Proteome Changes in *Penicillium expansum* Grown in a Medium Derived from Host Plant

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**Introduction**

The fungal pathogen *Penicillium expansum* causes blue mold rot, a prevalent postharvest disease of pome fruit, and is also the main producer of the patulin. However, knowledge on the molecular mechanisms involved in this pathogen-host interaction remains largely unknown. In this work, a two-dimensional gel electrophoresis-based proteomic approach was applied to probe changes in *P. expansum* 3.3703 cultivated in apple juice medium, which was used to mimic the in planta condition. The results showed that the pH value and reducing sugar content in the apple juice medium decreased whereas the patulin content increased with the growing of *P. expansum*. A total of 28 protein spots that were up-regulated in *P. expansum* when grown in apple juice medium were identified. Functional categorization revealed that the identified proteins were mainly related to carbohydrate metabolism, secondary metabolism, protein biosynthesis or degradation, and redox homeostasis. Remarkably, several induced proteins, including glucose dehydrogenase, galactose oxidase, and FAD-binding monooxygenase, which might be responsible for the observed medium acidification and patulin production, were also detected. Overall, the experimental results provide a comprehensive interpretation of the physiological and proteomic responses of *P. expansum* to the host plant environment, and future functional characterization of the identified proteins will deepen our understanding of fungi-host interactions.

**Keywords:** *Penicillium expansum*, apple juice medium, proteomics, fungi-host interaction

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genomic sequence stimulated functional genomic research of this pathogen [9–11]. For instance, Ballester et al. [9] reported a global gene expression analysis of *P. expansum* during the pathogen-host interaction, which provided a new insight into the possible involvement of patulin biosynthesis and secondary metabolism in this pathogenic fungal infection.

Proteomics has developed to incorporate techniques and protocols that allow high-throughput analysis of proteins encoded by a genome [12]. In recent years, the proteomic approach has been successfully carried out in order to explore the microbe-host interactions [13, 14], pathogenic processes and toxin biosynthesis [15], and responses of microorganisms to environmental factors [16–18]. Although the pathogen-host interaction at the initial stage of infection is vital for the phytopathogenesis, it is difficult to detect the expression of fungal genes or proteins since very few fungal cells are normally present at that stage. In the present work, we investigated the cell growth, and physiological and proteomic changes of *P. expansum* grown with deproteinized apple juice as the sole nutrient source to simulate the nutritional status of this fungus in planta. Proteomic analysis of differentially expressed proteins in *P. expansum* grown under this condition will enable us to better understand the molecular mechanism underlying fungi-host interaction, which could be useful in postharvest control of *P. expansum*-caused fruit diseases.

**Materials and Methods**

**Fungal Strain and Growth Conditions**

*P. expansum* 3.3703 isolated from decayed apples was provided by China General Microbiological Culture Collection Center (CGMCC), and its abilities to cause blue mold rot in harvested apple fruits and produce patulin have been proved in our previous studies [19]. The isolate was grown on potato dextrose agar (PDA) medium at 25°C. Conidia of *P. expansum* were collected from sporulating 7-day-old PDA cultures by flooding with sterile distilled water. After filtration through three layers of cheesecloth, the conidia were resuspended in distilled water with a final concentration of 10⁷ conidia per milliliter.

Apples, purchased from a local market, were juiced using a liquidizer. The resulting apple juice was heated to 80°C for 3 min followed by filtering through three layers of cheesecloth to remove the heat-denatured protein precipitate. The sterilized apple juice (50 ml, pH 5.0) was inoculated with 10⁸ conidia and incubated at 25°C on a rotary shaker at 180 rpm. Biomass formation, medium pH, reducing sugar, and patulin content were determined over 2, 4, 6, and 8 days. Samples were taken in triplicates for subsequent biochemical and proteomic analyses.

**Biomass, pH, and Reducing Sugar Determination**

At each sampling time, fungal mycelia in the Erlenmeyer flasks were collected. After drying at 60°C for 48 h, the mycelia dry weight was measured on a standard balance. The pH value of the medium was determined with a pH meter. Total titratable acidity (TA) acidity was determined by titrating 10 ml of the medium with 0.1 mol/l NaOH to pH 7.0, and expressed as the volume consumed in milliliters of 0.1 mol/l NaOH per milliliter sample. The reducing sugars content was determined using the 3,5-dinitrosalicylic acid method with a spectrophotometer [20].

**Patulin Determination**

Patulin was extracted and analyzed as described previously [19] with slight modifications. Briefly, a 25 ml aliquot of cell-free medium was extracted twice with 25 ml of ethyl acetate by vigorous vortexing. The upper ethyl acetate layers at each extraction step were pooled and cleaned up with 1.5% Na₂CO₃. The resulting solvent extracts were evaporated to dryness at 40°C and then dissolved in 1 ml of 0.2 mol/l acetic acid prior to high-performance liquid chromatography (HPLC) determination of patulin content. The HPLC apparatus was a Shimadzu Prominance system (Shimadzu, Japan) that consisted of a LC-20A solvent delivery unit and a SPD-M20A photo-diode array detector. Liquid chromatography was conducted with a Shim-pack VP-ODS C18 column (250 × 4.6 mm i.d., 5 μm particle size). Water:acetonitrile (90:10 (v/v)) was used as the mobile phase at a flow rate of 1 ml/min. An aliquot of 20 μl was injected, and patulin was measured at 276 nm.

**Protein Extraction**

Protein was extracted following the method of Wang et al. [21]. The mycelia were ground in liquid nitrogen to a fine powder, and suspended in 10% trichloracetic acid in ice-cold acetone for 2 h at 4°C. After centrifugation at 10,000 × g for 10 min, the precipitate was washed three times with 80% ice-cold acetone and air dried at room temperature. Then, the protein pellet was dissolved in 8 M urea, 2 M thiourea, 4% 3-(3-cholamidopropyl) dimethylammonio)-1-propane sulfonate, 20 mM DTT, and 1% carrier ampholine (pH 4–7). Finally, the resultant lysate was centrifuged for 10 min at 16,000 × g at 4°C after vortexing overnight at room temperature. Protein concentration was determined by the Bradford assay using bovine serum albumin as the standard.

**Two-Dimensional Gel Electrophoresis**

Two-dimensional gel electrophoresis (2-DE) was carried out according to the method of Cai et al. [22]. Briefly, 800 μg of total protein extracts were applied to IPG strip gels (pH 4–7, 17 cm) (Bio-Rad, USA) by passive rehydration for 12 h. Isoelectric focusing (IEF) was conducted on a PROTEAN IEF Cell (Bio-Rad, USA) for a total of 60 kVh at 17°C. After IEF, the IPG strips were first incubated in 10 ml of equilibration buffer consisting of 7 M urea, 2% (w/v) SDS, 0.075 M Tris–HCl (pH 8.8), and 30% (v/v) glycerol together with 1% (w/v) DTT for 15 min, and subsequently dried.
with the same buffer containing 2.5% (w/v) iodoacetamide for 15 min. The second dimensional SDS-PAGE was performed on 12% polyacrylamide gels with an Ettan DALT System (GE Healthcare, USA). Protein spots were visualized by colloidal Coomassie blue staining; images were acquired with a GS-900 scanning densitometer (Bio-Rad, USA) and analyzed with PDQuest software (Bio-Rad, USA) according to the manual protocol. The individual protein spot intensity was normalized as a percentage of the total quantity of the valid spots present in each gel. By comparing the normalized intensity of each protein spot between fungal cultures, only those protein spots that showed greater than 1.5-fold increase \( (p < 0.05) \) in quantitative intensity were considered as induced proteins.

In-Gel Digestion and Protein Identification

The selected protein spots were manually punched from gels. After washing twice with distilled water and 50 mM \( \text{NH}_4\text{HCO}_3/50\% \ (v/v) \) acetonitrile, the excised gel pieces were first reduced by incubation with 10 mM DTT for 45 min at 55°C. After cooling, the gel pieces were alkylated by incubation with 55 mM iodoacetamide for 45 min at room temperature in the dark. Subsequently, the gel pieces were dehydrated with 50 mM \( \text{NH}_4\text{HCO}_3/50\% \ (v/v) \) acetonitrile, followed by a complete centrifugal lyophilization. Finally, 10 \( \mu l \) of 10 mg/l trypsin (Sigma–Aldrich, USA) was added and incubated with the dried gel pieces overnight at 37°C. The digestion solution was carefully collected for later mass spectrometry analysis.

Peptide mass fingerprinting (PMF) analysis was performed using an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker, Germany). The obtained peak lists were queried against the NCBInr database using the MASCOT program (http://www.matrixscience.com). The search parameters were set as NCBInr other fungal, one trypsin missed cleavage, carbamidomethylation of cysteine as fixed modification, monoisotopic, mass value MH\(^+\), and a maximum peptide tolerance of \( \pm 0.2 \) Da.

Statistical Analysis

Data from the experiment were analyzed by one-way analysis of variance using the software package SPSS ver. 17.0. Duncan’s multiple range test was adopted for mean comparison at the \( p = 0.05 \) level.

Results and Discussion

Growth Response of \( P. \text{expansum} \) to Apple Juice

During the entire incubation period, the biomass of \( P. \text{expansum} \) mycelia in apple juice medium exhibited an increasing trend up to day 6, followed by a moderate decrease (Fig. 1A). The growth curve of \( P. \text{expansum} \) in the apple juice observed in this experiment is quite similar to that reported by Taguchi et al. [23]. We also monitored the changes in reducing sugar content in the medium during

![Fig. 1](image-url)

**Fig. 1.** Growth assay of \( P. \text{expansum} \) in apple juice medium. (A) Biomass; (B) reducing sugar content; (C) pH value and total acidity; and (D) patulin content.
cultivation (Fig. 1B), and the results indicated that this increased slightly during the first 2 days of cultivation, which might be explained by the secretion of invertase that catalyzes sucrose hydrolysis, and was followed by a gradual decrease with the fungal growth and carbon source consumption [24].

The pH values of the medium decreased gradually with respect to the time for all the samples (Fig. 1C); coordinately, the total titratable acidity progressively increased over the incubation and attained the final average acidity of 0.32 ml of 0.1 mol/l NaOH per milliliter of medium (Fig. 1C). With respect to patulin, the major mycotoxin produced by *P. expansum*, it was observed that the content increased along the incubation and reached a maximum value of about 18.7 µg/ml on day 8 (Fig. 1D). Patulin accumulation showed a good correlation to the decrease of pH value of the culture medium. In agreement with our findings, Vilanova et al. [7] reported a rapid acidification in *Penicillium digitatum*-decayed oranges and *P. expansum*-decayed apples. They suggested that the ability of pathogenic fungi to modify the host pH is required to ensure the optimal pH conditions for the mycotoxin production and the fungal pathogen virulence. Moreover, a recent report by Zhang et al. [25] clearly indicated that the expression of cell wall-degrading enzymes in *P. digitatum* was regulated by PacC, a transcription factor related to pH signaling, suggesting a crucial role of the pH signal in pathogenesis of this phytopathogen.

2-DE Protein Profiles of *P. expansum* Grown in Different Culture Media

To further investigate the cellular processes possibly involved in fungi-host interaction, comparative proteomic analyses of *P. expansum* grown in Czapek–Dox medium, which was used as a control, and the apple juice medium, which was used to mimic the in planta condition, were carried out. Patulin accumulation in apple juice medium increased dramatically after 48 h of cultivation (Fig. 1D). Moreover, a previous report indicated that a number of differentially expressed genes in *P. expansum* grown in medium supplemented with apple pectin were observed at 3 days after inoculation, by differential display RT-PCR [26]. Therefore, to explore the cellular events at the early stage of pathogen-host interaction, the samples were collected at 48 h after inoculation to perform proteomic analysis. 2-DE was conducted using IPG strips with linear pH 4−7 and 12% SDS-PAGE, and about 450 protein spots could be reproducibly detected by CBB staining (Fig. 2). After normalization by the total density of all the valid spots on the respective 2-DE gel, quantitative comparison indicated 37 protein spots with increased expression abundance of greater than 1.5-fold in *P. expansum* after 48 h of cultivation in the apple juice medium. These induced protein spots were excised and subjected to MS analysis, and 28 protein spots were successfully identified by querying the PMF data against the NCBInr protein database (Table 1), achieving an identification rate of 76%.

![Fig. 2](image-url) Two-dimensional electrophoresis of *P. expansum* cultivated for 48 h in Czapek–Dox and apple juice media. Numbers on the spots refer to proteins identified by peptide mass fingerprinting and are explained in Table 1. Mr, Molecular weight; pl, isoelectric point.
Based on their possible involvements in cellular processes, all the identified proteins could be mainly allocated to seven functional categories, including carbohydrate metabolism, energy production, secondary metabolism, redox homeostasis, protein biosynthesis, protein degradation, signal transduction, and unclassified (Fig. 3). Among the 28 identified proteins, 16 protein spots were found to be involved in carbohydrate
metabolism, energy production, and secondary metabolism (Fig. 4), suggesting these physiological processes are major priorities for *P. expansum* in response to the host plant environment (Fig. 5). This is accordance with previous results reported by Tremblay *et al.* [27] at the transcriptome level, and they found that many genes related to carbohydrate metabolism and oxidative phosphorylation were preferentially expressed in *Phakopsora pachyrhizi* during the development of soybean rust.

In addition, it is worth to notice that spot 7 corresponding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), an enzyme catalyzing the transformation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate, was found to be apparently induced in *P. expansum* after exposure to host plant tissues. In addition to its function in glycolysis for energy production, GAPDH has also been considered as a

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**Fig. 3.** Pie chart showing the functional categorization of identified proteins. The classification was based on the MIPS functional catalog database (http://mips.helmholtz-muenchen.de/proj/funcatDB/) and related literature data.

**Fig. 4.** Enlarged views of proteins related to carbohydrate metabolism, energy production, and secondary metabolism (A), and bar graphs of their relative abundance in *P. expansum* after cultivation for 48 h in Czapek-Dox and apple juice media (B).
bacterial virulence factor, since this protein was observed only on the surface of pathogenic E. coli cells but not on nonpathogenic cells [28]. Recently, GAPDH has been demonstrated to be an adherence factor in Penicillium marneffei by mediating the adhesion of conidia to host cells during the initial development of infection [29]. The increased expression of this enzyme in P. expansum cultivated in the apple juice, which is predicted to be a secreted protein (Fig. 4), suggested that GAPDH might be involved in the host tissue colonization by P. expansum, although further experiments are required to test this hypothesis.

Glucose dehydrogenase (spot 10) and galactose oxidase (spot 3), enzymes known to catalyze the oxidation of glucose and galactose to their corresponding alduronic acid, were up-regulated 2.0- and 4.6-fold in P. expansum when cultivated in apple juice medium, which might explain the observed obvious acidification of the culture medium during the incubation (Fig. 1C). As suggested by Barad et al. [30], accumulation of gluconic acid, the oxidation product of glucose, might modulate patulin biosynthesis as a direct precursor under dynamic pH conditions regulating the subsequent activation of virulence factors, which contributes to the establishment of infection by P. expansum.

Protein spot 13 was identified as lactamase and functioned in the degradative detoxification of benzoazolinones, a compound that belongs to the phytoalexins produced by plants in defense response to various biotic stresses [31]. The involvement of lactamase in pathogen infection was recently confirmed by Yang et al. [32], when they found lactamase was regulated by NagZ, which was required for full pathogenicity in Xanthomonas campestris. Cytochrome P450 monooxygenase is known to be a type of FAD-binding monooxygenase [33] with a role in the hydroxylation of m-cresol and m-hydroxybenzylalcohol during patulin biosynthesis [34]. Protein spots 13 and 14 were identified as FAD-binding monooxygenases, and their expression levels were significantly up-regulated in P. expansum when cultivated in the apple juice medium, which might be responsible for the rapid accumulation of patulin in the culture medium.

Three families of peptidase, including peptidase family aspartic, M16, and M20, were also found to be highly expressed in P. expansum cultivated in the fruit juice medium. In plant pathogens Valsa mali and Diplodia corticola, aspartic peptidase and other types of peptidases were induced during fungal infection, which might be involved in the hydrolysis of the plant cell wall, impairing the proteins in the host plant and aiding fungal hyphal penetration [35, 36].
Proteins with antioxidant activity are known to be associated with the pathogenicity of *P. expansum* [37]. Interestingly, the increased expression of two antioxidant protein species, catalase (spot 26) and isoflavone reductase (spot 24 and 25), was indeed observed in this study and showed good agreement with the very recent findings by Ballester et al. [9] at the transcriptome level. Therefore, it can be suggested that the modulation of reducto status in *P. expansum* and subsequent adaption to a stressful redox environment could be of great importance in the colonization of host tissues by the fungal pathogen.

In summary, this study describes a biochemical and proteomic analysis of the phytopathogenic fungus *P. expansum* grown with the presence of host plant tissues. It was found that the pH value of the culture medium and reducing sugar content decreased, whereas the patulin content increased, with the *P. expansum* growing. Differential proteomic analysis revealed that 28 proteins were up-regulated in *P. expansum* after 48 h of cultivation in apple juice medium. An important number of the identified proteins corresponded to pathogenesis, such as glyceraldehyde-3-phosphate dehydrogenase, catalase, and peptidase, and some of them have been related with secondary metabolism and patulin biosynthesis regulation, such as glucose dehydrogenase and FAD-binding monooxygenase. An interpretation of the physiological and proteomical changes, a host plant environment-responsive protein network in *P. expansum*, was proposed. The future elucidation of the involvement of the proteins identified in this study in the development of infection by *P. expansum* using target gene mutant experiments could provide new insights into the interplay between the fungus and host.

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**References**


