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Chapter 6

Determination of Staphylococcal Phenol-Soluble Modulins (PSMs) by a High-Resolution HPLC-QTOF System

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Abstract

Phenol-soluble modulins (PSMs) are multifunctional, amphipathic, α-helical peptides produced by virtually all staphylococcal strains. They have recently drawn much attention owing to the key contribution of some PSM peptides to staphylococcal virulence, in particular in highly virulent *Staphylococcus aureus*. High concentration of PSMs may cause cytolysis, damaging neutrophils, immune cells, red blood cells and white blood cells. Furthermore, all PSMs contribute to biofilm structuring and the dissemination of biofilm-associated infection. Here we describe a method for PSM analysis in *S. aureus* by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry system (HPLC-QTOF).

Keywords: phenol-soluble modulins, HPLC-QTOF, *Staphylococcus aureus*, determination

1. Introduction

Phenol-soluble modulins (PSMs) are a family of multifunctional, amphipathic and α-helical peptides produced by virtually all staphylococcal strains [1–3]. They have recently drawn much attention owing to the key contribution of some PSM peptides to staphylococcal virulence, in particular in highly virulent *Staphylococcus aureus* [4]. In addition, they have antibacterial activity, likely to compete with other environmental rival bacteria such as streptococci [5, 6], as well as biofilm structuring and dissemination functions [7, 8]. *Staphylococcus aureus* is an important and versatile opportunistic human pathogen that can cause a wide range of acute and chronic diseases, which range from superficial infections to invasive and life-threatening ones [9, 10].

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PSMs were first identified in 1999 by the group of Seymour Klebanoff with the description of a "pro-inflammatory complex". They were isolated by hot phenol extraction from *S. epidermidis* culture filtrate [11]. The peptides were named PSMα, PSMβ and δ-toxin. Afterwards, the PSM compositon of *S.aureus* was also analyzed more systematically, which include the shorter α-type (four PSMα1-PSMα4 peptides and δ-toxin) and the longer β-type (the PSMβ1 and PSMβ2 peptides). The PSMs are encoded at three different locations in the genome. Four PSMα1-PSMα4 peptides are encoded in the PSMα operon. PSMβ1 and PSMβ2 are encoded in the PSMβ operon. δ-toxin is encoded within the coding sequence for RNAIII [12, 13].

S. aureus are considered to be second to salmonella as important foodborne pathogens, which have been frequently reported as agents leading to outbreaks of diseases caused by enterotoxins in ready-to-eat food and food products. The pathogenic mechanisms of *S. aureus* have been examined extensively through different stages of infection. Various staphylococcal components contribute to virulence with an improved understanding of specific functions [14–16].Proteomics studies have revealed that the production of virulence factors by different isolates of *S. aureus* is diverse and only a few of these seem to be invariantly produced [17]. PSMs are the most commonly identified staphylococcal virulence factors, especially in the community-associated (CA)-MRSA lineages. Recently, it was found that many *in vitro* PSM phenotypes were strongly inhibited by serum lipoproteins, and can exert their contribution to pathogenesis by intracellular killing and participate in neutrophil killing after phagocytosis [18]. In addition, PSMs at low concentration can lead to immune cell chemotaxis and inflammatory reaction. High concentration of PSMs may cause cytolysis, damaging neutrophils, immune cells, red blood cells and white blood cells [19, 20]. Thus, it is important to develop a rapid, specific and accurate method to detect PSMs in food products for the prevention of transmission.

At present, there are only a few methods for determination of PSMs, such as imaging mass spectrometry and liquid chromatography-ion trap or quadrupole mass spectrometry [11, 21]. The sample pre-treatment of the method using imaging mass spectrometry is quite cumbersome and time-consuming. The resolution of the method by liquid chromatography-ion trap or quadrupole mass spectrometry is too low to separate interfering substances with similar charge-to-mass ratios. Here, we describe a simple and effective method with higher sensitivity and selectivity for PSM analysis in *S. aureus* by high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry system (HPLC-QTOF).

2. Materials

2.1. Reagents and chemicals

1. Tryptone soy broth (TSB).
2. *Staphylococcus aureus* strains.

3. 1-butanol.
4. Acetonitrile.
5. Formic acid.
6. Ultrapure water.
7. Eluent A: 0.1% formic acid in water, Eluent B: 0.1% formic acid in acetonitrile.

2.2. Instrument and equipment

1. Chromatograph: High-performance liquid chromatography-quadrupole time-of-flight mass spectrometry system (1260 HPLC system connected to a 6530 Accurate-Mass spectrometer Q-TOF/MS, Agilent, America).
2. Columns: Zorbax 300SB-C8 (2.1 μm, 4.6×150 mm, Agilent, America).
3. Mass Hunter software (Agilent, America).
4. Centrifuge (3-18K, Sigma, Germany).
5. Autoclave (DB-200, Systec, Germany).
6. Incubator shaker (ZWY-211B, ZhiCheng, China).
7. N-EVAP (Organomation, America).

3. Methods

3.1. Extraction of PSMs

Pre-cultures grown overnight from a liquid culture are used to inoculate bacterial cultures at 1:100 dilution.

3.1.1. Extraction of PSMs from TSB for qualitative analysis

1. The *Staphylococcus aureus* cells were removed by centrifugation for 10 min (5000 rpm, 4°C) after overnight growth (~16 h) in 100 mL of culture medium (TSB) at 37°C with shaking at 200 rpm.
2. Transfer supernatants to a 100-mL volumetric flask.
3. Add 1/3 (v/v) of 100% 1-butanol to the supernatants to make 25% (v/v) of 1-butanol.
4. Shake the solutions vigorously at 37°C for 2 h.
5. After brief centrifugation, collect upper (butanol) phases.
6. Dry the extract under nitrogen and redissolve the dried sample in ultrapure water.
7. Filter the solution through a 0.22-μm filter using a syringe before LC-MS analysis.

3.1.2. Extraction of PSMs from TSB and beverage for quantitative analysis

1. The *Staphylococcus aureus* cells were removed by centrifugation (5000 rpm, 10 min, 4°C) after overnight growth (~16 h) in 4 mL of culture medium (TSB or beverage) at 37°C with shaking at 200 rpm. The "beverage" means pure juice or juice beverage. Soda, water and coffee were not included.
2. Transfer 1.5 mL supernatants to 4-mL centrifuge tubes.
3. The supernatants were incubated at a 1:1 ratio with 1-butanol for 2 h (37°C, 200 rpm).
4. Transfer 1.0 mL supernatants to 2-mL centrifuge tubes.
5. Dry the extract under nitrogen and redissolve the dried sample in 0.5 mL ultrapure water.
6. Filter the solution through a 0.22-μm filter using a syringe before LC-MS analysis.

3.1.3. Extraction of PSMs from milk for quantitative analysis

1. The *Staphylococcus aureus* cells were precipitated by acetonitrile after overnight growth (~16 h) in 4 mL of culture medium (milk) at 37°C with shaking at 200 rpm.
2. Remove fat by frozen centrifugation technology (5000 rpm, 10 min, 0°C).
3. Extract water layer 1.5 mL to 4-mL centrifuge tubes.
4. The extract was incubated at a 1:1 ratio with 1-butanol for 2 h (37°C, 200 rpm).
5. Transfer 1.0 mL supernatants to 2-mL centrifuge tubes.
6. Dry the extract under nitrogen and redissolve the dried sample in 0.5 mL ultrapure water.
7. Filter the solution through a 0.22-μm filter using a syringe before LC-MS analysis.

3.1.4. Extraction of PSMs from pork for quantitative analysis

1. The *Staphylococcus aureus* cells were precipitated by acetonitrile after overnight growth (~16 h) in 4 mg of culture medium (pork) at 37°C with shaking at 200 rpm.
2. Add 1.5 mL 1-butanol and remove fat by frozen centrifugation technology (5000 rpm, 10 min, 0°C) after extraction of 2 h (37°C, 200 rpm).
3. Transfer 1.0 mL supernatants to 2-mL centrifuge tubes.
4. Dry the extract under nitrogen and redissolve the dried sample in 0.5 mL ultrapure water.
5. Filter the solution through a 0.22-μm filter using a syringe before LC-MS analysis.

3.2. Analysis of PSMs

3.2.1. Liquid chromatography methodology

1. Transfer the supernatants into glass sample vials. Cool down the sample tray to 10°C and put the sample vials in it.

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2. An Agilent high-performance liquid chromatography-electrospray ionization-qudropole time-of-flight mass spectrometry (HPLC-ESI-QTOF/MS) system was used to analyze the PSMs qualitatively and quantitatively.
3. The chromatographic separation was performed with a Zorbax 300SB-C8 column (2.1 μm, 4.6×150 mm, Agilent) in series with a flow rate of 0.5 mL/min.
4. The gradient program can be described as follows: 10% eluent B for 2.5 min; 50% eluent B for 2.5 min; a linear gradient from 50% to 90 % eluent B for 15 min.
5. Injections (10 μL) were made using an autosampler, and the column temperature was set at 30°C.

3.2.2. Qualitative analysis of PSMs from culture

1. Transfer the supernatants into glass sample vials. Cool down the sample tray to 10°C and put the sample vials in it.
2. The ESI experiments were carried out by Dual AJS ESI ion source in a high-resolution instrument mode with a mass range of 3200 m/z and a positive scan mode with gas temperature at 325°C, drying gas flow rate at 5 L/min, sheat gas temperature at 350°C, and sheat gas flow rate at 7.5 L/min. The nebulizing gas was produced by a nitrogen generator.
3. The capillary voltage was kept at 4000 V and the fragmentor voltage was set to 175 V. Signals acquired in MS mode were used to make a preliminary identification, and data obtained in targeted MS/MS mode were searched from the online Mascot database, which could determine the PSMs accurately.
4. The peak area based on extracted ion chromatogram (EIC) can be used to quantitate the PSMs sensitively and rapidly.

4. Results and discussion

Examples of preset m/z ratios and retention times of *S. aureus* PSMs for qualitative and quantitative analyses are shown in **Table 1**. Retention times may vary according to the LC system and MS detector. (d), (t) and (q) stand for doubly, triply and quadruply charged ions, respectively.

The chromatograms of PSMs extracted from the culture are shown in **Figure 1**. The result indicates that the new method has a high selectivity. Good linearity of the PSMs was achieved in the range 0.5~100 μg/L ($R^2 > 0.99$). Compared with other analytical methods, the pre-treatment of the new method is simple and rapid, and the high resolution makes the method highly sensitive and selective. The method has been used successfully for the determination of PSMs extracted from different culture mediums, such as milk, beverage, vegetable and meat.

PSM	MS (m/z)	RT (min)	MS/MS (m/z)
PSMα1	1144.6842(d)	14.353	330.1491, 302.1539
PSMα2	1153.6964(d)	12.727	330.1475, 302.1538
PSMα3	1318.2228(d)	11.705	289.0861, 436.1542
PSMα4	1100.6982(d)	20.250	344.1674, 316.1711
δ-toxin	1002.8844(t)	15.010	474.1661, 587.2490
PSMβ1	1131.8610(q)	15.437	346.1059, 459.1892
PSMβ2	1121.8514(q)	11.353	318.1080, 702.3068

Table 1. m/z ratios and retention times of *S. aureus* PSMs.

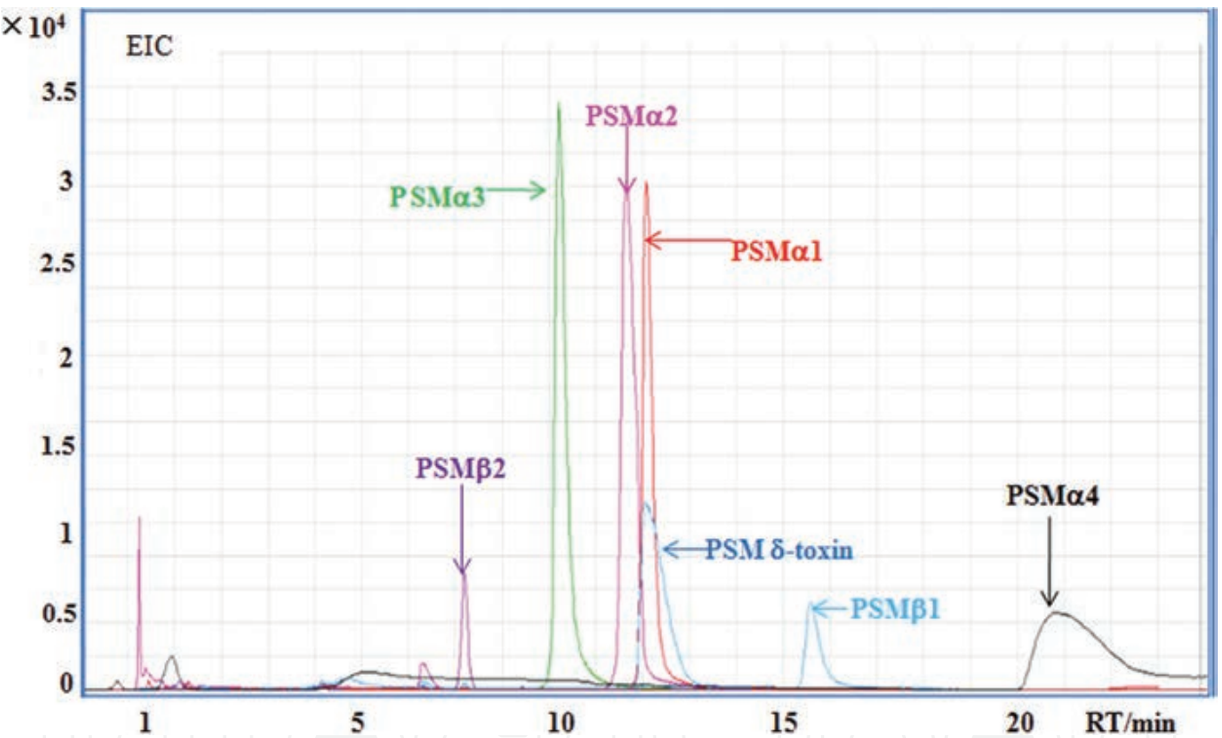


Figure 1. Chromatograms of PSMs extracted from the culture.

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