

Utilization of Random Peptide Library with Natural Neurotoxin  
Scaffold and Development of Directed-evolution Technologies

January 2013

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**Utilization of Random Peptide Library with Natural Neurotoxin  
Scaffold and Development of Directed-evolution Technologies**

**A Dissertation Submitted to  
the Graduate School of Life and Environmental Sciences,  
the University of Tsukuba  
in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy in Biotechnology  
(Doctoral Program in Life Sciences and Bioengineering)**

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## Abbreviations

<b>3F</b>	<b>three finger neurotoxin protein</b>
<b>Ni-NTA</b>	<b>nickel nitroloacetic acid</b>
<b>PCR</b>	<b>Polymerase Chain Reaction</b>
<b>PBS</b>	<b>Phosphate Buffered Saline</b>
<b>SDS-PAGE</b>	<b>sodium dodecyl sulfate polyacrylamide gel electrophoresis</b>
<b>EMCS</b>	<b>N-(6-maleimidocaproyloxy) succinimide</b>
<b>SPR</b>	<b>Surface Plasmon Resonance</b>
<b><math>K_d</math></b>	<b>dissociation constants</b>
<b>EC<sub>50</sub></b>	<b>term half maximal effective concentration</b>
<b>IVC</b>	<b><i>in vitro</i> compartmentalization</b>
<b>BDNF</b>	<b>the growth factor brain-derived neurotrophic factor</b>
<b>TrkB</b>	<b>tropomyosin-related kinase receptor type B</b>
<b>MGSA/CXCL1</b>	<b>melanoma growth stimulatory activity factor</b>

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# Chapter I

## Preface

Antibodies, as a class of biological drugs, are most expected as one of the effective therapeutic tools against various diseases. They constitute the most rapidly growing class of human therapeutics and the second largest class of drugs after vaccines. However, it has become obvious that antibodies suffer from some fundamental disadvantages with increasing application in research, biotechnology, and medical therapy. For example, Immunoglobulins (Igs) are rather large molecules, thus limiting tissue penetration. Moreover, it costs a lot in commercial basis for antibody production, and not always successful. In the last decade, a minimizing antibody by rearranging antibody fragments has been developed to overcome the problems. Recently, various kinds of protein scaffolds derived from non-immunoglobulin proteins have also been shown to be useful as alternatives of antibodies. In particular, these proteins are smaller than Igs offering advantages in production and clinical effectiveness. Directed evolution has become a very popular strategy for improving or altering the biophysical properties of proteins, and generating proteins with novel functions. Disulfide-containing scaffolds, such as serine protease inhibitor (EETI-II, knottin family), bovine pancreatic trypsin inhibitor (BPTI; Kunitz domain),  $\alpha$ -amylase inhibitor (tendamistat) and related proteins recently are popular targets of directed molecular evolution. Attention has been paid to their nature as small, defined structures and remarkably stable proteins. These scaffolds are considered to be able to present surface loops of varying sequence and length, including exposed hydrophobic residues. The property make scaffolds excellent candidates for novel binding specificities generating without significant changes in the structural framework. These

candidates have been successfully engineered for various purposes, such as for generating protease inhibitors, and ligands for macromolecules such as receptors.

The first part of my study is functional characterization of trypsin protease inhibitors identified from a three-finger (3F) DNA library. The 3F scaffold used in this study is a novel snake  $\alpha$ -neurotoxin, MicTx3, isolated from the South American coral snake, *Micrurus corallinus*. Due to their properties as small size, remarkably high temperature stability, resistance to proteases and low immunogenicity, 3F neurotoxins have broad spectrum in target molecules in nature, such as ion channels, receptors, proteases, etc. In the previous work, Naimuddin et al. prepared 3F random cDNA library based on the MicTx3 neurotoxin as a template and succeed to generate regulatory molecules against interleukin-6 receptor (IL-6R), and also achieved size reduction of 3F which can be conveniently prepared by chemical synthesis. Here, trypsin was chosen as target molecule to obtain protease inhibitors from the same library. Trypsin is a serine protease, which is used for numerous biotechnology processes in a range of laboratory tests because of its ability to hydrolyze proteins. These procedures include cell culture, separating tissues into constituent cells, sample preparation for genetic analysis, and protein studies. Clinical beneficial activities of trypsin inhibitors with therapeutic actions have been extensively investigated in acute pancreatitis, acute migraine, ischemic, cancers and so on. However, inhibitors also have some potentially adverse effects in special circumstances. I demonstrated the potential of the 3F scaffold in generating novel molecules for trypsin inhibitors by *in vitro* evolution using cDNA display.

In second part of my study, *In vitro* compartmentalization (IVC) offers an alternative strategy based on partitioning reactions in water droplets dispersed to form microscopic compartments in water-in-oil emulsions. Because of the small size of

compartments, very large protein libraries ( $10^8$ – $10^{12}$  variants) can be expressed, and sequence variants with altered functions can be captured by a variety of selections. Previous study reported that a number of variants of the basic IVC selection method have been used for the identification of affinity reagents. The growth factor brain-derived neurotrophic factor (BDNF) and its receptor TrkB mediate influence neuronal activity, function and survival throughout life. BDNF shows therapeutic potential in the treatment of various neurodegenerative diseases, such as Parkinson disease (PD), Alzheimer disease (AD) and amyotrophic lateral sclerosis (ALS). However, the therapeutic application of BDNF has been greatly limited because of its poor permeability blood-brain barrier (BBB). In order to generate small BDNF mimicking peptides, I prepared CXC scaffold random library based on CXCL1 to obtain specific ligand to target receptor TrkB by IVC selection. CXCL1 was chosen as template due to its small size (8 kDa), 2 disulfide bonds of four cysteine residues in conserved, and functions in biological processes of angiogenesis, inflammation, wound healing and tumorigenesis. I expect to produce CXC-based peptide probes to study the molecular mechanisms of neuronal development. Further study on generating small peptides targeting to TrkB receptor with potent neurotrophic activities, which may be of potential application in the treatment of various neurodegenerative diseases, is under progress.

## Chapter II

### Directed evolution of three-finger toxin to produce serine protease inhibitors

#### 1. Abstract

Directed evolution has become a very popular strategy for improving biophysical properties and even for generating proteins with novel functions. Recent advances in combinatorial protein engineering mean it is now possible to develop protein scaffolds that could substitute for whole antibody-associated properties as emerging therapeutic proteins. Protein, RNA, and DNA are the major targets of directed molecular evolution. In particular, disulfide-rich proteins are attractive templates for directed evolution in the search for novel molecules because they can regulate the activities of receptors, enzymes, and other molecules. Previous work from our group demonstrated that functional regulatory molecules against interleukin-6 receptor (IL-6R) could be obtained by directed evolution of the three-finger toxin (3F) scaffold. In the present study, trypsin was selected as a target for directed evolution to further explore the potential use of the 3F cDNA display library. After seven rounds of selection, the DNA sequences converged. The recombinant proteins produced by the selected candidates had inhibitory activity against trypsin ( $K_i$  of 33–450 nM). Three out of six groups had  $K_i$  values that were comparable to bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor. Two of the candidates also had inhibitory effects against chymotrypsin and kallikrein. This study suggests that 3F protein is suitable for the preparation of high-diversity libraries that can be utilized to obtain protease inhibitors. In addition to previous successful targeting of IL-6R, the technique developed in previous and present studies may have wide applications in



the generation of regulatory molecules for targets of interest, such as receptors, enzymes for research, diagnostic applications, and therapeutic uses.

## 2. Introduction

Antibody-based medicine is expected to be one of the most effective novel therapeutic tools for combating various diseases, because this approach involves direct and specific interactions with disease-related molecules (1, 2). However, antibody production is not always successful and it may incur vast costs on a commercial scale (3). In the last two decades, approaches have been developed to overcome these problems, which involve minimizing antibodies by rearranging their fragments. Recently, various types of protein scaffolds derived from non-immunoglobulin (non-Ig) proteins have also been shown to be useful alternatives to antibodies (4, 5). In particular, these proteins are smaller than immunoglobulin (Ig), which is advantageous in terms of production and clinical effectiveness. These proteins present surface loops with variable sequences and lengths, including exposed hydrophobic residues, but without significant changes in the structural framework (6). Disulfide-containing scaffolds, such as  $\alpha$ -amylase inhibitor (tendamistat), bovine pancreatic trypsin inhibitor (BPTI; Kunitz domain), and serine protease inhibitor (EETI-II, knottin family), have been utilized as templates for different purposes to generate protease inhibitors (7, 8) and ligands for receptors (9). Previously, we utilized the three-finger toxin (3F) scaffold to prepare a cDNA library and we successfully generated regulatory molecules (antagonist and agonist) with activity

against the interleukin-6 receptor (IL-6R) (10). These proteins are 30–40 times smaller than Igs and the scaffolds are stabilized by disulfide bonds (11). The 3F scaffold used in this study is a novel snake  $\alpha$ -neurotoxin, MicTx3, isolated from the South American coral snake *Micrurus corallines*. 3F protein families are found in a variety of organisms such as the elapidae snake and mammals, including humans (12), knotted together by 4-5 disulfide bridges. 3F proteins exhibit high temperature stability, resistance to proteases and low immunogenicity (13). 3F families are attractive because of their small size (7–8 kDa),  $\beta$ -structure(s), and the conservation of cysteine frameworks with several loops (the variable domain, which corresponds to the 3F structure). By exploiting the structural features of 3F toxins, new ligands for G protein-coupled receptors (GPCRs) with novel functions were generated by loop grafting 3F muscarinic toxins (14). The strict conservation of the cysteine frameworks and the high sequence diversity in the loops may allow the production of 3F proteins with a broad spectrum of target molecules, such as ion channels, receptors, and enzymes (15), and potentially allow 3F proteins to serve as excellent scaffolds for directed evolution. The specificity of 3F proteins for their respective receptors, which is conferred by the residues on the tips of the loops, has been investigated extensively (16, 17). In the present study, trypsin was used as the target molecule to obtain protease inhibitors from the 3F cDNA display library. Trypsin is a serine protease,

which is found in the digestive system of many vertebrates where it hydrolyses proteins. Trypsin is produced in the pancreas as the inactive proenzyme trypsinogen (18). Trypsin inhibitors reduce the availability of biologically active trypsin to cells or proteins and are important for research, diagnostics, and therapeutics (19, 20). The clinically beneficial activities of trypsin inhibitors with therapeutic effects have been investigated extensively in serious acute pancreatitis, migraine, ischemia, cancers, and other diseases (21-23). Aprotinin, or BPTI, is known to inhibit plasmin, and has been used for clinical purposes to reduce blood loss during heart and liver surgery (24). However, trypsin inhibitors also have potentially adverse effects in specific circumstances (25, 26). For example, the use of BPTI in diabetic patients may be related to an increased risk of renal dysfunction. Acetylcholinesterase inhibitors have been isolated from 3F toxins, i.e., faciculins, but trypsin inhibitors have not been reported from 3F toxins. The present study demonstrates the potential use of the 3F scaffold for generating novel molecules that act as serine protease inhibitors via the directed evolution of a cDNA display library.

### **3. Materials and methods**

#### ***3.1 Preparation of the 3F library and target protein selection***

The full method for the construction of the three-finger (3F) protein library, cDNA display and selection of target protein were described previously by Dr. Mohammed Naimuddin (10).

##### ***3.1.1 Construction of the 3F library***

Briefly, MicTx3 cDNA was used as a template and the 3F cDNA library was constructed by introducing (NNS)s into each finger tip [Residues T5–P10 in loop 1 (six residues), K25–V34 in loop 2 (ten residues), and A46–H52 in loop 3 (seven residues)] (Fig. 3A). The 3F genetic construct (Fig. 1C) was prepared by joining the fragments by overlap PCR to facilitate the formation and purification of cDNA displayed proteins. Briefly to the sequence of the 3F library, SP6 fragment containing the SP6 promoter, cap site, *Xenopus* globin untranslated sequence (UTR) and translation initiation site was added at the 5' end, while the (G3-S) spacer, C-terminal 6xHis, (G3-S) spacer and Y-tag sequences were added at the 3' end. The final library was expressed and purified by Ni-NTA resin (Qiagen, CA, USA) and the quality of the library was assessed by sequencing 20 randomly selected clones after cloning them into the TA cloning vector (Invitrogen, CA, USA).

##### ***3.1.2 Transcription of DNA library***

The DNA library was subjected to urea polyacrylamide gel electrophoresis (PAGE), purified from the gel, annealed by heating at 94 °C, and gradient cooling to form the correctly paired double-stranded DNA. The DNA templates were transcribed using SP6 RNA polymerase with a RiboMAX Large Scale Production Systems (Promega,

WI, USA). The reactions were terminated by adding DNase I and the products were purified using the phenol/chloroform method. The RNA concentration was measured at 260 nm using a spectrophotometer.

### ***3.1.3 Synthesis of the puromycin-linker and ligation to mRNA***

The puromycin-linker was synthesized as described previously (10), by cross-linking two modified oligonucleotides with the hetero-bifunctional reagent, N-(6-maleimidocaproyloxy) succinimide (EMCS). The modified oligonucleotides, Puro-F-S [5'-(S)-TC (F)-(Spec18)-(Spec18)-(Spec18)-(Spec18)-CC-(Puro)-3'] and Biotin-loop were custom synthesized (BEX Co, Tokyo, Japan). The mRNA was annealed to the biotinylated puromycin-linker DNA (1:1 ratio) via the Y-tag sequence in 1× ligase buffer (Takara, Kyoto, Japan) by heating at 94 °C and cooling slowly. The mRNA and linker were ligated via the addition of T4 kinase (3 units) and T4 RNA ligase (20 units) (Takara) at 25 °C for 1 h, then the conjugated product was purified using an RNeasy Kit (Qiagen). The ligation efficiency and the purity of the products were checked by PAGE using FITC and/or Vistra Green (Molecular Probes, USA) staining with a fluoroiager (Bio-Rad, Hercules, CA, USA).

### ***3.1.4 cDNA display***

The mRNA-puromycin linker DNA (3-5 picomoles) was translated in 25 µl using a Retic Lysate IVT Kit (Ambion, Austin, TX, USA) at 30 °C for 10 min in the presence of protein disulfide isomerase (1:1 ratio of display protein/PDI) (27). After translation, the proteins were covalently linked to the puromycin-linker in the presence of 65 mM MgCl<sub>2</sub> and 750 mM KCl at 37 °C for 2 h. The translation products conjugated with the corresponding mRNA were purified using oligo-dT. cDNA synthesis was followed by M-MLV reverse transcriptase (Takara) priming from the 'built-in' primer included in the linker. After the displayed proteins were

released by restriction digestion with PvuII, the full-length displayed proteins were purified via the C-terminal 6×His-tag using Ni-NTA resin (Qiagen). The overall process was scaled up for library preparation where 200 picomoles were used for translation. The subsequent rounds (R) were reduced to 1/5th for R2–R5 and 1/10th for R6–R7.

### **3.1.5 Preparation of immobilized enzymes**

Porcine pancreas trypsin (Sigma–Aldrich, Saint Louis, USA) were immobilized on NHS-activated Sepharose 4 Fast Flow (GE Healthcare, Milwaukee, UK) by utilizing the amine coupling chemistries to form a chemically stable amide bond, according to the manufacturer’s instructions. The coupling efficiency was estimated from the absorbance measurements of the initial and unbound protein at 280 nm. The activity of the immobilized enzyme was measured using an activity assay (see below, “*Protease inhibition activity assay*”). Non-coated beads were also prepared using the same derivatization procedure, but in the absence of the enzyme.

### **3.1.6 Selection of target protein (trypsin) binders**

The 3F library was screened to identify molecules that bound trypsin selectively. Each round of selection was performed using non-coated beads and trypsin-coated beads in a batch-selection process with phosphate-buffered saline (PBS), which contained an additional 100 mM NaCl and 0.1% Tween-20. The initial round contained 2 nM cDNA displayed protein and 300 nM trypsin. Before selecting the trypsin binders, non-specific Sepharose binders were excluded by preincubating the library with non-coated beads for 1 h. This was followed by incubation of the precleared library with trypsin-coated beads for 1 h. After incubation, the trypsin beads were washed several times with the PBS/NaCl/Tween-20 buffer and eluted

using the same buffer containing 100 mM dithiothreitol (Sigma). The eluted products were desalted and PCR amplified (denaturation for 20 s at 94 °C, annealing for 15 s at 60 °C, and extension for 30 s at 72 °C) with forward and reverse PCR primers, purified, and used as templates for PCR amplification with the SP6 fragment. The DNA products were gel-purified, transcribed, mRNA-ligated to the puromycin-linker, translated, transformed into display proteins, reverse transcribed, Ni-NTA affinity-purified, quantified, and used in the next round of selection. The trypsin concentration and the incubation period were reduced gradually, whereas the number of washes was increased progressively during further rounds of selection to apply selection pressure. After seven rounds of stringent selection, the DNA pool was amplified using primers that encoded the initiation methionine region and the C-terminal region. The PCR products were cloned into the vector pCR2.1-TOPO using a TOPO TA cloning kit and transformed into electrocompetent cells, TOP10. Positive clones with the correct insert size were sequenced (Operon Biotechnologies, Tokyo, Japan).

### ***3.1.7 Assessment of the selection***

Direct binding analysis using displayed proteins: Selected Round 7 library (R7) was prepared by the cDNA display method and purified by spin columns (Bio-Rad). One of the fractions was denatured by treatment with 100 mM dithiothreitol (DTT) for 1 h and purified (designated as R7-D). Purified display proteins (R7 and R7-D; 5 µl) were mixed with non-coated beads and 250 nM of trypsin coated beads in PBS-BSA (0.01% BSA) and incubated at 25 °C for 1 h. The mixtures were washed with PBS-T (0.1% Tween) and subsequently incubated with Penta.His-HRP (Qiagen) in PBS-T for 30 min. at 25 °C. The mixtures were then washed several times with PBST followed by addition of the substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB; Sigma). After color development was complete, the reaction was stopped by the addition of



H<sub>2</sub>SO<sub>4</sub>, the sample was centrifuged, and absorbance measured at 450 nm.

### **3.2 Preparation of selected candidate proteins**

The recombinant proteins were prepared using a pBAD/TOPO ThioFusion Expression Kit (Invitrogen), as described by Naimuddin *et al.* (10) with several modifications. The selected candidate clones were PCR amplified using primers encoding the N-terminal methionine and C-terminal 6×His, before being subcloned into the vector pBAD/Thio-TOPO and transformed. Clones with the correct orientation were cultured overnight at 37 °C in Luria-Bertani (LB) medium containing 50 µg/ml ampicillin. A small portion of the culture was transferred to fresh LB-ampicillin medium and grown to an OD<sub>600</sub> of 0.5 at 37 °C, then induced with 0.02% arabinose for 4 h at 37 °C. The cells were pelleted by centrifugation at 3000 rpm for 20 min at 4 °C and resuspended with Bugbuster Protein Extraction Reagent (Novagen, San Diego, USA) in the presence of 25 U/ml Benzonase, 1000 U/ml rLysozyme, and protease inhibitor cocktail (Sigma) at 25 °C for 30 min. The lysate was centrifuged, separated into the supernatant (soluble fraction) and the pellet (insoluble fraction), and analyzed by Tris-glycine sodium dodecyl sulfate-PAGE (SDS-PAGE). Four of the candidate proteins were expressed mainly as soluble proteins, whereas the other two proteins were expressed as insoluble forms in inclusion bodies. The insoluble forms of the proteins were recovered from the inclusion bodies using a solubilizer (50 mM CAPS, pH 11.0, 0.3% N-lauroylsarcosine and 1 mM dithiothreitol), and refolded with a Protein Refolding Kit (Novagen), according to the supplier's protocol. The soluble and refolded proteins were purified using TALON metal affinity resin (Clontech, Canada, USA), which had an affinity for the 6×His-tag of the expressed proteins under native conditions, according to the manufacturer's instructions. The buffers were exchanged with PD-10 desalting columns in the following protease assay. The

proteins were also confirmed by Western blotting for 6×His using a HRP conjugated anti Penta-His antibody (Qiagen). The concentrations were measured using a BCA Protein Assay Kit (Takara) at 595 nm with bovine serum albumin (BSA) as the standard, and based on densitometry measurements of the protein bands on SDS-PAGE gels using known protein concentration standards, such as BSA. Thioredoxin (Trx) derived from the vector pBAD/Thio-TOPO was prepared and used as a negative control in the experiments.

### ***3.3 Binding activity against trypsin***

Dissociation constants ( $K_d$ ) of candidate peptides were measured by Surface Plasmon Resonance (SPR) with a Biacore X (GE Healthcare) according to the manufacturer's instructions. Bovine pancreas trypsin (Sigma) was immobilized on the surface of a CM5 sensor chip flow cell, in concentration of 50  $\mu\text{g/ml}$  by the Biacore amine coupling kit (Biacore) according to the procedure suggested by Biacore. Candidate peptides and soybean trypsin inhibitor (STI; Wako Pure Chemical Industries, Osaka, Japan) were dissolved in HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20) to various concentrations (0-10  $\mu\text{M}$ ) and used as analytes. Candidate peptides and soybean trypsin inhibitor were injected over the trypsin surface or the blank surface. All the above experiments were run at RT, using HBS EP Buffer as running buffer. The trypsin surface was regenerated by two injections of 10 mM NaOH. The blank sensorgram was subtracted from each sample sensorgram and the binding response evaluated. The binding responses, were measured in the three set of experiments, were plotted to find out the relative potencies of aptamers in binding trypsin. Data were analyzed with the affinity analysis model in the BIA evaluation software.

### 3.4 *Protease inhibition activity assay*

Protease assays were performed with the following pairs of enzyme and substrate: bovine pancreas trypsin (Sigma) and Bz-Arg-MCA (Peptide Institute, Osaka, Japan); bovine pancreas chymotrypsin (Sigma) and Suc-Ala-Ala-Pro-Phe-MCA (Peptide Institute); porcine pancreas kallikrein (Sigma) and Z-Phe-Arg-MCA (Peptide Institute), in 85 mM Tris-HCl (pH7.5) buffer containing 112.5 mM NaCl, 1.5% PEG 6000, and 15 mM CaCl<sub>2</sub>. To compare the inhibitory activities, 100 nM BPTI (Santa Cruz Biotechnology, USA) was used. After incubating 50 nM trypsin with the candidate proteins for 30 min at 37 °C, the trypsin activities were measured by adding 400 μM of the fluorogenic substrate Bz-Arg-MCA (28). The time-courses of the fluorescence changes were monitored using a Spectra MAX Gemini (Molecular Devices, Sunnyvale, CA) with excitation at 355 nm and emission at 460 nm (29). The data were analyzed and plotted using Origin 8 (OriginLab, Northampton, MA). The half-maximal effective concentration (EC<sub>50</sub>) values were estimated from plots of the percentage inhibition versus the inhibitor concentration. The apparent  $K_{iS}$  ( $K_i^{app}$ ) was calculated using the equation:  $K_i^{app} = EC_{50} / [(1 + S/K_m)]$ , where S represents the substrate concentration (30). The  $K_m$  of trypsin for the substrate Bz-Arg-MCA was 200 μM. The data were plotted and each data point represents the mean from a single experiment, which was performed in triplicate. In the data plots, the error bars represent the standard deviation based on three replicate reactions.

## 4. Results

### 4.1 Selection of novel trypsin inhibitor candidates by directed evolution

Proteins with trypsin inhibition activity were selected by directed evolution using cDNA display, as described previously (10). The selection procedure is illustrated in Fig. 2. The mRNA (genotype) and its protein (phenotype) were linked via puromycin, followed by rapid conversion of the mRNA to cDNA by reverse transcription, and specific *in vitro* protein labeling at the C-terminus.

The selection procedures were repeated for seven rounds and the conditions were made progressively more stringent during this process. After seven rounds, the selected DNA pool was subcloned. Three-hundred transformants were randomly selected and the plasmid DNA products were sequenced. The fully translated protein sequences were classified into six groups based on variations in their predicted amino acid sequences (Fig. 3). Group 1 sequences comprised 20.6% of the total, followed by group 2 with 8.8%, group 3 with 5.1%, group 4 with 2.5%, and group 5 and group 6, each with 0.6%. Mutations were observed in the non-randomized portions, which may have been due to failed polymerase reactions in the 385 cycles (31). Point mutations and silent mutations (different coding sequences that encoded the same amino acid) were also observed in the loop sequences, which indicated that different amino acid residues may have been present in these regions. The group 1 sequence was almost the same as that of group 2, except that the mutations R9 (group 1) and Q9 (group 2) were present in random residues at the tip of loop1. Group 3 was almost the same as group 5, except that the mutations T52 (group 3) and K52 (group 5) were present in random residues at the tip of loop 3. The cysteine framework was intact in most of the clones.

## ***4.2 Expression of candidate proteins***

The recombinant candidate proteins were expressed as thioredoxin and 6×His fusion proteins using the vector pBAD/Thio-TOPO. The expressed proteins from group 2, group 3, group 4, and group 5 were expressed mainly as soluble proteins, whereas the proteins from group 1 and group 6 were expressed as insoluble forms in inclusion bodies. The expressed proteins of groups 2-5 were purified using TALON metal affinity resin in native conditions. The insoluble forms of the group 1 and group 6 proteins were recovered from the inclusion bodies with solubilization buffer, with a solubilizer, refolded in the presence of reduced and oxidized glutathione (a redox pair), and purified with TALON metal affinity resin. The proteins were analyzed by SDS-PAGE (Fig. 4) and Western blotting (data not shown). The purities were 70–90% and the average yield of the recombinant proteins from bacterial culture was 0.7 mg/100ml.

## ***4.3 Biochemical characterization of the recombinant proteins***

### ***4.3.1 Inhibitory activities against trypsin***

First, the inhibitory activities of the candidates against trypsin were examined using *in vitro* biological assays. The recombinant proteins from group 1, group 2, group 5, and group 6 had effective dose-dependent inhibitory activities against trypsin, whereas the proteins in group 3 had lower activities and the proteins in group 4 had essentially no effects (Fig. 5). The  $EC_{50}$  and  $K_i^{app}$  (apparent inhibition constants) were estimated and are shown in Table 1. The effective inhibitory activity of proteins in group 1 was double that of those in group 2 (102 nM vs. 260 nM). The two candidate groups could be distinguished based on their different amino acid residues, i.e., R9 (group 1) and Q9 (group 2). The effective inhibitory activity of proteins in group 5

was almost 15 times that of those in group 3 (66 nM vs. 900 nM), and these two candidate groups could be distinguished based on their different amino acid residues, i.e., T52 (group 3) and K52 (group 5). The inhibitory activities of selected proteins were also compared with those of Kunitz-type protease inhibitors, i.e., BPTI and STI. The  $K_i^{\text{app}}$  values of BPTI and STI were 25 nM and 29 nM, respectively. The inhibitory activities of the group 5 and the group 1 proteins against trypsin (33 nM and 51 nM, respectively) were comparable to those of BPTI and STI. To investigate the specificity of the inhibitory effects, the effects of the recombinant proteins from group 1 and group 5 were tested using two other serine proteases, i.e., chymotrypsin and kallikrein (Fig. 6). The group 1 and group 5 proteins had dose-dependent inhibitory activities against kallikrein, where the  $K_i$  values of group 1 and group 5 were 50 nM and 29 nM, respectively. Group 5 had a more potent inhibitory activity against kallikrein than trypsin. The activity of chymotrypsin was suppressed by up to 80–85% using the group 1 (500 nM) and group 5 (100 nM) proteins. It was remarkable that the compensatory recovery of chymotrypsin activity occurred in a dose-dependent manner with higher concentrations of the proteins.

#### **4.3.2 Binding activities of candidate peptides**

Binding activities of candidate peptides were measured by Surface Plasmon Resonance (SPR). The amount of trypsin in concentration of 50  $\mu\text{g/ml}$  immobilized on the CM5 sensor chip surface of the flow cell was 1709.6 RU. Tested aptamers, candidate proteins and STI were dissolved to various concentrations and used as analytes. Proteins group 1 at variable concentration 0.5, 1, 2, 4.5 and 9  $\mu\text{M}$ , and group 3 at 0.2, 2, 4, 8 and 16  $\mu\text{M}$  were performed, kinetic analysis gave dissociation constants ( $K_d$ ) of 3.67  $\mu\text{M}$  (group 1) and 7.39  $\mu\text{M}$  (group 3). As positive control, trypsin inhibitor STI at 1, 10, 100 and 1000 nM were performed, kinetic analysis gave

a  $K_d$  of 20 nM (Data not shown). Previous report shows the  $K_d$  for the interactions of STI-bovine pancreatic trypsin and BBI-bovine pancreatic trypsin were determined, respectively, to be 47.0 nM and 83.6 nM, which is comparable to the  $K_d$  of STI in our study. The binding activities values of our recombinant peptides data were about 100 times lower than that of the Kunitz-type inhibitors.

## 5. Discussion

Antibodies are the most rapidly growing class of human therapeutic molecules and the second largest class of drugs after vaccines. Antibodies have undergone significant evolution since entering clinical application, with remarkable success. However, it is obvious that antibodies have several fundamental disadvantages, including their large size, steric hindrance restriction of their tissue penetration (solid tumors and poorly vascularized tissues), and planar binding interfaces, which raise doubts in their practical applications in research, biotechnology, and medical therapy. In the past two decades, over 50 different protein scaffolds (Ig-like scaffolds and non-Ig-based scaffolds) have been developed and utilized to overcome some of these constraints. Protein scaffolds that serve as alternatives to whole antibodies have proved successful as therapeutic candidates with appropriate clinical efficacy (32, 33). Disulfide-containing scaffolds and related proteins have been utilized as templates to generate protease inhibitors and ligands for macromolecules. Previously, we successfully utilized the 3F scaffold to generate regulatory molecules (antagonist and agonist) against IL-6R (10). In the 3F-scaffold library, 23 accessible positions were randomized within the three exposed loops.

Applications of trypsin inhibitors in therapeutic activities have been investigated extensively in acute pancreatitis, acute migraine, ischemia, cancers, and other diseases

(21). Although they have been demonstrated to be effective, these drugs have potentially adverse effects in special circumstances (25, 26). In the present study, the 3F cDNA display library was preferentially screened by targeting trypsin. After selection by affinity to immobilized trypsin, selected candidate proteins were expressed as Trx fusion proteins and four exhibited effective inhibitory activities against trypsin. The inhibitory activities were in the nanomolar range, with  $K_i$  values ranging from 33 nM to 133 nM. In the present study, the  $K_i$  values were higher, but comparable to those reported previously for BPTI (25 nM) and STI (29 nM) (28). The effective inhibitory activity of the candidate proteins in group 1 was twice that of those in group 2. The two candidates could be distinguished based on their different amino acid residues, i.e., R9 (group 1) and Q9 (group 2). The effective inhibitory activity of the candidate proteins in group 5 was almost 15 times that of those in group 3, and the two candidates could be distinguished based on their different amino acid residues, i.e., T52 (group 3) and K52 (group 5). It is interesting to note that the presence of a basic amino acid residue Lys (K) at position 52 instead of Thr (T) in random residues at the tip of loop 3 may play an important role in determining the inhibitory activity against trypsin. This is because typical serine protease inhibitors bind to the reactive centers of proteases via positively charged amino acid residues (34). It has been reported that the Kunitz-type protease inhibitors BPTI and STI can inhibit chymotrypsin activity and the active site of STI is analogous with respect to trypsin and chymotrypsin (35). The investigation of the inhibitory activity of the candidates against chymotrypsin produced interesting results, where the candidate proteins in group 1 and group 5 had high inhibitory effects at low doses and low inhibitory effects at high doses.



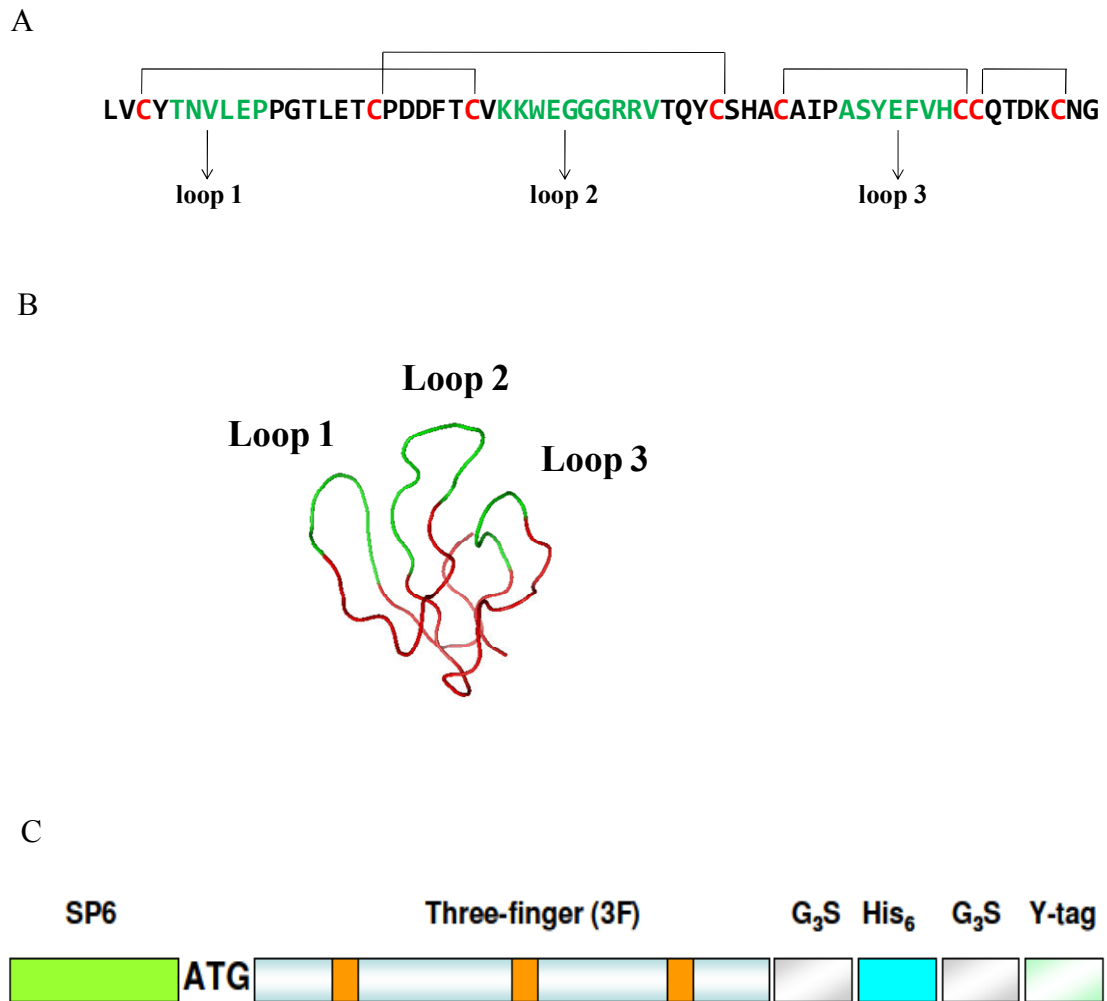
Kallikrein belongs to the family of serine proteases and is structurally related to trypsin. Plasma kallikrein plays a major role in producing bradykinin, which is a vasodilator that increases vascular permeability, activates inflammation, and produces pain (36). The leading cause of morbidity and mortality in hereditary angioedema (HAE) patients is plasma kallikrein-mediated excessive production of bradykinin. This is the rationale for the development of potent inhibitors of kallikrein (37, 38). Plasma kallikrein inhibitors also have potential therapeutic uses in the treatment of sepsis and septic shock (39). The group 1 and group 5 recombinant proteins generated via the directed evolution of the 3F library were effective inhibitors of kallikrein with  $K_i$  values of 50 nM and 29 nM, respectively. The present study demonstrates that the 3F protein library may provide a new platform for discovering potent small molecule inhibitor(s), where the effectiveness may be optimized to treat kallikrein-mediated inflammatory disorders while minimizing the adverse effects.

Previously, we demonstrated that functional regulatory molecules with activity against IL-6R could be generated via the directed evolution of the 3F scaffold. The present study extended the application of this technique by targeting trypsin. Selected proteins had a broad range of inhibitory activities against trypsin and some also had inhibitory effects against other proteases. Some protein protease inhibitors have been reported to inhibit multiple proteases, such as trypsin and chymotrypsin (35), or trypsin and kallikrein (40), which are assumed to share a conserved molecular environment in the active pocket of the proteases. The advantage of our techniques is that additional conditions can be incorporated during the selection step to include (positive selection) and/or exclude (negative selection) binding proteins. In future studies, this technique could be used to generate inhibitors, which may facilitate the

strict discrimination of inhibitors of trypsin, kallikrein, and plasmin with broad applications in research, diagnostics, and therapeutic uses.

## **6. Conclusions**

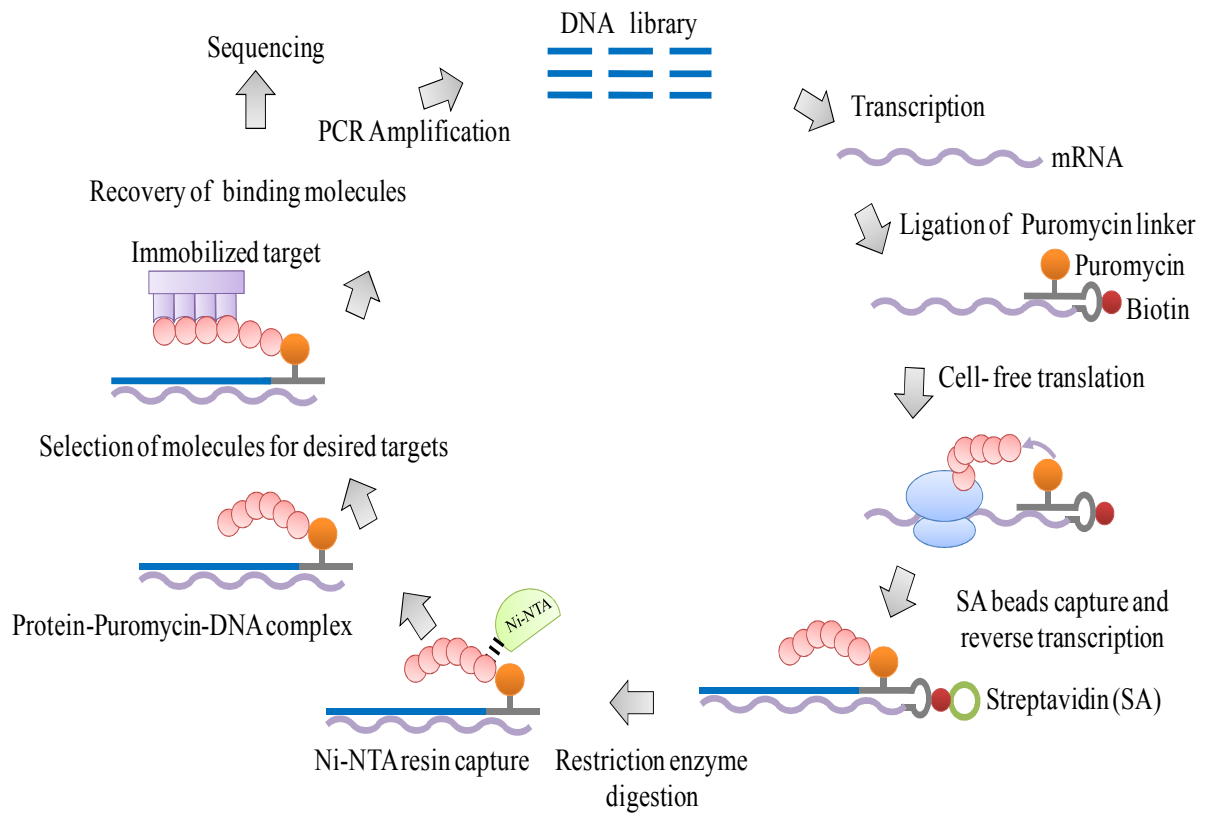
In the present study, selected candidate proteins with effective serine protease inhibition activities were generated via the directed evolution of a 3F library. This procedure was based on a high-diversity library where the 3F scaffold was utilized as a template and the tips of the loops were randomized. In addition to our previous success in targeting IL-6R, the technique described in the present study may have wide applications in the generation of regulatory molecules that target molecules of interest, such as receptors, as well as enzymes for research uses. Recent research has shown that various scaffolds may potentially be used as substitutes for whole antibodies and their associated properties have been validated, which has allowed them to be translated into biological agents with drug-like properties in clinical development. Thus, the 3F cDNA library, which can be used as a directed evolution template to target proteases and receptors, may be useful for generating viable drugs with diagnostic and therapeutic applications in a post-antibody landscape.



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**Fig. 1.** Construction of the three-finger (3F) cDNA library

(A) Primary structure (B) 3D structure of the 3F protein. The four disulfide bonds are formed between 8 conserved cysteines. The loops are shown in color and were randomized to generate the libraries. (C) Genetic construct adopt for cDNA display SP6 contains the sp6 promoter, capping site and *Xenopus* globin untranslated sequence (UTR). ATG is the translation initiation codon. The 3F gene with the three randomized loops provides the basis of the library. G<sub>3</sub>S is spacer, 6XHis facilitates affinity purification by Ni-NTA, and Y-tag facilitates ligation of mRNA to the puromycin linker which contains the complementary Y-tag sequence.

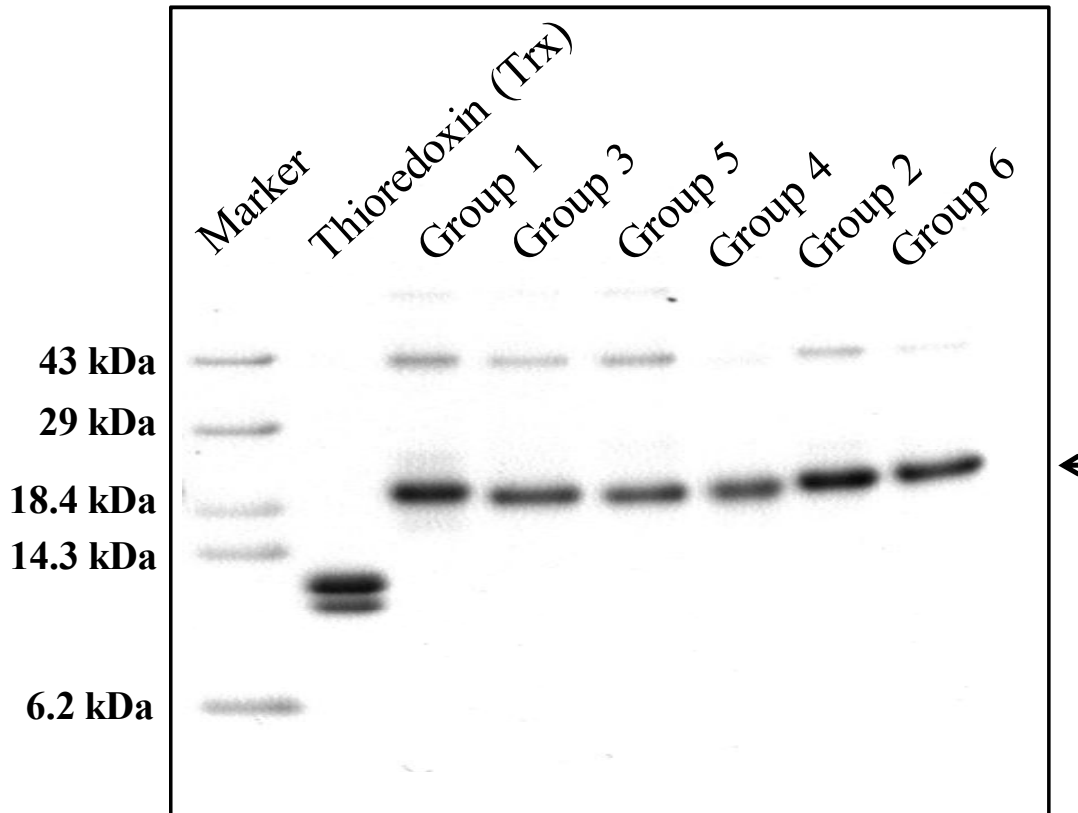


**Fig. 2.** Overview of the cDNA display method used for evolution *in vitro*.

cDNA display was based on covalent fusion of the expressed protein (phenotype) and the encoding cDNA (genotype) via puromycin attached to an oligonucleotide linker. The library was prepared using the construct required for cDNA display, transcribed into mRNA, and ligated to the puromycin-linker. The mRNA-linker conjugates were translated in a cell-free translation system. After purification, the cDNA products were synthesized by reverse transcription and the full-length synthesized proteins were purified using Ni-NTA resin. After selecting the molecules using the immobilized targets, the bound molecules were recovered, purified, amplified, and employed in the next round of selection.

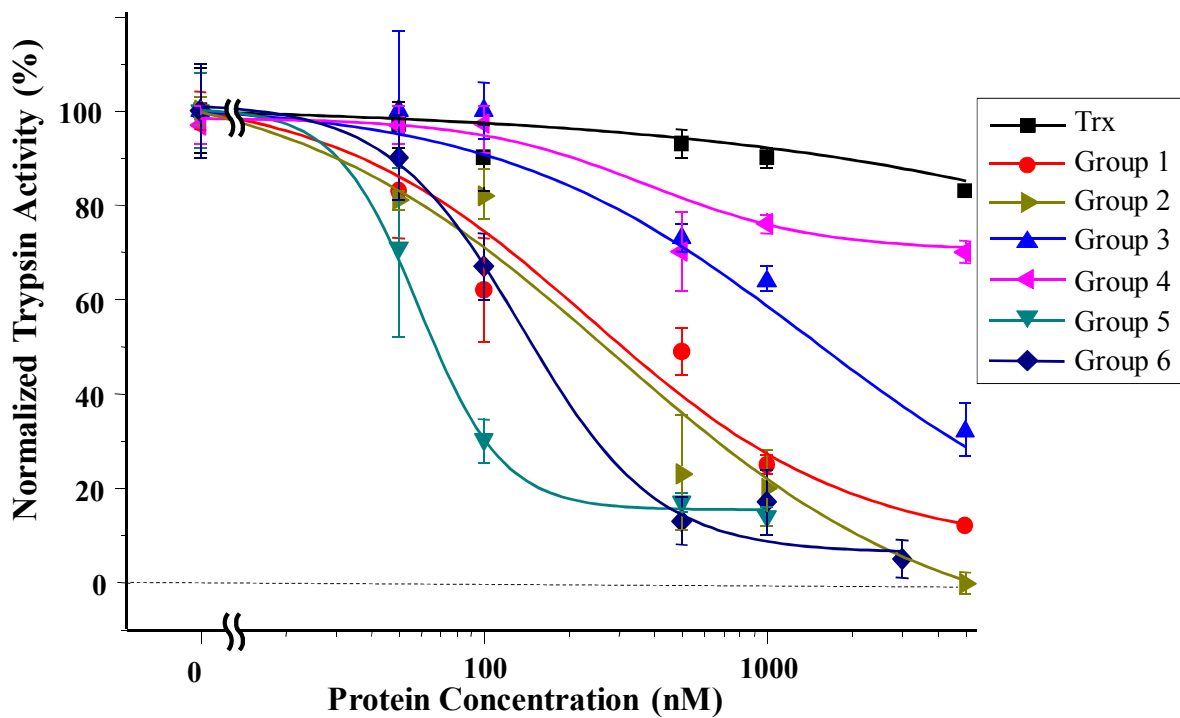
Wt	LVCY <u>TNVLEP</u> PGTLET <u>CPDDFTCV</u> <u>KKWEGGRRV</u> TQYCSHACAIP <u>ASYEFVH</u> <u>CCQTDK</u> CNG			
R0	<b>XXXXXX</b>	<b>XXXXXXXXXX</b>	<b>XXXXXX</b>	% population
Group 1	<b>LRVFR<u>Y</u></b>	<b>TTSPASEHTM</b>	<b>PRKRQSP</b>	20.6
Group 2	<b>LRVF<u>Q</u>Y</b>	<b>TTSPASEHTM</b>	<b>PRKRQSP</b>	8.8
Group 3	<b>APPVCD</b>	<b>ARKILSGCHS</b>	<b>KCHQL<u>Y</u>T</b>	5.1
Group 4	<b>MAVPRL</b>	<b>NGSDHTMSG</b>	<b>SADNAGD</b>	2.5
Group 5	<b>APPVCD</b>	<b>ARKILSGCHS</b>	<b>KCHQL<u>Y</u>K</b>	0.6
Group 6	<b>LGPVGT</b>	<b>GRNRTLSPHH</b>	<b>PLATQRS</b>	0.6

**Fig. 3.** Sequence alignment of the selected proteins with the template 3F protein, MicTx3. The sequence of Mic Tx3 was used as the template for the 3F library, which is indicated by 'wt,' whereas the initial 3F library is indicated as 'R0.' The cysteine residues are shown in red. The conserved disulfide bonds in 3F proteins are indicated by black lines. Randomized sequences in the tips of loops are shown as underlined letters in 'wt' and are indicated by 'X' in 'R0'. The sequences of the candidate clones were assigned to six groups based on their sequence identities. The same sequences of selected clones that appeared in the tips of different groups are shown in the same color. Mutations in the same color are indicated by the underlined residues. The E9 and H52 residues were found to be mutated to R (group 1)/Q (group 2) and T (group 3)/K (group 5) residues, respectively. The percentage population indicates the proportion of the selected molecules (300 clones) that were sequenced.

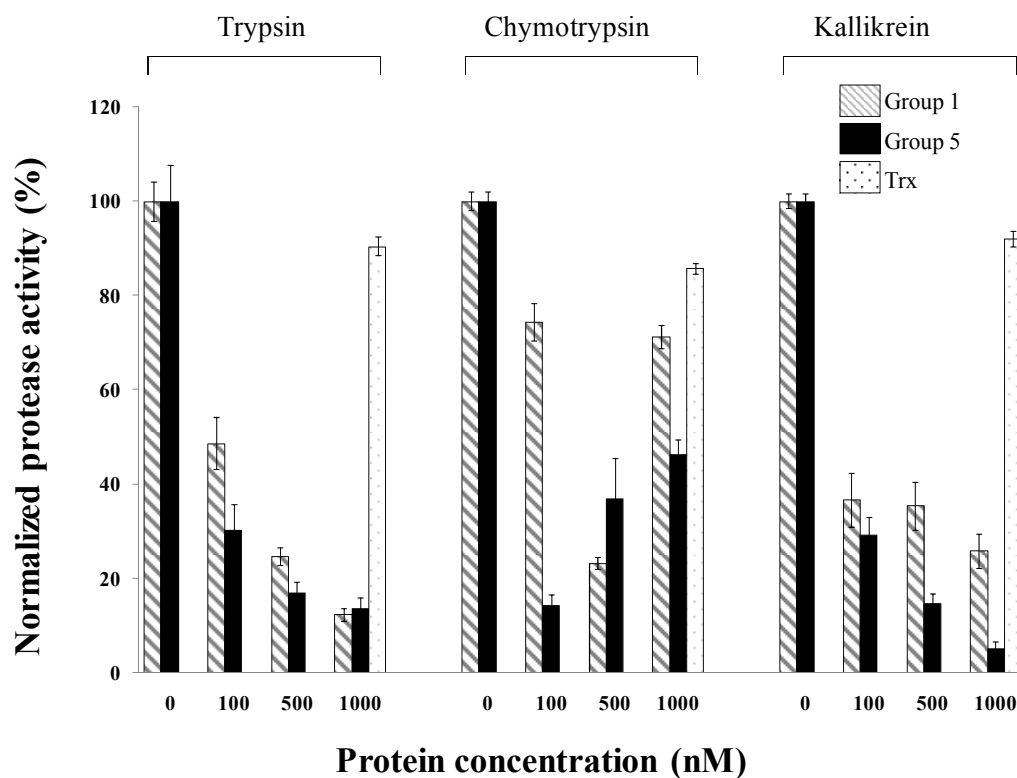


**Fig. 4.** Purification of the recombinant 3F candidate proteins.

The selected candidate proteins were expressed as 6×His and thioredoxin (Trx) fusion proteins, and purified using TALON metal affinity resin. The elution buffer was replaced with PD-10 desalting columns and the purified proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, before staining with Coomassie Brilliant Blue R-250. Trx without candidate protein (molecular weight 13.1 kDa) was prepared as the negative control for the protease assay. The arrows indicate the *bands* with the expected size of purified candidate proteins (average molecular weight 21.5 kDa). The upper bands were approximately twice the size of the candidate proteins and they may have corresponded to dimers of the candidate proteins (average molecular weight 43.0 kDa). Some 3F proteins, including kappa-bungarotoxin and alpha-cardiotoxin, tend to form dimers in solution. The samples loaded in the respective lanes were as follows: molecular weight markers (lane 1); Trx (lane 2); group 1 (lane 3); group 3 (lane 4); group 5 (lane 5); group 4 (lane 6); group 2 (lane 7); group 6 (lane 8).



**Fig. 5.** Dose-dependent inhibitory effects of the selected 3F proteins on trypsin. Trypsin (50 nM) was incubated with the indicated concentrations (from 0 nM to 5  $\mu$ M) of candidate recombinant proteins (group 1 to group 6) for 30 min at 37  $^{\circ}$ C and the trypsin activities were measured using fluorogenic substrates. Thioredoxin was used as a negative control in the protease assay. The asymptotic value of the sigmoid curve was defined as the 0% trypsin activity. The 100% trypsin activity value was determined based on measurements in the absence of candidate protein. The broken line indicates the asymptotic value of the sigmoid curve. Each data point represents the mean from a single experiment, which was performed in triplicate. The error bars represent the standard deviation.



**Fig. 6. Effects of the selected 3F proteins on serine proteases.**

Trypsin (50 nM), chymotrypsin (10 nM), and kallikrein (20 nM) were incubated with the indicated concentrations (0 nM, 500 nM, and 1  $\mu$ M) of candidate and thioredoxin proteins for 30 min at 37 °C. The protease activities were measured using fluorogenic substrates. Each data point represents the mean from a single experiment, which was performed in triplicate. The error bars represent the standard deviation.



**Table 1.** Comparison of the trypsin inhibition activities of selected 3F proteins

Protease	Substrate	$K_m$ ( $\mu$ M)	$EC_{50}$ <sup>a</sup> (nM)						$K_i$ <sup>b</sup> (nM)						BPTI	STI
			3F proteins						3F proteins							
			1	2	3	4	5	6	1	2	3	4	5	6		
Trypsin (bovine)	Bz-Arg-MCA	200	102	260	900	NI <sup>c</sup>	66	140	51	130	450	NI <sup>c</sup>	33	70	25 <sup>d</sup>	29 <sup>d</sup>

<sup>a</sup>  $EC_{50}$ = the half-maximal effective inhibitory concentration

<sup>b</sup>  $K_i = EC_{50}/[(1+S/K_m)]$

<sup>c</sup> NI = non-inhibitory

<sup>d</sup> Reference 28

## Chapter III

### **In vitro evolution of CXC-based peptides to study the molecular mechanisms of neuronal development**

#### **Summary**

Directed evolution has now been applied to a wide range of protein functions, including ligand and protein binding, and regulatory functions in the past few years (41, 42). The cDNA display technology utilized a cell-free translation system was performed in second chapter to generate trypsin inhibitor from a 3F neurotoxin cDNA library. In this chapter, I employed an entirely different strategy based on *in vitro* compartmentalization (IVC) in emulsion. This strategy was used to physically link proteins (phenotype) to the DNA (genotype) that encodes them of reactions in the aqueous droplets of water-in-oil via microbeads (43, 44). *In vitro* compartmentalization (IVC) offers an alternative strategy dispersed to form microscopic compartments in water-in-oil emulsions based on partitioning reactions in water droplets (46, 47). Water-in-oil emulsions allow individual genes were transcribed and translated in femtoliter-sized aqueous reaction vessels (48, 49). Small size of the compartments, large protein libraries ( $10^8$ – $10^{12}$  variants) can be expressed, and sequence variants that lead to altered function can be captured by a variety of selections and screens (50). Previous study reported that a number of variants of the basic IVC selection method have been used for the identification of affinity reagents. For example, Sepp et al. demonstrated that genes encoding peptides bearing a FLAG tag could be enriched by co-immobilizing the genes and an anti-Flag antibody on a

microbead. Following translation, compartments were broken and bead-bound peptides were fluorescently-labeled and isolated by FACS (43, 51). Using a different approach, Yanagawa's lab reported the affinity enrichment of peptide-SA (streptavidin) fusions and the biotinylated genes that encoded them. SA-fusions translated within a compartment associated tightly with their genes (52).

Here, I expect to generate novel small peptides targeting to the growth factor brain-derived neurotrophic factor (BDNF) receptor/TrkB from CXC-based random cDNA library by IVC selection in emulsion. Proteins are classified as chemokines according to shared structural characteristics such as small size (8-10 kDa), and the presence of four cysteine residues in conserved locations that are key to forming their 3-dimensional shape, functions as a growth regulator and potent chemoattractant for neutrophils (45). Members of the chemokine family are divided into two major families (CXC or  $\alpha$  chemokines, and CC or  $\beta$  chemokines) and two minor families (C or  $\gamma$  chemokines, and CX3C or  $\delta$  chemokine) depending on the spacing of their first two cysteine residues. Chemokines promote angiogenesis (the growth of new blood vessels), or guide cells to tissues that provide specific signals critical for cellular maturation. CXCL1 is a polypeptide hormone of 73 residues with two disulfide bonds between C1 and C3, C2 and C4. It plays a role in spinal cord development and is involved in biological processes of angiogenesis, inflammation, wound healing and tumorigenesis (53, 54). These properties may make CXC family as candidates for *in vitro* evolution to generate specific ligand for target molecules. BDNF receptor, TrkB was used as target molecules for directed evolution of CXC random library in present study. BDNF and its receptor TrkB mediate neuronal survival, differentiation, synaptic plasticity, and neurogenesis (55), show therapeutic potential in the treatment of various neurodegenerative diseases, such as Parkinson' disease (PD), Alzheimer's

disease (AD) and amyotrophic lateral sclerosis (ALS) (56-58). However, the therapeutic application of BDNF has been greatly limited because of its poor permeability blood-brain barrier (BBB). While several completely *in vitro* display systems have recently been used for protein minimization to develop peptide drugs, selection of low-molecular-weight BDNF-mimicking peptides with good permeability and superior pharmacokinetic properties has met with little success. In order to select low-molecular-weight BDNF-mimicking peptides, random cDNA library was prepared based on CXCL1 to produce specific ligand to target receptor TrkB by IVC selection in emulsion. After construction, *in vitro* transcription and translation of CXCL1 random library, physiological potentials of the library were first investigated by biological assay using primary cell culture of hippocampal neurons. Peptide pools not only promoted neuronal survival, but also enhanced neurites outgrowth, which indicate that CXC-based peptides may mimic cell signaling pathways identical to or similar to that of BDNF and/or NGF. Ligand-binding domains (LBD) of target protein TrkB which expressed in *E.coli* had been prepared. The recombinant TrkB had binding activity to BDNF, and the value was comparable to previous reports. After 7-10 rounds selection of CXCL1-based peptides targeting to TrkB, the selected DNA pools will be recovered, sequenced, expressed and characterized by proceeding to biological assays.

Present data showed that the peptide pools prepared based on CXC scaffold not only significantly promoted neuronal survival, but also enhanced neurite outgrowth. The result indicated that the CXC-based peptides may mimic cell signaling pathways identical to or similar to that of BDNF and/or NGF, may be of potential application in the treatment of various neurodegenerative diseases. The IVC strategy based on creating repertoires of microbeads (each displaying a gene and the protein it encodes)

were described in present study. Microbead-display libraries can be selected on the basis of the binding activity of the displayed polypeptide in the strategy. Present study of IVC proceeds well, and can be performed to further study. Further investigation on *in vitro* evolution of CXC random library by using IVC, may help in the development of small molecule compounds such as low-molecular-weight BDNF-mimicking peptides of potential utility in the treatment of a variety of neurological diseases.

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## **Acknowledgment**

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever.

I owe my deepest gratitude to Dr. Tai Kubo, Prof. Norio Ishida, Dr. Hidetoshi Inagaki, and Dr. Kimihiko Kameyama for their continuous support, encouragement and guidance throughout my project work. I hope that one day I would become as good an advisor to my students as they have been to me. A special thanks to Dr. Mohammed Naimuddin for his kind support and guidance throughout my project work.

It is a pleasure to thank those who made this thesis possible, all my friends from Drs. Kameyama/Kubo's group and Dr. Doi's group. In addition, I would like to give my thanks to all members of Ishida Group of Clock Gene for their kind support. Their support and care helped me overcome setbacks and stay focused on my study. I greatly value their friendship and I deeply appreciate their belief in me.

Most especially to my parents, and my sister. Words alone cannot express what I owe you for your encouragement and patient love, which enabled me to complete my study.

Finally, I appreciate the financial support from University of Tsukuba and National Institute of Advanced Industrial Science and Technology (AIST) helped make the completion of my graduate work possible.