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Peptides derived from tryptic hydrolysate of *Bacillus subtilis* culture suppress fungal spoilage of table grapes

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ABSTRACT

This study confirmed the anti-fungal effect of trypsin-treated *Bacillus subtilis* culture (BC) (tryptic hydrolysate, TH) on mold growth on Kyoho grapes. We examined the anti-fungal activity of TH by identifying TH peptides and performing a computational docking analysis. TH was more potent than untreated BC in suppressing fungal growth on grapes. Specifically, TH maintained grape freshness by inhibiting respiration and rachis browning, maintaining firmness, and preventing weight loss. Thirty-six inhibitory peptides against β-1,3-glucan synthase (GS) were screened from 126 TH peptides identified through proteomic analysis. Among them, 13 peptides bound tightly to GS active pockets with lower binding energies than that of GppNHp. The most potent peptides, LFEIDEELNEK and FATSDLNDLYR, were synthesized, and further experiments showed that these peptides had a highly suppressive effect on GS activity and *Aspergillus niger* and *Penicillium chrysogenum* growth. Our results confirm that tryptic treatment is effective for improving the anti-fungal activity of BC.

1. Introduction

The table grape (*Vitis vinifera* L.) is cultivated throughout the world and is one of the most important fruits for humans because it is rich in vitamins, carotenoids and phenolics. However, every year 30–40% of post-harvest table grapes are lost due to softening, weight loss, and in particular, fungal contamination, which can be hazardous to human health (Jiang, Shi, Liu, & Zhu, 2014; Romanazzia, Lichterb, Gablerc, & Joseph, 2012). Various strategies have been postulated to control post-harvest table grape decay and weight loss. Of these methods, chemicals such as SO_2 have been used to prolong shelf life during cold storage (Youssef et al., 2015). However, these chemical fungicides are currently avoided because of consumer health and environmental concerns (Waewthongraka, Pisuchpenb, & Leelasuphakula, 2015). Therefore, the effect of safe treatments, such as those involving the use of sodium alginate and lysozyme (Hu & Zhou, 2011), vanillin-enriched alginate (Takma & Korel, 2017), chitosan (Adel & Mohamed, 2015), polyamines (Mirdehghan & Rahimi, 2016) and

Abbreviations: AGC, automatic gain control; AOAC, Association of Official Analytical Chemists; BC, *Bacillus subtilis* culture; CFU, colony-forming units; CIE, Commission Internationale de L'Eclairage; DIZ, the diameter of inhibition zone; DRBC, ichloran rose Bengal chloramphenicol agar; FDR, false discovery rate; GS, β-1,3-glucan synthase; HCD, higher energy collision-induced dissociation; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; PDA, potato dextrose agar medium; PDB, Protein Data Bank; TH, tryptic hydrolysate of *B. subtilis* culture.

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pulse ultraviolet radiation (Hu, 2016), on extending shelf-life are a primary focus for researchers and consumers.

Bacillus subtilis is a Gram-positive bacterium that has long been used to ferment soybean foods such as natto and touchi (Lee & Chang, 2017). Currently, it is widely used for the production of industrial enzymes and chemicals (Calvo, Marco, Blanco, Oria, & Venturini, 2017). Due to its strong anti-fungal activity, *B. subtilis* is also used as a biocontrol agent to kill spoilage fungi on crops, vegetables and fruits in the field (Xu, Park, Kim, & Kim, 2016; Zhao & Zhao, 2016). Recently, research on post-harvest fruit preservation (apple, grape and orange) has shown that this bacterium can inhibit *Botrytis cinerea* (Elmer & Reglinski, 2006), *Aspergillus carbonarius* (Jiang et al., 2014) and *Penicillium expansum* (Sun, 2009). Results from further studies showed that the bacterium produces anti-fungal proteins including bacteriocin, lipopeptides, peptide antibiotics and peptides (Alvarez et al., 2012; Zhang, Li, & Mao, 2016) that compete with molds for nutrition and space, thereby inducing plant resistance to some diseases. However, the anti-fungal substances found in *B. subtilis* culture (BC) vary depending on the strain and fermentative parameters. Therefore, strategies such as screening for strains, optimizing fermentative conditions, and even genetic modification have been used to acquire stable anti-fungal substances with high activity (Tian, 2013). Currently, protein proteolysis is an effective method to prepare peptides with a wide spectrum of bioactivities, including anti-fungal activity (Chen, 2015). However, this method has not been utilized to improve the anti-fungal activity of BC.

We hypothesized that tryptic hydrolysate (TH) from BC is more potent than untreated BC in suppressing fungal spoilage of post-harvest table grapes (*V. vinifera* L.). Therefore, we compared the anti-fungal and freshness-maintenance effects of BC and TH on post-harvest table grapes. We also investigated the *in vitro* effects of BC and TH on suppressing the growth of *A. niger* and *P. chrysogenum,* which typically grow on post-harvest grapes. β-1,3-glucan synthase (GS, EC 2.4.1.34) is essential for synthesizing the fungal cell wall polymer β-(1,3)-glucan (Qadota et al., 1996). To examine the anti-fungal effect and mechanism of action of the peptides, the inhibitory effects of BC and TH on GS production by *A. niger* and *P. chrysogenum* were assayed, and the peptide profile of TH was determined using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Finally, molecular modeling was performed to identify high-activity peptides from TH and to elucidate the molecular mechanisms underlying their anti-fungal activity.

2. Materials and methods

2.1. Materials

Ripe 'Kyoho' grapes were harvested at ripening stage (~16% total soluble solids) in Changping, Beijing in September 2016 and transported to the laboratory within 2 h. *B. subtilis* (strain EBL16), *P. chrysogenum* (ATCC strain 10106) and *A. niger* (ACCC strain 30557) were provided by the Institute of Microbiology, China Academy of Sciences (Beijing, China).

Potato dextrose agar (PDA), salt czapek dox agar and dichloran rose bengal chloramphenicol agar (DRBC) were purchased from Aobox (Beijing, China). Trypsin (4–6 units/mg from porcine pancreas) was obtained from Sigma-Aldrich (St. Louis, MO, USA), and ascorbic acid was purchased from Shanghai Ziqi Biotechnology Co., Ltd. (Shanghai, China). A BCA protein assay kit was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The LFEIDEELNEK (>96.8%) and FATSDLNDLYR (>98.0%) peptides were synthesized by China Peptides Co. Ltd. (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of BC and TH

The strain EBL16 was screened in advance from 11 *B. subtilis* strains for the storage experiment of table grapes. A suspension of *B. subtilis* EBL16 (approximately 1×10^8 colony forming units [CFU]/mL, measured using a hemocytometer) was prepared by washing cultures grown for 24 h at 37 °C on nutrient agar (NA; 20 g glucose, 5 g yeast extract, 10 g peptone, 5 g NaCl and 15 g agar in 1 L distilled water, pH 6.5–6.7) with sterile distilled water and then adjusting to the desired cell density. To obtain a *B. subtilis* liquid culture (1×10^8 CFU/mL), a 5-mL aliquot of the prepared cell suspension was inoculated into 100 mL nutrient broth (NA medium without agar) and incubated for 48 h at 32 °C with shaking at 200 rpm. Cell-free supernatant from the liquid culture (BC) was obtained by centrifuging the culture at 10,000*g* for 20 min and then filtering through a Millex-HV filter (0.22 μm, 13 mm diameter; Millipore, MA, USA). After determining the protein concentration using the BCA assay kit and adjusting the pH to 7.8, the BC was hydrolyzed with trypsin (protein:enzyme ratio of 50:1, w/w) for 12 h at 37 °C. After hydrolysis, TH was obtained by heating the BC for 15 min to inactivate trypsin.

2.3. Grape berry treatment

After arrival at the lab, the grape bunches were selected based on uniform color, size, absence of injuries, and healthy or greenish rachises within 30 min. The selected grapes with rachises were then immersed in BC or TH for 1 min, surface dried under a sterile airflow (20 °C), placed in a cardboard box and then stored at the optimum cold storage temperature (−1 to 0 °C) in a refrigerator. As a control, untreated grapes were also stored under the same conditions. Fungal contamination on grape surfaces and the biological indices were measured every 5 days for 30 days.

2.4. Microbial analysis

Microbiological analyses were performed according to Jiang et al. (2014). Briefly, five berries were randomly selected from each bunch of grapes, weighed and macerated in 50 mL sterile 0.1% (w/v) peptone. The mixture was vigorously shaken for 5 min to dislodge fungi from the surface of the grapes. The solution was serially diluted 10-fold in a 0.1% peptone solution to obtain 1/10, 1/100 and 1/1000 dilutions. Each diluted solution (0.1 mL) was plated onto DRBC medium (Jiang et al., 2014). Plates were incubated for 2 days at 25 °C, and the number of CFU was expressed per gram. Analyses were performed after 0, 5, 10, 15, 20, 25 and 30 days of storage.

2.5. Assays of respiratory rate, weight loss, firmness, color, titratable acidity and ascorbic acid

The respiratory rate of grapes was quantified using a fruit and vegetable breathing apparatus (GXH-3051, Beijing, China), and the results were expressed as CO_2 mg/kg·h. Weight loss was measured as the percentage loss of initial weight by recording the difference in weight of table grapes selected from the treatment and control groups during storage. Grape firmness was measured using the TVT-6700 Texture Analyzer (Perten, Beijing, China) with 25% deformation and a 5-mm diameter cylindrical probe at a pre-speed and post-speed of 1 mm/s. Measurements were performed on 10 grape berries for each treatment during storage, and firmness is expressed in Newtons (N).

The colors of the grape rachises were quantified using the DTQC-10A Chromameter with a 4-mm-diameter viewing aperture (Beijing Oriental Precision Technology Co., Beijing, China). The instrument was used to determine color using the three CIE