Exogenous glycine and serine promote growth and antifungal activity of Penicillium citrinum W1 from the south-west Indian Ocean

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One sentence summary: Exogenous glycine and serine elevate growth and antifungal activity of Penicillium citrinum W1 from the south-west Indian Ocean though metabolic modulation.

ABSTRACT

PcPAF is a novel antifungal protein identified by our recent study, which is produced by a fungal strain Penicillium citrinum W1 isolated from a south-west Indian Ocean sediment sample. The present study identified glycine as a potential metabolite which increased the fungal growth and promoted antifungal activity. Then, GC/MS based metabolomics was used to disclose the metabolic mechanism manipulated by glycine. With the aid of unsupervised hierarchical clustering analysis and supervised orthogonal partial least-squares-discriminant analysis, the intracellular metabolite profiles were distinguished among two glycine-treated groups and control. 43 and 47 significantly varied metabolites were detected in 2.5 mM or 5 mM glycine-treated groups and involved in seven and eight pathways, respectively. Furthermore, exogenous serine, which is converted from glycine, showed the same potential as glycine did. Our findings not only identify glycine and serine as nutrients which promoted P. citrinum W1 growth and increased antifungal activity, but also highlight the way to utilize metabolomics for an understanding of metabolic mechanism manipulated by an exogenous compound.

Keywords: metabolomics; glycine; serine; Penicillium citrinum; growth; antifungal activity

INTRODUCTION

Microbial products are attractive sources of chemotherapeutic agents and industry production. Manipulating for the growth conditions of microorganisms and understanding for the effects of the manipulation are a common strategy used by pharmaceutical companies to improve yields and diversity of secondary metabolites of therapeutic interest. Several studies have provided insight into metabolites of nitrogen source which affect fungal growth and compound production. Trametes trogii grown in medium with glutamic acid supplement produced...
the highest laccase and manganese peroxidase activities (Levin, Melignani and Ramos 2010). Urea caused maximum production of CMCase among the nitrogen sources in Fomitopsis sp. RCK2010 (Deswal, Khasa and Kuhad 2011). On the other hand, investigations on metabolic regulation are beneficial for understanding the effects of these manipulations. Hu et al. (2013) reported that arachidonic acid enhanced the maximum β-carotene production in Blakeslea trispora, which proceeded by increase of the glycolysis and some fatty acids and decrease of amino acids as a consequence of the arachidonic acid treatment. Qi et al. (2014) combined the mechanism of carotenoids production in three strains during different growth phases and found that slow the TCA cycle and amino-acid metabolism might promote energy and metabolic flux towards the carotenoids and fatty acids synthesis. These reports suggest that metabolomic profiling is a useful tool to gain insight into the metabolic pathways and shed light on molecular mechanisms of elevated production.

Penicillium citrinum is a potential application source of several industrial enzymes, such as cellulases and lipases, used in detergent and waste treatment applications (Maliszewska and Mastalerz 1992; Pimentel et al. 1996). Recently, we have reported a novel antifungal protein, namely PcPAF, which is produced by a fungal strain P. citrinum W1 isolated from a south-west Indian Ocean sediment sample. PcPAF displays well antifungal activity against Trichoderma viride, Fusarium oxysporum, Paeocilocybes variotii and Alternaria longipes with high thermostability and a certain extent of resistance to proteases and metal ions (Wen, Guo and Chen 2014). Improvement of PcPAF yields is required for the prospective production.

Here, we present an investigation of manipulating growth conditions by exogenous nutrients and understanding of the manipulation mechanism in P. citrinum W1. A GC/MS-based metabolomic approach and a multivariate analysis were combined to investigate the changes in the P. citrinum W1 cell metabolome at different concentration of glycine treatment. The role of glycine, serine and threonine metabolism was also confirmed by the subsequent functional investigation using replacement of glycine with serine. This study provides a better understanding of the molecular mechanism underlying glycine, serine and threonine metabolism promotes growth and antifungal activity of P. citrinum W1.

**MATERIALS AND METHODS**

**Media and culture conditions**

Penicillium citrinum W1 was isolated from the south-west Indian Ocean sediment sample (S 38.1329’, E 48.5975’) (Wen, Guo and Chen 2014). Seed culture was developed by inoculating single colony of P. citrinum W1 into 50 mL Vogel media (pH 7.0) containing 3.0% sucrose, 0.5% yeast extract, 0.1% K2HPO4, 3.0% NaNO3, 0.5% KCl, 0.5% MgSO4 and 0.01% FeSO4 and incubated on a rotary shaker at 200 rpm for 24 h at 28°C. Aliquot 1 mL seed culture was then diluted in 50 mL fresh Vogel medium in 250 mL flasks and grown for 12 days. Samples were collected, centrifuged at 9000 rpm for 10 min at 4°C. Precipitate was used to determine the dry cell mass and metabolite extract. For nutrition supplement, the following nitrogen sources were used: glycine, alanine, serine, glutamine, proline, arginine and inosine. All chemicals were purchased from Sigma-Aldrich.

**Measurement of dry cell weight**

To examine the effect of metabolites on dry cell weight (DCW), 5 mM metabolites including glycine, alanine, glutamine and inosine were added in Vogel media and grown for 12 days. Growth of P. citrinum W1 was expressed in terms of DCW per 50 mL of culture broth, which was harvested and dried to a constant weight in drying oven at 60°C for 48 h and then weighed. Four biological replicates were prepared each sample.

**Preparation of metabolome samples**

To investigate metabolic response to glycine, two doses of 2.5 and 5 mM were designated as low- (glycine-L) and high-doses (glycine-H), respectively. Sample preparation was performed as described previously (Lise et al. 2006). In brief, equivalent cells from these cultures were quenched using cold methanol at −40°C and collected by centrifugation at 8000 rpm for 5 min. Cellular metabolites were extracted with 1 mL cold methanol (Sigma) containing 10 µL 0.1 mg mL−1 ribitol (Sigma) as an analytical internal standard. Cells were lysed by liquid nitrogen and sonication for 10 min at a 10 W power setting and centrifuged for 10 min at 12 000 rpm at 4°C. A total of 500 µL of supernatant was transferred into a new 1.5 mL centrifugation tube and dried in a rotary vacuum centrifuge device (LABCONCO). The resulting samples were used for GC/MS analysis. Four biological repeats were prepared each sample.

**Metabolomics analysis**

**GC/MS analysis**

GC/MS analysis was carried out with a variation on the two-stage technique (Zhao et al. 2014). In brief, for derivatization, 40 µL of 20 mg mL−1 methoxyamine hydrochloride (Sigma-Aldrich) in pyridine was added to the dried samples through a 90 min, 37°C reaction. Then 80 µL N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich) was added to the samples, and the mixtures were incubated at 37°C for 30 min. Chromatography was performed on an Trace DSQ II (Thermo Scientific) equipped with a 30 m × 0.25 mm i.d. × 0.25 µm DBS-MS column. The derivatized sample of 1 µL was injected with splitless injection by the Agilent autoinjector. The initial temperature of the GC oven was held at 85°C for 5 min followed by an increase to 270°C at a rate of 15°C min−1 and then maintained at 270°C for 5 min. Helium was used as carrier gas at a constant flow rate of 1 mL min−1. Electron impact ionization (70 eV) at full scan mode (m/z 50–600) at a rate of 20 scans s−1 was used. The acceleration voltage was turned on after a solvent delay of 6 min. Ribitol served as an internal standard to monitor batch reproducibility and correct for minor variations that occurred during sample preparation and analysis.

**Data processing**

Spectral deconvolution and calibration were performed using AMDIS and internal standards. A retention time (RT) correction was performed for all the samples, and then the RT was used as reference against which the remaining spectra were queried and a file containing the abundance information for each metabolite in all the samples was assembled. Metabolites from the GC/MS spectra were identified by searching in National Institute of Standards and Technology Mass Spectral Library. Among the detected peaks of all the chromatograms, 167 peaks were considered as endogenous metabolites excluded internal standard ribitol. The resulting data matrix was normalized by the
Statistical analyses

Data transformations and manipulations were done using Excel, and multivariate statistical analysis was performed with SIMCA-P (Umetrics). Principal component analysis (PCA) and partial least-squares-discriminant analysis were applied to the data after mean-centering and Ctr-scaling. An unsupervised PCA was initially performed to obtain an overview of the GC/MS data from the different groups of the control and glycine-treated P. citrinum cells. Data were analyzed using SPSS13.0 software for Windows. Differences showing p-values less than 0.01 were considered statistically significant between control and glycine-treated groups. Hierarchical clustering was performed on the normalized data, completed in the R platform with the package gplots (http://cran.r-project.org/web/packages/gplots/) using the distance matrix. Prior to analysis, sets of metabolites data subtracted the median metabolites and scaled by the quartile range in the sample. Z-score analysis scaled each metabolite according to a reference distribution, and calculated based on the mean and standard deviation of reference sets control and glycine treatment groups. Detailed accounts of pattern recognition methods were described by Lindon (Lindon, Holmes and Nicholson 2001).

Measurement of antifungal activity

The assay for antifungal activity against the tested phytopathogenic T. viride was carried out as described previously (Wen, Guo and Chen 2014). Briefly, one 0.6 cm diameter piece of T. viride or P. citrinum was placed on the center of a 90 × 15 mm petri plates containing 10 mL of PDA medium. After the mycelial colony had developed, sterile blank paper discs of 0.65 cm diameter were placed at a distance of 0.8 cm away from the rim of the growing mycelial colony. Fifty microliter of culture supernatant collected at each time point was added to each paper disc. Only culture medium at each time point was used as blank controls. Plates were incubated at 28°C until mycelia growth enveloped the control discs and formed crescents of inhibition around discs containing samples with antifungal activity. The distance between the inhibited mycelial and the center of paper discs were measured as the radius of inhibition zone. The experiments were repeated three times. The radius of inhibition zone was expressed as the mean ± the standard errors of three experiments at each time point. The antifungal activity of culture supernatants of P. citrinum W1 at each time point was characterized by the radius of inhibition zone. Since antifungal spectrum of culture supernatants of P. citrinum W1 is well corresponding to that of the antifungal protein PcPAF purified from its culture supernatants. Thus, the increasing production of PcPAF by exogenous glycine/serine is represented by the increasing antifungal activity (widening inhibition zone radius) of culture supernatants of P. citrinum W1 at different time points after addition of glycine/serine.

RESULTS

Effect of exogenous glycine on growth and antifungal activity of P. citrinum W1

A total of 167 aligned individual peakswere identified across glycine addition. Intracellular metabolites were extracted and quantified using GC/MS analysis. Typical GC/MS total ion current (TIC) chromatograms from the control, 2.5 or 5 mM glycine-treated P. citrinum W1 cells were shown in Fig. 2A. A total of 63 metabolites were identified. Pearson correlation coefficient between two technical replicates varied between 0.996 and 0.999, which suggests that reproducibility of GC/MS was sufficient to ensure data quality in global metabolic profiling application (Fig. 2B). Metabolomics approach was used to identify dynamically metabolic responses to the increasing concentrations of glycine. The metabolite profiles were displayed as a heat map (Fig. 2C), yellow and dark blue indicated increase and decrease of the metabolites according to the color scale. Orthogonal partial least-squares-discriminant analysis (OPLS-DA), a supervised pattern recognition method, was carried out for the multivariate analysis. Obvious separation among the control group and glycine groups in three different quadrants of the PCA scores plot, and clear separations between 2.5 mM and 5 mM glycine (Fig. 2D). In all cases, 94.4% of the total variance in the data was described by the first two PCs as R²X = 0.944. This suggests that 94.4% of variation in the data set is attributable to the treatment, indicating that glycine is likely to have been responsible for the majority of the metabolic variation in the data.

Metabolic profiles of P. citrinum W1 in the presence or absence of glycine

To study metabolic mechanisms by which glycine promote the growth and antifungal activity of P. citrinum W1, we used GC/MS based metabolomics to investigate metabolic profile in response to glycine addition. Intracellular metabolites were extracted and quantified using GC/MS analysis. Typical GC/MS total ion current (TIC) chromatograms from the control, 2.5 or 5 mM glycine-treated P. citrinum W1 cells were shown in Fig. 2A. A total of 63 metabolites were identified. Pearson correlation coefficient between two technical replicates varied between 0.996 and 0.999, which suggests that reproducibility of GC/MS was sufficient to ensure data quality in global metabolic profiling application (Fig. 2B). Metabolomics approach was used to identify dynamically metabolic responses to the increasing concentrations of glycine. The metabolite profiles were displayed as a heat map (Fig. 2C), yellow and dark blue indicated increase and decrease of the metabolites according to the color scale. Orthogonal partial least-squares-discriminant analysis (OPLS-DA), a supervised pattern recognition method, was carried out for the multivariate analysis. Obvious separation among the control group and glycine groups in three different quadrants of the PCA scores plot, and clear separations between 2.5 mM and 5 mM glycine (Fig. 2D). In all cases, 94.4% of the total variance in the data was described by the first two PCs as R²X = 0.944. This suggests that 94.4% of variation in the data set is attributable to the treatment, indicating that glycine is likely to have been responsible for the majority of the metabolic variation in the data.

Varied metabolomes responsible for glycine and its dose

We further identified differential metabolites between the two glycine groups (glycine-L and glycine-H) and control using SPSS when p-values less than 0.01 were considered statistically significant between control and glycine-treated groups. 43 and 47 significant varied metabolites were obtained in the presence of 2.5 or 5 mM glycine, respectively. For visualizing the relationship among the abundances of metabolites, hierarchical clustering was used to arrange the metabolites on the basis of their relative levels across samples (Fig. 3A). Z-score displayed variations concentrations of added internal standards and the total intensity. This file was then used for subsequent statistical analyses.
of these metabolites based on control. Among the perturbed metabolites, 35 and 31 increased, 8 and 16 decreased in abundance in 2.5 and 5 mM glycine groups, respectively. (Fig. 3B and C). OPLS-DA was carried out for the multivariate analysis. Obvious separation among the control group and the two glycine addition groups were detected in three different quadrants of the PCA scores plot (Fig. 3D). Category of these differential metabolites was compared between the two glycine addition groups. Number of differential metabolites increased mainly in amino acids and fatty acids (Fig. 3E). Loading plots displayed variables positively correlated with score plots. The loading plot represented the impact of the significantly differential metabolites on the clustering results. The metabolites most responsible for the variance were indicated by their distance from the origin with red mark in the score plot (Fig. 3F and G). In the plots of predictive correlation p(corr)[1] and p(corr)[2] (∼0.5 or ∼0.5), the red triangles indicate that the differential metabolites have larger weight and higher relevance. These results indicate that exogenous glycine affects cell metabolism, which is related to glycine dose.

Metabolic pathways affected by glycine

For understanding metabolic pathways affected by glycine, ingenuity network analysis was used to perform more detailed analysis of pathways and networks influenced by glycine addition. We analyzed the significantly differential metabolites to their respective biochemical pathways as outlined in the Kyoto Encyclopedia of Genes and Genomes (KEGG, release 41.1, http://www.genome.jp/kegg) and MetPA (http://metpa.metabolomics.ca/MetPA/faces/Home.jsp). Seven and eight pathways were enriched in the 2.5 and 5 mM glycine groups, respectively. Shared and differential enriched pathways between them were visualized in Fig. 4A. Specifically, the six overlapping pathways included glycine, serine and threonine metabolism, galactose metabolism, alanine, aspartate and glutamate metabolism, cyanoamino metabolism, biosynthesis of unsaturated fatty acids and beta-alanine metabolism (Fig. 4A).

Out of these pathways, glycine, serine and threonine metabolism and cyanoamino metabolism displays elevated abundance and galactose metabolism shows decreased abundance in a glycine-dose-dependent manner (Fig. 4B), suggesting the metabolic characteristics in response to different doses of glycine treatment. Furthermore, the overview of carbon metabolism, amino acid metabolism and fatty acid synthesis affected by glycine was described in Fig. 4C. The results stressed on elevating abundances of metabolites from glycine, serine and threonine metabolism, alanine, aspartate and glutamate metabolism, citrate cycle, cyanoamino metabolism and fatty acid synthesis, while the metabolites of galactose metabolism decreased generally. These findings show that increased amino acid metabolism and decreased galactose metabolism form a
characteristic feature in response to exogenous glycine. In addition, increasing TCA cycle and synthesis of fatty acids are detected although these pathways are not enriched. These results indicate that glycine promotes growth and antifungal activity of \textit{P. citrinum} W1 through modulation of metabolic pathways, especially glycine, serine and threonine metabolism.

**Effect of exogenous serine on growth and antifungal activity of \textit{P. citrinum} W1**

To further demonstrate the importance of glycine, serine and threonine metabolism, replacement of glycine with serine was carried out for testing the effect. The similar results were obtained when serine was used but not proline or arginine (Fig. 5A).

Specifically, dry weight of \textit{P. citrinum} W1 increased in medium with 5 and 10 mM serine in comparison with medium without the amino acid (Fig. 5A). Antifungal activity significantly elevated during 6–12 days. Generally, the activity was higher in medium with 5 or 10 mM serine in 6 days than in medium without the amino acid in 10 days, suggesting that exogenous serine promotes synthesis of antifungal activity in advance. Similarly, exogenous serine increase antifungal activity by a dozens of times on the eighth day in comparison with medium without serine (Fig. 5B). Seemingly, antifungal activity is detected earlier in serine than glycine additions, which may be related to the flux from glycine to serine (Figs 1D and 5B). These results support the importance of glycine, serine and threonine metabolism in \textit{P. citrinum} W1 growth and antifungal activity.
Figure 3. Differential metabolomic profiling of *P. citrinum* W1 in response to exogenous glycine. (A) Heat map of unsupervised hierarchical clustering of differential metabolites (row). Glycine-L (2.5 mM glycine), glycine-H (5.0 mM group) yellow and dark blue indicate increase and decrease of the metabolites scaled to mean and standard deviation of row metabolite level, respectively (see color scale). (B) The score plot of the OPLS-DA (supervised orthogonal partial least-squares-discriminant analysis) model from variant metabolites. (B–C), z-score scatter diagrams of differential metabolites based on control. The data from tested groups are separately scaled to the mean and standard deviation of control. Each point represents one metabolite in one technical repeat and is colored by sample types. (D) Number of metabolites increased and decreased in different categories. (E–G) The distribution of differential abundance of metabolites’ weight from method of OPLS-DA to control and experimental samples. Triangles in metabolites’ scatter diagram marked with color own larger weight and higher relevance.
Figure 4. Features of differential metabolites. (A) Pathway enrichment analysis of differential metabolites. Significant enriched pathways are selected to plot. (B) The change of common metabolic pathway. (C) Common metabolic pathways of significantly changed metabolites in two tested groups based on control group. Red and green indicate increase and decrease of the metabolites, respectively. Black and gray indicate no change and no detection in GC/MS tests, respectively.
DISCUSSION

In the present study, we identify glycine as an effective nutrient supplement to elevate growth and antifungal activity of *P. citrinum* W1. The demonstration on elevation of both *P. citrinum* W1 growth and antifungal activity by glycine leads us to suppose that the elevation attributes to metabolic modulation. To test this idea, we use our recently developed functional metabolomics to understand metabolic regulation mechanisms (Guo et al. 2014; Zhao et al. 2014). In our functional metabolomics approach, first a crucial biomarker is identified and then the action of the crucial biomarker is assessed by exogenous addition. Finally, metabolic profile perturbed by the exogenous metabolite was analyzed for understanding the metabolic regulation mechanism. Here, following by the demonstration that glycine affects *P. citrinum* W1 growth and antifungal activity, the combination of GC/MS with multivariate statistical analyses is used to investigate the most statistically relevant metabolic pathway of *P. citrinum* W1 cells as a result of exposure to glycine, which results in identification of glycine, serine and threonine metabolism as the most affected metabolic pathway that contributes to the antifungal growth and activity. These results demonstrate the importance of glycine, serine and threonine metabolism in contributing to *P. citrinum* W1 growth and antifungal activity by glycine. Metabolomics has been used for understanding of metabolic regulation to compound supplements (Hu et al. 2013; Lee et al. 2014; Qi et al. 2014), but few research groups functionally validate the identified pathway. Compared with a single metabolite, pathway gives richer content. Thus, an understanding of a key pathway is beneficial to know a metabolic regulation.

Glycine is the simplest amino acid in nature and a major constituent in extracellular structural proteins in animals and microorganisms. However, much evidence shows that the amount of glycine synthesized in vivo is insufficient to meet metabolic demands (Wu 2009; Wang et al. 2013). Although mild insufficiency of glycine is not threatening for life, a chronic shortage may result in suboptimal growth, and other adverse effects on health and nutrient metabolism (Lewis et al. 2005). The glycine-dependent metabolic mechanism may be explained from the flux modulated by exogenous glycine. As shown in Fig. 4C, exogenous glycine increases levels of amino acids and fatty acids and decreases levels of the glycolysis, resulting in promotion of fatty acid synthesis and the TCA cycle and then contributing to *P. citrinum* W1 growth and antifungal activity. Increase flux of fatty acid synthesis and the TCA cycle are required for fungal growth (Strijbis and Distel 2010). The finding on the promotion of exogenous glycine to biosynthesis of fatty acids and the TCA cycle may be supported by the action of exogenous serine. Glycine is degraded into pyruvate in two steps. The first step is the reverse of glycine biosynthesis from serine with serine hydroxymethyltransferase. Serine is then converted to pyruvate by serine dehydratase. Pyruvate is converted into acetyl coenzyme A, which is the main input for a series of reactions known as biosynthesis of fatty acids and the TCA (Strijbis and Distel 2010; Hynes and Murray 2010). Thus, serine and glycine share similar actions.

CONCLUSION

The present study identifies glycine as an ideal nutritious supplement for improvement of antifungal activity. For an understanding of mechanisms by which glycine promotes the activity, a GC/MS-based metabolomics approach is used to investigate metabolic profile in the supplement. Glycine, serine and threonine metabolism is determined as the largest impact pathway. Elevated glycine, serine and threonine metabolism is determined as the largest impact pathway. The replacement of glycine with serine demonstrates the reliability of the identified glycine, serine and threonine metabolism. These results indicate that functional metabolomics is a powerful tool for understanding mechanisms manipulated by a supplement compound.

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