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SHORT CONTRIBUTION

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Development of a bisphenol A-adsorbing yeast by surface display of the *Kluyveromyces* yellow enzyme on *Pichia pastoris*

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Abstract A novel surface-engineered strain of yeast *Pichia pastoris* was constructed that displays at its surface *Kluyveromyces lactis* Yellow Enzyme (KYE) fused to the C-terminal half of *Saccharomyces cerevisiae* α-agglutinin. The expression of the fusion protein was controlled by the *AOX1*-promoter. The new strain showed an increased sorption of the xenoestrogen Bisphenol A (BPA). It was shown that sorption of BPA depended on the presence of methanol in the growth medium and on the pH of the binding assays. The binding kinetics were typical for binding at a surface. The present results demonstrate that the α-agglutinin surface display system can be used in the yeast *P. pastoris*.

Introduction

Bisphenol A (BPA) is widely used as monomer for the manufacture of polycarbonates, polystyrene and epoxy resins (Ben-Jonathan and Steinmetz 1998). It is numbered among the group of xenoestrogens or endocrine disruptors because it competes with estradiol for binding with estrogen receptors (Krishnan et al. 1993).

In the secondary effluents of sewage treatment plants, BPA concentrations between 80 and 1,500 ng/l have been found (Fürhacker et al. 2000; Spengler et al. 2001). In German rivers, BPA concentrations between 9 and 776 ng/l have been measured (Heemken et al. 2001). In this concentration range BPA shows adverse effects on growth, reproduction and gonadal maturation of young mice and water snails (Takao et al. 1999; Oehlmann et al. 2000). Therefore it is advisable to construct specific biological filters to remove BPA from sewage effluents.

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One approach towards this aim is the production of BPA-binding yeast cells. We constructed such yeast cells using the yeast surface display technique, whereby functional proteins are fused with the cell wall protein α-agglutinin and are then displayed at the cell surface (Schreuder et al. 1993; Yasui et al. 2002).

In the present study, we constructed yeast cells displaying the *Kluyveromyces* yellow enzyme (KYE) from *Kluyveromyces lactis* at their surface. KYE is encoded by the gene *KYE1* and is highly homologous to old yellow enzyme (OYE) from *Saccharomyces cerevisiae* (Miranda et al. 1995). Because OYE binds to phenolic compounds (Abramovitz and Massey 1976) it is likely that KYE is able to bind the phenolic xenoestrogen BPA. We displayed the functional protein not on the surface of *S. cerevisiae* but on that of the methylotrophic yeast *Pichia pastoris*. We considered this approach possible because α-agglutinin belongs to the group of GPI-proteins that are highly conserved in eukaryotic cells (Schreuder et al. 1993; Gaynor et al. 1999). The *P. pastoris* expression system offers advantages such as easily performable induction of expression of heterologous proteins by using methanol as carbon source, superior gene stability, and higher productivity of heterologous proteins (Hollenberg and Gellissen 1997; Cereghino and Cregg 2000).

Materials and methods

Strains and media

Escherichia coli DH5α [*F', endA1, hsdR17(r_K, m_K+), supE44, thi-1, λ, recA1, gyrA96, ΔlacU196, F80lacZΔM1*] was used as the host for recombinant DNA manipulation. *P. pastoris* GS 115 *his4* (purchased from Invitrogen, Carlsbad, Calif.) was used as the host for cultivation. *E. coli* was grown in Luria-Bertani medium (1% peptone, 0.5% yeast extract, 1% sodium chloride) with 100 μg ampicillin/ml where necessary. Yeast was cultivated in YPD medium (1% yeast extract, 2% peptone, and 2% glucose). For binding assays, yeast was precultivated in 20 ml MD (synthetic medium containing 0.5% glucose as carbon source, Sreekrishna and Kropp 1996). The yeast cells were then transferred into 100 ml MM (with 0.5% methanol as carbon source, Sreekrishna and Kropp

1996) or MD. Medium used for host strain *P. pastoris* GS115 also contained 0.004% L-histidine.

Isolation of genomic DNA

The genomic DNA used for PCR was isolated using the method described by Hoffman and Winston (1987).

Plasmids and transformation

The plasmid for the expression of the KYE/α-agglutinin fusion protein on the cell surface of *P. pastoris* GS115 was constructed as follows. The 3′-half of the open reading frame of the α-agglutinin-encoding gene AG*α1* from *S. cerevisiae* (1.0 kB) was amplified by PCR (primers 5′-AAAAAACCCGGGTACGCAATTCTCG-3′ and 5′-AAAAAAGCATGCGGCCGCC-GACTCAATGTACTAACT-3′), with genomic DNA isolated from *S. cerevisiae* as template, and inserted into the *Xma*I/*Sph*I site of pUC19 (Yanish-Perron et al. 1985) to give the plasmid pUA277-1. The open reading frame of the gene *KYE1* encoding the binding protein was amplified by PCR (primers 5′-AAAAAAGAATTCTACGCAATGTCGTTTAT-GAAC-3′ and 5′-AAAACCCGGGTTTCTTGTAACCCTTGGC-3′ with genomic DNA isolated from *K. lactis* as template and inserted into the *Xma*I/*Eco*RI site of pUC19 to give the plasmid pUK278-9. Plasmid pUA277-1 was then digested with *Xma*I/*Sph*I to reisolate the amplified 3′-half of *AGα1* (*3′-AGα1*). *3′-AGα1* was inserted into the *Xma*I/*Sph*I site of pUK278-9 giving the plasmid pUKA356-5.

The whole *KYE1-3′-AGα1* fusion gene was re-isolated by *Eco*RI/*Not*I digestion of pUKA 356-5 and inserted into the *Eco*RI/*Not*I site of pPIC9K (Invitrogen, Carlsbad, Calif.) to give the plasmid pPIKA373-2.

The plasmid pPIKA373-2 was linearised by *Sal*I digestion and introduced into the host strain *P. pastoris* GS115 using the Invitrogen transformation kit according to the procedure of the supplier. Transformants with HIS^+ phenotype were isolated. The resultant strain was named *P. pastoris* pPIKA.

Binding assays

For binding assays the yeast strain *P. pastoris* pPIKA was precultivated in MD before cultivation in medium containing 2.0% glucose/0.5% methanol as sole carbon source (see above). After overnight cultivation, an aliquot of 15 ml was taken, the cells were collected by centrifugation, washed twice with distilled water und resuspended in 15 ml carbonate/bicarbonate buffer pH 9.3. The cell suspension was added to 85 ml of an equilibrated solution of BPA in the same buffer. The binding assays were shaken at 25°C for 3 h.

BPA analysis samples (10 ml) were taken, the cells were separated from the solution by centrifugation and then discarded. BPA in the aqueous supernatant was extracted by solid-phase extraction using columns filled with Octadecyl Silica endcapped (C18 ec, Macherey-Nagel, Düren, Germany) and eluted with acetone. The acetonic solution was then derivatised and analysed by HPLC using a method described by Naassner et al. (2002).

The amount of BPA bound by the yeast cells (sorption performance) was calculated by the equation:

$$q=[V \times (c_i - c_f)]/S \quad (1)$$

where q is the sorption performance (μg BPA/g dry weight), V is the volume of the BPA-containing solution (in all assays: 0.1 l), c_i the initial concentration of BPA in the solution (μg/l), c_f the final (equilibrium) concentration of BPA in the solution (μg/l), and S the amount of the added biosorbent, i.e. the dry weight of the added yeast cells.

Results

Construction of a vector for yeast surface display of KYE

To display the KYE protein on the cell surface of *P. pastoris*, the open reading frame of the *K. lactis KYE1*-gene was fused C-terminally to the 3′-half of the open reading frame of the *AGα1*-gene of *S. cerevisiae*. This construct was cloned into the cloning site of the expression vector pPIC9K using the *Eco*RI and *Not*I sites. The correct construction was verified by DNA sequencing. The vector with the insert was transformed into *P. pastoris* GS115 as described above giving the novel strain *P. pastoris* pPIKA. Genomic DNA analysis showed that the fusion gene had been correctly incorporated into the *HIS4* locus of the chromosomal DNA of *P. pastoris* (data not shown).

BPA-sorption of the novel strain *P. pastoris* pPIKA and the host strain *P. pastoris* GS115

The host strain *P. pastoris* GS115 and the novel strain *P. pastoris* pPIKA harboring the *KYE1-3′-AGα1* gene under the control of the strong methanol-regulative *AOX1* (alcohol oxidase) promoter were cultivated both on glucose and methanol. The host strain and the new strain did not differ in their growth on glucose and methanol. The BPA sorption of these strains was measured, together with a control assay to determine unspecific BPA binding by glass vessels. The assays were carried out with an initial BPA concentration of 2 μg/l at pH 9.3. The experiment was performed three times using different amounts of cells. The host strain *P. pastoris* GS115 showed a BPA sorption performance of less than 3 μg/g dry weight whether grown on glucose or on methanol. The novel strain *P. pastoris* pPIKA grown on glucose showed also only a weak sorption performance, while the same strain grown on methanol gave a BPA sorption between 8 and 20 μg/g dry weight, depending on the amount of cells.

The effect of pH on BPA sorption

We performed four BPA-binding assays at four different pH values each (pH 7.0, pH 8.0, pH 9.3, pH 10.2) using the strain *P. pastoris* pPIKA. In all assays the highest BPA-sorption was measured at pH 9.3.

Kinetics of BPA sorption

Kinetic experiments were performed at pH 9.3 using 2 μg BPA/l as initial concentration. Samples were taken after 0 min, 10 min, 30 min, 1 h, 3 h and 6 h. A sorption vs time diagram is given in Fig. 1. Two experiments were performed using high amounts of cells (dry weights: 40.5

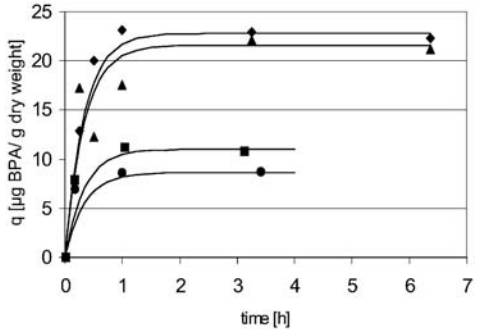

Fig. 1 Kinetics of bisphenol A (BPA) binding to *Pichia pastoris* pPIKA at pH 9.3, initial BPA concentration 2 µg/l. Two experiments with high dry weight: ■ 25.5 mg dry weight, ● 40.5 mg dry weight; two experiments with low dry weight: ◆ 7.1 mg dry weight, ▲ 5.3 mg dry weight

and 25.5 mg) and another two experiments with low amounts of cells (dry weights: 7.1 and 5.3 mg).

Figure 1 shows that sorption performance increases rapidly within 30 min and then stagnates at the same level. In plating assays we observed no change of viability of the cells. It can also be seen that sorption performance increases when the amount of cells (measured as dry weight) decreases.

From the binding experiments a number of 4×10^5 binding sites per cell was calculated.

Discussion

The present study examined the display of KYE from *K. lactis* on the cell surface of *P. pastoris* by using the AGα-surface display system from *S. cerevisiae* with the aim of constructing a BPA-binding yeast strain.

The synthesis of the KYE-AGα-fusion protein did not seem to influence the growth of the strain significantly as both the host strain and the new strain showed similar growth on glucose and methanol. The BPA binding ability of the novel strain *P. pastoris* pPIKA was confirmed by comparison of the host strain *P. pastoris* GS 115 with the new strain *P. pastoris* pPIKA; the host strain showed only a low BPA sorption due to unspecific binding at the cell wall while the new strain had a significantly increased sorption performance. In addition, induction of BPA binding by methanol showed that sorption of BPA was caused by a protein that was regulated by the methanol-dependent *AOX1* promoter, i.e. the novel strain *P. pastoris* pPIKA expressed the KYE-AGα fusion protein upon induction by methanol.

The influence of pH on BPA sorption as well as the kinetics of BPA sorption provided evidence that BPA was bound on the cell surface, because binding to inner compartments of the cell should not be influenced by changes in outer pH values.

The highest BPA sorption was measured at pH 9.3, being 0.3 pH units below the pK_a of BPA (9.6 according to Staples et al. 2000). This confirms that BPA is bound by KYE as phenolate anion because Abramovitz and Massey (1976) found similar results for the binding of 4-chlorophenol as phenolate anion to OYE, which is homologous to KYE. The binding of 4-chlorophenol is also dependent on pH, reaching its highest value at pH 8.7, i.e. 0.6 pH units below the pK_a of 4-chlorophenol (Abramovitz and Massey 1976).

The kinetics showed that BPA sorption was complete after 30 min (Fig. 1). These kinetics are typical for biosorption processes, which are regarded as passive reactions independent of metabolism (Mogollon et al. 1998). Thus, the sorption kinetics proved that BPA binding of *P. pastoris* pPIKA is a sorption process at the cell surface.

The decrease in sorption performance upon increasing the amount of cells (Fig. 1) was due to the lowering of availability of BPA molecules to cells. This phenomenon is typical of sorption processes at cell surfaces (Puranik and Paknikar 1999).

The number of binding sites per cell (4×10^5) agreed well with values obtained by Yasui et al. (2002) for surface display of estradiol receptors on *S. cerevisiae* cells.

The results clearly support the following conclusions: strain *P. pastoris* pPIKA binds BPA, binding is caused by sorption at the cell surface, and expression of the binding protein is induced by methanol. These results therefore demonstrate that the AGα cell-surface display system from *S. cerevisiae* can be used successfully for cell-surface display in the methylotrophic yeast *P. pastoris*. To the best of our knowledge, this study is the first example of AGα-surface display on *P. pastoris*, thus offering the possibility of combining yeast surface display with the advantages of the *P. pastoris* expression system.

The novel strain *P. pastoris* pPIKA also offers an approach to construct biological filters for removal of the xenoestrogen BPA from wastewater. For this purpose it will be necessary to optimise the new strain by mutation in order to create cells which bind undissociated BPA in a pH-independent manner, as observed for some phenols by Abramovitz and Massey (1976).

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