Pichia pastoris INO1 gene expression affects intracellular maltase activity and MAL1+ gene expression in Schizosaccharomyces pombe

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In our previous study, we found that the transformant Sch.p944 which contains the plasmid pADH-INO synthesized higher inositol than the transformant Sch.p1025 which contains the plasmid pSPIN-22. In this study, we examined the influence of INO1 gene expression on intracellular maltase activity and MAL1+ gene expression in these two transformants. The highest specific maltase activity was observed when Sch.p944 was grown in the synthetic medium containing initial 2.0% (w/v) glucose, whereas glucose repression of maltase activity occurred when Sch.p1025 was cultivated in the same medium containing glucose more than initial 0.5% (w/v), suggesting that synthesis of the maltase was derepressed in Sch.p944. Also, higher mRNA encoding intracellular maltase activity was found in Sch.p944 than that in Sch.p1025 after 32 h of cell growth in the media containing initial 2.0% glucose, indicating that the derepression occurred at the transcriptional level. Furthermore, higher phosphatidylinositol (PI) content was detected in Sch.p944 cells grown in the medium containing initial 2.0% (w/v) glucose for 32 h than in Sch.p1025 cells grown under the same conditions, indicating that PI might be involved in derepression of MAL1+ gene expression and intracellular maltase activity.

Keywords: MAL1+ gene expression, Maltase activity, Schizosaccharomyces pombe, phosphatidylinositol, Glucose repression

A large set of genes that function in carbon source utilization can be repressed by glucose in yeasts12. Furthermore, Schizosaccharomyces pombe is also one of the ideal eukaryotic microorganisms for biochemical and genetic studies34. Although some genes such as INV1+ gene encoding secreted invertase are repressed by glucose in S. pombe3, little is known about glucose repression mechanism in S. pombe. Earlier, we reported inositol and phosphatidylinositol (PI)-mediated glucose derepression, gene expression and invertase secretion in Saccharomyces and Schizosaccharomyces36-11. We found that invertase secretion and SUC2 gene expression were derepressed, when the cells of Saccharomyces sp. W4 growing in the synthetic medium with initial 2.0% (w/v) glucose contained higher PI content6.

In another study8, it was found that higher concentration of added inositol in the synthetic medium derepressed invertase secretion and INV1+ gene expression in S. pombe. We thought that the derepression was related to higher content of PI in the yeast cells and PI-type signaling pathway might be involved in activation of transcription of INV1+ gene in this yeast9. In recent study10, we found that the increase in inositol in the cells of S. pombe also caused rise in PI content and invertase secretion and synthesis of mRNA encoding invertase was derepressed in the cells containing higher PI content. In addition, intracellular maltase activity and MAL1+ gene expression in S. pombe was also derepressed, when the cells contained higher PI content11.

Some strains of S. pombe are naturally inositol-requiring organisms, as the genome in this yeast does not contain INO1 gene encoding inositol-phosphate synthase, so that its growth fails in the absence of inositol9. Pichia pastoris INO1 gene alone is found to confer inositol prototrophy to wild type S. pombe when the plasmid with INO1 gene is transformed into the cells4. Therefore, the aim of this study is to know how produced inositol by the transformants of...
S. pombe with the plasmids pADH-INO and pSPIN-22, respectively affects glucose repression, intracellular maltase activity, MAL1+ gene expression and phospholipid synthesis, in order to get another evidence to show that PI may be involved in derepression of intracellular maltase activity and its corresponding gene expression at the transcriptional level.

Materials and Methods

Yeast strain

Schizosaccharomyces pombe, a naturally inositol-requiring yeast strain was kindly supplied by Dr. Olaf Nielsen at University of Copenhagen, Denmark. The strain was maintained at 4°C on YPD agar (w/v) which contained 2.0% glucose, 2.0% peptone, 1.0% yeast extract and 2.0% agar.

Plasmids

Plasmids pADH-INO and pSPIN-22 which contained INO1 gene from Pichia pastoris were kindly supplied by Dr. Anand K Bachhawat, Institute of Microbial Technology, Chandigarh, India.

Medium

A completely synthetic medium without inositol addition at pH 5.5 was used in this study.

Growth conditions for effects of produced inositol on intracellular maltase activity, mRNA biosynthesis, phospholipids synthesis and cell growth

To determine the effect of produced inositol on intracellular maltase activity, mRNA biosynthesis, phospholipids biosynthesis and cell growth of S. pombe, different concentrations of glucose were added to the synthetic medium. Thereafter, the cells were shaken vigorously on a rotary shaker (170 rpm) at 30°C for 44 h.

Transformation of S. pombe

The wild type cells of S. pombe were transformed with plasmids pADH-INO and pSPIN-22, respectively using the lithium acetate method as described. The transformants containing the plasmids pADH-INO and pSPIN-22 were named Sch.p944 and Sch.p1025, respectively.

Determination of maltase activity

Intracellular maltase activity was determined in situ by following the conversion of p-nitrophenyl glucoside (PNPG) to p-nitrophenol (PNP) at 410 nm. Briefly, pelleted cells were permeabilized by freezing in liquid nitrogen for 30 s and thawing at 37°C. The reaction mixture containing 50 µl of the permeabilized cells suspended in 50 mmol/L phosphate buffer (pH 6.8) and 1.0 ml of 5.0 mmol/L PNPG in the same buffer was incubated at 30°C. The reaction was stopped by addition of 1.0 ml of 0.1 mol/L Na2CO3. After a brief low-speed centrifugation, release of PNP was followed by measuring the absorbance at 410 nm. One unit of maltase activity was defined as the amount of enzyme that hydrolyzed PNPG to liberate one microgram PNP per min at 30°C and pH 6.8. The specific maltase activity was defined as units per g of cell dry weight.

Determination of reducing sugar

The amount of reducing sugar in the supernatant of culture was determined as described previously.

Determination of cell growth

The yeast cells culture (5.0 ml) was harvested by centrifugation and washed two times with distilled water. The washed cells were then suspended in 5.0 ml of distilled water and cell density of the suspension was determined at 600 nm using the Spectrophotometer. To determine cell dry weight, the washed cells were dried at 100°C until constant weight was maintained.

Lipid extraction and phospholipid analysis

Yeast cells in the culture were harvested by centrifugation and washed three times with deionized water. The washed cells were resuspended in 10.0 ml of deionized water. The cell disruption and lipid extraction were carried out according to the previously described methods. The lipid sample was stored at -20°C.

Individual phospholipids in the lipid extract were separated by two-dimensional thin-layer chromatography (TLC) in the first direction with chloroform-methanol-25% ammonia (65: 35: 5 by vol.) and in the second direction with chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5 by vol.) Spots of different phospholipids were located by exposing the plates to iodine vapor and phospholipid amount of each spot on the plate was scanned and determined at 375 nm by using TCL Scanner (CS-9301PC, Shimadzu, Japan) and the percentage of each phospholipid was calculated.
Determination of MAL1+ gene expression by RT-PCR

The cell culture (5.0 ml) was collected and washed by centrifugation at 5000 × g for 10 min. Total RNA in the yeast cells was extracted by using Rnasey Mini Protocols for isolation of total RNA from yeast (purchased from Qiagen Co.) according to the instructions of the manufacturer. RNA content in the sample was measured at 260 nm. The amount of total RNA was adjusted to 500 pg in each reaction, when the RNA was used as the template for RT-PCR.

MAL1+ gene expression was determined by using SuperScript™ One-Step RT-PCR with Platinum®Taq DNA Polymerase kit (Invitrogen). The primers for RT-PCR were: primer K1: 5’-CGGGATCCATGAAAGTCGTGCCAAG-3’ and primer K2: 5’-GCACTGCAGTCAGACAGACCCCAAC-3’. The conditions for RT-PCR amplification were as follows: initial denaturation at 94°C for 2 min, denaturation at 94°C for 15s, annealing temperature at 50°C for 30s, extension at 72°C for 1.5 min, and final extension at 72°C for 10 min. The RT-PCR was run for 30 cycles and RT-PCR cycler was GeneAmp® PCR System 2400 (Perkin-Elmer).

Results

Effects of varying glucose concentrations on intracellular maltase activity and residual glucose concentration

In previous study10, we reported the highest specific invertase activity in Sch.p944, when it was grown in the synthetic medium containing initial 1.0% (w/v) glucose, and repression of invertase secretion in Sch.p1025, when it was cultivated in the same medium containing glucose more than initial 0.2% (w/v). The search for the secretion leader in the deduced protein sequence of cloned MAL1+ gene in S. pombe used in this study using the methods as described in SignalP3.0 Server at http://www.cbs.dtu.dk/services/SignalP revealed no secretion leader in the deduced protein sequence. Therefore, the maltase in this yeast strain was an intracellular enzyme11.

Fig. 1 shows that the specific intracellular maltase activity was significantly higher in transformant Sch.p944 than in transformant Sch.p1025, when they were grown in the same medium with 2.0% (w/v) glucose, respectively, suggesting that the activity was greatly repressed in the latter, when initial glucose concentration in the synthetic medium was 2.0% (w/v). However, we found that both the transformants consumed most of glucose and almost the same amount of glucose remained in the media with 2.0% (w/v) of initial glucose concentration, when grown for 44 h (data not shown). This might imply that residual glucose in the culture had no effect on the derepressed intracellular maltase activity in Sch.p944.

Changes in intracellular maltase activity, cell growth and residual glucose during the cell growth

Fig. 2 shows the time course of intracellular maltase activity during the cell growth. In Sch.p944, the activity reached the highest (Fig. 2), when the cell growth reached late log phase after growth for 44 h (Fig. 3), whereas in Sch.p1025, the activity was significantly less than in Sch.p944 when it was cultivated for 44 h and reached the highest when the cells were grown for 62 h (Fig. 2). Results in Fig. 3 also suggested that cells of Sch.p1025 grew more slowly than those of Sch.p944, as the former synthesizes less inositol than the latter10. Results in Fig. 4 also show that cells of both the transformants used most of glucose and almost the same amount of

![Fig. 1](image1.png)  
Fig. 1—Effect of different glucose concentrations on specific maltase activity of the transformants Sch.p944 (□) and Sch.p1025 (▲) [Values represent mean ± SD of three replications]

![Fig. 2](image2.png)  
Fig. 2—Time course of maltase activity during batch cultivation of transformants Sch.p944 (□) and Sch.p1025 (▲) [Values represent mean ± SD of three replications]
residual glucose in the medium remained after grown for 44 h, suggesting that higher intracellular maltase activity in Sch.p944 than in Sch.p1025 was not related to residual glucose concentration in the culture.

Changes of percentage of phospholipids during the cell growth

The monitoring of changes of percentage of phospholipids in the two transformants showed that only PI content increased, while other phospholipids decreased during the cell growth (data not shown). Although PI content in both the transformants was almost the same within 32 h of cell growth, it increased more rapidly in Sch.p944 than in Sch.p1025 after 32 h of cell growth (Fig. 5). Combining the results of Figs 2 and 5, it could be clearly observed that PI might be involved in increase in intracellular maltase activity in Sch.p944.

Changes in the amount of mRNA encoding intracellular maltase

Results in Fig. 6 show that amount of mRNA encoding intracellular maltase in both the transformants increased during the cell growth. Although the amount of mRNA encoding intracellular maltase in both the transformants was almost the same within 24 h of cell growth, it increased more quickly in Sch.p944 than in Sch.p1025 after 32 h of cell growth and was significantly higher in Sch.p944 than in Sch.p1025 after 44 h of cell growth.

It may be emphasized that the total RNA was extracted from the Sch.p944 and Sch.p1025 cells grown in the synthetic medium with 2.0% (w/v) glucose at different times of cultivation, respectively. The same amount of the total RNA was used the template for RT-PCR.

Discussion

The above results clearly demonstrated that Sch.p944 containing plasmid pADH-INO had significantly higher intracellular maltase activity, the amount of mRNA encoding maltase and PI content than Sch.p1025 containing pSPIN-22, suggesting that PI might be involved in increase in MAL1+ gene expression and intracellular maltase activity in Sch.p944 at the transcriptional level.
In this study, we got another evidence to show that glucose derepression, maltase gene expression and intracellular maltase activity in the fission yeast were also mediated by produced PI. Based on earlier studies\textsuperscript{1,2}, we believed that increase in PI content might cause phosphorylation of Scr1p activator, which has the same function as Mig1 in \textit{S. cerevisiae} by PI-type signaling pathway\textsuperscript{5,20,21}. The phosphorylated activators then activate transcription of \textit{MAL1}\textsuperscript{+} gene and cause increase in mRNA encoding intracellular maltase even in the presence of high concentration of glucose (Figs 1, 2 and 6). Therefore, glucose derepression in \textit{Sch.p}\textsubscript{944} which produced higher concentration inositol in its cells\textsuperscript{10} occurred at initial glucose concentration below 2.0\%, when the transformant was grown in the synthetic medium (Fig. 1 and 2) due to high content of PI in the cells (Fig. 5).

When \textit{Sch.p}\textsubscript{1025}, which produced lower concentration of inositol in its cells was grown in the synthetic medium with initial concentration of 2.0\% (w/v) glucose\textsuperscript{10}, decrease in PI content (Fig. 5) might cause dephosphorylation of Scr1p and the dephosphrylated Scr1p in the nucleus repressed the transcription of \textit{MAL1}\textsuperscript{+} gene, so that decrease in mRNA encoding intracellular maltase happened in the presence of high concentration of glucose (Fig. 6). Therefore, the specific maltase activity decreased continuously as initial glucose concentration increased from 0.5 to 4.0\% (w/v) (Figs 1 and 2). But, it is still unclear how glucose derepression and gene expression in yeasts were mediated by the PI-type signaling pathway. Therefore, the further work on whether the produced inositol and PI-mediated glucose derepression, intracellular maltase activity and \textit{MAL1}\textsuperscript{+} gene expression in \textit{S. pombe} are related to PI-type signaling pathway, is being undertaken in the laboratory by using the relevant inhibitors, such as neomycin and compound 48/80\textsuperscript{22,23}.

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**References**