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Proteomic analysis of the inhibitory effect of chitosan on *Penicillium expansum*

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Abstract

The antimicrobial effect of chitosan on *Penicillium expansum*, a major postharvest pathogen of pome fruit, and the possible mechanisms involved in its effect were examined in this study. Chitosan strongly inhibited spore germination and hyphal growth of *P. expansum*. Light microscopy and transmission electron microscopy observations revealed that chitosan also caused morphological changes in hyphae and conidia, such as abnormal branching and vacuolation. Proteomic changes in *P. expansum* after chitosan treatment were analyzed by two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) analysis, and 26 proteins were ambiguously identified and categorized based on their putative biological function. Proteins related to DNA or protein biosynthesis, carbohydrate metabolism and energy production were decreased in relative protein abundance, while proteins involved in antibiotics resistance and defense response increased in relative protein abundance. Changes in abundance of these identified proteins were in accordance to the observed physiological and morphological changes of the fungi cells. Altogether, these experimental results provide a detailed illustration of the responses to chitosan in *P. expansum*, and widen our knowledge on the potential antifungal mechanisms of chitosan.

Keywords: chitosan; *Penicillium expansum*; antifungal activity; proteomics.

Practical application: Antifungal activity of chitosan against *P. expansum* and its possible mechanisms.

1 Introduction

The fungal pathogen *Penicillium expansum* is the causal agent of a major postharvest disease in pome fruits known as blue mold decay. In addition to causing economic losses, *P. expansum* also can produce patulin mycotoxin, a highly toxic compound that is harmful to both humans and animals therefore raising potential public health significance (Andersen et al., 2004; Puel et al., 2010). Currently, chemical fungicides are still the main strategy to control postharvest diseases; however, the appearance of resistance in pathogens together with growing public concern about environmental safety and human health associated with the use of chemical substances have impose necessity to explore new alternatives for postharvest diseases management (Zhang et al., 2011; Liu et al., 2013).

Chitosan, the deacetylation product of chitin, has been recently used as a promising alternative postharvest diseases management. Diverse studies have shown the efficiency of chitosan in controlling postharvest rot of horticultural commodities caused by plant pathogenic fungi, such as *Aspergillus flavus* (Dias et al., 2018), *Botrytis cinerea* (Lopes et al., 2014), *Ceratocystis fimbriata* (Xing et al., 2018), *Pilidiella granati* (Munhuweyi et al., 2017) and *P. expansum* (Wang et al., 2014). In addition to its potential to elicit plant defense by increasing the production of defense-related secondary metabolites and promoting the expression of defense-related enzymes (Hadwiger, 2013), chitosan also exhibits direct antimicrobial activities against plant pathogens (Xing et al., 2015). A number of researches have revealed that the antimicrobial mechanisms of chitosan can be partially explained by electrostatic interaction between the protonated amino group ($-HN_3^+$) on chitosan chain and the negatively charged components at the microbial surfaces, thereby increasing the plasma membrane permeability and causing the death of microorganisms (Garcia-Rincon et al., 2010; Olicón-Hernández et al., 2015). Furthermore, chitosan is presumed to be able to cross microbial cell membrane, destroy intracellular components, disrupt the normal physiological processes, or directly bind to genetic materials, interfere with DNA replication and ultimately resulting in the death of pathogens (Palma-Guerrero et al., 2009; Xing et al., 2015). Nevertheless, the responses to chitosan in plant pathogenic fungi are far from well known, especially the responses occurred at omics level.

Proteomic profiling by two-dimensional gel electrophoresis (2-DE) combined with mass spectrometry (MS) has been successfully used to analyze the global changes of protein expression in biological organisms under various environmental conditions (Wang et al., 2013; Yuan et al., 2014). In the present work, our aim is (i) to investigate the effect of chitosan on fungal growth, morphological and ultrastructural characteristics of *P. expansum*, (ii) to reveal the proteomic response of *P. expansum* to chitosan and elucidate the possible mechanisms of antifungal activity of chitosan.

2 Materials and methods

2.1 Penicillium expansum strain

P. expansum (CGMCC 3.3703) isolated from rotten fruits was obtained from the China General Microbiological Culture Collection Center, and its ability to cause postharvest decay of

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apples and the accumulation of patulin in diseased fruits have been discussed in our previous report (Li et al., 2015).

2.2 Chitosan solution

Chitosan, with an average molecular weight of 100 kDa and an approximate deacetylation degree of 93%, was provided by Golden-Shell Pharmaceutical Co., Ltd. (Yuhuan, China). Chitosan stock solution was prepared by dissolving 1 g of chitosan in 100 mL of 1% (w/v) acetic acid under stirring overnight and adjusting pH to 5.6 with 1 mol/L NaOH. After filtration, the resulting solution was used to prepare chitosan amended fungal culture media at different concentrations.

2.3 Spore germination assay

Conidia of *P. expansum* used in this experiment were harvested by flooding with sterile water from 7-day-old sporulated potato dextrose agar (PDA) plate. Test samples containing 10^4 spores mL^{-1} in potato dextrose broth (PDB) medium amended with chitosan at different concentrations (0.01%, 0.05% and 0.1%) were incubated at 25 ∘C for 12 h in darkness. PDB medium amended with diluted acetic acid solution (pH 5.6) served as control. Spores were observed under an Eclipse Ci-L light microscope (Nikon) by using a haematocytometer. A spore with germ tube length higher than 1.5 times the spore diameter was considered germinated (Plascencia-Jatomea et al., 2003).

2.4 Fungal growth measurement

Agar disc (diameter 0.5 cm) cut from the edge of actively growing culture of *P. expansum* was placed mycelium side down in the center of a fresh PDA culture plate supplemented with chitosan at different concentrations. After incubation at 25 °C for 7 days, growth of *P. expansum* mycelium was assayed by measuring colony diameter. Mycelium from the margin of the colony was collected for light microscope observation.

One milliliter of conidial suspension adjusted to 10^7 spores mL^{-1} was added to conical flasks containing 50 mL of PDB medium amended with chitosan at different concentrations. After 7 days incubation on a rotary shaker at 180 rpm and 25 °C, the culture liquids were filtered and the biomass obtained with each concentration was weighed.

2.5 Transmission electron microscopy

Conidia of *P. expansum*, either treated or untreated with 0.05% chitosan for 6 h, were fixed by immersion in cold 2.5% (v/v) glutaraldehyde at 4 °C for 8 h. After washing three times with phosphate buffered solution (0.2 mol/L, pH 7.4), conidia were postfixed with 0.1% osmium tetroxide for 2 h at room temperature and washed three times again with the same buffer. Subsequently, samples were dehydrated in a graded acetone series (50%, 60%, 70%, 80% and 90%, v/v) with 15 min per change, and then embedded in Epon 812 (Electron Microscopy Sciences, Fort, WA, USA). 60-nm-thick ultrathin sections were made using a diamond knife blade and collected on Formvar-coated copper grids. Sections were stained in uranyl acetate for 30 mins and in lead citrate for 10 mins. The grids were examined with a Philips CM100 transmission electron microscopy (FEI Company, Darmstadt, Netherlands).

2.6 Protein extraction

Total protein extraction was performed according to trichloroacetic acid (TCA)-acetone precipitation protocol as described by Cai et al. (2014). In brief, mycelia were grounded to fine powder using a pre-cooled mortar and pestle under liquid nitrogen. The samples were suspended in ice-cold 10% (w/v) TCA in acetone and kept at 4 °C for 2 h. The precipitates were collected by centrifugation at 10 000 g for 10 min and washed three times with 80% ice-cold acetone. After air dried to near dryness at room temperature, the pellets were suspended for protein solubilization in a small volume of lysis buffer containing 8 M urea, 2 M thiourea, 20 mM DTT, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-pro-panesulfonate (CHAPS) and 1% Carrier Ampholyte (pH 4–7). Finally, the resulting lysates were vortexed overnight at room temperature followed by centrifugation at 16 000 g for 10 min, at 4 °C. Protein content was estimated following the method of Bradford, with bovine serum albumin (BSA) as a standard.

2.7 Two-dimensional gel electrophoresis

A total of 800 μg of protein were loaded into IPG strips (17 cm, pH 4–7 linear gradient) using in-gel passive rehydration at 17 °C for 12 h. The first isoelectric focusing (IEF) was carried out on a PROTEAN IEF Cell (Bio-Rad Co., Ltd., Hercules, USA) with a gradually increasing voltage: 0-250 V, 30 min; 250-1000 V, 30 min; 1000-8000 V, 120 min and to a total of 60 kVh with a maximum voltage of 8,000 V at 17 °C. After focusing, the IPG strips were subjected to reduction and alkylation by incubating in equilibration buffer consisting of 0.075 M Tris–HCl pH 8.8, 7 M urea, 2% (w/v) SDS, 30% (v/v) glycerol together with 1% (w/v) DTT for 15 min, and subsequently in the same buffer containing 2.5% (w/v) iodoacetamide for 15 min. The second dimension electrophoresis was run on 12% polyacrylamide gel with a PROTEAN Plus Dodeca Cell (Bio-Rad Co., Ltd., Hercules, USA). 2-DE gels were stained with colloidal Coomassie blue G-250 overnight. Gel images were captured using a GS-900 densitometer (Bio-Rad Bio-Rad Co., Ltd., Hercules, USA). Scanned images were analyzed using PDQuest v7.4 image analysis software (Bio-Rad Co., Ltd., Hercules, USA) according to the manual protocol. The intensity of each protein spot was normalized as a percentage of the total quantity of valid spots detected on the gel. Normalized abundance of each spot between the treatments and controls were compared, and only those protein spots showed at least a 1.5 fold variation in relative intensity ($p < 0.05$) were considered as differentially expressed.

2.8 Protein identification

Spots of interest were excised from gels manually and transferred into microtubes. After destaining with 50% (v/v) acetonitrile containing 50 mM NH_4HCO_3, gel pieces were reduced in 50 μL

of 10 mM DTT in 100 mM NH_4HCO_3 at 55 °C for 45 min, and then subjected to alkylation by incubating in 50 µL of 55 mM iodoacetamide in 100 mM NH_4HCO_3 at room temperature in the dark for 45 min. Afterwards, gel pieces were dehydrated by 50% (v/v) acetonitrile containing 50 mM NH_4HCO_3 and dried in a vacuum centrifuge. Finally, 0.1 µg of sequencing grade trypsin (Sigma-Aldrich (Shanghai) Trading Co. Ltd., Shanghai, China) in sufficient 100 mM NH_4HCO_3 was added into the microtube to submerge the gel piece in the solution. After tryptic digestion at 37 °C overnight, peptide mixtures were carefully collected.

Peptide mixtures were analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The peptide mass fingerprinting (PMF) data resulting from the MS analysis were queried against non-redundant NCBI database using the MASCOT searching program (http://www.matrixscience.com). All searches were performed with the following parameters: taxonomy restrictions to other fungi, allowance of one missed cleavage, cysteine carbamidomethylation as fixed modification, mass value MH^+, monoisotopic and fragment mass tolerance of 0.2 Da.

2.9 Statistical analysis

Data of chitosan treatments and the control were subjected to one way ANOVA analysis using SPSS version 17.0 software (SPSS Inc., Chicago, USA) for Windows. Means comparison was performed using Duncan's multiple range test with $P< 0.05$.

3 Results and discussion

3.1 Effect of chitosan on spore germination and fungal growth

The results obtained for fungal spore germination were presented in Figure 1A. The fungistatic effect of chitosan gradually increased with the chitosan concentration, resulting in about 39% of spore germination rate in media with 0.05% chitosan and 9% of that in media with 0.1% chitosan, whereas the spore germination rate in the control was nearly 92%.

Effect of chitosan on mycelial growth was estimated by measuring the colony diameter of *P. expansum* on PDA supplemented with different chitosan concentrations. As shown in Figure 1B, the degree of inhibition was directly correlated to the chitosan concentration. After 7 days of incubation, fungal colony grew up to 41.2 mm in diameter on the control PDA, but the colony diameter was only 37.5, 31.4 and 23.9 mm on PDA supplemented with 0.01%, 0.05% and 0.1% chitosan, presenting inhibition rate of 9.0%, 23.8% and 42.0% respectively (Figure 1B).

When a liquid PDB culture was used, the mycelial biomass of untreated cultures reached 6.6 g after 7 days of incubation, whereas the mycelial biomass in PDB amending 0.05% and 0.1% chitosan was only 2.6 g and 0.4 g with approximately 60.6% and 93.9% growth inhibition, respectively (Figure 1C). *P. expansum* grown in liquid medium exhibited more sensitive to chitosan than those grown in solid medium. Similar results were reported by Prapagdee et al. (2007) for *Fusarium solani*

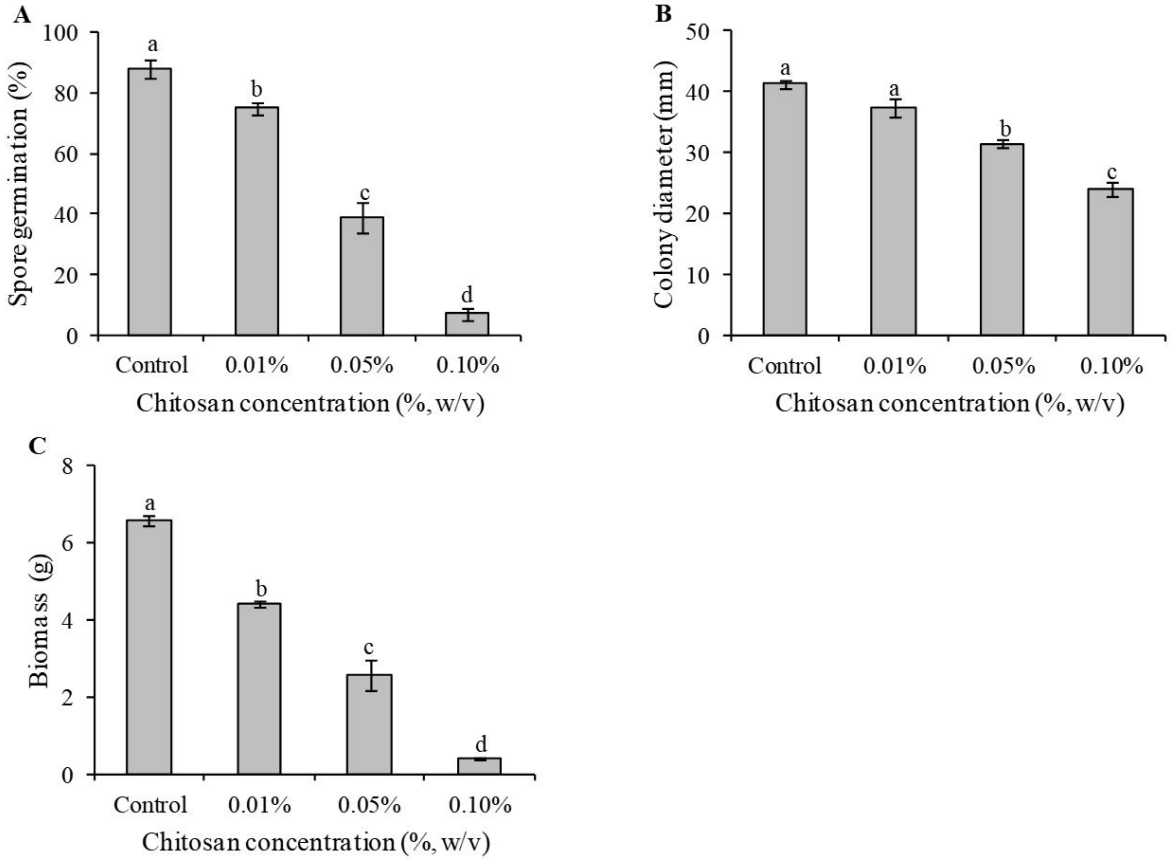

Figure 1. Effect of chitosan on spore germination (A), mycelial growth (B) and biomass accumulation (C) of *P. expansum*. Values represent the mean ± SD of three biological replicates and the different letters within each parameter indicate statistically significant differences ($p<0.05$).

and Al-Hetar et al. (2011) for *F. oxysporum*, possibly due to the cells cultured in liquid medium were entirely in contact with the added chitosan (El Hassni et al., 2004).

Both spore germination and mycelial growth were inhibited by chitosan clearly demonstrating its effects on various growth and development stages of *P. expansum*. Similar antifungal activities of chitosan against other plant pathogenic fungi have been observed, such as *F. oxysporum* (Palma-Guerrero et al., 2008), *A. parasiticus* (Cota-Arriola et al., 2011) and *A. flavus* (Dias et al., 2018). Moreover, about 60% spore germination and mycelium growth of *P. expansumin* in liquid medium were inhibited by chitosan at concentration of 0.05%, which is very close to the observed 50% inhibition concentrations of chitosan for *Alternaria alternate*, *F. oxysporum* and *Pythium debaryanum* (Rabea & Steurbaut, 2010).

3.2 Morphological changes in response to chitosan

As shown in Figure 2, chitosan treatment induced remarkable changes in *P. expansum* morphology characterized by hyphal agglomeration, presence of large vesicles in the hyphae, and abnormal shapes, such as bifurcation and swollen. Without chitosan treatment, hyphae showed even and branches formed at considerable distance from the apical tip of hyphal cell. While the conidia subjected to chitosan, intense vacuolization inside the cell and membrane rupture were observed (Figure 3). Microscopy observations showed that both conidia and mycelium of *P. expansum* were severely damaged by chitosan treatment. Similar effects were reported for *F. oxysporum* (Palma-Guerrero et al. 2008) and *Ustilago maydis* (Olicón-Hernández et al. 2015). The antifungal activity of chitosan might be attributed to its structural capability to bind to the negatively charged components presented on fungal cell surface via electrostatic interactions, which resulted in membrane leakage (Xing et al., 2015). Therefore, abnormal morphological changes, cell membrane disruption and loss of cellular materials could account for the inhibitory effect of chitosan on spore germination and mycelial growth.

3.3 Changes in protein expression profiles in response to chitosan

Cell growth and morphological analysis indicated that chitosan with concentration of 0.05% (w/v) could exert effective inhibition on fungal growth both in liquid and solid medium. Therefore, to gain insight into the antifungal activity of chitosan

Figure 2. Light micrographs of *P. expansum* mycelia after 7 days of cultivation with or without 0.05% chitosan treatment.

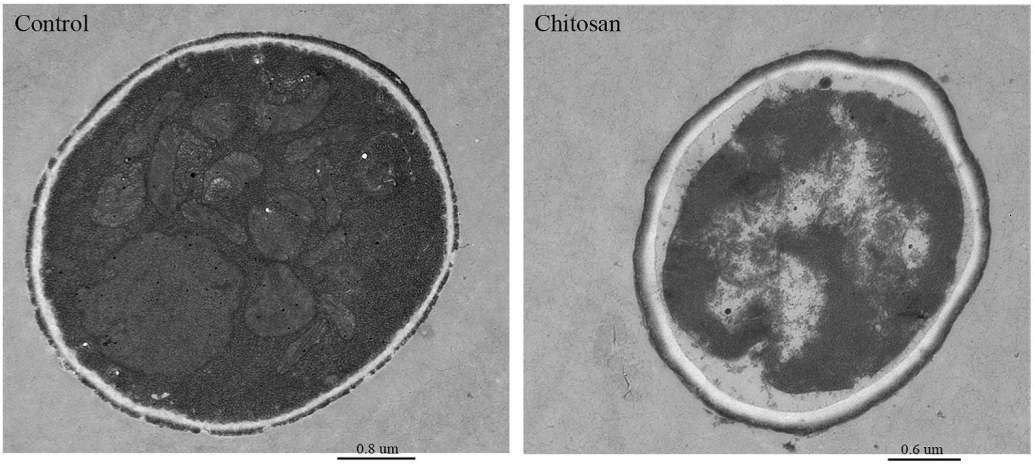

Figure 3. Transmission electron micrographs of *P. expansum* conidia after 6 h of cultivation with or without 0.05% chitosan treatment.

against *P. expansum*, spores after incubation with or without 0.05% chitosan for 12 h were collected for further proteomic analysis. Each CBB stained 2-DE gel revealed that about 500 protein spots were well resolved and detected in pH 4–7 and molecular weight 14–110 kDa (Figure 4), and a total of 42 spots showed at least 1.5 fold difference in relative abundance in response to chitosan treatment. All the differentially expressed proteins were analyzed by MALDI-TOF MS followed by MASCOT database search, and 26 of them were identified unambiguously with an identification rate of 62% (Table 1). According to their putative biological function, these identified proteins were classified into 6 categories, including DNA or protein biosynthesis, carbohydrate metabolism and energy production, antibiotic resistance and defense response, protein transport, secondary metabolism and unknown.

Among the identified proteins, several proteins involved in DNA or protein biosynthesis with reduced expression level were identified, including nucleic acid-binding (spot 2), aminoacyl-tRNA synthetase (spot 4 and 12), ribosomal protein S14 (spot 1), ribosomal protein S2 (spot 17), ribosome assemble protein

Table 1. List of differentially expressed proteins in *P. expansum* after chitosan treatment.

Spot	Protein name	Accession Number	Mr (kDa)	pI	Score	Matched peptides	Sequence coverage (%)	Fold change	Species
DNA/protein biosynthesis									
1	Ribosomal protein S14	KGO56193.1	72.8	5.6	79	7	17	0.55	*P. expansum*
2	Nucleic acid-binding, OB-fold	XP_016595516.1	61.0	5.4	80	6	22	0.49	*P. expansum*
4	Aminoacyl-tRNA synthetase, class II	XP_016603573.1	57.7	6.1	88	8	26	0.61	*P. expansum*
5	Translation elongation/initiation factor/ribosomal	KGO42256.1	48.3	5.0	121	22	60	0.17	*P. expansum*
6	Ribosome assembly protein	ENH78528.1	57.0	6.7	87	6	27	0.21	*Colletotrichum orbiculare*
12	Aminoacyl-tRNA synthetase	OQE12468.1	42.3	6.4	78	6	14	0.55	*P. vulpinum*
17	Ribosomal protein S2	KGO50333.1	32.4	4.8	90	7	37	0.43	*P. expansum*
24	Translation elongation factor, IF5A	CRL29786.1	23.2	7.0	77	7	38	0.39	*P. expansum*
Carbohydrate metabolism and energy production									
3	Thiamine pyrophosphate enzyme, TPP-binding	XP_016597049.1	63.4	5.3	154	21	47	0.63	*P. expansum*
7	ATPase	KGO37099.1	47.1	5.2	135	19	53	0.25	*P. expansum*
8	Hexokinase	KGO52770.1	54.4	5.2	107	20	46	0.19	*P. expansum*
11	Phosphoglycerate kinase	KGO47626.1	43.7	5.6	90	13	43	0.65	*P. expansum*
15	Aldose 1-/Glucose-6-phosphate 1-epimerase	KGO37445.1	34.9	4.9	83	7	46	0.52	*P. expansum*
16	Aldose 1-/Glucose-6-phosphate 1-epimerase	KXG45800.1	32.8	5.1	93	7	40	0.42	*P. expansum*
18	NAD binding NADP oxidoreductase coenzyme	ATY61637.1	32.3	6.4	86	7	49	0.53	*Cordyceps militaris*
19	Glyceraldehyde/Erythrose phosphate dehydrogenase	KGO37673.1	31.4	4.7	88	7	38	0.38	*P. expansum*
20	Short-chain dehydrogenase/ reductase SDR	XP_003835031.1	30.5	5.4	83	6	56	0.57	*Leptosphaeria maculans*
Antibiotic resistance and defense response									
13	Aminoglycoside phosphotransferase	XP_016598064.1	41.4	5.6	89	8	42	1.59	*P. expansum*
22	Peroxiredoxin, AhpC-type	XP_016601946.1	24.7	5.2	83	7	51	1.62	*P. expansum*
25	ABC multidrug transporter	OQE72650.1	18.2	5.1	74	5	43	1.94	*P. nalgiovense*
Protein Transport									
14	Vacuolar protein sorting-associated protein 26	KGO49711.1	40.8	7.0	75	6	19	2.70	*P. expansum*
Secondary metabolism									
23	S-adenosylmethionine dependent methyltransferase	XP_016598675.1	24.6	5.0	84	8	49	1.91	*P. expansum*
Unknown protein									
8	hypothetical protein	OQD64464.1	45.1	6.7	64	6	24	0.60	*P. expansum*
9	hypothetical protein	OQD64464.1	45.1	6.8	84	8	33	0.55	*P. polonicum*
21	hypothetical protein	EPS25373.1	27.0	4.8	79	4	31	0.42	*P. oxalicum*
26	hypothetical protein	XP_016595490.1	18.1	5.6	118	9	60	2.41	*P. expansum*

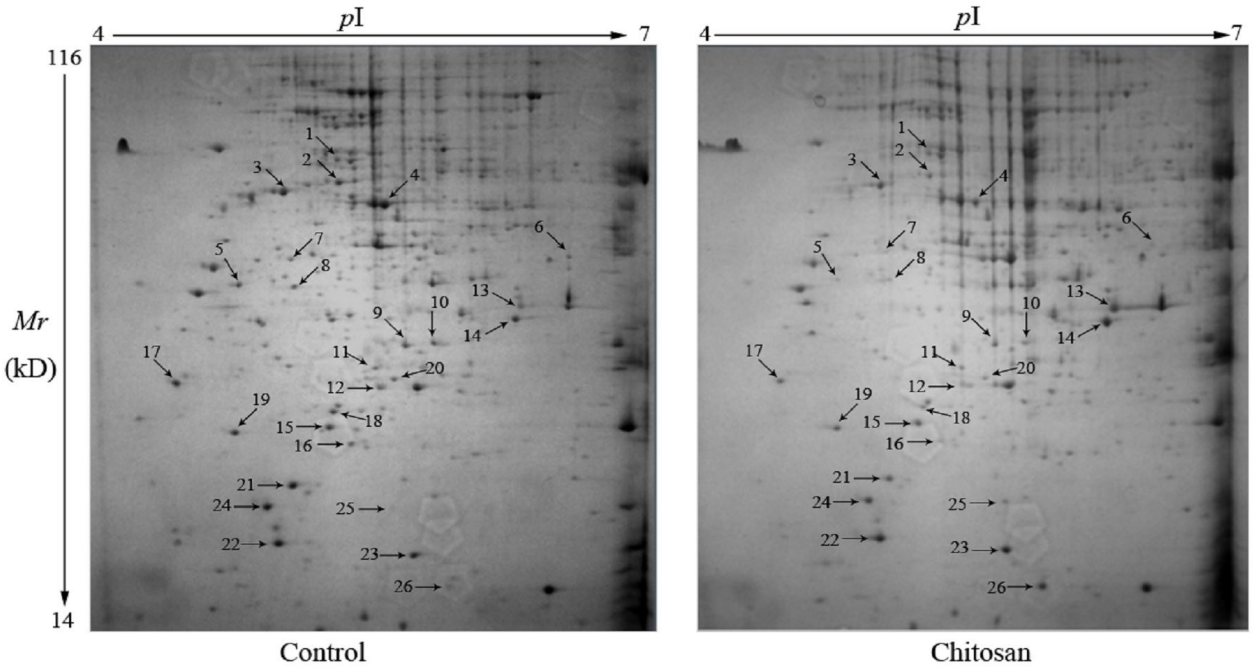

Figure 4. Two-dimensional electrophoresis of *P. expansum* with or without 0.05% chitosan treatment.

(spot 6) and translational elongation factor (spot 5 and 24). This finding is in accordance with the work of Márquez et al. (2013), in which gene deletion array was employed to screen and identify chitosan-regulated cellular pathway in *Saccharomyces cerevisiae* and the results clearly indicated that chitosan can directly interfere with fungi protein synthesis and cell membranes. It has been reported that electrostatic interactions is potentially occurred between positively charged $-NH_2$ groups on polysaccharide chain of chitosan and negatively charged phosphate groups on nucleic acid chain, or –COOH groups on protein (Boeris et al., 2011; Moussa et al., 2013). These interactions between chitosan and biomacromolecules in microbial cell could partially explain the inhibitory effect of chitosan on spore germination and mycelium growth and could also help to explain our proteomic findings that proteins involved in DNA or protein biosynthesis decreased in relative protein abundance after exposure to chitosan.

A number of proteins related to carbohydrate metabolism and energy production, including thiamine pyrophosphate enzyme (spot 3), ATPase (spot 7), hexokinase (spot 8), phosphoglycerate kinase (spot 11), aldose1-/glucose-6-phosphate 1-epimerase (spot 15 and 16), NAD binding NADP oxidoreductase coenzyme (spot 18) were found to be reduced by chitosan treatment. These enzymes are mainly involved in glycolytic pathway and Kreb's cycle, generating NADH and ATP. Spot 20 was identified as short-chain dehydrogenase/ reductase which plays a important role in lipid, carbohydrate, amino acid, cofactor and xenobiotic metabolism (Kavanagh et al., 2008), and was identified to be reduced by chitosan treatment in *Neurospora crassa* (Lopez-Moya et al., 2016). Consistent with our results, previous transcriptomic studies revealed that chitosan treatment could disrupt the cellular energy production and reduces the expression of genes associated with lipid, carbohydrate and coenzyme metabolism in *Staphylococcus aureus* (Raafat et al., 2008) and *S. cerevisiae* (Jaime et al., 2012).

Proteins related to antibiotics resistance and antioxidant defense response, including aminoglycoside phosphotransferase (spot 13), peroxiredoxin (spot 22) and ABC multidrug transporter (spot 25), increased in relative abundances after chitosan treatment. This finding is not unprecedented; for example, the expression levels of peroxiredoxin and a number of other proteins related to reactive oxygen species homeostasis in *N. crassa* were induced by chitosan treatment (Lopez-Moya et al., 2016). Oxidative stress was also observed in chitosan treated *U. maydis* (Olicón-Hernández et al., 2017); therefore, increased expression of these proteins may be associated with an imbalance of the intracellular redox state. Moreover, spot 25 was identified as an ABC multidrug transporter, which is known to be involved in extrusion of antifungal drugs (Cavalheiro et al., 2018). It has been assumed that chitosan could enter into fungi cell thereby causing physiological disturbance (Palma-Guerrero et al., 2009). Therefore, activation of this efflux system might contribute to protection of fungi cells from hostile environment (Sanglard et al., 2009).

S-adenosylmethionine dependent methyltransferase (spot 23), which plays a key role in the transfer of methyl groups to various biomolecules, increased in relative protein abundance after chitosan treatment. Increased expression of S-adenosylmethionine dependent methyltransferase at transcriptional level has been reported in chitosan-treated *S. cerevisiae*, which appears to be a possible stratagem employed by fungi cells for chitosan tolerance (Zakrzewska et al., 2005). Spot 14 was identified as vacuolar protein sorting-associated protein and known to be participated in the sorting and delivery of soluble vacuolar proteins to vacuole (Bonangelino et al., 2002). The vacuolar protein sorting-associated protein deletion strain of yeast *S. cerevisiae* exhibited more

sensitive than the wild type to chitosan, suggesting that induced expression of this protein might provide some protection against chitosan treatment (Jaime et al., 2012).

4 Conclusion

In summary, the results described in this paper demonstrate that chitosan strongly inhibits fungal growth and induces remarkable morphological and ultrastructural changes in *P. expansum*. Differential proteomic analysis reveals that proteins involved in DNA or protein biosynthesis and energy production decreased in relative protein abundance, while proteins associated with antibiotics resistance and defense response increased in relative protein abundance after chitosan treatment, which might explain the antifungal activity of chitosan against *P. expansum*.

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