Improvement of FGF7 Thermal Stability by Introduction of Mutations in Close Vicinity to Disulfide Bond and Surface Salt Bridge

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Abstract
Fibroblast Growth Factor 7 (FGF7), a growth factor specific to epithelial cells, has attracted attention as a therapeutic protein. However, FGF7 has a limitation in its use due to low protein stability. Here, the mutations were designed to increase the stability of FGF7 by analyzing its 3D structure and sequence of other FGFs. Palifermin, N-terminal truncated FGF7 is known to have improved stability and was used as control protein in our study. The K126 and K178 were substituted into glutamate to form salt bridge with the neighboring residue R175 respectively and A120C mutation was introduced in close vicinity to disulfide bond between C133 and C137. The data of Circular Dichroism (CD) showed that all mutant proteins tested had higher $T_m$ value than Palifermin and $T_m$ of A120C/K126E/K178E FGF7 mutant protein was 15.24 °C higher than that of Palifermin. The results of cell proliferation activity and soluble protein analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) after 37 °C or 45 °C incubation exhibited that the stability of A120C mutant protein and A120C-including mutant proteins was improved. These results suggest that the mutation of amino acid in close vicinity to disulfide bond and the salt bridge at the surface of FGF7 enhanced thermal stability and make FGF7 more useful for pharmaceutical and cosmetical application.

Keywords Salt bridge · Thermal stability · Fibroblast growth factor 7 · KGF · FGF7

Introduction
Fibroblast growth factor 7 (FGF7) or exogenous keratinocyte growth factor (KGF1) is one of the 22 FGF families (Eswarakumar et al. 2005; Harmer et al. 2004; Hui et al. 2018; Imamura 2014; Ornitz and Itoh 2001) and belongs to subfamily of FGF3/7/10/22. FGF7 plays an important role in regulation of embryonic development, cell differentiation and cell proliferation by binding to epithelial cell-specific FGFR 2IIIb with heparin or heparan sulfate (Jang et al. 1997). Due to its cell proliferative activity, FGF7 has been developed as therapeutics for wound healing and tissue regeneration. Currently, recombinant FGF7 in its N-terminally truncated form, called Palifermin, is prescribed to remedy oral mucositis caused by chemotherapy and radiation therapy used to treat cancers of blood or bone marrow (Spielberger et al. 2004). Palifermin has been reported to effectively reduce the incidence and duration of oral mucositis (Henke et al. 2011) and increase thermal stability and activity than wild-type FGF7 (Hsu et al. 2006). However, the biological half-life of Palifermin is not long enough yet, so its stability improvement is required for better usefulness.

All FGFs protein have core FGF domain that contains $\beta$-trefoil fold consisting of 12-stranded $\beta$-sheet structure, therefore FGFs share similar structure (Bellosta et al. 2001; Eriksson et al. 1991; Osslund et al. 1998; Plotnikov et al. 2001; Zhu et al. 1991). This structural similarity of FGFs helps to study and improve the stability and activity by comparing differences between FGFs. Generally, introduction of disulfide bond in protein can increase thermal stability
can bind to heparin (Kan et al. 1993; Plotnikov et al. 1999). Most of FGFs have positive charge clusters that affect stability, there have been several results to overcome the problem. Reducing agent.

The bond between C133 and C137, which was due to the presence of cysteine (Lee and Blaber 2009). The substitution of Ala 66 (120 for FGF7) of FGF1 into Cys 66 yielded to a disulfide bond between 66 and 83th amino acid, which increased stability (Lee and Blaber 2010, 2013). Even though A120 (FGF1 mutation A66C) of FGF7 is not cysteine, a disulfide bond between C133 and C137 (FGF1 C83) was already formed (Hsu et al. 1998), which was unusual to the structure of typical FGF family. However, the crystal structures of FGF7 (PDB: 1qql, 1qqk) exhibited no disulfide bond (120 for FGF7) of FGF1 into Cys 66 yielded to a disulfide bond in the same location of FGF8, because the amino acid of these FGFs corresponding to 109th residue of FGF8 is not cysteine (Lee and Blaber 2009). The substitution of Ala 66 (120 for FGF7) of FGF1 into Cys 66 yielded to a disulfide bond between 66 and 83th amino acid, which increased stability (Lee and Blaber 2010, 2013). Even though A120 (FGF1 mutation A66C) of FGF7 is not cysteine, a disulfide bond between C133 and C137 (FGF1 C83) was already formed (Hsu et al. 1998), which was unusual to the structure of typical FGF family. However, the crystal structures of FGF7 (PDB: 1qql, 1qqk) exhibited no disulfide bond between C133 and C137, which was due to the presence of reducing agent.

Since FGF7 can be utilized in various fields but has low stability, there have been several results to overcome the problem. Most of FGFs have positive charge clusters that can bind to heparin (Kan et al. 1993; Plotnikov et al. 1999). Addition of negatively-charged heparin to FGFs with positively-charged clusters brings electrostatic stability through the binding between the two molecules. And the additives such as heparin, osmolyte, and salt increased the melting temperature and elongated half-life of native FGF7 (Chen and Arakawa 1996; Chen et al. 1994). The replacement of R175 in FGF7 with Glu or Gln increased protein stability. The improvement of protein stability by R175 mutation may result from ionic interaction around the residue. However, as a result of analyzing the 3D structure, it is expected that stability can be further improved through mutations around R175.

In this study, in order to increase the stability of FGF7, we introduced mutations into N-terminal truncated FGF7, Palifermin because N-terminal deletion of FGF7 was known to increase the stability (Hsu et al. 2006). The effects of salt bridge and disulfide bond in mutant proteins on stability were examined because two methods were generally used to enhance thermal stability of protein. Although the formation of a disulfide bond between C133 and C137 has been reported, A120C mutation may affect the disulfide bond between C133 and C137 because the cysteine in the same position of FGF8 form disulfide bond with C137 of FGF8 (Olsen et al. 2006). We investigated whether this substitution increase thermal stability of FGF7. In addition, when lysine is replaced with glutamate at 126th and 178th, it is expected that the electrostatic interaction with R175 will be strengthened and the thermal stability will be improved. Based on sequence comparison and 3D structure, 3 amino acids were replaced in Palifermin and we found out that these substitutions enhance the thermal stability of protein, which can improve the value of FGF7 as an industrial and medical material.

Materials and Methods

Cloning, Expression, and Purification of FGF7 Proteins

The gene encoding residues 32–194 of hFGF7(∆31N_hFGF7) and residues 55–194 of hFGF7(∆54N_hFGF7) was inserted into the expression vector pCold I (Takara, Japan) to have additional 17 residues including 6 histidines at its N-terminus (MNHKVHHHHHHIEGRHM). The coding genes of ∆31N_hFGF7 and ∆54N_hFGF7 were chemically synthesized (BIONICS, Republic of Korea). After verifying the DNA sequence, plasmid DNA was transformed into Escherichia coli BL21(DE3).

The transformed cells were grown to an A600 of ~0.6 in Luria–Bertani medium containing 50 g/mL at 37 °C and then the expression of FGF7 was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside. After an 18 h induction at 20 °C, cells were harvested and resuspended in a 20 mM Tris buffer (pH 8.0), 200 mM NaCl and disrupted by sonication and then centrifuged at 13,000 rpm for 40 min at 4 °C. The resulting supernatant was loaded onto a Ni–NTA column (Qiagen, Germany) with washing buffer 20 mM Tris–HCl (pH 8.0) with 30 mM imidazole and eluted with the same buffer containing 500 mM imidazole. The peak fractions were applied to a heparin column (GE Healthcare, USA) with 20 mM Tris buffer (pH 8.0), 100 mM NaCl and eluted with a linear gradient of 500–1800 mM NaCl in 20 mM Tris buffer (pH 8.0). The elution of bound FGF7 was performed using a gradient of NaCl from 500 to 1800 mM containing 20 mM Tris buffer (pH 8.0). The eluted fractions containing FGF7 were applied to a Superdex 75 HR 16/60 column (GE Healthcare, USA) equilibrated with buffer containing 1X PBS buffer.

3D Prediction Model, Structure Analysis and Sequence Comparison

The human FGF7 prediction protein structure model was designed by substituting the human sequence in the crystal structure of FGF7(1qqk) and FGF7/1 chimera(1qql) using the SWISS MODEL server (http://swissmodel.expasy.org). To compare the similarity of the 3D structures, the PyMOL program was used to superimpose the two protein structures and then calculated the root mean square deviation (RMSD). Protein sequence alignment was calculated using MultAlin (Corpet 1988) and ESPript 3.0 (Robert and Gouet 2014) servers.
Homology Modeling and Prediction of Protein Formulation Properties

The 3D structure of human FGF7 was required to be constructed using homology modeling method for lack of its crystal structure. As the amino acid sequence of human FGF7 was retrieved and imported to the Basic Local Alignment Search Tool (BLAST) for protein in NCBI to obtain a suitable protein template, 1QQK in the Protein Data Bank (PDB) database, respectively. Furthermore, all procedure, from the sequence alignment between the target and template sequences, align by structure similarity, analyze structure, build homology models, verify protein using MODELLER and protein formulation properties at pH 6.0 were performed using Discovery Studio 2021 software (BIOVIA, USA).

Docking and Analysis of Protein Complexes

Since the receptor known to bind FGF7 is FGFR2 (P21802), 1DJS, one of the crystal structures of FGFR2, was used to calculate the mutual binding energy. Using the ZDOCK module of Discovery Studio 2021, the interaction between FGF7 model and FGFR2 (PDB ID: 1DJS) was simulated. The ranking of the poses was done by ZDOCK score. The 2000 predicted docked conformations were subjected to refinement and re-ranking using RDOCK module in order to remove clashes and optimize polar and charge interactions that helps to pick up a near-native conformation.

Site-Directed Mutagenesis

The mutant proteins of human FGF7 protein have been produced (Bionics, Republic of Korea) by variety of mutations; A120C, A120C/K126E, A120C/K178E, A120C/K126E/K178E. The mutant proteins of FGF7 used in the manuscript are listed in Table 1.

Mass Spectrometry Analysis

100 µg of FGF7 sample was digested in solution. Briefly, samples were denatured with 8 M urea and reduced/non-reduced with/without 10 mM dithiothreitol for 30 min at 37°C, followed by alkylation with 25 mM iodoacetamide for 30 min at room temperature. For enzyme digestion, trypsin enzymes were used and added at a ratio of 1 µg trypsin: 50 µg protein and then samples were incubated overnight at 37°C. After incubation, samples were desalted by C18 SPE cartridge. The extracted peptides were analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) coupled with an Easy-nLC system (Thermo Fisher Scientific). Peptides were reconstituted in 0.1% formic acid and separated on analytical column (C18, 2 µm particle size, 50 µm id × 15 cm length, Thermo Fisher Scientific). Samples were eluted with a linear gradient of solvent B (100% Acetonitrile, 0.1% formic acid); 5–45% over 45 min, 45–80% over 2 min at a flow rate of 300 µl/min. The separated peptide ions were entered into the mass spectrometer at an electrospray voltage of 2.3 kV. All MS/MS spectra were obtained in a data-dependent mode for fragmentation of the fifteen most abundant peaks from the full MS scan with 30% normalized collision energy. The dynamic exclusion duration was set at 30 s and the isolation mass width was 1.2 m/z. MS spectra were acquired with a mass range of 350–2000 m/z and 70,000 resolution at m/z 200. MS/MS resolution was acquired at a resolution of 17,500.

For identification of disulfide-bonded peptides, we used the BioPharma Finder 4.0 software (Thermo) in the reduced and non-reduced protein digests. The mass tolerance of precursor ions and fragment ions were set to 5 ppm and 0.5 Da, respectively. Carbamidomethylation of cysteine (+ 57.021 Da) was specified as a fixed modification, and oxidation of methionine (+ 15.995 Da) was included as variable modifications. For BioPharma Finder search, S/N threshold was set to 100 and ms noise level was defined by ms signal threshold to be 0.01% intensity of the base peak chromatogram peak top. Trypsin was selected for digestion. The maximum peptide length was set to 50 amino acids. Confidence score was set by higher than 99% for the analysis.

Circular Dichroism Spectroscopy

Circular dichroism (CD) measurements were performed by a Jasco J-1500 equipped with Jasco PTC-517 Peltier thermostatted cell holder (Jasco Corporation, Japan) and bath circulator (JEIOTECH, Korea). The protein concentration

| Table 1 | Indication of the FGF7 name described in the manuscript |
|---------|---------------------------------------------------------|
| Analog class | Abbreviation | Full name |
| Control | Wild-type | Δ31N_hFGF7 |
| Palifermin | | Δ54N_hFGF7 |
| Amino acid substitutions | A120C | Δ54N_hFGF7(A120C) |
| | A120C/K126E | Δ54N_hFGF7(A120C/K126E) |
| | A120C/K178E | Δ54N_hFGF7(A120C/K178E) |
| | A120C/K126E/K178E | Δ54N_hFGF7(A120C/K126E/K178E) |
was ~0.1 mg/ml in quartz cuvette with a path length of 0.1 cm. All data were averaged over three scans with signal subtraction by protein-free PBS buffer.

The far-UV CD spectra were performed over the wavelength range of 190 to 260 nm at 20 °C. The data were collected by continuous scanning at data pitch and bandwidth of 1 nm, a scanning speed of 200 nm/min and a digital integration time (D.I.T.) of 1 s, respectively. The influence of differences in concentration between proteins was minimized in the representation of the spectra by the mean residue molar ellipticity (Woody 1996).

Thermal denaturation experiment was measured at 195 nm with a heating rate of 1 °C/min, raising the temperature interval of 5 °C from 20 to 85 °C. Transition curves indicated the native and fully denatured states of proteins in the range of 0–100% by the standard equation: % CD\textsubscript{denaturation} = ((θ\textsubscript{20} − θ\textsubscript{temp})/(θ\textsubscript{20} − θ\textsubscript{85})) × 100; θ\textsubscript{temp} represents the ellipticity observed on specific temperature. Each T\textsubscript{m} was calculated using the OriginPro 2021 program (OriginLab Corporation, USA) through a non-linear adjustment by the Boltzmann method, where the inflection point of the curve corresponds to the value of Tm (Moro Perez et al. 2019).

Thermal Stability of Proteins Assessed by SDS-PAGE

The protein samples (0.5 mg/ml, volume 40 μl) were mixed with 1X PBS and incubated for 7 or 15 days at 37 or 45 °C. Soluble proteins were collected after centrifugation (12,000 rpm, 10 min). Protein samples were prepared in SDS sample buffer (10% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 0.25 M Tris–HCl (pH 6.8), 0.1% bromophenol blue, and 30% glycerol), boiled for 3 min, and resolved by 15% SDS-PAGE. Quantification reflects the relative amount of soluble protein as the ratio of each protein band using image J.

Cell Culture and Cell Proliferation Assay

HaCaT cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO\textsubscript{2} incubator. To determine the cell proliferation activity of FGF7s, cells (0.6 X 10\textsuperscript{4} / well) were seeded on 96 well plate and treated with FGF7 wild-type or FGF7 mutant proteins in DMEM contained with 0.03% BSA and heparin (10 μg/mL) for 48 h. The levels of proliferation were measured using cell counting kit-8 (CCK8; Dojindo, Gaithersburg, MD, USA) according to the manufacturer’s protocol. The results were expressed as the mean ± standard error (SEM). Statistical significance was calculated using one-way ANOVA followed by Tukey’s post hoc test. A P-value of <0.05 was considered statistically significant.

In Silico Analysis of Stabilization Energy

All the residues in the models of A120 of human FGF7 were mutated to remaining 19 amino acids after analysis with the software Calculate mutation energy (Stability) in Discover studio 2021. The mutation energy of every amino acid residue was calculated, and the effect of mutation was classified as neutral, stabilizing and destabilizing. The top three residues that caused destabilizing effects were selected for site-directed mutagenesis experiments.

Results

Designing Mutation of FGF7 to Increase Thermal Stability

Disulfide bonds are known to play an important role in protein stability (Fass 2012). Cross-linking by cystine disulfide is usually closer than ~5.5 Å distance between Cβ and Cβ (Dombkowski et al. 2014) and the CS-SC dihedral angle is ±90° (Van Wart et al. 1973). This criteria for bond distance and angle was adopted to make stable FGF1 mutant protein by replacement of Ala into Cys in 66th amino acid of FGF1 leading to disulfide bond with C83 (Lee and Blaber 2009, 2010). And the two residues containing cysteine at the same position in other native FGFs such as FGF8 formed disulfide bond (Lee and Blaber 2009). The stabilization strategy of FGF1 by disulfide bond formation can be applied to FGF7 that have the same β-trefoil fold form. The RMSD value of FGF7 (PDB: 1qqk) and disulfide bond-induced FGF1 (PDB: 3hom) is 0.888Å, which means that they may have similar structure. Comparison of amino acid sequence and structure between FGF1 and FGF7 revealed that two amino acids (A66C and C83) of FGF1, which are involved in disulfide bond, and the two amino acids (A120C and C137) of FGF7 are located in the same position (Fig. 1). And the distance between Cβ and Cβ of the two amino acids of FGF7 (A120C and C137) is 4.74 Å, which is within the acceptable disulfide bond range (Fig. 1a). Therefore, it was likely that a disulfide bond would form between A120C and C137 of FGF7. However, mass analysis of FGF7 had revealed that native FGF7 had already disulfide bond between C133 and C137 (Hsu et al. 1998), while 3D structure of FGF7 retains reduced form at C133 and C137. We examined that alanine to cysteine substitution at position 120 of FGF7 can perturb the structure and/or stability of the native FGF7.

Salt bridge in protein is formed by electrostatic attraction between oppositely charged residues and also contributes to enhance protein stability (Alsop et al. 2003; Dominy et al. 2004; Xiao and Honig 1999). It was reported that the stability of FGF7 increased when R175 was replaced with Glu and there is ionic interaction between the positive residues.
of adjacent amino acids and the negative polarity of the substituted E175 (Hsu et al. 2006). In this study, attention was paid to 3 residues K126, K177, and K178 that have positive properties around R175 residues to increase stability. However, FGF7 structure (1qqk pdb) analysis showed that NZ1 of K177 already formed a salt bridge between the OE1 atoms of E126 (Fig. 1a), and thus was excluded from mutation candidates. Glu substitution of K126 and K178 is expected to increase stability through ion bonding with R175 (Fig. 1a and Supplementary Fig. 1a–d).

Fig. 1 3D structure and sequence alignment analysis for protein engineering. a To confirm the structural similarity, FGF7 and FGF1 were superimposed with the pymol program and shown as a ribbon. Residues are shown as sticks. FGF7 and FGF1 are shown in green and magenta colors respectively. b Sequence comparison of human FGF1 and human FGF7. The sequence of FGF7 contains 32 signal peptides. The start of the Palifermin sequence is indicated by a black arrow. Residues related to disulfide bonds are indicated by black boxes. Residues related to the salt bridge are marked with green boxes.
To confirm whether FGF7 mutant were properly designed, modeling of FGF7 proteins was performed by MODELLER installed in the Discovery studio 2021. The scheme of modeling process was summarized as Supplementary Fig. 2. The validation of mutants was expressed by Discrete Optimized Protein Energy (DOPE) score and normalized DOPE (Eramian et al. 2008; Shen and Sali 2006). DOPE score is used to choose better protein model and the lower the DOPE score is, the better the model is. The DOPE score and normalized DOPE score of mutant A120C/K126E/K178E are lower than that of wild type, which means that the mutant has more stable conformation and the model of the mutant was validated (Supplementary Fig. 3).

**Mass Analysis of Wild-Type and FGF7 Mutant Proteins**

To see whether the recombinant FGF7 proteins were produced correctly, the mass of FGF7 proteins were determined. The result of identified free thiol residue and disulfide-linked peptides was shown as Table 2. Two cysteines (C34 and C83) were modified by carbamidomethylation (57.02146 Da) in the non-reduced condition, which represent the free thiol residue and the other two cysteines (C96 and C100) were linked by disulfide-bridge. The base peak chromatograms obtained for the reduced and non-reduced samples showed that peptides (14.4, 26.3, and 32.63 min) relate to the disulfide linkage (Supplementary Fig. 4). Figure 2 clearly demonstrated the representative detailed fragment spectrum of disulfide-linked peptides (ECNEDCNFK, 550.194 Da, +2 charge). These masses were in agreement with the previous mass spectrometry data (Hsu et al. 1998) and indicate that in the absence of reducing agent, the wild-type FGF7 recombinant protein contains a disulfide-bonded cystine residue involving positions C133 and C137. Mass spectrometry showed A120C did not disturb C133-C137 disulfide bond in native FGF7.

**Thermodynamic Stability of FGF7 Mutant Proteins**

To compare the thermal stability between Palifermin and FGF7 mutant proteins, their mid-point transition

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**Table 2** Identified free thiol residues and disulfide-linked peptides

| N  | Peptide sequence       | Modification          | Delta (ppm) | Confidence Score | RT  | M/Z  | Charge State | Mono Mass Exp |
|----|------------------------|-----------------------|-------------|------------------|-----|------|-------------|---------------|
|    | Identified free thiol residues |                       |             |                  |     |      |             |               |
| 1  | VRRLFCRTQWYLRIDK       | Carbamidomethylation  | −0.51       | 100              | 22.33 | 443.049 | 5 | 2209.204   |
| 2  | VHHHHHHIEGRHMSYDY-     | Carbamidomethylation  | −0.43       | 100              | 25.66 | 643.945 | 8 | 5140.498   |
| 3  | HMSYDYMEGGDIRVRLF-     | Carbamidomethylation  | −0.89       | 100              | 28.82 | 654.662 | 6 | 3919.926   |
| 4  | LTSFCRTQWYLRIDK        | Carbamidomethylation  | −1.11       | 100              | 29.97 | 754.173 | 5 | 3763.825   |
| 5  | HMSYDYMEGGDIRVRLF-     | Carbamidomethylation  | −1.26       | 100              | 31.15 | 1476.648 | 1 | 1475.641   |
| 6  | HMSYDYMEGGDIRVRLF-     | Carbamidomethylation  | −1.42       | 100              | 38.6  | 810.425 | 3 | 2427.251   |
| 7  | TVAVGIVAIKGVESEFYLCMNK | Carbamidomethylation  | 2.33        | 100              | 41.81 | 851.23  | 5 | 4249.114   |
| 8  | GTQMENYNYNIMEIRT-      | Carbamidomethylation  | 1.78        | 100              | 43.84 | 895.206 | 4 | 3574.792   |
| 9  | NNNIMEIRTVAVGIVAIK-   | Carbamidomethylation  | 1.78        | 100              | 43.84 | 895.206 | 4 | 3574.792   |
|    | Identified disulfide-linked peptide |                 |             |                  |     |      |             |               |
| 1  | ECNEDCNFK              | 1ss [C133=C137]       | −1.89       | 100              | 14.4  | 550.194 | 2 | 1098.373   |
| 2  | ECNEDCNFK              | 1ss [C133=C137]       | −2.11       | 100              | 14.4  | 1099.38 | 1 | 1098.373   |
| 3  | KECNEDCNFKELLEN-       | 1ss [C133=C137]       | −0.66       | 100              | 26.32 | 744.589 | 4 | 2973.325   |
| 4  | KECNEDCNFKELLEN-       | 1ss [C133=C137]       | −0.49       | 100              | 26.32 | 595.873 | 5 | 2973.325   |
| 5  | ECNEDCNFKELLENHYNTYASAK| 1ss [C133=C137]       | 5.3         | 100              | 32.63 | 913.022 | 5 | 4558.067   |

M/Z, mass-to-charge ratio; RT, retention time; ‘Mono Mass exp’, experimental mass
temperatures \((T_m)\) were estimated from thermal denaturation curves (Fig. 3). The \(T_m\) values of Palifermin and the wild-type FGF7 were 51.9 \(^\circ\)C and 45.5 \(^\circ\)C, respectively (Table 3), which is consistent with previous findings that Palifermin is more stable than the wild-type FGF7 (Hsu et al. 2006). The \(T_m\) values of the A120C, A120C/K126E, A120C/K178E, and A120C/K126E/K178E mutant proteins were estimated to be 52.63 \(^\circ\)C, 60.49 \(^\circ\)C, 58.85 \(^\circ\)C and 67.20 \(^\circ\)C, respectively. The increased \(T_m\) values of the four mutant proteins suggest that they have enhanced thermal stabilities compared with Palifermin. It should also be noted that each mutation contributed to the thermal stability in an additive manner.

### The Thermal Stability of FGF7 Mutations Determined by SDS-PAGE

To examine the thermal stability of FGF7 mutant proteins, proteins were stored at 37 \(^\circ\)C and 45 \(^\circ\)C during indicated time periods and centrifuged to eliminate protein aggregates and then the supernatants were subjected to SDS-PAGE. The thermal stability was quantified by portion of soluble FGF7. The results at 37 \(^\circ\)C (Fig. 4a and b) showed that soluble portion of Palifermin reduced more slowly than wild-type FGF7. Palifermin (53%) had 1.65 times more soluble protein than wild-type FGF7 (32%) at 4 days. However, there was a little difference between the two proteins at 45 \(^\circ\)C (Fig. 4c and d). Four mutations (A120C, A120C/K126E, A120C/K178E, A120C/K126E/K178E) designed to enhance stability increased the amount of soluble protein compared to wild-type FGF7 and Palifermin at 37 \(^\circ\)C and 45 \(^\circ\)C. A120C, A120C/K126E, A120C/K178E, and A120C/K126E/K178E FGF7 protein remained 58%, 79%, 101%, and 119% soluble, respectively when incubated at 37 \(^\circ\)C for 15 days (Fig. 4a and b). In the result at 37 \(^\circ\)C, the cleaved and diffused protein band in SDS-PAGE were prominent leading to problem that the soluble portions of proteins exceeded 100%. At 45 \(^\circ\)C after 3 days, only 6% of A120C remained soluble. And A120C/K126E, A120C/K178E, and A120C/K126E/K178E were found to have 22%, 9% and 79% soluble proteins for 15 days, respectively (Fig. 4c and d). The replacement of Ala into Cys at 120th amino acid of FGF7 did not change the disulfide bond between C133 and C137 but enhanced the protein stability. The results of long-term storage at 37 \(^\circ\)C and 45 \(^\circ\)C showed that A120C/K126E/K178E has the most extended half-life, which suggested that the mutation in close vicinity to disulfide bond and salt-bridge in FGF7 improved the stability of long-term storage at hash temperature.

### Table 3 Melting temperatures for wild type and variants of FGF7

| Sample          | \(T_m\) (\(^\circ\)C) ± SE | \(r^2\) |
|-----------------|--------------------------|--------|
| Wild-type       | 45.50709 ± 0.26723       | 0.99606|
| Palifermin      | 51.96353 ± 0.29975       | 0.99762|
| A120C           | 52.63137 ± 0.36424       | 0.99431|
| A120C/K126E     | 60.49222 ± 0.4089        | 0.99659|
| A120C/K178E     | 58.85455 ± 0.50375       | 0.9919 |
| A120C/K126E/K178E | 67.19709 ± 0.59711       | 0.99353|

\(T_m\), melting temperature; SE, standard error; \(r^2\), coefficient of determination.
Cell Proliferation Activity of FGF7 Mutant Proteins at 45 °C

FGF7 has a variety of biological functions including cell proliferation so cell proliferation activity in HaCaT cell was monitored to see whether mutations affect the biological activity of FGF7 at harsh condition. FGF7 is called as KGF1 (keratinocyte growth factor 1) because it is mitogen specific for keratinocyte and epithelial cell. HaCat cell, human keratinocyte cell line has been used to evaluate the biological activity of FGF7. FGF7 mutant proteins showed similar cell proliferation activity with wild-type FGF7 (Fig. 5a), which means that the mutations of FGF7 in this study did not change the biological activity. After incubation of FGF7 proteins at 45 °C for indicated duration, cell proliferation activity of proteins was determined. After 4-day’s incubation, the activity of FGF7 wild-type disappeared while Palifermin and mutant proteins still remained active (Fig. 5b). The levels of proliferation by mutant proteins were significantly higher than that of Palifermin in the whole time period (Fig. 5b). All mutant proteins remained active even though the mutant proteins were incubated at 45 °C after 8 days and A120C/K126E/K178E did not lose its cell proliferation activity (Fig. 5b). These results suggest that the mutations tested in this study enhance the thermal stability judged by remaining activity and does not influence biological activity itself.

The Energy Change Caused by A120C to Explain the Increase of Protein Stability

The introduction of disulfide bond in proteins generally increase thermal stability but, the relation of thermal stability and the environmental change in close vicinity to disulfide bond has not been established well. We estimated the energy change caused by mutation of A120C of FGF7 using in silico analysis. Structural stability of FGF7 was calculated and compared by substituting A120 with 19 amino acids including Cys. First, as a result of the analysis using the FGF7 model made from 1qqk as a template, the stabilization of the protein was improved when A120 were changed to Phe, Gln, Leu, Cys and Asn (Table 4). And using 1qql as a template in the same way, stabilization energy increased when A120 were replaced with Asn, Phe, Gln, Glu, and Cys (Table 4). In silico analysis of change of stabilization energy caused by A120C mutation in both models can explain why
the thermal stability of A120C mutant protein increased without the formation of disulfide bridge.

The Effect of Thermal Stability of FGF7 Mutants on the Stability of pH

The stability of protein is largely affected by pH as well as temperature. We investigated whether pH stability of thermal stable FGF7 mutant proteins was changed using calculation. The introduction of mutation into FGF7 to increase stability of temperature did not affect pH of maximal stability (Supplementary Fig. 5). However, the data of solubility score and developability index indicated that the pH stability of mutant protein A120C/K126E/K178E decreased. Interestingly, it was reported that the mutation to reduce protein charge can increase pH stability (Boroujeni et al. 2021).

Discussion

FGF7 is the only FDA-approved medicine for oral mucositis and used as cosmetic ingredient for anti-aging (de Araujo et al. 2019). In spite of the versatile usefulness of FGF7, its thermal instability restricts practical use at room temperature. We have also shown here that the wild-type FGF7 is unstable than Palifermin. In this study, two strategies of mutation were attempted using Palifermin as template to improve the stability of FGF7. The introduction of novel disulfide bonds into proteins has been used generally to enhance thermal stability. First, FGF7 A120C mutation was investigated, which was the same location with FGF1 A66C mutation with increased stability (Lee and Blaber 2009). The results of CD indicated that the Tm value increased by 0.67 °C compared to Palifermin (Table 3, Fig. 3). Moreover, the amount of soluble protein remaining (Fig. 4) and the biological activity (Fig. 5b) after long-term storage at high temperature confirmed the CD result of A120C mutant protein. However, mass results showed that a disulfide bond was formed between C133 and C137 not between A120C and C137 of FGF7 (Table 2), which is consistent with the
previous report (Hsu et al. 1998). Although disulfide bond was not formed between A120C and C137 of FGF7, CD analysis and the solubility of long-term storage at high temperature showed increased stability of A120C FGF7, suggesting that the A120C mutation did not form disulfide bond but contributed to stability. The increase of Tm caused by A120C mutation seems trivial (Table 3), but the enhancement of soluble A120C mutant protein at 37 °C and the remaining activity after incubation at 45 °C was obvious (Fig. 4a, b and 5b). These results can be explained through in silico analysis of mutation energy (Table 4). Therefore, it was suggested that the introduction of Cys into A120 of FGF7 did not induce disulfide bonds as intended, but brought structural stability by itself.

In addition to introduction of disulfide bond, salt bridge in proteins generally contributes to stability (Bosshard et al. 2004) and increasing salt bridge is suggested as efficient strategy to enhance stability of protein (Lee et al. 2014). It was reported that the thermal stability was improved by replacing the positive residue Arg175 with a neutral or negative residue, Gln or Glu to change ionic interaction (Hsu et al. 2006). Here, we were interested in the positive amino acid residues around R175 so we investigated the effect of the K126 and K178 mutations around R175 substituted with a negative residue Glu to make surface salt bridges. Based on the 3D structure, R175 was located in the middle between K126 and K178, and the distance between amino acids was close enough to form a salt bridge with R175 (Fig S1 and S6). The K126E mutation model predicted by the SWISS MODEL server maintained the distance between the anionic OE2 of K126E and the cation NH2 of R175 at 2.56 Å. And the distance between OE1 of K178E mutant protein and NH1 of R175 was maintained at 2.76 Å. These predictions suggest that the positively charged guanidinium group of 175R can interact with the negatively charged carboxyl groups of 126E and 178E to form a salt bridge, a combination of two non-covalent interactions: hydrogen bonding and ionic bonding (Fig S6). In the experiment, the mutant proteins to form salt bridge were generated using Palifermin with A120C mutation. As a result of the CD experiment, Tm values of A120C/K126E and A120C/K178E increased by 8.53 °C and 6.89 °C (Table 3), respectively, compared to Palifermin. In addition, A120C/K126E/K178E, in which both mutations were introduced simultaneously, showed a 15.24 °C rise (Table 3) in Tm value compared to Palifermin. It was confirmed that the A120C/K126E/K178E mutant protein with the greatest increase in Tm value remained soluble (Fig. 4) and active state (Fig. 5) longer in long-term stability experiments (Fig. 4) and longer activity in cell proliferation experiments (Fig. 5). These results showed that the stability of K126E/K178E protein was increased more than that of K126E or K178E single mutant proteins. It also suggests that the surface salt-bridge of the FGF7 mutant protein is involved in the interaction with R175, making the protein more robust. Even though the mutant proteins were designed to form salt-bridge, it can be suggested that the negative charge of protein increased the thermal stability. The more negative charge protein has, the more stable protein is (Tm: K126E/K178E > K126E, K178E > K126/K178 (palifermin)). It cannot be ruled out the possibility that there is correlation between negative charge of protein and its thermal stability.

## Conclusion

We have observed that the mutations tested in this study exhibited higher thermal stability than wild-type FGF7 and Palifermin. Although A120C introduced for disulfide bond did not form a disulfide bond with C133 predicted by MS analysis, enhancement of thermal stability of A120C/ K126E/K178E mutations including A120C and A120C was proved by Tm determined by CD, the amount of soluble protein and the cell proliferation activity after 45 °C incubation. The structural characteristics of FGF7 and the role of salt bridge provided insight into improvement of thermal stability and this increased stability must enlarge the usefulness of FGF7.

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s10989-022-10394-1.

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## Data Availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Declarations

### Conflict of interest

The authors declare no conflict of interest.

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