Regulation of chitin synthase activity in the dimorphic fungus *Benjaminiella poitrasii* by external osmotic pressure

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Abstract

The effects of changes in external osmotic pressure on chitin synthase activity of a dimorphic fungus, *Benjaminiella poitrasii*, have been investigated. Mycelial and yeast cells incubated in medium of low osmolality (distilled water, 0 mOsm) for 10 min had 2–3-fold higher specific activities of native chitin synthase in mixed membrane preparations than cells that had been subjected to a high osmolality medium (1.2 M sorbitol in distilled water, 1612 mOsm). Cells suspended in media of different osmolalities for 10 min were also affected in the extent of germ tube formation. Germ tube formation was highest in cells incubated in low osmolality medium. The addition of protein phosphatase inhibitors (cyclosporin A, 1.2 μg/ml; cantharidin, 20 μM) abolished the effect of hypo-osmotic stress on chitin synthase activation of yeast mixed membrane preparations. The presence of protein kinase inhibitors (genistein, 40 μg/ml; H-7, 100 μM) and a Ca^{2+} channel blocker (verapamil, 50 μM) reduced chitin synthase activity to 50–60% of that observed in cells under hypo-osmotic shock. These inhibitors also inhibited germ tube formation. This suggests that chitin synthase activity and yeast hyphal morphogenesis are both subject to regulation by osmotic pressure, phosphorylation and calcium.

Keywords: *Benjaminiella poitrasii*; Chitin synthase activation; Osmotic stress; Signal transduction inhibitor

1. Introduction

The yeast- mycelium transition in *Benjaminiella poitrasii* is accompanied by significant changes in the chemical composition of the cell wall [1,2]. The level of chitin, one of the main cell wall constituents, is three times higher in mycelium than in the yeast form cells. This difference in chitin levels suggests that chitin synthase (EC 2.4.1.16) may have a regulatory role in the yeast- mycelium transition in *B. poitrasii*.

Various regulatory processes have been suggested for temporal and spatial control of chitin synthase activity in fungi [3,4]. The chitin synthase activity present in cell-free extracts of the majority of fungi is largely in a zymogenic form and differential activation of chitin synthase may be one of the factors which control morphogenesis in fungi [3,4]. The activities of membrane-bound enzymes can be affected significantly by changes in membrane fluidity. It has been suggested that chitin synthase could be regulated locally by membrane stress or local stretching [3,5]. In *Candida albicans* and *Coprinus cinereus*, cells which were subjected to hypo-osmotic stress had in-
increased native chitin synthase specific activities [6]. In the present communication, this hypothesis has been investigated in B. poitrasii in both mycelium and yeast cells.

2. Materials and methods

2.1. Chemicals

Uridine diphosphate-[\textsuperscript{14}C]\textsuperscript{}N-acetylglucosamine (UDP\textsuperscript{14}C-GlcNAc, specific activity, 250 \textmu Ci/\mu mol) was from Amersham International, UK. Cyclosporin A was a gift from Sandoz, Switzerland. Biochemicals were purchased from Sigma-Aldrich, UK. All other chemicals used were of analytical grade.

2.2. Organism

Stock cultures of B. poitrasii parent strain were maintained by subculturing weekly on slants of YPG agar (yeast extract, 0.3%; peptone, 0.5%; glucose, 1.0%; agar, 2%) [1]. To get mycelial form cells, YP (without glucose) medium was inoculated with a sporangiospore suspension and was incubated under shaking conditions (180 rpm) at 30°C for 24 h. Yeast cells were obtained under identical conditions of incubation in YP5G (5% glucose) medium.

2.3. Yeast-mycelium transition

The yeast-mycelium transition experiments were carried out as described earlier [7,8]. Yeast inoculum was grown in YP5G medium for 24 h at 30°C and the transition was studied in YP medium at 30°C. The morphology (yeast/mycelium) was determined as described earlier [8].

To study the effect of osmotic stress on the yeast-mycelium transition, yeast cells were incubated in different media for 10 min at 30°C. The osmolalities of the media were (mOsm): distilled water, 0; YP, 57; 0.3 M sorbitol in distilled water, 351; YP5G, 383; 1.2 M sorbitol in distilled water, 1612. The cells were then incubated in YP medium at 30°C for 4 h and the percentage of cells forming germ tubes was assessed as described before [8].

To study the effect of signal transduction inhibitors on the yeast-mycelium transition, yeast cells were grown in YP5G for 24 h at 30°C, then were incubated in YP medium containing exogenously added inhibitors (40 \mu g/ml genistein, 100 \mu M H-7, 20 \mu M cantharidin, 0.3 and 1.2 \mu g/ml cyclosporin A or 50 \mu M verapamil) at 30°C for 18 h and the percentage germ tube formation was assessed. Stock solutions of inhibitors were prepared in DMSO.

2.4. Exposure of whole cells to osmotic shock

To study the effect of osmotic stress on the chitin synthase activity of membrane fractions, whole yeast or hyphal cells were pre-incubated in the media of different osmolalities for 10 min at 30°C then processed for the isolation of mixed membrane fractions.

Table 1

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Chitin synthase activity (pkat/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dist.water (0 mOsm)</td>
</tr>
<tr>
<td>Mycelium</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>2.16 ± 0.44</td>
</tr>
<tr>
<td>Trypsinized</td>
<td>3.0 ± 1.14</td>
</tr>
<tr>
<td>Activation ratio</td>
<td>1.38</td>
</tr>
<tr>
<td>Yeast cells</td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>2.25 ± 0.61</td>
</tr>
<tr>
<td>Trypsinized</td>
<td>3.5 ± 0.71</td>
</tr>
<tr>
<td>Activation ratio</td>
<td>1.55</td>
</tr>
</tbody>
</table>

Results are average ± S.D. of 3 sets of triplicate experiments.
Whole cells and mycelia were incubated in the respective media for 10 min at 30°C. Mycelium and yeast cells were grown in YP and YP5G, respectively, for 24 h at 30°C.
The effect of pre-incubation with signal transduction inhibitors on chitin synthase activity of the membrane fraction was studied in yeast cells (grown in YP5G medium for 24 h at 30°C) incubated in the same medium containing respective chemicals dissolved in DMSO for 15 min at 30°C, separated by centrifugation and incubated in distilled water containing DMSO (40 µl/ml) with and without inhibitors for 10 min at 30°C. Controls showed that this concentration of solvent did not affect chitin synthase activity or germ tube formation.

2.5. Preparation of mixed membrane fraction

All the manipulations were carried out at 4°C, unless otherwise stated. After the respective treatments, cells were harvested by centrifugation (3000 x g, 5 min) and 1 g fresh weight of pellet was washed twice with TM (50 mM Tris-HCl, pH 7.5; 2.5 mM MgCl₂) with 1 mM EDTA, resuspended in TM buffer (1 ml for 250 mg wet weight) and homogenised with a glass homogeniser. The homogenate was centrifuged (2000 x g, 5 min) to remove cell wall debris and then at 100 000 x g for 40 min to separate the mixed membrane fraction and cytosol. The membrane pellet was resuspended in 250 µl TM buffer containing 30% glycerol. For digitonin solubilisation,

Table 2
Effect of pre-incubation with various signal transduction inhibitors on native chitin synthase activities of yeast membrane fractions of Benjaminiella poitrasi

<table>
<thead>
<tr>
<th>Medium</th>
<th>Chitin synthase activity (pkat/µg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>1.1 ± 0.42</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1.66 ± 0.53</td>
</tr>
<tr>
<td>Genistein (40 µg/ml)</td>
<td>0.9 ± 0.6</td>
</tr>
<tr>
<td>H-7 (100 µM)</td>
<td>1.2 ± 0.44</td>
</tr>
<tr>
<td>Cantharidin (20 µM)</td>
<td>0.8 ± 0.44</td>
</tr>
<tr>
<td>Cyclosporin A (1.2 µg/ml)</td>
<td>0.46 ± 0.22</td>
</tr>
<tr>
<td>Verapamil (50 µM)</td>
<td>0.9 ± 0.5</td>
</tr>
</tbody>
</table>

Results are average ± S.D. of 2 sets of triplicate experiments.

Yeast cells were grown in YPG (5% glucose) for 24 h at 30°C. The cells were pre-incubated in the same medium containing respective chemicals dissolved in DMSO for 15 min at 30°C then incubated in distilled water containing DMSO (40 µl/ml) with and without inhibitor for 10 min at 30°C. Further operations to prepare and assay mixed membrane fraction were carried out as described in Section 2.

*Control, cells grown for 24 h in YP5G.

Table 3
Effect of signal transduction inhibitors on yeast-myelium transition in Benjaminiella poitrasi

<table>
<thead>
<tr>
<th>Medium</th>
<th>Germ tubes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control*</td>
<td>95 ± 5</td>
</tr>
<tr>
<td>Genistein (40 µg/ml)</td>
<td>76 ± 5</td>
</tr>
<tr>
<td>H-7 (100 µM)</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>Cantharidin (20 µM)</td>
<td>88 ± 6</td>
</tr>
<tr>
<td>Cyclosporin A (0.3 µg/ml)</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Cyclosporin A (1.2 µg/ml)</td>
<td>0</td>
</tr>
<tr>
<td>Verapamil (50 µM)</td>
<td>57 ± 7</td>
</tr>
</tbody>
</table>

Results are average ± S.D. of 3 sets of experiments.
The yeast cells, grown in YP5G for 24 h at 30°C, were incubated in YP medium at 30°C for 18 h and the percentage of cells forming germ tube or budding was assessed. All the solutions were prepared in DMSO.

*YP with DMSO (40 µl/ml).

the mixed membrane fraction was resuspended in 1% digitonin in TM buffer, incubated at 0°C for 30 min, and centrifuged (165 000 x g, 1 h).

2.6. Trypsin treatment of enzyme preparations

Trypsin (porcine pancreas) was added to membrane preparations to activate chitin synthase, at a final concentration of 50 µg/ml, and incubated at 25°C for 10 min. The reaction was stopped by adding soya bean trypsin inhibitor (final concentration, 75 µg/ml).

2.7. Enzyme assay

Each assay was 50 µl containing 10 µl of enzyme preparation and final concentrations of Tris-HCl (30 mM, pH 7.4 and pH 8.0 for yeast and mycelium enzyme preparations, respectively), MgCl₂ (10 mM), N-acetylglucosamine (25 mM), UDP-GlcNAc (1 mM) containing 25 nCi UDP-¹⁴C-GlcNAc. The assay mixture was incubated at 37°C for 1 h and the reaction was terminated by the addition of 66% (v/v) ethanol. The assay mixture was filtered through glass microfibre filters (presoaked with 10% TCA; Whatman GF/C, 2.5 cm) on a Millipore Manifold system. The reaction tubes were washed twice onto the filter with 1 ml 1% Triton X-100 and the filters were washed with 4 ml 66% ethanol. The filters were dried at 80°C for 2 h and transferred to scintillation vials containing 3 ml scintillation fluid (4 g PPO (2,5-
diphenyloxazole) and 0.1 g POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene) in 1 l toluene for radioactive counting. The total and specific activities were calculated as described earlier [9]. The results showed quite wide variation in the specific activities between experiments. The data presented are therefore the average of three sets of triplicate experiments, unless otherwise stated.

2.8. Protein determination

Protein was estimated using the Bradford method with bovine serum albumin as standard [10].

3. Results

3.1. Properties of chitin synthase preparations

The pH optima for the chitin synthase activities of mixed membrane fractions were 7.4 and 8.0 for yeast and mycelial cells, respectively, and so further assays were performed at the appropriate pH. Chitin synthase activities from both mycelium and yeast cells showed some activation after treatment with trypsin (Table 1). Trypsin activation in the mixed membrane preparation was rapid and maximal activity was observed after 10 min treatment at 25°C followed by progressive loss of activity (not shown). In all the mixed membrane preparations of B. poitrasii, there was no appreciable increase in the native activity in the digitonin-solubilised fraction. Total activities after treatment with digitonin and treatment with trypsin of solubilised fraction and pellet were, however, five- and two-fold higher than the untreated samples for mycelial and yeast cells, respectively.

3.2. Effect of osmotic stress on chitin synthase activity and on yeast-mycelium transition in B. poitrasii

The effect of external osmotic pressure changes on chitin synthase activity in B. poitrasii was studied in whole cells (Table 1). When incubated in media of different osmolalities, the 24 h mycelial and yeast cells showed higher native specific activities of chitin synthase in membrane preparations from cells which had been subjected to low osmolality than from cells subjected to high osmolality media. Although the results showed quite wide variation between experiments, the specific activities of chitin synthases in membranes prepared from whole cells decreased as the osmolality of the medium increased (Table 1). For mycelial cells there were no marked differences in the activation ratio of the trypsin-treated enzyme preparations from the cells incubated in low or high osmolalities. For yeast cell preparations, however, the activation ratio was higher from cells incubated at higher osmotic pressure (Table 1).

The influence of osmotic stress in the initial period of yeast-mycelium transition was also studied. Yeast cells pre-incubated for 10 min in media of various osmolalities exhibited different extents of germ tube formation at 4 h. Germ tube formation of cells pre-incubated in YP medium was 50 ± 7.0% within 4 h. In the same period of time, the cells that had been suspended in medium of low osmolality (distilled water, 0 mOsm) showed 64 ± 5% germ tube formation, while the cells incubated in high osmolality medium (1.2 M sorbitol in distilled water, 1612 mOsm) exhibited significantly reduced germ tube formation (20 ± 3.0%).

3.3. Effect of signal transduction inhibitors on chitin synthase activity and on yeast-mycelium transition in B. poitrasii

Inhibitors of protein kinases (40 μg/ml genistein, tyrosine protein kinase inhibitor; 100 μM H-7, inhibitor of cyclic nucleotide-dependent protein kinase and protein kinase C), protein serine/threonine phosphatase, type 1A and 2A inhibitor (20 μM cantharidin) and type 2B inhibitor (1.2 μg/ml cyclosporin A) and Ca²⁺ channel blocker (50 μM verapamil) were studied to determine their effects on the activation of chitin synthase from yeast mixed membrane preparation (Table 2). Cyclosporin A and cantharidin abolished the effect of membrane stress on the chitin synthase activation in B. poitrasii yeast mixed membrane fraction (Table 2). Protein kinase inhibitors and verapamil reduced chitin synthase activity to 50–60% compared to the native activity after exposure of cells to hypo-osmotic stress.

The above signal transduction inhibitors also affected the yeast-mycelium transition (Table 3). Cyclosporin A (0.3–1.2 μg/ml) significantly reduced germ tube formation, while growth by budding was
unaffected. Only $36\pm4\%$ germ tube formation was observed within 18 h with 0.3 μg/ml cyclosporin A, germ tube formation was totally inhibited above 1.2 μg/ml. However, cantharidin did not show the same extent of inhibition. The protein kinase inhibitors genistein and H-7 reduced germ tube formation by 25–60%.

4. Discussion

The saponin digitonin is thought to solubilise chitin synthase activity that complexes with membrane sterols. Gooday and de Rousset-Hall [9] observed that chitin synthase activity of C. cinereus was solubilised by digitonin treatment, with an increase in specific activity and stability. In B. poiratii, there was no appreciable solubilisation of native chitin synthase activity after digitonin treatment. However, total activities of the digitonin-treated samples, i.e. activities in trypsin-treated solubilised and pellet fractions, were higher than in the untreated samples. This can be attributed to the increase in the stability of the enzyme due to digitonin treatment.

Osmotic stress imposed by growing whole cells in media of different osmolalities influenced native chitin synthase activities of mixed membrane preparations. Cells suspended at low osmolality (0 mOsm) exhibited increased germ tube formation compared to cells incubated at high osmolality (1612 mOsm). This correlates with a three-fold increase in native chitin synthase activity in the yeast cells subjected to hypo-osmotic stress (Table 1).

The relationship between membrane stress and the activation of zymogenic chitin synthase by trypsin treatment is still unclear. It has been observed in C. albicans that the specific activities of the trypsin-treated enzyme preparations were higher in protoplasts incubated in high osmolality media than in protoplasts in low osmolality media [6]. Similar effects have been observed in B. poiratii yeast cells; i.e. the difference in specific activity between the two treatments was reduced (Table 1). However, in membrane preparations of mycelial cells, the activation ratios (trypsin-treated:native activity) were similar in all the treatments (Table 1).

Eukaryotic cellular functions depend on a variety of signalling mechanisms, in which second messengers such as Ca$^{2+}$ and cAMP translate external stimuli into specific intracellular responses [11,12]. Many signalling pathways involve protein kinases and phosphatases. Using various signal transduction inhibitors [13,14], correlations between the yeast-mycelium transition, the presence of various inhibitors of signal transduction pathways and chitin synthase activation due to hypo-osmotic stress were studied. Cyclosporin A, a protein phosphatase type 2B inhibitor, abolished the effect of hypo-osmotic stress on chitin synthase activity of the yeast membrane fraction (Table 2) and also reduced the yeast-mycelium transition to a greater extent (Table 3). Correlations between the effect of other signal transduction inhibitors and chitin synthase activation and germ tube stimulation by hypo-osmotic stress were not observed. This study, however, indicates that chitin synthase activity in B. poiratii is regulated in part both by external osmotic pressure and by phosphorylation. These results are almost certainly the net effects of treatments on a family of chitin synthase isozymes [15].

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References


