration was increased stepwise by adding set volumes of concentrated stock solution in 0.05 M phosphate buffer solution (PBS) pH 6.0 at 30 °C. The resulting steady-state current responses were recorded at the rotating electrode (500 rpm) modified with the PPO/MgAl–Cl LDH film at $E_{app} = -0.2$ V. These experimental conditions correspond to optimized catechol determination on a PPO/ZnAl–Cl LDH biosensor (Shan et al., 2003). Sensitivity was calculated from the slope of the linear part of the calibration curve. The detection limit was defined as a signal-to-noise ratio of 3. A pseudo-plateau occurs in the current/concentration curve (J_{max}) when the active sites of all enzyme molecules are saturated.

These biosensor characteristics corresponding to the determination of monophenol L-tyrosine, diphenol L-DOPA and N-Ac-Tyr-OEt (the product of the enzymatic reaction of TK) are compared in Table 1. Sensitivity was highest with N-Ac-Tyr-OEt, and was five times higher than for L-tyrosine.

Apparent Michaelis–Menten constants (K_M^{app}) were evaluated from electrochemical Lineweaver–Burk plot analysis of the calibrations curves (Table 1). K_M^{app} values for L-tyrosine and L-DOPA were compared with those obtained with PPO in solution ($K_M = 0.21$ and 0.8 mM for L-tyrosine and L-DOPA, respectively; Espin et al., 2000). For both substrates, the values are around four times lower than in solution. The decrease of K_M^{app} values for immobilized enzyme can be explained by the fact that *o*-quinone was involved in an electrochemical recycling process *via* diphenol oxidation by PPO, providing a local increase in substrate concentration together with signal amplification (Scheme 1). The K_M^{app} of N-Ac-Tyr-OEt was similar to the value calculated for L-tyrosine, however the J_{max} was

Table 2
Current responses for N-Ac-Tyr-OEt determinations in 5 mL 0.05 M MOPS pH 7.2, 100 μM ribose-5-P, 2 mM TPP, 3 mM $MgCl_2$, 1 U/mL TK (25 °C, E_{app} = −0.2 V).

TK	BSA (mg/mL)	I^a (nA)	I^b (nA)	N-Ac-Tyr-OEt formed (μM)	% of transformed TK probe
Pure	2	140 ± 21	174 ± 15	1.60 ± 0.10	8.0 ± 0.5
Crude	2	156 ± 9	214 ± 16	1.50 ± 0.03	7.3 ± 0.1
Crude	–	129 ± 10	186 ± 39	1.41 ± 0.19	7.0 ± 0.9

[a] TK probe (20 μM).
[b] N-Ac-Tyr-OEt (2 μM).

higher (Table 1). It is known that K_M^{app} values does not show the same dependence on molecular size as the V_{max} values, because molecular size and charge of the side-chain has different effects on the binding rate constants involved in K_M^{app} and on the transformation rate constants involved in V_{max} (Espin et al., 2000). As shown by Serra et al. (2002), the catalytic efficiency ($C_{eff} = J_{max}/K_M^{app}$) gives a trend of PPO biosensor sensitivity for different substrates, depending on enzyme immobilization matrices. Indeed there is a good correlation between the catalytic efficiency of the enzyme reaction and the measured sensitivities (Table 1), with C_{eff} values of 847, 165 and 725 mA/M cm^{-2} for N-Ac-Tyr-OEt, L-tyrosine and L-DOPA, respectively. Moreover, the use of protected tyrosine prevents the formation of dopachrome and melanin by-products, which are spontaneously formed from isomerization of dopaquinone (Otavio de Faria et al., 2007) and cause electrode fouling. This factor will also cause a decrease of J_{max} for L-tyrosine.

Determination of N-Ac-Tyr-OEt using a PPO/MgAl–Cl biosensor proved sensitive enough to meet our objective. The experimental conditions (applied potential, pH, and electrolyte nature) could almost certainly be optimized to improve biosensor performance. The dependence of the biosensor response on applied potential was investigated between 0 and −0.3 V; measurements were carried out in the presence of 1 μM N-Ac-Tyr-OEt in 0.05 M PBS (pH 6.0). The response was maximal at −0.2 V and decreased for more negative potentials due to the increase in oxygen reduction, causing oxygen depletion within the biomembrane. Therefore, −0.2 V was selected as the applied potential in subsequent experiments. Since the optimum pH of TK was around 7, we investigated the influence of buffer type at pH 7.0 using three different buffers (PBS, HEPES, and MOPS), commonly used in enzyme assays. The highest response was obtained in MOPS, which was consequently used for further experiments.

The repeatability of the current response of the PPO/Mg_2Al–Cl biosensor was studied in MOPS (pH 7.0, 25 °C and E_{app} = −0.2 V) for 10 successive additions of 1 μM N-Ac-Tyr-OEt; the relative standard deviation (RSD) was 2.8%. The repeatability of the analytical response was also examined using the same biosensor for 10 independent measurements in 1 μM N-Ac-Tyr-OEt, giving an RSD of 4.4%. Reproducibility of the biomembrane fabrication process was tested using four different modified electrodes constructed by the same procedure. The calibration curves of N-Ac-Tyr-OEt had an average sensitivity of $S = 1052 \pm 86$ mA M^{-1} cm^{-2} (RDS = 8.2%) and $J_{max} = 141 \pm 10$ μA cm^{-2} (RDS = 6.7%) with a linear range between 8×10^{-8} and 2×10^{-5} M.

3.2. Detection of TK activity

The N-Ac-Tyr-OEt calibration curve was then established in the medium used for the TK assays containing: 100 μM D-ribose-5-P, 2 mg/mL BSA, 2 mM TPP, 3 mM $MgCl_2$ and 1 U/mL TK in MOPS pH 7.2 at 25 °C (Fig. 1). Under these conditions, the time required to reach equilibrium at applied potential was increased to 4 h, due to environmental changes at the biomembrane/electrolyte interface occurring within this complex medium. However, the biosensor response remained stable and rapid with t_{95} = 10 s and biosensor performance decreased by about 50% in sensitivity and maximum

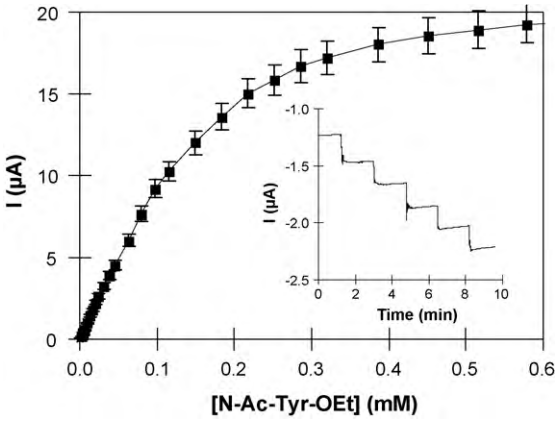


Fig. 1. Calibration curve of N-Ac-Tyr-OEt in 0.05 M MOPS solution (pH 7.2) containing 100 μM D-ribose-5-P, 2 mg/mL BSA, 2 mM TPP, 3 mM $MgCl_2$ and 1 U/mL TK. Inset shows steady-state current–time responses for increasing N-Ac-Tyr-OEt concentration by 2 μM steps (E_{app} = −0.2 V, 25 °C).

current ($S = 556$ mA M^{-1} cm^{-2}, $J_{max} = 101$ μA cm^{-2}) with a linear range between 5×10^{-7} and 3×10^{-5} M.

To avoid any artifacts due to possible electrode passivation or denaturation of PPO in this complex assay medium, we decided to run a normalized double determination with five additions of 20 μM TK probe, followed by five subsequent additions of 2 μM N-Ac-Tyr-OEt (Fig. 2a). In this case, the biosensor response time to the additions of TK probe was also rapid (t_{95} = 12 s). The amount of N-Ac-Tyr-OEt released by the enzymatic transformation of TK probe **1** can be calculated from the current ratio $I_{TK\ probe}/I_{N\text{-}Ac\text{-}Tyr\text{-}OEt}$ normalised in molar concentration (Table 2). Under these biosensing conditions, 7% of TK probe was transformed by 1 U/mL of crude TK

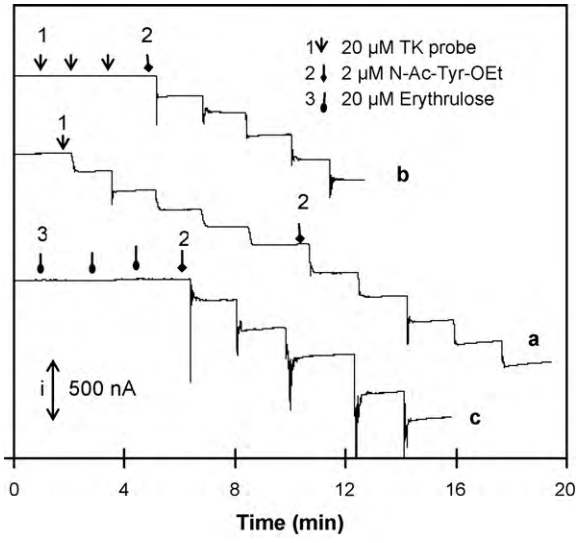


Fig. 2. Current responses to (a) TK probe **1** and N-Ac-Tyr-OEt additions in crude TK extract medium (b) in biological filtrate without TK, (c) erythrulose and N-Ac-Tyr-OEt additions in crude TK extract medium (experimental conditions as in Fig. 1).

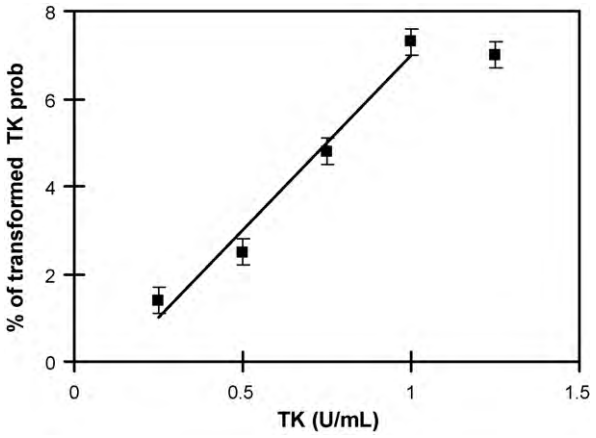


Fig. 3. Influence of the amount of TK on % TK probe **1** transformed in the enzymatic reaction (experimental conditions as in Fig. 1).

extract present in the medium. This percentage increased slightly to 8% when the same volume of filtered TK, devoid of crude extract metabolites after centricon pass-through, was added (Table 2). This result confirms that the transformation of compound **1** is specifically catalyzed by TK.

Two blank experiments were performed. The first corresponded to the same experiments as curve (a) in Fig. 2 but replacing TK with the crude extract devoid of TK. Arrows show analyte additions. As expected, there was no current response following TK probe additions (Fig. 2b). Similarly, no current response was observed when erythrulose, a commonly used TK donor substrate, was added in the TK assay (Fig. 2c). Indeed, transformation of erythrulose by TK leads to non-electroactive compounds that cannot be detected at the PPO biosensor. These experiments confirm the selectivity of our biosensing assay. The amount of N-Ac-Tyr-OEt released, and consequently the percentage of transformed TK probe, are dependent on the TK concentration (U/ml) present in the electrolyte medium, with saturation at 1.25 U/mL (Fig. 3). The reproducibility of these experiments was verified for at least two independent experiments (RDS 4%).

Finally, the role of BSA was also investigated. Using a PPO/Mg_2Al–Cl biosensor, 7% of TK probe was transformed without BSA (Table 2). This percentage fell to 2% with a PPO/Zn_2Al–Cl biosensor without BSA, but reached 8% in the presence of BSA. These results suggest that the basic strength of MgAl LDH played a role in the alkaline catalysis of β-elimination leading to the formation of N-Ac-Tyr-OEt; which was not the case with the neutral LDH (ZnAl–Cl). This fact is currently under verification in batch synthesis experiments.

4. Conclusion

In this preliminary study, we have demonstrated the possibility to measure TK activity by means of a tyrosinase biosensor. Sensitive and stable response obtained for N-Ac-Tyr-OEt determination confirms the good biocompatibility of LDH material to immobilize tyrosinase. Optimized conditions were obtained for enhancing the biosensor sensitivity to detect N-Ac-Tyr-OEt released during the *in vitro* enzymatic reaction catalyzed by TK. Further improvements, in particular concerning the possible interferent effects, will be proposed in future applications. The common strategy to eliminate interferences is to cover the bioelectrodes with permselective membranes (such as nafion or PTFE). An alternative solution should be the use of composite membranes (LDH–alginate or LDH–chitosan) as enzyme immobilization matrices. Development of this type of PPO biosensors is currently under investigation in our laboratory. The co-immobilization of TK with PPO will be investigated to extend the applications to clinical monitoring and biomedical research, such as for screening human TK inhibitors which are candidate targets for study in many diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2010.05.023.

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