Soluble prokaryotic expression and purification of crotamine using an N-terminal maltose-binding protein tag

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Crotamine is a peptide toxin found in the venom of the rattlesnake Crotalus durissus terrificus. Interestingly, crotamine demonstrates promising anticancer, antimicrobial, and antifungal activities. The crotamine peptide can also deliver plasmids into rapidly dividing cells, such as cancer and stem cells, and demonstrates potent analgesic effects. Efficiently producing crotamine in mammalian cells is difficult because it is both cell-permeable and cytotoxic. Prokaryotic expression of this peptide is also difficult to maintain because it does not fold properly in the cytoplasm, resulting in aggregation and in the formation of inclusion bodies.

In our current study, we show for the first time that N-terminal fusion with three protein tags—N- utilization substance protein A (NusA), protein disulide isomerase b'α domain (PDIb'α), and maltose-binding protein (MBP)—enables the soluble overexpression of crotamine in the cytoplasm of Escherichia coli. MBP-tagged crotamine was purified using Ni affinity, anion exchange, and MBP chromatography. The tag was cleaved using TEV protease, and the final product was pure on a silver-stained gel. In total, 0.9 mg pure crotamine was obtained from each liter of bacterial culture with endotoxin level approximately 0.15 EU/μg, which is low enough to use in biomedical applications. The identity and intramolecular disulfide bonds were confirmed using MALDI-TOF MS analysis. Purified crotamine inhibited the hKv1.3 channel (but not hKv1.5) in a dose-dependent manner with IC50 value of 67.2 ± 44.7 nM (n = 10), indicating the correct protein folding. The crotamine product fused with MBP at its N-terminus also inhibited the hKv1.3 channel, suggesting that the N-terminus is not involved in the channel binding of the toxin.

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1. Introduction

Crotamine is a major component in the venom of the South American rattlesnake Crotalus durissus terrificus (Laure, 1975; Giglio, 1975). It belongs to the myoneurotoxin protein family, and induces skeletal muscle spasms at an LD50 value 33 μg/g (this is considered mild in comparison with other protein family members) (Nicastro et al., 2003). Initially, voltage-gated sodium channels were proposed as the molecular target of crotamine (Chang and Tseng, 1978; Matavel et al., 1998), though this was later disputed (Rizzi et al., 2007). Subsequently, voltage-gated K+ (Kv) channels were suggested as potential targets of crotamine according to computational docking analyses (Yount et al., 2009). Recently, it was shown that crotamine selectively inhibits hKv1.1, hKv1.2, and hKv1.3 channels at an IC50 value of approximately 300 nM, but other K channels are not affected (Peigneur et al., 2012). The structure of crotamine was determined using NMR (Fadel et al., 2005) and X-ray crystallography (Coronado et al., 2013), which...
revealed three intramolecular disulfide bonds. The overall structure resembles the β-defensin-like superfamily, which is typical for cysteine-stabilized antimicrobial polypeptide (Yount et al., 2009).

Interestingly, crotamine demonstrates promising anti-cancer activities. The toxin induces cell death in a dose-dependent manner in several actively proliferating cancer cells, but not in normal cells (Pereira et al., 2011). Crotamine cytotoxicity is related to the disruption of lysosomes and the activation of intracellular proteases (Hayashi et al., 2008). Crotamine also increases intracellular calcium and induces the loss of mitochondrial membrane potential (Nascimento et al., 2012) It effectively reduced tumor size in a mouse model of melanoma (Oguiura et al., 2011; Nascimento et al., 2012). Crotamine demonstrates strong antifungal activity against yeast Candida spp. Crotamine also shows modest activities against both Gram-negative and –positive bacteria (Yamane et al., 2014). Crotamine has been purified from its native form in rattlesnake venom (Li et al., 1993; Toyama et al., 2000; Coronado et al., 2012). Crotamine contains many basic amino acids, so it is positively charged and has a binding affinity for DNA (Radis-Baptista and Kerkis, 2011; Chen et al., 2012). Due to its ability to rapidly penetrate highly proliferative cells (Kerkis et al., 2004), it can be used for the targeted delivery of plasmid DNA and drugs into tumor cells and also as a marker for tumor cells (Hayashi et al., 2008).

Crotamine is also a potent analgesic that is >30-fold more effective than morphine (Giorgi et al., 1993; Mancini et al., 1998). Due to these potential biomedical applications, the efficient production of crotamine is desirable for further clinical research.

Crotamine has been purified from its native form in rattlesnake venom (Li et al., 1993; Toyama et al., 2000; Coronado et al., 2012). However, the amount of crotamine in venom is small, and the purity can be problematic so that it is not appropriate for mass production for biomedical applications. Eukaryotic overexpression is also an obstacle because the protein is both cell-permeable and cytotoxic. Hence, there have been few studies of the heterologous production of crotamine, even though its sequence has been known for almost 40 years (Laure, 1975). Recently, recombinant crotamine with the hexahistidine (His6) tag was expressed in Escherichia coli (E. coli), but the peptide was easily denatured and readily formed inclusion bodies (IBs) (Hayashi et al., 2012). Solubilization and refolding of the IB protein resulted in a nonfunctional protein, suggesting that proper folding was not achieved. In addition, purification of the IB protein is often not reproducible, and the yield is also often low (Fahnert et al., 2004). The soluble expression of crotamine in E. coli is therefore desirable for efficient production.

In our current study, we evaluated the effects of seven protein tags—glutathione S-transferase (GST), His6, maltose-binding protein (MBP), protein disulfide bond isomerase (PDI), thioredoxin (Trx), the b’α domain of PDI (PDlb’a’), and N-utilization substance protein A (NusA)—on the soluble overexpression of crotamine in E. coli. Interestingly, all of these tags produced increased crotamine solubility >50%. In particular, NusA, PDlb’a’, and MBP demonstrated higher solubility than the other tags at 20°C. Among the tagged fusion proteins, MBP-crotamine was chosen because of its size and convenient purification. The overexpressed MBP-tagged crotamine was effectively purified using Ni affinity, anion exchange, and MBP chromatography. Crotamine was cleaved from the MBP tag using tobacco etch virus (TEV) protease. The purified protein inhibited the hKv1.3 channel (but not the hKv1.5 channel) expressed in Xenopus oocytes.

2. Materials and methods

2.1. Plasmid construction

The DNA sequence that encodes the 42 amino acids of crotamine was chemically synthesized by a gene synthesis service (Bioneer, Daejon, Korea) in order to construct an entry vector, which was followed by constructing attB1-TEVrs-crotamine-attB2 that was then inserted into the pUC57 vector by EcoRV. The codons of the synthesized gene had been optimized to improve expression in E. coli. The attB1 (5’-agc gag aag ttt gta cca aac agc gtt c-3’) and attB2 sites (5’–acc cag ctt tct tgt tca aag tgg tcc cc-3’) for the BP recombination reaction (recombination between attB and attP sites yields attL and attR sites) were inserted at the 5’- and 3’-ends, respectively. The sequence encoding a TEV recognition site (TEVs: ENLYFQ; G) for cleaving the tag from crotamine was inserted between the attB1 site and crotamine. First, the entry vector—named pENTR-TEVs-crotamine—was constructed using the BP recombination reaction (Invitrogen, Carlsbad, CA), which integrates the synthesized DNA into pDONOR207 vector. Then, TEVs-crotamine in pENTR-TEVs-crotamine was recombined into the destination vectors pDEST-HGW, pDEST-HGGWA, pDEST-HMGWA, pDEST-HNGWA, pDEST-HXGWA, pDEST-PDI, and pDEST-PDlb’a’, which encode His6, GST, MBP, NusA, Trx, PDI, and PDlb’a’ tag, respectively, using an LR recombination reaction (recombination between attL and attR sites) (Invitrogen). The correct construction sequences that resulted from the BP and LR reactions were confirmed using a DNA sequencing service (Macrogen, Daejon, Korea). All constructs contained the His6 or His8 tag (for PDlb’a’ and PDI) in front of each tag for ease of purification.

2.2. Expression and solubility testing

Overexpression of the seven hybrid constructs was tested in bacteria. Each expression vector was transformed into competent E. coli BL21 (DE3) host cells, and the transformants were cultured at 37°C in Luria–Bertani medium (containing 50 μg/mL ampicillin) until the A600 optical density reached 0.4–0.6. The expression system was induced using 0.5 mM 1-thio-β-d-galactopyranoside (IPTG) for 3 h at 37°C or 18 h at 20°C. The harvested cells were sonicated with Tris buffer, and then the soluble and insoluble parts were separated using centrifugation at 18,000 × g for 15 min at 4°C. Expression and solubility were assessed using SDS-PAGE and 10% tricine gels. The protein levels in the SDS-PAGE gels were measured using ImageJ software (http://imagej.nih.gov/ij). Expression and solubility levels were calculated using the following formulas:

Expression level = S’/I′

Solubility = S”/(S” + P’)

where S’ is the amount of fusion protein; I’ is the total amount of cellular protein following IPTG induction; S” is the amount of the fusion protein in the supernatant section (S) and P’ is the amount of the fusion protein in the pellet section (P).

2.3. Purification

The induced E. coli were cultured at 20°C for 18 h and collected by centrifugation at 3,600 × g for 30 min at 4°C, and then the pellets were stored at −20°C until further study. The frozen cell pellets were homogenized in lysis buffer (20 mM Tris–HCl, 200 mM NaCl, 5% glycerol [v/v], 5 mM β-mercaptoethanol; pH 8.0) at a 12.5 mL/g ratio, and the lysate was sonicated 25 times (on/off for 20/10 s) to disrupt the cells. The soluble protein in the supernatant was obtained by centrifugation at 34,500 × g for 10 min at 4°C, filtered using a 0.45 μm pore size filter (Chromocore, Seoul, Korea), before purification. The filtered supernatant was applied to a 5 ml HisTrap FF affinity column (GE Healthcare, Piscataway, NJ) that was equilibrated in the lysis buffer using an AKTA Prime (Amersham Pharmacia, Piscataway, NJ). The column was washed with 40 column volumes (CV) of buffer A (20 mM Tris–HCl, pH 8.0) and eluted with a linear gradient of 20–250 mM imidazole in buffer A until the target protein eluted at a final concentration of 250 mM imidazole. The protein was concentrated and dialyzed against buffer B (20 mM Tris–HCl, pH 8.0, 250 mM NaCl).
200 mM NaCl, 5% glycerol [v/v], 5 mM β-mercaptoethanol, 50 mM imidazole; pH 8.0) to remove any nonspecific binding, and the elution step was performed using buffer B (20 mM Tris–HCl, 200 mM NaCl, 5% glycerol [v/v], 5 mM β-mercaptoethanol, 500 mM imidazole; pH 8.0). A 14 kDa cut-off membrane (Viskase Corporation, Darien, IL) was used to reduce the salt concentration of the purified MBP-crotamine in buffer C (20 mM Tris–HCl, 5% glycerol [v/v], 100 mM NaCl, 2 mM DTT; pH 8.0) for TEV cleavage. Purified TEV was added to the sample at a ratio of 1:10 (TEV:fusion protein [w/w]) and incubated for 24 h at 25°C with shaking at 85 rpm. After TEV treatment, the precipitate was removed by centrifugation at 1,500 × g for 30 min at 4°C, and the supernatant was collected. The TEV-treated supernatant sample was concentrated to 5–10 mL and then dialyzed in buffer D (50 mM MES, 5% glycerol [v/v], 2 mM DTT; pH 6.0) using a dialysis membrane with a 3,500 Da cut-off membrane (Viskase, Darien, IL) for 5 h at room temperature (RT). The sample was filtered using a 0.45 μm pore size filter (Chemocore, Seoul, Korea) before application onto a 10% tricine gels. Protein concentrations were measured using the Bradford assay. Under reducing conditions, 2 μg of recombinant crotamine, two kinds of samples were prepared under reducing and non-reducing conditions with/without dithiothreitol (DTT). Under reducing conditions, 2 μg of recombinant crotamine was exchanged with the PBS buffer via cation steps were checked using SDS-PAGE with 50% HCl, 200 mM NaCl, 2% SDS, 1 mM EDTA; pH 8.0), which included 10 mL and then dialyzed in buffer D (50 mM MES, 5% glycerol [v/v], 2 mM DTT; pH 6.0) using a dialysis membrane with a 3,500 Da cut-off membrane (Viskase, Darien, IL) for 5 h at room temperature (RT). The sample was filtered using a 0.45 μm pore size filter (Chemocore, Seoul, Korea) before application onto a 10% tricine gels. The band of the recombinant crotamine was cut off, and the solution was treated with 10 mM DTT for 15 min at RT, and the solution was applied to the oocytes by continuous chamber perfusion while electroplating. The purification steps were checked using SDS-PAGE with 10% tricine gels. Protein concentrations were measured using the BCA protein assay (Pierce Biotechnology, Rockford, IL) using BSA as the standard (Smith et al., 1985).

2.4. MS analysis

To identify and confirm the intra-disulfide bonds of the purified recombinant crotamine, two kinds of samples were prepared under reducing and non-reducing conditions with/without dithiothreitol (DTT). Under reducing conditions, 2 μg of recombinant crotamine was treated with 10 mM DTT for 15 min at RT, and the solution was evaporated using the vacuum drying. The precipitate was resuspended in iodoacetamide (IAA) buffer (0.5 M Tris–HCl, 5% glycerol [v/v], 100 mM NaCl, 2% SDS, 1 mM EDTA; pH 8.0), which included 50 mM IAA, and left at RT for 1 h. Samples were then loaded onto 10% tricine gels. The band of the recombinant crotamine was cut off, and trypsin. MALDI-TOF was conducted using Voyager-DE STR (Applied Biosystems, South San Francisco, CA). Data were interpreted using Data Explorer software (Applied Biosystems). The obtained peptide masses were then confirmed using the computed peptide mass values used in the PeptideMass program (http://web.expasy.org/peptide_mass).

2.5. Electrophoresis and silver staining

The proteins from all purification steps were confirmed using SDS-PAGE. Proteins were denatured in 5× sample buffer (200 mM Tris–HCl, 48% glycerol, 16% SDS, 0.04% Coomassie blue G-250, 0.4 M DTT; pH 6.8) for 15 min at 150°C. Proteins were stained with Coomassie brilliant blue R-250 staining (AMRESCO, Solon, OH). Crotamine purity was evaluated using the Silver Stain Plus kit (Bio-Rad Laboratories, Hercules, CA). The reaction was completed by adding 5% acetic acid [v/v] for 15 min when the bands were obviously visible. Gels were washed with abundant water and maintained in solution with 5% glycerol [v/v] and 0.02% sodium azide (w/v).

2.6. Endotoxin assay

The amount of lipopolysaccharide (LPS) in the final purified crotamine was calculated using the quantitative Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) test (Lonza, Basel, Switzerland). 50 μL of 1 μg/mL purified crotamine or an endotoxin standard (0.1, 0.25, 0.5, or 1.0 EU/mL) was first placed into prewarmed (37°C) microplate wells. Then, 50 μL LAL reagent was deposited into each well. After 10 min of incubation at 37°C, 100 μL chromogenic substrate solution was allocated to each well and allowed to incubate at 37°C for 6 min. Subsequently, 100 μL of reaction termination reagent (25% acetic acid [v/v]) was finally allocated, and the absorbance of each well was measured at 405 nm using a spectrophotometer. The endotoxin units were determined based on standard curves obtained from standards.

2.7. hKv1.3 and hKv1.5 expression in oocytes

Human hKv1.3 (hKv1.3, GenBank: BC0350501) and hKv1.5 (hKv1.5, GenBank: BC0996653) were obtained from PlasmID (Harvard Medical School, Boston, MA) (clone IDs: HsCD0043746 and HsCD0377041, respectively). The genes were transferred to the pEF5/FRT/VS-DEST vector (Invitrogen). cRNA was synthesized using mMESSAGE mMACHINE T7 kits (Ambion, Austin, TX) and stored in nuclease-free water at −80°C. Stage V–VI oocytes were manually isolated from the thecal and follicular layers of female Xenopus laevis (Nasco, Modesto, CA), and then cRNAs were injected into the oocytes. Modified Barth’s Solution contained the following (in mM): 88 NaCl, 1 KCl, 0.4 CaCl2, 0.33 Ca(NO3)2, 1 MgSO4, 2.4 NaHCO3, 10 HEPES (pH 7.4), and 50 μg/mL gentamicin sulfate for storing the oocytes. Currents were measured 3–6 days after injection. These experiments were conducted according to the Research Guidelines of Kangwon National University IACUC (Institutional Animal Care and Use Committee).

2.8. Solution and voltage-clamp recordings of the oocytes

Normal Ringer’s solution contained the following (in mM): 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.4). This solution was applied to the oocytes by continuous chamber perfusion while recording. Solution exchanges were completed within 3 min, and the current was recorded 15 min after solution exchange. The current was measured at room temperature (20–23°C) using a 2-microelectrode voltage clamp amplifier (Warner Instruments, Hamden, CT). Electrodes were filled with 3 M KCl and had a resistance of 2–4 MΩ and 1–2 MΩ for voltage-recording electrodes and current-passing electrodes, respectively. Stimulation and data acquisition were controlled using an AD–DA converter (Digital 1200; Axon Instruments [Warner Instruments, Hamden, CT]) and pCLAMP software (v5.1; Axon Instruments). The dose-response inhibition of the hKv1.3 current by crotamine was determined using the following non-linear equation:

$$ Re = 1 - \left( \frac{IC50}{conc} \right)^{H} $$

where Re is the hKv1.3 channel current; IC50 is the concentration that inhibits the current by 50%; conc is the concentration of the protein; and H is the Hill coefficient of inhibition.

2.9. Statistical analysis

All data are shown as the mean ± SEM (n ≥ 3). The student t test, 2-tailed paired test, and analysis of variance (ANOVA) were used to analyze the data. Statistical significance is defined as p < 0.05.
3. Results

3.1. Plasmid construction and solubility testing

In order to identify the protein tags that could increase the solubility and expression level of crotamine in E. coli cytoplasm, we constructed seven different expression plasmids that over-expressed the recombinant fused crotamine using His6, Trx, GST, PDIb′, MBP, PDI, and NusA (the Gateway system [Invitrogen, Carlsbad, CA] was used to construct these combinations) (Fig. 1). AttP1 and attP2 in the pDONR207 vector were recombined with attB1 and attB2 in the pUC57-crotamine vector in order to exchange the ccdB and Cm(R) genes in the pDONR207 vector for the TEVrs-crotamine gene in the pUC57-crotamine vector. As a result, pENTR-crotamine constructed attL1-TEVrs-crotamine-attL2 was generated. AttL1 and attL2 were then recombined with attR1 and attR2 in pDEST-HGWA, pDEST-HGGWA, pDEST-HMGA, pDEST-HNGWA, pDEST-HXGWA, pDEST-PDI, and pDEST-PDIb′. In this step, ccdB and Cm(R) in the each destination vector were also exchanged for TEVrs-crotamine in the pENTR-crotamine gene. Finally, seven different expression plasmids under the control of the T7 promoter were generated.

The seven plasmids were transformed into E. coli BL21(DE3) cells and induced by IPTG at two different temperatures: 37°C and 20°C. Then, the proteins in the whole-cell extracts, soluble fraction, and insoluble fraction were analyzed using SDS-PAGE (Fig. 2), and the expression and solubility levels were quantified (Table 1). Temperature had minor effects on expression or solubility. The expression and solubility of crotamine were as low as 10% and 38%, respectively, at 20°C when using the His6 tag (the smallest tag). Three tag proteins—PDIb′, MBP, and NusA—increased these expression level to >50% at 20°C, and the MBP tag produced the largest increase. Solubility also increased to >70% using GST, PDIb′, MBP, and NusA. The MBP-crotamine construct was chosen for further purification and functional analysis because of its high expression and solubility level, its smaller size in comparison with NusA, and the availability of the MBP-affinity column (Table 2).

3.2. Purification

Fig. 3A depicts the purification procedures. The whole-cell lysates of E. coli expressing MBP-tagged crotamine were applied to the Immobilized Metal Affinity Chromatography (IMAC) column that was charged with nickel to bind to the His6 tag at the N-terminus of each hybrid construct. The bound proteins were eluted with 500 mM imidazole, and the eluent contained mostly MBP-crotamine (Fig. 3B, lane 4). To determine the optimal digestion conditions for the TEV protease, several temperatures, times, and ratio conditions were tested (Fig. S2). The optimal digestion conditions were found to be 25°C for 24 h using a 1:10 ratio of TEV protease: recombinant crotamine. Under these conditions, there were high levels of precipitation, mainly consisting of MBP and TEV protease (Fig. S2). To remove the MBP and TEV protease more efficiently, the sample was shaken at 75 rpm during enzymatic digestion (Fig. 3B, lane 5). After TEV protease digestion, the solution was applied to the HiTrap SP HP cation exchange column. During this step, the buffer pH was lowered from 8.0 to 6.0 to facilitate crotamine binding. Lowering the pH partially precipitated the MBP tag and other endogenous proteins, whereas crotamine remained soluble. The cleaved crotamine demonstrated a particularly high affinity for the cation exchange column, with the protein starting to elute at 500 mM NaCl and was completely eluted using 1 M NaCl. 50 CVs of washing buffer were used to remove endotoxin with a residual level estimated to be 0.15 EU/μg. After the final step of the purification procedure, MBP chromatography, a yield of 0.9 mg pure crotamine (Fig. 3B, lane 6) per liter of bacterial culture was determined (Table 1). Silver staining under reducing conditions indicated that the final product contained a single band corresponding to 5 kDa crotamine (Fig. 3C) with a purity of 99% after the final step.

The purified crotamine exhibited strong thermal stability. After heating to 55°C for 20 min, the crotamine remained in the soluble fraction (data not shown).

3.3. MALDI-TOF MS analysis

To confirm the identity of the purified protein and characterize its disulfide bond status and connectivity, proteins were analyzed...
using MALDI-TOF MS. Proteins were treated with DTT to cleave the disulfide bonds then treated with IAA to prevent the reformation of disulfide bonds. Under these reduced conditions, most of the critical fragments containing alkylated cysteine and free cysteine were detected (T2, T7, T2+IAA, T7+IAA, T4+IAA, and T6+IAA), thereby confirming that the purified protein was indeed crotamine (Fig. 4B). The T10 fragment (composed of two adjacent cysteines) was not detected because its mass is outside the detection range. Under non-reducing conditions, all alkylated fragments and fragments containing free cysteine disappeared (Fig. 4C) and two peaks representing two correct disulfide bonds between T2 and T10 (866 Da) and between T4 and T7 (1268 Da) were instead detected. The presence of the T6+IAA peak under reducing conditions, and the absence of this peak under non-reducing conditions, suggests the formation of a third disulfide bond between T6 and T10.

3.4. Functional assay

To test the biological activity of the purified crotamine, its effects on hKv1.3 and hKv1.5 currents were measured using the Xenopus oocyte expression system. hKv1.3 channel currents were elicited using a series of 1 s voltage pulses from −90 mV to +60 mV, which were increased by 10 mV every 10 s from a holding potential of −80 mV. Fig. 5A indicates the voltage-clamp recordings obtained in Xenopus oocytes and representative current traces obtained at +60 mV both under control conditions and after exposure to crotamine, which illustrates that crotamine decreased the hKv1.3 channel currents (Fig. 5A). Fig. 5B shows that crotamine inhibited the hKv1.3 channel peak currents by 40.31 ± 4.72% at 3 nM (Fig. 5B). To determine the concentration-dependent inhibition of crotamine on hKv1.3 channel currents, hKv1.3 channel currents were recorded under control conditions and in the presence of 30 pM, 300 pM, 3 nM, 30 nM, 300 nM, or 3 mM crotamine. The IC50 value of crotamine on the hKv1.3 channel was 67.2 ± 44.7 nM and the Hill coefficient was 0.36 ± 0.03. However, Kv1.5 channel currents were not inhibited by crotamine (Fig. S1). A series of 2 s voltage pulses from −80 mV to +50 mV that were increased by 10 mV every 10 s from the holding potential of −100 mV elicited the hKv1.5 channel current (Fig. S1A). Treatment with 3 nM crotamine did not change the current traces, which were evoked by +60 mV pulses (Fig. S1B). Crotamine also did not change the hKv1.5 channel currents throughout the entire test from −80 mV to +50 mV. Our present results indicate that crotamine selectively blocks the hKv1.3 channel, but not the hKv1.5 channel. Interestingly, the fusion protein, MBP-crotamine, also inhibited the hKv1.3 currents (Fig. 6). The IC50 of MBP-crotamine was measured at 477.6 ± 147.5 nM and the Hill coefficient at 0.81 ± 0.24.

4. Discussion

Crotamine is a promising anticancer, antibacterial, antifungal protein (Oguiura et al., 2011; Nascimento et al., 2012) and potential nanocarrier (Hayashi et al., 2012; Chen et al., 2012). However, its efficient production for biomedical applications is hampered by its cytotoxicity towards eukaryotic cells and its low expression and insolubility in prokaryotic cells. The aim of this study was to improve the soluble expression of crotamine in E. coli cytoplasm using tagged proteins fused to its N-terminus and, thereby, efficiently produce functional recombinant crotamine.

At both high and low temperatures, MBP was found to be the most efficient tag for the soluble overexpression of crotamine in E. coli cytoplasm. Generally, MBP functions as a molecular chaperone and assists in appropriately folding target proteins (Bach et al., 2001). In addition, MBP may also act as a holdase that preserves the fused proteins in an aggregation–resistant state, which thereby promotes proper folding (Raran-Kurussi and Waugh, 2012). It has been suggested that MBP could interact with hydrophobic residues to expose misfolded proteins and protect them from aggregation (Kapust and Waugh, 1999). Moreover, MBP is thought to be resistant to proteolysis and, thus, prevent the degradation of fused proteins.
PDI, an enzyme that catalyzes disulfide formation and isomerization, is composed of 4 domains: a, b, b', and a'. The a and a' domains are responsible for both chaperone and redox-active catalytic activities, while the b and b' domains only demonstrate chaperone properties (Appenzeller-Herzog and Ellgaard, 2008). The PDB\(b'\)a' construct represents the full activity of PDI, and its small size has been successfully applied to produce fibroblast growth factor 2 (Song et al., 2013). In our current study, PDB\(b'\)a' was found to be the most efficient partner for the solubilization of target proteins at low temperatures (Table 1). However, we chose the MBP tag to produce crotamine because of its higher expression level and availability for purification.

A low expression temperature successfully increases the solubility of many of the proteins expressed in E. coli (Shirano and Shibata, 1990; Ferrer et al., 2004; Vera et al., 2007; Kim et al., 2012; Yi et al., 2009; Imsoonthornruksa et al., 2011). However, the solubility of crotamine was not greatly affected by expression temperature. Instead, crotamine demonstrated similar, and sometimes lower, solubility at low expression temperatures (Table 1). Crotamine contains three intramolecular disulfide bonds between Cys4-Cys36, Cys11-Cys30, and Cys18-Cys37. The MALDI-TOF data confirmed two of the expected disulfide bonds and suggested the presence of a third disulfide bond in purified crotamine (Fig. 4). This is consistent with the purified crotamine exhibited strong thermal stability, even though crotamine had previously been demonstrated to have high intrinsic flexibility by NMR (Fadel et al., 2005).

Crotamine inhibited the hKv1.3 channel (but not the hKv1.5 channel), which is consistent with a previous study that reported that crotamine selectively inhibits hKv1.1, hKv1.2, and hKv1.3 channels, but not hKv1.4 or hKv1.5 channels (Peigneur et al., 2012). This functionality suggests that the purified recombinant protein is correctly folded. Crotamine purified from crude venom exhibits an IC\(_{50}\) value for hKv channel inhibition of approximately 300 nM (Peigneur et al., 2012). The crotamine purified here from E. coli inhibited hKv1.3 characterized by an IC\(_{50}\) value of 67 nM, which is approximately 4 times more active in comparison with crotamine

### Table 1

<table>
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<th>Fusion (kDa)</th>
<th>Expression level (%)</th>
<th>Solubility (%)</th>
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<td>53</td>
<td>82</td>
</tr>
</tbody>
</table>

### Table 2

<table>
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<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Concentration (mg/mL)</th>
<th>Total protein (mg)</th>
<th>Purity (%)</th>
<th>Crotamine (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial culture</td>
<td>1000</td>
<td>–</td>
<td>32,000</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Supernatant</td>
<td>50</td>
<td>4.5</td>
<td>225</td>
<td>23</td>
<td>5.3</td>
<td>100</td>
</tr>
<tr>
<td>1st IMAC column</td>
<td>45</td>
<td>1.4</td>
<td>63</td>
<td>76.7</td>
<td>4.9</td>
<td>92.45</td>
</tr>
<tr>
<td>2nd SP column</td>
<td>16</td>
<td>0.2</td>
<td>3.2</td>
<td>89.4</td>
<td>2.8</td>
<td>53.9</td>
</tr>
<tr>
<td>3rd MBP column</td>
<td>4.5</td>
<td>0.2</td>
<td>0.9</td>
<td>99.5</td>
<td>0.9</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Crt: Crotamine.
IMAC: immobilized metal affinity chromatography.
SP: cation-exchange.
MBP: maltose-binding protein.

![Fig. 3](image-url)
purified from snake venom. The high IC50 value of crotamine obtained from snake venom may elevate due to partial denaturation by the harsh conditions during the purification from crude venom or due to contamination.

The anticancer effects of crotamine are caused by endocytosis, lysosome disruption, and the activation of intracellular proteases (Hayashi et al., 2008), but it also increases intracellular calcium and decreases the mitochondrial membrane potential (Nascimento et al., 2016).

Fig. 4. Mass analysis. MALDI-TOF MS analysis of the purified crotamine obtained from the MBP-TEVrs-crotamine construct. (A) Tryptic peptide map of crotamine (43 amino acids). (B) MALDI-TOF MS analysis of the purified crotamine under reducing conditions. (C) MALDI-TOF MS analysis of the purified crotamine under oxidizing conditions.

Fig. 5. Effect of crotamine on hKv1.3 channel currents in Xenopus oocytes. (A) Superimposed current traces elicited using a series of 1 s voltage pulses from −90 mV to +60 mV (voltage was increased 10 mV every 10 s from the holding potential of −80 mV). (B) Current–voltage (I–V) relationship curves in the absence or presence of 3 nM crotamine. (C) Dose-dependent curve of the inhibition of the peak current by crotamine. The solid line was fitted to the data points using the Hill equation. Data are shown as the mean ± SEM (n = 10). Normal Ringer’s solution without crotamine was used as the control.
et al., 2012). However, cytotoxicity might also be related to the inhibition of several hKv channels, as Kv channels are well expressed and play a role in the cell proliferation of many types of cancer (e.g., breast cancer, prostate cancer, melanoma) (Peigneur et al., 2012; Wang et al., 2004). In addition, some autoimmune diseases are ameliorated by the inhibition of hKv1.3 in T-lymphocytes (Beeton et al., 2001), thereby widening the application potential of crotamine.

Surprisingly, noncleaved MBP-crotamine also was capable of inhibiting the hKv1.3 channel current (Fig. 6). Although the IC\textsubscript{50} value of MBP-crotamine was found to be higher than that of crotamine, it was still several hundred nM. Despite the size of the MBP tag (40 kDa) being much larger than that of crotamine (5 kDa), the fact that the noncleaved form can inhibit the hKv1.3 channel suggests that the N-terminus of crotamine does not play a role in binding to the hKv1.3 channel. Because crotamine is cytotoxic to not only cancer cells but also to stem cells in the bone marrow, spleen, liver and lungs, it may be useful to attach to a signal such as the RGD sequence (a tumor homing peptide) in order to target cancer cells (but not stem cells), at this N-terminus.

Endotoxins produced from the cell membranes of Gram-negative bacteria (mainly LPS) induce side effects such as endotoxin shock and tissue injury. Hence, proteins purified from Gram-negative bacteria must be <1.0 EU/µg LPS for use in biomedical applications. Because LPS is negatively charged, anion exchange chromatography or Triton X-114 is often used to decrease LPS levels (Magalhaes et al., 2007). Since crotamine is highly basic (pI = 10.3), it bound very tightly to the cation-exchange column. With sufficient washing, the purified crotamine demonstrated a very low LPS level without the use of anion-exchange chromatography or Triton X-114.

5. Conclusion

Using prokaryotic soluble overexpression and simple fusion techniques, we were able to efficiently produce bioactive crotamine. The recombinant crotamine demonstrated the correct disulfide bonding connectivity and high levels of activity. Altering the N-terminal region of crotamine did not abolish its bioactivity, suggesting a convenient site for future modification to enhance cell targeting.

Ethical statement

Handling Xenopus laevis was conducted according to the Research Guidelines of Kangwon National University IACUC (Institutional Animal Care and Use Committee).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.10.017

Transparency document

Transparency document related to this article can be found at http://dx.doi.org/10.1016/j.toxicon.2014.10.017

References


