CHAPTER 2

MATERIAL AND METHOD

1. Materials

1.1. Instruments

Instrument	Model	Company
Autoclave	ES-315	Tomy
Automat DNA sequencer	ABI PRISM 377	Applied Biosystem
Balance (4 digit)	AB204-S	Mettler
Balance (2 digit)	PG5002-S	Mettler
Centrifuge, refrigerated	5804R	Eppendorf
	18/80 (MSE)	Harrier
Gel Document (Labworks 4.0)	C-80	UVP
Gel Document	Biodoc-It™ System	UVP
Horizontal Electrophoresis	B3	Owl Scientific
Microcentrifuge	260D	DENVILLE
	SD220	Clover
Micropipettes		Gilson,Labnet, Labmate, Nichipet, SOCOREX
Oven	240 litre	Binder
Orbital shaking incubator,		Gallen Kamp
Refrigerated	SH 30	FINEPCR
Orbital shaker	713	Metrohm
PCR	Master cycle	Eppendorf
	PTC-200	MJ RESERCH

Instrument	Model	Company
pH meter	ELITE 300 plus	Wealtec
Power supply	PAC 3000	Bio-Rad
Preparative gel electrophoresis	491	Bio-Rad
Slab gel electrophoresis	MINI PROTEIN II	Bio-Rad
	AE-6450	Atto
Spectrophometer	8453	Hewlett-Packard
	Thermo spectronic	GENESYS 20
	UV-160A	Shimadzu
Stirrer		Corning
Ultra-speed centrifuge	Avanti J301, J2-21	Beckman
Vortex mixer	VX100	Labnet
Water bath		GallenKamp
	EcoTempTW20	Julabo

1.2. Chemicals

1.2.1. Analytical grade

Chemical	Company
Absolute ethanol	BDH
Acetic acid	Lab Scan
Acrylamide	Fluka
Agar	Merck
Biotin	Fluka
Bis-acrylamide	Fluka
Boric acid	Merck
Calcium chloride	Merck

Chemical	Company
D-glucose	Univar
Dipotassium hydrogen phosphate	Univar
Dithiothreitol	Bio-Rad
Ethylene diamine tetraacetic acid (EDTA)	Carlo
Formaldehyde	Sigma
Formamide	Sigma
Glycerol	BHD, Univar
Glycine	Fisher
Hydrochloric acid	Merck
Methanol	Lab Scan, Merck
Peptone	Merck
Phenol-chloroform-isoamyl alcohol	Sigma
Silver nitrate	Carlo, Merck
Sodium acetate	CARLO EBRA
Sodium carbonate	Ajex, Merck
Sodium chloride	Lab Scan
Sodium Dodecyl Sulfate (SDS)	Finechem
Sorbitol	Sigma
Sodium hydroxide	Lab Scan
Sucrose	USB
Tris (Hydoxymethyl)- methylamine	USB
Triton X-100	Pharmacia Biotech
Tryptone	Merck
Yeast extract	Merck
Yeast nitrogen base	Fluka

1.2.2. Molecular biology grade

Chemical	Company
Agarose	Gene Pure LE
Ampicilin	Calbiochem
BamH I	GIBCO
Deoxynucleotide triphosphate	Promega
100 bp DNA ladder	NEB Biolabs, Promega
EcoR I	GIBCO, NEB Biolabs
Ethidium bromide	Promega
Hind III	NEB Biolabs
Lambda DNA	NEB Biolabs
pGEM-T Easy vector	Promega
Proteinase K	Invitrogen
T4 DNA ligase	NEB Biolabs, Promega
T4 DNA kinase	Promega
Taq DNA polymeras	Invitrogen
Sal I	NEB Biolabs
X-gal	Promega

1.2.3. Reagent kits

Reagent	Company
GeneEditor site-direct Mutagenesis kit	Promega
Qiagen®PCR cloning kits	QIAGEN
QIA kit for plasmid purification	QIAGEN
QIA kit for PCR purification	QIAGEN
QIA kit for gel extraction	QIAGEN

1.3. Samples

1.3.1. Blood samples

EDTA-whole blood samples were collected from 4 healthy Thai volunteers and 48 patients with mental retardation admitted at the Rajanukul Institute, Bangkok, Thailand.

1.3.2. Lymphoblast cells

Lymphoblast cell lines of 44 healthy Thai people were obtained from Veerayuth Praphanphoj, M.D., the Rajanukul Institute, Bangkok, Thailand.

1.3.3. Yeast and Bacterial cells

Pichia pastoris strain GS115 and *E.coli* strain JM109 are products of Invitrogen. *E.coli* DH5α was a gift from Professor Schreiber, Australia. *E.coli* strain JM109 and BMH 71-18 *mutS* are products of Promega.

2. Methods

2.1. Leukocyte and lymphoblast preparations

Leukocytes were isolated from peripheral blood by lysis of red blood cells with a lysis buffer I (0.32 M sucrose, 10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ and 0.75% Triton X-100). Then, the leukocyte cells were collected by centrifugation at 3,500 rpm for 15 min.

Lymphoblasts were collected from a culture media and washed twice with PBS (50 mM Phosphate buffer, 0.15 M NaCl).

2.2. Genomic DNA preparation

Genomic DNAs were isolated from the leukocytes or lymphoblasts by a standard extraction method using phenol and chloroform. In brief, the leukocytes or lymphoblasts were digested overnight at 55°C with lysis buffer II (20 mM Tris-HCl, pH 7.5, 4 mM EDTA, pH 8.0 and 100 mM NaCl) supplemented with proteinase K (188 µg/ml) and SDS (0.5%). Then, DNA was extracted with phenol-chloroform-

isoamyl alcohol (25:24:1) and the clear aqueous phase was transferred to a new tube. To precipitate DNA, 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of ice-cold ethanol was added and the solution was incubated overnight at -80°C. The DNA pellet was collected by centrifugation at 14,000 rpm for 20 min, and it was washed once with 70% ethanol, dried up and dissolved in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The DNA were analyzed and visualized for quality check on 0.8% agarose gel stained with ethidium bromide prior to being stored at 4°C until used.

2.3. Amplification of TTR gene fragments by polymerase chain reaction (PCR)

Specific oligonucleotide primers were used in amplifying DNA fragments of TTR exon2, 3 and 4. The nucleotide sequences of the primers, as previous reported (Nichols and Benson, 1990), are shown in Table 2.1.

The PCR was performed by a standard protocol, using purified genomic DNAs from leukocytes or from lymphocytes as a template. An aliquot of the DNA (300 ng) was amplified in 100 µl of reaction mixture containing a PCR buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 250 mM $MgCl_2$, 25 mM dNTPs, 2.5 Unit of Taq DNA polymerase and 25 pmol of the specific primers. The amplification was started with an initial denaturation step at 94°C for 4 min, followed by 50 cycles of denaturation at 94°C for 1 min, annealing at annealing temperature (Ta) of the primers used (Table 2.1) for 1 min and extension at 72°C for 1 min. The final extension was carried out for 1 cycle at 72°C for 1 min. The PCR products were electrophoretically analyzed on 1% agarose gel and the DNA bands were visualized by staining with 0.4µg/ml ethidium bromide.

Table 2.1 Oligonuclectide primers used for amplification of TTR exon2, 3 and 4

TTR fragment	Fragment size (bp)	Type	Primer sequence (5' → 3')	Ta (°C)
Exon2	311	forward reverse	TCTTGTTTCGCTCCAGATTTC CAGATGATGTGAGCCTCTCTC	60
Exon3	205	forward reverse	GCCACTGCAGTCCTCCATGCGTAAC TTAAT CTCGAAGGTCTGTATACTC	60
Exon4	258	forward reverse	TAGGTGGTATTCACAGCC GTGCCTTTCACAGGAATG	62

2.4. Single-stranded conformation polymorphism (SSCP)

SSCP was performed as described by Connor *et al*., 2004. In brief, genomic DNA was amplified for TTR exon 2, 3 and 4 by PCR using specific nucleotide primer pairs (Table 2.1). The PCR product (100 ng) was incubated with 2.5 volumes of SSCP buffer containing 98% formamide, 10 mM NaOH, 10 mM EDTA, 0.05% xylene cyanol and 0.05% bromphenol blue, at 95°C for 10 min before the mixture was chilled on ice and analyzed on 15% polyacrylamide gel containing 10% glycerol. The samples were electrophoresis in Mini PROTEIN II (Bio-Rad, Hercured CA) using TBE buffer pH 8.3 at 4°C with a constant voltage (150 volts) for 2 h. Temperature was kept cool through the run of gel. Thereafter, the gel was stained with silver nitrate.

2.5. Cloning of TTR gene fragment

Purified TTR gene fragment was ligated to *E.coli* clonig vector, pGEM-T Easy (promega) or pDrive (QIAGEN® PCR cloning Kit) according to the method described by the company. In brief, the ligation reaction mixer (10 µl in total volume), comprising of 20 ng of purified DNA and 25 ng of the cloning vector, was incubated at 37°C for 1 to 2 min prior to chilling on ice and then 2 units of T4 DNA ligase was added, and the incubation was continued at room temperature for 2 h. The

reaction mixture was immediately transformed into a bacterial cell as described in section 2.7.1 or kept at 4°C until used.

2.6. DNA sequencing

Nucleotide sequences of DNA inserted plasmid or PCR product was determined by the Scientific Equipment Center, Prince of Songkla University (PSU) with a modification of the chain termination sequencing method of Sanger *et al.* (1977), using a Dye Terminator Cycle Sequencing Kit (Applied Biosystems). DNA separation according to size in polyacrylamide gel under denaturation and the data processing were performed using ABI Prism 377 Automated DNA sequencer (Applied Biosystems). An alignment program was used to check the nucleotide sequences obtained.

2.7. Preparation of competent cells and transformation of DNA

2.7.1. *E.coli* competent cells

Competent cells were prepared from *E. coli* strain DH5α and JM109 using calcium chloride as described by Cohen *et al.* (1972). A single colony was picked from colonies freshly grown overnight on a Luria-Bertani (LB) agar plate and grown in 5 ml of LB broth in a 50-ml tube at 37°C. Subculture was performed using 50 µl of the overnight culture, and cells were grown until OD_{600} of the culture reached 0.4 to 0.5 (2 to 3 h). Then, cells were transferred to a polypropylene tube, cooled down on to 0°C and collected by centrifugation at 2,500 rpm for 10 min, at 4°C. All supernatant was removed and tubes were inverted for 1 to 2 min to drain away traces of media. Cell pellets were then suspended in ice-cold 0.1 M calcium chloride (10 ml per 50 ml original culture) and cooled to 0°C. After centrifugation and draining, cells were suspended in ice-cold 0.1 M calcium chloride (2 ml/50 ml original culture). The competent cells were aliquoted and glycerol was added to the final concentration of 15.5% (v/v). Cells were quickly frozen in liquid nitrogen and immediately kept at -80°C as glycerol stock.

To transform cells with a foreign DNA, 50 µl of the competent cell suspension was mixed with the DNA (approximately 0.5 µg) or an aliquot (10 µl)

of ligation mixture in polypropylene tube and stored on ice for 10 min. Then cells were subjected to heat-shock at 42°C for exactly 90 s without shaking prior to cooling down on ice for 1 to 2 min. Thereafter, SOC medium (100 µl) was added and the cells were incubated at 37°C for 1 h prior to being plated onto a LB agar plate, supplemented with X-gal (150 µg/ml agar) and ampicillin (100 µg/ml agar). The cells were grown overnight at 37°C. Five to ten single white colonies, expected to contain the DNA inserted plasmid, were selected for isolation of the plasmid.

2.7.2. BMH competent cells

To prepare the BMH 71-18 *mutS* competent cell, a single freshly grown overnight colony on LB-tetra (12.5 µg/ml tetracycline), was dispersed into 1 ml LB by a vortex-mixer, and cells were inoculated into 50 ml LB in a 500-ml flask. The overnight culture (2.5 µl) was inoculated in 250 ml LB in a 1-liter flask and incubated at 37°C in an orbital shaking incubator (240 rpm) until the OD_{550} reached 0.75 (approximately 3-4 h). The cells were collected by centrifugation at 2,500 rpm for 15 min, at 4°C, and suspended in an equal volume (250 ml) of chilled 10% glycerol while keeping the cells on ice. Then, cells were collected and suspended once with 250 ml of chilled 10% glycerol. Finally, cells were suspended in 190 µl of 10% glycerol to give the OD_{550} to 0.75 (200-250 cells/ml). The competent cells were divided into aliquots of 50 µl, quickly frozen in liquid nitrogen and stored at -80°C.

Transformation of a plasmid into the BMH competent cells was carried out by an electroporation using GenePulser (Bio-Rad). An aliquot (50 µl) of BMH71-18 *mut*S competent cells was mixed with 0.5 µg of DNA, and the mixture was transferred to a 0.2 cm electroporation cuvette. Cells were incubated on ice for 5 to 10 min and eletroporation was carried out at voltage of 2,500 volts, capacitance of 25 µF and resistance of 200 ohms, generating pulse length of 5 milliseconds with field strength ~2,300 V/cm. Immediately thereafter, 1 ml of SOC medium was added to the cuvette and cells were flushed for a couple times prior to growing at 37°C with moderate shaking for 1 h. Then, 100 µl of the cell culture was inoculated into LB containing the GeneEditor™ Antibiotic selection (Promega), and cells were grown overnight at 37°C.

2.7.3. *Pichia* competent cells

To prepare competent cells of *P. pastoris*, the *Pichia* strain GS115 from fresh yeast extract peptone dextrose (YPD) agar plate was grown overnight in 5 ml of YPD broth at 30°C. Then 100 µl of the overnight culture was transferred to 120 ml of the fresh medium in a 1-liter flask and continuously grown to an OD_{600} of 1 to 1.2. Cells were collected by centrifugation at 2,000 rpm for 5 min at 4°C, and the cells pellet was suspended in 22.5 ml of ice-cold water. Thereafter, cells were collected and suspended in 1.8 ml of ice-cold 1.0 M sorbitol, and finally in 135 µl of ice-cold sorbitol. The competent cells were aliquot and immediately used in transformation.

Transformation of plasmid into the *Pichia* competent cell was carried out by an electroporation as described by Scorer *et al* (1994), using GenePulser (Bio-Rad). Aliquot (80 µl) of the *Pichia* cells was mixed with 0.56 µg of the DNA linearized with *Sal*I and transferred to a 0.2 cm electroporation cuvette. Cells were incubated on ice for 5 to 10 min and electroporation was carried out at a voltage of 1,500 volts, capacitance of 25 µF and resistance of 400 Ω, generating pulse length of ~9 milliseconds with a field strength ~1300 V/cm. Immediately thereafter, 1 ml of ice-cold 1 M sorbitol was added to the cuvette. The cells were flushed for a couple of times and then spread onto a minimal dextrose medium (MD) agar plate and incubated until the yeast colonies appeared.

2.8. Purification of PCR product

The DNA fragment amplified by PCR was purified using a QIAquick PCR Purification Kit (Qiagen), following a protocol described by the company. In brief, 5 volumes of a binding buffer were added to the reaction mixture, and the entire mixture was applied onto a spin column. The DNA bound to the column while other impurities were washed out with a buffer containing ethanol. In the final step, the plasmid was eluted out with 30 to 50 µl of 10 mM Tris-HCl, pH 8.5 and stored at -20°C.

2.9. Purification of DNA plasmid

2.9.1. By using the QIAprep Miniprep kit

Plasmids were isolated from bacterial cell by an alkaline lysis, using a QIAprep Spin Miniprep Kit (Qiagen) with a procedure according to the company (Birnboim and Doly, 1979). In brief, bacterial cells from 2 to 5 ml of an overnight culture with antibiotic were collected by centrifugation and resuspended in a buffer containing RNase A. Then, membrane of the cells was broken down with an alkaline buffer for 5 min at room temperature. Thereafter, the cell lysate was neutralized and adjusted with a chaotropic salt (1.6 M guanidine hydrochloride) for binding to silica material with a neutralizing buffer (4 M guanidine hydrochloride, 0.5 M Potassium acetate, pH 4.2). The precipitates of chromosomal DNA and cell debris that occurred were removed by centrifugation then the supernatant was loaded onto a spin column. Plasmid was purified by its absorption to the surface of silica filter of the column and was separated from unbound impurities in washing step with a solution of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80% methanol. The plasmid was eluted out from the column with 10 mM Tris-HCl, pH 8.5, and stored at -20°C until used.

2.9.2. By using a QIAquick PCR purification Kit

Plasmid in an enzymatic reaction was purified with the same method described for purification of PCR product in section 2.8, using a QIAquick PCR Purification Kit (Qiagen).

2.10. Isolation and purification of DNA from agarose

DNA plasmid or PCR product separated on an agarose gel can be isolated and purified using a QIAquick Gel Extraction Kit (Qiagen), following a protocol of the company. After electrophoresis, the gel area containing the DNA of interest was excised with a sterile razor blade. The gel slice was dissolved in solution containing guanidine thiocyanate with a ratio of gel : solution at 1 : 3 (v/v). Thereafter, 1 gel volume of isopropanol was added and the entire mixture was applied onto a QIA spin column. The DNA was adsorbed onto a membrane of the column while other impurities were washed out with a buffer containing ethanol. Finally, the

DNA was eluted with an appropriate volume of 10 mM Tris-HCl, pH 8.5 and stored at -20°C until used.

2.11. Restriction analysis of a DNA inserted plasmid

To determine size of the DNA inserted in a recombinant plasmid, the plasmid was digested with an appropriated restriction endonuclease. In general, the enzymatic reaction mixture (20 μl) comprising 200-5,000 ng of plasmid and 10 units of each enzyme. The reaction was carried out at 37°C for 1.5 to 2 h. Thereafter, the mixture was analyzed by agarose gel electrophoresis and DNA bands were visualized by staining with ethidium bromide.

2.12. Determination of DNA concentration

The concentration of DNA was determined by spectrophotometric absorption at 260 nm. One OD_{260} was assumed to correspond to 50 μg/ml of double stranded DNA. The purity DNA was determined from the ratio of OD_{260}/OD_{280} in which 1.8 to 1.9 is the normal range (Sambrook *et al*., 1989).

2.13. Preparation of *Pichia* clone of human TTR variant

2.13.1. Preparation of human TTR DNA template for mutagenesis

PCR was used to amplify and generate human TTR DNA with compatible restriction ends for ligation into the pPIC 3.5 (BamHI and EcoRI ends). Fifty nanograms of human TTR cDNA in a bacterial plasmid (a gift from Professor Schreiber, University of Melbourne, Australia) were amplified using 40 pmol of forward primer (5′ AGGATCCAGGATGGCTTCTCATCG3′) and reverse primer (5′ AGGAGTGAATTCTCATCCTTGGGATTGG 3′) in 100 μl of the reaction mixture. The amplification was started with an initial denaturation at 94^{o}C for 5 min, and then was followed by 30 cycles of 94 ^{o}C for 30 s, 55 ^{o}C for 30 s and 72 ^{o}C for 1 min. The final extention was carried out for 1 cycle of 72 ^{o}C for 5 min. The DNA was purified using the PCR purification kit (QIAGEN). Thereafter, it was ligated to pGEM-T Easy vector and transformed into *E. coli* DH5α as described in section 2.7.1.

The TTR DNA inserted plasmid was prepared followed by double digestion with EcoRI and BamHI for 2 h. Ligation of the DNA to *Pichia* expression vector pPIC 3.5, previously linearized with EcoRI and BamHI was carried out with T4 DNA ligase (Promega) in a 10 µl total reaction volume.

2.13.2. Construction of human TTR variant DNA

A site-directed mutagenesis and PCR were used to generate and amplify DNA of TTR variant, Leu110Pro (leucine at position 110 was replaced by proline). Mutagenesis was performed by using GeneEditor *in vitro* Site-Directed Mutagenesis System (Promega). The reaction involved annealing of the selected oligonucleotide, which encodes mutation that create a resistance to the antibiotic mixture so as to facilitates selection of the desired mutation, and the mutagenic oligonucleotide of Leu110Pro, 5′ATTGCCGCCCCGGCTGAGCCC 3′, to the human TTR DNA (in pPIC3.5) template. The mutant strand was synthesized with T4 DNA polymerase prior the hetero-duplex DNA strands were transformed into the BMH 71-18 *mut*S, and cells were grown in a selective media. Plasmid prepared from the transformant that was resistant to the antibiotic selection mix was transformed and *in vivo* amplified in *E. coli* DH5α. The plasmid was prepared from the *E. coli* DH5α and checked for the sequence by DNA sequencing, using *5′AOX primer* (5′-GACTGGTTCCAATTGACAAGC-3′). The pPIC3.5 containing Leu110Pro cDNA was, then, linealized and transformed into *Pichia* GS115 cell by an electroporation as described in section 2.14 and 2.7.3, respectively. The cell suspension was spread onto the minimal dextrose medium (MD) agar plate and incubated at 30^{o}C for 3 days.

The pPIC3.5 containing TTR variant DNA was linearized by digestion with *Sal*I at 37^{o}C for 1.5 h, and the plasmid was purified using QIAquick PCR purification kit (QIAGEN) prior to being transformed into the *Pichia* GS115. Electroporation was conducted using Gene Pulser (Bio-Rad), following the protocol recommended by the company. In brief, the linearized plasmid (0.56 µg) was mixed with competent *Pichia* cells (80 µl) and transferred to an ice-cold 0.2 cm electroporation cuvette. The electroporation was carried out at 1.5 kV, 25 µF, and 400 Ω, generating pulse lengths of 9 milliseconds with a field strength ~1300 V/cm. Then,

the cells were flushed for a couple times with 1 ml of ice-cold 1 M sorbitol. The cell suspension was spread onto the minimal dextrose medium (MD) agar plate and incubated at 30^{o}C until colonies appeared (approximately 3 days).

2.14. Linealization and transformation of the Leu110Pro DNA plasmid into *P. pastoris*

The Leu110Pro DNA inserted pPIC3.5 was linearized by digestion with *Sal*I at 37°C for 1.5 h. Then, the plasmid was purified by phenol/chloroform extraction prior 0.56 μg of the linearized plasmid was mixed with 80 μl of competent *Pichia* cell and the cell mixture was subjected to transformation as described in section 2.7.3. The entire cell suspension was spread on the minimal dextrose medium (MD) agar plate and the plate was incubated at 28°C until colonies appeared (approximately within 3 days).

2.15. Screening for Mut^{+} transformants

The *Pichia* transformants with phenotype His^{+}Mut^{+} (histidine synthesis and methanol utilization plus) and His^{+}Mut^{s} (histidine synthesis and methanol utilization slow) can be separated from each other by growth on the minimal dextrose medium (MD) and minimal methanol medium (MM) agar plates. A colony of His^{+} transformant from MD plate was picked with a sterile toothpick and patched onto MM, and then onto MD plate. Screening was performed with 50 to 100 His^{+} transformants and cells were allowed to grow at 28°C for 3 days. His^{+}Mut^{+} transformants showed the same growth on both MD and MM plates. Twenty to fifty colonies of the His^{+}Mut^{+} transformants were selected for the protein synthesis in small scale.

2.16. Synthesis of recombinant Leu110Pro

2.16.1. The small scale synthesis

The *Pichia* clone containing Leu110Pro DNA was induced to synthesize and extracellularly secrete the recombinant protein into the culture medium. A single colony of the *Pichia* clone on yeast extract peptone dextrose (YPD)

plate was inoculated into 5 ml of a buffer medium containing glycerol (BMGY) and cells were grown at 30°C in orbital shaking incubator (200 rpm) until the absorbance at 600 nm reached 2 to 6. Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature and transferred to grow in 5 ml of a buffer medium containing methanol (BMMY), at an OD_{600} of the cell suspension at the start point was approximately to 1, for 6 to 7 days. Methanol was added every 24 h to maintain the level of methanol in the culture to 0.5%. After induction, the culture supernatant was collected by centrifugation at 5,000 rpm for 5 min and was analyzed for secreted proteins by SDS-PAGE. The protein band was detected by silver staining. The supernatant was kept at -20°C until used.

2.16.2. Scaled up production of the recombinant Leu110Pro

Large-scale preparation of the recombinant Leu110Pro was performed in a shake-flask using an orbital shaking incubator at 200 rpm and with the same culturing condition as described for the small scale synthesis. A single colony of the *Pichia* clone from YPD plate was inoculated into 10 ml BMGY, and cells were grown overnight. Ten milliliters of the overnight cell culture was transferred into 300 ml of BMGY in a 1-liter flask and culturing was continued until OD_{600} reached 3 to 4. Thereafter, cells were harvested by centrifugation at 2,500 rpm for 5 min at room temperature and suspended in 300 ml of BMMY in a 2-liter flask at an OD_{600} of the cell suspension equal to 1. Induction with methanol was carried out for 7 days. Then, the culture supernatant was collected by centrifugation at 5,000 rpm for 10 min at 4°C.

2.17. Purification of recombinant Leu110Pro from yeast culture supernatant

The recombinant Leu110Pro was purified from the *Pichia* culture supernatant by preparative native polyacrylamide gel electrophoresis (preparative native PAGE), using the Prep Cell model 491 (Bio-Rad). Discontinuous gel (12% of resolving gel and 4% of stacking gel) was chosen for the separation, and electrophoresis was performed as recommended by the company. The culture supernatant of *Pichia* was concentrated by ultra-filtration and centrifuged at

10,000 rpm for 5 min prior to load onto gel. Eluting fractions were collected with flow rate of 1 ml/min, and proteins in the fractions were analyzed by native PAGE and visualized by silver staining. The fractions containing Leu110Pro were pooled and stored at -20°C for analysis.

2.18. SDS-PAGE and non-denaturing PAGE

For analysis under denaturing conditions, proteins were separated in ventrical sodium dodecyl sulfate polyacrylamide (SDS-PAGE) slab gel (12% acrylamide resolving gel and 4% acrylamide stacking gel) and with discontinuous buffer system of Laemmli and Favre (1973). Protein samples were denatured by incubated with β-mercaptoethanol at 100°C for 1 h prior to separation in 12% resolving gel and 4% stacking gel. The protein bands were detected by staining with silver nitrate (Morrissey, 1981).

Non-denaturing PAGE for protein was performed using 10% polyacrylamide gel and 0.05 M Tris-glycine, pH 8.6, while that for DNA was performed using 15% polyacrylamide gel and TBE buffer. After analysis, protein and DNA bands were detected by silver staining.

2.19. Silver staining

Polyacylamide gel staining with silver nitrate was performed using the method of Morrissey, 1981. After electrophoresis, gel was placed in a solution containing 9.2% acetic acid and 45.4% methanol for 30 min and transferred to a solution containing 7.5% acetic acid and 5% methanol for 30 min. Then, it was rinsed with distilled water for 3 to 4 changes prior to being soaked in solution of DTT (500 µg/ml) for 30 min. Thereafter, DTT solution was discarded and 0.1% silver nitrate was added, and gel was soaked for 20 min. To develop the gel, a developing solution containing 3% (v/v) sodium carbonate and 0.02% (v/v) formaldehyde was added, and gel was soaked until bands appeared. To stop the reaction, 50% acetic acid was added until a few air bubbles occurred. Gel was washed several times with distilled water prior to be packed in a plastic bag and stored at 4°C. The gel was photographed by gel document (Labworks 4.0, Bio-Rad).

2.20. Determination of protein concentration

The concentration of protein was measured by Bradford assay (Bradford, 1976). The reaction mixture comprised 0.1 ml of protein sample and 1.0 ml of an assay reagent (0.085 mg/ml Coomassie blue G-250, 5% methanol, and 5.06% H_3PO_4). The complex formation was allowed at room temperature for 2 to 3 min before the optical density at 595 nm of the mixture was measured. Protein standards were prepared in the same buffer as the sample to be assayed. The standard curve was generated using bovine serum albumin (BSA) at amounts of 0, 5, 10, 15, 20, and 40 µg, and used to determine the protein concentration of unknowns.

2.21. Determination of subunit mass

Subunit mass of recombinant TTR was determined by SDS-PAGE as described in section 2.18. The purified TTR was incubated with a solution containing 2% SDS and 2.5% of β-mercaptoethanol at 100^{o}C for 1 h before loading on gel. The protein separation was carried out using a 12% acrylamide resolving gel, pH 8.8, and 4% acrylamide stacking gel, pH 6.8, and the electrophoresis was performed at constant voltage of 100 volts. The protein band was visualized by silver staining.