SHORT COMMUNICATION

Separation, determination and antifungal activity test of the products from a new Bacillus amyloliquefaciens

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Flow chart of the process of separation and purification of bacilysin and chlorotetaine.

A new Bacillus amyloliquefaciens named ZJU-2011 was discovered, and the culture supernatant showed a strong inhibitory effect against Candida albicans. In this study, a novel method was developed to purify the antifungal compounds in high purity. The obtained products were analysed by high performance liquid chromatography and proven to be of high purity. Mass spectrometry showed that the molecular weights of the two bioactive components were 270 and 288, respectively, and their structures were determined to be bacilysin and chlorotetaine by using \textsuperscript{1}H and \textsuperscript{13}C nuclear magnetic resonance spectroscopy. To the best of our knowledge, this is the first time that B. amyloliquefaciens has been reported to produce bacilysin and chlorotetaine simultaneously. The minimum inhibitory concentration of chlorotetaine against six common fungal pathogens were determined to be in the range of 1.8–7.8\,\mu g/mL.

Keywords: isolate; bacilysin; chlorotetaine; Bacillus amyloliquefaciens; antifungal

1. Introduction

During the past decades, overuse and misuse of antibiotics has posed great challenges to the ecosystem, leading to an explosion of drug-resistant bacteria and public concerns over pesticide hazards. Therefore, it is imperative to identify novel effective drugs and to develop environmentally friendly alternatives to the extensively used chemical drugs. Recently, a great number of marine microbial metabolites of diverse chemical structures have been identified with antimicrobial or other bioactive properties (Erwin et al. 2010). Among them, antimicrobial peptides (AMPs) have drawn growing interest in drug development as a new generation of
antibiotics for their great advantage over conventional antibiotics (Peters et al. 2010; Onaizi & Leong 2011). In the search for novel antibiotics, a new strain of Bacillus amyloliquefaciens named ZJU-2011, showing strong inhibitory activity against Candida albicans was isolated from the East China Sea. In this study, a combined adsorption chromatograph was applied to separate and purify the antifungal substances, which were determined to be bacilysin and chlorotetaine – two of the simplest AMPs. Finally, for the first time, the minimum inhibitory concentrations (MICs) of chlorotetaine were determined, which are beneficial for development of novel drugs.

2. Results and discussion

2.1. Identification of ZJU-2011 by 16S rRNA sequence analysis

The 16S rRNA gene (1399 bp) obtained (Figure S2) was used to identify ZJU-2011, and the results showed that it was 99% similar to B. amyloliquefaciens FZB42. The phylogenetic tree of ZJU-2011 based on the 16S rRNA gene sequences was constructed with BLAST pairwise alignments (Figure S3). Based on this, it is reasonable to conclude that ZJU-2011 was a novel strain of B. amyloliquefaciens.

2.2. Separation and purification of the antifungal compounds

In the pre-processing process (Table S1), around 84.6% of the impurities were taken out with almost no loss of activity. Next, through gradient elution of an MCI-GEL CHP-20P column, the bioactive products were easily obtained in the latter phase due to the decreased polarity. In the final reversed-phase chromatography with C18, it was clearly shown that a mixture of antifungal components had been obtained (Figure S4). By a second reversed-phase chromatography, products collected in peak 1 and peak 2 were purified. The purity of the final products was determined by HPLC-CAD (high performance liquid chromatography-charged aerosol detector) detection and the purity of the target product was up to 90% (shown in Figures S8 and S9).

2.3. Structural determination of the antifungal components

From Figure S6, it can be concluded that the molecular weight of this component was 270 Da, giving a molecular formula of C_{12}H_{18}O_{5}N_{2} with five degrees of unsaturation. The $^{13}$C, $^1$H NMR (Table S3) revealed that 14 of the protons were attached to 12 carbons, including one methyl, three methylenes and five methines. The remaining three carbons were assigned as non-protonated centres including two ketone carbonyls ($\delta_C$ 170.08; 178.44) and one anhydride carbonyl ($\delta_C$ 209.52). Also, it can be seen that this component contains at least one double bound, one carbonyl group and two rings. Based on these, the structure of this component was determined as bacilysin (Figure 1(a)).

From Figure S7, it can be concluded the molecular weight of this component was 288 Da. The $^{13}$C NMR, $^1$H NMR (Table S4) revealed that 14 protons and 12 carbons exist in this structure containing one methyl, three methylenes, three carbonyl and one double bond. Due to the presence of nitrogen, it can be determined that there are at least two nitrogens in this component. Thus, the possible molecular formula is C_{12}H_{17}ClN_{2}O_{4} (if Cl in this molecule) or C_{13}H_{25}O_{4}N_{4}. Based on the information in the NMR, the final structure of this component was determined as chlorotetaine (Figure 1(b)).

According to papers published to date, only three strains of bacteria can produce chlorotetaine, including two Bacillus subtilis strains and one B. amyloliquefaciens strain (Rapp et al. 1988; Phister et al. 2004; Arguelles-Arias et al. 2009). ZJU-2011 is the fourth strain that
can produce chlorotetaine, and more importantly, it is the first *B. amyloliquefaciens* reported to produce bacilysin and chlorotetaine simultaneously.

### 2.4. Antifungal activity of chlorotetaine

Although more work has been done on the bioactivity and the biosynthesis of bacilysin, no further work on chlorotetaine has been done. To further determine the bioactivity of chlorotetaine, another four pathogens of *Candida* spp. and one strain of *Aspergillus niger* were tested. All of the strains tested were highly sensitive to chlorotetaine (Table S2), and the MICs were in the range of 1.8–7.8 μg/mL, which meets the National Committee for Clinical Laboratory Standards. Compared with the popular antifungal drug, fluconazole, used today, the antifungal activity of chlorotetaine is almost the same as fluconazole when it comes to *Candida krusei* CBS573. Considering its great water-solubility, excellent bioactive activity, smaller molecular weight and the naturally derived nature without any chemical modifications, chlorotetaine will be a better and more promising antifungal candidate.

### 3. Conclusions

In this study, a novel method for the separation and purification of water-soluble components with small molecular weights was developed, which was simple, rapid and reproducible. For the first time, it was found that *B. amyloliquefaciens* can produce bacilysin and chlorotetaine simultaneously, like other two *B. subtilis* strains. In addition, to further study the antifungal activity of chlorotetaine, a detailed antifungal test against six common fungal pathogens was performed and the MICs were determined, which is the crucial data for the development of novel antifungal drugs. When it comes to *C. krusei*, chlorotetaine was shown to be nearly as effective as fluconazole.

**Supplementary material**

Experimental details relating to this article are available online at [http://dx.doi.org/10.1080/14786419.2015.1048246](http://dx.doi.org/10.1080/14786419.2015.1048246).

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