Penicillium expansum, a widespread filamentous fungus, is a major causative agent of fruit decay and may lead to the production of mycotoxin that causes harmful effects on human health. In this study, we compared the cellular and extracellular proteomes of P. expansum in the absence and presence of borate, which affects the virulence of the fungal pathogen. The differentially expressed proteins were identified using ESI-Q-TOF-MS/MS. Several proteins related to stress response (glutathione S-transferase, catalase, and heat shock protein 60) and basic metabolism (glyceraldehyde-3-phosphate dehydrogenase, dihydroxy-acid dehydratase, and arginase) were identified in the cellular proteome. Catalase and glutathione S-transferase, the two antioxidant enzymes, exhibited reduced levels of expression upon exposure to borate. Because catalase and glutathione S-transferase are related to oxidative stress response, we further investigated the reactive oxygen species (ROS) levels and oxidative protein carbonylation (damaged proteins) in P. expansum. Higher amounts of ROS and carbonylated proteins were observed after borate treatment, indicating that catalase and glutathione S-transferase are important in scavenging ROS and protecting cellular proteins from oxidative damage. Additionally, we find secretory proteins that contribute to the virulence, we studied the extracellular proteome of P. expansum under stress condition with reduced virulence. The expression of three protein spots were repressed in the presence of borate and identified as the same hydrolytic enzyme, polygalacturonase. Molecular & Cellular Proteomics 6:425–438, 2007.

Penicillium expansum is a widely spread fungal pathogen that causes blue mold rot in a variety of fruits, including apples, pears, peaches, and cherries (1). It causes significant economic losses to the fruit industry and is also of potential public health concern because it produces toxic secondary metabolites, including patulin, citrinin, and chaetoglobosins (2). Patulin is produced by various filamentous fungi with P. expansum being generally regarded as one of the main producers (3). Because of the potential carcinogenic effects of this mycotoxin, Europe and the United States have established a maximum limit for patulin contamination in apple-based products (3). Control of decay caused by P. expansum has become important for ensuring the quality and safety of various fruits. Currently, the disease caused by P. expansum is mainly controlled by the intensive use of synthetic fungicides. However, concerns about public health and the development of fungicide resistance by pathogens have prompted the search for alternative methods (4). This requires more knowledge about the pathogenesis at the biochemical and molecular level.

During the course of invasion, a plant pathogen encounters the defense strategies of the host, including the accumulation of barrier-forming substances and the production of antimicrobial compounds that act directly to prevent pathogen invasion (5). In addition, the pathogen will also face the attack of reactive oxygen species (ROS), primarily superoxide (O2•-) and hydrogen peroxide (H2O2), generated by the host at the site of infection (5). These ROS may cause oxidative damage to cell components including proteins, lipids, and nucleic acids (6, 7). To protect cells against ROS, pathogens have developed several defense mechanisms, including enzymes (e.g., catalase and superoxide dismutase) as well as the nonenzymatic protective molecules such as glutathione and thioredoxin (8, 9). Davidson et al. (10) reported that yeast mutants deleted for the genes encoding catalase, superoxide dismutase, and cytochrome c peroxidase were more sensitive to the oxidative stress than the isogenic wild-type yeast, demonstrating that antioxidant enzymes play an important role in oxidative stress resistance. Under protection by defense mechanisms from adverse environmental conditions in the site of invasion, a pathogen infects the host by crossing through the cell wall of the plant, which is composed of a complex matrix of polysaccharides, proteins, lipids, and other components.
Identification of Virulence Proteins Based on Proteomics

charides. Extracellular proteins of the plant pathogen play a crucial role in this process. Through genetic and biochemical approaches, many secretory proteins have been characterized, such as pectate lyases (11, 12) and polygalacturonases (13, 14). However, these studies have mainly focused on the identification, purification, and characterization of single secreted proteins. Few publications on the global analysis of fungal secretory proteins are available (15).

In recent years, proteomics analysis has proven to be a powerful method for studying the changes of protein expression profiles in response to various stresses in yeast (16–18) and bacterium (19–22). On the other hand, there is still a lack of information about proteomics analysis from filamentous fungi (23, 24). P. expansum is very poorly characterized at the protein level. In a preliminary study, we found that borates, which are distributed widely in the environment, could significantly reduce the virulence of P. expansum in various fruits. Borates are essential plant micronutrients that help plant growth and have been used extensively in industry and agriculture as a safe method for control of fungi, bacteria, and many insects. However, there is no information about using borates to control fruit decay. In the present study, comparative analysis of the cellular and extracellular proteome was performed in P. expansum under normal condition and borate stress to gain insights into the pathogenesis of this fungal pathogen. Particular attention was paid to the antioxidant proteins and hydrolytic enzymes that are differentially expressed upon exposure to borate. To our knowledge, this is the first report for identifying virulence proteins in P. expansum on the basis of proteomics analysis.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—0.67% yeast nitrogen base without amino acids plus 2% dextrose (YNB) was from Difco. Urea was purchased from ICN (Aurora, OH). Carrier ampholyte was from Amersham Biosciences. Potato dextrose broth (PDB), thiourea, DTT, CHAPS, SDS, and 2,3-dichloro-5,6-dinitrophenylfluorescein diacetate (DCFH-DA) were purchased from Sigma. PMSF, acrylamide, bisacrylamide, TEMED, ammonium persulfate, β-mercaptoethanol, and Nonidet P-40 were from Amresco (Solon, OH). Two-dimensional SDS-PAGE standard was from Bio-Rad. Sequencing grade trypsin was from Promega (Madison, WI). Water was prepared using a Milli-Q system (Millipore, Bedford, MA).

Fungal Strain and In Vitro Antimicrobial Effect Assays—The fungal pathogen P. expansum Link (CGMCC3.3703) was used in this study. The fungus was inoculated and reisolated from apple fruit to maintain pathogenicity. The isolates were routinely grown on potato dextrose agar (PDA) or 0.1% potassium tetraborate. YNB instead of PDB was used to avoid the contamination of proteins from PDB. Galacturonase at 0.2% was added to the culture medium to induce pectinase synthesis (26), and the pH of the medium was adjusted to 5.8 with NaOH. The fungus was grown on a rotary shaker at 200 rpm for 7 days at 25 °C with an initial concentration of 1 × 10^6 spores/ml. After incubation, hyphae in the suspension were removed by filtration through 0.2-μm-pore size membrane. The extracellular proteins in the cell-free filtrate were precipitated with cold acetone as described for the intracellular protein fraction.

Two-dimensional Gel Electrophoresis—Two-dimensional (2D) gel electrophoresis was carried out according to the method of Abbasi and Komatsu (30) with minor modification. The IEF gel mixture contained 8% urea, 3.3% (w/v) acrylamide, 0.19% (w/v) bisacrylamide, 2% (v/v) Nonidet P-40, 5% (v/v) carrier ampholytes (pH 3.5–10.0; pH 5.0–8.0 = 1:1), 0.015% (w/v) ammonium persulfate, and 0.1% (v/v) TEMED. The gels were polymerized in glass tubes (Daichi Pure Chemicals, Tokyo, Japan) to obtain gels of 13.5-cm length and 3-mm diameter. Approximately 500 μg of intracellular proteins and 400 μg of extracellular proteins determined by the method of Bradford (31) were applied to the gel. IEF was performed at 200 V for 30 min, 400 V for 15 h, and then 800 V for 1 h. The cathode buffer was 20 mM NaOH, and the anode buffer was 20 mM H_2PO_4^- . Following IEF, gels were incubated in equilibration buffer containing 10% (v/v) glycerol, 2.5% (w/v) SDS, 125 mM Tris-HCl (pH 6.8), and 5% (v/v) β-mercaptoethanol with gentle agitation at room temperature for 15 min with two changes. SDS-PAGE in the second dimension was conducted using 15% polyacrylamide gels with 5% stacking gels. The gel strip of the first dimension was placed onto the stacking gel and sealed with 0.5% agarose melted in equilibration buffer without β-mercaptoeth-
anol. The running buffer contained 25 mM Tris (pH 8.3), 195 mM glycine, and 0.1% (w/v) SDS. The gels were stained with colloidal Coomassie Brilliant Blue (CBB) R-250 solution containing 50% (v/v) methanol, 15% (v/v) acetic acid, and 0.1% (w/v) CBB R-250. The pI and Mᵋ of each protein was calibrated using 2D electrophoresis markers.

Image Analysis of 2D Gels—The colloidal CBB-stained gels were scanned using a flatbed scanner (Amersham Biosciences) with 300 dpi resolution and saved in TIF format. Comparison of protein expression in 2D gel images was performed using Image Master 2D Elite software (Amersham Biosciences). To account for experimental variation, at least triplicate gels, resulting from protein extracts obtained from independent experiments, were analyzed for each treatment. Spot detection was carried out automatically, and those spots showing faint intensity near the detection limit of colloidal CBB were not included in the comparisons. Prior to automatic matching of spots between gel images, one gel was selected as the reference gel of each treatment. The amount of a protein spot was calculated based on the volume of that spot. To reflect the quantitative variations in each treatment, the instrument was externally calibrated using the fragmentation spectrum of the doubly charged ions of fibrinopeptide B. The peptides were identified by the addition of 1 volume of neutralization solution. Proteins were stained in the gels to monitor equal loading. Gel digest was performed as described by Shen et al. (32). Coomassie Blue-stained protein spots were manually excised from the gels and cut in about 1-mm² pieces. Gel slices were destained with 50 mM NH₄HCO₃ in 50% (v/v) methanol for 1 h at 40 °C. The step was repeated until the color disappeared. Gel pieces were then mixed with 10 mM DTT in 100 mM NH₄HCO₃ for 1 h at 60 °C to reduce the proteins. The gels were dried in a vacuum centrifuge for 30 min prior to incubating with 40 mM iodoacetamide in 100 mM NH₄HCO₃ for 30 min at ambient temperature in the dark to alkylate the proteins. The gel pieces were washed several times with water and completely dried in a vacuum centrifuge. Enzymatic digestion was carried out by adding gel pieces into the digestion buffer containing 100 mM NH₄HCO₃ and 5 ng/µl trypsin. The reaction mixture was kept at 37 °C for 16 h. Digested peptides were extracted by three changes of 0.1% TFA in 50% acetonitrile. The collected solutions were concentrated to 10 µl and then desalted with ZipTipC₁₈ (Millipore). Peptides were eluted from the column in 2 µl of 0.1% TFA in 50% acetonitrile.

ESI-MS/MS was performed for the purified tryptic digests using a quadrupole time-of-flight mass spectrometer (Q-TOF-2; Micromass, Altrincham, UK) equipped with a z-spray source (29). Before loading the digested peptide samples, the instrument was externally calibrated using the fragmentation spectrum of the doubly charged 1571.68-Da (785.84 m/z) ion of fibrinopeptide B. The peptides were loaded by nanoelectrospray with gold-coated borosilicate glass capillaries (Micromass). The applied spray voltage was 800 V with a sample cone working on 30 V. Dependent on the mass and charge state of the peptides, the collision energy was varied from 14 to 40 V. Peptide precursor ions were acquired over the m/z range 400–1900 Da in TOF-MS mode. Multiply charged (2⁺ and 3⁺) ions rising above predefined threshold intensity were automatically selected for MS/MS analysis, and product ion spectra were collected from m/z 50–2000. Tandem MS data were processed using MaxEnt 3.0 (Micromass) to create peak lists. The generated peak lists were uploaded to Mascot MS/MS Ions Search program (Mascot version 2.0) on the Matrix Science public web site, and protein identification was performed against the National Center for Biotechnology Information non-redundant (NCBInr) protein databases (version, April 14, 2006; 3,570,920 sequences) or EST database (version, April 29, 2006; 140,695,050 sequences) with a taxonomy restriction to “Other Fungi.” Trypsin was specified as the proteolytic enzyme, and one missed cleavages was allowed. Variable modifications selected for searching included carboxymethylation of cysteine, oxidation of methionine, and N-terminal pyroglutamine. A peptide tolerance of ±2.0 Da for the precursor ions and an MS/MS tolerance of ±1.2 Da for the fragment ions were set (33). Peptide charges of +2 and +3 and monoisotopic mass was chosen, and the instrument type was set to ESI-QUAD-TOF. Mascot uses a probability-based “Mowse score” to evaluate data obtained from tandem mass spectra. Mowse scores were reported as −10 log₁₀(p) where p is the probability that the observed match between the experimental data and the database sequence is a random event. This means that the best match is the one with the highest score. Mowse scores greater than 44 were considered significant (p < 0.05). To confirm protein identification, a minimum of three observed peptides that were selected for MS/MS was required if the proteins were identified across species. For proteins that were identified with MS/MS spectra matched to less than three peptides from proteins in other species, MS/MS spectra were subjected to de novo sequence analysis using PEAKS Version 4.0 software (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada), and the generated peptide sequences were used for homology searching using available on-line tools (MS BLAST, European Molecular Biology Laboratory) as described by Shevchenko et al. (34) with the default search settings and the nrdb95 database. MS BLAST uses a scoring scheme based on precomputed threshold scores that are set conditionally on the number of retrieved high scoring segment pairs and the total number of fragmented precursors (34, 35).

Measurement of Reactive Oxygen Species—The oxidant-sensitive probe DCFH-DA was used to assess the intracellular ROS levels in P. expansum according to the methods described previously (36, 37). Spores of P. expansum were cultured in PDB medium supplemented with 0 or 0.1% potassium tetraborate as described above and collected after 2, 4, and 6 h of incubation. The spores were washed with 10 mM potassium phosphate buffer (pH 7.0) and incubated for 1 h in the same buffer containing 10 µM DCFH-DA (dissolved in dimethyl sulfoxide). After washing twice with potassium phosphate buffer, spores were examined under a Zeiss Axioskop microscope (Carl Zeiss, Oberkochs, Germany) using a fluorescein 2’,7’-dichlorodihydro-dio-specific filter. Immunodetection of Carbonylated Proteins—Carbonylated proteins from P. expansum were detected using the chemical and immunological reagents of the OxyBlot™ Protein Oxidation Detection kit (Chemicon) (38, 39). Briefly 1 volume of sample containing 20 µg of proteins was added to an equal volume of 12% SDS. Protein samples were derivatized to 2,4-dinitrophenylhydrazones (DNPs) by incubation with 1 additional volume of 2,4-dinitrophenylhydrazine for 15 min at room temperature. The derivatization reaction was stopped by the addition of 1 volume of neutralization solution. Proteins were separated by 12% SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore) using an electroblotting apparatus (Bio-Rad). The oxidatively modified proteins were detected with anti-DNP antibodies and the chemiluminescence blotting substrate ECLplus (Amersham Biosciences). Colloidal CBB was used to stain the proteins in the gels to monitor equal loading.

Supplemental Material—The detailed information of Mascot searching including the information of the mass spectra matching, individual ions scores, and sequence coverage diagrams are given in Supplemental Data 1. Peak lists generated from the primary mass.
spectra are provided as Supplemental Data 2. The results of MS BLAST for proteins that were identified across species with MS/MS spectra matched to less than three peptides are provided as Supplemental Table S1.

RESULTS

Antifungal Activity of Borate against P. expansum—The inhibitory effect of borate on the mycelial growth of P. expansum is shown in Fig. 1. The culture medium was supplemented with borate and adjusted to pH 5.8 to eliminate the influence of pH value on the efficacy of borate. It was observed that borate at 0.1% was effective to inhibit conidial germination and germ tube elongation of P. expansum, and the inhibitory effect was not significantly influenced by the pH value of the solution (Fig. 1). Spores began to germinate after incubation for 12 h at 25 °C under borate stress, whereas extensive mycelial growth was observed in culture medium without borate at the same time. After 17 h of incubation, when mycelial ramification was evident in the control treatment, abnormal germ tubes and distorted mycelium were observed in culture medium supplemented with borate.

Comparative Analysis of P. expansum Intracellular Proteome—Intracellular protein expression profiles of P. expansum under normal condition and borate stress were analyzed using high resolution 2D gel electrophoresis (Figs. 2 and 3). The concentration of borate and the time of contact were determined to ensure that a sufficient living population can be collected for protein extraction.

Approximately 500 protein spots were detected on individual gels of control and stressed hyphae after ignoring very faint spots and spots with undefined shapes and areas. A total number of 66 proteins whose expression was induced or
repressed under borate stress were detected in this study, and we focused on 25 differentially expressed protein spots with relatively high abundance. Due to the lack of genome sequence information for this species, protein identification provided an analytical challenge. We used MS/MS, which has been reported to be the most successful technique to correctly identify proteins from organisms with the whole DNA sequence unknown (40), to identify these proteins. MS/MS spectra were submitted for database searching in Mascot search engine. Of the 25 excised spots, 10 had no MS/MS data, whereas one did not fit with the database. Their identities need to be further confirmed. Fourteen proteins were identified with Mowse scores greater than the threshold. Table I summarizes the apparent molecular mass and relative pl for the 14 differentially expressed proteins as well as their relative levels of expression in the control and stressed con-

**Fig. 3.** Close-up views of intracellular proteins showing differential expression between control and borate-treated gels at indicated times. *P. expansum* was incubated in potato dextrose broth medium supplemented with 0 and 0.1% potassium tetraborate for 48, 60, and 72 h at 25 °C. Proteins were extracted from mycelium of the pathogen with magnesium/Nonidet P-40 extraction buffer by sonification as described under “Experimental Procedures.” Total protein (500 μg) was separated on 2D gels (pl 3–10) and stained with colloidal Coomassie Brilliant Blue R-250. The number of each protein spot (In-1–15) corresponds to its listing in Table I.
### Identification of intracellular proteins showing differential expression under borate stress using MS/MS analysis

Spot, spot number corresponding to spots in Figs. 2 and 3; Change, the average change in abundance expressed as intensity means ± S.D. from three independent treatments; Protein name, matched protein description; NCBI accession, accession number from NCBI database of matched protein; Theo. M, (kDa)/pI, theoretical molecular mass and isoelectric point based on amino acid sequence of the identified protein; Expt. M, (kDa)/pI, experimental molecular mass and isoelectric point estimated from the 2D gels; Species, the species of the matched protein; Mascot score, score obtained from Mascot for each match; NP, the number of matched peptides; PD, peptides detected; SC, amino acid sequence coverage for the identified proteins; , control; ■, treatment with borate.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Change</th>
<th>Protein name</th>
<th>NCBI accession</th>
<th>Theo. M</th>
<th>Expt. M</th>
<th>Species</th>
<th>Mascot score</th>
<th>NP/PD</th>
<th>MS/MS peptide sequence</th>
<th>Expt. m/z (charge)</th>
<th>SC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-1</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>67903760</td>
<td>54.0/6.30</td>
<td>61/5.8</td>
<td>Aspergillus nidulans</td>
<td>60</td>
<td>2/11</td>
<td>MLDADWFDTR YPGAPFLLTVK</td>
<td>655.39 (2)</td>
</tr>
<tr>
<td>In-2</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>88185990</td>
<td>53.2/4.83</td>
<td>41/4.6</td>
<td>Chlamydovirus phages</td>
<td>49</td>
<td>1/7</td>
<td>NGLYNTAFSLK</td>
<td>748.48 (2)</td>
</tr>
<tr>
<td>In-3</td>
<td></td>
<td>glutathione-S-transferase</td>
<td>gi</td>
<td>70988679</td>
<td>20.4/6.72</td>
<td>32/5.6</td>
<td>Aspergillus fumigatus</td>
<td>51</td>
<td>1/20</td>
<td>RBYLYEINPR</td>
<td>628.99 (2)</td>
</tr>
<tr>
<td>In-4</td>
<td></td>
<td>catalase</td>
<td>gi</td>
<td>1857716</td>
<td>79.9/5.65</td>
<td>77/5.8</td>
<td>Aspergillus fumigatus</td>
<td>92</td>
<td>2/12</td>
<td>NNYQIQR LFSVLITQQR</td>
<td>659.17 (2)</td>
</tr>
<tr>
<td>In-5</td>
<td></td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>gi</td>
<td>12692699</td>
<td>36/27.67</td>
<td>37/6.4</td>
<td>Aspergillus oryzae</td>
<td>243</td>
<td>4/18</td>
<td>VHSADAPM/VG/MVNNK</td>
<td>70.33 (3)</td>
</tr>
<tr>
<td>In-6</td>
<td></td>
<td>Coecidioidea sp.</td>
<td>gi</td>
<td>00093134</td>
<td>62/7.58</td>
<td>62/5.3</td>
<td>Coecidioidea inamisis</td>
<td>73</td>
<td>1/13</td>
<td>SDNPTGTITYR</td>
<td>654.78 (2)</td>
</tr>
<tr>
<td>In-7</td>
<td></td>
<td>vacuolar serine protease</td>
<td>gi</td>
<td>14215732</td>
<td>52.3/6.05</td>
<td>38/7.2</td>
<td>Penicillium chrysogenum</td>
<td>258</td>
<td>4/5</td>
<td>ELSFQWFK VSNGC1MSSVVK</td>
<td>571.87 (2)</td>
</tr>
<tr>
<td>In-8</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>90300942</td>
<td>37.9/7.95</td>
<td>45/5.6</td>
<td>Coecidioidea inamisis</td>
<td>135</td>
<td>3/18</td>
<td>APYEEVVK</td>
<td>597.8 (2)</td>
</tr>
<tr>
<td>In-9</td>
<td></td>
<td>unnamed protein product</td>
<td>gi</td>
<td>83766142</td>
<td>38.9/6.77</td>
<td>48/6.2</td>
<td>Aspergillus oryzae</td>
<td>132</td>
<td>3/24</td>
<td>MVLGQHGDGVK</td>
<td>675.92 (2)</td>
</tr>
<tr>
<td>In-10</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>67515665</td>
<td>27.3/4.90</td>
<td>59/4.1</td>
<td>Aspergillus nidulans</td>
<td>117</td>
<td>1/7</td>
<td>VWVNWAAADYVNGEGEPAQTALR</td>
<td>824.22 (3)</td>
</tr>
<tr>
<td>In-11</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>67901476</td>
<td>32.3/5.04</td>
<td>39/5.7</td>
<td>Aspergillus nidulans</td>
<td>253</td>
<td>4/14</td>
<td>EIMEAVTIVMAK</td>
<td>716.90 (2)</td>
</tr>
<tr>
<td>In-12</td>
<td></td>
<td>dihydroxy-acid dehydrogenase 3</td>
<td>gi</td>
<td>56313548</td>
<td>59.9/5.45</td>
<td>60/5.7</td>
<td>Aspergillus nidulans</td>
<td>62</td>
<td>1/13</td>
<td>YFSEDESSRGAMAR</td>
<td>813.86 (2)</td>
</tr>
<tr>
<td>In-13</td>
<td></td>
<td>heat shock protein 60</td>
<td>gi</td>
<td>67539838</td>
<td>61.8/5.53</td>
<td>60/5.2</td>
<td>Aspergillus nidulans</td>
<td>289</td>
<td>4/12</td>
<td>EDFDILNENGK</td>
<td>638.80 (2)</td>
</tr>
</tbody>
</table>

---

**Molecular & Cellular Proteomics 6.3**
The change in abundance is presented as grouped histograms with error bars, which represent the intensity means ± S.D. of gels from three independent experiments (Table I). Of the differentially expressed proteins, three are related to stress response, including glutathione S-transferase (In-3), catalase (In-4), and heat shock protein 60 (Hsp60, In-13). Most notably, we found that the expression levels of glutathione S-transferase (In-3) and catalase (In-4) were apparently reduced when cells were exposed to borate. Close-up views of the gels indicated that these proteins were also differentially expressed when the time of exposure to borate was prolonged (Fig. 3). The roles of these proteins in protecting cells from oxidative damage were confirmed by further analysis of ROS levels and protein carbonylation in cells subjected to borate in subsequent studies. Of the remaining proteins, three were enzymes involved in basic metabolism, including glyceraldehyde-3-phosphate dehydrogenase (In-5), dihydroxy-acid dehydratase 3 (In-12), and arginase (In-14). The other proteins were vacuolar serine pro-

---

**TABLE I—continued**

<table>
<thead>
<tr>
<th>Spot</th>
<th>Change</th>
<th>48h 60h 72h</th>
<th>Protein name</th>
<th>NCBI accession</th>
<th>Theo. Mr (kDa)</th>
<th>Expt. Mr (kDa)</th>
<th>Species</th>
<th>Mascot score</th>
<th>NP/PD</th>
<th>MS/MS peptide sequence</th>
<th>Expt. m/z (charge)</th>
<th>SC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-14</td>
<td></td>
<td></td>
<td>arginase</td>
<td>gi</td>
<td>0099514</td>
<td>35.4/5.32</td>
<td>43/5.4</td>
<td>Aspergillus fumigatus</td>
<td>55</td>
<td>1/10</td>
<td>AF581948/AF581949/AF283665</td>
<td>546.33 (2)</td>
</tr>
<tr>
<td>In-15</td>
<td></td>
<td></td>
<td>ND</td>
<td>—</td>
<td>—</td>
<td>30/6.0</td>
<td>—</td>
<td>—</td>
<td>02</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Mascot scores greater than 44 are statistically significant (p < 0.05).
* OM represents protein modifications (oxidation of methionine).
* ND, no match with database.

**FIG. 4.** Two-dimensional pattern of extracellular proteins of *P. expansum* cultured under normal condition and borate stress. *P. expansum* was cultured for 7 days in yeast nitrogen base medium supplemented with 0 and 0.1% potassium tetraborate at 25 °C. The mycelium in the suspension was removed by filtration through nylon membrane and 0.2-μm-pore size membrane. Secretory proteins in the cell-free filtrate were precipitated with ice-cold 20% (w/v) TCA. Total protein (400 μg) was separated on 2D gels (pI 3–10) and stained with colloidal Coomassie Brilliant Blue R-250. Arrows indicate proteins that are differentially expressed under borate stress. The protein spots are numbered, corresponding to the numbers in Table II.

**FIG. 5.** Close-up views of extracellular proteins showing differential expression between control and borate-treated gels as indicated in Fig. 4. The number of each protein spot (Ex-1–15) corresponds to its listing in Table II.
### Identification of Differentially Expressed Extracellular Proteins under Borate Stress Using MS/MS Analysis

<table>
<thead>
<tr>
<th>Spot</th>
<th>Change</th>
<th>Protein Name</th>
<th>NCBI Accession</th>
<th>Theo. Mr (kDa)/ pI</th>
<th>Expt. Mr (kDa)/ pI</th>
<th>Species</th>
<th>Mascot Score</th>
<th>NP/PD</th>
<th>MS/MS Peptide Sequence</th>
<th>Expt. MW (Charge)</th>
<th>SC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex-1</td>
<td></td>
<td>cell wall synthesis protein</td>
<td>gi</td>
<td>543923441</td>
<td>44.7/4.56</td>
<td>68.4/9.9</td>
<td><em>Penicillium chrysogenum</em></td>
<td>93 1/20</td>
<td>VTVSQYYYYPK</td>
<td>635.56 (2)</td>
<td>2</td>
</tr>
<tr>
<td>Ex-2</td>
<td></td>
<td>unnamed protein product</td>
<td>gi</td>
<td>503060918</td>
<td>35.4/5.17</td>
<td>55/5.2</td>
<td><em>Klosterella lactis</em></td>
<td>58 1/8</td>
<td>NVPIJNQIQK</td>
<td>528.92 (2)</td>
<td>2</td>
</tr>
<tr>
<td>Ex-3</td>
<td></td>
<td>S-acetylcholine synthetase</td>
<td>gi</td>
<td>675518128</td>
<td>42.2/5.3</td>
<td>42/5.6</td>
<td><em>Aspergillus nidulans</em></td>
<td>211 3/14</td>
<td>VYPAELDDDR FVQRNHFAGLGTGNDGSFRLPDITK</td>
<td>570.86 (2)</td>
<td>2</td>
</tr>
<tr>
<td>Ex-4</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>46112769</td>
<td>36.8/5.71</td>
<td>35/5.8</td>
<td><em>Gibbonella zeae</em></td>
<td>156 2/13</td>
<td>NSETLSSQGR LQIDTLQPPKP</td>
<td>575.20 (2)</td>
<td>6</td>
</tr>
<tr>
<td>Ex-5</td>
<td></td>
<td>polygalacturonase</td>
<td>gi</td>
<td>29353384</td>
<td>38.0/6.98</td>
<td>38/6.9</td>
<td><em>Penicillium expansum</em></td>
<td>113 2/3</td>
<td>GSSITGLNIK VIFGETTGFFYK</td>
<td>546.20 (2)</td>
<td>6</td>
</tr>
<tr>
<td>Ex-6</td>
<td></td>
<td>polygalacturonase</td>
<td>gi</td>
<td>29353384</td>
<td>38.0/6.98</td>
<td>38/6.9</td>
<td><em>Penicillium expansum</em></td>
<td>290 5/8</td>
<td>GSSITGLNIK WIDGEQSSFVIFGETTGFFYK</td>
<td>546.32 (2)</td>
<td>29</td>
</tr>
<tr>
<td>Ex-7</td>
<td></td>
<td>polygalacturonase</td>
<td>gi</td>
<td>29353384</td>
<td>38.0/6.98</td>
<td>38/7.1</td>
<td><em>Penicillium expansum</em></td>
<td>248 4/13</td>
<td>GIDQFYFAFIR RGDTGFGFAFR</td>
<td>619.35 (2)</td>
<td>11</td>
</tr>
<tr>
<td>Ex-8</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>76597627</td>
<td>32.6/6.64</td>
<td>20/5.5</td>
<td><em>Aspergillus fumigatus</em></td>
<td>121 2/13</td>
<td>VITLVFK VAVNITTVASCERF</td>
<td>619.35 (2)</td>
<td>4</td>
</tr>
<tr>
<td>Ex-9</td>
<td></td>
<td>hypothetical protein</td>
<td>gi</td>
<td>76593638</td>
<td>42.3/5.6</td>
<td>40/5.8</td>
<td><em>Aspergillus fumigatus</em></td>
<td>273 4/35</td>
<td>LAQWPEHFLRLENDQRQNDVDAVQMR QVIAH</td>
<td>1089.27 (3)</td>
<td>13</td>
</tr>
<tr>
<td>Ex-10</td>
<td></td>
<td>malate dehydrogenase, NAD-dependent</td>
<td>gi</td>
<td>76988899</td>
<td>35.9/9.08</td>
<td>34/6.1</td>
<td><em>Aspergillus fumigatus</em></td>
<td>248 4/13</td>
<td>IFQGDEKVK VSLAEYTVLR LGYTVLQVR DLQPTMVASVLR</td>
<td>546.56 (2)</td>
<td>12</td>
</tr>
<tr>
<td>Ex-11</td>
<td></td>
<td>3-phosphoglycerate kinase</td>
<td>gi</td>
<td>298258</td>
<td>44.0/6.07</td>
<td>44/6.1</td>
<td><em>Penicillium citrinum</em></td>
<td>1176 21/35</td>
<td>KELEYAK ELPVVAALSSK</td>
<td>514.86 (2)</td>
<td>52</td>
</tr>
</tbody>
</table>

**TABLE II**

Identification of Virulence Proteins Based on Proteomics
tease (In-7), unnamed protein product (In-9), and hypothetical protein (In-1, -2, -6, -8, -10, and -11). To confirm the protein identifications performed by Mascot searching with MS/MS spectra matched to less than three peptides (In-1–4, In-6, -10, -12, and -14), MS BLAST was performed. The result indicated that seven of these proteins (In-1, -2, -4, -6, -10, -12, and -14) could be identified by MS BLAST (see Supplemental Table S1). For proteins that were not identified by MS BLAST, peptide MS/MS data were searched against publicly available EST database. Nam et al. (41) reported that EST database searches combined with MS/MS analysis could be used to identify proteins from organisms for which the full genome database is not yet available. We performed the conventional BLAST searching with the query of matched sequences found in the EST database. Spot In-3 was successfully identified with this method.

**Comparative Analysis of P. expansum Secretory Proteome**—Comparative analysis of the extracellular proteome was performed to investigate the secretory protein profiles of *P. expansum* between control and stressed cells (Figs. 4 and 5). Changes in abundance of protein were measured in control gels compared with borate treatment in three independent replicates. To avoid the contamination of proteins from the culture medium, YNB medium without amino acids was chosen to culture the fungus. The fungus was incubated up to 7 days to obtain enough proteins for separation by 2D electrophoresis. Several methods for extracting proteins from culture supernatant of *P. expansum* were tried. In our case, as acetone precipitation resulted in a poor yield and the precipitates were difficult to be solubilized (data not shown), we used 20% (w/v) TCA to precipitate the secretory proteins in the cell-free filtrate to obtain well resolved gel electrophoresis.

Most extracelllular protein spots on the gel have an acidic pl value in the range from pH 4 to 7 and a molecular mass between 15 and 80 kDa. About 200 protein spots could be detected on 2D gels after ignoring very faint spots and spots with undefined shapes and areas. Image analysis revealed that 26 proteins were differentially expressed under borate stress. Using ESI-MS/MS, we analyzed 18 of these protein spots that showed relatively high abundance. Three of these differentially expressed proteins did not show MS/MS data, and the remaining 15 were submitted for database searching in Mascot search engine. Among these 15 protein spots, 14 representing 12 different proteins were identified with signifi-
cant Mowse scores (p < 0.05), whereas one (Ex-15) did not fit with the database. It is noteworthy that three protein spots (Ex-5–7) were identified as the same protein, polygalacturonase (Table II). Their location in the gels differed slightly in molecular mass and pI (Figs. 4 and 5), indicating that they might be isoforms of polygalacturonase or have different posttranslational modifications. Their expression was repressed under borate stress. In addition, four proteins were identified as enzymes involved in basic metabolism, including S-adenosylmethionine synthetase (Ex-3), malate dehydrogenase (Ex-10), 3-phosphoglycerate kinase (Ex-11), and glyceraldehyde-3-phosphate dehydrogenase (Ex-12). Also we identified a cell wall synthesis protein (Ex-1) and six proteins with unknown function (Ex-2, -4, -8, -9, -13, and -14). Among these proteins, six (Ex-1, -2, -4, -8, -13, and -14) were identified across species with MS/MS spectra matched to less than three peptides. MS BLAST analysis confirmed the identifications of spot Ex-8. Proteins that were not identified by MS BLAST were searched against EST database, and the result showed that no homologous sequences were found for the remaining protein spots (Ex-1, -2, -4, -13, and -14). The identification of these proteins remains tentative.

Detection of Reactive Oxygen Species with DCHF-DA—To further investigate the role of catalase and glutathione S-transferase in scavenging ROS in cells, we used a fluorescent dye, DCHF-DA, to determine whether ROS levels changed when P. expansum was exposed to borate (Fig. 6). DCHF-DA
Identification of Virulence Proteins Based on Proteomics

has been extensively used to assess ROS in yeast cells under oxidative stress (36, 37). In our study, spores of P. expansum were incubated in the absence and presence of borate for different time at 25 °C. The spores were collected before germination (less than 8 h), stained with DCHF-DA, and observed under a fluorescence microscope. After 2 h of incubation, the cells showed no staining with DCHF-DA, implying that there were few oxidizing molecules (ROS) produced at that time. However, with the increased period of incubation, ROS were detected in spores of P. expansum. Compared with control, more cells under borate stress were stained with the DCHF-DA, indicating that an increasing amount of oxidizing molecules was produced in cells exposed to borate. The basal levels of ROS in cells are generated by aerobic metabolism (7). We speculate that the increased levels of ROS in cells of P. expansum treated with borate are likely due to the suppression of expression of antioxidant enzymes, such as catalase and glutathione S-transferase, following borate stress.

Assay of Oxidative Damage to Cellular Proteins of P. expansum with Western Blot Analysis—Because the expression of two oxidative stress-specific proteins, catalase and glutathione S-transferase, were repressed in the intracellular proteomics analysis, an assessment of the oxidative damage of cellular proteins in P. expansum was undertaken by immunoblot assay with anti-DNP antibodies (Fig. 7A). Protein carbonylation is a widely used marker of oxidative protein damage, and immunoassay has been extensively used as a sensitive method for the detection of carbonylated proteins (38, 39, 42, 43). By comparing the CBB stain pattern of cellular proteins with the anti-DNP staining pattern, it is clear that protein carbonylation occurred in both control and stressed hyphae. The basal levels of protein carbonyl content are caused by ROS generated in cells through normal metabolic activity, such as mitochondrial respiration (44). Multiple protein bands were selectively modified (Fig. 7A) perhaps because they are especially sensitive to oxidative damage. The levels of protein carbonylation increased when the hyphae were exposed to borate. These proteins ranged in size of apparent molecular masses from 30 to 60 kDa. With the duration of the exposure to borate increased, the amounts of carbonyl groups in cell proteins of borate-treated hyphae were not enhanced significantly. Thus, we conclude that P. expansum cells were under an increased level of oxidative damage due to the suppression of expression of catalase and glutathione S-transferase by borate stress.

DISCUSSION

In this study, we explored proteins relevant to virulence of P. expansum on the basis of comparative analysis of cellular and extracellular proteomes under normal condition and borate stress. Through ESI-MS/MS analysis, 14 differentially expressed proteins were identified successfully in the cellular proteome (Table I). Among these identified proteins, the most notable was the reduced expression of two proteins, catalase (In-4) and glutathione S-transferase (In-3), which are involved in stress response. Catalase, the enzyme that catalyzes the degradation of H₂O₂ into water and oxygen, has been considered as one of the major H₂O₂-scavenging enzymes in all aerobic organisms (45). It is present from lower to higher organisms and has gained a lot of attention recently due to its link to cancer, diabetes, and aging in humans and animals (46, 47). Similarly glutathione S-transferase, the enzyme that detoxifies hazardous compounds such as fatty acid peroxides by conjugating glutathione to these toxic compounds, is important in protecting cells from oxidative stress (48–50). These two antioxidant enzymes may act together to fight against the ROS produced under lethal oxidative environments. In the present study, the expression of these two antioxidant defense proteins was found to be repressed when the fungus was subjected to borate stress (Figs. 2 and 3), implying that the ability of the P. expansum cells to scavenge ROS may decrease under borate stress. We then analyzed the ROS production in P. expansum exposed to borate using the oxidant-sensitive probe DCHF-DA. As expected, more cells were stained with the dye, indicating that more ROS were produced in borate-treated cells (Fig. 6). Undesirable accumulation of ROS can cause oxidative damage to cell components including proteins, lipids, and nucleic acids (6, 7). To verify whether such oxidative modifications occurred to proteins when P. expansum was under borate stress, we further studied the content of protein carbonylation using anti-DNP antibody. The patterns of protein oxidation presented in Fig. 7A revealed that, compared with control cells, more oxidatively damaged proteins were detected in cells exposed to borate. Taken together, these data suggest that borate causes oxidative damage to P. expansum by suppression of the expression of the antioxidant enzymes catalase and glutathione S-transferase. These proteins may be critical for the virulence of the fungal pathogen.

A proteomics approach has been widely applied to compare the protein expression patterns in organisms under different stress conditions. Stress-responsive proteins are often found to be up-regulated in response to stress treatments (16, 20). However, the results described here showed that the expression of two antioxidant defense proteins, catalase and glutathione S-transferase, were repressed in P. expansum after long term exposure to borate stress (Figs. 2 and 3). This may be explained by the difference in exposure time of organisms to stresses in the different experiments (17). The over-expression of antioxidant enzymes often occurs in the studies where cells are exposed to stresses for a short time (always less than 12 h), whereas in our experiment hyphae of P. expansum were treated with borate for 48–72 h. This result is consistent with the work of Chuang et al. (21), who observed that the expression levels of antioxidant enzymes were decreased in the Gram-negative microaerophilic bacterium Helicobacter pylori after long term incubation under oxidative...
stress. Interestingly we also found that the profile of the catalase changes in proteomics analysis was more complex at different incubation stages. The expression of catalase was significantly repressed after exposure to borate for 48 h. However, this repression decreased after 60 and 72 h of incubation in borate, suggesting that catalase could play an important role in protecting *P. expansum* from protein oxidative damage at the early stage of borate exposure. With the increased incubation period, other antioxidant proteins such as glutathione S-transferase and superoxide dismutases may work to fight against the ROS (48, 51).

Although the expressions of catalase and glutathione S-transferase were repressed, the expression of Hsp60 (In-13) in *P. expansum* was induced under borate stress. Hsp60 is a molecular chaperone with a wide variety of functions, such as protein folding, degradation, and the assembly of large protein complexes (52). Exposure of cells to elevated temperatures or other harsh treatments that damage cell proteins could induce the expression of Hsp60 (39). In the present study, we hypothesize that Hsp60 is likely involved in the degradation of oxidatively damaged proteins, which were produced upon long term exposure to borate stress. It is possible that as the fungus was exposed to borate Hsp60 was effectively induced to take part in the selective breakdown of these potentially toxic proteins, preventing cell death upon lethal stress environments. Together with the repressed expressions of catalase and glutathione S-transferase, these findings further indicate the complexity of cellular defense mechanisms against stresses.

In recent years, many studies have focused on the molecular mechanisms with which pathogens infect their hosts. Identification of secretory proteins, especially hydrolytic enzymes, is crucial to understand the pathogenesis of pathogens. A proteomics approach has been proved as a powerful technique for identifying novel virulence determinants (53, 54). To investigate the changes of secretory proteins of *P. expansum* under stress condition, we analyzed the extracellular proteome of this pathogen. In the protein spots that were found to be differentially expressed, we identified a hydrolytic enzyme, polygalacturonase. Three protein spots (Ex-5–7) with a slight difference in Mr and pI were identified as the same protein. This may be explained as a result of protein isoforms or posttranslational modifications (19). In the study of plant-infecting pathogen *Aspergillus flavus* and *Botrytis cinerea*, polygalacturonase was demonstrated as a colonization and virulence factor because the disruption of polygalacturonase genes reduces virulence (55, 56). Polygalacturonase splits the long pectin chains into smaller units of galacturonic acid, leading to breakdown of the gel-like pectic compounds that cement the plant cells. As shown in Figs. 4 and 5, the expression level of polygalacturonase was reduced when *P. expansum* was exposed to borate.

Interestingly several proteins associated with carbohydrate metabolism, such as S-adenosylmethionine synthetase (Ex-3), malate dehydrogenase (Ex-10), 3-phosphoglycerate kinase (Ex-11), and glyceraldehyde-3-phosphate dehydrogenase (Ex-12), were identified in the culture supernatant of *P. expansum* (Table II). The occurrence of these proteins, which are generally regarded as being of cellular origin, was considered to be the result of contamination by cell lysis or other reasons (57). However, in several other works, the cytoplasmic proteins were also associated with the cell surface or secreted into the external environment (58–60). Hughes et al. (59) reported that many of the major surface-bound proteins such as phosphoglycerate kinase, glyceraldehyde-3-phosphosphate dehydrogenase, enolase, and glucose-6-phosphate isomerase in the oral pathogen *Streptococcus agalactiae* had glycolytic or chaperonin functions. Among these proteins, glyceraldehyde-3-phosphate dehydrogenase is of particular interest. In the study of *Streptococcus pyogenes* (60), it was postulated to be a virulence determinant that contributed to the infection of the host.

Additionally two proteins (Ex-8 and -9), which did not display any significant similarity to proteins with known function in the protein database, corresponded to hypothetical proteins. They may be potential virulence factors, and their biological functions need to be elucidated.

In summary, we have applied a comparative proteome analysis to find two antioxidant enzymes, catalase and glutathione S-transferase, in *P. expansum* among the identified intracellular proteins. These two proteins may be critical for the virulence of this fungal pathogen because the suppression of expression of these proteins resulted in a higher amount of ROS and an increased level of carbonylated proteins in cells. Additionally polygalacturonase was identified in the extracellular proteome, and its expression was repressed in the presence of borate. These findings may be useful for understanding the mechanisms of pathogenicity and exploring potential targets for new antifungal agents.

Acknowledgments—We thank Dr. Shihua Shen for his advice in the proteomics experiment and Mei Huang for her help in the analysis of MS/MS. We also thank Dr. Li Li for her valuable suggestions and careful correction of the manuscript.

* This work was supported by National Natural Science Foundation of China Grants 30430480 and 30500351 and Ministry of Science and Technology of China Grant 2006CB101900. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Key Laboratory of Photosynthesis and Environmental Molecular Physiology, Inst. of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Haidian District, Beijing 100093, China. Tel.: 86-10-62836559; Fax: 86-10-82594675; E-mail: tsp@ibcas.ac.cn.

REFERENCES


sum: consistent production of patulin, chaetoglobosins, and other secondary metabolites in culture and their natural occurrence in fruit products. J. Agric. Food Chem. 52, 2421–2428


25. Molecular & Cellular Proteomics 6.3

437
tive stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 275, 27393–27398


