Application of A Yeast Oestrogen Screen for the Detection of Oestrogen-like Response of Environmental Samples

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Introduction

As the public awareness of endocrine disrupting chemicals is growing, great efforts are undertaken to identify such substances especially those which activate the oestrogen receptor [1]. Bioassays based on genetically modified yeasts are very promising among the wealth of test systems available for the determination of oestrogenic activity, because of their physiological simplicity, easy handling and low costs [2-5]. In general yeast test systems rely on yeast constructs expressing an (human) oestrogen receptor which upon binding of suited substrates acts as a transcriptional enhancer for an oestrogen responsive DNA element-controlled reporter gene, in most cases bacterial β-galactosidase. The activity of the enzyme is determined photometrically and serves as a measure for the oestrogenic potency of the sample under investigation.

In this study we used a yeast construct, which neither expresses a complete oestrogen receptor nor contains a reporter gene under the control of oestrogen responsive elements. Instead this strain expresses a fusion protein carrying only the hormone binding domain of the human oestrogen receptor connected to the yeast GAL4-DNA binding domain [6]. Upon binding of an appropriate compound to the hormone binding domain the fusion protein recognises a responsive DNA element upstream of a β-galactosidase reporter gene.

Until now the application of yeast oestrogen screens has in most cases been limited to pure substances or mixtures of pure compounds [3, 7, 8]. However, from the risk assessment point of view it would be very important to apply such tests for environmental monitoring to identify emitters, actual environmental loads and environmental reservoirs of (xeno)oestrogenic compounds.

The experiments described were performed to investigate the (xeno)oestrogen responsiveness of this distinct yeast construct and to study the applicability of yeast bioassays for the analysis of environmental samples.

Materials and Methods

Chemicals of the highest purity available were purchased from Merck, Darmstadt, Germany, Aldrich, Steinheim, Germany and Fluka, Neu-Ulm, Germany. Yeast Nitrogen Base and Bacto agar were obtained from Difco, Augsburg, Germany.

Toluene extracts of environmental samples were prepared according to Schwirzer et al. [9]. Shortly before use in the yeast bioassay, suited amounts of the toluene extracts were evaporated to dryness using a rotary evaporator at 40°C, 50 mbar. The residues were dissolved in DMSO and stored at 4°C as were all chemicals to be investigated in the yeast bioassay.

The yeast strain was a kind gift of D. Picard, University of Geneva, Switzerland [6]. It was grown at 30°C, 130 rpm in Erlenmeyer flasks with one notch using SC medium prepared in HPLC-grade water as described in Kaiser et al. [10] without histidine. Stock cultures prepared from exponentially growing cultures by adding DMSO up to a final concentration of 15% (v/v) were kept frozen at -80°C in 0.5 mL aliquots.

For performing the oestrogen bioassay exponentially growing overnight cultures were diluted with SC medium to an OD_{600nm} of 0.75. Ten-millilitre aliquots were distributed into 100 mL-Erlenmeyer flasks with one notch. and received 100 µL of DMSO (negative control), 100 µL of DMSO-dissolved 17β-oestradiol (1 µM, positive control) or DMSO-dissolved samples. Test cultures were incubated at 30°C, 130 rpm for 2 h. Growth was determined afterwards by measuring the OD_{600nm} of the fivefold diluted test cultures. The β-galactosidase activity was determined with all solutions prepared in HPLC-grade water according to Miller [11]. In brief: 200 µL of the test cultures were transferred into Eppendorf reaction vessels. After addition of 600 µL of Z-buffer ($Na_2HPO_4 \cdot 7H_2O$, 60 mM; $NaH_2PO_4 \cdot H_2O$, 40 mM; KCl, 10 mM; $MgSO_4 \cdot 7H_2O$, 1 mM; β-mercaptoethanol 35 mM), 20 µL SDS solution (0.1 % (w/v)) and 50 µL chloroform, the assays were carefully mixed (Vortex, three times 15 s) and preincubated for 5 min at 28°C in a shaking water bath. The enzyme reaction was started by adding 200 µL of *o*-nitrophenyl-β-D-galactopyranoside 0.4% (w/v) dissolved in Z-buffer. The assays were incubated at 28°C until a significant yellow colour developed in the 17β-oestradiol (E2) induced positive controls (≤ 20 min) and samples (≤ 120 min). After stopping the reaction by adding 500 µL of Na_2CO_3 (1M), cell debris was pelleted by centrifugation (25,500·g, 15 min) and the Ex_{420nm} of the supernatants determined.

Relative β-galactosidase induction factors in the environmental sample screening were defined as the ratio of the β-galactosidase activity in a sample and of the corresponding negative controls which received DMSO only.

Results and Discussion

To determine the response specificity of the yeast strain thirty different compounds, some of them known or suspected environmental oestrogens, were screened for their activity.

Under the test conditions selected, the strain showed a clear selectivity for oestrogenic compounds, as it responded to 17β-oestradiol and all tested artificial oestrogens at a concentration of 10 nM whereas no response was seen with other mammalian steroid hormones even at a nominal concentration of 1 mM. Positive results were obtained for all tested phenols carrying *p*-substituents with more than 5 C-atoms. Chlorinated pesticides with the exception of *p,p*-DDE did not elicit any response.

The sensitivity of the yeast construct was investigated in more detail by recording dose response curves for E2, 4-tert.-octylphenol and bisphenol A. The corresponding EC_{50} values as calculated from curve fitting were E2: 1.5 nM, 4-tert.-octylphenol: 6.7 µM and bisphenol A: 104 µM.

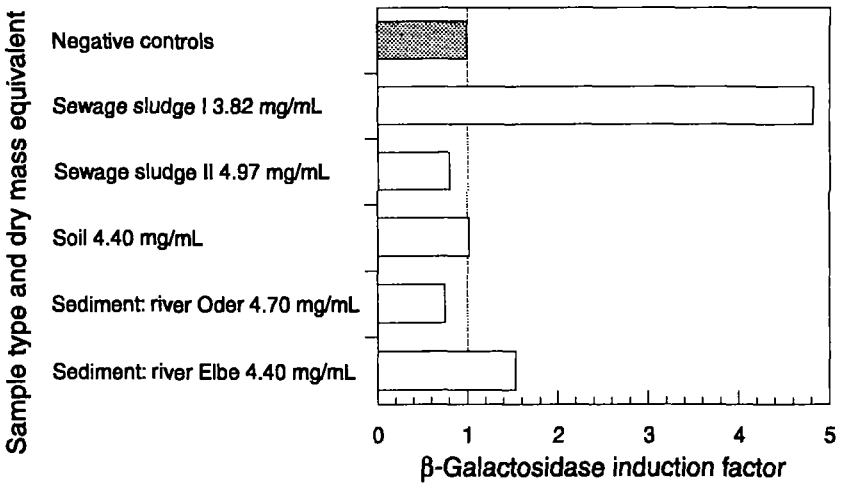

FIGURE 1 Oestrogen-like activity in toluene extracts of selected environmental samples determined as β-galactosidase induction factor to allow for a better comparison of different experiments.

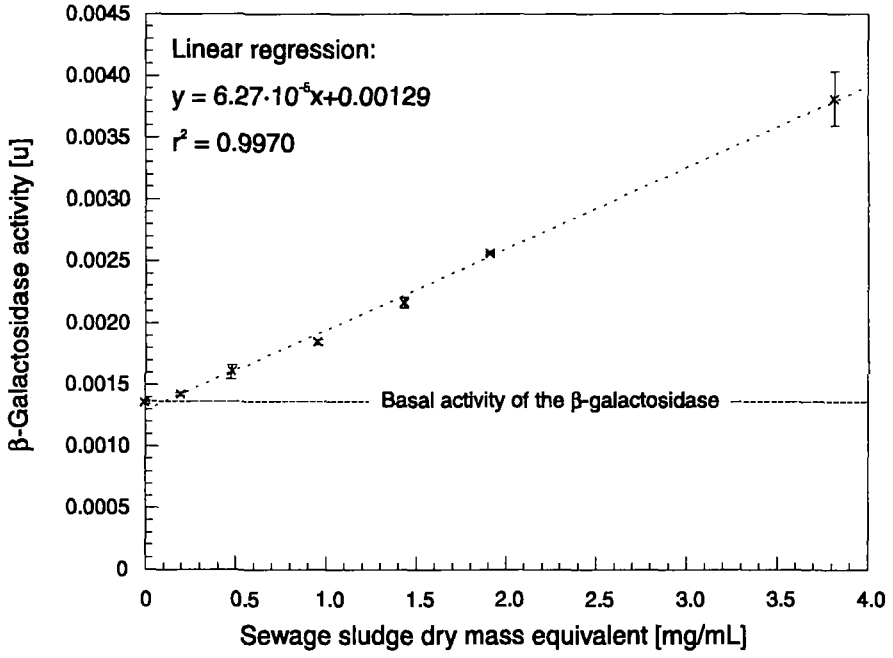

FIGURE 2 Oestrogenic activity in the toluene extract of sewage sludge I in the yeast oestrogen bioassay. All concentrations were analysed in triplicate. Error bars indicate the standard deviation.

The applicability of the bioassay on complex samples was studied screening DMSO dissolved toluene extracts from different environmental matrices, like river sediment, sewage sludge and soil. (Figure 1). The toluene extract of sewage sludge I showed a pronounced oestrogen-like activity and was analysed in more detail. The oestrogenic activity was dose dependent in a linear manner (Figure 2). Almost all yeast oestrogen bioassays described so far are based on yeast constructs expressing whole oestrogen receptors which upon binding of appropriate substrates interact with EREs to activate the transcription of a reporter gene [2-4, 7, 12]. Only a construct used by Chen et al. [13] produces a fusion protein (LexA-hER) which in its activated form enhances reporter gene transcription via LexA-responsive DNA-elements. But this fusion protein still encompasses the complete human oestrogen receptor whereas the fusion protein produced by the strain used in our experiments only contains the oestradiol binding domain of the human oestrogen receptor (amino acids 282-576) [6].

The screening experiments gave results which were mainly consistent with data reported for other yeast constructs [3, 8, 13, 14]. Exceptions encompassed 4-tert.-amylphenol, dieldrine, toxaphene, endosulfan and methoxychlor which exhibited no activity in our bioassay. In the case of methoxychlor this may be explained by the fact that this compound needs a metabolic activation prior to being active [15], transformations which might not be carried out properly by our yeast strain. For the remaining four compounds the reason for their inactivity remains unclear at the moment, however among other the artificiality of our „oestrogen receptor" could be involved in this phenomenon.

In summary, despite the high artificiality of the signalling cascade, the yeast construct applied in our bioassay showed a detection range similar to that reported for other yeast constructs.

The EC_{50} determined for E2 (1.5 nM) was similar to that found by Chen et al. and Maier et al. [13, 16] and about one order of magnitude higher than that observed by Arnold et al. and Gaido et al. [3, 17].

The dose response curves for 4-tert.-octylphenol and bisphenol did not show a saturation plateau but a optimum course which might be due to toxic effects exerted on our yeast strain by this compounds at higher concentrations. However, the EC_{50} based ratios of the oestrogenic activity of 17b-oestradiol, 4-tert-octylphenol and bisphenol A ($1 : 2.23 \cdot 10^{-4} : 1.44 \cdot 10^{-5}$) were comparable to values found with another yeast construct [3] and with MCF7 breast cancer cells [18].

The screening of DMSO dissolved toluene extracts from different environmental matrices served as a first test for the applicability of the bioassay for the investigation of any environmental sample. The toluene extract of sewage sludge I gave a significant response which could be demonstrated to be linearly in the concentration range investigated. The signal represented the sum activity of all oestrogen receptor-oestrogen binding domain-coupling substances present in the extract which might originate from natural oestrogens excreted in the urine, artificial oestrogens from *the* pill [19] and to a lesser extent (due to their low potency) from phytooestrogens and xenooestrogens like alkylphenols and bisphenol A.

Several additions and modifications will be made to improve the efficiency of the test procedure for environmental monitoring: As the toluene extraction mainly covers non polar compounds an important range expansion for coverable substances will be achieved by applying more polar extraction solvents e. g. acetone. To allow the identification of active compounds the bioassay has to be supplemented by a extract fractionation procedure coupled to a suited instrumental analysis like GC-MS and HPLC-MS. Furthermore the yeast bioassay itself will be improved by introducing an additional bioactivation step to enable the detection of substances like methoxychlor which act only after bioactivation. Finally a miniaturisation and automatisation of the test will allow for a higher number of samples to be analysed in one experiment.

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