A new pH-responsive peptide tag for protein purification

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Contents

Chapter 1.

General Introduction

1.1. Overview of method for protein production

Proteins having a variety of functions are widely utilized for commercial uses such as pharmaceutical products [1] and industrial enzymes [2], and also for research uses in which proteins are used to elucidate various biological phenomena. Thus, proteins are substances essential in improvements in quality of life and scientific advancements. As means for producing these various proteins in large quantities at a low cost with a high reproducibility, techniques for producing proteins using recombinant organisms and techniques for purifying the proteins have been developed. The techniques for producing proteins using recombinant organisms utilize animal cells [3] and microorganisms [4]. The animal cells used include Chinese Hamster Ovary (CHO) cells, while the microorganisms used include *Escherichia coli*, yeasts [5], and so on. For example, there is a protein secretory production system using *Corynebacterium glutamicum* (hereinafter may be abbreviated as *C. glutamicum*) as the microorganism [6–8]. Main methods for purifying proteins produced by recombinant organisms include a method utilizing properties of a protein itself, and a method by adding a few amino acid sequence, called purification tag, used for purification to a protein and utilizing properties of the purification tag.

1.2. Challenges for protein purification

The method utilizing properties of a protein itself includes chromatography and liquid-solid separation [9]. The chromatography uses chromatographic matrixes having various properties. The chromatography utilizes an interaction between a protein and a chromatographic matrix [8], or the molecular sieving effect of the chromatographic matrix [10]. The interaction between a protein and a chromatographic matrix includes electrostatic interaction, hydrogen bond, hydrophobic interaction, specific interaction [11]. The liquid-solid separation is a separation method including: insolubilizing (i.e., making solid) a protein in a solubilized state by changing the solution conditions, obtaining a solid component by a simple process such as centrifugation, and bringing the separated solid component into a solubilized state again. Specific examples of the means for insolubilizing the protein include isoelectric point precipitation [12], salting out [13], precipitation using an organic solvent [14], precipitation using a water-soluble polymer. The isoelectric point precipitation utilizes a property in which the solubility of a protein becomes lowest at an isoelectric point thereof. The salting out, the precipitation using an organic solvent, and the precipitation using a water-soluble polymer utilize a property in which the solubility of a protein is decreased in the presence of the salt, the organic solvent, or the water-soluble polymer [15], each of which is at a high concentration. Another insolubilizing means is protein aggregation [16–18]. The protein aggregation may be particularly effective means when it is possible to select

conditions for aggregating a protein other than a protein to be purified while leaving the protein to be purified in a solubilized state. In order to overcome these problems, additional sequences having various properties, namely purification tags, have been developed [19].

The method by adding a purification tag used for purification to a protein and utilizing properties of the purification tag includes a method utilizing properties of the purification tag itself, and a method utilizing an interaction between the purification tag and a substance immobilized on the matrix. In the method utilizing an interaction between the purification tag and a substance immobilized on matrix, the substance is often disposed on a chromatographic matrix [11,19–21]. The interaction between the purification tag and the substance other than the tag includes electrostatic interaction, hydrogen bond, hydrophobic interaction, specific interaction [22].

Now, many kinds of purification tags and immobilized affinity ligands are commercially available; however, the scale-up of affinity chromatography can represent a major cost in the production of the final protein product at the industrial scale. So, new peptide tags are in demand for use in costless and simple methods for protein purification.

1.3. Strategy for development of new purification tag

The elastin-like polypeptide (ELP) tag is a widely used tag for non-chromatographic purification of several proteins, including calmodulin, catalase, and thioredoxin [23–25]. The ELP tag comprises thermally responsive polypeptides that undergo reversible aggregation above an intrinsic transition temperature (Tt), and this aggregation is caused by hydrophobic interaction [20–22]. ELP fusion proteins are soluble in aqueous solutions below Tt, whereas they form aggregates when the temperature is raised above Tt. The aggregation (precipitation) process of ELP fusion proteins is fully reversible. Accordingly, the precipitation–redissolution cycle of the ELP fusion protein controlled by temperature can be used for purification without the need for chromatography. However, the typical Tt of the ELP tag is 30°C–40°C, which may causes irreversible unfolding of heat-labile proteins. On the other hand, this type of purification tag for non-chromatographic purification of fusion protein has a lot of merits. So, I performed development of a new purification tag which can undergo reversible aggregation according to the environmental condition change by using cell surface protein B (CspB) which known as self-assembled protein in nature [26–28].

1.4. Objective of this thesis

This thesis investigated the development of a new purification tag for protein purification. Chapter 2 describes a newly developed CspB-tag which insolubilizes-solubilizes in response to pH. Chapter 3 describes development of production process for pharmaceutical protein using CspB-tag. Chapter 4 describes an effect of small additive on the pH response behavior of CspB fusion protein. Subsequently, chapter 5 and chapter 6 present the overview and discussion of this thesis including the future work to be studied.

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Chapter 2.

Development of A New pH-Responsive Peptide Tag for Protein Purification

2.1. Introduction

Recombinant proteins are widely used in many applications, as exemplified by pharmaceuticals and industrial enzymes. A number of protein expression systems have been designed to increase the purification efficiency of recombinant proteins incorporating purification tags, which are peptides or proteins that display highly specific and reversible binding with a ligand [1,2]. Such a fusion protein is typically purified from host cell proteins by affinity chromatography, which is both simple and efficient. Many kinds of purification tags and immobilized affinity ligands are commercially available [3]; however, the scale-up of affinity chromatography can represent a major cost in the production of the final protein product at the industrial scale.

Peptide tags are in demand for use in costless and simple methods for protein purification. The elastin-like polypeptide (ELP) tag is a widely used tag for nonchromatographic purification of several proteins, including calmodulin, catalase, and thioredoxin [1,4,5]. The ELP tag comprises thermally responsive polypeptides that undergo reversible aggregation above an intrinsic transition temperature (Tt), and this aggregation is caused by hydrophobic interaction [6]. ELP fusion proteins are soluble in aqueous solutions below Tt, whereas they form aggregates when the temperature is raised above Tt. The aggregation (precipitation) process of ELP fusion proteins is fully reversible. Accordingly, the precipitation–redissolution cycle of the ELP fusion protein controlled by temperature can be used for purification without the need for chromatography. However, the typical Tt of the ELP tag is 30°C–40°C, which may causes irreversible unfolding of heat-labile proteins [4].

This paper describes a novel purification tag based on cell surface protein B (CspB) of Corynebacterium glutamicum. It has been shown that C. glutamicum strains have a surface (S) layer outside the normal cell wall, which comprises a single protein assembled in two-dimensional (2D) paracrystalline arrays [5]. The protein

CspB (also known as PS2) has been identified as a major secreted protein of several C. glutamicum strains and forms the S-layer in these species [7,8]. Electron microscopy and atomic force microscopy have shown that the S-layer of C. glutamicum formed from CspB assembles into hexameric complexes in a hexagonal 2D lattice structure mainly as a result of hydrophobic interactions [8–14]. The hexagonal lattice has been shown to be stable in 3% sodium dodecyl sulfate (SDS) at temperatures up to 60°C. Considering that (i) CspB is secreted in soluble form in physiological condition, (ii) the self-assembled structure of CspB forms in physiological condition and is highly stable [9], and (iii) the C-terminal portion of CspB works for anchoring to the cell wall, I assumed that a N-terminal partial sequence of CspB can be used as a stimuli responsive peptide and thus as a protein purification tag for precipitation and redissolution in physiological conditions. According to the above hypothesis, I prepared several gene constructs, which consist of various lengths of CspB as tags, and a proinsulin as a model pharmaceutical protein. Next, I investigated the behavior of the expressed fusion proteins in various solution conditions. Finally, I evaluated the versatility of the new CspB tag using other types of pharmaceutical proteins, Teriparatide and Bivalirudin.

2.2. Materials and Methods

Bacterial strains, plasmids, and the design of fusion proteins

CspB fusion proteins were prepared according to a standard protocol for protein expression and secretion described previously[8], using a Corynebacterium glutamicum expression system, named CORYNEX® from the underlined characters which provided as a protein expression service from AJINOMOTO Co., Inc. The bacterial strain and plasmid used in this study were YDK010, a derivative of wild-type C. glutamicum ATCC13869, and pPK4, respectively [15–21]. The amino acid sequence of CspB (also known as PS2) has already been determined (Genbank Accession No. BAV24076.1). The amino acid sequence of mature CspB protein is shown in Figure 2.1A. The N-terminal portions of CspB of 5, 6, 17, 50, and 250 amino acid residues were selected as a tag to be fused and evaluated and were termed as CspB5, CspB6, CspB17, CspB50, and CspB250, respectively.

The amino acid sequences of proinsulin (PIns), Teriparatide, and Bivalirudin have been determined as Genbank Accession Nos. 2KQP_A, AAQ51502.1, and ACW47046.1, respectively. For PIns, CspB5, CspB6,

CspB17, CspB50, and CspB250 fusion constructs were designed as shown in Figure 2.1B and termed as CspB5-PIns, CspB6-PIns, CspB17-PIns, CspB50-PIns, and CspB250-PIns, respectively. For Teriparatide, a CspB50 fusion construct, which includes a TEV protease recognition site, ENLYFQ, in between was designed, and termed as CspB50TEV-Teriparatide. For Bivalirudin, a sequence lacking N-terminal D-Phe and Pro (named as Biva18) was selected, and a CspB50 fusion construct, which includes a trypsin recognition site, lysine, in between was designed and termed as CspB50Lys-Biva18. The DNA sequences encoding these CspB fusion proteins were designed by incorporating the C. glutamicum codon bias. DNA fragments containing the promotor of cspB from C. glutamicum and the signal sequence of cspA from C. ammoniagenes were fused to the CspB fusion protein genes. These gene constructs were inserted into pPK4.

Expression of fusion proteins

C. glutamicum transformants expressing the PIns or CspB fusion PIns series were cultured in test tubes at 30°C for 72 h in MM liquid media (120 g of glucose, 0.4 g of magnesium sulfate heptahydrate, 30 g of ammonium sulfate, 1 g of potassium dihydrogen phosphate, 0.01 g of iron sulfate heptahydrate, 0.01 g of manganese sulfate pentahydrate, 200 µg of thiamine hydrochloride, 500 µg of biotin, 0.15 g of DL-methionine, and 50 g of calcium carbonate, adjusted to 1 L with water and to pH 7.5) containing 25 mg/L of kanamycin.

C. glutamicum transformants expressing the CspB50TEV-Teriparatide and CspB50Lys-Biva18 were cultured at 30°C for 3 days in a 1-L capacity jar fermenter to which 300 mL of an MMTG liquid medium (120 g of glucose, 2 g of calcium chloride, 3 g of magnesium sulfate heptahydrate, 3 g of ammonium sulfate, 1.5 g of potassium dihydrogen phosphate, 0.03 g of iron sulfate heptahydrate, 0.03 g of manganese sulfate pentahydrate, 450 µg of thiamine hydrochloride, 450 µg of biotin, 0.15 g of DL-methionine, and 50 g of calcium carbonate, adjusted to 1 L with water and to pH 6.7) containing 25 mg/L of kanamycin had been charged, and the pH was maintained at 6.7 by the addition of gaseous ammonia.

In the both of test tube and jar fermenter cultivation, the fusion proteins were constitutively expressed and secreted into the culture broth by *C. glutamicum*. After culturing by a test tube or jar fermenter was completed, the culture broth were transferred to microtubes and centrifuged at 12,000 g for 10 min to separate the bacterial

cells. The resulting supernatants were filtered through a sterile filter, and the culture supernatants were obtained, which were used for further experiments.

Characterization of CspB fusion proteins

The pH-dependent precipitation–redissolution behavior of the CspB fusion proteins was characterized as follows. The culture supernatant (1 mL) was adjusted to various pH values by adding 0.5 M H2SO4 or 0.5 M NaOH. After mixing well, the pH-adjusted culture supernatant was left for 10 min. The pH-adjusted culture supernatant was then centrifuged at 12,000 g for 5 min to separate precipitates formed by the pH change. The supernatant was transferred to another microtube, and an equal volume of 100 mM Tris-HCl buffer (pH 8.5) was added to the remaining precipitate. The precipitate dissolved immediately and a precipitation–redissolution sample was obtained. The supernatant and precipitation–redissolution samples obtained at various pH values were analyzed by SDS-PAGE using AnykD Mini-PROTEAN® Precast Gel (Bio-Rad Laboratories, Inc.), and the bands of CspB fusion proteins were detected by staining with SYPRO®Ruby (Life Technologies Japan Ltd.). The band intensity was quantified as an intensity using a Multi Gauge (FUJIFILM Corporation), and the precipitation ratio (P/(S+P) × 100, where P and S denote the band intensities of the CspB fusion protein in supernatant and precipitation–redissolution samples, respectively) of each CspB fusion protein at each pH was calculated.

2.3. Results

Reversible pH responses of CspB fusion proinsulin

Figure 2.1A shows the full sequence of mature CspB, which comprises 469 amino acid residues. I designed various lengths of the N-terminal CspB sequence with 5, 6, 17, 50, and 250 amino acid residues as the precipitation–redissolution tag for protein purification, named CspB5, CspB6, CspB17, CspB50, and CspB250, respectively (Fig. 2.1B). The model protein used was proinsulin (PIns), which is a precursor of a therapeutic protein comprising 86 amino acid residues; its isoelectric point is pH 5.2, and it contains 34% α-helical structure at neutral pH [22].

First, I investigated the solution states of CspB250-PIns, CspB50-PIns, CspB17-PIns, CspB6-PIns, and CspB5-PIns at neutral and weak acidic pH. The culture supernatants typically at around pH 7.8 were used, and the pH was lowered to around 4.6 by the addition of H_2SO_4. The precipitates formed by the pH change were separated by centrifugation and then mixed with 100 mM Tris-HCl buffer (pH 8.5). As a result, all the precipitates redissolved immediately and clear solutions (i.e., precipitation–redissolution samples) were obtained. Figure 2.1C shows the SDS-PAGE analyses of the supernatant (Sup.) and precipitation–redissolution (Ppt.) samples at neutral and weak acidic pH for each CspB fusion PIns. At weak acidic pH, CspB6-PIns, CspB17-PIns, CspB50-PIns, and CspB250-PIns formed precipitates, whereas PIns alone and CspB5-PIns did not form precipitates (Fig. 2.1C). Interestingly, six amino acid residues of CspB (CspB6) induced the precipitation of PIns depending on pH.

Figure 2.1D shows the precipitation ratio as a function of the length of CspB fused to PIns. The precipitation ratio increased with increasing CspB peptide length from 37% precipitation by six residues (CspB6) to 65% precipitation by 250 residues (CspB250). Taken together, it was revealed that the small fragments of CspB fused to PIns can induce precipitation at weak acidic pH and that the precipitate formed can easily dissolve at a higher pH, i.e., in 100 mM Tris-HCl (pH8.5), suggesting that these CspB fragments can add a protein reversible pH-responsive character, which controls soluble and precipitated states of the protein simply by pH. I further investigated the CspB50 fragment as a pH-responsive tag fused to other types of pharmaceutical proteins.

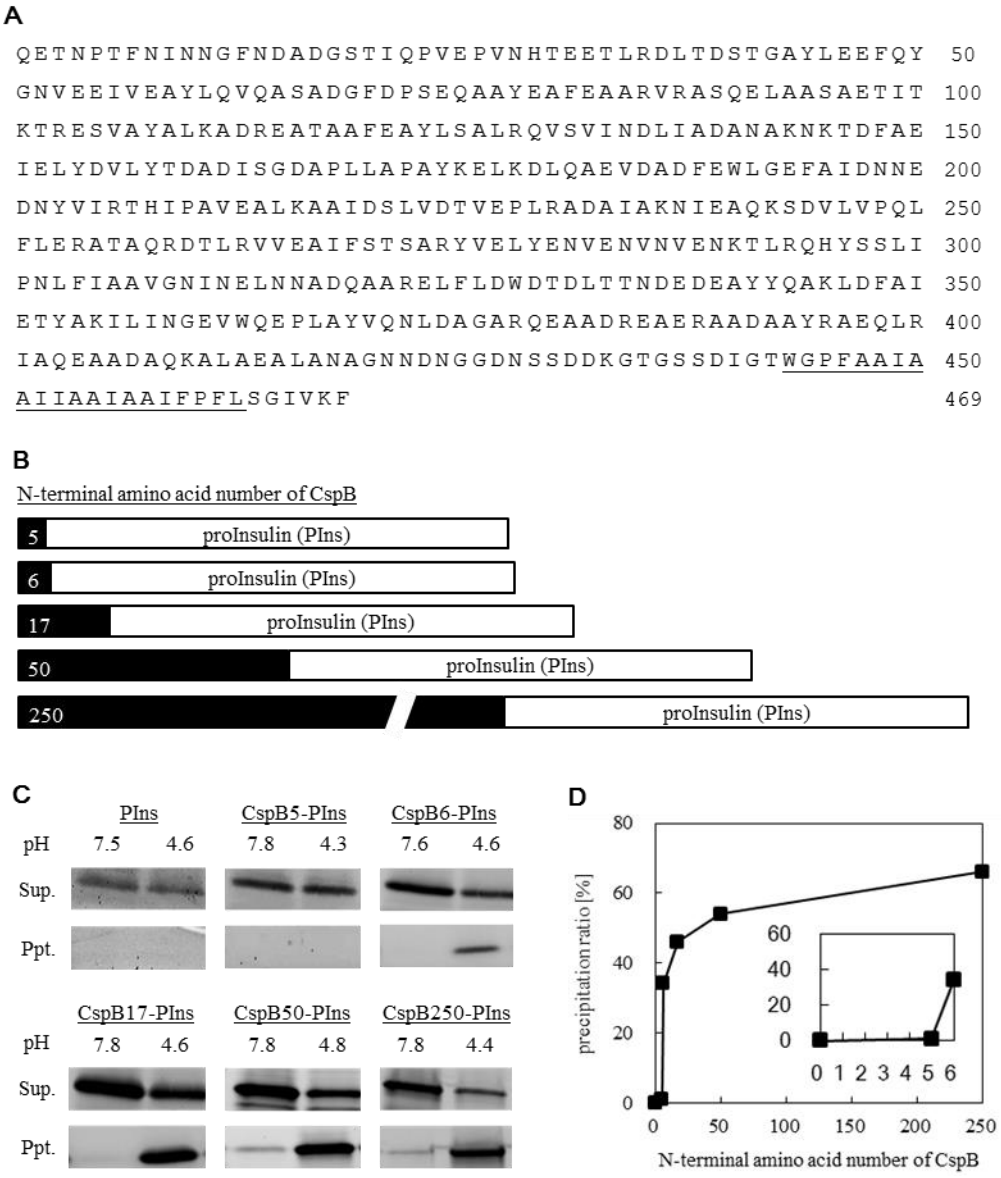

Fig. 2. 1. Construction of CspB fusion proinsulin and evaluation of pH response. (A) Amino acid sequences of mature CspB. The underlined C-terminal amino acid sequence is thought to be a transmembrane region with the anchor on the cell wall of *C. glutamicum*. (B) Schematic structures of CspB fusion proinsulin are shown. Various lengths of the N-terminal portion of CspB, the length of which is indicated by the numbers, are fused with proinsulin (PIns). (C) SDS-PAGE analysis of the supernatant (Sup.) and precipitation–redissolution (Ppt.) samples obtained from the culture supernatant of CspB fusion protein at weak basic and weak acidic pH. (D) Plot of the relationship between the length of the CspB tag and the precipitation ratio of each CspB fusion proinsulin. Inset is the same plot focusing on the short CspB.

Reversible pH responses of CspB50TEV-Teriparatide and CspB50Lys-Biva18

I selected the CspB50 fragment and investigated it as a pH-responsive tag for Teriparatide and Biva18 (Fig. 2. 2). Teriparatide is a pharmaceutical protein with 34 residues that is used to treat osteoporosis [23]. Biva18 is a pharmaceutical protein fragment comprising 18 residues that is a precursor of an anticoagulant Bivalirudin [24,25]. The two kinds of pharmaceutical proteins were fused to CspB50 with small cleavage sites (TEV and Lys) for TEV protease (for Teriparatide) or trypsin (for Biva18), named CspB50TEV-Teriparatide and CspB50Lys-Biva18, respectively (Fig. 2. 2A and 2B).

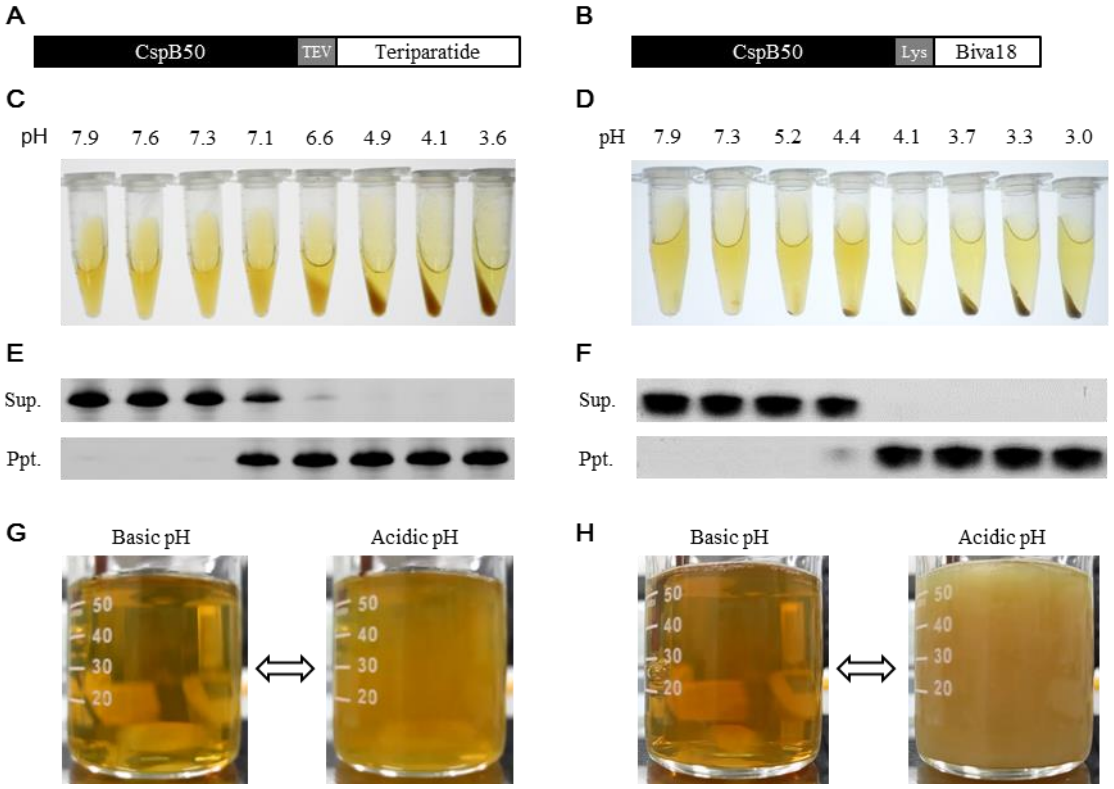

Fig. 2. 2. Construction of CspB50 fusion Teriparatide and Bivalirudin and evaluation of pH response.

(A, B) Schematic structures of the CspB50 fusion proteins CspB50TEV-Teriparatide and CspB50Lys-Biva18. The cleavage sites (TEV and Lys) recognized by TEV protease and trypsin were also fused. (C, D) Images of pH-dependent precipitation. (E, F) SDS-PAGE analysis of the supernatant (Sup.) and precipitation–redissolution (Ppt.) samples prepared from the samples shown in C and D. (G, H) Snapshots of the pH response of each CspB50 fusion protein at the 50 mL scale (see Supplemental Data Fig. 2. 2. S1 for the original movie).

The fusion proteins of CspB50TEV-Teriparatide and CspB50Lys-Biva18 were expressed in *C. glutamicum*, and then the culture supernatants were collected. After that, the solution pH values of the culture supernatants were adjusted using 0.5 M NaOH or 0.5 M H_2SO_4. Figure 2. 2C and 2D shows photographs of the culture supernatants of CspB50TEV-Teriparatide and CspB50Lys-Biva18 adjusted at various pH values taken after centrifugation. As expected, precipitates were observed depending on pH for both the fusion proteins. Figure 2. 2E and 2F show the SDS-PAGE analyses of the supernatant (Sup.) and precipitation–redissolution (Ppt.) samples prepared from the samples shown in Figures 2. 2C and 2D, respectively. CspB50TEV-Teriparatide was fully in a soluble state at pH 7.9–7.3, whereas it was precipitated at below pH 7.1 and redissolved by 100 mM Tris-HCl (pH8.5) (Fig. 2. 2E). Similarly, CspB50Lys-Biva18 was fully in soluble state at pH 7.9–5.2, whereas it was precipitated at below pH 4.4 and redissolved by 100 mM Tris-HCl (pH8.5) (Fig. 2. 2F). The reversibility between soluble (redissolved) and precipitated states of the fusion proteins was monitored at the 50 mL solution scale (Fig. 2. 2G and 2H). CspB50TEV-Teriparatide and CspB50Lys-Biva18 formed clear solutions at a weak basic pH of around 9.0. The clear solution became cloudy on adding 0.5 M H_2SO_4 to the solution. After that, the cloudy solution became clear on adding 0.5 M NaOH (see Supplemental Data for the original video for Fig. 2. 2G and 2H). Taken together, CspB50 was suggested to work as a versatile pH-responsive tag for reversible precipitation–redissolution of various proteins.

Effect of the type of acid

As shown in the above analyses, three kinds of CspB50 fusion proteins showed reversible precipitation–redissolution behavior that depended on the solution pH. Here, I investigate the effect of the type of acid on the precipitate formation of CspB50TEV-Teriparatide at various pH values (Fig. 2. 3). All types of acids tested, i.e., sulfuric acid, hydrochloric acid, and acetic acid, formed precipitates of the fusion proteins at weak acidic pH (Fig. 2. 3A). Figure 2. 3B shows the pH dependence of the precipitate formation of CspB50TEV-Teriparatide. All types of acids tested yielded very similar transition curves for precipitation–redissolution in the pH range of 6.6–7.0. These results indicate that the reversible precipitation–redissolution of the fusion protein depends only on the solution pH and not on the type of acid used.

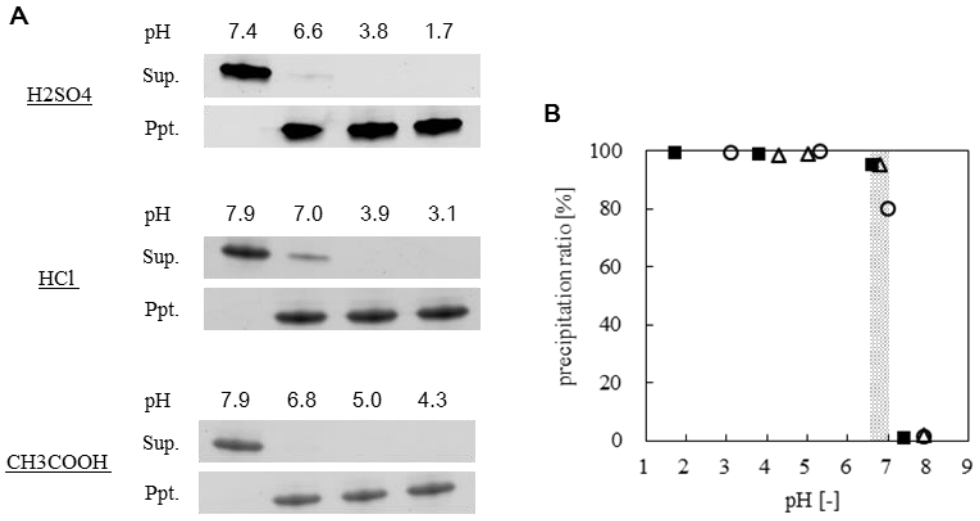

Fig. 2. 3. Effect of the type of acid on the transition pH of a CspB50-fusion protein. (A) SDS-PAGE analysis of the supernatant (Sup.) and precipitation–redissolution (Ppt.) samples prepared from culture supernatant of CspB50TEV-Teriparatide samples with various pH, adjusted by three different types of acid. (B) Plot of the relationship between the pH and the precipitation ratio of CspB50TEV-Teriparatide. The shaded area corresponds to the transition pH of precipitation–redissolution of CspB50TEV-Teriparatide.

2.4. Discussion

This paper proposes a new peptide tag based on CspB derived from *C. glutamicum*. The advantages of the CspB tag are (i) a sharp pH response centered around neutral pH, (ii) a relatively short amino acid sequence for versatile applications, and (iii) a simple costless method for precipitation–redissolution by using weak acidic and weak basic solutions. These advantages are discussed in the following subsections.

Sharp pH response

The most important advantage of the CspB based peptide tag is the sharp pH response centered around neutral pH. The transition occurs within a narrow range of only 0.5 pH units, as shown in Figure 2. 3B, and the recovery after redissolution of the precipitated CspB50TEV-Teriparatide was almost 100% (Fig. 2. 3B). In contrast, the thermally responsive ELP tag has a broad transition temperature range of around 30°C–40°C [26].

The transition of the ELP tag from precipitation to redissolution usually requires an increase in temperature of 15°C and also the higher end is sometimes harsh to proteins. Accordingly, the sharp transition of CspB tag near neutral pH with high recovery is a useful property for protein purification without protein denaturation from an industrial application point of view. For example, the purification of Teriparatide usually involves three chromatographic processes, including cation-exchange chromatography followed by two types of reversed-phase chromatography [27]; the overall recovery is around 48% for the conventional purification process. In contrast, the CspB tag can be used to skip at least one chromatographic process because CspB50TEV-Teriparatide can easily be isolated from a cell lysate of *E. coli* or a culture supernatant of *C. glutamicum*, yeast, or Chinese hamster ovary (CHO) cells with high recovery through the precipitation–redissolution process by simply adjusting the pH near the sharp transition.

Versatile applications due to a short amino acid sequence

The three proteins with different characters showed pH-responsive behavior by fusing the CspB50, suggesting that this tag can be used for a wide variety of proteins. Note that all original proteins tested do not form precipitates without CspB tag. Considering the fact that the pH- responsive behavior is achieved even when its length is short, i.e., six residues, it is considered that the length of the tag might be optimized at a broad range for every target protein to be fused. CspB6 (or longer) -PIns formed precipitates at weak acidic pH but CspB5 PIns did not. The amino acid sequences of CspB5 and CspB6 are QETNP and QETNPT, respectively. It is suggested that the character built by the six amino residues is essential for pH-responsive behavior, but the role of each residue is currently unknown. One of the approaches is to use several solvent additives, such as salt, denaturant, and detergent to understand the mechanism of protein-protein interactions. According to the molecular mechanism, the CspB variant tag will be developed for various types of proteins in future. Actually, a thermally responsive ELP tag comprises simple amino acid residues with VPGXG (X stands for all amino acids other than proline). In addition, many kinds of ELP variant tags have been developed by changing the repeat number of the VPGXG sequence or by changing the guest residue (X), leading to favorable properties of the fusion proteins [28,29]. For example, the length of the ELP tag influences the transition temperature of aggregation of the fusion protein and the particle size of the aggregates [30], and the

role of the surface hydrophobicity of ELP and ELP fusion proteins has been investigated [6]. Furthermore, prediction of the transition temperature of ELP fusion proteins has been reported [31]. The CspB variant tag might offer different transition pH suitable for each target protein.

Costless method compared with chromatography

The development of efficient conventional chromatography methods involves examining and screening various types of conditions such as pH, conductivity, and the kinds of buffer and resin for both adsorption and elution [32]. To avoid this complicated development and cost required for actual production, various types of affinity tags have been developed, represented by His tag and MBP tag [1]. However, affinity chromatography also requires resins with corresponding ligands for affinity-tag purification, which raises the production cost, and sometimes other problems related to the ligand occur, such as its dissociation and/or regeneration. Thus, purification processes that do not require resins are in demand for industrial-scale purification due to their low cost. The pH-responsive CspB tag can be considered as one approach for purification without resins, and also as one approach for protein concentration process.

Figure 2. 4 shows a schematic of a possible purification process based on CspB fusion proteins. The CspB fusion protein is harvested in culture supernatant (step 1), and then the pH of the culture supernatant is lowered by the addition of acid (step 2). The cloudy solution is next centrifuged to obtain the precipitate (step 3). The supernatant of the solution of the pH-adjusted solution, including soluble impurities is removed and rinsed, after which the precipitate is redissolved by the addition of a buffer with a high pH (step 4). The supernatant of the redissolved solution, including purified CspB fusion protein, is collected (step 5), leaving irreversibly precipitated insoluble impurities. Note that the highly reversible pH-dependent aggregation is used as a protein concentration method [33,34]. When a small amount of buffer is added in steps 3–4, the CspB fusion protein is easily concentrated.

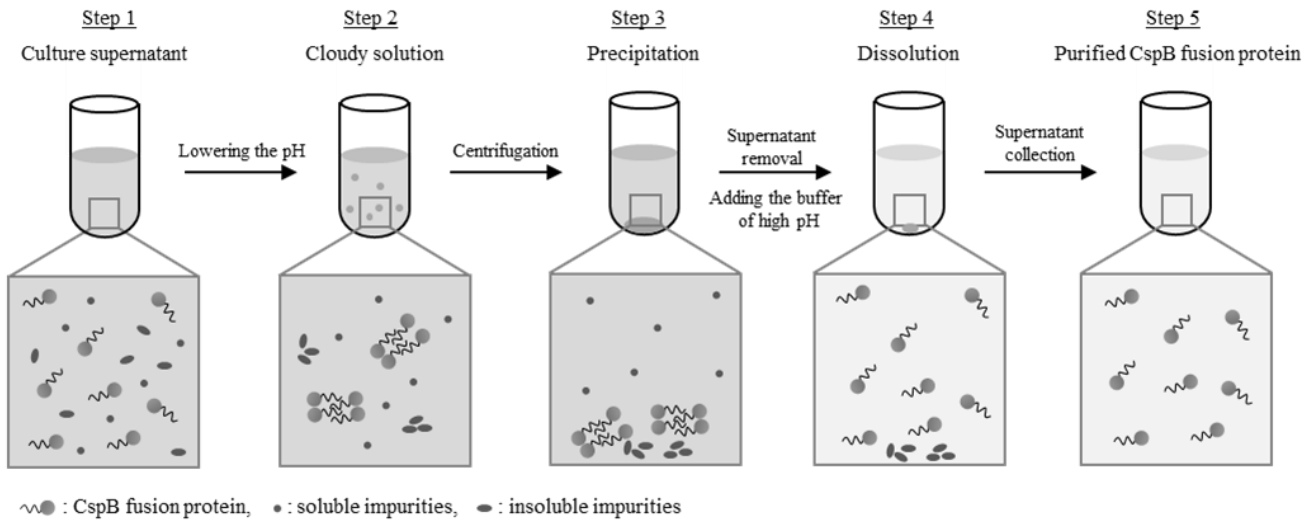

Fig. 2. 4. Schematic illustration of a possible purification method making use of the precipitation–redissolution behavior of a CspB fusion protein.

2.5. Conclusion

In conclusion, CspB fragment was investigated as a pH-responsive tag for proinsulin, Teriparatide, and Bivalirudin. The advantages of the CspB tag are (i) full reversibility of the aggregated-state protein, (ii) pH responsivity centered around neutral pH, (iii) sharp pH responsivity within 0.5 pH units, (iv) versatility of application to pharmaceutical proteins at various pH values, and (v) utilization by a simple concentration method. I believe that the pH-dependent reversible precipitation–redissolution response can be used for the purification of various proteins.

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Chapter 3.

Non-Chromatographic Protein Purification with A pH-Responsive CspB Tag

3.1. Introduction

Recombinant proteins are widely used in many applications such as pharmaceuticals and industrial enzymes. Many protein expression systems have been designed to increase the purification efficiency of recombinant proteins incorporating purification tags, which are peptides or proteins that exhibit highly specific and reversible binding to a particular ligand [1–3]. A variety of purification tags and immobilized affinity ligands are commercially available [4]. Such protein tags are typically purified from the other proteins of the host cell by affinity chromatography. This purification process is both simple and efficient on a laboratory scale, but the scale-up of affinity chromatography can introduce a significant cost in the industrial-scale production of a protein.

Thus, there is a demand for peptide tags that facilitate the non-chromatographic purification of proteins. An elastin-like polypeptide (ELP) tag has been used to purify several proteins including calmodulin, catalase, and thioredoxin [1,5,6]. The ELP tag is a thermally responsive polypeptide that is soluble in aqueous solutions at temperatures below an intrinsic transition temperature (T_t) but undergoes reversible aggregation above T_t because of hydrophobic interactions [7,8]. Thus, ELP fusion proteins are soluble in aqueous solutions below T_t, whereas they form aggregates when the temperature is raised above T_t. The aggregation (or precipitation) process of ELP fusion proteins is fully reversible. Therefore, precipitation–redissolution cycles of the ELP fusion protein can be controlled by cycling the temperature and can be leveraged for protein purification without the need for chromatography. However, the typical T_t of the ELP tag is 30–40 °C, which may induce irreversible unfolding of heat-labile proteins [6].

The CspB tag is derived from cell surface protein B (CspB) of *Corynebacterium glutamicum*. It is a pH-responsive polypeptide that undergoes reversible precipitation and dissolution according to the pH of the solution [9–12]. Thus, CspB tag has been used as an alternative purification tag for the non-chromatographic purification of CspB fusion proteins [10,11]: the CspB fusion protein is harvested in the culture supernatant of the *C. glutamicum*. The pH of the culture supernatant is lowered by adding acid. The resulting cloudy solution is centrifuged to precipitate the CspB fusion protein while the supernatant, which contains other impurities, can be decanted. The precipitate was redissolved in a 1.0 M arginine solution of pH 8.0. The pH-driven precipitation–redissolution cycle with arginine was repeated three times in order to remove the pigments and other impurities through the precipitation–redissolution cycles. The final precipitate is then redissolved by adding a neutral buffer. However, in order to obtain an intact target protein, further steps are necessary to digest the CspB tag and purify the intact target protein. In this study, I investigated the purification of Teriparatide, which is a biologic treatment for osteoporosis, as a model target protein using the pH-responsive CspB tag fusion system. This is the first report of the purification of an intact target protein from the pH-responsive CspB fusion protein.

3.2. Materials and Methods

Bacterial strain, plasmid, and the design of fusion protein

CspB50TEV-Teriparatide comprised N-terminal 50 amino acid residues of CspB, the cleavage site (six amino acid residues; ENLYFQ) of TEV protease, and Teriparatide (Fig. 3. 1A). CspB50TEV-Teriparatide was prepared according to a standard protocol for protein expression and secretion described previously [13], using a *Coryne*bacterium *glutamicum* expression system, named CORYNEX® from the underlined characters which provided as a protein expression service from AJINOMOTO Co., Inc. The amino acid sequences of CspB and Teriparatide were derived from GenBank (accession numbers BAV24076.1 and AAQ51502.1, respectively) [10]. The corresponding DNA sequence encoding CspB50TEV-Teriparatide was designed by incorporating the *C. glutamicum* codon bias (GenScript, Piscataway, NJ, USA). DNA fragments containing the promoter sequence of CspB from *C. glutamicum* and the signal sequence of CspA from *C. ammoniagenes* were fused to the CspB50TEV-Teriparatide gene. This synthesized construct was then inserted into the pPK4 plasmid and

incorporated into the YDK010 bacterial strain, which is a derivative of wild-type *C. glutamicum* (ATCC 13869) [14–21].

Expression of the CspB50TEV-Teriparatide

The *C. glutamicum* transformant expressing CspB50TEV-Teriparatide was cultured at 30 °C for 3 days in a 1 L-capacity jar fermenter. The transformant was cultured in 300 mL of an MMTG liquid medium (120 g of glucose, 2 g of calcium chloride, 3 g of magnesium sulfate heptahydrate, 3 g of ammonium sulfate, 1.5 g of potassium dihydrogen phosphate, 0.03 g of iron sulfate heptahydrate, 0.03 g of manganese sulfate pentahydrate, 450 µg of thiamine hydrochloride, 450 µg of biotin, 0.15 g of DL-methionine, and 50 g of calcium carbonate, adjusted to 1 L with water and to pH 6.7) containing 25 mg/L of kanamycin had been charged, and the pH was maintained at 6.7 by the addition of gaseous ammonia. In the jar fermenter cultivation, the CspB fusion protein was constitutively expressed and secreted into the culture broth by *C. glutamicum*. After the cultivation was completed, the culture broth was centrifuged at 12,000 g for 10 min to separate the bacterial cells. The resulting supernatant was filtered through a Stericup (Merck) for sterile filtration, and the culture supernatant was obtained, which was used for further experiments.

Isolation of CspB50TEV-teriparatdie from the culture supernatant

The 30 mL of culture supernatant was adjusted to pH 5.0 by adding a small amount of HCl to precipitate the CspB fusion protein. The sample was then centrifuged at 10,000 g for 5 min to pellet the precipitate. After the supernatant was removed, the precipitate was redissolved by adding an equal volume of a 1.0 M arginine solution (pH 8.0) to supernatant as described previously [11]. This pH-driven precipitation–redissolution cycle with arginine was repeated three times. The obtained precipitate was finally redissolved in 10 mL of 20 mM Tris–HCl buffer (pH 8.0). The initial culture supernatant and the finally obtained isolated CspB50TEV-Teriparatide sample were subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) as described in Section 2.6 to measure the CspB50TEV-Teriparatide concentrations. Then, the yield of the isolation process was calculated as the ratio between the amounts of CspB50TEV-Teriparatide in the isolated sample and initial culture supernatant (expressed as a percentage).

Enzymatic digestion of CspB50TEV-Teriparatide by TEV protease

The enzymatic digestion of CspB50TEV-Teriparatide was carried out according to the standard protocol for the TEV protease used here, proTEV plus (Promega). Briefly, CspB50TEV-Teriparatide was treated by urea solutions of 0–6 M at 25 °C for 1 h. Then, each enzymatic digestion solution was diluted to 0.5 M urea by adding 20 mM Tris-HCl buffer (pH8.0). The enzymatic digestion solution comprised 1 mM DTT, 1.3×10^{-3} unit/mL of TEV protease, 20 mM Tris–HCl (pH 8.0), and 0.5 M urea in the CspB50TEV-Teriparatide solution (which had a CspB50TEV-Teriparatide concentration of approximately 0.7 mg/mL). The enzymatic digestion process was performed in 5 mL scale for 24 h at 25 °C.

The digested samples were subjected to RP-HPLC as described in Sections 2.6, to determine digestion efficiency. The digestion efficiency was calculated as the ratio between the amount of undigested CspB fusion protein in the digested solution and the initial amount of CspB fusion protein before the digestion (expressed as a percentage).

Purification of Teriparatide by changing the solution pH

The enzymatic digestion solution, which had a pH of 8.0 after the reaction, was aliquoted, and the aliquots were adjusted to pH 6.0, 5.0, 4.0, and 3.0 by adding small amounts of HCl. The pH-adjusted samples were incubated at 25 °C for 1 h and centrifuged at 10,000 g for 5 min to separate the supernatant and the precipitate formed by the pH change. The supernatants were then subjected to SDS-PAGE and RP-HPLC to calculate the purity and yield, respectively. The purity of the experimentally obtained Teriparatide was calculated as the ratio between the intensity of the band associated with Teriparatide and the total of the intensities of the bands for all detected proteins in the final sample (expressed as a percentage). The yield was calculated as the ratio between the amounts of Teriparatide in the samples of pH 3.0 and 8.0 (expressed as a percentage).

RP-HPLC

RP-HPLC was performed using a Waters Alliance PDA system with a YMC-Triart C18 column (φ4.6 × 100 mm, a particle diameter of 5 μm, and a pore diameter of 12 nm) at 30°. Into the column, 30 μL of

the sample was injected. The mobile phases were 10 mM ammonium acetate with 10% acetonitrile (A) and 10 mM ammonium acetate with 80% acetonitrile (B). The flow rate was 1.0 mL/min, and elution gradient was 0–25% B applied over 25 min. The absorbance was measured at 280 nm. A calibration curve was created from the peak areas in samples with different concentrations of a Teriparatide reference standard (Bachem). The concentration of Teriparatide was quantified from the peak areas based on the calibration curve of Teriparatide reference standard. On the other hand, CspB50TEV-Teriparatide and Teriparatide have a value of 0.947 and 1.336, as absorbance values at 280 nm of 1.0 mg/mL, respectively. CspB50TEV-Teriparatide is underestimated about 1.4-fold (1.336/0.947) when Teriparatide is used as standard. Thus, I used the following equation when the concentration of CspB50TEV-Teriparatdie is calculated by RP-HPLC using Teriparatide as a reference standard. Actual concentration of CspB50TEV-Teriparatide [mg/mL] = estimated concentration by RP-HPLC [mg/mL] × 1.4.

SDS-PAGE

SDS-PAGE was performed using a 16.5% mini-PROTEAN peptide gel (Bio-Rad Laboratories, Inc.). The resulting protein bands were detected by applying a Bio-Safe™ Coomassie stain (Bio-Rad Laboratories, Inc.). Unstained polypeptide SDS-PAGE standards (Bio-Rad Laboratories, Inc.) and a Teriparatide reference standard (Bachem) were used as standards. The band intensities were quantified using Image Quant TL (GE Healthcare).

Protein sequencing

The RP-HPLC eluate containing Teriparatide was subjected to protein sequencing. The sequencing was performed using a protein sequencer, PPSQ-10 (Shimadzu Corporation), based on Edman degradation.

Mass spectrometry

The RP-HPLC eluate containing Teriparatide was subjected to mass spectrometry. The analysis was performed using AXIMA-TOF2 (Shimadzu Corporation) based on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Circular dichroism

The far-ultraviolet (far-UV) circular dichroism (CD) spectra were obtained for 0.1 mg/mL solutions of a Teriparatide reference standard (Bachem) and the experimentally obtained Teriparatide, each in 20 mM sodium acetate (pH 4.0). The far-UV CD spectra were measured at 25 °C using a J-720 spectropolarimeter (Jasco) in the range of 250 to 200 nm at a scanning rate of 100 nm/min. Each sample was loaded in a quartz cell with a 1-mm path length.

3.3. Results

Expression of CspB50TEV-Teriparatide

Figure 3. 1A shows the amino acid sequences of the pH-responsive CspB tag and Teriparatide. CspB50TEV-Teriparatide was composed of CspB50, which was derived from 50 amino acid residues starting at the N-terminus of CspB, a digestion sequence of six residues that are recognized and digested by TEV protease (underlined), and the target protein, Teriparatide, which includes 34 residues (bold). Secretory expression using *C. glutamicum* was carried out, and the obtained culture supernatant and a Teriparatide reference standard (0.1 mg/mL) were analyzed by SDS-PAGE. Figure 3. 1B shows that a band specific to CspB50TEV-Teriparatide could be detected in the culture supernatant. Based on the quantitative analysis by RP-HPLC, the accumulation of CspB50TEV-Teriparatide in the culture supernatant was 3.0 g/L (equivalent to approximately 1.2 g/L Teriparatide).

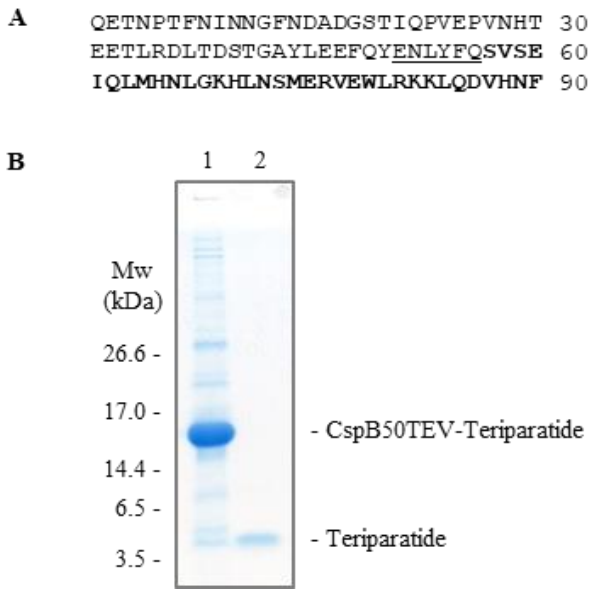

Fig. 3. 1. Expression of CspB50TEV-Teriparatide by *C. glutamicum*. (A) Amino acid sequence of CspB50TEV-Teriparatide: 50 residues from the N-terminus of the CspB sequence, six-residue spacer that is recognized and digested by TEV protease (underlined), and Teriparatide, which contains 34 residues (bold). (B) SDS-PAGE analysis of the culture supernatant (lane 1) and 0.1 mg/mL of Teriparatide as a reference standard (lane 2).

Isolation of CspB50TEV-Teriparatide

Figure 3. 2 shows the process used to isolate CspB50TEV-Teriparatide by cycling the pH: the pH of the culture supernatant was adjusted to pH 5.0, and the precipitate was redissolved in a 1.0 M arginine solution of pH 8.0. The pH-driven precipitation–redissolution cycle with arginine was repeated three times. The observed color change was due to the removal of the pigments and other impurities through the precipitation–redissolution cycles in the presence of arginine [11] as arginine increases the solubilities of aromatic compounds [22] and insoluble aggregation-prone proteins [23]. Then, the obtained precipitate was redissolved in 20 mM Tris–HCl buffer (pH 8.0). As a result, CspB50TEV-Teriparatide was successfully isolated, whereas the impurities were removed by the arginine effect. Figure 3. 2B shows the RP-HPLC results of the initial culture supernatant and the final sample of isolated CspB50TEV-Teriparatide. The final isolated CspB50TEV-Teriparatide was eluted in a single peak around 19 min. In contrast, the culture supernatant exhibited various

peaks in addition to the 19 min peak associated with CspB50TEV-Teriparatide. Based on the quantitative RP-HPLC data, the yield of the CspB50TEV-Teriparatide isolation process was about 98.9%.

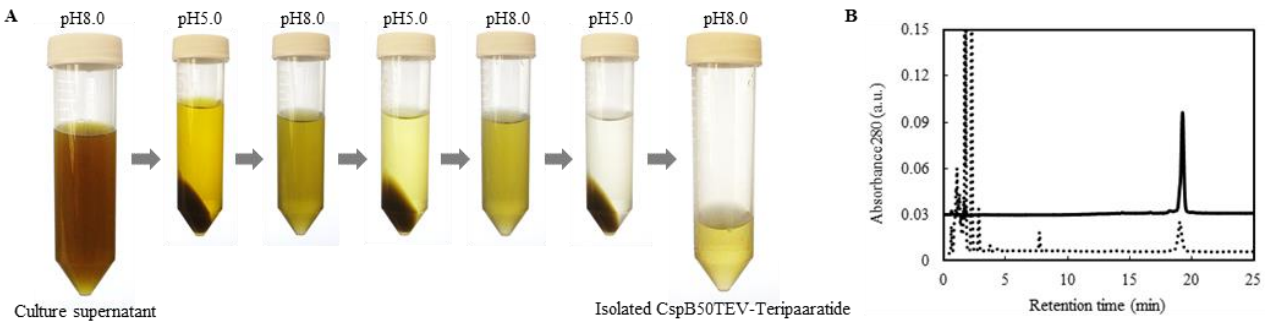

Fig. 3. 2. Isolation of CspB50TEV-Teriparatide by pH-driven precipitation–redissolution cycles. (A) Actual images of the samples over the precipitation–redissolution cycles. (B) RP-HPLC analysis of the initial sample, the culture supernatant (dotted line), and the final sample, isolated CspB50TEV-Teriparatide (solid line). A peak corresponding to CspB50TEV-Teriparatide is seen at a retention time of approximately 19 min.

Enzymatic digestion of CspB50TEV by TEV protease

In order to remove the CspB50 tag from Teriparatide, the CspB50TEV-Teriparatide was treated with TEV protease. It is possible that the TEV sequence was not exposed to the surface of the CspB50TEV-Teriparatide; thus, urea was added to loosen the tertiary structure of the CspB50TEV-Teriparatide (i.e., unfold the protein) to improve the digestion efficiency (Figure 3. 3). The digestion efficiency was only 5% without the urea treatment but was improved with increasing urea concentrations (1–6 M), reaching 98% with the 6 M urea pretreatment.

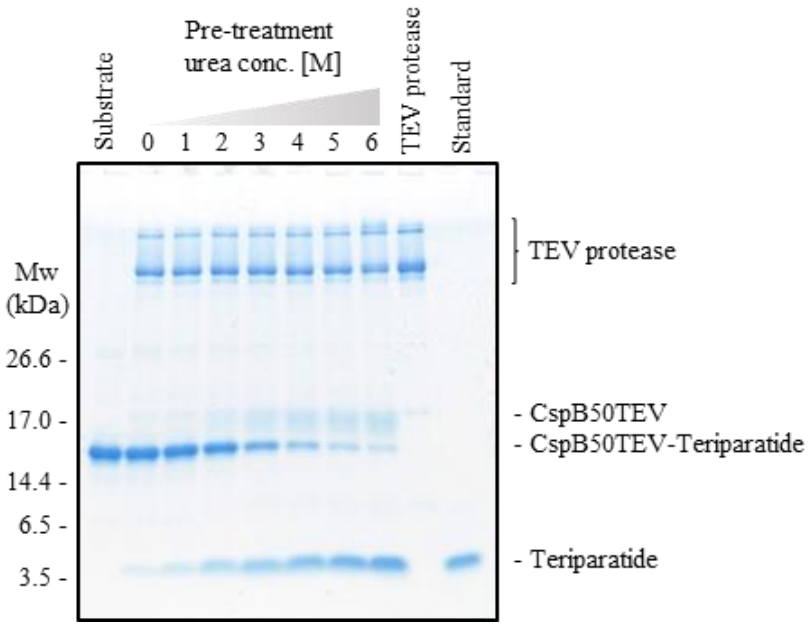

Fig. 3. 3. SDS-PAGE analysis of the TEV protease digestion of CspB50TEV-Teriparatide into CspB50TEV and Teriparatide. The samples were pre-treated with various concentrations of urea before the digestion step.

Purification of Teriparatide

After enzymatic digestion, the solution contains Teriparatide, CspB50TEV, non-digested CspB50TEV-Teriparatide, and a small amount of TEV protease. At this point, the CspB50TEV-Teriparatide and CspB50TEV can be easily insolubilized by reducing the pH due to the pH-responsive behavior of the CspB tag. Figure 3. 4 shows the results of the SDS-PAGE analysis of the solution digested by TEV protease and adjusted to various pH values (pH 8.0, 6, 5, 4, and 3). The results show that most of the impurities, but not Teriparatide, were insolubilized in the enzymatic digestion solutions of pH <5.0. Interestingly, TEV protease was also insolubilized at acidic conditions due to the acid-induced aggregation. This was an unexpected but favorable effect that further facilitated the non-chromatographic purification. Accordingly, highly pure Teriparatide was easily obtained in the supernatant with minimal loss.

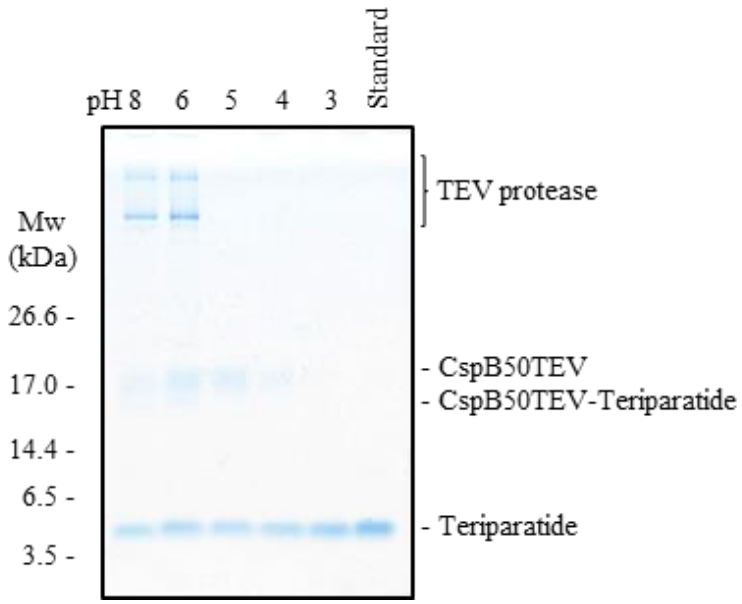

Fig. 3. 4. Purification of Teriparatide by pH changes. Teriparatide purification upon changing the pH of the solution following enzymatic digestion from 8.0 (i.e., the initial sample) to acidic pH values of 6–3 (i.e., the supernatants of pH-adjusted and centrifuged samples).

Evaluation of the purification steps and the final product

Figure 3. 5A shows the results of the SDS-PAGE analysis of the samples are different steps of the purification processes of Teriparatide from CspB50TEV-Teriparatide. The accumulation of the CspB50TEV-Teriparatide was 3.0 g/L (equivalent to 1.2 g/L as Teriparatide) in the culture supernatant following secretory expression using *C. glutamicum* (lane 1). Using the pH-responsive properties of the CspB tag, the CspB50TEV-Teriparatide was recovered from the culture supernatant by three precipitation–redissolution cycles with a final yield of 98.9% (lane 2). The sample was then treated with 6 M urea followed by the addition of a buffer and TEV protease. The enzymatic digestion yielded CspB50TEV, undigested CspB50TEV-Teriparatide, TEV protease, and Teriparatide with an efficiency of 97.6% (lane 3). The pH of the digested solution was then adjusted to 3.0 to insolubilize all components aside from Teriparatide. Finally, I obtained highly purified Teriparatide with a purity of 96.0% and a yield of 96.5%. The final productivity of the Teriparatide was 1.1 g/L of *C. glutamicum* culture.

In order to confirm the identity of the purified product, it was analyzed using a protein sequencer, MALDI-TOF-MS, and CD spectroscopy. The purified sample had the amino acid residues (SVSEIQLMHN) that were identical to the N-terminal sequence of Teriparatide. In addition, the purified sample had a mass of 4118.1 Da as measured by MALDI-TOF-MS, which is close to the theoretical mass (4117.7 Da) of Teriparatide. The far-UV CD spectrum of the purified sample was identical to that of the commercially available Teriparatide reference standard (Figure 3. 5B) with minima around 205 and 222 nm [24–28]. Although CspB50TEV-Teriparatide was treated by 6 M urea for 1 hour at enzymatic digestion process, the final urea concentration was adjusted to 0.5 M. Accordingly, it is naturally thought that Teriparatide can form an appropriate secondary structure during digestion process. Thus, it was concluded that the obtained protein was Teriparatide and that it retained the primary and tertiary structures associated with bioactive Teriparatide.

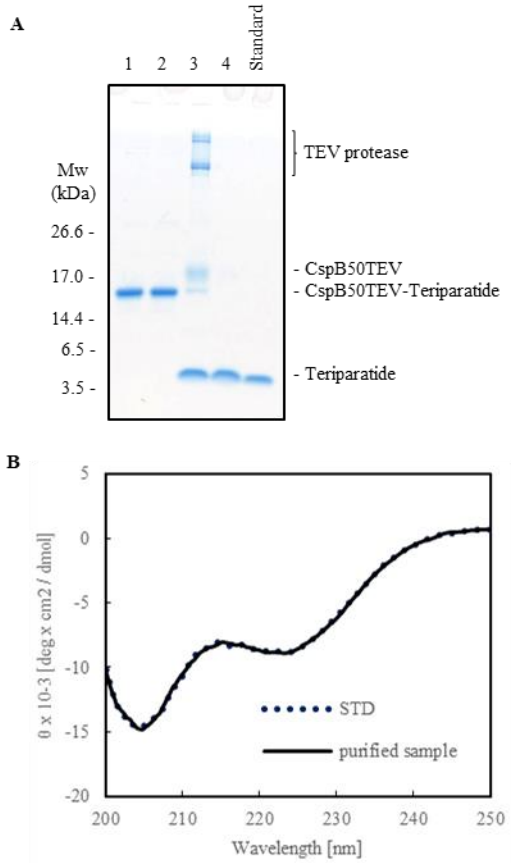

Fig. 3. 5. Evaluation of the effectiveness of the process and the obtained Teriparatide. (A) SDS-PAGE analysis of the samples obtained from the various stages in the protocol: culture supernatant with CspB50TEV-Teriparatide (lane 1), CspB50TEV-Teriparatide isolated via precipitation–redissolution cycles (lane 2), digested sample containing Teriparatide (lane 3), and acid-treated Teriparatide (lane 4). (B) Far-UV CD spectra

of a Teriparatide reference standard (dashed line) and the purified Teriparatide sample that was obtained experimentally (solid line).

3.4. Discussion

Here, I demonstrated a novel protocol for the purification of Teriparatide involving the expression of the CspB fusion protein, isolation of the CspB fusion protein, enzymatic digestion of the CspB tag, and purification of the target protein. The most valuable feature of this purification protocol is that it only requires pH adjustments and a pH meter and solid–liquid separation using a centrifuge.

The purification of fusion proteins is usually performed by chromatography, but chromatography can be expensive and time-consuming [2]. For example, the standard method for Teriparatide purification involves the expression of histidine-tagged Teriparatide in *Escherichia coli* cells followed by Ni-NTA chromatography [29–33]. After the fused tag is digested, ion-exchange chromatography and reverse-phase chromatography are used for further purification of the Teriparatide. This affinity-based process yields Teriparatide with a purity of 99.5% and productivity of 0.5 g Teriparatide/L of culture [31] and with a purity of 95.0% and productivity of 0.3 g Teriparatide/L of culture [29]. On the other hand, the non-chromatographic CspB-fusion technique proposed here is relatively simple and yields Teriparatide with a comparable final purity (98.0%) and productivity of 1.1 g Teriparatide /L of culture.

I have previously reported the versatility of the CspB tag as a pH-responsive purification tag for proinsulin (an insulin precursor) and a bivalirudin derivative [10]. Here, I demonstrated a non-chromatographic purification process that yields a tag-free protein. Moreover, various types of CspB fusion proteins can be created to achieve similar pH-responsive purification. However, the key to the purification process is the stability of the target protein denaturation in acidic condition. The CspB50TEV and TEV protease were insolubilized at pH <4.0 (Fig. 3. 4), and the Teriparatide alone remained soluble under the acidic pH. However, this characteristic cannot be guaranteed for all target proteins. Thus, only target proteins that are stable at pH 4.0 can be purified from the CspB fusion protein by the proposed non-chromatographic process. This condition is not so hard for various proteins, including monoclonal antibodies and peptide drugs.

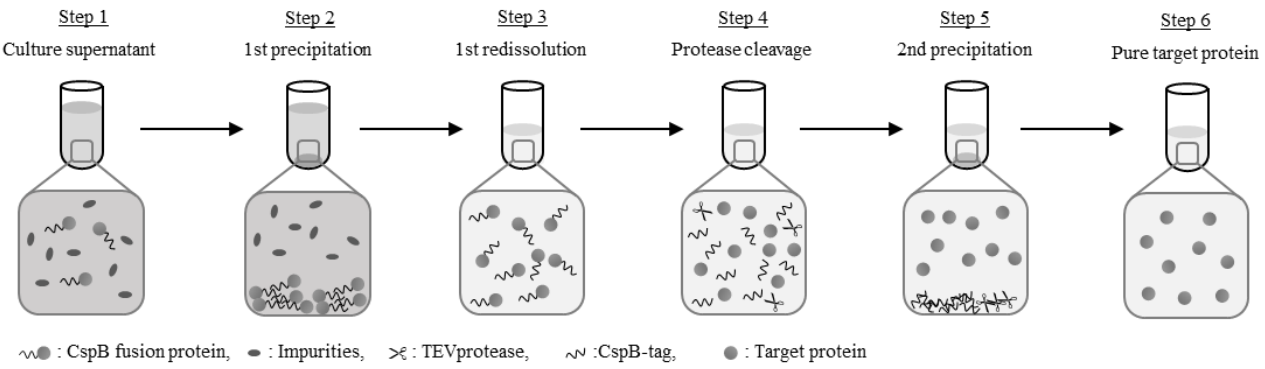

Fig. 3. 6. Schematic of the proposed process for purifying intact target protein using a CspB tag.

Figure 3. 6 shows a schematic illustration of the purification process that can potentially be used to purify other intact target proteins using the CspB tag fusion system. The target protein with the CspB tag is expressed and secreted into the culture supernatant by *C. glutamicum*, which serves as a host cell (Step 1). The pH of the culture supernatant is then reduced by adding acid, thus insolubilizing the CspB fusion protein so it can be recovered as a precipitate by centrifugation (Step 2). After the supernatant is removed, the precipitated CspB fusion protein is redissolved in an alkaline solution (Step 3). The use of arginine as an additive in this solution and repeating the precipitation–redissolution cycle facilitate the removal of impurities. Next, the CspB fusion protein is digested by TEV protease, yielding the CspB tag and the target protein (Step 4). The pH is again lowered by adding acid to insolubilize the CspB fusion protein, CspB tag, and TEV protease (Step 5). Finally, the sample is centrifuged, and the supernatant contains the highly purified target protein (Step 6).

3.5. Conclusion

Here, I developed a novel purification system for Teriparatide involving the expression of the protein with a pH-responsive CspB tag by *C. glutamicum*, an isolation of the CspB fusion protein, an enzymatic digestion of the CspB tag, and a final purification of the target protein from the digested components. The advantage of this non-chromatographic protein-purification method is its simplicity at the precipitation–redissolution cycles are actuated by adjusting the pH. Moreover, there is potential for this protocol to be broadly applied for protein preparation on laboratory and industrial scales.

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Chapter 4.

Effect of Small Additives for pH-Responsive Behavior of CspB Fusion Protein

4.1. Introduction

Protein purification process is complicated. In order to solve this problem, various type of purification tags have been developed so far [1–3]. Histidine tags are representative purification tags [4]. It has become possible to specifically purify the histidine tagged protein by specific interaction with nickel ions immobilized on the resin surface. However, the cost of the resin and the related equipment are the major cost on the industrial scale. In recent years, a new purification tag, CspB-tag that do not require resin, have been reported [5–7]. The CspB fusion protein has precipitation–redissolution properties in response to the pH of the solution. As a result, CspB fusion protein can be purified by simple pH adjustment and solid-liquid separation.

A certain concentration of salt is often added to the solution for the purpose of preventing nonspecific adsorption of the target protein to the purification instrument [8]. In addition, PPC has been reported as a method for precipitation and concentration of the proteins using a polymer electrolyte [9,10]. However, precipitation does not occur in the presence of salt. On the other hand, it has not been investigated whether the pH response of the CspB fusion protein is maintained even in the presence of various additives.

In this study, I investigated the effect of small additives on pH response of CspB fusion protein.

4.2. Materials and Methods

Preparation of CspB50TEV-Teriparatide

CspB50TEV-Teriparatide was expressed same way in Chapter 3, and then the 30 mL of culture supernatant was adjusted to pH 5.0 by adding a small amount of HCl to precipitate the CspB50TEV-Teriparatide. The sample was then centrifuged at 10,000 g for 5 min to pellet the precipitate. After the supernatant was removed,

the precipitate was redissolved by adding an equal volume of a 1.0 M arginine solution (pH 8.0) to supernatant as described previously [11]. This pH-driven precipitation–redissolution cycle with arginine was repeated three times. The obtained precipitate was finally redissolved to 1.0 mg/mL in 20 mM Tris–HCl buffer (pH 8.0).

Effect of small additives for pH-responsive behavior

Samples were prepared in which the concentration of CspB50TEV-Teriparatide was 0.5 mg / mL, and the concentration of each additive (NaCl, Na_2SO_4, Arg-HCl, urea, Gdn-HCl) was 0.5 M, respectively. The pH of the solution was adjusted to pH 4.0 by adding 2 M HCl to each sample, and then it was held at 25 °C for 1 hour. Each sample was centrifuged at 10000 g for 5 min, and the supernatant was subjected to 0.22 um filtration. The obtained filter permeate was subjected to RP-HPLC, and the concentration of CspB50TEV-Teriparatide remaining in the supernatant of each sample was calculated. Based on this concentration, the precipitation ratio was calculated by the following calculation formula. The precipitation ratio [%] = (1 - concentration of supernatant [mg/mL] / 0.5 [mg/mL]) × 100.

Effect of denaturant for pH-responsive behavior

Samples were prepared in which the concentration of CspB50TEV-Teriparatide was 0.5 mg / mL, and the concentration of each denaturant (urea, Gdn-HCl) was 0-6 M, respectively. And then, far-UV CD spectra at each denaturant concentration was obtained. After that, the pH of the solution was adjusted to pH 4.0 by adding 2 M HCl to each sample, and then it was held at 25 °C for 1 hour. Each sample was centrifuged at 10000 g for 5 min, and the supernatant was subjected to 0.22 um filtration. The obtained filter permeate was subjected to RP-HPLC, and the concentration of CspB50TEV-Teriparatide remaining in the supernatant of each sample was calculated. Based on this concentration, the precipitation ratio was calculated. On the other hand, these denaturants were removed from samples containing 6 M urea or Gdn-HCl using ultrafiltration membranes (molecular weight cut-off : 5 kDa). These renatured samples were subjected to the measurement of far-UV CD spectra. After that, by adding 2 M HCl to each sample and adjusting the pH to 4.0, the precipitation ratio was calculated in the same manner as described above.

4.3. Results

Effect of small additives for pH-responsive behavior

The effect of various additives on pH response of CspB50TEV-Teriparatide was investigated. Various additives (NaCl, Na2SO4, Arg-HCl, urea, Gdn-HCl) were added to a final concentration of 0.5 M. Thereafter, the pH of the solution was adjusted from 8.5 to 4.0. The resulting aggregation was precipitated by centrifugation, and then the concentration of CspB50TEV-Teriparatide remaining in the centrifugal supernatant was quantified by subjecting to RP-HPLC analysis. As a result, no CspB50TEV-Teriparatide was detected in the centrifugal supernatant of any sample containing any additive tested. So, it was found that CspB50TEV-Teriparatide completely precipitated at pH4.0, even in the presence of each additives (Fig. 4. 1).

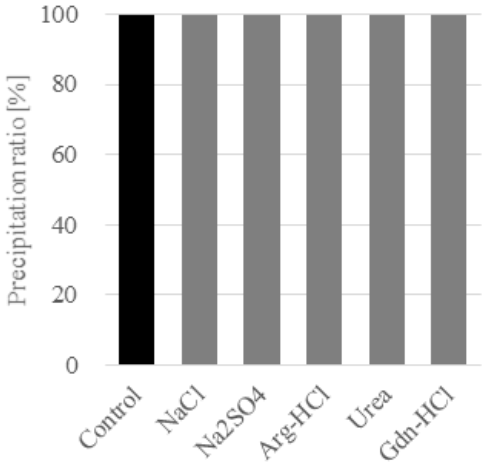

Fig. 4. 1. The effect of various additives on precipitation ratio of CspB50TEV-Teriparatide

Effect of denaturant for pH-responsive behavior

Next, by adding urea and Gdn-HCl known as a protein denaturant to a final concentration of 6 M, the relationship between pH response behavior and secondary structure of CspB50TEV-Teriparatide was investigated. As a result, in the addition of urea, the precipitation ratio decreased at 3 M, and it appeared that the pH response of CspB50TEV-Teriparatide was lost because no precipitate was formed at 4 M or more (Fig. 4. 2A). On the other hand, the results from the far-UV CD spectra, the secondary structure was lost as the urea concentration became higher, and almost the same CD spectrum was observed at 4 M or more (Fig. 4. 2C). A similar trend was observed when plotting 222 nm meaning the presence of α-helix (Fig. 4. 2E). Similarly, in

the addition of Gdn-HCl, the precipitation ratio decreased at 1 M, and it appeared that the pH response of CspB50TEV-Teriparatide was lost because no precipitate was formed at 4 M or more (Fig. 4. 2B). On the other hand, the far-UV CD spectra were significantly different in the presence of 1 M Gdn-HCl and the absence condition. The secondary structure was lost as the Gdn-HCl concentration became higher, and almost the same CD spectra was observed at 4 M or more (Fig. 4. 2D). A similar trend was observed when plotting 222 nm meaning the presence of α-helix (Fig. 4. 2F). In both denaturant treatment, pH response was lost at 4 M or more and at the same time the secondary structure was also disappeared. However, for urea and Gdn-HCl, the CD spectra at each denaturant concentration and the plot at 222 nm were significantly different.

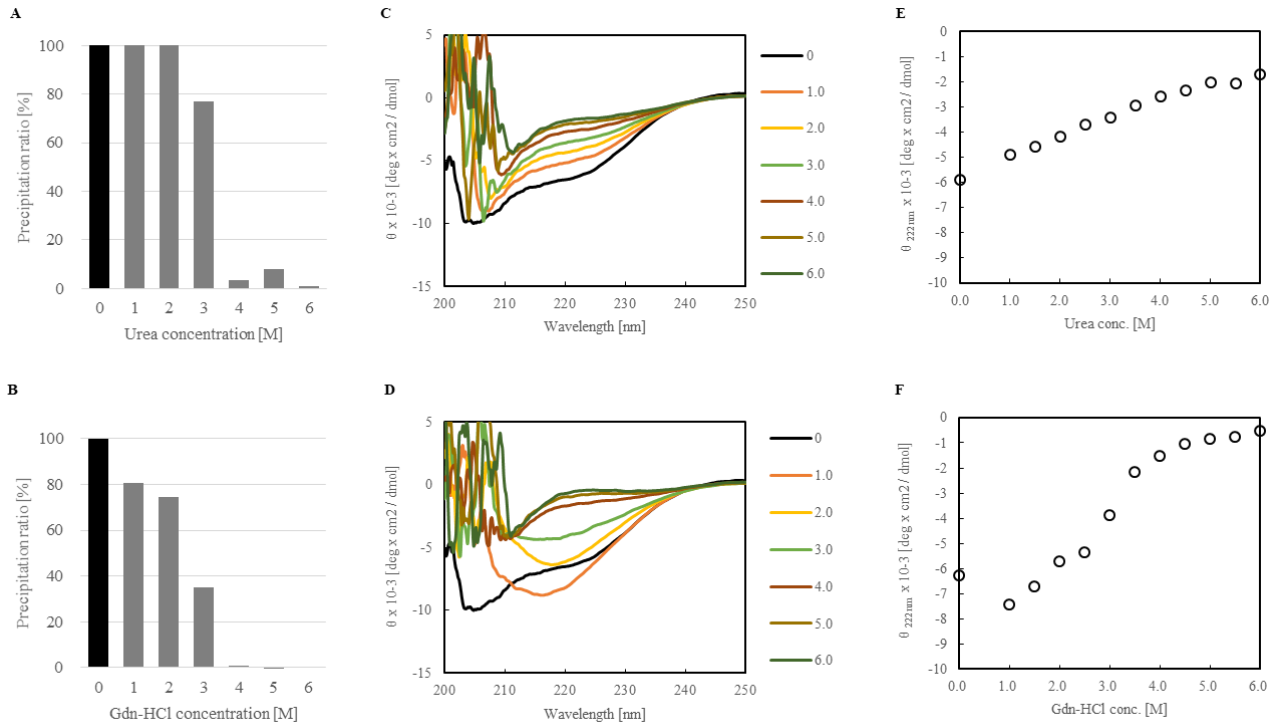

Fig. 4. 2. Effect of denaturant for pH-responsive behavior of CspB50TEV-Teriparatide. (A, B) Precipitation ratio at pH4.0 in the presence of urea and Gdn-HCl, respectively. (C, D) Far-UV CD spectra at pH8.0 in the presence of urea and Gdn-HCl, respectively. (E, F) Plot at 222nm of far-UV-CD spectra at pH8.0 in the presence of urea and Gdn-HCl, respectively.

Next, it was investigated whether the pH response was restored by removing the denaturant by buffer exchange from denatured CspB50TEV-Teriparatide with 6 M Urea or Gdn-HCl. As a result, CspB50TEV-

Teriparatide in the renatured sample completely precipitated at pH 4.0 and pH response was restored. The secondary structure, which had been greatly reduced in the presence of 6 M denaturant, showed a similar CD spectra with original CspB50TEV-Teriparatide (Fig. 4. 3).

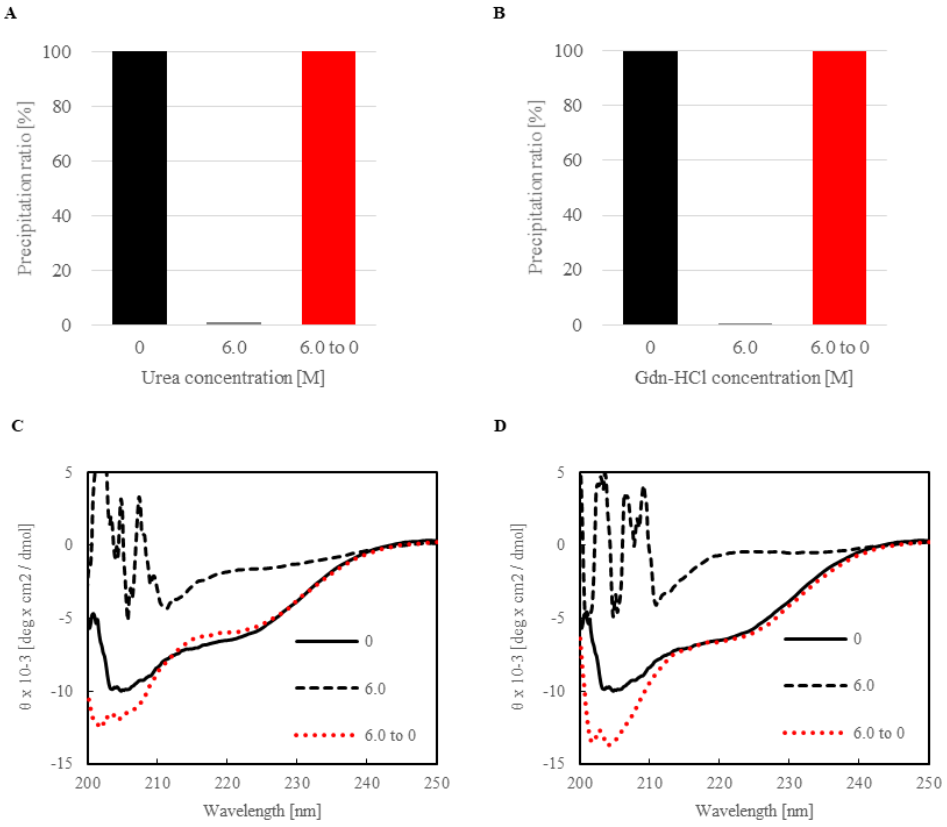

Fig. 4. 3. Comparison of pH-responsive behavior of original, denatured and renatured CspB50TEV-Teriparatide. (A, B) Precipitation ratio at pH4.0 of the original (0 M), denatured (6.0 M) and renatured (6.0 to 0 M) CspB50TEV-Teriparatide by urea and Gdn-HCl, respectively. (C, D) Far-UV CD spectra at pH8.0 of the original (0 M), denatured (6.0 M) and renatured (6.0 to 0 M) CspB50TEV-Teriparatide by urea and Gdn-HCl, respectively.

4.4. Discussion

For several additives that could coexist in the purification process, I examined the effect of CspB fusion protein on pH response. It was found that the pHresponse of the CspB fusion protein was not lost even in the presence of 0.5 M in the additives tested this time, so stable pH response was obtained. From the study using

a denaturant, the secondary structure and the pH response was lost under the high denaturant concentration. It was suggested that a specific secondary structure derived from CspB-tag is necessary to express the pH response. The pH response was restored after removal of the denaturant and its secondary structure also showed a far-UV CD spectra similar to the original one, suggests the importance of the specific secondary structure of CspB-tag.

It is interesting to show different far-UV CD spectra when urea and Gdn-HCl are used as denaturant. Especially in the presence of relatively low denaturant concentration of 1 to 3 M, CD spectra are largely different, showing own properties of each additive more than the denaturation effect, for example, as a salt of Gdn-HCl. By observing the pH response, secondary and tertiary structure in the presence of additives other than tested in this report, it is possible to elucidate the pH responsive mechanism of CspB-tag and become a more versatile technique.

4.5. Conclusion

Various salts that may be present in the purification process did not inhibit the pH response of the CspB fusion protein. The secondary structure is important to express pH response, and removal of the denaturant restored the lost secondary structure and pH response. From the results, it was found that purification of CspB fusion protein by precipitation–redissolution utilizing pH response can be stably performed.

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Chapter 5.

General discussion

Protein is a useful material owing to its functionality. Notably, the pharmaceutical application of protein is promised field. However, the complexity and high cost of purification process of protein is still an issue. In this thesis, I provide the knowledge of purification tag as the method of purifying the pharmaceutical protein. In this section, the overview, perspective and future study of this thesis are described.

The thermally responsive ELP-tag is known as a purification tag not requiring chromatography. I was able to develop a new pH-responsive CspB-tag as a first alternative of ELP-tag. It is known that native CspB forms an S-layer by self-organization, and covering the cell surface of microorganisms. It is quite interesting to be able to develop purification tags with pH response by using partial fragments of CspB. That is, it was suggested that it is possible to develop new purification tags from the protein which forms structures in nature, such as proteins of viral envelope or fragments thereof. Based on these points of view, it is expected that the research field of purification tags that become insoluble and solubilized in response to environmental changes will become more active.

Using the CspB fusion protein as a raw material, it was shown that the target protein can be obtained non-chromatographically through enzyme cleavage process. On the other hand, in the enzyme cleavage process, pretreatment with high concentration urea was necessary to improve cleavage efficiency. It is considered that the TEV sequence recognized by the cleavage enzyme was exposed by relaxing the structure of the entire CspB fusion protein. Likewise, it is conceivable that the structure of the target protein is relaxed, and the essential secondary structure and tertiary structure may be lost. In this report, a fusion protein (CspB50TEV-Teriparatide) linked with CspB-tag, TEV sequence and target protein is used, but add a flexible linker (e.g. 10 residues of Glycine) behind the CspB-tag sequence, it is thought that the TEV sequence will be exposed without urea pretreatment. As a result, it can be considered that it can be applied as a method for producing various proteins in which secondary structure or tertiary structure plays an important role.

The mechanism of pH responsiveness of CspB-tag developed this time has not been elucidated at the moment. Since it responds to pH change, it is considered to be related to the charge state of the amino acids of the CspB-tag. High concentration electrolytes (i.e. salts) were added to the solution for the purpose of inhibiting such interaction due to charging, but pH response of the CspB fusion protein was not lost. On the other hand, the CspB fusion protein denatured by denaturant lost pH response. I hope that by clarifying changes in secondary structure, tertiary structure, association state and pH responsiveness in the presence of various additives, I can gradually elucidate the mechanism concerning pH response of CspB-tag.

Chapter 6.

General Conclusion

This thesis investigated the development of a new purification tag for protein purification. Chapter 2 describes a newly developed CspB-tag which insolubilizes-solubilizes in response to pH. In conclusion, CspB fragment was investigated as a pH-responsive tag for proinsulin, Teriparatide, and Bivalirudin. The advantages of the CspB tag are (i) full reversibility of the aggregated-state protein, (ii) pH responsivity centered around neutral pH, (iii) sharp pH responsivity within 0.5 pH units, (iv) versatility of application to pharmaceutical proteins at various pH values, and (v) utilization by a simple concentration method. Chapter 3 describes development of production process for pharmaceutical protein using CspB-tag. Here, I developed a novel purification system for Teriparatide involving the expression of the protein with a pH-responsive CspB tag by *C. glutamicum*, an isolation of the CspB fusion protein, an enzymatic digestion of the CspB tag, and a final purification of the target protein from the digested components. Chapter 4 describes an effect of small additive on the pH response behavior of CspB fusion protein. I hope that by clarifying changes in secondary structure, tertiary structure, association state and pH responsiveness in the presence of various additives, I can gradually elucidate the mechanism concerning pH response of CspB-tag. I believe that the pH-dependent reversible precipitation–redissolution response can be used for the purification of various proteins. And the advantage of this non-chromatographic protein-purification method is its simplicity at the precipitation–redissolution cycles are actuated by adjusting the pH. Moreover, there is potential for this protocol to be broadly applied for protein preparation on laboratory and industrial scales.

List of Publications

Related Publications

1. **T. Nonaka,** N. Tsurui, T. Mannen, Y. Kikuchi, K. Shiraki, Non-chromatographic purification of Teriparatide by pH-responsive CspB tag, Protein Expr. Purif. 155 (2019) 66–71.
2. **T. Nonaka**, N. Tsurui, T. Mannen, Y. Kikuchi, K. Shiraki, A new pH-responsive peptide tag for protein purification, Protein Expr. Purif. 146 (2018) 91–96.

Other Publications

1. S. Oki, **T. Nonaka**, K. Shiraki, Specific solubilization of impurities in culture media: Arg solution improves purification of pH-responsive tag CspB50 with Teriparatide, Protein Expr. Purif. 146 (2018) 85–90.
2. **T. Nonaka**, T. Mannen, N. Tsurui, [Method for producing protein by precipitation], WO2014/126260 A1 (21. 08. 2014)

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