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Original article

A dilution method to increase the yield of engineered basic fibroblast growth factor

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ABSTRACT

Background: *Genetically engineered chimeric proteins have potential applications as components of dental materials and can be synthesized recombinantly in various cells, including bacteria, yeast, insects and mammals. However, increasing the yields of functionally active products remains a challenge.* ***Purpose:*** *This study focused on basic fibroblast growth factor fused with pentadeca $(GGGGS)_3$ peptide as a linker and hexahistidine as an affinity tag (bFGF-PH). The objective was to enhance the yield of bFGF-PH expressed in bacteria by employing a dilution method.* ***Methods:*** *Escherichia coli was used to express bFGF-PH in a soluble form, which was then purified using metal chelate affinity chromatography. The protein solution was diluted 100-fold with a buffer solution to promote spontaneous refolding. Subsequently, the protein solution was concentrated using metal chelate affinity chromatography. Circular dichroism (CD) spectroscopy was used to analyze the protein's structure, assessing its correct folding by comparing it to a reference spectrum obtained through computer-based simulation.* ***Results:*** *The dilution method prevented bFGF-PH aggregation, and CD spectroscopy suggested that the protein was correctly folded. As a result, a total of 3.0 mg of bFGF-PH was obtained per liter of lysogeny broth medium, which was higher than the yield achieved using the conventional method.* ***Conclusion:*** *The dilution method examined in this study increased the yield of correctly folded bFGF-PH.*

Keywords: *dental materials; tissue engineering; recombinant protein; genetic engineering; protein aggregation; medicine*
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INTRODUCTION

Chimeric proteins consist of two or more tandem polypeptides, each with distinct biofunctionalities. They have potential applications as components of dental materials, particularly in tissue engineering scaffolds for the restoration of bone defects caused by tumor resection and periodontitis-associated bone resorption.[1–5] Additionally, chimeric proteins with growth factor-like biofunctionality and the ability to bind to solid substrates can be used to fabricate biologically active materials beneficial in stem cell engineering.[6,7] These applications rely on recombinant protein synthesis technologies.

Recombinant chimeric proteins can be expressed in various cells, including bacteria, yeast, insects and mammals. Among these, bacterial expression systems are the most straightforward and efficient. However, exogenous proteins are often deposited in cytosolic inclusion bodies, necessitating solubilization with chaotropic agents such as urea and guanidine hydrochloride. To obtain biologically active products, dialysis is conventionally performed to remove these denaturing agents and refold the proteins. Even when proteins are expressed in bacteria in a soluble form, misfolding and aggregation frequently occur during dialysis for buffer exchange.

The misfolding and aggregation of proteins considerably decrease the yield of active products. Numerous studies have been conducted to overcome this problem,[8] including the use of additives[9] such as sucrose and arginine chloride and stabilizers[10] such as polysorbates and Pluronic to prevent protein aggregation. Other groups have reported that the solubility of a recombinant protein increased

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upon overexpression as a fusion protein with glutathione S-transferase.[11,12] Furthermore, diluting protein solutions has been shown to enhance the recovery of soluble and active enzymes.[13]

In this study, we examined a dilution method in which proteins were expressed in bacteria, purified using column chromatography and diluted 100-fold. We hypothesized that chimeric proteins would have a lower propensity to aggregate in dilute solutions, resulting in a higher yield of the active form. To verify this hypothesis, we prepared a recombinant chimeric protein – basic fibroblast growth factor fused with a pentadeca peptide as a linker and hexahistidine as an affinity tag (hereafter referred to as bFGF-PH). To assess the correct folding of the bFGF domain, the prepared bFGF-PH was analyzed using circular dichroism (CD) spectroscopy, and the resulting spectrum was compared with a computationally predicted reference spectrum.

MATERIALS AND METHODS

Figure 1 shows the amino acid sequence of the engineered bFGF prepared in this study. At the C-terminus of bFGF, pentadeca $(GGGGS)_3$ and hexahistidine peptides were added as a linker and an affinity tag, respectively. We expected that the nonionic glycine (G) peptide would help maintain the distance between the two segments while serine (S) would provide flexibility to the linker.

To express these proteins in bacteria, a DNA insert encoding bFGF-PH was synthesized using the GeneArt Strings DNA Fragments service (Thermo Fisher Scientific, Waltham, MA, USA). The obtained DNA fragment was inserted into the multiple cloning sites of pET-22b(+) (69744, Novagen, Madison, WI, USA) using the seamless cloning method,[14] employing an In-Fusion HD cloning kit (Z9648N, Clontech Laboratories, Inc., Mountain View, CA, USA). Sequence accuracy was confirmed by sequencing. The plasmid harboring the bFGF-PH gene was transferred to *Escherichia coli* strain BL21-CodonPlus (DE3)-RIPL (230280, Agilent Technologies, Inc., Santa Clara, CA, USA) and cultured in 200 mL lysogeny broth (LB) medium with 100 µg/mL ampicillin (02739-32, Nacalai Tesque, Kyoto, Japan) at 37°C. When the medium turbidity reached 0.4–0.8, 1 mM isopropyl β-D-thiogalactopyranoside (06289-67, Nacalai Tesque) was added to induce protein expression, followed by incubation at 37°C for an additional 3 hours.

The cells were lysed using BugBuster reagent (70584-4, EMD Millipore Corp., Burlington, MA, USA) containing 10 µg/mL lysozyme (L6876, Sigma-Aldrich, St. Louis, MO, USA) and 10 U/mL benzonase (70746-3, Novagen). Subsequently, bFGF-PH in the soluble fraction was purified by metal chelate affinity chromatography using a HisTrap HP column (17524801, Cytiva, Chicago, IL, USA). The protein solution was dialyzed against 20 mM phosphate buffer (PB) containing 500 mM NaCl (pH 7.4; 191-01665, Fujifilm Wako Pure Chemical Corp., Osaka, Japan), followed by dialysis in 20 mM citrate buffer (pH 5.0),[6,15] with or without 500 mM NaCl. This was followed by another round of dialysis in 20 mM PB containing 500 mM NaCl (pH 7.4). Alternatively, a bFGF-PH solution obtained immediately after affinity purification was diluted 100-fold with 20 mM citrate buffer (pH 5.0) to initiate protein refolding. Then, 500 mM NaCl was added to the solution. The protein was subsequently concentrated using a HisTrap HP column, yielding a final bFGF-PH solution in 20 mM PB containing 500 mM NaCl (pH 7.4).

The concentration of bFGF-PH was determined using a Micro BCA protein assay kit (23235, Thermo Fisher Scientific Inc., Waltham, MA, USA). Purity and molecular size were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining. All recombinant DNA experiments were conducted with the approval of the Hiroshima University Safety Committee for Living Modified Organisms.

The secondary structure of bFGF-PH was analyzed by CD spectroscopy using a JASCO J-850 spectropolarimeter (JASCO Corp., Tokyo, Japan). The far-ultraviolet CD spectrum of bFGF-PH was recorded in 20 mM PB containing 500 mM NaCl (pH 7.4) at 20°C. To determine whether bFGF-PH was correctly folded, an ideal CD spectrum was generated using the PDBMD2CD web server,[16,17] and compared with the experimental spectra. The server generated a CD spectrum based on an atomic coordinate file obtained through structural prediction using the AlphaFold2 program,[18,19] an open-source tool available at https://github.com/deepmind/alphafold.

bFGF-PH

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        10         20         30         40         50         60
AAGSITTLPA LPEDGGSGAF PPGHFKDPKR LYCKNGGFFL RIHPDGRVDG VREKSDPHIK

        70         80         90        100        110        120
LQLQAEERGV VSIKGVCANR YLAMKEDGRL LASKCVTDEC FFFERLESNN YNTYRSRKYT

       130        140        150        160        170
SWYVALKRTG QYKLGSKTGP GQKAILFLPM SAKSGGGGSG GGGSGGGGSH HHHHH
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Figure 1. Amino acid sequence of bFGF-PH, shown from N- to C-terminus. Ala1–Ser154: bFGF domain (blue); Gly155–Ser169: pentadeca peptide linker (red); His170–His175: hexahistidine tag (green).

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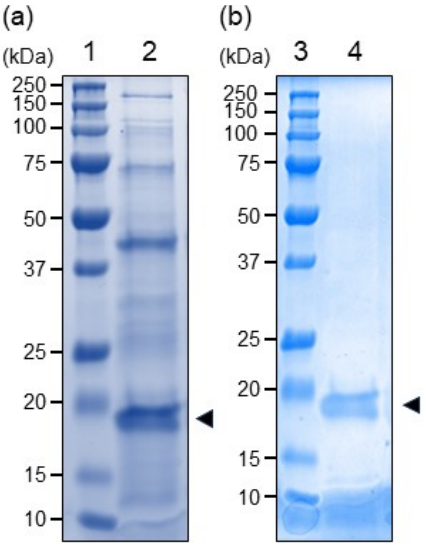

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of bFGF-PH. (a) Lane 1: molecular weight standard; lane 2: bFGF-PH obtained after affinity purification. (b) Lane 3: molecular weight standard; lane 4: bFGF-PH refolded using the dilution method and concentrated via affinity chromatography. Arrowheads indicate the bFGF-PH band.

RESULTS

Figure 2a shows the results of the SDS-PAGE analysis of the protein after purification by metal chelate affinity chromatography. The protein separated into a single bold band at 19 kDa. This molecular weight matched the predicted value based on the amino acid sequence of bFGF-PH, leading us to conclude that bFGF-PH was prepared as expected. However, several faint bands were also observed. These bands were attributed to endogenous bacterial proteins, which were reduced using the dilution method (Figure 2b), as described later.

The bFGF-PH obtained as described above was dialyzed against 20 mM citrate buffer (pH 5.0) containing 500 mM NaCl. The final product concentration was 243 µg/mL. Compared with the CD spectrum generated for the computationally predicted bFGF-PH structure (Figure 3a), the experimental CD spectrum of this protein (Figure 3b) had a remarkably different shape. The experimental spectrum showed a small but distinct Cotton effect at approximately 208 and 220 nm, suggesting the presence of an α-helical structure.

In contrast, the CD spectrum of bFGF-PH dialyzed against 20 mM citrate buffer (pH 5.0) without 500 mM

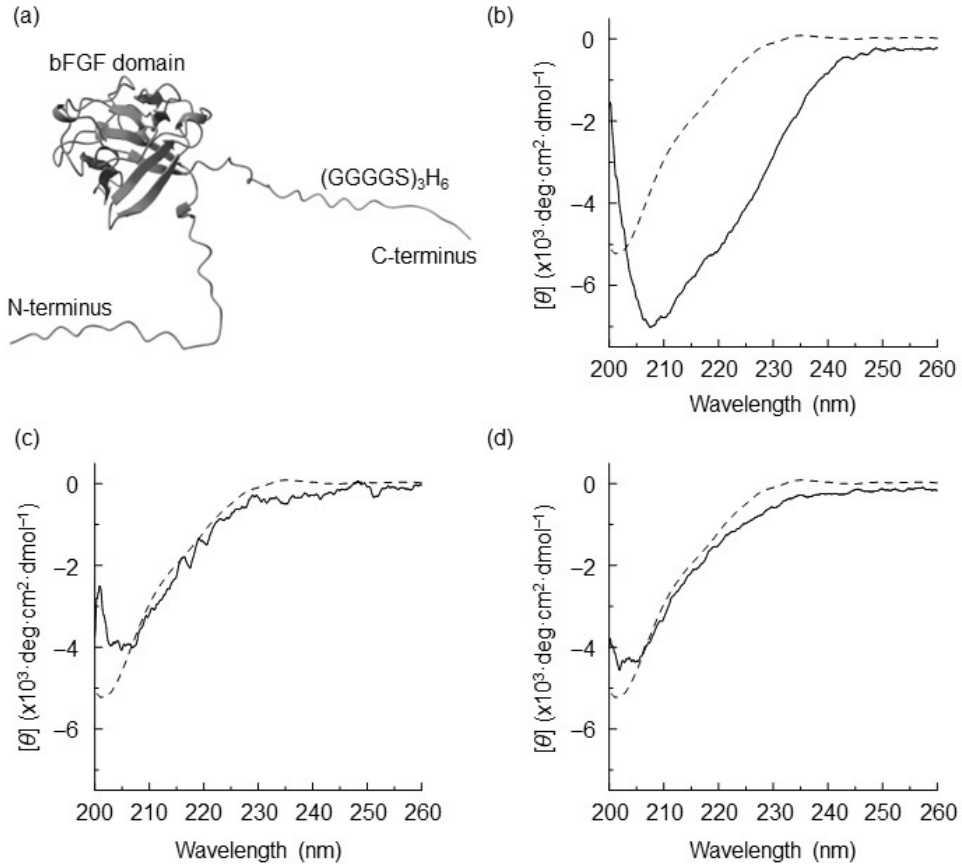

Figure 3. Far-ultraviolet CD spectra of bFGF-PH. (a) bFGF-PH structure predicted by AlphaFold2. The CD spectrum was generated as a reference using the atomic coordinate file of this structure. (b–d) Circular dichroism spectra recorded for bFGF-PH dialyzed in 20 mM citrate buffer (b) with or (c) without 500 mM NaCl and (d) bFGF-PH refolded in a dilute solution and concentrated via affinity chromatography. The computationally predicted reference spectrum was adjusted to an arbitrary scale and is shown as a dotted line.

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NaCl had a shape similar to the predicted one (Figure 3c). However, the yield of soluble bFGF-PH was extremely low (38.4 µg/mL) due to substantial aggregation and precipitation during dialysis in the low-ionic-strength buffer solution.

Conversely, the CD spectrum of bFGF-PH obtained using the dilution method closely resembled the predicted spectrum (Figure 3d). The diluted bFGF-PH was subjected to metal chelate affinity chromatography to concentrate bFGF-PH and eliminate bacterial proteins (Figure 2b). The final concentration of soluble bFGF-PH was 120 µg/mL, corresponding to a yield of 3.0 mg per liter of LB medium.

DISCUSSION

High-yield preparation of recombinant chimeric proteins is a major challenge for downstream applications. In this study, we demonstrated that the yield of engineered bFGF (bFGF-PH) was drastically increased using the dilution method. This may be due to the effect of diluting the protein solution, which increases the average protein–protein distance and decreases the probability of monomer association into aggregates.

According to our previous experience with recombinant protein synthesis, the solubility of bFGF is relatively high.[14] However, bFGF-PH had a propensity to aggregate and precipitate, particularly when dialyzed for refolding and buffer exchange. This tendency was likely due to the presence of the pentadeca-peptide linker and hexahistidine tag. These peptides may reduce the hydrophilicity of bFGF and increase the likelihood of interactions between neighboring segments. However, hexahistidine is useful for protein purification via metal chelate affinity chromatography and for the surface tethering of proteins.[6,20] The linker peptide is expected to increase the mobility of the bFGF domain, potentially preventing steric hindrance.

Protein aggregation is influenced by many factors, including protein structure, chemical degradation, temperature and solution conditions.[10] In this study, we attempted to optimize solution conditions by using a citrate buffer (pH 5.0) instead of a neutral PB and by adding 500 mM NaCl to buffer solutions. These modifications were based on the understanding that protein association involves several forces, such as electrostatic attraction, hydrogen bonding, van der Waals forces and hydrophobic interactions.[10] However, despite these efforts, we were unable to recover large amounts of soluble and correctly folded bFGF-PH.

In contrast, when the bFGF-PH concentration was reduced 100-fold, the protein remained soluble during dialysis and folded into its correct structure. Accordingly, we found this procedure to be simple yet effective. The average protein–protein distance in solution increases as concentration decreases, which likely reduces the probability of monomer association and oligomer growth into aggregates.[10] The diluted bFGF-PH could also be easily reconcentrated using metal chelate affinity chromatography.

In this study, we determined protein concentration using a standard protein assay, while correct folding was assessed by CD spectroscopy. This spectroscopic method is useful for analyzing the secondary structures present in a protein of interest, although it does not provide information on tertiary structure. In CD spectroscopic analysis, a reference spectrum of a correctly folded protein is required to validate the structure of sample proteins. However, no such reference spectrum exists for bFGF-PH, as its sequence is artificial and unique. For this reason, we performed a computer-based prediction of a CD spectrum using the PDBMD2CD program.[16] This program provides highly accurate CD spectra for a wide variety of protein samples.[21]

The Cotton effect observed in the predicted spectrum was approximately three times larger than those in the experimental spectra across the entire wavelength range, most likely due to the presence of denatured bFGF-PH in the sample solutions. To facilitate direct comparison, we used an arbitrary unit for the predicted spectrum in Figure 3 (dotted line), allowing the spectral shapes to be compared with each other. This approach ultimately enabled us to identify the conditions under which soluble and correctly folded bFGF-PH could be obtained in sufficiently high yields.

In conclusion, the dilution method used in this study effectively increased the yield of correctly folded bFGF-PH. Protein aggregation is a major issue that frequently arises during the purification and refolding of recombinant proteins. The results of this study provide further evidence that the dilution method can enhance the yields of correctly folded products. While this study focused on a specific case with bFGF-PH, optimal dilution conditions may vary depending on protein structure. Further studies on a broader range of chimeric proteins will be necessary to verify the versatility of this method.

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