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Purification and Characterisation of a Fungicidal Peptide from *Bacillus amyloliquefaciens* NCPSJ7

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Abstract

Wang J., Zhao S., Qiu J., Zhou Q., Liu X., Xin X., Guo D., Yudina T.G., Wang Y., Sun H., Chen X., Chen L. (2017): Purification and characterisation of a fungicidal peptide from *Bacillus amyloliquefaciens* NCPSJ7. Czech J. Food Sci., 35: 113–121.

Bacillus amyloliquefaciens NCPSJ7 could secrete extracellular antimicrobial substances, showing potent antifungal activities. An active peptide AFP3 was isolated from the fermentation supernatant. After chromatography, the purified peptide was tested for the fungicidal activity, molecular mass, and stability. The results indicated that the peptide with a molecular mass of around 3.3 kDa, showed discernible inhibition of the pathogen *Fusarium oxysporum* f.sp. *niveum* with the minimum fungicidal concentration of 31 μg/ml. It also exhibited excellent inhibition of some representative pathogenic fungi at a low concentration. Moreover, the peptide remained active at a wide range of temperatures and pH. Ion Na^+ may even increase the antifungal activities. At the same time, the peptide could well tolerate the treatment with trypsin. Electron microscopy was used to investigate the effect of the peptide on the pathogens. The peptide inhibited the growth of pathogens by disrupting the integrity of the hyphal membranes, resulting in their lysis. The potent fungicidal activities and stability made the peptide be a candidate for a biopreservative.

Keywords: antifungal peptide; mechanism; stability; biopreservative

Bacillus amyloliquefaciens is a member of free-living soil bacteria known to promote plant growth and suppress plant pathogens. There are a lot of reports on the antifungal activities of *B. amyloliquefaciens* (Chen *et al*. 2007; Arguelles Arias *et al*. 2013; Li *et al*. 2013; Zhang *et al*. 2013; Zhao *et al*. 2014; Han *et al*. 2015; Nam *et al*. 2015; Yamamoto *et al*. 2015). It is known to produce various potent antimicrobial substances which provide an alternative approach to protect the food from pathogenic organisms (Sumi *et al*. 2015). Among them, lipopeptides such as bacillomycin D, surfactin, iturin, and fengycin were mostly studied for the antagonistic activities (Ongena & Jacques 2008). They have a

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well-recognised potential to be used in biocontrol because of their antifungal activities and surfactant properties. The lipopeptides could also induce the plant defence responses to conquer the pathogens (Li *et al*. 2015). Moreover, some peptides were also reported to be able to inhibit the pathogen growth, such as lantibiotics, bacteriocin, and LCI protein (Sutyak *et al*. 2008; Herzner *et al*. 2011; Meng *et al*. 2012; Arguelles Arias *et al*. 2013; Scholz *et al*. 2014; Rasimus-Sahari *et al*. 2015). The good stability and inhibitory activities of the peptides pointed out their potential in the plant protection and food preservation. Besides, *B. amyloliquefaciens* could also secrete some small molecular weight compounds with excellent inhibitory activities, including the macrolactin (Chen *et al*. 2007), plantazolicin (Scholz *et al*. 2011), difficidin (Wu *et al*. 2015), and some volatile compounds (Yuan *et al*. 2012). With the outstanding antimicrobial activities, some active substances were tested for the abilities to suppress postharvest disease development on stored fruits, while some could decrease the disease incidence in a pot experiment, which supported the active substances to be developed as biocontrol agents (Arrebola *et al*. 2010; Wu *et al*. 2014).

By now, all reported antifungal peptides secreted by *B. amyloliquefaciens* were still under research in the laboratory. There is no available active antimicrobial agent as a biopreservative for the pathogen infection. It demonstrated that more effort should be made to promote the development and application of such antimicrobial peptides. Our previous study has identified a potent *B. amyloliquefaciens* strain NCPSJ7 with strong inhibition of the growth of various pathogenic fungi. Furthermore, the crude extract could protect the postharvest pears and apples from infection caused by *Penicillium* sp. (Qiu *et al*. 2014). To address these active substances in more details, a further research on their purification, characterisation, and antifungal mechanism would be discussed in this study.

MATERIAL AND METHODS

Bacterial strains and culture media. The *B. amyloliquefaciens* strain NCPSJ7 was isolated from a ginger field and has been deposited in China Centre for Type Culture Collection (CCTCC No. M 2013098). Pathogenic fungi *F. oxysporum* f.sp. *niveum* and *Alternaria bokurai* were purchased from Agricultural Culture Collection of China (ACCC No. 30024 and 30001). The other used fungi (Table 1) were donated by the State Key laboratory of Microbial Technology of Shandong University.

Potato Dextrose Agar medium (PDA, pH 5.6) was of commercial origin, containing 0.6% potato extract, 2% glucose, and 2% agar (Qingdao Hope Bio-Technology Co., Ltd., China). Nutrient agar (NA) included 1% peptone, 0.5% NaCl, 0.3% beef extract, and 2% agar, while nutrient broth (NB) with no agar, pH 7.0–7.2, was also used. Fermentation medium contained 0.5% glucose, 0.75% yeast extract, 0.75% peptone, 0.5% $(NH_4)_2SO_4$, and 0.5% NaCl, pH 7.0. Microbial growth was monitored by optical density measurement using Eppendorf biophotometer at 600 nm (OD600).

Preparation of the antifungal peptide. The bacterial strain taken from storage was activated on NA at 37°C for 24 hours. Then, a single colony was transferred to NB and cultured at 37°C until the OD_{600} reached 0.6 (optical path = 2 mm). Then, 4.06 ml of the NB culture was inoculated to 100 ml of fermentation medium and cultured at 150 rpm for 6 days at 33°C.

Centrifuged at 8000 *g* for 10 min after fermentation, the supernatant was added to ammonium sulphate ($(NH_4)_2SO_4$) until it reached 80% saturation at 0°C. The precipitate was collected after standing overnight at 4°C and redissolved in 50 mmol/l PBS and dialysed against 50 mmol/l PBS for 48 hours.

The resulting solution was purified on an anion-exchange column (DEAE-Sepharose FF; GE Healthcare) using gradient elution. The initial eluent was 50 mmol/l PBS, followed by 0.25 mol/l NaCl in 50 mmol/l PBS, then 0.4 mol/l NaCl, and last 0.6 mol/l

Table 1. Broad-spectrum antifungal activities of purified peptide AFP3

Indicator pathogens fungi	Concentration (mg/ml)			
	0.0625	0.125	0.25	0.5
Fusarium graminearum	++	++	+++	++++
Alternaria bokurai	++	++	+++	+++
Fusarium graminearum Schwabe	–	+	++	++
Macrophoma kawatsuka	+	++	+++	+++
Verticillium dahliae	+	++	+++	++++
Pythium ultimum	+	++	+++	+++

Inhibition diameters at different concentrations (mm): – = < 9 (no inhibition); + = 9–13; ++ = 14–18; +++ = 19–25; ++++ = > 25, showed obvious inhibition

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NaCl. After that, a gel filtration column (Superdex 75 pg; GE Healthcare) was employed for the next purification using 50 mmol/l PBS as the eluent. Further purification was operated on a hydrophobic column (Octyl Sepharose 4 FF; GE Healthcare) using linear gradient elution from 50 mmol/l PBS to ddH_2O. All fractions were monitored at 280 nm for the chromatography on a real-time UV detector. All peaks were collected and tested for antagonistic activities after dialysed against 50 mmol/l PBS while 50 mmol/l PBS was used as a control.

Tricine-sodium dodecyl sulphate-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE). Tricine-SDS-PAGE was used to analyse the molecular mass of antifungal peptide. According to Schagger (2006), a protocol was performed using a 16% separating gel with Coomassie staining. The molecular masses of marker proteins (Solarbio®) were indicated as: 20.1, 14.4, 7.8, 5.8, and 3.3 kDa.

Susceptibility to heat, pH, metal ions, and proteases. To estimate the susceptibility to heat, pH, metal ions, and proteases, antifungal assays were applied to evaluate the fungicidal activities after the antifungal peptide was treated in various conditions.

Heat sensitivity was evaluated after incubation at various temperatures (including 4, 25, 40, 60, 80, and 100°C) for 0.5, 1, and 2 h, respectively. For pH stability testing, the samples were adjusted to 1, 2, 3, 5, 7 (initial value, with no adjustment as control test – CK), 9, 10, 11, 12, and 13 with 2 mol/l HCl or 2 mol/l NaOH and placed at 4°C for 4 hours. Then the pH value was adjusted to 7.0 before antifungal assays. To 1 ml sample, 5 mol/l metal ion in 50 mmol/l PBS was added until the final ion concentrations reached 0.05 mol/l and 0.1 mol/l, respectively. Then the samples stood at 4°C for 8 h before antifungal assays. The ions dissolved in 50 mmol/l PBS were used as the blank control while 50 mmol/l PBS instead of the metal ion was used in the control test (CK). The metal ions included Na^+, K^+, Ca^{2+}, Fe^{3+}, Mg^{2+}, and Zn^{2+}, which came from NaCl, KCl, $CaCl_2$, $FeCl_3$, $MgSO_4$, and $ZnSO_4$, respectively. The effect of enzymes on the antifungal activities was assessed by incubating the peptide with various enzymes (trypsin, pepsin, proteinase K, and papain) at a final concentration of 1 mg/ml for 60 minutes. Then the mixture was boiled for 10 min to inactivate the enzyme. The antifungal peptide AFP3 was replaced with 50 mmol/l PBS in the blank test while the AFP3 was incubated with PBS instead of the proteases in the control test (CK).

Antifungal assays and the minimum fungicidal concentration (MFC). After activation, the pathogen (5 mm diameter) from the margin of a growing fungal culture (activated on PDA at 28°C for 3 days) was resuspended in 400 µl sterile water. The bacterial suspension (50 µl/plate) was spread onto a fresh PDA plate (6 cm). An oxford cup with 200 µl sample, which contained 100 µl analyte and 100 µl 2.5 mg/ml chloramphenicol, was placed on the pathogen plate. Then the plates were cultured at 28°C for 2–3 days. The analyte was replaced with sterile water in the blank test. Each assay was repeated at least three times. The size of the inhibition zone was analysed using the means of the three replicates.

To estimate the MFC, the initial protein concentration was analysed with Coomassie Brilliant blue G-250. After successive twofold dilution, all samples were tested as described above. The minimum protein concentration that caused the formation of a clearly distinguishable inhibition zone was regarded as MFC.

Scanning electron microscopy (SEM) and Transmission electron microscope (TEM) (Gerhardt 1981). A fungicidal test was conducted according to the description in antifungal assays. The fungus from the margin of inhibition zone was fixed in 2.5% glutaraldehyde. The (human) tripcasin was added to a concentration of 0.02%. Two hours later, the samples were treated with increasing concentrations of ethanol and absolute acetone successively. After that, dehydrated samples were dried to the critical point (Revina *et al*. 2005). In the control experiment, the hyphae were incubated without antifungal peptides and processed in the same way. The samples powdered with platinum were examined using Amray-1830 (Amray, Inc., USA) and Hitachi (Hitachi Ltd., Japan) scanning electron microscopes.

The sample observed under TEM was treated in the same way as that for SEM. After dehydration, the samples were embedded in Epon and the ultrathin sections were made (0.1 µm). After coating on copper grids and being stained with 3% uranyl acetate and lead citrate, the grids were examined using a LEO-Libra 120 transmission electron microscope (Carl Zeiss, Germany).

RESULTS AND DISCUSSION

In the preliminary test, NCPSJ7 was found to be able to inhibit the growth of *F. oxysporum* f.sp. *niveum* (Figure 1A). Moreover, it could reduce the spoilage of

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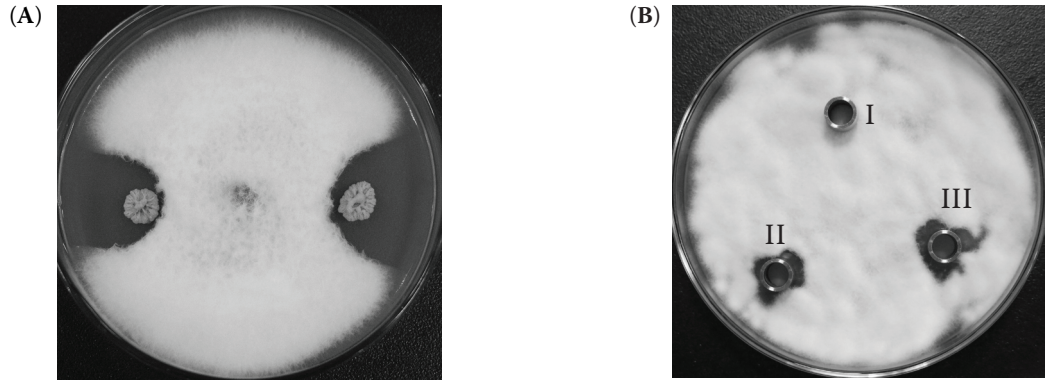
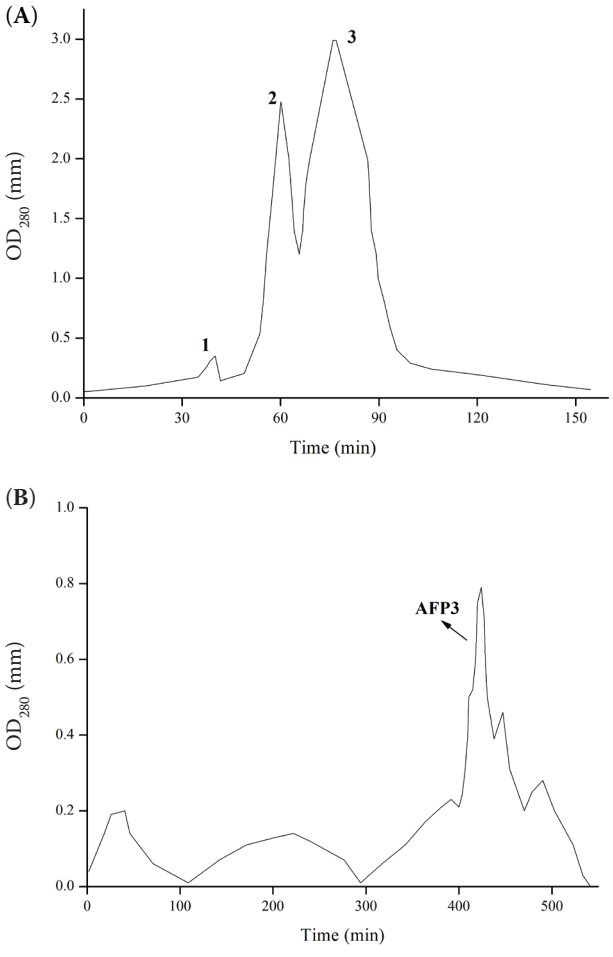


Figure 1. Inhibitory activity of *B. amyloliquefaciens* NCPSJ7 against *F. oxysporum* f.sp. *niveum*: (**A**) strain showed antagonism to pathogenic fungus; (**B**) the effect of fermentation on the growth of *F. oxysporum* f.sp. *niveum*; (I) fermentation medium as control; (II) *B. amyloliquefaciens* NCPSJ7 bacterial suspension in cultivation broth; (III) cell-free fermentation supernatant of *B. amyloliquefaciens* NCPSJ7

apples and pears, being of value to fruit and vegetable preservation (Qiu *et al*. 2014). Based on Sumi *et al*. (2015), the antimicrobial substances made the major contribution to the inhibition of fungi and bacteria by the *B. amyloliquefaciens* strains. To promote the development of potent *B. amyloliquefaciens* strain NCPSJ7, the active substances from fermentation were studied.

After being activated on the NA plate and fermented at 33°C for 6 days, the cell-free supernatant showed a significant inhibition of the fungus in antifungal assays



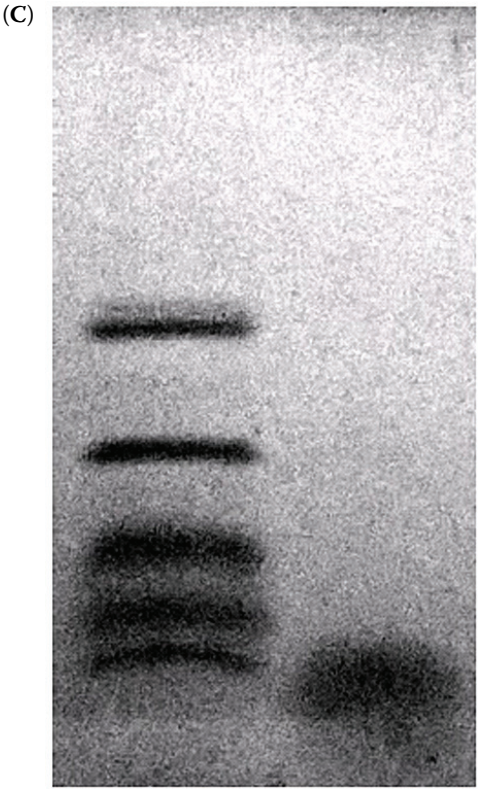


Figure 2. Purification and characterisation of the antifungal peptide: (**A**) gel filtration chromatography; (**B**) hydrophobic chromatography; (**C**) 16% Tricine-SDS-PAGE of isolated antifungal peptide

Left lane – protein marker; right lane – purified peptide AFP3.

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(Figure 1B). It means that NCPSJ7 strain could secrete extracellular antimicrobial substances to inhibit the fungus growth. Then, the fermentation supernatant was used to obtain the antifungal substances.

After ammonium sulphate precipitation, the precipitate underwent an ion-exchange chromatography. The elution peak obtained by 0.4 mol/l NaCl in 50 mmol/l phosphate buffer saline (PBS) showed the most prominent inhibition in the antifungal assay. So fractions of this peak were pooled and purified in gel filtration chromatography and hydrophobic chromatography (Figures 2A and 2B). After exclusion chromatography, the third peak showed the best inhibitory activities against the fungus. Then, the purified peptide was analysed using gel electrophoresis. According to the results of Tricine-SDS-PAGE, the molecular mass of the antifungal peptide was around 3.3 kDa (Figure 2C).

Then, the purified peptide named AFP3 was tested for the minimum fungicidal concentration and stabilities. In the MFC test, 0.5 mg/ml was prepared as initial concentration. After twofold dilution, six dilutions (0.5, 0.25, 0.125, 0.063, 0.031, and 0.016 mg/ml) were tested for the fungicidal activities. The results indicated that 31 μg/ml purified peptide showed obvious antifungal activities, while being inactive at a

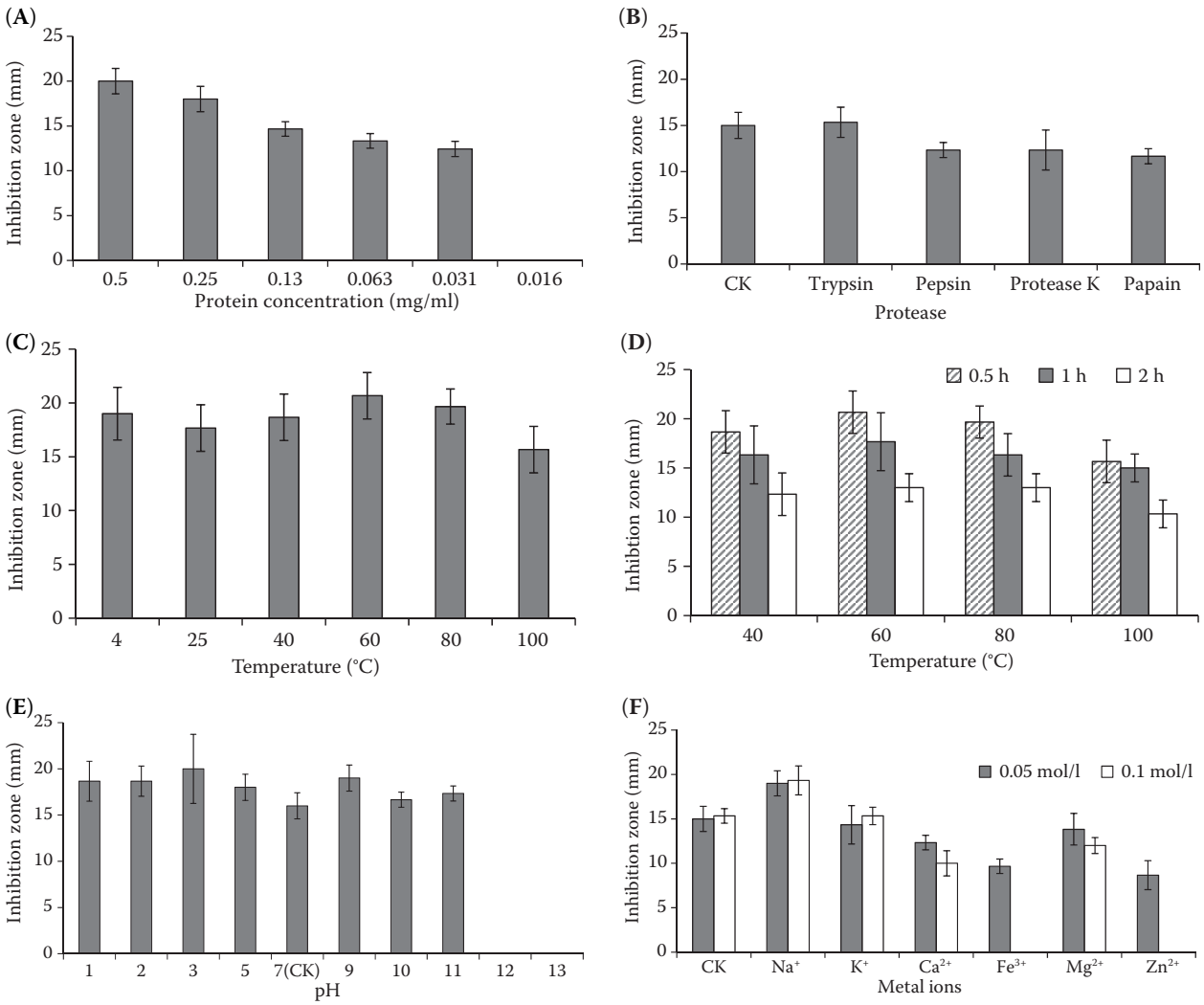

Figure 3. The properties of the peptide AFP3: (**A**) minimum fungicidal concentration of AFP3 to the pathogenic fungus *F. oxysporum* f.sp. *niveum*; (**B**) inhibition of peptide incubated with 1 mg/ml proteases indicated in the figure, for 60 minutes; (**C**) antifungal activities of peptide being processed at different temperatures for 30 minutes; (**D**) inhibitory activities of peptide incubated at 40, 60, 80, and 100°C for 0.5, 1, and 2 h, respectively; (**E**) inhibition of peptide to the *F. oxysporum* was tested in a pH range of 1–13 (CK – control test sample); (**F**) inhibitory activities of AFP3 incubated with various metal ions at a concentration of 0.05 and 0.1 mol/l, respectively

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concentration of 16 µg/ml (Figure 3A). The minimum protein concentration that caused the formation of a clearly distinguishable inhibition zone was 31 µg/ml. So, 31 µg/ml was adopted to be the minimum fungicidal concentration. In stability studies, the peptide was found to bear well the trypsin digestion without changes in activities. However, the other three types of proteases, pepsin, proteinase K, and papain, slightly decreased the inhibitory activities of AFP3 (Figure 3B). For thermosensitivity, the inhibitory activities showed no significant changes after half a year at 4°C (data not shown). It demonstrated that AFP3 could keep the activity at 4°C. The inhibitory activities maintained stability from 25°C to 80°C within 30 minutes. However, the diameter of the inhibition zone was reduced while the peptide was incubated at 100°C for half an hour. The situation was more serious as time passed. It was apparent that the peptide treated at 100°C for 2 h showed little suppression of the growth of the pathogenic fungus *F. oxysporum* f.sp. *niveum*. And one day later, the peptide showed no inhibition at all (Figures 3C and 3D). The peptide showed the equal inhibition of *Fusarium oxysporum* at pH 1–11, while it was inactive when the pH was over 11 (Figure 3E).

For the antimicrobial substances secreted by *B. amyloliquefaciens*, most peptides were tested to be active against fungi. The most common class may be lipopeptides with small molecular mass, broadly including surfactin (~1.36 kDa), iturin (~1.1 kDa), and fengycin (~1.5 kDa), which have potent applications in food industry, therapeutics, plant protection, and insect control (Zhao *et al*. 2014; Guo *et al*. 2015; Meena & Kanwar 2015). Among the other fungicidal peptides from *Bacillus amyloliquefaciens*, a smaller peptide with the mass of 852.4 Da was purified and it showed activities against pathogenic fungi (Han *et al*. 2015). Kim *et al*. (2015) purified a peptide PT14-4a (1495 Da), which showed antagonistic activities to the pathogenic fungi *Fusarium solani* and *Fusarium oxysporum*. LCI protein is an antifungal peptide having the obvious inhibition of *Streptomyces* spp. and being stable under a wide range of temperatures and pH. But it could be reduced by all tested proteases (Meng *et al*. 2012). The reported active peptides suggest that *B. amyloliquefaciens* is a rich measure for

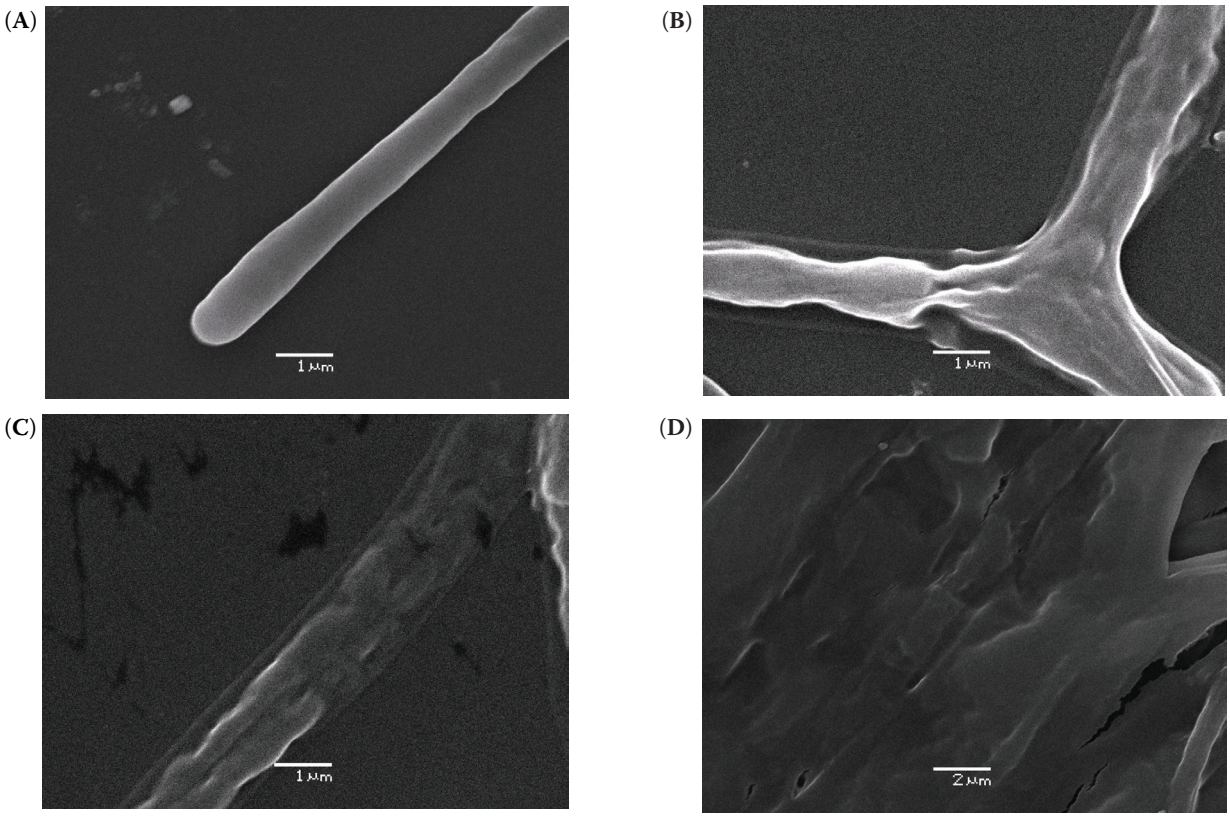

Figure 4. Scanning electron microscopy (SEM) of *F. oxysporum* hypha incubated with antifungal peptide (0.5 mg/ml); (**A**) *F. oxysporum* hyphae not treated with antifungal peptide; (**B**, **C**, and **D**) different degrees of damage of the hypha incubated with the active peptide

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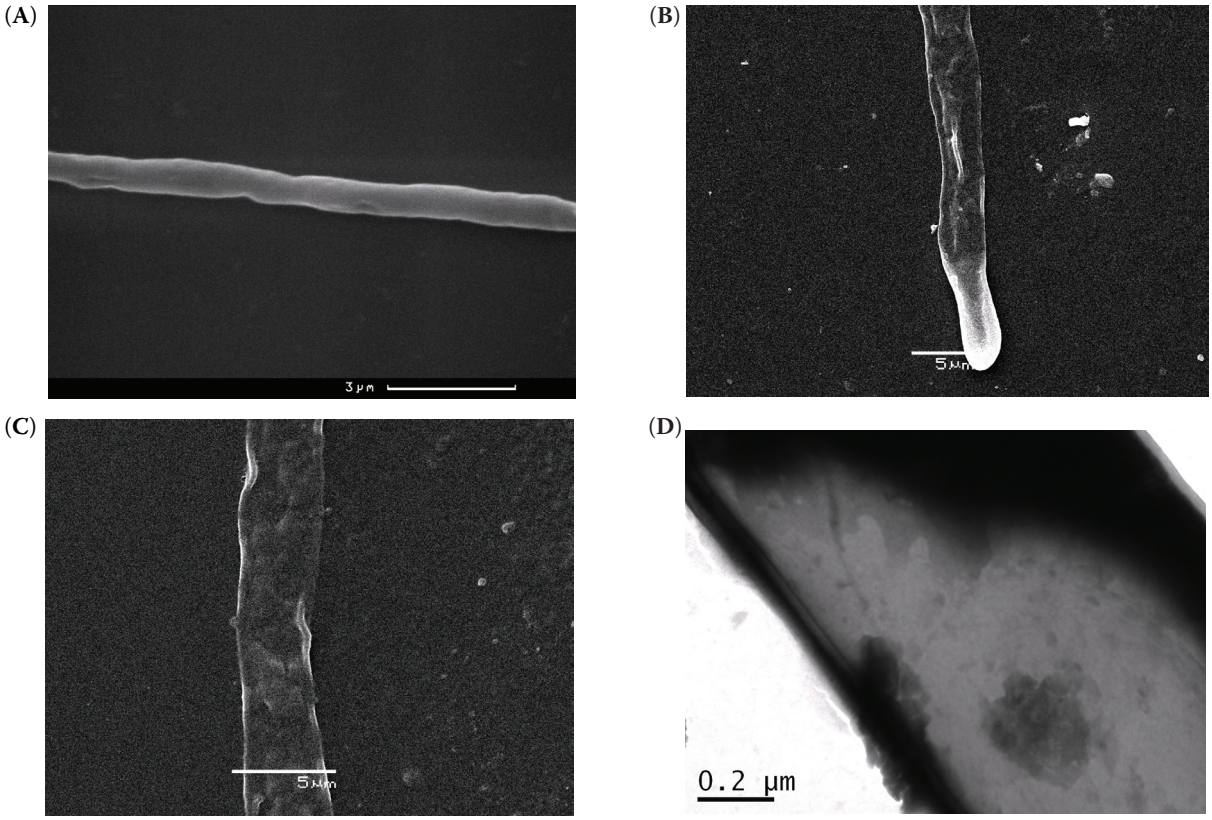

Figure 5. Electron microscopy of *Alternaria bokurai* hypha incubated with antifungal peptide (0.5 mg/ml); (**A**) SEM image of untreated *Alternaria bokurai*; (**B** and **C**) SEM micrographs of *Alternaria bokurai* incubated with purified peptide (fragments caused by hypha broken tagging with white arrow); (**D**) imaging results of treated hypha under Transmission electron microscope (TEM)

the effective biocontrol agents developed in food safety and plant protection. From the NCPSJ7 fermentation, a fungicidal peptide (around 3.3 kDa) was purified to be effective against *F. oxysporum*. As we know, the peptides as biocontrols are limited because they are inherently sensitive to many conditions, usually temperature, pH, proteases, and so on. But the purified peptide AFP3 was stable in a wide range of temperatures and pH with *F. oxysporum* as indicator fungus. The proteases even could not inactivate the peptide, showing that it was more stable compared with most peptides. Due to its stabilities, it is convenient for both production and application. The stabilities and fungicidal activities of the peptide AFP3 make us believe that the bacteria and the peptide may be candidates for the application in food preservation after further research.

From the pH stability results, the analyst (CK) without pH changing showed lower activities to *Fusarium oxysporum*. The changes of ion concentration may be the reason leading to the results. So different metal ions were added to the peptide AFP3 and tested for their influence on the fungicidal activities (Figure 3F). At a lower ion concentration (0.05 mol/l), Na^+ enhanced the inhibitory activities. K^+ and Mg^{2+} had no effect on the fungicidal activities while Ca^{2+}, Fe^{3+}, and Zn^{2+} inhibited them. At a higher concentration (0.1 mol/l), Na^+ regulated the activities positively and K^+ showed no influence, while Mg^{2+} slightly decreased the antifungal activities. For the other three types of ions, Ca^{2+} significantly inhibited the activities and the analysts with Fe^{3+} and Zn^{2+} caused the complete loss of the antifungal activities. It is understandable that the ions Ca^{2+}, Fe^{3+}, and Zn^{2+} may decrease the activities through chelation when K^+ led to no changes because no chelating interaction existed. But why could Na^+ increase the inhibition? Some scientists found that the addition of high concentrations of NaCl (> 100 mmol/l) could inhibit the activity of many cationic antimicrobial peptides (Hancock & Sahl 2006; Nagao *et al.* 2016). This unusual observation suggests that the peptide AFP3 may not be a cationic antimicrobial peptide. But it is still unclear how Na^+ can enhance the inhibitory activities. For the application, sodium

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ions may be used as an additive to increase the antifungal activities.

In addition, the peptide also showed broad-spectrum antifungal activities. The peptide showed the inhibition of most pathogenic fungi at a low concentration, especially of *Fusarium graminearum* and *Alternaria bokurai* (Table 1). The potent fungicidal activities showed that AFP3 had the potential as a biopreservative.

The electron microscopy was used to observe how the peptide inhibits the growth of the fungi. After being treated with the antifungal peptide, the hyphae generally changed by shrinkage, wrinkling, disturbance, collapse, squash, non-homologous surface, and lysis (Jasim *et al*. 2016; Sajitha *et al*. 2016; Sellamani *et al*. 2016). From the micrographs, the normal hyphae showed smooth surface and intact membrane. After being treated with the antifungal peptide, the shrinkage and collapse were observed with *F. oxysporum* hyphae. The lysed hyphae even fused to one piece (Figure 4). Beside the damaged membrane, the fragments could be observed caused by broken hyphae (Figure 5C). The hypha lysis was also confirmed on TEM. In Figure 5D the hypha membrane was undergoing lysis. The micrographs showed that AFP3 could damage the structure of fungal hyphae to control the fungal growth.

CONCLUSION

Bacillus amyloliquefaciens was reported to secrete various active substances that inhibit the growth of many microbes. In this study, an antifungal peptide AFP3 was isolated from *B. amyloliquefaciens* NCPSJ7 and exhibited significant inhibitory activities against the pathogenic fungi by decomposing the integrity of the membrane. The molecular mass is approximately 3.3 kDa with the MFC of 31 μg/ml. At the same time, it showed tolerance to temperature (4–80°C), pH (1–11), and trypsin digestion. With the broad fungicidal activities and well stabilities, AFP3 showed a potential to control diseases caused by fungi. How to analyse the novel peptide and increase the production for biopreservative application would be carried out in the further study.

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