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Fluorimetric measurement of 7-ethoxyresorufin-O-deethylase (EROD) activity.

Caution about the spectral characteristics of the standards used.

G. RADENAC ^{1†}, G. COTEUR ², B. DANIS ², P. DUBOIS ² and M. WARNAU ^{2*}

¹ Laboratoire de Biologie & Environnement Marins, EA 3168 – Université de La Rochelle, La Rochelle, France.

² Laboratoire de Biologie Marine, Université Libre de Bruxelles, Brussels, Belgium.

[†] Corresponding author: LBEM, EA 3168 – Université de La Rochelle, Institut de la Mer & du Littoral, Port des Minimes, F-17000 La Rochelle, France. gradenac@univ-lr.fr

^{*} Present address: IAEA - Marine Environment Laboratory, 4 Quai Antoine 1^{er}, BP 800, MC-98012 Principality of Monaco

Abstract

The activity of the enzyme 7-ethoxy-resorufin-O-deethylase (EROD) has been widely used in biomonitoring studies for more than a decade. Although the analytical procedure for this activity is quite simple, it is often poorly characterised. In this study, spectral properties of particular standard compounds used for EROD activity measurement (viz. ethoxyresorufin – EthR – and resorufin – Res–, standards from Molecular Probes®) were tested in order to optimise excitation and emission wavelengths to be used in the fluorimetric assay of EROD activity. The optimal excitation wavelength for the detection of Res was found to be 560 nm. Indeed, at this wavelength, the excitation represents only 37% of its maximum level for EthR while it represents 86% for Res. This allows to discriminate between the fluorescence emitted by both standards favouring the formed product (Res). Our results demonstrate that any analytical work using spectrofluorometry to measure EROD activity should be preceded by a precise determination of the spectral characteristics of each set of standards used.

Keywords : EROD measurement, ethoxyresorufin, resorufin, spectral characteristics, fluorimetry, biomarker

Introduction

The use of the enzyme 7-ethoxy-resorufin-O-deethylase (EROD) activity as an environmental biomarker was suggested some thirty years ago (Burke and Mayer 1974). From that time, the main objective of the numerous studies dealing with EROD activity has been to assess the effects of contamination by specific pollutants on target organisms in the marine environment (Addison and Edwards 1988, Galgani *et al.* 1991, Holdway *et al.* 1994). Indeed, a few organic compounds such as polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) specifically induce the activity of EROD, an enzyme that is involved in the detoxication of these organic pollutants (Sulaiman *et al.* 1991). Because of the good sensitivity of this biomarker in marine vertebrates, it has been widely used in biomonitoring studies for more than a decade (Galgani *et al.* 1992).

The analytical procedure for EROD activity measurement is quite simple and consists in determining (generally using spectrofluorimetry) the efficiency of a given biological sample to convert an experimentally-added substrate (ethoxy-resorufin) into a specific product (resorufin) (Grzebyk and Galgani 1991). However, several studies highlighted the lack of knowledge on the "natural" variability of this enzymatic activity (Galgani and Payne 1991). More recently, it has been shown that factors such as season (Eggens *et al.* 1995), sexual maturity stage in female individuals (Burgeot *et al.* 1994) or growth (Sleiderink *et al.* 1995) strongly influence EROD activity. In addition, it is now well known that experimental parameters (e.g., storage temperature, pH of extraction buffers, incubation temperature) governing in the laboratory during sample treatments may also affect this activity (Grzebyk and Galgani 1991, Burgeot *et al.* 1994). Surprisingly, optimal wavelengths for the measurement of this enzymatic activity have never been fully characterised, even if several

technical reports questioned the purity of the standards used (Eggens and Galgani 1992, Munkittrick *et al.* 1993, Stagg and McIntosh 1998).

When testing for EROD activity, low amounts of resorufin are formed compared to the amounts of ethoxyresorufin added to the samples. Moreover, these two products have similar chemical (and thus spectral) properties making it difficult to accurately and specifically measure the end-product resorufin. Therefore, the aim of the present work was to test the spectral properties of the reference standards (*viz.* ethoxyresorufin and resorufin) used for EROD activity determination, in order to determine the optimal excitation and emission wavelengths which should be used to measure accurately the apparition of the resorufin in the incubation mixture.

Methods

The standards tested in this study (R-352 for ethoxyresorufin –EthR– and R-363 for resorufin –Res –; references from Molecular Probes®) were stored under optimal conditions: refrigerated (4°C) and protected from light in order to avoid any degradation.

The concentrations of the stock solutions were first determined by spectrophotometry using an extinction coefficient of $54.0 \text{ cm}^{-1} \text{ mM}^{-1}$ for Res (absorbance read at $\lambda = 572 \text{ nm}$) and of $16.0 \text{ cm}^{-1} \text{ mM}^{-1}$ for EthR (absorbance read at $\lambda = 494 \text{ nm}$) (Molecular Probes-®, unpublished data). Homogenisation of the Res stock solution was extended overnight and was facilitated using ultrasonication during 10 minutes at room temperature (Kennedy and Jones 1994). Working solutions were freshly prepared in a 0.1M phosphate buffer, pH = 7.6 and kept at 4°C in the dark. The final concentrations of EthR and Res used in this study were $2 \mu\text{M}$ and 2.5 nM , respectively.

Fluorimetric measurements were performed during the first minute of incubation within a 2ml cuvette at a temperature of 20°C, using a RF-5001 PC Shimadzu fluorimeter.

Excitation and emission spectra of the tested standards (EthR and Res) were carried out in order to determine the maximum excitation and emission wavelengths for each chemical.

Results and discussion

A wide range of excitation wavelengths for EROD activity measurement are reported in published studies: e.g. 510 nm (Galgani and Payne 1991), 530 nm (Holdway *et al.* 1994, Brumley *et al.* 1995), 535 nm (Stagg and McIntosh 1998), 537 nm (Hewitt *et al.* 1998), 538 nm (Gunther *et al.* 1997), 544 nm (Burke and Mayer 1974, Grzebyk and Galgani 1991, Burgeot *et al.* 1994). In order to determine the optimal excitation wavelength to be used for the detection of Res in a mixture of EthR and Res, excitation spectra of both compounds were recorded with a fixed emission wavelength corresponding to the peak emission of Resorufin (i.e. 584 nm).

Maximum excitation was reached at $\lambda = 494$ nm for EthR and $\lambda = 572$ nm for Res (figure 1). The shape of each spectrum differed quite significantly. Res showed a narrow excitation spectrum whereas, for EthR, the spectrum displayed a large plateau from 494 nm (the maximum of excitation) to 535 nm: at this wavelength, the excitation of EthR still represented 86% of its maximum value.

[Insert figure 1 about here]

This plateau was not detected in another study (Galgani and Payne 1991) testing the same products purchased from another company (Sigma®). These authors recorded a narrower peak characterised by a maximum of excitation at 457 nm while less than 20% of the maximum excitation remained at 535 nm. In our case, it appears that an excitation of 535 nm

(which is the most commonly used wavelength) would be most inappropriate to detect Res in the reaction mixture. Indeed, EthR would be the main excited (and thus detected) compound.

This hypothesis was tested by comparing emission spectra of both molecules excited at 535 nm (figure 2). They showed very close wavelengths of maximal emission: 576 nm for EthR and 584 nm for Res. This is consistent with the similarity of their chemical conformation. Moreover, the emission at $\lambda = 584$ nm from the EthR was greater than the one from the product to be detected, i.e. Res (figure 2).

[Insert figure 2 about here]

It seems that an optimal detection of the reaction product would consist of exciting at a wavelength close to the peak of excitation of Resorufin (572 nm) but not close to the emission peak (584 nm) in order to prevent any interference by the excitation beam. For this reason, standards were excited at $\lambda = 560$ nm; emission at $\lambda = 584$ nm due to EthR was about four times lower (23%) than that of resorufin (figure 3). At this wavelength, the excitation represents only 37% of its maximum level for EthR and 86% for Res (figure 1). Therefore, although close to the emission wavelength, this excitation wavelength (560 nm) would be efficient to discriminate between the set of standards tested here. To solve the problem of wavelength proximity, slits were reduced, thereby preventing any interference between excitation and emission beams (3 and 5 nm respectively).

[Insert figure 3 about here]

Conclusion

In conclusion, among the "normal" variability of the enzymatic response, spectral characterisation of standards used should be carefully checked before any assay of EROD activity using EthR as substrate. The lack of such control would question the validity of the assay. For the standard used in the present study (references from Molecular Probes®), optimum excitation wavelength was determined to be 560 nm, a wavelength which is much higher than those used in previous works.

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Captions to figure

- Figure 1 - Excitation spectra of ethoxyresorufin (2 μ M) and resorufin (2.5 nM) standards from Molecular Probes® - Emission at 584 nm
- Figure 2 - Emission spectra of ethoxyresorufin (2 μ M) and resorufin (2.5 nM) standards from Molecular Probes® - Excitation at 535 nm
- Figure 3 - Emission spectra of ethoxyresorufin (2 μ M) and resorufin (2.5 nM) standards from Molecular Probes® - Excitation at 560 nm

Figure 1.

Figure 2.

Figure 3.