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Purification of a Pore-forming Peptide Toxin, Tolaasin, Produced by *Pseudomonas tolaasii* 6264

Kwang-Hyun Cho#, Sung-Tae Kim and Young-Kee Kim*

Department of Agricultural Chemistry, Chungbuk National University, Cheongju, Chungbuk, 361-763, Korea

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Tolaasin, a pore-forming peptide toxin, is produced by *Pseudomonas tolaasii* and causes brown blotch disease of the cultivated mushrooms. *P. tolaasii* 6264 was isolated from the oyster mushroom damaged by the disease in Korean. In order to isolate tolaasin molecules, the supernatant of bacterial culture was harvested at the stationary phase of growth. Tolaasin was prepared by ammonium sulfate precipitation and three steps of chromatograpies, including a gel permeation and two ion exchange chromatographies. Specific hemolytic activity of tolaasin was increased from 1.7 to 162.0 HU mg^{-1} protein, a 98-fold increase, and the purification yield was 16.3%. Tolaasin preparation obtained at each purification step was analyzed by HPLC and SDS-PAGE. Two major peptides were detected from all chromatographic preparations. Their molecular masses were analyzed by MALDI-TOF mass spectrometry and they were identified as tolaasin I and tolaasin II. These results demonstrate that the method used in this study is simple, time-saving, and successful for the preparation of tolaasin.

Keywords: Peptide toxin, Pore formation, *Pseudomonas tolaasii*, Tolaasin

Introduction

Tolaasin is a pore-forming peptide toxin. It causes a brown blotch disease on the cultivated mushrooms by forming membrane pores and disrupting cellular membrane structure. It is produced by *Pseudomonas tolaasii*, a world-wide pathogenic bacterium to the various mushrooms. *P. tolaasii* was isolated as a causal organism of brown blotch disease of *Agaricus bisporus* (Tolaas, 1915). An extracellular toxin was purified by Peng (1986) and its molecular structure was determined by Nutkins *et al*. (1991). It consists of 18 amino acids, and its molecular mass is 1,985 Da. Two isomers of tolaasin, Tol I and Tol II, were isolated, and their primary structures were determined. Both isomers have a β-hydroxyoctanoic acid covalently linked to N-terminus. Many isomers were identified from various virulent strains of *P. tolaasii* and their structures were determined (Shirata *et al.,* 1995; Bassarello *et al.,* 2004). Since tolaasin has two positive charges at C-terminus, it is an amphipathic peptide.

Biological toxicity of tolaasin was investigated and tolaasin was proved to be responsible for the brown blotch disease (Rainey *et al.,* 1991). Tolaasin-induced membrane disruption has been explained by both pore formation and surfactant property of tolaasin molecule (Hutchison and Johnstone, 1993). It is generally accepted that the former property of tolaasin is more responsible for cell disruption at low concentration of tolaasin. Ion channel formation of tolaasin was demonstrated in a planar lipid bilayer and two types of ion channels were observed (Cho and Kim, 2003). The slope conductances of these channels were 150 and 500 pS. Characteristics of tolaasin-induced pore have been investigated by measuring tolaasin-induced hemolysis and the effects of various osmotic protectants on hemolysis (Rainey *et al.,* 1991; Cho *et al.,* 2000). Some of metal ions, such as Zn^{2+} and Gd^{3+}, inhibit the activity of tolaasin channel as well as hemolysis.

Since brown blotch disease causes very serious problem in domestic cultivation of oyster mushroom, purification of tolaasin molecules and evaluation of its toxicity are very important to prevent the disease from spreading. For the purification of tolaasin, Peng's method is used with minor modifications (Peng, 1986) and it is successful and still being widely used. However, this method is a long-time procedure and it consists of two steps of precipitations, three steps of extractions, many desalting steps, and several other steps, such as lyophilization, gel filtration chromatography, and centrifugation. In this

#Present address: Department of Physiology, College of Medicine, The Catholic University of Korea, Seocho-gu, Seoul, 137-701, Korea

*To whom correspondence should be addressed.
Tel: 82-43-261-2560; Fax: 82-43-271-5921
E-mail: ykkim10@chungbuk.ac.kr

study, an alternative method of tolaasin preparation has been designed and a four-step procedure was simple, time-saving and successful for the isolation of tolaasin molecules.

Materials and Methods

Materials. Tricine [N-{Tris(hydroxymethyl)methyl}glycine], Tris-Tricine precast gel, and molecular weight markers for peptide gel electrophoresis were purchased from Bio-Rad Laboratories. Materials for gel permeation, ion exchange chromatographies, and all other chemicals were obtained from Sigma-Aldrich Co..

Isolation and culture conditions of *Pseudomonas tolaasii*. *P. tolaasii* 6264 was originally isolated from the fruiting bodies of oyster mushrooms severely damaged with brown blotch disease in Korea (Lee *et al.,* 1997). The mushroom tissues were homogenized and the homogenate was extracted with sterilized distilled water. The extract was evenly spread onto a PAF (*Pseudomonas* agar F) agar plate and *P. tolaasii* 6264 was isolated and identified by white line test and pitting test on mushroom tissue (Wong and Preece, 1979) as well as by bacteriological characteristics (Tsuneda *et al.,* 1995). The isolated bacterium was stored at −80°C in PAF broth with 20% (v/v) glycerol until use.

Hemolysis. Hemolytic activity of tolaasin was measured with rat erythrocytes. Defibrinated rat erythrocytes were washed with HBS (HEPES-buffered saline; 5 mM HEPES and 150 mM NaCl, pH 7.4) three times and diluted with the same buffer to get 10% solution. Tolaasin preparation properly diluted with HBS was added to the erythrocyte solution and incubated for 30 min at 37°C. Hemolysis was monitored by measuring the absorbance changes at 600 nm using a spectrophotometer (U-2000, Hitachi Ltd.). One hemolytic unit (HU) of tolaasin was defined as the amount of tolaasin, which is able to induce complete hemolysis of 1% erythrocyte within 30 min.

Purification of tolaasin. Tolaasin molecules were purified from 0.5 l of bacterial culture obtained at late stationary phase of growth. Bacteria were removed by low speed centrifugation and tolaasins in the supernatant were collected by ammonium sulfate precipitation. Ammonium sulfate was added to the supernatant and final concentration was adjusted to 30% (17.6 g/100 ml). The solution was incubated for 1 h at 4°C and tolaasin was precipitated by ultracentrifugation for 1 h at 20,000 rpm (50,000 × *g*) in a type SW-28 rotor (Beckman Instruments Inc.). The precipitate containing tolaasin molecules was resuspended in 10 mM sodium phosphate buffer (pH 7.0) and dialyzed for 8 h with benzoylated dialysis tubing (Sigma-Aldrich Co.) against the same buffer. The preparation was homogenized with a glass-Teflon homogenizer, applied to Sephadex G-75 column (∅1.4 × 45 cm), and eluted at a flow rate of 1 ml min^{-1}. Protein concentration of each eluted fraction was monitored by measuring absorbance at 220 nm. Fractions containing proteins were collected and assayed for hemolytic activity.

Fractions having hemolytic activity were pooled together and further analyzed by a Dowex 1X8-50 column (∅1.4 × 24 cm). After anionic components were removed, the fractions containing tolaasin were pooled together and loaded onto a column (∅1.4 × 22 cm) packed with CM-cellulose pre-equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was eluted at a flow rate of 0.25 ml min^{-1} with five step gradients of NaCl from 10 to 200 mM. The fractions containing purified tolaasin were collected and concentrated by lyophilization. The tolaasin was resuspended and dialyzed for 8 h against 10 mM sodium phosphate buffer (pH 7.0). The concentration of protein was determined by the Lowry method (Lowry *et al.,* 1951). Purity of tolaasin preparation was analyzed by HPLC using an elution solution containing 10 mM sodium phosphate buffer (pH 7.0). A gel permeation column, Protein-Pak™ 60 column (Waters Co.), was used and the purified tolaasin was stored at −80°C.

SDS-PAGE. SDS gel electrophoresis of tolaasin peptide was performed by the method described by Schägger and Jagow (1987) with partial modification. Tolaasin preparations were diluted with 2 parts of SDS-PAGE sample buffer containing 200 mM Tris-HCl, pH 6.8, 40% glycerol, 2% β-mercaptoethanol, and 2% SDS. After heating at 95°C for 5 min, the solutions were loaded onto a Tris-Tricine precast gel (16.5% acrylamide and 3.3% crosslinker). Running buffer consisted of 100 mM Tris, 100 mM Tricine, pH 8.3, and 0.1% SDS. Electrophoresis was conducted at 100 V of constant voltage for 1.5 h. Apparent molecular mass of tolaasin was determined by manufacturer's procedure using molecular weight standard (#161-0326, Bio-Rad), containing triosephosphate isomerase (26.6 kDa), myoglobin (16.9 kDa), α-lactalbumin (14.4 kDa), aprotinin (6.5 kDa), oxidized form of insulin β-chain (3.5 kDa), and bacitracin (1.4 kDa). Protein bands were visualized with silver staining reagent (Bio-Rad Lab.) according to manufacturer's instructions (Merril *et al*., 1981).

MALDI-TOF mass spectrometry. Molecular masses of the purified peptides were determined by MALDI-TOF MS (Axima-CFRplus, Shimadzu/Kratos; Krittanai and Panyim, 2004). The purified tolaasin (1 μl) was spotted on a dried droplet of matrix on a stainless steel target plate (384-well plate, Shimadzu Co.). The α-cyano-4-hydroxycinnamic acid (CHCA) matrix was prepared by dissolving 10 mg of CHCA in 1 ml of 50% ACN/0.1% TFA solution. Mass spectra were recorded in positive ion mode with an acceleration voltage of 20 kV. The spectra of 150 laser shots were averaged. For external calibration, reflectron mode was used with two calibrators, angiotensin II (1046.5423 Da) and ACTH fragment 18-39 (2465.1989 Da).

Results

Purification of tolaasin. When *P. tolaasii* 6264 was incubated in PAF media, secretion of tolaasin molecule was increased at late logarithmic phase of bacterial growth and the concentration of tolaasin in the culture media was maximal at stationary phase (Cho *et al.,* 2000). In order to purify tolaasin molecules, culture supernatant was obtained at late stationary phase and tolaasin was collected by ammonium sulfate precipitation. The precipitated tolaasins were harvested by ultracentrifugation

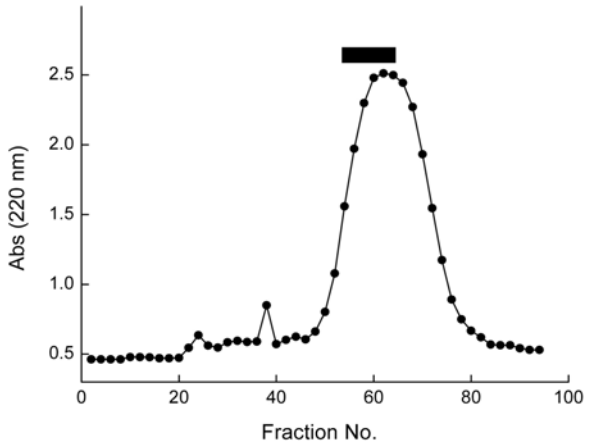

Fig. 1. Isolation of tolaasin by a gel permeation chromatography. Tolaasin prepared by ammonium sulfate precipitation was analyzed by a gel permeation chromatography. Fractions of high hemolytic activity were marked with horizontal bar on the top of the chromatogram.

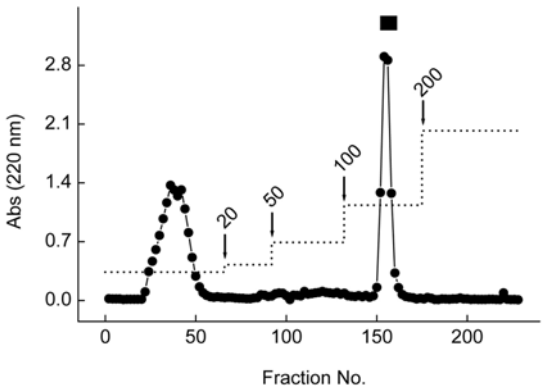

Fig. 2. Purification of tolaasin by cation-exchange column chromatography. Crude tolaasin preparation was applied to CM-cellulose column. Fractions were eluted by a series of discontinuous NaCl gradient from 10 to 200 mM. The concentrations of NaCl in elution buffer were indicated with downward arrows. Flow rate was 0.25 ml min^{-1} and fractions having high hemolytic activity were marked with horizontal bar on the top of chromatogram.

and more than 60% of the original tolaasin activity was recovered during these initial procedures.

The crude tolaasin preparation was analyzed by gel permeation chromatography. In Sephadex G-75 column chromatography, the eluted fractions containing tolaasin were identified by measuring hemolytic activity (Fig. 1). Hemolytic activity was observed from the early fractions of the major peptide peak. The fractions containing tolaasin activity were pooled together and concentrated by lyophilization.

The obtained tolaasin preparation was further analyzed by a Dowex-1 ion exchange column chromatography. A single peak of tolaasin was isolated with high hemolytic activity (data not shown). In the subsequent purification of tolaasin by CM-cellulose chromatography, the column was eluted with five-step concentration gradient of NaCl from 10 to 200 mM as shown in Fig. 2. Two peaks were separated, the first one eluted with a normal buffer and the second with a buffer containing 100 mM NaCl. Hemolytic activity was only observed from the second peak. Fractions from No. 150 to No. 156 were pooled together and concentrated for further analysis.

Table 1 summarizes the yield and purity of the tolaasin calculated at each step of the purification. The specific activity of tolaasin was increased by four-step purification procedure from 1.7 HU mg^{-1} protein of the crude preparation to 162.0 HU mg^{-1} protein, and it was a 98-fold increase. The purified peptide obtained was 2.9 mg and the yield was 16.3%.

Analysis of the purified tolaasin preparation. Tolaasins obtained from each purification step were analyzed by HPLC and SDS-PAGE. In the HPLC analysis, the crude tolaasin preparation obtained by ammonium sulfate precipitation was separated into many components. Among these, three major components were identified and their retention times were 14.8, 16.4, and 17.0 min (data not shown). However, only two peptides were detected from the purified tolaasin preparation and the retention times of the first and second peptides were 15.4 and 17.3 min, respectively (Fig. 3). The second peptide was major component and its peak height was four times bigger than that of the first peptide. The retention times of these two peptides did not match to any of major components obtained from the crude tolaasin preparation.

In the analysis by SDS-PAGE, many proteins were observed from the ammonium sulfate-precipitated preparation (Fig. 4. lane 1). When tolaasin fractions obtained from gel permeation

Table 1. Purification of tolaasin from the culture of *Pseudomonas tolaasii* 6264

Purification step	Total protein (mg)	Total hemolytic activity (HU)*	Specific activity (HU mg^{-1})	Yield (%)	Purification (Fold)
Culture supernatant	1741.1	2875.0	1.7	100.0	1.0
Ammonium sulfate	31.4	1750.0	55.8	60.9	32.8
Sephadex G-75	6.6	705.9	107.6	24.6	65.2
Dowex 1X8-50	5.4	626.4	116.7	21.8	70.7
CM-cellulose	2.9	468.3	162.0	16.3	98.2

*Hemolytic activity is defined under Materials and Methods.

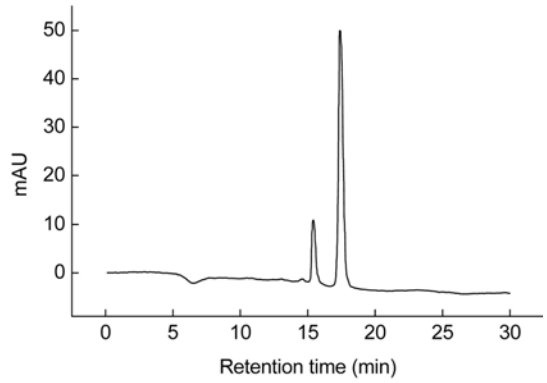

Fig. 3. HPLC analysis of the purified tolaasin preparation. The purified tolaasin was analyzed by HPLC with 10 mM sodium phosphate buffer (pH 7.0). Flow rate was 0.5 ml min^{-1} and absorbance was measured at 220 nm.

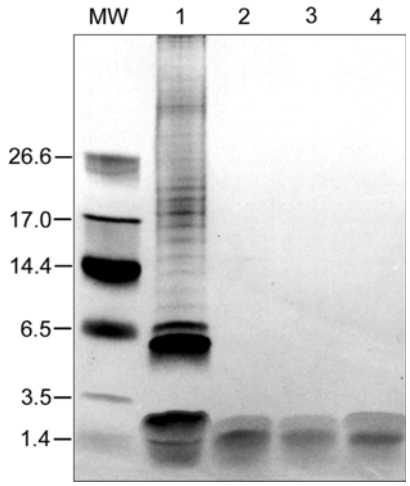

Fig. 4. SDS-PAGE analysis of tolaasin preparations. Tris-Tricine precast gels were prepared for the analysis of small peptides. After electrophoresis, the gel was stained by silver staining method. MW, molecular weight markers (kDa); lane 1, tolaasin preparation obtained from ammonium sulfate precipitation; lane 2, tolaasin prepared by gel permeation chromatography; lane 3, tolaasin by anion exchange chromatography, and lane 4, tolaasin by cation exchange chromatography.

chromatography were subjected to analysis by SDS-PAGE, most of high molecular weight proteins were not detected and two major peptide bands were identified by silver staining. Their approximate molecular masses were near 1,900 Da. During the purification steps of both anion and cation exchange chromatographies, these two peptides were always detected along with hemolytic activity. Therefore, no further purification step was necessary.

Molecular mass analysis. Since two peptides were detected by both SDS-PAGE and HPLC, molecular masses of these peptides were analyzed by MALDI-TOF mass spectrometry. As shown in Fig. 5, two peptides were detected and their

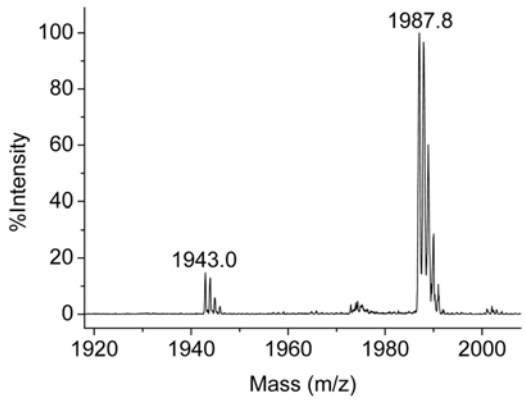

Fig. 5. Mass spectra of the purified tolaasin preparation measured by MALDI-TOF mass spectrometry.

average molecular masses were determined as 1943.0 and 1987.5 Da. The peptide exhibited a molecular ion peak at *m/z* 1943.0 $(M+H)^+$ matched to the calculated molecular mass of tolaasin II isomer plus one proton. The molecular mass of the second peptide was *m/z* 1987.8 $(M+H)^+$ and this also matched to the molecular mass of tolaasin I isomer. Although molecular mass of tolaasin I has been determined as 1985 Da (Nutkins *et al.*, 1991), average mass of tolaasin I is predicted to 1987 Da after considering the sum of the average atomic weight of naturally occurring isotopes in the tolaasin molecule.

Discussion

An extracellular toxin, tolaasin, of *P. tolaasii* was purified by Peng (1986). The procedure for the isolation of tolaasin consists of many steps of precipitation, extraction, and chromatographies. Although this method takes a lot of efforts, it has been widely used because of no available alternative method for the purification of tolaasin. In this study, a four-step procedure was designed for the purification of tolaasin and it was time-saving and very successful for the isolation of tolaasin molecules. Fast isolation of tolaasin also made it possible to contribute to the fast evaluation of brown blotch disease. After the gel permeation chromatography, tolaasin was already purified enough and no major impurities were detected by the analyses of SDS-PAGE and HPLC. Since the biological activity of tolaasin can be evaluated within 1 h by measuring the hemolytic activity, fast preparation, evaluation, and biological application will be advantages of this preparation method. Therefore, this could be an alternative method for the tolaasin preparation along with Peng's method.

Recently, two research groups purified tolaasin molecules according to modified methods of Peng's (1986). The yields of tolaasin peptide preparations reported by Jourdan *et al.* (2003) and Bassarello *et al.* (2004) were 42.3 mg from 1.35 l and 143.5 mg from 6.75 l of culture solutions, respectively. The amount of tolaasin purified in this study was 2.9 mg from

0.5 l of culture solution. Although the yield of our preparation is 20-30% of those reported by others, it is difficult to compare the specific activities of all preparations since specific activities are not shown.

Several isomers of tolaasin peptide have been identified from the cultures of various *P. tolaasii* strains. Tolaasin I and II were isolated from *P. tolaasii* Paine (Nutkins *et al.,* 1991). Tolaasin II is a structural analogue of tolaasin I since it is identical to tolaasin I except a substitution of homoserine at 16 amino acid sequence by glycine. Eight isomers of tolaasin I were identified from the culture of *P. tolaasii* isolated from Japan (Shirata *et al.,* 1995). In structural analyses, the isomer-4 and -6 were identified as tolaasin I and II, respectively. The tolaasin I was the only major peptide in the preparation. Seven isomers of tolaasin were also isolated from the culture of the strain type NCPPB2192 of *P. tolaasii* (Bassarello *et al.,* 2004). Two of these isomers were also matched to tolaasin I and II, and some were to those characterized by Shirata *et al*. (1995). In this study, *P. tolaasii* 6264 isolated from a domestic farm produced two types of tolaasin peptides. Based on the molecular masses obtained from mass spectrometry and retention times of HPLC analysis, the major peptide was identified as the tolaasin I and the minor one as the tolaasin II. Therefore, *P. tolaasii* 6264 is similar to the strain, *P. tolaasii* Paine, characterized by Nutkins *et al.*, rather than the strains secreting 7 or 8 peptides, characterized by Shirata and Bassarello.

Membrane pore formations of small peptide toxins were explained by the aggregations of peptide molecules in the range of 2-6 (Duclohier *et al.,* 1989; Matsuzaki *et al.,* 1994; Leetachewa *et al.,* 2006). During the purification of tolaasin molecules by a gel permeation chromatography, hemolytic activity was observed from the fractions of molecular masses, ranged from approximately 1,700 to 6,000 Da (Fig. 1). When these fractions were analyzed by SDS-PAGE, proteins of M.W. bigger than 2,500 were not found in silver-stained gels (Fig. 4). Similar results were also obtained from HPLC analysis. Therefore, the fractions of molecular mass above 4,000 Da obtained by gel permeation chromatography may indicate the formation of tolaasin aggregates (Shai and Oren, 2001). Forming molecular aggregate is important because it is prerequisite for membrane pore formation. Although it is not well studied where tolaasin forms aggregates in solution or cellular membrane, our data may indicate that tolaasin molecules form dimeric or trimeric aggregates in aqueous solution. Since tolaasin has two positive charges at C-terminus, molecular aggregation could occur by the hydrophobic interactions between/among nonpolar N-termini of tolaasin molecules. Recently, the changes in the secondary structure of tolaasin I was observed when the peptide was passed from aqueous buffer to lipid membrane (Coraiola *et al.,* 2006), suggesting that there is difference in the aggregate structure between these two phases.

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