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ORIGINAL RESEARCH PAPER

Proteomic insights into the stimulatory effect of Tween 80 on mycelial growth and exopolysaccharide production of an edible mushroom *Pleurotus tuber-regium*

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Abstract Proteomic analysis was applied to investigate the mechanism of the stimulatory effect of Tween 80 on the mycelial growth and exopolysaccharide production by an edible mushroom *Pleurotus tuber-regium.* 32 differentially expressed proteins were identified by one-dimension gel electrophoresis. Combined with our previous findings, the up-regulation of heat shock proteins might help to maintain cellular viability under environmental stress. The up-regulation of ATP:citrate lyase isoform 2 could suppress the activity of tricarboxylic acid cycle and, consequently, stimulate exopolysaccharide production. The present results provide important insight to the mechanism by which stimulatory agents (Tween 80) can increase the production of useful fungal metabolites and also fill the gap of our knowledge on the under-developed mushroom proteomics.

Keywords Exopolysaccharide · Mushroom · One-dimensional gel electrophoresis · *Pleurotus tuber-regium* · Proteomic analysis · Tween 80

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Introduction

In our previous study, the exopolysaccharide (EPS) isolated from the fermentation broth of an edible mushroom, *Pleurotus tuber-regium*, was found to have anti-tumor activity (Zhang and Cheung 2011a, b). To increase the efficiency of the submerged fermentation process, Tween 80 was applied as the stimulatory agent by which the mycelial growth and EPS production of *P. tuber-regium* could be significantly enhanced by 51 and 42 %, respectively (Zhang and Cheung 2011a). Moreover, a preliminary study on the underlying mechanisms of the stimulatory effect of Tween 80 had revealed that the mechanism by which Tween 80 could affect fungal metabolism might be associated with the enhanced nutrient consumption rate, the intact structure of mycelial cells and transport activity across the mycelial membrane (Zhang and Cheung 2011b).

By use of proteomic analysis, the relationship between chemical conditions of the mycelial

fermentation and fungal metabolism could be further illuminated in terms of cellular and molecular mechanisms (Francesca et al. 2010; Oda et al. 2006; Svensater et al. 2000; Wilkins et al. 2001). One-dimensional (1D) gel electrophoresis (GE), followed by enzymatic hydrolysis and nanoelectrospray ionization-LC-MS/MS (nESI-LC-MS/MS) analysis, is an efficient method for proteomic analysis (Graves and Haystead 2002; Xiong et al. 2005).

The aim of this study was to analyze the proteome changes of *P. tuber-regium* during submerged fermentation with and without addition of Tween 80. As a result, it could provide significant insights into the stimulatory effect of Tween 80 on the mushroom mycelial growth and EPS production in terms of cellular and molecular mechanisms. Moreover, such study will facilitate a deeper understanding on the underdeveloped mushroom proteomics.

Materials and methods

Strain and growth conditions

The strain of *P. tuber-regium* was from Fungi Perfecti Ltd. Co. (Olympia, USA) and the culture conditions used were the same as in our previous work (Zhang and Cheung 2011a). The mycelial cells obtained from the fermentation broth with and without addition of Tween 80 (0.3 %, w/v, added on the fifth day of fermentation) were used for the proteomic analysis comparatively by the following steps.

Extraction of total protein

Total protein of *P. tuber-regium* mycelia was extracted by using phenol with some modifications (Horie et al. 2008; Hurkman and Tanaka 1986). The extracted proteins were quantified by the PlusOne Quant Kit (GE Healthcare) and stored at −80 °C. The details are shown in Supplementary information.

1D-GE, in-gel digestion and peptide extraction

The proteins were separated by SDS-PAGE using gels (4 % stacking gel and 12 % separating gel) on a vertical electrophoresis unit at 110 V. After SDS-PAGE, the gels were stained with Coomassie Blue. After distaining, each lane of sample was cut into ~1 mm cubes and further treated with acetonitrile (ACN), dithiothreitol (DTT) and iodoacetamide (IAA), respectively. Then the pieces were digested with trypsin (sequencing grade modified trypsin, Promega) and finally the peptides were extracted by extraction buffer (50 % ACN/5 % formic acid). The details are shown in Supplementary information.

nESI-LC-MS/MS analysis

Just before MS analysis, the trypsin-digested peptides were desalted by using the ZipTip$_{u\text{-}C18}$ (Millipore) treatment. The desalted peptides were separated by a C18 reverse-phase column and analyzed on a nano-electrospray ionization mass spectrometer (nESI-LC-MS/MS). Acquired data were searched against the National Center for Biotechnology Information (NCBI) non-redundant protein database (fungi) using the MASCOT software package (Version 2.3, Matrix Science, UK; www.matrixscience.com). The protein hit with a confidence level higher than 95 % ($p < 0.05$) and an expectation score smaller than 10^{-3} was considered as an identified protein.

Results and discussion

The SDS-PAGE of mycelial proteins of *P. tuber-regium* producing during submerged fermentation with and without the addition of Tween 80 is shown in Fig. 1. In general, the major bands of proteins appeared in the gels were similar while there were also some differences in the expression of several protein bands with the addition of Tween 80 (Fig. 1).

1D-GE-based proteomic analysis identified 32 differentially expressed proteins, among which 16 proteins could only be identified on the SDS-PAGE gel from *P. tuber-regium* mycelial cell produced without the addition of Tween 80 while 16 proteins only appeared on the gel with the addition of Tween 80 (see Supplementary information, Table 1). According to their functions in biological processes, these proteins can be classified into ten functional categories (see Supplementary information, Table 1). The followings are the details of the possible functional roles of several important identified proteins which may be closely related to the stimulatory mechanism of Tween 80.

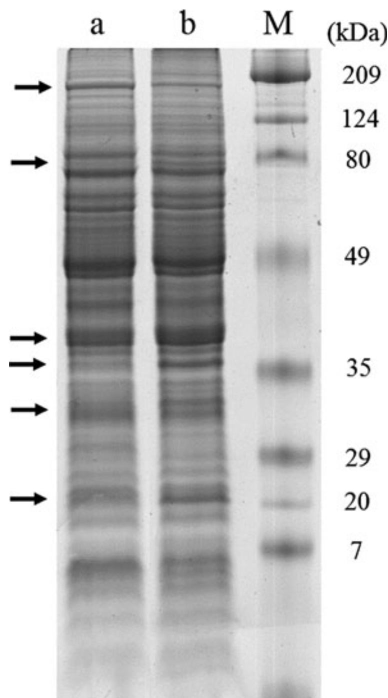

Fig. 1 SDS-PAGE of mycelial proteins of *P. tuber-regium* by submerged fermentation without (*a*) and with (*b*) the addition of Tween 80. *M* markers. Some protein bands with differential expression level by the addition of Tween 80 were denoted with the *arrows*

Translation, ribosomal structure and biogenesis related proteins

There were 14 translation, ribosomal-structure and biogenesis-related proteins differentially expressed in the two comparative gels with and without the addition of Tween 80 (see Supplementary information, Table 1). Translation elongation factor proteins associate with ribosomes cyclically during the elongation phase of protein synthesis, and catalyze formation of the acyl bond between the incoming amino-acid residue and the peptide chain (Parker 2001). The differential expression level in these proteins may be attributed to translational channeling and compartmentalization of protein synthesis in higher eukaryotic cells and they may contribute to the coordinate regulation of multiple cellular processes including growth, division, and transformation (Negrutskii and El'Skaya 1998). In general, ribosomal proteins are involved in ribosome biogenesis and/or for different stages of the translation process (Ferreira-Cerca et al. 2005). Accordingly, ribosomal proteins might be very important in transport of the ribosomal precursors, RNA folding, protein assembly, rRNA processing, stabilization of the subunit structure, and/or interaction with other factors required for either ribosome biogenesis or translation. Furthermore, they can also play a key role in co-translational processes like co-translational translocation or the interaction with protein folding factors at the exit tunnel of the ribosome (Ferreira-Cerca et al. 2005). Ribosomal proteins can also contribute to important enzymatic activities for ribosome function like the mRNA helicase activity of bacterial ribosomes (Takyar et al. 2005). Hence, the differential expression level in these translation-related proteins suggested the protein synthesis was affected by the addition of Tween 80.

Heat shock proteins

There was a trend of an increasing expression of heat shock proteins from *P. tuber-regium* mycelial cells produced with the addition of Tween 80 (see Supplementary information, Table 1). Heat shock proteins were either constitutively expressed or activated when exposed to elevated temperatures or other environmental stress conditions, such as contacted with toxins, infection, starvation or other nutrition deficiency (Narberhaus 2002). Apart from the stress response ability, HSP70 plays an essential role under normal condition including assisting folding of newly synthesized proteins, guiding translocation of proteins across organelle membrane, disassembling oligomeric protein structures and facilitating proteolytic degradation of unstable proteins (Bakau and Horwich 1998). Heat shock protein was also found to be responsible for assembly of peptides that were imported into mitochondria, as well as export of certain secretory proteins (Plesofsky-Vig and Brambl 1995).

In the late growth phase, the environmental stresses in the submerged fermentation of the *P. tuber-regium* mycelial cells were increased and might result in the increase number of damaged proteins or other cellular components. Consequently, the demand for heat shock proteins had to be increased to provide additional repair of misfolded proteins, protecting cellular proteins, and maintaining cellular viability under conditions of intensive stress. In our previous study, addition of Tween 80 extended the growth of the mycelia possibly by maintaining the intact structure of

the mycelial pellets and preventing their disintegration caused by the shearing forces during the shake-flask experiments (Zhang and Cheung 2011b). Combining all these results, it can be suggested that the addition of Tween 80 might protect *P. tuber-regium* mycelial cells by up-regulating the expression of some heat shock proteins such as HSP70.

Tricarboxylic acid (TCA) cycle-related proteins

As mentioned in our previous studies, the addition of Tween 80 could significantly increase the EPS production in the submerged fermentation of *P. tuber-regium* (Zhang and Cheung 2011a). Hence, it is necessary to investigate the proteins involved in the function of carbohydrate transport and metabolism which may be closely related to the EPS production. In this functional category, ATP:citrate lyase isoform 2 (only appeared in the gel with the addition of Tween 80) was involved in the TCA cycle and associated with the aerobic respiration in all living cells. ATP:citrate lyase isoform 2 has the citrate lyase activity which catalyzes the reaction of citrate and CoA to form oxaloacetate and acetyl-CoA, along with the hydrolysis of ATP. Hence, the up-regulation of ATP:citrate lyase isoform 2 with the addition of Tween 80 suppresses the TCA cycle. Activity of TCA cycle enzymes can be influenced by the nutritional condition of the cell and by a variety of stress-inducing stimuli (Sadykov et al. 2008; Vuong et al. 2005). Synthesis of PIA (an *N*-acetylglucosamine EPS) by *Staphylococcus epidermidis* was increased when the TCA cycle activity was suppressed by changing the environmental and nutritional conditions (Vuong et al. 2005). It had been proposed that the mechanism by which *S. epidermidis* can perceive external environmental change is through alterations in its TCA cycle activity leading to changes in the intracellular levels of biosynthetic intermediates, ATP, or the redox status of the cell (Sadykov et al. 2008; Vuong et al. 2005).

In the present study, the activity of TCA cycle was suppressed due to the increasing expression of ATP citrate lyase isoform 2 with the addition of Tween 80, which might further exert a stimulatory effect on the production of EPS. This finding was consistent with previous studies and was very important for the understanding the mechanism of the stimulatory effect of Tween 80 (Sadykov et al. 2008; Vuong et al. 2005). More in-depth investigations on the intracellular levels of this bioprocess such as how these differential expressed TCA related proteins affect the EPS production by mushroom mycelial cells are required.

Based on our previous studies and the above results by comparative 1D-GE-based proteomic analysis, the plausible mechanism by which the addition of Tween 80 could stimulate the *P. tuber-regium* mycelial cell

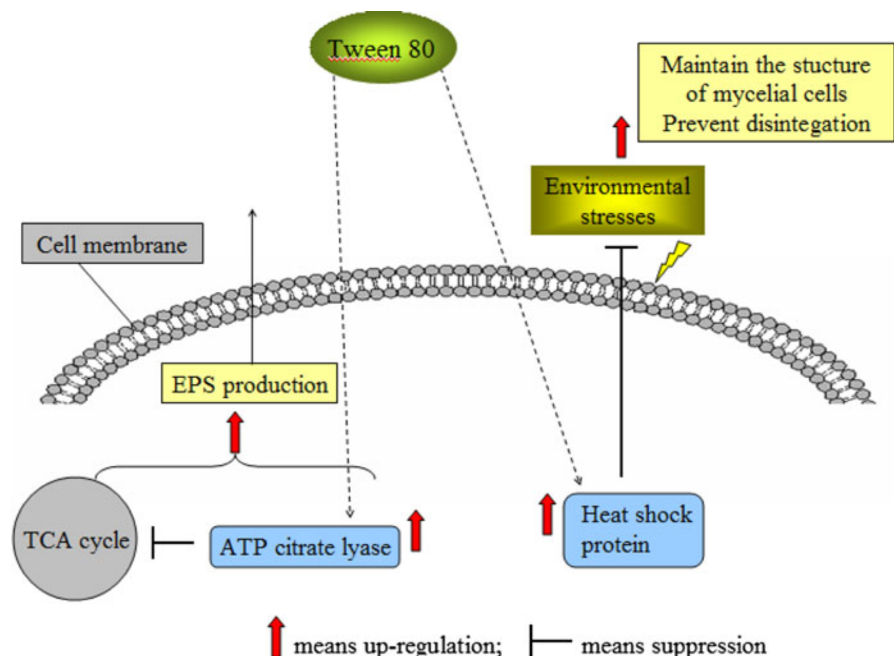

Fig. 2 A partial model showing the proposed mechanisms by which Tween 80 influences the growth of *P. tuber-regium* mycelia and EPS production

growth and EPS production was proposed as shown in Fig. 2. On one hand, the addition of Tween 80 up-regulates the expression of ATP:citrate lyase, which suppresses the TCA cycle activity and results in an increase of EPS production. On the other hand, the up-regulation of heat shock proteins can reduce the environmental stresses on the mycelial cells and consequently maintains its intact structure and prevents its disintegration.

In conclusion, we have for the first time successfully applied comparative 1D-GE-based proteomic analysis to investigate the mechanism by which Tween 80 can enhance the mushroom mycelial biomass and EPS production. However, due to the limited availability of genome sequences for filamentous fungi especially the lack of information on mushroom genome and proteome, many proteins isolated from the 1D-gels are still with hypothetical or unknown functions. Hence, functional genomic study on *P. tuber-regium* is underway to provide a more in-depth understanding on the stimulatory mechanisms of Tween 80.

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