CHAPTER IV
MATERIALS AND METHODS

1. Purified pertussis toxin (PT)

Purified pertussis toxin (PT) was kindly provided by BIKEN (The Research Foundation of Microbial Disease of Osaka University, Japan) in lyophilized ampoule containing 100 μg of PT protein.

2. *Escherichia coli* strains

The *E. coli* strains used in this study included:

2.1 *E. coli* BL21 [F – *ompT hsdSB* (*rB– mB–*) *gal dcm*]

2.2 *E. coli* TG1 [supE thi-1 D(lac-proAB) hsdD5(F' traD36 proAB+ lacIq lacZD M15)]

2.3 *E. coli* HB2151 (ara, Mlac-pro, thi/F'. proA+B+, lacIq, lacZAMJS)

2.4 *E. coli* JM109 [(endA1 glnV44 thi-1 relA1 gyrA96 recA1 $mcrB^+$ Δ(lac-pro AB) glnV44 e14- (F' traD36 $proAB^+$ $lacI^q$ lacZΔM15] hsdR17($r_K^-m_K^+$)]

3. Murine hybridoma clone PT6-2G6

A hybridoma clone that secretes monoclonal antibody (MAb) specific to PT, namely clone PT6-2G6, was produced in 1994 (Buddhirakkul, 1994) and maintained in the Molecular Immunology and Microbiology Laboratories, Faculty of Allied Health Sciences, Thammasat University, Rangsit Center. This hybridoma was derived from the fusion between immunoglobulin non-secreting myeloma cells (P3-x63-Ag8.653) and the spleen cells of a mouse immunized with 1 μg of purified PT for three times at an interval of two weeks by the method of Koehler and Milstein (1975). The MAbPT6-2G6 was IgG1 subclass, with kappa light chain. The antigen recognized by this MAb was determined by Western-blot analysis and was found to be specific to S1 component (Buddhirakkul, 1994).

4. Preparation of MAbPT6-2G6

PT6-2G6 hybridoma cells were grown in serum free medium (CD medium, GIBCO®, New York, USA) to late log phase. The cell suspension was centrifuged at 3,500 × *g* at 4°C for 10 min and the supernatant was pooled. The pool of spent culture medium containing monoclonal antibody was concentrated by pressure filtration method (Amicon, USA). The concentrated PT6-2G6 monoclonal antibody was dialyzed again phosphate buffered saline (PBS) pH 7.4 (**Appendix B**) at 4°C overnight. Antibody solution was centrifuged at 8,500 × *g* at 4°C for 20 min and filtered through 0.45 μm filter (Amicon, USA). Then the MAb solution was subjected to purification by protein G affinity column chromatography.

5. Purification of MAbPT6-2G6 by protein G affinity column chromatography

The concentrated MAbPT6-2G6 was loaded onto a HiTrap Protein G HP column (size 1 ml, GE Healthcare, USA) pre-equilibrated with 10 ml of binding buffer (20 mM sodium phosphate, pH 7.0, **Appendix I**). After MAbPT6-2G6 was applied to the column, unbound materials were washed with 10-20 column volumes of the binding buffer. Then the IgG was eluted out with 6 ml of an elution buffer (0.1 M glycine-HCl, pH 2.7, **Appendix I**) and immediately neutralized by adding 200 μl of 1 M Tris-HCl, pH 8.2 (**Appendix I**) per ml of the elution fraction. The OD of IgG in each fraction was measured at OD_{280} nm.

6. PT neutralizing activities of the MAbPT6-2G6

6.1 *In vitro* assay: hemagglutination assay (HA) and hemagglutination-inhibition (HI) test

The hemagglutination assay (HA) was performed as the following: 50 μl aliquots of a 0.7% suspension of washed chicken erythrocytes in PBS were added to individual wells of the serially diluted PT in PBS in a V-bottom well plate, mixed and the plate was kept at 25°C for about 30 min. The least amount of the PT in the well that caused complete agglutination of the red blood cells (hemagglutination dose 100; HD100) was determined.

For hemagglutination inhibition (HI) assay, one HD100 of PT in 25 μl of PBS was incubated with various amounts of MAb (in 25 μl PBS) at 25°C for 30 min. Fifty

µl of 0.7% chicken erythrocyte suspension was added to each incubation mixture and the preparation was kept at 25°C for 30 min. The lowest amount of the MAb that could completely inhibit the hemagglutination caused by the HD100 of PT was recorded.

6.2 *Ex vivo* test: CHO clustering inhibition (CCI) assay

The procedure as described by Hewlett *et al*. (1983) was followed with some modification. CHO cells (strain K1) were cultured in RPMI-1640 medium (GIBCO®, New York, USA) containing 10% fetal bovine serum and 1% antibiotic solution (complete RPMI medium, Appendix A) at 37°C in 5% CO_2 for 2-4 days until a confluent cell growth was obtained. PT was two-fold serially diluted in complete RPMI medium and 50 µl of each dilution was added to each well. Complete RPMI medium was used as a negative control. The CHO-cell suspension (200 µl, 10^5 cells/ml) was added to each well. The plate was gently shaken, incubated at 37°C in 5% CO_2 for 1-2 days, and the examined for CHO-clustering. The CHO-clustering dose (CCD) were determined and used in the CCI assay.

For neutralization experiments (CCI assay), PT at a CCD_{50} was incubated with equal volume of serially diluted MAbPT6-2G6 at 25°C for 30 min. Fifty microliters of the reaction mixture was added to CHO cells in a 96-well microplate. Eighteen hours thereafter, clustering of the cells was observed under a phase contrast microscope and the clustering was scored as 0 (no clustering), 1 (25% CC), 2 (50% CC), 3 (75% CC), or 4 (100% CC).

6.3 *In vivo* test: leukocytosis-promotion (LP) inhibition test

EDTA-blood samples were taken individually from 30 female BALB/c mice, aged 6 weeks old, on day 0 and the number of white blood cells (WBC) in each sample was counted as the baseline data. The mice were then divided into 5 groups (groups 1-5). Mice of groups 1 and 2 were each injected intravenously with 100 µl PBS alone (negative LP control) or PBS containing 500 µg of MAb specific to cholera toxin plus 100 ng PT (positive LP control). Mice of groups 3-5 were injected with the same amount of PT previously incubated with three different concentrations of MAbPT6-2G6. Four days post-PT injection (day 3), all mice were bled and their

WBC counts were re-determined. The WBC count of each mouse on day 3 was subtracted with baseline WBC count of day 0 of that particular mouse to obtain the ΔWBC. The ΔWBC of mice of groups 3-5 were compared with the ΔWBC of groups 1 and 2 by using Mann-Whitney U test. Difference at $p < 0.05$ was statistically significant.

7. Determination of the mimotope of MAbPT6-2G6 by using a peptide phage display library

7.1 Random heptapeptide peptide T7 phage library

Mimotope of the specific MAb to PT was determined using a T7 phage display peptide library. The library was constructed by G. Froman, Department of Medical Microbiology, Uppsala University, Sweden, using the T7 select415 kit from Novagen (Wisconsin, USA). Library construction started by synthesizing the random heptapeptide inserted DNA. The inserted DNA was derived from degenerated oligonucleotides, synthesized chemically by adding mixtures of nucleotides to the growing nucleotide chain. The synthetic oligonucleotides were designed to give a seven-residue long random amino acid sequence flanked by cysteine residues. To limit the occurrence of in-frame stop codons, the seven position of degenerated sequence NNK was used; each N was an equal mixture of A, G, C and T, while each K was an equal mixture of G and T. For each NNK, a mixture of 32 nucleotide triplets could be formed, include codons for all 20 natural amino acids, and one stop codon (TAG). Each synthesized oligonucleotide was ligated to the T7 vector arm. Target peptides were expressed as fusion to the C-terminus of the 10B capsid protein and were displayed on the virion surface, where they were accessible for interaction with other proteins or ligands. The displayed peptide was situated between cysteine residues, and therefore, formation of a disulfide bridge would join the ends of the heptapeptide. The fusion polypeptide was present in ~415 copies of each phage particle. It had an original size of 3.3×10^7 pfu/ml, but before use it was amplified to a titer of 2.6×10^{10} pfu/ml. The library has been successfully used to map mimotopes of MAb against *Leptospira* spp. (Tungtrakanpoung *et al.*, 2006) and cockroach allergen (Sookrung *et al.*, 2006).

7.2 Bio-panning

For phage bio-panning, MAb at 10 μg/ml PBS was used to coat wells in microtiter plate (100 μl per well), incubated in a humid box at 37°C for 1 h and then kept at 4°C overnight. The MAb-coated wells were washed three times with PBS-T (PBS with 0.05% Tween-20) and blocked with 5 % bovine serum albumine (BSA) in PBS (**Appendix F**). Excess blocking reagent was removed by washing with PBS-T, then the T7-peptide phage library was added to MAb-coated-well and incubate at 25°C under gentle agitation for 45 min. Unbound phages were washed off; and bound phages were eluted by incubating the well with 100 μl of 1 % SDS. The eluted phages were used to transfect *E. coli* strain BL21 to produce a peptide-phage sublibrary for the next bio-panning round. To increase phages affinity to the MAb, three more bio-panning rounds were done with decreasing the duration of the binding step and increasing the time of washing. Finally, the phages in the fourth sub-library were cloned by plaque isolation technique. Thirty single plaques of T7 phages were randomly picked by cutting out the gel plugs. The individual phage clones were eluted by disperse the gel plugs in 100 μl of 10 mM EDTA, pH 8.0 (**Appendix F**). DNA was extracted from the individual T7 phage clone preparation by heating at 65°C for 10 min and used as templates for DNA amplification by PCR.

7.3 PCR and DNA sequencing of selected T7 phages

In order to analyze the peptide sequences of the MAb bound phages, a DNA segment of the T7 phage encoding the 10B capsid protein was amplified using the T7 select up (5'-AGC TGT CGT ATT CCA GTC A-3') and down (5'-ACC CCT CAA GAC CCG TTT A-3') sequence as primers according to the protocol of the manufacturer (Novagen 2000). The total PCR reaction mixture (50 μl) consisted of the following reagents: 2 μl each of T7 selected up and down primers (10 pmol/μl), 5 μl of 10 x buffer, 3 μl of 25 mM $MgCl_2$, 0.4 μl of *Taq* DNA polymerase (5 units/μl), 4 μl of dNTP (25 mM), 32.6 μl H_2O, and 1 μl of phage DNA. The DNA amplification was performed in Thermal Cycler (Mastercycler gradient, Eppendorf, Germany) using the following program: one cycle at 94°C for 2 min, 35 cycles of (94°C for 20 sec, 50°C for 20 sec and 72°C for 45 sec) and final-cycle complete extension at 72°C for 4

min. The PCR product was purified using the QIA quick PCR purification kit (Qiagen, Valencia, CA, USA). The PCR products of the selected T7 phages clones were subjected to an automated DNA sequencing procedure.

7.4 Local alignment of selected phage sequences with S1 sequence

Amino acid sequences were deduced from the so-obtained DNA sequences. The deduced amino acid sequences were aligned with previously reported "target-unrelated peptides" (TUP) (Menendez and Scott, 2005) and also compared with vector sequences in GenBank using Vecscreen software (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen. html). These MAb mimotope sequences were subsequently aligned with the S1 peptide sequence of the GenBank database using SIM software (http://www.expasy.ch/tools/ sim-prot.html). The criterion of matching was set at ≥ 40 % homology between the mimotope sequences containing 5 or more amino acids with the S1 amino acid sequence. The consensus peptide sequence was subjected to map the amino acids on the 3D structure of S1 protein using PyMOL program in order to localize the MAb epitope on the S1 protein.

7.5 Locating the epitope recognized by MAbPT6-2G6 on the S1 model

To study the location of epitope recognized by MAbPT6-2G6 on the S1 molecule of PT, the mimotope were compared with S1 model obtained from The Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB) (http://www.rcsb.org/pdb/static.do?p=fileformats/pdb/index.html) using PyMOL 0.99rev8 software (DeLano Scientific LLC, USA).

8. DNA amplification and CDR identification of VH and VL genes of murine hybridoma clone PT6-2G6

8.1 Preparation of RNA of PT6-2G6 hybridoma

PT6-2G6 hybridoma cells were grown in RPMI-1640 medium supplemented with 10 % fetal bovine serum (FBS) at 37^{o}C in a 5% CO_2 incubator (Shel Lab, USA). Log phase hybridoma cells were harvested by centrifugation at 250 × *g* for 10 min. Hybridoma cells (6-10 × 10^6) were subjected to RNA isolation by adding with 1 ml of Trizol® reagent (Invitrogen, USA). Cells were homogenized by repetitive pipetting.

The homogenized sample was incubated at 25°C for 5 min to permit the complete dissociation of nucleoprotein complexes. Two-hundred microliters of chloroform were added. The homogenized sample was shaked vigorously by hand for 15 sec and incubated at 25°C for 2 min. The sample was centrifuged at 12,000 × *g* for 15 min at 4°C. After centrifugation, a lower red, phenol-chloroform phase, an interphase, and colorless upper aqueous phase containing RNA was obtained. The upper aqueous phase was transferred to a sterile new microtube. RNA was precipitated by adding 0.5 ml isopropyl alcohol, at 25°C for 10 min and the preparation was centrifuged at 12,000 × *g* for 10 min. The supernatant was discarded. The RNA pellet was washed with 75% ethanol and air-dried. RNA was resuspended in RNAse-free water and stored at -70°C until use.

8.2 cDNA synthesis

Complementary DNA of PT6-2G6 hybridoma cells was synthesized by using RevertAid™ H Minus kit (Fermentas, USA). The synthesis mixture (20 µl) consisted of 1 µg of total RNA and 1 µl of oligo $(dT)_{18}$ primer (0.5 µg/µl). The reaction mixture was incubated at 70°C of 5 min and then chilled on ice. The mixture (7 µl) contained 4 µl 5x reaction buffer, 1 µl of 20 units/µl ribonuclease inhibitor and 2 µl of dNTP (10 mM) each was added to the reaction mixture and incubated at 37°C for 5 min. One microliter of reverse transcriptase (200 units/µl) was added and incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and chilled on ice. The cDNA was stored at -20°C until use.

8.3 PCR amplification of hybridoma *PT6-2G6-VH* and *VL* genes

The VH and VL genes of the hybridoma were amplified by using the cDNA as a template and VH-F (AGG ATT CGC GGC CGC TGA CGA GAC GGT GAC TGA GGT), VH-R (GGC GGC GGC TCC GGT GGT GGT GGA TCC GAG GTC CAG CTG) and VL-F (GGA GCC GCC GCC GCC AGA ACC ACC ACC AGA ACC ACC ACC), VL-R (AGC CGG CCG AYA TTG TGM TSA CCC AAA CTC CA) as primers for VH and VL gene amplification, respectively. Fifty microliters of DNA amplification reaction mixture containing 2 µl each of forward and reverse primers (20

pmol/μl), 5 μl of 10 x buffer, 3 μl of 25 mM $MgCl_2$, 0.4 μl of *Taq* DNA polymerase (5 units/μl, Fermentas, USA), 4 μl of dNTP (2.5 mM each), 32.6 μl H_2O, and 1 μl of cDNA, was prepared. The DNA amplification was performed in a Mastercycle gradient (Effendorf, Germany) using the following condition: 1 cycle of denaturation 95°C for 5 min 29 cycles of denaturation 95°C for 45 sec, annealing 50°C for 45 sec and extension 72°C for 45 sec and 1 cycle of final extension 72°C for 5 min. The PCR product was subjected for agarose gel electrophoresis and ethidium bromide staining**.**

8.4 Determination of hybridoma PT6-2G6-VH and VL coding DNAs by agarose gel electrophoresis

Agarose gel electrophoresis was used to detect the *VH* and *VL* amplicons. Briefly, the 1 % DNA grade agarose (USB, Cleveland, OH, USA) in Tris-acetate-EDTA (TAE, **Appendix E**) buffer was prepared. The agarose solution was cooled down to approximately 50°C before pouring into the gel casting apparatus with appropriate gel comb insertion in order to make wells. The gel was allowed to solidify at 25°C. Five microliters of each DNA amplicon was mixed with 1 μl of 5x DNA loading dye (**Appendix E**). The mixture was loaded into the designated well. Electrophoresis was done in a chamber filled with 1× TAE buffer at 100 Volts until the dye front reached ~70% of the gel length. Then, the power was turned-off; the agarose gel was removed and stained with 0.5 μg/ml of ethidium bromide solution for 10 min. After destaining in TAE buffer, the specific bands of amplicons were visualized and photographed by using UV transilluminator (UVP Transilluminator, USA).

8.5 Cloning of PT6-2G6-VH and VL genes

The *VH* and *VL* amplicons of the hybridoma PT6-2G6 were purified from the agarose gel using GENECLEAN® II KIT (Q-BIO gene, USA). Purified VH and VL genes were ligated individually into pGemT® Eazy Vector (Promega, USA). The ligation reaction (10 μl) consisted of 3 μl of ultrapure distilled water, 2 μl 5x ligation buffer, 1 μl of pGemT® Eazy Vector, 1 μl of T4 DNA ligase and 3 μl VH or VL DNA. The ligation mixture was incubated at 25°C for 1 h and then at 4°C overnight. The ligated vectors were used to transform *E. coli* strain JM109 by adding 3 μl of the

ligation mixture to TSS treated-*E. coli* competent cells and heated-shock the reaction in 42°C water bath for 45 sec. The transformed *E. coli* were recovered by adding 2× YT medium (**Appendix H**) and kept at 37°C with gentle shaking at 250 rpm for 1 h. The transformed cells were grown on LB agar containing 100 μg/ml amplicilin/ IPTG/ X-Gal. The agar plate was incubated at 37°C overnight. The white colonies were randomly picked, inoculated into LB medium containing 100 μl/ml ampicillin and incubated at 37°C with shaking at 250 rpm. Plasmid DNA was prepared from each culture according to alkaline lysis plasmid miniprep protocol in Molecular Cloning: A Laboratory Manual**.** The plasmid DNA was electrophoresed in 0.7% agarose gel at 100 Volts and then visualized after ethidium bromide staining as above.

Plasmid vectors containing VH and VL genes were subjected to DNA sequencing in order to ensure that they were murine VH and VL DNA sequences.

8.6 CDR identification of the murine PT6-2G6-VH and VL coding DNA sequences

VH and VL DNA sequences of the hybridoma PT6-2G6 were deduced to amino acid sequences. CDRs of the deduced peptide sequences were identified using IMGT/V-QUEST (http://imgt.cines.fr/).

8.7 Selection of the most matched-human VH and VL sequences to the murine VH and VL sequences

In order to select the most matched-human VH and VL sequences to the murine counterparts, the amino acid sequences of the latter were aligned with the human VH and VL amino acid sequences of the database.

9. Production of humanized-murine *scFv* sequence (*huscFv*)

9.1 Construction of humanized-murine *scFv* sequence

The murine DNA sequences encoding the three *CDRs* were grafted onto the respective human immunoglobulin frameworks (*FRs*) by using splice overlapped extension PCR (SOE-PCR). For production of the humanized-murine *VL*, four DNA segments were constructed. The first segment was N-terminus *Sfi*I restriction site-human *VL FR1*-murine *CDR1* sequence; the second segment was murine *CDR1*-

human *FR2*-murine *CDR2* sequence; the third segment was murine *CDR2*-human *FR3*-murine *CDR3* sequence; and the fourth segment was murine *CDR3*-human *FR4*-$(Gly_4Ser)_4$ nucleotide sequence. The SOE-PCR was then used in linking the first to the second segments and the third to the fourth segments, respectively. Finally, the yielded two contiguous DNA segments were joined to form a humanized-murine *VL* sequence, also by the SOE-PCR. Humanized-murine *VL* sequence was similarly constructed. However, the N-terminus of the first segment of the *VH* sequence was designed to start with the $(Gly_4Ser)_4$ nucleotide sequence and the last sequence at the *VH* C-terminus had a *Not*I restriction site. The humanized-murine *VL* and *VH* segments were linked *via* the $(Gly_4Ser)_4$ nucleotide sequence to make a complete *huscFv* with *Sfi*I at the 5' and *Not*I at the 3' (Figure 14).

Primer sets used for constructing *huscFv* DNA are shown in Table 1.

9.2 Cloning of PT6-2G6-*huscFv* DNA to phagemid vector

The amplified PT6-2G6-*huscFv* DNA from 9.1 from several reaction tubes were pooled and concentrated with sodium acetate and ethanol precipitation. Briefly, two volumes of 70% ethanol and 0.1 volume of 3 M sodium acetate pH 4.8 were added to the pool of PT6-2G6-*huscFv* DNA. The mixture was chilled at -20°C for 1 h. PT6-2G6-*huscFv* DNA was pelleted by centrifugation at 12,000 × *g* at 4°C for 30 min. The DNA pellet was washed by adding 1 ml of 70% ethanol. Then supernatant was discarded and DNA pellet was dried. DNA was resuspended with TE buffer. The PT6-2G6-*huscFv* DNA was cut with *Sfi*I and *Not*I restriction enzymes (Fermentas, USA). Then PT6-2G6-*huscFv* DNA was cloned into pCANTAB5E phagemid vector (GE Healthcare, USA). The ligation reaction (10 µl) consisted of 3 µl of ultrapure water, 2 µl of 5x ligation buffer, 1 µl of pCANTAB5E vector, 1 µl of T4 DNA ligase (New England Biolab, UK) and 3 µl of PT6-2G6-*huscFv* DNA. The ligation mixture was incubated at 16°C overnight. The recombinant phagemids were introduced into competent TG1 *E. coli* by adding 3 µl of the ligation mixture to TSS-treated *E. coli* cells, incubating in ice bath for 20 min and heating-shock the cell suspension in 42°C water bath for 2 min. The transformed *E. coli* were recovered by adding 2× YT medium and kept at 37°C with shaking at 250 rpm for 1 h. The transformed cells were

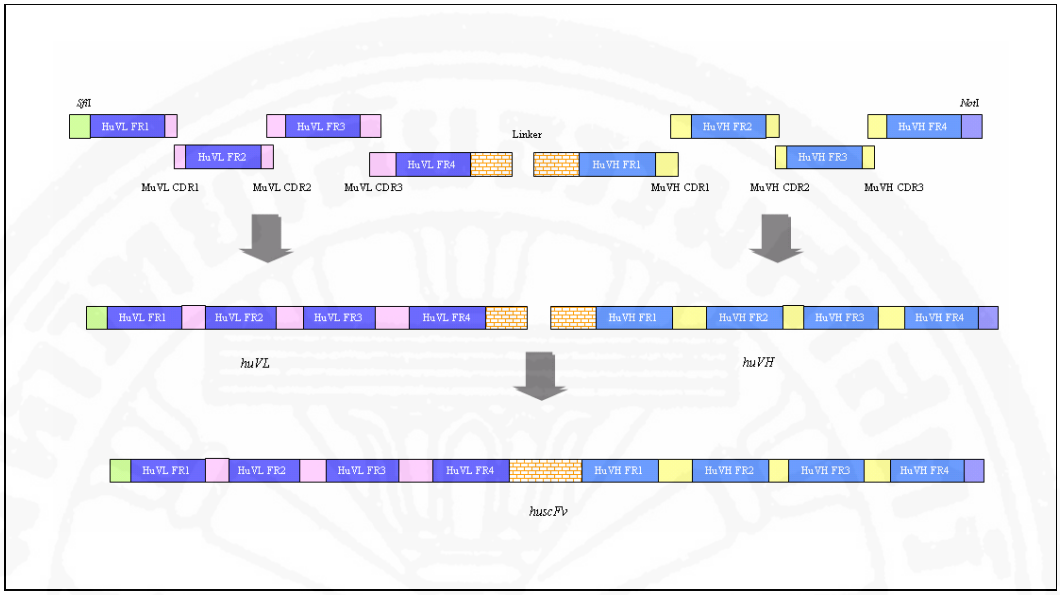

Figure 14 Schematic diagram for production of humanized-PT6-2G6-HuScFv coding sequence**.**

FR(s), immunoglobulin framework(s)

CDR(s), complementarity determining region(s)

plated onto 2× YT agar containing 100 μg/ml amplicilin and 2 % glucose (2× YT-AG agar, **Appendix H**). The agar plate was incubated at 37°C overnight and kept at 4°C. 0

10. Production of ScFv displaying phages

10.1 Growth of *E. coli* containing the recombinant phagemid

Bacterial colonies were harvested from the phagemid transformation plates of 9.2 by flooding each plate with 5 ml of 2× YT medium containing 2% glucose and 100 μg/ml ampicillin (2× YT-AG medium; **Appendix H**) and scraping the cells into the medium using a sterile glass spreader. The bacteria cells from several plates were pooled and the volume of the medium was adjusted to 50 ml in a 250-ml flask. The cell suspension was incubated at 37°C with shaking at 250 rpm for 1 h and then subjected to phage rescue by M13KO7 helper phages.

10.2 Rescue of ScFv displaying phages by M13KO7 helper phages

To rescue phages carrying HuScFv, the M13KO7 helper phages were used. The M13KO7 helper phages were added to the phagemid infected *E. coli* suspension (8×10^8 pfu/ml) and incubated at 37°C with shaking at 250 rpm for 1 h. The cells were pelleted by centrifugation at 2,500 × *g* for 10 min. They were resuspended in 50 ml of 2× YT containing 100 μg/ml ampicillin and 50 μg/ml kanamycin (2× YT-AK; **Appendix H**) and transferred to a 250-ml flask. The cell suspension was incubated at 37°C with shaking at 250 rpm overnight. The bacterial cells were pelleted by centrifugation at 2,500 x *g* for 10 min. The supernatant containing complete phages was subjected to phage precipitation.

10.3 Phage precipitation by PEG/NaCl

Ten-ml of PEG/NaCl (**Appendix H**) was added to 50 ml of recombinant phage supernatant. The solution was mixed and placed in ice bath for 60 min. Recombinant phages were pelleted by centrifugation at 8,500 × *g* at 4°C for 30 min (Sorvall, USA). The supernatant was discarded. The tube was inverted over the clean paper towel to entirely remove the buffer. The phage pellet was resuspended in 1 ml of sterilized PBS pH 7.4.

Table 1 List of nucleotide primers for construction of humanized-*VH* and *VL* sequences

Primer	Nucleotide sequences	Tm (^{o}C)
VL		
>6VLF1	GCG GCC CAG CCG GCC GAC ATC GTG ATG ACC CAG	50
>6VLF2	AAT AGT AGC AAT CAA AAG AAC TAT TTA GCT TGG TAC CAG CAG	48
>6VLF3	TTT GCA TCT ACC CGG GAA TCC GGG	50
>6VLF4	CAA CAT TAT AGC GCT CCG TAC ACG TTT GGC CAG GGG ACC	48
>6VLB1	TTG ATT GCT ACT ATT TAA AAG GCT CTG GCT GGA CTT GCA GTT G	46
>6VLB2	CCG GGT AGA TGC AAA GTA AAT GAG CAG CTT AGG AG	50
>6VLB3	AGC GCT ATA ATG TTG CTG ACA GTA ATA AAC TGC CAC ATC	49
>6VLB4	AGA GCC ACC TCC GCC TGA ACC GCC TCC ACC CCG TTT GAT CTC CAG CTT	48
VH		
>6VHF1	TCA GGC GGA GGT GGC TCT GGC GGT GGC GGA GAG GTC CAG CTG GTA	**45**
>6VHF2	TCA TTC ACT GGA TAC TAC ATG CAC TGG GTG CAG	**45**
>6VHF3	TGT TAC AAT GGT GCT ACT ATA TAC GCA GAG AAG TTC	**43**
>6VHF4	TAC TAC GTG AGT AGC TTC TGG TAC TTC GAT GTC TGG GGC CAG GGA ACC	**50**
>6VHB1	TCC AGT GAA TGA GTA ACC AGA AAC CTT GCA GGA GAT	**46**
>6VHB2	ACC ATT GTA ACA ACT AAT AAG TCC CAT CCA CTC	**42**
>6VHB3	GCT ACT CAC GTA GTA ATT AGG GGA TTG GGT TCT TGC ACA GTA ATA CAC G	**43**
>6VHB4	G CGC ACC TGC GGC CGC GGT GGA GGC TGA GGA	**47**

Underlined alphabets indicate the annealing site

Tm, melting temperature

011. Selection of pertussis toxin specific phages by bio-panning

Purified PT was used to select phages that display PT specific HuScFv. The purified PT was diluted to 1 μg/ml in coating buffer (carbonate-bicarbonate buffer pH 9.6). One hundred μl of diluted PT were added to each well of an 8-well EIA strip (Costar, Corning, USA) and incubated at 37°C in a humid chamber for 1 h. The PT-coated wells were washed three times with PBS-T. The coated wells were blocked with 3% BSA in PBS (100 μl per well) and incubated at 37°C for 1 h. The excess blocking buffer was washed-off as mentioned above. To reduce non-specific protein-protein interaction, the phage suspension (50 μl) was diluted with 50 μl of 3% BSA in PBS containing 0.1% Triton X-100 and incubated at 25°C for 15 min. The diluted phages were added (100 μl) into the PT-coated well and incubated at 37°C for 1 h. After removing of the unbound phages by washing several times with PBST, one hundred μl of phage elution buffer (0.1 M HCl pH 2.2; **Appendix I**) were added to each well and the plate was kept at 25°C for 10 min. The eluted phages were used to infect TG1 *E. coli* cells by adding eluted phages to 5 ml of log phage TG1 *E. coli* and incubating at 37°C with shaking at 250 rpm for 1 h. Ampicillin and glucose were added to the cell suspension to 100 μg/ml and 2%, respectively. Then, phage infected *E. coli* cells were subjected to rescuing by co-infecting with M13KO7 helper phages. Complete recombinant phages were precipitated by the PEG/NaCl method as described above. The phage particles were resuspended with PBS and the preparation was ready to the next round of the bio-panning. The bio-panning was repeated three more rounds (four phage sub-libraries were collected). The phages of the fourth round were individually cloned and used in screening for the phage clones displaying HuScFv specific to PT.

12. Screening of specific phages from the enriched phage-sub-library

12.1 Transfection of *E. coli* with the enriched phage clones

The pooled phages from the fourth sub-library were cloned by infecting TG1 *E. coli* cells as the following: the phages in the sub-library were diluted in 2× YT medium to 10^7-10^9 pfu/ml. Five μl of the diluted phages were added to 50 microliters of log phage TG1 *E. coli* cells. The *E. coli* suspension was incubated at 25°C for 20

min to allow transfection of the *E. coli*. Phage-infected cells were spread on 2× YT-AG agar plate. The plate was incubated at 37°C overnight. Bacterial colonies on the plate were randomly picked, inoculated individually into 1 ml of 2× YT-AG broth in 15 ml-tubes and incubated at 37°C with shaking 250 rpm for 2 h. One hundred microliters of 2× YT-AG medium containing ~ 2×10^7 pfu of M13KO7 phages was added to each culture and allowed to infect the phagemid containing cell by incubating at 37°C with shaking at 250 rpm for 1 h. The bacterial cells were pelleted by centrifugation at 1,500 × *g* (Sorvall, USA) for 20 min. The supernatant was discarded and bacteria cells were resuspended with 1 ml of 2× YT-AK. The bacterial suspension was incubated at 37°C with shaking at 250 rpm overnight. Bacterial cells were pelleted by spinning as above and supernatant was transferred to new tube and kept at 4°C unit use.

12.2 Phage ELISA

The purified PT and BSA diluted to final concentration at 1 μg/ml in coating buffer were added to appropriate wells in a microtiter plate (100 μl per well) and the plate was incubated at 37°C for 1 h. The plate was washed three times with PBST and then blocked with 3% BSA in PBS (200 μl per well) at 37°C for 1 h. The selected phage clones mixed equally with blocking buffer were added (100 μl per well) and incubated at 37°C for 1 h. After the incubation, all wells were washed three times with washing buffer. The anti-M13 monoclonal antibody-horseradish peroxidase conjugate (GE Healthcare, USA) was added (100 μl per well) to appropriate wells. The plate was incubated at 37ºC for another hour then the wells were washed three times with PBS-T followed by adding freshly prepared ABTS substrate solution (Zymed, USA) to each well (100 μl per well). The plate was kept in the dark at 25°C for 30 min. The optical density (OD) of the content of each well was read with ELISA reader (Multiskan, *EX* labsystem, Helsinki, Finland) against PBS blank included in the same plate at wavelength of 405 nm. The positive reaction was the PT-coated well that gave OD two-times higher than the well coated with BSA instead of PT.

13. Production of soluble humanized-murine ScFv (HuScFv)

13.1 Infection of HB2151 *E. coli* cells with phage displaying specific HuScFv to PT

Recombinant phages displaying ScFv to PT were used to transfect non-suppressor *E. coli* strain, *i.e.* HB2151, for producing soluble ScFv molecules. The log phase culture of HB2151 *E. coli* was prepared as the following: one colony of HB2151 *E. coli* from an overnight culture agar plate was inoculated into 5 ml of 2× YT medium and incubated at 37°C with shaking at 250 rpm overnight. Five hundred µl of the overnight culture was inoculated into 50 ml of fresh 2× YT and incubated at 37°C with shaking at 250 rpm until the culture reached an $OD_{600\ nm}$ of 0.5. The selected phage clones were used to infect HB2151 *E. coli* by adding 2 µl of the recombinant phages to 400 µl of log phase culture of HB2151 *E. coli* and incubating at 37°C for 30 min. A loopful of the infected *E. coli* was streaked onto 2× YT agar containing 100 µg/ml ampicillin, 100 µg/ml nalidixic and 2% glucose (2× YT-ANG, **Appendix H**). The plate was incubated at 37°C overnight.

13.2 Small scale expression of soluble HuScFv

A colony of the infected HB2151 *E. coli* from 13.1 was inoculated into 2 ml of 2× YT-AG medium in a 15 ml-glass tube and incubated at 37°C with shaking at 250 rpm overnight. One hundred µl of the overnight culture was inoculated into 10 ml of 2× YT-AG broth and incubated in 37°C with shaking at 250 rpm for 2 h. The bacterial cells were pelleted by centrifugation at 1500 × *g* for 10 min then the supernatant was discarded. The bacterial cells in the pellet was resuspended in 10 ml of fresh 2× YT containing 100 µg/ml and 1 mM isopropyl-thio-ß-D-galactopyranoside (IPTG, USB, USA) (2× YT-AI, **Appendix H**) and transferred to a 50 ml-tube. The cell suspension was incubated as mentioned above for 3-5 h. The bacteria cells were harvested by centrifugation then resuspended in 1 ml of PBS. The cells were lysed by sonication (LABSONIC®, Sartorius, Germany) at 40% amplitude, 2 sec pulse-on, 2 sec pulse-off, for a total of 5 min. Bacterial homogenate was centrifuged at 8,500 × *g* at 4°C for 30 min. The supernatant containing soluble *E. coli* fraction was collected and subjected

to dot blot ELISA and Western blot analysis using an anti-E tag antibody/HRP conjugate.

13.3 Verification of soluble PT6-2G6-HuScFv

To determine the bacterial fraction containing soluble PT6-2G6-HuScFv, bacterial culture supernatant (medium), cytoplasmic and periplasmic fractions were obtained from the 50 ml of bacterial culture. After five hours of IPTG induction at 30ºC with shaking at 250 rpm, bacterial culture supernatants were separated from cell pellet by centrifugation at 1,500 × *g* for 20 min and subjected to ethanol precipitation (Hansson SF *et al.*, 2004). For periplasmic protein extraction, bacterial cells were resuspended in 1 ml of 1× Tris-EDTA-sucrose solution (TES, **Appendix H**) followed by adding with 1.5 ml of 1/5× TES (**Appendix H**). The preparation was kept on ice for 30 min and then centrifuged at 8,500 × *g* at 4°C for 20 min. The supernatant containing periplasmic protein was collected. The pellet was resuspended in 1 ml of PBS pH 7.4 and subjected to sonication as mention previously. To obtain cytoplasmic protein, the lysate was centrifuged at 8,500 × *g* at 4°C for 20 min and the supernatant was transferrred to new tube. All fractions of bacterial protein were subjected to SDS-PAGE and Western blot analysis with anti E-tag antibody /HRP conjugate.

13.4 Determinating the reactivity of PT6-2G6-HuScFv against PT by soluble HuScFv-ELISA

Coating purified PT and BSA was coated on the surface of well as described in 12.2. The plate was blocked with 3% BSA in PBS (200 µl per well) at 37^{o}C for 1 h. The periplasmic fractions were added (100 µl per well) and incubated at 37^{o}C for 1 h. After the incubation, all wells were washed three times with washing buffer. The anti-E-tag antibody/HRP conjugate was added (100 µl per well) to appropriate wells. The plate was incubated at 37ºC for another hour then the wells were washed three times with PBST followed by adding freshly prepared ABTS substrate solution (Zymed) to each well (100 µl per well). The plate was kept in the dark at 25^{o}C for 30 min. The optical density (OD) of the content of each well was read against PBS blank included

in the same plate at wavelength of 405 nm. The positive reaction was the PT-coated well that gave OD two-times higher than the well coated with BSA instead of PT.

14. Large scale expression and purification of soluble PT6-2G6-HuScFv

A colony of HB2151 *E. coli* harboring the recombinant phagemids that display specific HuScFv to PT (S1) was inoculated to 5 ml of of 2× YT-AG medium at 37°C with shaking at 250 rpm overnight. The overnight culture was added to 500 ml of 2× YT-AG and incubated at 37°C with shaking at 250 rpm until OD at 600 nm was 0.4-0.5. Bacterial cells were pelleted by centrifugation at 1,500 × g at 4°C for 20 min. the bacterial pellet was resuspended in 2 l of freshy prepared 2× YT-AI. The culture was incubated at 30°C with shaking at 250 rpm for three to five hours. Bacterial cells were harvested after centrifugation of the culture at 3,500 × *g* for 20 min. To obtain periplasmic protein, cells were resuspended in 40 ml of 1× TES followed by adding with 60 ml of 1/5× TES. The preparation was kept on ice for 30 min with occasional stirring and then centrifuged at 8,500 × *g*, 4°C, 20 min. The supernatant (*E. coli* periplasm) containing crude humanized-murine ScFv (crude HuScFV) was collected. The HuScFv was purified from the *E. coli* periplasm by using a HiTrap® Anti-E Tag column (GE Healthcare, USA). Briefly, the pH of crude HuScFv preparation was adjusted to 7.0 with HCl and filled through 0.45 μm filter before loading onto Anti-E Tag column. Unbound materials were washed with 10-20 column volumes of the binding buffer (0.02 M phosphate buffer pH 7.0, **Appendix I**). Then HuScFv was eluted out with 5 ml of an elution buffer (0.1 M glycine-HCl, pH 2.7) and immediately neutralized by adding 500 μl of 1 M Tris-HCl, pH 8.2 per ml of the elution fraction. PT6-2G6-HuScFv containing fractions were determined by SDS-PAGE with Coomassie Brilliant Blue R250 (CBB) staining. The preparation was then subjected to Western blot analysis.

15. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of 1% (w/v) sodium dodecyl sulfate (SDS-PAGE) was used to analyze the complexity of protein profile of a preparation prior to electroblotting onto a nitrocellulose membrane (NC) for Western blot (WB) analysis or staining the gel directly for direct visualization of the separated components. The technique described by Laemmli (1970) was followed with some modifications. The 8.0 × 7.3 cm vertical slab gel was prepared by using the casting apparatus (Mini-PROTEAN® 3 Cell, Bio-Rad, USA) while electrophoresis was done in an electrophoretic chamber and with an electric power supply, *i.e*. model 3,000/300, Bio-Rad, USA. A 4% acrylamide stacking gel and 12% acrylamide separating gel were used in this process (**Appendix C**).

15.1 Preparation of samples for loading into a slab gel

Samples were prepared by diluting with four volumes of sample loading buffer (Appendix A) and heating at 100^{o}C for 4 min before carefully loaded into the slots in the stacking gel. The desired amount of each sample was carefully applied into each well. Prestained SDS-PAGE broad range standard (Bio-Rad, USA) was included in one slot of each gel slab. Care was taken not to contaminate the adjacent wells with the sample.

15.2 Running the gel

Electrophoresis was carried out in an electrophoretic chamber at 10 mAmp/gel during the first 15 min and at 20 mAmp/gel until the tracking dye reached the lower edge of the gel.

After electrophoresis, the gel was removed from the glass. It was then either stained and destained for direct visualization of the separated components or proceeded to the electro-transblotting onto the nitrocellulose membrane for Western blot analysis.

15.3 Staining and destaining of protein bands

Protein bands were revealed by soaking the gel in CBB staining solution and destained solution (**Appendix C**) as follows:

The slab gel from SDS-PAGE containing separated proteins was placed in a CBB staining solution at 25°C for 1-2 h on a rocking platform. Thereafter, the stain was removed and the gel was destained in a destaining solution until the background color was adequately reduced by changing the destaining solution frequently. When the destaining was complete, the gel was either immediately photographed, scanned and saved to a computer or dried on a Cellophane backing membrane (Bio-Rad, USA).

16. Western blot analysis

The proteins resolved in the polyacrylamide gel after electrophoresis were electrotransblotted onto a sheet of nitrocellulose membrane (ECL, USA). After blotting, the empty sites on the NC were blocked by soaking in the blocking buffer (3% BSA, 0.5% gelatin in PBS, pH 7.4, **Appendix D**) at 25°C with gentle rocking for 1 h. The NC was then washed to remove the excess BSA and gelatin with three changes of washing buffer over a period of 15 min. It was then placed in the solution of antibody preparation at 25°C on the rocking platform for 1 h. The NC was washed three times with the washing buffer and was put in a solution containing appropriated antibody conjugate at 25°C with continuous shaking for 1 h. After excessive washing (5 times for 5 min each) with the washing buffer, the NC was placed in a freshly prepared substrate solution (2, 6-sodium dichlorophenol indophenol solution or BCIP/NBT solution for alkaline phosphatase or horseradish peroxidase systems, respectively), until the protein bands appeared. Then the membrane was washed with distilled water until the background was cleared.