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Aspects of the progesterone response in *Hortaea werneckii*: Steroid detoxification, protein induction and remodelling of the cell wall

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ABSTRACT

Progesterone in sublethal concentrations temporarily inhibits growth of *Hortaea werneckii*. This study investigates some of the compensatory mechanisms which are activated in the presence of progesterone and are most probably contributing to escape from growth inhibition. These mechanisms lead on the one hand to progesterone biotransformation/detoxification but, on the other, are suggested to increase the resistance of *H. werneckii* to the steroid. Biotransformation can detoxify progesterone efficiently in the early logarithmic phase, with mostly inducible steroid transforming enzymes, while progesterone biotransformation/detoxification in the late logarithmic and stationary phases of growth is not very efficient. The relative contribution of constitutive steroid transforming enzymes to progesterone biotransformation is increased in these latter phases of growth. In the presence of progesterone, activation of the cell wall integrity pathway is suggested by the overexpression of Pck2 which was detected in the stationary as well as the logarithmic phase of growth of the yeast. Progesterone treated *H. werneckii* cells were found to be more resistant to cell lysis than mock treated cells, indicating for the first time changes in the yeast cell wall as a result of treatment with progesterone.

1. Introduction

Steroid hormones are signalling molecules that regulate a host of organismal functions, exerting their actions by binding to the plasma membrane or intracellular receptors of the target cells. Steroids are hydrophobic and relatively stable molecules which cannot be degraded and used as a source of energy in higher organisms. After fulfilling their task they are deactivated, transformed into more soluble forms and excreted via urine and bile into the environment [1].

In contrast to higher organisms microorganisms are capable of biotransformation and even mineralization of steroids [2,3]. Numerous microorganisms that inhabit human and animal tissues, such as the skin and urinary and gastrointestinal tracts [4,5], come into contact with steroid hormones and can transform them [6–10]. After their release from the organisms, steroids are further degraded by mixtures of microorganisms in the sewage treatment plants and the environment [11–14]. These processes are very important because of the effects that undegraded steroid hormones can have on wildlife and on

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man [15,16]. However, the environmental fate and the rate of mineralization of naturally produced steroid hormones are so far poorly characterized.

Biotransformation of steroid hormones in the environment takes place with mixtures of microorganisms, mostly bacteria, and the contribution of individual species is not known precisely. The importance of individual microorganisms for the production of commercially important steroids was first realized in 1952 when Peterson and Murray of the Upjohn Company patented the process of 11α-hydroxylation of steroids by Rhizopus species [17]. Certain of the synthetic chemistry steps have since been replaced by specific microbial transformations which are able to achieve stereo- and regiospecific conversions of the substrates [18–22]. A currently important area is the use of genetic engineering to improve the capabilities of microorganisms as steroid-transforming agents [19,23]. In addition, microorganisms which are adapted to extreme environments have recently gained great interest due to their specific characteristics [24].

The primary interest in steroid biotransformation was at first focused on steroids and the production of commercially valuable products. With time it became evident that steroid hormones can provoke changes in microorganisms. Interest in these changes was increased when the levels of steroid hormones were suggested to affect susceptibility to fungal infection [25–32]. Experiments with exogenous steroids have confirmed the effects of steroids on fungal growth [33–40]. During exposure of microorganisms to steroid hormones, the expression of several members of various gene families is increased [41–43]. Steroid transforming enzymes were connected with defence against steroids. Microorganisms are thought to induce enzymes that detoxify steroids by transforming them into more soluble and less toxic forms [33]. The contribution of constitutive steroid transforming enzymes to steroid detoxification is presently not clear [44].

Our studies of progesterone/yeast interaction have been focused on the halophilic black yeast *Hortaea werneckii*. The natural habitat of this eukaryotic microorganism is hypersaline water [45] but it is also known as the causative agent of *Tinea nigra*, a nonpathologic dermal change of the palms, more frequently observed in females and children [46]. Recently, analysis of the *H. werneckii* proteome suggests the interaction of progesterone with the cell growth and reproduction signalling pathways [42]. In the present study we have focused on progesterone biotransformation and its contribution to progesterone detoxification in different phases of growth of the yeast. We provide evidence for the activation of some of the processes which might, like induction of proteins and remodelling of the cell wall, contribute to adaptation of the yeast to the presence of progesterone and hence its effective escape from growth arrest.

2. Experimental

2.1. Strains and materials

The B-763 *H. werneckii* strain from the microbial culture collection of National Institute of Chemistry, Ljubljana, Slovenia (MZKI) was maintained and grown as described [47]. Steroids were obtained either from Sigma (St. Louis, MO) or from Steraloids Inc. (Wilton, U.S.A.). (1,2,6,7-^{3}H) progesterone was supplied by NEN Du Pont (Dreiech, Germany). Lyticase, a β-1, 3-glucanase from *Arthrobacter luteus*, was obtained from Sigma. All other chemicals were of analytical grade and obtained from standard suppliers.

2.2. Biotransformation of progesterone and product identification

H. werneckii was grown at 28 °C in YNB growth medium until early or late logarithmic and stationary growth phase. Yeast cells were obtained by centrifugation and resuspended in the growth medium to an optical density of $A_{600} = 2.5$. After the addition of progesterone in *N*,*N*-dimethyl formamide (DMF) to 64 μM final conc., biotransformation took place at 28 °C for 24 h in the absence or presence of cycloheximide, 5 μg/ml final conc. Steroids were extracted with chloroform and separated by TLC [48]. The Rf values of the biotransformation products were compared to those of standard steroids. The respective areas were scraped off, eluted with chloroform/methanol (1:1) and the structures of the biotransformation products confirmed by GC–MS analysis as described [48].

2.3. Determination of progesterone biotransformation profile

To elucidate the progesterone biotransformation profile the (1,2,6,7-^{3}H) progesterone, 16 kBq/ml final conc., was submitted to biotransformation in the presence of 16 μM unlabelled progesterone, under the same conditions as described above. The steroids were extracted with chloroform and separated by TLC as above. Radioactive compounds were visualized by fluorography after spraying the air-dried plates with 10% 2,5-diphenyloxazolone [48].

In a parallel experiment TLC separated labelled steroids were scraped off and evaluated quantitatively in a liquid scintillation counter (LKB 1214 Rackbeta liquid scintillation counter).

2.4. Growth inhibition assay

The growth inhibition assay was performed by growing *H. werneckii* on YNB agar plates at 26 °C in the presence of selected steroids. Yeast was grown to the logarithmic phase of growth and diluted to an optical density at 600 nm (OD_{600}) of 0.2. Identical volumes of 0.2 OD_{600} culture, as well as of 1:10 and 1:100 serial dilutions were spotted onto agar plates containing 32 and 320 μM final conc. of tested steroids. Tested compounds were dissolved in DMF and controls were prepared with the appropriate amount of the respective solvent.

2.5. Water solubility of steroids

Water solubility of steroids expressed as Interactive Analysis (IA) log W was obtained by the IA LogP and LogW predictor website http://www.logp.com/.

2.6. Lyticase sensitivity assay

Structural changes in the cell wall of *H. werneckii* were monitored as described [49], using lyticase as the enzyme source. *H. werneckii* cells in the early logarithmic phase were exposed to 320 μM steroids (progesterone and other selected steroids), 32 μM progesterone, DMF (control) or Calcofluor White in a 100 μg/ml final conc. (positive control) for 4 h. After treatment the cells were harvested, washed with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and resuspended in 1.5 ml of the same buffer at OD_{600} of approximately 0.2. After the addition of lyticase in 220 U/ml final conc., cell lysis was followed at room temperature by measuring OD_{600} of the cell suspensions every 10 min up to 1 h.

2.7. Protein sample preparation and protein labelling

H. werneckii cultures growing in the stationary phase of growth were either exposed to 32 μM progesterone or mock treated with DMF (control). After 4 h, progesterone treated cells and the control were harvested, washed with PGSK buffer (3.8 mM NaH_2PO_4, 49.4 mM Na_2HPO_4, 48.4 mM NaCl, 5 mM KCl, 61 mM glucose), frozen in liquid nitrogen, pelleted and mechanically disintegrated as described [42]. Powdered frozen lysates were then resuspended in lysis buffer (4% (w/v) CHAPS, 2 M thiourea, 7 M urea, 30 mM Tris–HCl, pH 8.5), sonicated and centrifuged at maximum speed for 10 min at 4 °C in a tabletop centrifuge.

Proteins from cell lysates were delipidated and desalted by chloroform/methanol extraction, washed with methanol and dissolved in lysis buffer. Protein concentration was determined with Bradford reagent (BIO-RAD). Proteins were labelled with CyDye DIGE Fluors Cy3 and with Cy5 (Amersham Biosciences, Upssala, Sweden); the two samples were combined and analyzed as described [42].

2.8. Protein separation and visualization

Proteins were separated and visualized as described [42]. They were separated in the first dimension on IPG gels of pIs in the pH 4–7 range on an IPGpfor (Amersham Biosciences, Uppsala, Sweden). After isoelectric focusing the IPG strips were transferred on to 12% polyacrylamide gels and electrophoresed in an Ettan DALT*twelve* system (Amersham Biosciences, Uppsala, Sweden) overnight at 2 W per gel at 18 °C.

2D gels were scanned directly between glass plates with a Typhoon Imager (Amersham Biosciences, Upssala, Sweden) with the excitation and emission wavelengths for Cy3 and Cy5. Scanned gels were fixed in 10% methanol, 7% acetic acid for 2 h, stained in Sypro Ruby stain (BIO-RAD) overnight, destained (10% methanol, 7% acetic acid) for 1 h and scanned with the appropriate wavelength for Sypro Ruby stain. Differentially expressed proteins were determined as described [42].

2.9. Mass spectrometry analysis

H. werneckii proteins from the stationary phase of growth were separated on 2D gels and compared to those of the previously identified proteins from the logarithmic phase of growth. The identity of the spots of interest was confirmed using mass spectrometric analysis as described [42].

2.10. Isolation of RNA and Northern hybridisation

H. werneckii in the early logarithmic, late logarithmic and stationary phases of growth was exposed to 320 μM progesterone or mock treated with DMF (control) for 10, 25 or 50 min. Total RNA was isolated as described [50]. The electrophoresis of RNA, reversible staining of RNA blots with methylene blue and hybridisation using 0.5 kb PvuII fragment of cDNA encoding Hsp-1 of fungus *Rhizopus nigricans* were carried out as described [43].

3. Results

3.1. Progesterone biotransformation profiling

Progesterone biotransformation was followed using radioactively labelled progesterone in the absence and presence of cycloheximide with *H. werneckii* cells from the early logarithmic, late logarithmic and stationary phases of growth. Progesterone was transformed into products with 98% efficiency with *H. werneckii* cells from the early logarithmic phase (Fig. 1, line 1). Quantitatively the main product was 11α-hydroxy-progesterone which represented 34% of all the

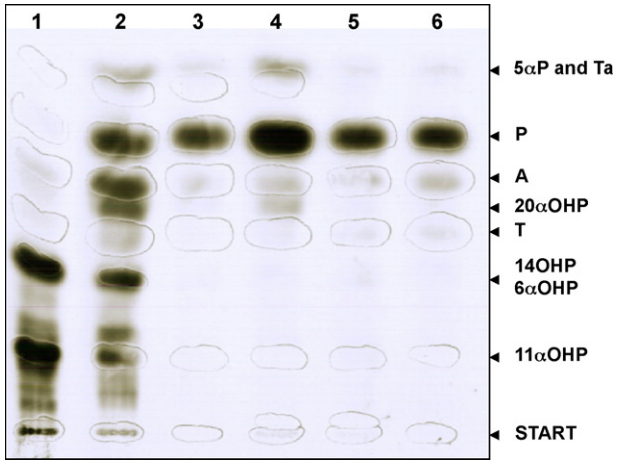

Fig. 1 – Autoradiogram of the progesterone biotransformation products separated by TLC. Lines represent biotransformation profiles of radiolabelled progesterone with *H. werneckii* from the early (1 and 2), late logarithmic (3 and 4), and stationary (5 and 6) phases of growth. The biotransformations were performed in the absence (1, 3 and 5) and presence (2, 4 and 6) of cycloheximide. 5αP: 5α-pregnane-3,20-dione; Ta: testosterone acetate; P: progesterone; A: androstenedione; 20αOHP: 20α-hydroxy-progesterone; T: testosterone; 14OHP: 14-hydroxy-progesterone; 6αOHP: 6α-hydroxy-progesterone; 11αOHP: 11α-hydroxy-progesterone mark the Rf values of the main identified products.

Table 1 – Some characteristics of progesterone biotransformation by *H. werneckii*

Type of biotransformation	Identified progesterone biotransformation products	Effect of cycloheximide	Prevailing growth phase
Hydroxylation	11α-Hydroxy-progesterone 14-Hydroxy-progesterone 6α-Hydroxy-progesterone	I	Early logarithmic
Side chain cleavage	Testosterone acetate Androstenedione Testosterone	NSI	Late logarithmic and stationary
Reduction	4-Pregnen-20α-ol-3-one 5α-Pregnane-3,20-dione Pregnan-3-ol-20-one Pregnane-3,20-diol	NSI	All phases

I – significant inhibition of the respective biotransformation detected; NSI – no significant inhibition of the respective biotransformation detected.

recovered steroids. The involvement of inducible steroid transforming enzymes in progesterone biotransformation was suspected on the basis of the changed biotransformation profile in the presence of cycloheximide (Fig. 1, line 2). The presence of this inhibitor resulted in an increase of the percentage of untransformed progesterone from 2% to 35%, the amount of the recovered 11α-hydroxy-progesterone was lowered to 8% while the amount of recovered androstenedione increased to 20% of the recovered steroids. In contrast, in the late logarithmic and stationary phases of growth, progesterone was transformed less effectively, with only 30% efficiency (Fig. 1, lines 3 and 5). Androstenedione was found to be the most important product, representing 15% of all the recovered steroids, while the amount of 11α-hydroxy-progesterone was lowered to 3%. The presence of cycloheximide did not significantly affect the biotransformation with *H. werneckii* cells from the late logarithmic or stationary phase of growth (Fig. 1, lines 4 and 6).

Analysis of the respective biotransformation products revealed three main types of reactions: hydroxylation at different sites of the steroid nucleus, cleavage of the progesterone side chain at C17 and reduction of the 3- and/or 20-keto groups and Δ^4-double bond (Table 1, Fig. 1). Hydroxylation prevailed in the early logarithmic phase of yeast growth and was catalyzed by inducible enzymes, while side chain cleavage occurred predominantly in the late logarithmic and stationary phases of growth and was not affected by the presence of cycloheximide (Fig. 1, Table 1). Reduction was a minor reaction and was catalyzed by constitutive enzymes in all phases of growth of *H. werneckii* (Table 1).

Seven additional progesterone biotransformation products were identified; most of them were formed with *H. werneckii* from the early logarithmic phase. Pregnane-3,6,20-trione, pregna-4,7-diene-3,20-dione, pregna-4,16-diene-3,20-dione, methyl-testosterone, pregnane-3,20-dione, 21-hydroxy-progesterone and pregnenolone were present in small amounts and were not expected to contribute significantly to progesterone detoxification. The presence of very small amounts of additional steroids in the respective TLC areas (Fig. 1) cannot be excluded.

3.2. *The contribution of different steroid transforming enzymes to progesterone detoxification*

The contribution of the individual biotransformation reactions to progesterone detoxification was estimated by comparing the inhibitory action of selected progesterone biotransformation products on the growth of *H. werneckii* with that of progesterone. All the tested progesterone derivatives were found to be less toxic than progesterone. Hydroxylation of progesterone, e.g., at the 11α position, prevails in the early logarithmic phase (Table 1) and can be considered as effective detoxification (Table 2). Induction of hydroxylases in this phase of growth contributes to the defence of the yeast. The most problematic modification of progesterone from the viewpoint of detoxification is the cleavage of the side chain of progesterone. Androstenedione, as one of the important products of this modification, was found to be only slightly less toxic than progesterone (Table 2). This steroid was detoxified when androstenedione was reduced to non-toxic testosterone in a reaction catalyzed by a constitutive 17β-hydroxysteroid dehydrogenase (17β-HSD) from *H. werneckii* (Table 2). Since the equilibrium of the reaction with the 17β-HSD was found to be shifted towards reduction [51], constitutive 17β-HSD contributes to steroid detoxification. The third type of reaction, e.g., reduction of the 20-keto group of progesterone, was only slightly less effective than hydroxylation of the steroid (Table 2).

3.3. *Polarity of progesterone biotransformation products and their toxicity*

Hydroxylation of progesterone was found to increase water solubility of the steroid, as suggested by the predicted IA log W values for the hydroxylated derivatives of progesterone. At the same time, a decrease of toxicity was observed, as shown for 11α-hydroxy-progesterone (Table 2).

The connection between the polarity of progesterone derivatives and their toxicity was further investigated by growth inhibition assays with a water-soluble form of progesterone (progesterone WS) and pregnenolone. Neither steroid, with higher water solubilities than progesterone, was found to inhibit *H. werneckii* growth (Table 2). The same effect was

Table 2 – Water solubility of progesterone biotransformation products and other selected steroids and their potency in inhibiting *H. werneckii* growth

Steroid	IA log W	*H. werneckii* growth inhibition
Substrate		
Progesterone	−4.72	I
Biotransformation products		
11α-Hydroxy-progesterone	−3.44	NSI
14-Hydroxy-progesterone	−3.76	nd
6α-Hydroxy-progesterone	nd	NSI
Testosterone acetate	−4.64	nd
Androstenedione	−4.40	I
Testosterone	−4.20	NSI
4-Pregnen-20α-ol-3-one	−4.57	i
5α-Pregnane-3,20-dione	−4.84	NSI
Pregnan-3-ol-20-one	−4.75	NSI
Pregnane-3,20-diol	−5.00	NSI
Other selected steroids		
Pregnenolone	−4.17	NSI
Progesterone WS	Water soluble	NSI
11β-Hydroxy-androstenedione	−3.42	NSI
5α-Androstane-3,17-dione	−4.43	I
5β-Androstane-3,17-dione	−4.43	I
17α-Methyl-testosterone	−4.42	i
4-Estrene-3,17-dione	−4.02	I

I – stronger inhibition of *H. werneckii* growth at 320 μM and weak at 32 μM concentration of the respective steroid; i – weak inhibition of *H. werneckii* growth at 320 μM concentration and no significant inhibition at 32 μM concentration of the respective steroid; NSI – no significant inhibition detected; nd – not determined; IA log W – predicted water solubility of steroids as obtained by interactive analysis (IA) predictor website http://www.logp.com/.

observed when the toxicity of androstenedione was compared to that of 11β-hydroxy-androstenedione (Table 2). On the other hand an additional nonpolar methyl group of 17α-methyl-testosterone lowered water solubility and resulted in slightly increased toxicity (Table 2). These results are in accordance with the above hypothesis that reactions which increase water solubility of the steroid decrease its toxicity.

A closer look at the products of the second group of reactions, which lead to C19 steroids, shows that the transformed products are also more water soluble than progesterone, although the increase is much smaller than that for hydroxylated derivatives of progesterone. Of these derivatives, androstenedione was still found to inhibit growth of *H. werneckii* only slightly less efficiently than progesterone.

The reduction reactions of progesterone from the third group of progesterone biotransformations resulted in products with only slightly increased, as for 4-pregnen-20α-ol-3-one, or even decreased water solubility (Table 2). None of these products was found to be an inhibitor of *H. werneckii* growth.

3.4. *Common structural characteristics of H. werneckii inhibitory steroids*

Progesterone and androstenedione, both toxic to *H. werneckii*, contain a 3-keto-4-ene structure and an additional keto group at C20 or C17. Therefore a steroid with the same functional groups from the estrane series, 4-estrene-3,17-dione, was tested for its toxicity on the yeast. It was also found to be toxic (Table 2), suggesting some of the common structural characteristics of steroids toxic to *H. werneckii*.

In addition, two steroids of alternative stereochemistry, 5α-androstane-3,17-dione and 5β-androstane-3,17-dione, were found to inhibit growth of *H. werneckii* with almost the same efficiency (Table 2).

3.5. *The expression of the selected proteins in H. werneckii in the presence of progesterone*

We studied the effect of progesterone on the expression of *H. werneckii* proteins during the logarithmic and stationary phases of growth. Protein kinase C-like 2 (Pck2) and proliferating cell nuclear antigen (PCNA) were overexpressed in the presence of progesterone during the stationary phase of growth (Fig. 2). This is in accordance with our previous results showing an increase during logarithmic phase [42]. Hsp70 was not observed as one of the identified proteins with changed expression level during stationary phase. Its level did not change significantly in response to the steroid (Fig. 2), in contrast to Hsp70 overexpression in the presence of progesterone during the logarithmic phase of growth [42].

To further evaluate the changes following application of progesterone, we determined the *H. werneckii* Hsp70 transcript level by Northern blot analysis. Temporal analyses of Hsp70 mRNA abundance were carried out during treatment of the yeast grown to the early logarithmic, late logarithmic and stationary phases with progesterone (Fig. 3). The Hsp70 mRNA level was nearly undetectable when DMF was added to the growth medium in the early logarithmic and late logarithmic phases of growth, while the transcript level of Hsp70 was increased within 10 min of exposure of yeast to progesterone. In contrast, in the stationary phase of *H. werneckii* growth the amount of Hsp70 mRNA is nearly the same before and after exposure of the yeast to progesterone (Fig. 3).

3.6. *Changes in the cell wall of H. werneckii in the presence of progesterone*

Possible structural changes in the cell wall of *H. werneckii* as a result of exposure to progesterone were investigated using the lyticase sensitivity assay. The results indicate decreased sensitivity to lyticase for *H. werneckii* cells treated with progesterone (Fig. 4). The effect was most clearly seen with cells from the early logarithmic phase of growth, but could only just be observed with cells from the stationary phase of growth (data not shown). The effect was found to be specific for the free form of progesterone, being only just observed with the water-soluble form of progesterone (Fig. 4A). Exposure of *H. werneckii* cells to progesterone resulted in an effect similar to that on exposure to Calcofluor White and concentration dependent (Fig. 4B) but was only just observed with the non-toxic steroid 11α-hydroxy-progesterone (Fig. 4A). Intra-assay variability of the results of duplicate experiments was low (data not shown) while inter-assay/inter-sample variability was higher. The results suggest some differences in the cell

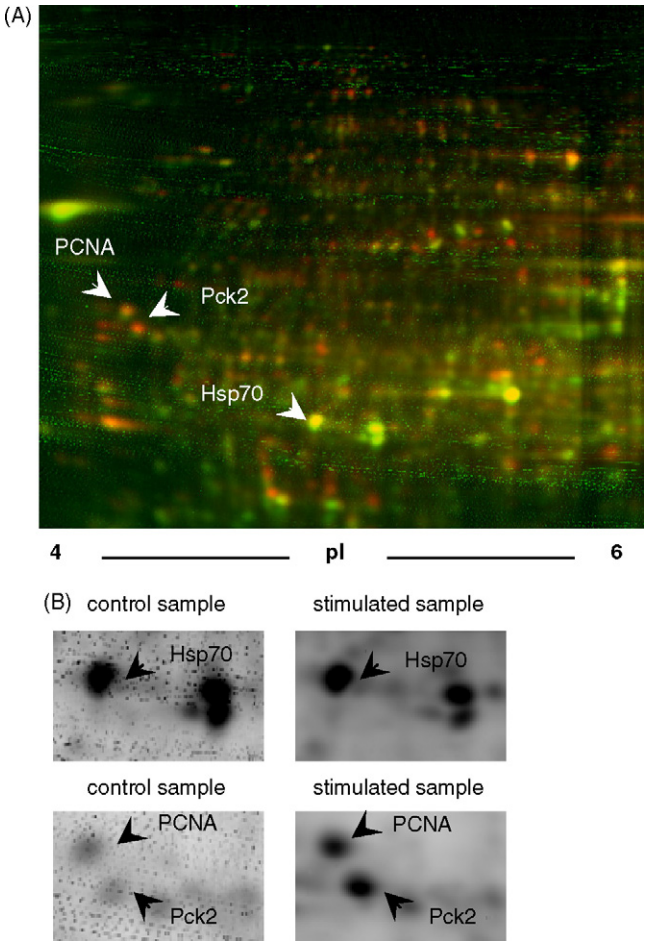

Fig. 2 – The effect of progesterone on the expression of proteins in *H. werneckii* during the stationary phase of growth. (A) Overlay of 2D-GE images of control sample (green) and progesterone-stimulated sample (red). (B) Enlarged regions of the identified proteins. On the left are images of gels loaded with control sample and on the right gels loaded with progesterone-stimulated sample. The arrows point to heat shock protein 70 (Hsp70), proliferating cell nuclear antigen (PCNA), and protein kinase C-like 2 (Pck2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

wall composition between the cultures grown for each individual experiment (Fig. 4).

4. Discussion

The biotransformation of progesterone has been studied extensively during the last decades [3,20,35,52–65]. It was found to affect the growth of different eukaryotic microorganisms [33–40], provoke the induction of steroid hydroxylating enzymes [33,66–68], trigger the induction of heat shock proteins [43] and ABC transporters [69–71]. In an expression profiling of the progesterone response in *Saccharomyces cerevisiae*, 163 genes were found to be upregulated and 40 downregulated [41].

In our previous study progesterone was found to cause a transitional arrest of cell growth and a change in gene expression in *H. werneckii* [42]. Here, we were interested in progesterone biotransformation/detoxification by the same microorganism. The major progesterone biotransformation products suspected to have the greatest effect on progesterone detoxification were identified from different phases of *H. werneckii* growth (Table 1). We compared the amounts of identified products obtained during biotransformation in the absence and presence of cycloheximide. In this way we were able to study the contribution of different types of biotransformation to progesterone detoxification and suggest the role of constitutive and inducible steroid transforming enzymes. The results show that induction of steroid transforming enzymes by progesterone constitutes an effective part of the defence of the yeast in the early logarithmic phase of growth. Our data confirm the hypothesis that reactions with inducible steroid transforming enzymes, e.g. hydroxylases, increase the polarity of steroids and effectively contribute to their detoxification (Tables 1 and 2). This type of defence is less effective in the late logarithmic and stationary phases of growth of *H. werneckii*. The yield of biotransformation products in these phases of growth is much lower and some of the products, e.g. androstenedione, are still toxic (Fig. 1 and Table 2). The yeast appears not to be able to respond, by increasing the expression of detoxifying enzymes, to the same extent as in the early logarithmic phase of growth. In the late logarithmic and stationary phases the relative contribution of constitutive steroid transforming enzymes, e.g., 17β-HSD, is increased. These enzymes are less effective in detoxifying progesterone and its derivatives. At the same time we could not establish a clear relationship between the water solubility of biotransformation products of constitutive steroid transforming enzymes and their toxicity (Table 2).

Steroids that inhibit growth of *H. werneckii*, e.g., progesterone, androstenedione, 4-estrene-3,17-dione, possess common structural characteristics. Our results show that partial or complete detoxification of progesterone or its derivative androstenedione can be achieved by e.g., reduction of 20- or 17-keto groups, which appear to be important for the toxicity of the respective steroids. However, the stereochemistry of steroids has almost no effect on the effectiveness of *H. werneckii* growth inhibition, as shown for the stereochemically different 5α-androstane-3,17-dione and 5β-androstane-3,17-dione (Table 2).

Progesterone biotransformation is just one of the processes that might contribute to the escape of the *H. werneckii* from growth arrest. Recently, *H. werneckii* cells treated with progesterone in the logarithmic phase of growth were investigated and the overexpression of Pck2 was noted [42]. In the present study we show that progesterone provokes an increase in Pck2 levels in *H. werneckii* in the stationary phase of growth (Fig. 2). These results are in accordance with the observation that quiescent cells are still able to respond to environmental signals by inducing the expression of genes similar to those in proliferating cells, despite their increased resistance to environmental stress [72,73].

Pck2 is a member of the cell wall integrity pathway which is activated under the conditions that jeopardize cell wall stability [74]. The mechanism by which progesterone stimu-

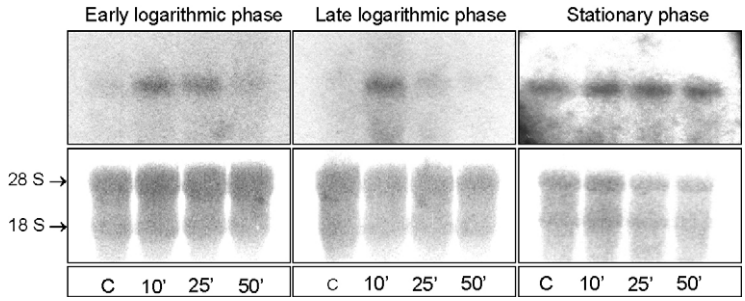

Fig. 3 – Temporal analysis of Hsp70 mRNA levels during treatment of *H. werneckii* with progesterone. The *H. werneckii* cells in early, late logarithmic and stationary phases of growth were exposed to N,N-dimethylformamide (DMF) alone (C) and, for the indicated times (min), to 320 μM progesterone. Total RNA blots were prepared, stained reversibly (lower panels) and hybridised (upper panels) as described in Section 2.10.

lates the induction of Pck2 and its activation in *H. werneckii* is not known. Steroid hormones are most probably, because of their low solubility, present in aqueous medium as a suspension [75]. During the process of crossing the cell wall and cell membrane, progesterone and other toxic steroids might therefore cause an acute stretching of the plasma membrane and/or otherwise modify the plasma membrane, thus activating specific cell surface sensor proteins [74]. On the other hand perturbation of the plasma membrane affects the activity of the plasma membrane-bound enzymes involved in cell wall synthesis and indirectly weakens the cell wall [76]. It has been shown that chitosan, as a plasma membrane perturbing substance, which can bind membrane phospholipids, affects the cell wall integrity pathway [77]. In our study we were interested in changes that progesterone might provoke in the plasma membrane of *H. werneckii*. Measurements of the fluidity of the *H. werneckii* membrane showed no detectable changes following the addition of progesterone (M. Turk and M. Žakelj-Mavrič, unpublished results).

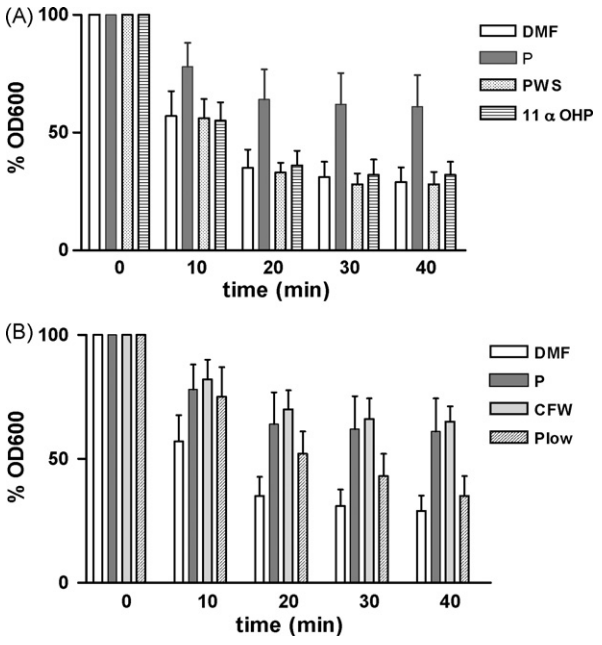

Fig. 4 – The effect of selected steroids on the susceptibility of *H. werneckii* to lytic action by lyticase. Yeast cells from the early logarithmic phase were treated for 4 h with: (A) progesterone (P), 11α-hydroxy-progesterone (11αOHP), water soluble form of progesterone (PWS) or N,N-dimethylformamide (DMF) alone; (B) progesterone (P), lower concentration of progesterone (P_{low}), Calcofluor White (CFW) or DMF alone. The steroid concentrations were 320 μM; progesterone P_{low} was 32 μM. The percentage of the initial absorption A_{600} after the addition of lyticase (220 U/ml final conc.) is presented. Data are means ± standard deviations of at least three independent experiments performed in duplicate.

Cell wall stress provokes a global transcriptional response in *S. cerevisiae* [78]. The cell wall integrity pathway is activated with the help of specific plasma membrane sensor proteins, leading finally to alterations in the cell wall [74,78–80]. In *S. cerevisiae* and *Schizosaccharomyces pombe* the components of the cell wall integrity pathway, e.g. protein kinase C homologues pkc1 and pck1p/pck2p, respectively, have been extensively studied and their cell wall targets identified [74,78,81–85]. The pathway was suggested to be conserved in several yeasts and filamentous fungi [86–88]. Although in *H. werneckii* the cell wall integrity pathway has not yet been investigated, the overexpression of Pck2, and most probably its increased activity in the presence of progesterone, suggested that changes in the cell wall could be one of the possible effects of progesterone action. In our study we showed for the first time that structural changes occur in the cell wall of *H. werneckii* treated with progesterone, treated *H. werneckii* cells being more resistant to cell lysis (Fig. 4). The effect is specific for the free form of progesterone and was not detected with *H. werneckii* cells exposed to 11α-hydroxy-progesterone. The effect of progesterone is comparable to that of Calcofluor White which has been shown to hinder normal cell wall assembly in *S. cerevisiae* and consequently activate the cell wall integrity pathway [89]. Our results suggest that changes in the *H. werneckii* cell wall induced by progesterone most probably contribute to an adaptation of the yeast to the presence of the steroid.

Another protein whose biosynthesis is stimulated in the presence of progesterone in the logarithmic [42] and stationary phases of growth is proliferating cell nuclear antigen (PCNA) (Fig. 2). PCNA has an essential role in the nucleic acid

metabolism of all eukaryotes. It can interact with a large number of proteins and is involved in several processes, e.g., DNA replication, DNA repair, cell cycle regulation, and apoptosis [90]. The role of increased expression PCNA in progesterone stimulated *H. werneckii* cells however remains unclear.

Although cells of *H. werneckii* in the stationary phase of growth are able to respond to stress triggered by progesterone by increasing the expression of proteins such as Pck2 and PCNA, they are not able to increase the levels of Hsp70 mRNA and protein (Figs. 2 and 3). One explanation is that cells of *H. werneckii* in the stationary phase of growth are most probably stressed due to lack of nutrients. This could be the reason for the increased levels of Hsp70 mRNA and protein in untreated cells from the stationary phase of growth. These levels do not increase when progesterone is added.

Finally, the question remains as to whether the response to progesterone is unique for *H. werneckii*. On the basis of preliminary experiments on *S. cerevisiae* (data not shown) and some published data [32,41,91,92] we believe it is not unique to *H. werneckii*, although the extent of the response is most probably yeast cell specific.

The results presented in this paper lead to the conclusion that progesterone is toxic to *H. werneckii*, inhibiting its growth. Progesterone biotransformation contributes to an escape from growth arrest of the yeast, most efficiently in the early logarithmic phase of growth. The overexpression of Pck2 in the presence of progesterone suggests the cell wall as one of the possible targets of progesterone action. The progesterone induced changes observed in the yeast cell wall most probably contribute to an adaptation of the yeast to the presence of the steroid.

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