<Research Article>

Development of a blocking peptide fragment from the hyperthermophilic bacterium *Thermotoga* sp.

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Summary A blocking peptide fragment (BPF) was used as the blocking reagent for immunoassays. In this study, a BPF derived from the hyperthermophilic bacterium *Thermotoga* sp. (T-BPF), which was expected to be more thermostable than the conventional *Escherichia coli* BPF (E-BPF), was developed. Part of the *Thermotoga* DnaK/heat shock protein 70 homolog was identified as the substrate-binding domain T-BPF. T-BPF was cloned, highly expressed, and purified to homogeneity. The circular dichroism spectral profiles indicated that T-BPF was stable at 95˚C for 2 h although E-BPF was not. The high thermal stability of T-BPF was attributed to intramolecular electrostatic interactions. The surface structure of T-BPF mainly contains hydrophobic regions, whereas that of E-BPF contains hydrophilic regions. The opposing properties of both BPFs may be reflected in their various practical applications, such as binding to a wide variety of solid phases and other proteins.

Key words: Blocking, Thermal stability, DnaK, Hsp70, *Thermotoga*

1. Introduction

In clinical testing, various protein components are used to improve reagent stability, reduce nonspecific reactions, and avoid interfering substances. These include albumins, such as bovine serum albumin (BSA), milk casein, and silk sericin proteins[1-6]. Unlike enzymes, these proteins do not catalyze specific reactions and they exert their effects by increasing the protein concentration in the solution or by binding to the solid phase[6-9]. These functional proteins utilize readily available natural proteins effectively.

In contrast, recombinant proteins can be used as functional proteins. The blocking peptide fragment (BPF), prepared by genetic engineering techniques, consists of the substrate-binding domain of the DnaK protein from *Escherichia coli* (Fig. 1)[10-13]. DnaK is an ATP-dependent chaperone protein, also known as heat shock protein 70 (Hsp70), which is highly expressed in high-temperature environments and refolds denatured proteins into their normal structures[14,15]. DnaK/Hsp70 is universally expressed in living organisms. In contrast, BPF is an artificially produced component of DnaK with no chaperone function. The tertiary structure of BPF is composed of hydrophilic α-helical and hydrophobic β-sheet

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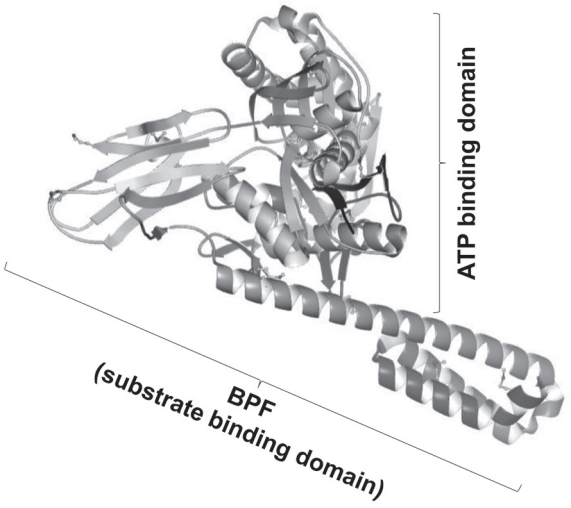

Fig. 1. *E. coli* DnaK/Hsp70 open structure (PDB ID: 4B9Qa). Two configuration domains, the ATP-binding domain and substrate-binding domain (BPF), are illustrated.

regions and has surfactant-like characteristics. It is a useful blocking reagent for enzyme-linked immunosorbent assays and immunochromatography and it offers an alternative to BSA and sericin[11]. Furthermore, its effectiveness as a stabilizer for analytical enzymes and conjugates has been demonstrated[16-18]. However, the practical use of *E. coli* BPF (E-BPF) for analysis under high-temperature conditions, such as in polymerase chain reactions (PCR), is unknown because it is derived from a mesophilic bacterium. Its long-term storage stability has not yet been investigated.

In this study, a BPF derived from the hyperthermophilic bacterium *Thermotoga* sp. (T-BPF) was developed with the aim of achieving high thermal stability.

2. Materials and Methods

Reagents, chemicals, bacterial strains, plasmids, and culture conditions.

E-BPF was purchased from Toyobo Co., Ltd. (Osaka, Japan). The compounds and reagents were purchased from Nacalai Tesque (Kyoto, Japan) and Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). *E. coli* BL21(DE3) (BioDynamics Laboratory Inc., Tokyo, Japan) and plasmid pET-29a(+) (5,371 bp, Km^r) (Novagen, Madison, WI, USA) were used for recombinant strain preparation and plasmid construction, respectively. Bacteria were grown in Luria-Bertani Broth (LB), in Terrific Broth (TB), or on LB agar (LB plus 1.5% agar) at 37°C[19]. Kanamycin (30 μg/mL) was used as an antibiotic.

DNA manipulation and recombinant plasmid construction

Plasmid isolation, DNA cleavage with restriction enzymes, and DNA ligation with T4 DNA ligase were performed as aforementioned[19]. *E. coli* cells were transformed using chemical method[19].

The T-BPF-encoding gene (567 bp, encoding amino acid residues 401 to 589 of the NCBI reference sequence: WP_004083187 [total 596 residues]) was artificially synthesized with codon preference of *E. coli* by Eurofins Genomics Inc. (Tokyo, Japan) and cloned into *Nde*I and *Bam*HI sites of pET-29a(+). The DNA sequences were verified by sequencing. The constructed plasmid, designated pET-T-BPF, was transformed into *E. coli* BL21(DE3) cells.

T-BPF expression and purification

The pET-T-BPF carriers were cultured in 5 mL of LB at 37˚C and 200 r/min shaker speed for 18 h. 100 mL of TB, into which 1 mL of the culture was inoculated, was subcultured at 37˚C and 200 r/min shaker speed. Recombinant cells were induced in order to synthesize the gene product by adding 100 μL of 100 mmol/L isopropyl-L-D-thiogalactopyranoside to the culture medium when the turbidity (OD_{590}) was within the range of 0.5 to 0.8.

The cells cultured for 18 h were collected by centrifugation at 4˚C and 13,000 xg for 5 min and suspended in buffer A (20 mmol/L potassium phosphate, pH 7.5). The cells were sonicated on ice, and the cell extracts were centrifuged at 4˚C and 13,000 xg for 20 min. The supernatant was heat treated at 80˚C for 1 h and then re-centrifuged. The supernatant was applied to a HiTrap Q HP anion-exchange chromatography column (Cytiva Life Sciences, Marlborough, MA, USA) equilibrated with buffer A. The bound proteins were eluted using a gradient of buffer B (20 mmol/L potassium phosphate and 1

mol/L NaCl, pH 7.5). The protein solution was dialyzed against buffer A in order to remove the NaCl. Finally, T-BPF was purified to homogeneity. Molecular weights were estimated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was quantified using the Bradford assay (Takara Bio Inc., Shiga, Japan).

Peptide mass fingerprinting (PMF)

T-BPF fragments were identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The purified protein was digested with 20 μg/mL of MS-grade trypsin (Fujifilm Wako Pure Chemical Corp.) in 50 mmol/L of NH_4HCO_3 at 37˚C overnight. The resulting peptides were mixed with 5.0 mg/mL of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid and subjected to MALDI-TOF-MS (AXIMA-Performance, Shimadzu Corp., Kyoto, Japan)[20].

Circular dichroism (CD) analysis

The CD spectra of the BPFs (20-50 μmol/L) were obtained using a J-805 spectropolarimeter (Jasco, Tokyo, Japan) at 37˚C using a 0.5 mm water-jacketed cylindrical cell[20].

Computational analysis

Homology modelling, protein surface analysis, prediction of interactions, and molecular visualization were performed using Molecular Operating Environment software (MOE ver.2022.02, Chemical Computing Group Inc., Montreal, Quebec, Canada). Energy minimization was applied to the models to achieve further structural refinement. Before the energy minimization, the force field Amber10:EHT was used to add hydrogen atoms and partial charges at pH 7 according to the manufacturer's instructions[21].

3. Results and Discussion

Production of T-BPF

The genus *Thermotoga* is well known as a typical hyperthermophilic bacteria[22]. Its proteins are extremely thermostable and are therefore useful for various industrial processes, such as chemical industries[23]. To identify T-BPF, putative orthologs of the *E. coli* Dnak protein were detected using the BLASTp (protein database search program, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). The NCBI reference sequence, WP_004083187, composed of 596 amino acid residues, was identified as a *Thermotoga* DnaK/Hsp70 homolog. An amino acid sequence homology search indicated that the peptide fragment encoding amino acid residues 401–589 in the sequence corresponded to T-BPF. It had a high sequence identity (approximately 44%) with E-BPF (Fig. 2).

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T-BPF 401' PVRKSKIFTT VEDGQTEVEI RVYQGERPIA RENIFLGSFK LVGIPPAPRG VPQIEVTFDI
           *...*..*.* .**.*..*.* .* **** *  .*  ** *. * ** ***** .**********
E-BPF 419" PTKHSQVFST AEDNQSAVSI HVLQGERKRA ADNKSLGQFN LDGINPAPRG MPQIEVTFDI

T-BPF 461' DSDGIVHVSA KDLGSGKEQS MVVTGRHKLS EDEIKRMIED AKRYEEQDKR LKEEIELKNR
           *.***.**** ** .*****. ......  *. ****..*. * *.  .*.*.. ..* .. .*.
E-BPF 479" DADGILHVSA KDKNSGKEQK ITIKASSGLN EDEIQKMVRD AEANAEADRK FEELVQTRNQ

T-BPF 521' ADDLAYSVEK TLKEHGDKIP ADLKSRLEDM IRELRDAINR NDIPKVKMLF DDLQKESMKI
           .*.* .*.  *  ..* ***.* ** *. .*.  . .* .*..  .* . ..  . ..*.. * *.
E-BPF 539" GDHLLHSTRK QVEEAGDKLP ADDKTAIESA LTALETALKG EDKAAIEAKM QELAQVSQKL

T-BPF 581' GEYLYKSAT
           *   .
E-BPF 599" MEIAQQQHA
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Fig. 2. Pairwise alignment of T-BPF (upper) and E-BPF (lower). The amino acids in both the BPFs were numbered from the first Met of DnaK/Hsp70. Identical and similar residues are indicated with asterisks and dots, respectively. Partial amino acid sequences of T-BPF determined by PMF are underlined.

The T-BPF gene was cloned and then expressed in a recombinant *E. coli* strain carrying the expression plasmid pET-T-BPF as described in the Materials and Methods section. The gene product T-BPF, with a molecular weight of 21.7 kDa was purified to homogeneity, as described in the Materials and Methods section. Finally, 84.7 mg of T-BPF was obtained. The SDS-PAGE patterns for the anion-exchange chromatography fractions of T-BPF purification are presented in Fig. 3. Highly purified T-BPF was obtained from fraction numbers 9-10 and used for subsequent analyses.

The four partial amino acid sequences of the purified T-BPF were determined by PMF (Fig. 2), resulting in complete correspondence with the internal sequences of T-BPF. As a starting point for understanding the structural differences between

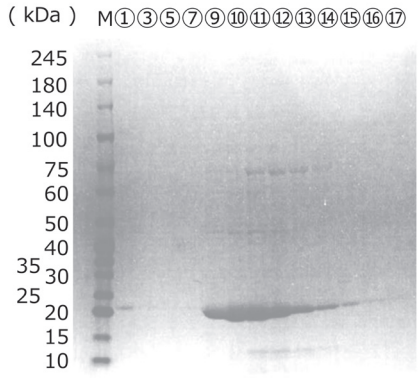

Fig. 3. Anion exchange chromatography fractions for T-BPF purification. Fraction numbers 1-17 were analyzed using 5-20% gradient SDS-PAGE and Coomassie Blue staining. (M) Molecular weight markers.

T-BPF and E-BPF, their amino acid compositions were compared (Table 1). T-BPF contained more Val (hydrophobic), Ile (hydrophobic), and Arg (hydrophilic) and less Ala (hydrophobic), Asn (neutral), and Gln (neutral) than that present in E-BPF. This suggests the difference in intramolecular interactions between the T-BPF and E-BPF structures.

Table 1 Comparison of amino acid compositions

Amino acid	T-BPF	E-BPF
[hydrophobic]		
Gly	11(5.82%)	10(5.29%)
Ala	9(4.76%)	24(12.70%)
Val	13(6.88%)	9(4.76%)
Leu	14(7.41%)	14(7.41%)
Ile	16(8.47%)	11(5.82%)
Met	5(2.65%)	4(2.12%)
Phe	5(2.65%)	4(2.12%)
Trp	0	0
Pro	8(4.23%)	5(2.65%)
[neutral]		
Ser	11(5.82%)	12(6.35%)
Thr	7(3.70%)	9(4.76%)
Asn	4(2.12%)	8(4.23%)
Gln	6(3.17%)	16(8.47%)
Cys	0	0
[hydrophilic]		
Asp	17(8.99%)	15(7.94%)
Glu	21(11.11%)	18(9.52%)
Lys	20(10.58%)	17(8.99%)
His	3(1.59%)	6(3.17%)
Arg	14(7.41%)	7(3.70%)
Tyr	5(2.65%)	0
Total A.A.	189	189
M.W.	21.7 kDa	20.7 kDa

A.A., amino acid; M.W., molecular weight.

Thermal stability

The secondary structures of T-BPF and E-BPF were identified by CD spectroscopy. Both proteins exhibited different CD spectra (Fig. 4) and were therefore predicted to have distinct secondary structural compositions using the BeStSel program (https://bestsel.elte.hu/index.php) (data not shown). The difference in both CD spectrum profiles is thought to reflect some structural alterations, although the details are unknown.

The 200-240 nm spectral profile of T-BPF was found to be almost the same after heat treatment at 95°C for 2 h, whereas that of E-BPF was clearly altered after heat treatment at 95°C for 1 h. Thus, T-BPF was structurally stable enough to be used for PCR testing. In contrast, E-BPF was not suitable for PCR, even though it was not completely denatured by heat treatment.

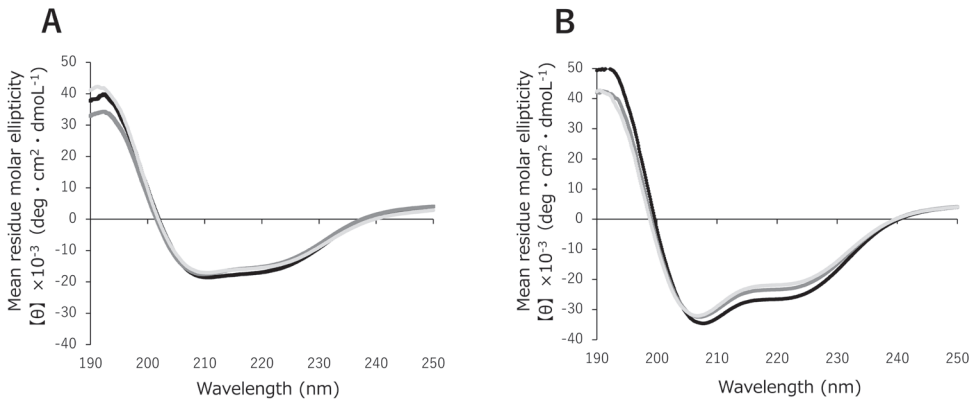

Fig. 4. CD spectral profiles of T-BPF and E-BPF. 50 μmol/L-buffer A concentration was used for the measurement of each protein. Black, dark grey, and light grey represent 95°C treatment of 0 (no treatment), 1 h, and 2 h, respectively. A: T-BPF, B: E-BPF.

Structural analysis

The tertiary structure of T-BPF was constructed using homology modelling, as aforementioned in the Materials and Methods section, based on the structure of the substrate-binding domain of DnaK/Hsp70 in *E. coli* (PDB ID: 4HY9b). No outliers were observed in the Ramachandran plots. The three-dimensional structure modeled in the present study was of high quality and could thus could enhance the understanding of the structure–function relationship.

The T-BPF model structure was superposed well on E-BPF, with root mean square deviations for atomic Cα positions of 0.83 Å (185 amino acid residues). Interaction prediction explained the difference in the thermal stabilities of both BPF proteins (Fig. 5),

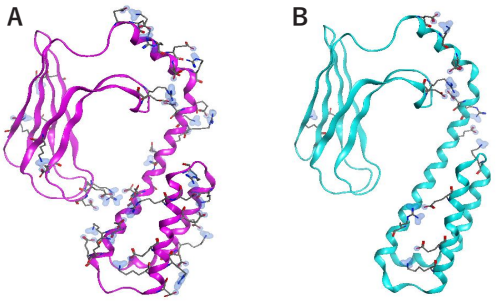

Fig. 5. Comparison of intramolecular electrostatic interactions between T-BPF and E-BPF structure models. Amino acid residues involved in electrostatic interactions are shown as stick drawings. The interacting atoms are indicated by blue balls. A: T-BPF, B: E-BPF. The electrostatic interaction numbers of T-BPF and E-BPF are estimated to be 29 and 9, respectively.

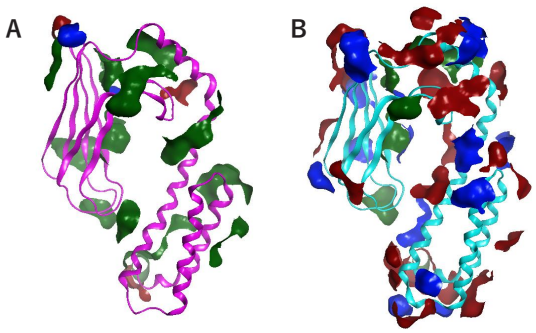

Fig. 6. Comparison of the molecular surface hydrophobic/hydrophilic regions between the T-BPF and E-BPF structure models. The hydrophobic, negative, and positive surface patches are indicated in green, brown, and blue, respectively. A: T-BPF, B: E-BPF.

although there was almost no conformational difference between T-BPF and E-BPF. The electrostatic interaction numbers of T-BPF and E-BPF were estimated to be 29 and 9, respectively. Among these, strong bonds with low free energies, which were below -10 kcal/mol, were limited to 12 in T-BPF and two in E-BPF. The high thermal stability of T-BPF was attributed to numerous strong electrostatic interactions.

Protein surface patch analysis revealed that the surface hydrophobic/hydrophilic region distribution of T-BPF was significantly different from that of E-BPF. T-BPF and E-BPF contained mainly hydrophobic and hydrophilic (positive and negative) regions, respectively (Fig. 6). The opposing properties of both BPFs may be reflected in their various practical applications, such as binding to a wide variety of solid phases and other proteins. Application of T-BPF as a functional protein is currently in progress.

Conflicts of interest

The authors declare no conflict of interest.

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