Preparation of Specific Polyclonal Antibody Against the Recombinant Mutacin Produced by sfGFP Fusion Protein Technology

Lamis Al-Homsi1, Souad Al-Okla2 and Abdul Q. Abbady3,*

1Department of Biotechnology, Faculty of Agriculture, Damascus University, Syria
2Department of Animal Biology, Faculty of Sciences, Damascus University, Syria
3Department of Molecular Biology and Biotechnology, Atomic Energy Commission of Syria (AECS), Damascus, Syria

Abstract: Mutacin I, a bacteriocin produced by streptococcus mutans, displays an antimicrobial activity against many gram positive and some gram negative bacteria. Because of its medical importance, production of this short peptide in large scale for future applications is a significant challenge. This work described the improvement of a novel system to produce the recombinant mutacin using fusion protein technology.

The short peptide was expressed directly as a fusion protein with a superfolder form of the green florescent protein (sfGFP), resulting in a high yield expression of soluble sfGFP–mutacin fusion protein (30 kDa) in the cytoplasm of E. coli. Mutacin was released from the fusion by enzymatic cleavage at the tobacco etch virus (TEV) protease recognition site and separated from the carrier sfGFP by nickel affinity and gel filtration chromatography. An additional advantage of this fusion system was tested in the generation of mutacin-specific polyclonal antibodies. Specific anti-mutacin IgGs were affinity purified, and were able to recognize the mutacin-sfGFP fusion protein or the cleaved forms of mutacin.

Even though it was efficiently produced (25 mg/L) by this method, pure mutacin was devoid of antibiotic activity. Fourier transform infrared spectroscopy (FTIR) analysis revealed the absence of thioether bonds in the purified mutacin, which are critical for final structure and function of this antibiotic. Determining whether the activity of pure mutacin could be recovered by the reformation of such structures by chemical reaction needs more investigations. The development of this system will provide large quantities of mutacin for future studies and applications as broad spectrum antibacterial peptide.

Keywords: Bacteriocin, fusion protein, mutacin, sfGFP, TEV,

1. INTRODUCTION

Bacteriocins are proteinaceous toxins produced by streptococcus mutans, which is an indigenous bacterial inhabitant of the human oral cavity [1]. The diversity of bacteriocin from S. mutans has been recently highlighted, as they represent 40% of the listed streptococcal antimicrobial peptides [2]. Historically, the importance of mutacins was based on the role they play in the colonization of the oral cavity and their usefulness as possible anti-caries agents [3]. New applications are developing for mutacins as potential food preservatives [4] and as new effective antibiotics [5]. Mutacin I is produced by S. mutans CH43 and UA140 [6, 7]. The mature peptide is composed of 24 amino acids with a molecular mass of 2364 Da. Mutacin I belongs to the lantibiotic type IA and epidermin group. The biosynthetic operon which is located in the bacterial chromosome was reported to encompass 14 ORFs [7]. Mutacin I could be used in different conditions due to its pH, thermostability and hydrophobic nature, which can be advantageous for coating the surface of catheters and preventing adhesion of bacteria to the surface [8].

Many lantibiotics have not been extensively tested for their potential usefulness in treating infectious diseases even though they are known to be both potent and have a broad spectrum of activity, notably against gram positive species [9]. The principle reason for this is the difficulty in obtaining these molecules in high purity and in sufficient, cost effective amounts to enable their testing and commercialization. For production of these peptides to be valuable in industry, they must be produced in an environmentally safe and cost effective manner [10]. Recombinant DNA technology provides means for an economical protein production, thus many antimicrobial peptides have been successfully produced through recombinant expression in various heterologous hosts [10]. E. coli and yeast are the two major systems used to produce recombinant antimicrobial peptides, which together account for over 95% of all reported cases [10, 11]. Antimicrobial peptides produced in E. coli are often expressed as fusion with carrier proteins, a strategy necessary to mask these peptides lethal effect
Recombinant Mutacin Produced by sfGFP Fusion Protein Technology

The development of efficient detection and purification procedures for bacteriocins could greatly facilitate their use as food preservatives or alternative antibiotics [32]. Antibodies are an important tool used by many investigators in the research and have led to many advances. In biochemical and biological research, Antibodies are routinely used as ligands for the preparation of immunoaffinity columns [33] and as coating or labeling reagents for the detection of bioactive molecules in a variety of assays, such as the enzyme linked immunosorbent assay (ELISA) and immunoblotting [34]. The generation of antibodies against bacteriocins may provide sensitive and specific methods for the identification and detection of mutacin I producing strains and for the quantification of bacteriocins cleaving in different substrates by the use of immunochemical assays [35].

We present here a bacterial expression system that uses sfGFP as a fusion partner with the short peptide that was released by enzymatic cleavage using the TEV protease recognition site and purified by means of the internal 6×His tag using nickel affinity chromatography. An additional advantage of this fusion system was in the generation of mutacin–specific polyclonal antibodies. Antibodies were produced after rabbit immunization by the pure fusion protein in order to increase the immune response against the displayed short peptide.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Growth Conditions and Plasmid

E. coli strains TOP10 (Invitrogen) and BL21 (DE3) Rosetta (Novagen) were used in cloning and protein expression after transformation with electroporation with the plasmid constructs pT7–His–sfGFP–mutA and pT7–His (kindly provided by Prof. Yu Ding, Fudan University, China). For general maintenance and protein expression, E. coli were grown in Luria Broth (LB; 1% Tryptone, 0.5% yeast extract, 171 mM NaCl) (Bio Basic INC) with the required antibiotic at 100 µg/ml (Ampicillin; Applichem) at 37°C.

2.2. Construction of the Plasmid pT7–His–sfGFP–mutA

The coding sequence of sfGFP in the plasmid pT7–sfGFP–mutA [36] was replaced by the DNA fragment His–sfGFP from the plasmid pRSET–sfGFP [37]. This was achieved by subcloning using a double digestion with Ndel/BamHI (Fermentas) of both plasmids, and the long linearized DNA fragment from the first plasmid was ligated with the small insert from the second using T4 DNA ligase (Fermentas). Freshly prepared electro–competent E. coli TOP10 cells were transformed with the new plasmid construct pT7–His–sfGFP–mutA by electroporation. Colony PCR screening for positive His–sfGFP clones was performed using specific primers (T–F/T–R), and the successful cloning was confirmed by digestion with restriction enzymes and by sequencing.

2.3. Expression and Purification of sfGFP–mutacin Fusion Protein

The confirmed plasmid construct was used to transform by electroporation E. coli BL21 (DE3) Rosetta cells. Protein expression of sfGFP–mutacin was performed in 250 ml shake flasks by growing the bacteria in LB medium until an optical density of 0.5 to 0.7 was reached, and then expression was induced with 0.5 mM Isopropyl β–D–thiogalactoside (IPTG; Promega) for 16 h at 19°C. After pelleting the cells, the pellet was resuspended in PBS 1×, then lysed by sonication (Lab Sonic) on ice and the lysate was cleared by 20 min centrifugation at 8,000 rpm and 4°C. Using fast protein liquid chromatography (FPLC) with AKTAprime plus system (GE Healthcare), recombinant sfGFP–mutacin was purified from this cytoplasmic extract using a 5 ml column of nickel charged Nitrilotriacetic acid (NTA) superflow Sepharose (Qiagen). After washing, the bound proteins were eluted from the column with a 500 mM imidazole buffer. The eluted fraction was concentrated on
Vivaspin concentrators with a molecular mass cutoff of 10kDa (Vivasience). Protein samples were separated by SDS-PAGE using a Bio-Rad mini-Protean II system following the manufacturer’s instructions. Gels were prepared using stacking gel 5% and running gel 12%. After electrophoresis, the gel was stained in coomassie blue for 2h then destained.

2.4. Cleavage of sfGFP–mutacin Fusion Protein by TEV Protease

Recombinant sfGFP–TEV protease was expressed from pT7–His plasmid and purified by nickel affinity chromatography using the standard method [23]. The purified protease, at a protein concentration of ~1.24 mg/ml, was used in the cleaving reaction of the substrate sfGFP–mutacin (1 mg), which contained a TEV cleavage site at the N-terminal of mutacin. Protease digestion was performed in a mass ratio of substrate to enzyme 100:4 (w:w) in a reaction buffer (75 mM NaCl, 5.0 mM EDTA, 25 mM Tris pH8, 10% glycerol and 100 mM DTT) at 30°C for 16h. The extent of cleavage of the samples was determined or compared by SDS–PAGE analysis. The cleavage product was purified by nickel affinity chromatography to eliminate His tagged proteins (sfGFP and TEV protease).

2.5. Purification of Short Peptide by Gel Filtration

The short peptide resulted from TEV protease digestion was purified using Superdex 200 10/300 GL column (GE Healthcare). After washing, the bound proteins were eluted from the column with sodium phosphate buffer 1×(30.5 mM Na₂HPO₄, 19.5 mM NaH₂PO₄, 0.15 M NaCl, pH7). The purity of the short peptide was evaluated in SDS–PAGE stained in cold silver staining buffer (0.2% AgNO₃ and 0.03% formaldehyde) for 20min, then destaining was done with the stop solution (50% methanol, 12% glacial acetic acid).

2.6. Production of Polyclonal Antibody Against the Recombinant sfGFP–mutacin Protein

For the first immunization, purified sfGFP–mutacin protein (500μg) in PBS (1ml) was mixed with an equal volume of Freund’s complete adjuvant (Bio Basic Inc.) to form a stable emulsion. One white rabbit was injected subcutaneously at 2 to 4 different sites. Three booster injections were given with 250μg recombinant protein mixed with incomplete Freund’s adjuvant at 15 day intervals. To evaluate the immune response, blood samples (0.5–1ml) were collected before each injection and bleeding (30ml) was done 10 days after the last boost. Polyclonal antibodies (IgGs) were purified from rabbit serum by affinity chromatography on a 5ml HiTrap Protein A column (GE Healthcare) according to the manufacturer's instructions. Binding was performed in 0.02 M sodium phosphate, pH 7.0, and elution was in 0.1 M citric acid, pH 3.0. Eluted IgGs were collected and immediately neutralized to physiological pH by adding 1M Tris–HCl buffer, pH 9 and then concentrated to 1 mg/ml using 30kDa–molecular mass cutoff concentrators.

2.7. Affinity Purification of Anti–mutacin Specific IgG

Two N–hydroxysuccinimide (NHS) activated Sepharose Columns (GE Healthcare) were used for affinity purification of mutacin–specific IgGs from the rabbit total antibodies. Concentrated sfGFP and sfGFP–mutacin (20 mg) in coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH8.3) were applied onto two NHS–activated columns, respectively, and allowed to bind for 30 min at 20°C. Any excess of active NHS groups that have not been coupled were deactivated by sequential washing with (0.5 M ethanolamine, 0.5 M NaCl, pH8.3) and (0.1 M sodium acetate, 0.5 M NaCl, pH4). Protein A purified IgGs from rabbit serum were first passed through the sfGFP–column. Purified sfGFP–specific IgG sample was concentrated and stored at -20°C. Unbound IgG, which was empty of sfGFP–specific IgGs, was washed away with the washing buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH7), and then it was re-purified by passing it through the sfGFP–mutacin column in order to obtain pure mutacin–specific IgGs which were eluted with 3–5 column volumes of 0.1 M citric acid buffer and then neutralized with 1 M Tris–HCl buffer, pH9.0. Purified mutacin–specific IgG sample was buffer exchanged with 0.05 M sodium phosphate buffer pH7, and then concentrated down to 1 mg/ml.

2.8. Mutacin Detection by ELISA Assay

An indirect ELISA format was employed for the analysis of sera and purified IgG. The basic protocol was as follows: sfGFP, sfGFP–mutacin, and mutacin antigens were diluted in carbonate coating buffer and 100μl were added to the wells of an ELISA microplate at 4°C overnight. After washing (phosphate-buffered saline containing 0.05% Tween 20) the plate was washed 3 times to remove unbound antigen. 150μl of blocking buffer (1% bovine serum albumin (BSA), 3% skimmed milk in 1 PBS) were applied to all the wells for one hour at 37°C. After the removal of blocking buffer, 100μl of rabbit sera or IgGs diluted in 1% blocking buffer were added per well for 1h at RT. After 3 washes, goat anti rabbit IgG conjugated to horse-radish peroxidase (HRP) was diluted (1:2000) in 1% blocking buffer, added (100μl per well), and allowed to bind to captured rabbit IgG for a further hour at RT. After an additional 5 washes, bound conjugate was detected with 50μl of 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma) substrate, the reaction was stopped after 8min with the addition of 50μl 1 M H₂SO₄. The spectroscopic absorbance of each well was measured in an automated plate reader at a wavelength of 450 nm.

2.9. Biological Activity of Recombinant Mutacin

Antibiotic potency of recombinant mutacin was tested on three different strains; *Streptococcus thermophilus*, *Bacillus thuringiensis*, *Lactobacillus bulgaricus*, by turbidimetric assay. Bacterial strains were cultured in LB medium until OD₆₀₀ reached 1, each strain was dilated to 0.1 in LB medium and cultured in 300μl/well in a 48 well microplate. Thirty μl of antibiotic stocks (1 mg/ml), mutacin or ampicillin, were added to the wells of each type of bacteria to obtain a final concentration of 100μg/ml. The culture microplate was incubated at 37°C for 3h with gentle shaking. The spectroscopic absorbance of each well was
measured in an automated plate reader at a wavelength of 600 nm.

2.10. FTIR Analysis

The IR spectrum of purified mutacin was determined using FTIR spectrometer (Thermo–Nicolet 6700) instrument at the Department of Chemistry in the AECS. Lyophilized sample (0.5 mg) was mixed with 200 mg of KBr in a clean glass pestle and mortar. The contents were dispersed in a KBr disk and then pressed into 1 mm pellets. The KBr disk was placed in a special sample holder in the IR beam of the spectrometer. Sample was analyzed in the wavenumber range of 400 and 4000 cm⁻¹ with a resolution 4 cm⁻¹ using 64 scans.

3. RESULTS

3.1. Construction of the Plasmid pT7–His–sfGFP–mutacin

In our previous work, the coding sequence of the mature mutacin (mutA) was constructed by Splicing by Overlap Extension (SOE) PCR using long overlapping primers, which cover the whole gene, and the DNA adapter was cloned in the plasmid pT7 [36]. Mutacin was produced as a part of a high solubility and productivity fusion protein (30 kDa), which was detectable by the colorimetric properties of sfGFP (Fig. 1A). However, the expressed fusion protein was mostly lost in the flow-through during nickel chromatography purification, resulting in a low yield purification. We surmised that the purification problem could be related to the inaccessibility of the internal 7xHis tag, that was added between the coding sequences of sfGFP and mutacin. To overcome the purification problem in the previous study, an extra 6xHis tag was added to the N-terminal of sfGFP–mutacin fusion protein, since it is impossible to eliminate this tag from mutacin if it was added to the C-terminal. To achieve this goal, the sfGFP gene in pT7–sfGFP–mutA plasmid construct was replaced with the His–sfGFP adapter from pRSET–sfGFP plasmid [37], by NdeI/BamHI double digestion and sub-cloning (Fig. 1A).

The new plasmid construct pT7–His–sfGFP–mutA was transformed into E. coli TOP10, then confirmed by colony PCR screening using T7F/T7R primers (Fig. 1B). PCR amplification of pT7–His–mutA containing colony (lane 1) resulted in a band of 1009 bp while in case of the new plasmid construct pT7–His–sfGFP–mutA (lane 2) a bigger band of 1051 bp was amplified from the colony. This was because of the presence of an additional N-terminal 6xHis tag in the new plasmid construct. To further confirm the difference between the two plasmid constructs, PCR products, from previous reaction, were digested with NheI.

![Fig. (1). Construction of the plasmid pT7–His–sfGFP–mutA](image-url)

(A) Schematic illustration of the plasmid pT7–His–sfGFP–mutA, with an enlarged part (up), which represents the coding region for the fusion protein sfGFP–mutacin. The most important elements of the plasmid are indicated. These include T7 promoter, His tags, TEV cutting site and the restriction sites used for cloning. Locations of T7 primers (F and R) used for PCR amplification and for sequencing are shown on the plasmid. (B) Agarose gel electrophoresis of PCR products using T7F/T7R primers and the plasmids pT7–His–mutA (lane 1) or pT7–His–sfGFP–mutA (lane 2) as DNA template. (C) DNA amplicons from the previous PCR were digested with NheI and separated by gel electrophoresis. DNA molecular weight marker was loaded in the first lane (M).
(Fig. 1C). Only amplified DNA fragment from the new construct contains a recognition site for NheI, resulting in the cleavage of this fragment into large and small bands of 926 bp and 125 bp, respectively (lane 2). To express the 6×His tagged sfGFP–mutacin, the new plasmid construct was transformed into the strain BL21 (DE3) and protein expression and purification was conducted by FPLC (Fig. 2). Obviously, the addition of N–terminal 6×His tag did not affect the solubility or the high level expression of the fusion protein, but resulted in its optimal purification (lane 5), as no further traces were remained in the flow–through sample (lane 4).

3.2. Purification of Mutacin After the Cleavage of sfGFP–mutacin Fusion Protein

Pure sfGFP–mutacin as well as sfGFP–TEV protease were prepared and their concentrations were determined by

![Fig. (2). Expression and purification of His–sfGFP–mutacin fusion protein](image)

(A) Diagram of purification procedure using nickel–charged column (5 ml). Continuous line represented the absorbance of the eluate, different purification steps are shown below and peaks of the flow–through sample and of purified sfGFP–mutacin are indicated. Dashed line represented conductivity of the eluate. (B) SDS–PAGE (acrylamide 12%) of protein samples from the different steps of expression and purification; total cytoplasmic extract before IPTG induction (lane 1), after 16 h of induction (lane 2), after sonication (lane 3), flow–through sample from nickel–charged column (lane 4) and purified sfGFP–mutacin fusion protein (lane 5). Protein molecular weight marker (in kDa) was loaded in the first lane (M), and side arrow referred to the expected location of the recombinant protein in the gel.

![Fig. (3). Cleavage, detection and purification of mutacin](image)

(A) Silver staining of SDS–PAGE (15 %) of protein samples from the cleavage mixture before nickel affinity purification (lane 1), pure fraction containing His tagged proteins; His–sfGFP and sfGFP–TEV (lane 2) and the flow–through sample containing pure mutacin (lane 3). Gel filtration of injected nickel–purified flow–through sample from the cleavage reaction of sfGFP–mutacin in the presence of active (B) or of heat–inactivated form (C) of sfGFP–TEV. Gel filtration was achieved by injecting protein sample (Inject) into a Superdex 200 column (10/30) at a flow rate of 1 ml/min, and the fraction peak of pure mutacin is shown.
the Bradford method and adjusted to 1 mg/ml. The mass ratio of the substrate His-sfGFP–mutacin; which contains the protease recognition site, to the sfGFP–TEV protease enzyme was 100:4, so that 25 mg of the substrate were incubated with 1 mg of the enzyme in the appropriate buffer for 16 h at 30 °C (Fig. 3). Protein samples from the cleavage reaction were prepared and then loaded onto 15 % SDS–PAGE gel for electrophoresis, which was finally visualized by staining with silver nitrate (Fig. 3A). In this result, liberated mutacin (~2kDa) could be observed after the cleavage reaction (Lane 1), then nickel affinity purification was applied to eliminate his tagged moieties from the reaction mixture (sfGFP–TEV and His–sfGFP) (Lane 2) and relatively pure mutacin was finally recovered in the flow–through fraction (Lane 3).

For more confirmation of the cleavage and to get highly pure mutacin, protein samples, from the flow–through fraction, before and after fusion protein cleavage and nickel affinity purification were concentrated and applied to a gel filtration column (Fig. 3B). The protein fractions eluted are detected by an in–line UV monitor and collected for subsequent specific analysis. When the flow–through sample from nickel affinity purification of a TEV-digested sfGFP–mutacin were added, pure mutacin was eluted from the gel filtration column as a last single peak (Fig. 3B). This peak was clearly absent in the control condition where samples were incubated with heat–inactivated TEV protease (Fig. 3C).

3.3. Production of Anti–mutacin Polyclonal Antibody

Polyclonal antibodies are produced in vivo in response to immunization of an animal, such as a rabbit, with different antigens. For small molecules, a carrier protein is required in order to be recognized by the immune system. In the fusion model of sfGFP–mutacin, the short peptide is already attached to a carrier immunogenic protein thus having the potential to elicit a good immune response [37]. For this aim, an adult female rabbit was immunized with four subcutaneous injections of purified sfGFP–mutacin, by two–weeks interval. Blood samples were collected at several time points from the start of immunization and tested in ELISA assay against sfGFP and mutacin to evaluate the rise of a specific immune response (Fig. 4A).

A solid phase ELISA test of the rabbit sera (dilution 1:5000) prepared from these samples revealed an increase in reactivity toward sfGFP and mutacin after six weeks of immunization (Fig. 4A). From this serum sample (at day 60), rabbit IgGs were purified by affinity chromatography using a protein A column. An automated purification procedure was established using the AKTA prime system allowing a direct flow–through sample of the previous step of protein A purification were loaded onto the column. Unbound IgGs, which contain the rest of antibodies including anti–mutacin, were recovered in the flow–through fraction. After column washing, specific anti–sfGFP antibodies were eluted from the column by low pH buffer as a pure fraction (Fig. 5A). Secondly, unbound IgGs in the flow–through of the first step, were filtered using an sfGFP–mutacin–column to obtain specific anti–mutacin antibodies in the pure fraction (Fig. 5B).

To evaluate the activity of purified antibodies against their antigens, indirect ELISA was performed on sfGFP,
The Open Microbiology Journal, 2015, Volume 9 Al-Homsi et al.

sfGFP mutacin and mutacin pre-coated wells and bound rabbit IgGs were detected with a goat anti-rabbit secondary antibody conjugated to HRP (Fig. 5C). As expected, pure total IgGs and anti-sfGFP were able to efficiently detect sfGFP and sfGFP mutacin at high dilution (1:5000). Interestingly, anti-mutacin as well as total IgGs, but not anti-sfGFP, were able to detect mutacin in its fusion or singular forms (Fig. 5C).

3.5. Mutacin Characterization

After mutacin was cleaved from the fusion protein and purified by gel filtration, its biological activity was tested by bacterial growth turbidimetry, using three different bacterial strains S. thermophilus, Bacillus thuringiensis, Lactobacillus bulgaricus. Turbidimetry by definition is the process of measuring the loss of intensity of transmitted light due to scattering effect particles suspended in the medium. Growth inhibitory effect of mutacin was tested in reference to ampicillin, and the LB medium was used as a negative control. All tested microorganisms appeared to be insensitive to mutacin, whereas the ampicillin was able to inhibit completely the growth of all used bacteria (Fig. 6A).

We supposed that the ineffectiveness of mutacin was due to the absence of one or more of its important bonds that affect its biological activity. As we mentioned previously, mutacin belongs to the lantibiotic type IA, which contains 4 thioether bridges (R–S–R) that are formed in post-translation modification. These additional structures are often critical for the biological activity of lantibiotics [38]. To test this idea, FTIR analysis; which is based on the analysis of functional groups and identification of compounds, was applied on lyophilized pure mutacin. The hallmark finding obtained from this analysis was that no significant peaks were observed around 600 and 800 cm\(^{-1}\) which may indicate the absence of thioether bonds [39]. Without such formations, recombinant mutacin structure could be malformed and thus devoid of activity [40] (Fig. 6B).

4. DISCUSSION

Antimicrobial peptides are now being looked at as an interesting alternative to combat antibiotic resistant strains of bacteria. Because of their medical importance, production of these polypeptides on a large scale for commercial development is a significant challenge. Different peptides have been successfully produced using several methods, including expression as fusion proteins in bacterial systems [41]. Mutacin consists of 24 amino acid residues and exhibits a wide spectrum antibacterial effect. A major obstacle for mutacin characterization and application is the difficulty of its purification from the original sources [7] and the high cost of its chemical manufacturing [42].

Fusion protein technology represents one of the best solutions to achieve rapid, efficient, and cost-effective protein expression and purification of recombinant proteins [43]. Several biological expression systems have been developed by fusing antimicrobial peptides to a partner protein to mask the intrinsic antimicrobial activity of the peptide inside variety of host cells such as bacteria [44],...
yeast [45] and insect cells [46, 47]. Many reports described the expression of different short peptides using variable strategies, including the application of GFP fusions in the expression of hard–to–fold proteins [48], toxins [49], proteases [50] and medical short peptides [36, 51] in both prokaryotic and eukaryotic cell types. Visibility of GFP is the most distinguishable element from other fusion tags [52]. Its unique green color (either visible blue light or UV light) allows us to monitor the existence of GFP fusion protein through purification steps even without any measurement apparatus like a UV spectrophotometer [53].

Cleavage of the fusion complex usually is necessary because of the possible interference with the structural or functional properties of the recombinant target protein. TEV protease represents the best choice for our system because of its high specificity and activity towards its cutting site [50]. The P1' position of TEV protease cleavage site (ENLYFQ|G) is relatively relaxed and many different residues can be substituted for glycine with little or no impact on the efficiency of cleavage [31]. Because we did not want to make any modification to mutacin structure, and as TEV protease cleavage leaves an additional one amino acid (glycine; G) to the C–terminal moiety of its cutting site, the last amino acid residue (G) of TEV cutting site was replaced by (phenylalanine, F), the first amino acid of mutacin.

Many previous works have described a method to produce TEV as soluble recombinant 6×His–tagged protein in E. coli [23, 54, 55]. In our model, purified sfGFP–fused protein could be cleaved with TEV protease, and the desired native protein could be recovered after eliminating TEV protease by nickel affinity chromatography together with the 6×His–tagged sfGFP portion. Gel filtration chromatography was finally conducted for cleaved mutacin in order to eliminate impurities and to obtain a highly purified short peptide.

One important advantage for fusion protein technology is for enhancing antibody production [56]. Antibodies are powerful tools for protein detection and purification by affinity chromatography. Such techniques have not found an application in the bacteriocin field, mainly because it is difficult to develop antibodies against such antimicrobial peptides. The generation of antibodies against bacteriocins is a main research target because it permits their detection and quantification in samples, as well as their purification in a single step through similar strategies [57]. Small proteins in general are bad immunogens, which fail to solicit a significant immune response alone and should be conjugated to a large carrier molecule for efficient presentation to the immune system of a vaccinated animal. In fusion structures, sfGFP could play the role of a carrier for these small molecules in order to enhance specific antibody production.
In our experiment, rabbit immune response against mutacin was induced by its fusion form with sfGFP, which has an important immunogenicity response in rabbits [37]. Rabbit immunization with sfGFP–mutacin resulted in a high titer immune response (~1:50000) towards sfGFP. To produce specific IgG FPLC fractions from immunized rabbit, recombinant sfGFP and sfGFP–mutacin were covalently immobilized on NHS–activated sepharose column by means of the N–terminus using free amine conjugation chemistry. The affinity chromatography column, which is created by this method, was successfully used to purify specific anti–sfGFP and anti–mutacin antibodies from total rabbit IgGs. FPLC purified polyclonal anti–sfGFP and anti–mutacin IgGs represent a valuable tool to detect GFP and mutacin in many biotechnological techniques.

An important production yield of pure mutacin was obtained using this system, estimated to be about 25 mg/L of bacterial culture, and the manufacturing cost was considerably lower, since all necessary elements, such as TEV protease and the specific antibodies, were prepared "in house". However, pure mutacin was inactive and failed to inhibit bacterial growth of three different gram–positive strains of bacteria. It was suggested that losing activity could be related to the malformation of mutacin during protein expression, cleavage or even purification. Furthermore, protein synthesis machinery, used in E. coli BL21 (DE3) strain, was not suitable for the production of properly structured mutacin. In S. mutans and after mutacin is ribosomally synthesized, the resulting translated protein must be modified before becoming active [7]. Genes coding for the enzymes that facilitate these post–translational modifications are usually in close proximity to the structural gene of mutacin [58]. A major mutacin structural modification is the formation of lanthionine bonds, which are thioether–based (R–S–R) ring structures critical for the biological activity of lantibiotics [59]. As expected, FTIR spectrum of pure mutacin revealed an obvious absence of thioether peaks. It would be of great interest to conceive a solution to correct the structure of mutacin either by chemical reaction or by in vitro enzymatic modification using a recombinantly produced lanthipeptide synthetase [60].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

The authors would like to thank the Director General of the Atomic Energy Commission of Syria and the head of the Molecular Biology and Biotechnology department for their continuous support throughout this work.

DISCLOSURE

The authors report no potential conflicts of interest in this work.

LIST OF ABBREVIATIONS

| Abbreviation | Definition |
|--------------|------------|
| BSA          | Bovine Serum Albumin |
| DTT          | Dithiothreitol |
| EDTA         | Ethylene Diamine Tetra Acetic Acid |
| ELISA        | Enzyme-Linked Immunosorbent Assay |
| FPLC         | Fast Protein Liquid Chromatography |
| FTIR         | Fourier Transform Infrared |
| HRP          | Horseradish Peroxidase |
| IPTG         | Isopropylthio–D–galactoside |
| NHS          | N–hydroxysuccinimide |
| NTA          | Nitrilotriacetic acid |
| ORF          | Open Reading Frame |
| PBS          | Phosphate Buffered Saline |
| PCR          | Polymerase Chain Reaction |
| SDS          | Sodium Dodecyl Sulfate |
| SDS-PAGE     | SDS-Poly Acrylamide Gel Electrophoresis |
| SOE          | Splicing by Overlap Extension |
| sfGFP        | Superfolder Green Fluorescent Protein |
| TEV          | Tobacco Etch Virus |
| TMB          | Tetra Methyl Benzidine |

REFERENCES

[1] Hamada S, Slade HD. Biology, immunology, and cariogenicity of Streptococcus mutans. Microbiol Rev 1980; 44: 331-84.
[2] Nes IF, Diep DB, Holo H. Bacteriocin diversity in Streptococcus and Enterococcus. J Bacteriol 2007; 189: 1189-98.
[3] Hamada S, Ooshima T. Production and properties of bacteriocins (mutacins) from Streptococcus mutans. Arch Oral Biol 1975; 20: 641-8.
[4] Nicolas G, Mota-Meira M, Lapointe G, Lavoie M. Mutacins and their potential use in food preservation. Food 2007; 2: 161-71.
[5] Mota-Meira M, Morency H, Lavoie MC. In vivo activity of mutacin B-Ny266. J Antimicrob Chemother 2005; 56: 869-71.
[6] Qi F, Chen P, Caufield PW. The group I strain of Streptococcus mutans, UA140, produces both the lantibiotic mutacin I and a nonlantibiotic bacteriocin, mutacin IV. Appl Environ Microbiol 2001; 67: 15-21.
[7] Qi F, Chen P, Caufield PW. Purification and biochemical characterization of mutacin I from the group I strain of Streptococcus mutans, CH43, and genetic analysis of mutacin I biosynthesis genes. Appl Environ Microbiol 2000;66:3221-9.
[8] Qi F, Caufield PW, Chen P. Mutacin I biosynthesis genes and proteins. US Patent 6,342,385 B1 2002.
[9] Hassan M, Kjos M, Nes IF, Diep DB, Lotfipour F. Natural antimicrobial peptides from bacteria: characteristics and potential applications to fight against antibiotic resistance. J Appl Microbiol 2012; 113: 723-36.
[10] Ingham AB, Moore RJ. Recombinant production of antimicrobial peptides in heterologous microbial systems. Biotechnol Appl Biochem 2007; 47: 1-9.
[11] Li Y, Chen Z. RAPD: a database of recombinantly-produced antimicrobial peptides. FEBS Microbiol Lett 2008; 289: 126-9.
[12] Li Y. Recombinant production of antimicrobial peptides in Escherichia coli: a review. Protein Exp Purif 2011; 80: 260-7.
[13] Shimomura O, Johnson FH, Saiga Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, Aequorea. J Cell Comp Physiol 1962; 59: 223-39.
Cabantous S, Cabantous S, Waldo GS, Wu X, Wu D, Lu Z, Chen W, Hu X, Ding Y. A novel method for protein folding efficiency by directed evolution using the GFP folding reporter. Methods Mol Biol 2003; 230: 345-59.

Magliery TJ, Wilson CG, Pan W, et al. Detecting protein-protein interactions with a green fluorescent protein fragment reassembly trap: scope and mechanism. J Am Chem Soc 2005; 127: 146-57.

Cabantous S, Terwilliger TC, Waldo GS. Protein tagging and detection with engineered self-assembly fragments of green fluorescent protein. Nat Biotechnol 2005; 23: 102-7.

Blokpoel MC, O'Toole R, Smeulders MJ, Williams HD. Development and application of unstable GFP variants to kinetic studies of mycobacterial gene expression. J Microbiol Methods 2003; 54: 203-11.

Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier PJ, Blokpoel MC, O'Toole R, Smeulders MJ, Williams HD. Efficient expression and purification of proteins. J Struct Funct Genom 2004; 5: 75-86.

Wu X, Wu D, Lu Z, Chen W, Hu X, Ding Y. A novel method for high-level production of TEV protease by superfolder GFP tag. J Biomed Biotechnol 2009; 2009: 1-8.

Cabantous S, Waldo G. In vivo and in vitro protein solubility assays using split GFP. Nat Methods 2006; 3: 845-54.

Pedrazaq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. Nat Biotechnol 2006; 24: 79-88.

Andrews BT, Schoenfish AR, Roy M, Waldo G, Jennings PA. The rough energy landscape of superfolder GFP is linked to the chromophore. J Mol Biol 2007; 373: 476-90.

Lee JH, Kim JH, Hwang SW, et al. High-level expression of antimicrobial peptide mediated by a fusion partner reinforcing formation of inclusion bodies. Biochem Biophys Res Commun 2000; 277: 575-80.

Li L, Wang YX, Zhao XF, Kang CJ, Liu N, Xiang JH, et al. High level expression, purification, and characterization of the shrimp antimicrobial peptide, Ch-penaeidin, in Pichia pastoris. Protein Expr Purif 2005; 39: 144-51.

Reichhart JM, Meister M, Dimarco JL, Zachary D, Hoffmann D, Ruiz C, et al. Insect immunity: developmental and inducible activity of the Drosophila diptericin promoter. EMBO J, [Research Support, Non-U.S. Govt] 1992; 11: 1469-77.

Andersson D, Engstrom A, Josephson S, Hansson L, Steiner H. Biologically active and amidated cecropin produced in a baculovirus expression system from a fusion construct containing the antibody-binding part of protein A. Biochem J 1991; 280 (Pt 1): 219-24.

Vasilijevic S, Ren J, Yao Y, Dalton K, Adamson C, Jones I. Green fluorescent protein as a reporter of prion protein folding. Virology J 2006; 3: 1-9.

Soler-Jover A, Blasi I, Gomez de Aranda I, et al. Effect of epsilon toxin-GFP on MDCK cells and renal tubules in vivo. J Histochem Cytochem 2004; 52: 931-42.

van den Berg S, Lofdahl PA, Hard T, Berglund H. Improved solubility of TEV protease by directed evolution. J Biotechnol 2006; 121: 291-8.

Skosyrev VS, Rudenko NV, Yakhnin AV, et al. EGFP as a fusion partner for the expression and organic extraction of small polypeptides. Protein Expr Purif 2003; 27: 55-62.

Taubenberger AM, Anderson KI. Recent advances using green and red fluorescent protein variants. Applied Microbiol Biotechnol 2007; 77: 1-12.

Lee CH. Recombinant green fluorescent protein derivatives as a fusion tag for in vitro experiments. Interdisciplinary Bio Central 2009; 1: 1-15.

Lucas LJ, Batey RT, Doudna JA. Large-scale purification of a stable form of recombinant tobacco etch virus protease. Biotechniques 2001; 30: 544-6, 8, 50 passim.
[56] Hall JP. Applying fusion protein technology to *E. coli*. BioPharm Internat 2007; 2007: 1-11.

[57] Suarez AM, Azcona JI, Rodriguez JM, Sanz B, Hernandez PE. One-step purification of nisin A by immunoaffinity chromatography. Appl Environ Microbiol 1997; 63: 4990-2.

[58] Yonezawa H, Kuramitsu HK. Genetic analysis of a unique bacteriocin, Smb, produced by Streptococcus mutans GS5. Antimicrob Agents Chemother 2005; 49: 541-8.

[59] Willey JM, van der Donk WA. Lantibiotics: peptides of diverse structure and function. Annu Rev Microbiol 2007; 61: 477-501.

[60] Zhang Q, Yu Y, Velasquez JE, van der Donk WA. Evolution of lanthipeptide synthetases. Proc Nat Acad Sci USA 2012; 109: 18361-6.

Received: September 07, 2014 Revised: February 19, 2015 Accepted: March 26, 2015

© Al-Homsi et al.; Licensee Bentham Open.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0/) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.