Electrochemical detection of protein–protein interactions using a yeast two hybrid: 17-β-Estradiol as a model

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Abstract

In this work we present a modified yeast two-hybrid bioassay for the highly sensitive detection of protein–protein interactions, based on the electrochemical monitoring of β-β-D-galactosidase reporter gene activity, using p-aminophenyl-β-D-galactopyranoside (PAPG) as a synthetic substrate. In a model system, the sensitive detection of 17-β-estradiol was achieved at concentrations as low as $10^{-11}$ M (approx 2 pg/ml) by monitoring 17-β-estradiol receptor dimerization after exposure to 17-β-estradiol. The sensitivity of this system was higher than that of standard optical methods by three orders of magnitude.

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The yeast two-hybrid system (YTH)\(^1\) was initially developed by Fields and Song [1] as a powerful technique for identifying novel protein–protein interactions. The method is based on the finding that many eukaryotic transcription factors can be divided into two separated functional domains that mediate DNA binding and transcriptional activation. In the YTH system, the “bait” is constructed by fusing a certain protein to the DNA-binding domain derived from a transcription factor, and the “prey” is constructed by fusing another protein to the activation domain of the same transcription factor. The bait and prey fusions are coexpressed in yeast, where the interaction of the two fused proteins leads to the reconstitution of a functional transcription factor. The reconstitution of the transcription factor is assayed by measuring the activity of one or more reporter genes, commonly the LacZ gene encoding β-galactosidase. Auxotrophic markers like HIS3 and LEU2, which allow the selection of interactions by monitoring growth on plates lacking histidine or leucine, are used as well. Hence, yeast is a very convenient organism for investigation of in vivo protein–protein interactions at low cost [2,3].

The YTH system has potential applications in many fields and has a significant impact on pharmacological research. Once a pharmacologically important interaction is demonstrated by YTH, the interacting protein partners can be scanned against a large number of compounds to dissociate undesirable interactions. Traditionally, the identification of such compounds relied on in vitro biochemical methods, such as in vitro affinity chromatography [4]. This approach, however, can detect only high levels of activity. High throughput screening of potential drug candidates in YTH is efficient and widely used, despite having certain problems. For example, the cell wall and membrane of Saccharomyces cerevisiae and its multidrug efflux systems have made this yeast insensitive to pharmaceutical compounds [5].

17-β-Estradiol, a hormone produced mainly in the ovaries, is essential for the normal development, maturation, and function of the female reproductive tract. Additional small amounts are secreted by the adrenal glands and by the male testes. 17-β-Estradiol regulates cellular events through a specific intracellular receptor
that functions as a ligand-inducible transcription factor. Hormone binding to the 17-β-estradiol receptor is followed by a series of events that include dissociation from heat shock proteins [6–8], dimerization [9–11] and binding to DNA at a 17-β-estradiol response element [12]. Because of its ability to detect protein–protein interactions, the YTH system can be used as a very convenient, reliable, and highly sensitive system for detecting 17-β-estradiol; consequently, several formats have been proposed for detecting this hormone. A system described by Wang et al. [13] relies on the detection of human 17-β-estradiol receptor (hERα) dimerization, cloned in yeast, after exposure to 17-β-estradiol. The binding of 17-β-estradiol to its receptor is cooperative; therefore, a significant response is generated even at low concentrations of the hormone [14]. hERα dimerization is not the only useful assay method. A system described by Nishikawa et al. [15] monitors the interaction between hERα and various cellular co-activators that interact with the receptor when exposed to the hormone.

The work presented here describes a new YTH assay, based on the electrochemical detection of β-galactosidase reporter gene activity, for the sensitive detection of 17-β-estradiol.

Materials and methods

Yeast cultivation and ligand treatment

The yeast strain Y-187 was purchased from Clontech. Plasmids Ppc-86 and Ppc-62, containing the cDNA of hERα fused to the GAL-4 DB (DNA binding) and GAL-4 AD (activating) domains, were a generous gift from Prof. Sohaib A. Khan (University of Cincinnati, OH, USA). Transformation of the yeast was carried out using the lithium acetate method with plasmid DNA, as described by the Clontech Matchmaker 3 system protocol. A scheme describing the YTH system with cloned and expressed hERα is illustrated in Fig. 1.

Figure 1. Scheme of the YTH system expressing hERα.

Yeast cells grown overnight were first separated by centrifugation for 10 min at 3000 rpm and then resuspended in 1 ml of PBS buffer, supplemented with 0.2% SLS, which served as a permeabilizing agent for the yeast membranes, according to the method described in [16]. We used a high concentration of the permeabilizing agent because cell viability is not necessary for the β-galactosidase assay. Consequently, the SLS served a dual purpose in this assay by allowing better permeability [1] during cell growth with 17-β-estradiol (low concentration) and [2] before the β-galactosidase assay (high concentration). The test tubes were vortexed intermittently for 30 min at room temperature. Alternative permeabilizing agents tested were ethanol, isopropanol, and toluene. The method used was similar to that described by Kondo et al. [17]. Briefly, the cells were harvested by centrifugation, resuspended in 1 ml of either solvent, and kept on ice for 10 min, with intermittent vortexing. The cells were then harvested again and resuspended in 1 ml of PBS buffer. As each test tube contained 4 ml of liquid medium with overnight-cultivated yeast, a fourfold preconcentration effect was obtained. For lower hormone concentrations (10⁻¹⁰ and 10⁻¹¹ M), the cells were concentrated ¼ before the assay.

β-Galactosidase electrochemical assay

The assay was performed with screen-printed disposable electrodes (Gwent, UK) with plastic microwells (300 μl volume) mounted on top of the electrodes, forming small electrochemical cells. Each electrochemi-
catal cell consisted of a carbon working electrode, a silver/silver-chloride reference electrode, and a carbon auxiliary electrode. Electrodes were used once only. Liquid mixing in each microwell was achieved by a suction/expulsion mechanism, operating at a frequency of approx 12 Hz, developed in our laboratory. Amperometric measurements were performed using a multichannelled potentiostat, manufactured at the Technion-IIT, Haifa, Israel, which is capable of analyzing eight samples simultaneously. $p$-Aminophenyl-$\beta$-D-galactopyranoside (PAPG) was used as a synthetic substrate for the $\beta$-galactosidase yeast reporter gene. The activity of $\beta$-galactosidase was detected by the anodic current generated by the oxidation of the product of the enzymatic reaction, $p$-aminophenol, at the carbon working electrode, at an applied potential of 220 mV vs Ag/AgCl. The final results were reported as $\Delta$current/time during the first 20 min of measurement.

The optical assay of $\beta$-galactosidase was done using o-nitrophenyl-$\beta$-D-galactopyranoside (ONPG), as described by Kippert [16]. Overnight-grown yeast cells were concentrated $\times$4 before permeabilization with SLS. Further concentration of the cells did not enhance the signal.

Results and discussion

Permeabilization methods

$\beta$-Galactosidase has been widely used as a reporter gene in *Saccharomyces cerevisiae* [18]. Two different substrates are commonly used for detecting reporter gene activity: X-gal for detecting enzyme activity on plates because this compound easily enters the cell and, conversely, ONPG for optical quantitative measurements because this compound cannot enter the cell. As the compound used by us, PAPG, is similar to ONPG in this respect, the cells must be permeabilized before the determination of enzyme activity. The most commonly used permeabilization method for yeast is a method described by Miller [19], which uses chloroform and SDS as permeabilizing reagents. The reproducibility of this method is poor, however [16]. Other useful permeabilization methods have been described, using solvents like ethanol or isopropanol [17] or detergents like SLS [16]. We tested several solvents and SLS for their yeast cell-permeabilization efficiency. Fig. 2 shows typical responses obtained using different permeabilization agents. Clearly, the most efficient agent was SLS. The data obtained are summarized in Table 1 ($n = 3$). Table 1 also clearly shows that when compared with other permeabilizing agents, the reproducibility of the SLS method is very good. It should also be noted that in all the cases tested, the application of permeabilization agents significantly increased the signal.

Optimization of PAPG concentration

To achieve the highest possible signal, we attempted to optimize the concentration of PAPG, the synthetic substrate for assaying $\beta$-galactosidase activity, by examining the concentration dependence of the substrate on the electrochemical signal. We checked increasing substrate concentrations, beginning with 0.5 mg/ml, using cells permeabilized with 0.2% SLS. The results, presented in graphic form in Fig. 3 and summarized in Table 2, show that the activity reached a plateau at concentrations between 4 and 8 mg/ml. Therefore, we chose a concentration of 8 mg/ml PAPG for all the following experiments.

Assay for estrogens

Any assay for 17-$\beta$-estradiol must be highly sensitive because this hormone can exhibit biological activity at the very low concentration of 1 nM [13] or lower [20]. High-affinity binding of the hormone to GAL-4-hER fusion proteins occurs in YTH systems [13]. Fig. 4 shows the response obtained after preconcentration of the cells.

Table 1

<table>
<thead>
<tr>
<th>Permeabilizer</th>
<th>Control</th>
<th>Toluene</th>
<th>Isopropanol</th>
<th>Ethanol</th>
<th>Sarcosinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Permeabilizer Current/time (nA/s)</td>
<td>1 $\times$ 10$^{-2}$ ± 3 $\times$ 10$^{-3}$</td>
<td>3.27 $\times$ 10$^{-2}$ ± 1 $\times$ 10$^{-2}$</td>
<td>8.2 $\times$ 10$^{-2}$ ± 1.5 $\times$ 10$^{-2}$</td>
<td>9.6 $\times$ 10$^{-2}$ ± 3 $\times$ 10$^{-2}$</td>
<td>1.3 $\times$ 10$^{-1}$ ± 5 $\times$ 10$^{-3}$</td>
</tr>
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</table>

Each result represents the mean of three measurements.
by a factor of 4 (see Materials and methods section). As shown, reporter gene activity reached almost maximal levels at very low hormone concentrations (10^{-6} to 10^{-7} M). The results are coherent with the data obtained in literature using optical methods, as reported by Wang et al. [13], where activity peaked at hormone concentrations of 10^{-5} to 10^{-6} M and by Le Guevel [21], where activity peaked at hormone concentrations of 10^{-7} to 10^{-8} M. As shown in Fig. 4, a detectable signal was obtained at hormone concentrations as low as 10^{-9} M. The reproducibility of the signal was very good, as shown by the error bar displayed in Table 3, which never exceeded ±10% of the mean result.

Lower hormone concentrations could be detected if a further preconcentration of overnight-cultivated yeast cells was done. Fig. 5 shows that a detectable signal was obtained with cells exposed to a hormone concentration of 10^{-10} M. Further enhancement of the signal was achieved by adding 10 mg/L of SLS to the growth medium. The latter step yielded a detectable signal at a hormone concentration of 10^{-11} M (see Fig. 6). The reproducibility of these signals was very good as well, as shown by the error of these measurements in Table 3.

We also tried to test the applicability of our system for the detection of estrogen-like chemicals. In preliminary experiments we found that exposure of yeast cells to bisphenol-A (10^{-5} M) yielded a detectable response (see Table 3). Our finding is in agreement with literature data [22] and indicates that the electrochemical yeast two hybrid may be a suitable bioassay for environmental xenoestrogens. Further investigation is currently being conducted.

The possibility of working with highly concentrated suspensions of permeabilized cells (and therefore very turbid) is a very significant advantage of the electrochemical system over standard optical systems because separation of the cells from the liquid bulk is not necessary. The absence of a separation step simplifies the working protocol, which can be crucial when a large number of samples must be analyzed simultaneously [21].

The electrochemical YTH assay is more sensitive than the standard optical method by three orders of magnitude (Table 3). The optical assay has a detection limit of 10^{-8} M under similar conditions used for permeabilization. The results reported for a similar YTH system by Wang et al. [13] showed a detection limit of 10^{-7} M using optical detection with ONPG. The authors used a standard permeabilization system comprising SDS and chloroform, a procedure that is time consuming and requires rigorous shaking of the test tubes, thus making this method unsuitable for analyzing a large number of samples. An improved method was described by Le Guevel and Pakdel [21], who used a permeabilization system based on the treatment of yeast cells with Lyticase enzyme, followed by hypotonic shock. This method did not require vigorous shaking and was found suitable for screening a large number of samples, but did require separation of the cells by centrifugation because the detection was optical. The de-
the detection limit with this system was approximately 1 nM 17-β-estradiol, which is still less sensitive than our electrochemical system. A nonyeast system for the detection of 17-β-estradiol using transformed human adenocarcinoma breast cells and Luciferase as reporter gene was recently described by Legler et al. [23]. The reported detection limit was in the picomolar range. The manipulation of transformed mammalian cells, however, is much more complicated than that of yeast cells.

**Conclusions**

We developed an improved YTH assay method, based on the electrochemical detection of reporter gene activity, using p-aminophenyl-β-D-galactopyranoside as a synthetic substrate for β-D-galactosidase. Sensitivity was enhanced by adding to the yeast growth medium 10 mg/L of sodium lauroyl sarcosinate, which most likely acts at such a low concentration by increasing the cell membrane permeability toward the hormone without being lethal. The assay described here is more sensitive than the conventional optical method by three orders of magnitude. The importance of a highly sensitive assay for the detection of weak protein–protein interactions is crucial because such interactions can have biological significance apart from the hERα [24]. Because separation of the cells from the liquid is not
required before the measurement, our assay is simpler to perform than the conventional optical method. Another advantage of this method is the simultaneous analysis of eight samples using disposable screen-printed electrodes.

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References