CHAPTER 3

RESULTS AND DISCUSSION

The human TTR gene has been localized in chromosome 18 (Wallace *et al.,* 1985) as a single copy (Sparke *et al*., 1987). The gene is found spanning to 6.9 kb with four exons and three introns. In this thesis, the TTR gene fragments were amplified from chromosomal DNA or genomic DNA of Thai people, these include the healthy Thai volunteers as controls and the cases of mental retardation with Down's syndrome who have ~ 4 times higher risk in developing an Alzheimer's disease and amyloidosis in brain than the normal human (6% risk) (Glenner, 1983). Their nucleotide sequences were determined for a possible single point mutation.

1. Preparation of human total genomic DNA

By the standard protocol, total genomic DNAs can be prepared from lymphoblasts and lymphocytes of 96 Thai people. These include 48 cases of mental retardation and 48 controls. Analysis on agarose gel of the DNAs isolated from the individual samples showed size of the purified DNAs close to that of the 23 kb fragment of lamda DNA marker (Figure 3.1). In the Human Genome Project, shotgun sequencing was used to generate the whole consensus sequence of the euchromatic portion of human genome. It revealed that human genome contains more than 3.2 billion base pairs (Venter *et al*., 2001). The size of the genomic DNAs purified from lymphoblasts and lymphocytes studied in this thesis, it indicated breaking down of the DNA molecule during the purification. The spectrophotometric measurement of absorbance at wavelengths of 260 nm and 280 nm provides an estimate of the purity of nucleic acids including DNA with respect to contaminants that absorb in the UV such as protein and phenol. A260 is frequent used to measure nucleic acid concentration and A280 is used to measure protein concentration. A ratio of A260/A280 less than 1.5 suggests protein contamination in a nucleic acid sample

(Sambrook *et al.*, 1989). The ratio of A_{260}/ A_{280} of the DNA samples obtained were ~1.3 to 2.00 (data not shown). These may indicate some protein contamination. However, physicochemical alterations produced by pH and temperature, and carcinogenic chemical modification may increase the absorbance ratio of 260/280 nm more than the normal range (1.8-1.9) (Wilfinger *et al*., 1997; Okamoto and Okabe, 2000; Kim *et al*., 2005). The quality of DNA was further assessed by agarose gel electrophoresis or by evaluating performance in an amplification reaction. As shown in Figure 3.1, only one major broad band of DNA was detected, and there was no a visible band with size less than 6.9 kb (size of the TTR gene). These should indicate sufficient quality of the DNAs to be used as templates in PCR.

2. Amplification of TTR gene fragments

The gene encoding for human TTR subunit consists of four exons and three introns. In this thesis, the TTR exon 2, 3 and 4 were amplified by PCR using the purified genomic DNA extracted from 96 Thai people as templates under conditions as described in section 2.3. The specific oligo-primers containing nucleotide sequences as shown in Table 2.1 were used to amplify fragments of the gene.

An example of an analysis of the PCR products obtained from the amplification on 1% agarose gel was shown in Figure 3.2. Only a single band of the DNA fragment was detected from each reaction. The fragment sizes of the DNAs were as previously reported (Nichols and Benson, 1990) i.e. 311 bp, 205 bp and 258 bp for the TTR exon 2, 3 and 4, respectively. Specificity of the amplification reactions was very high, evidently from high intensity of the amplified DNA bands obtained. These confirmed good quality of the purified DNA templates.

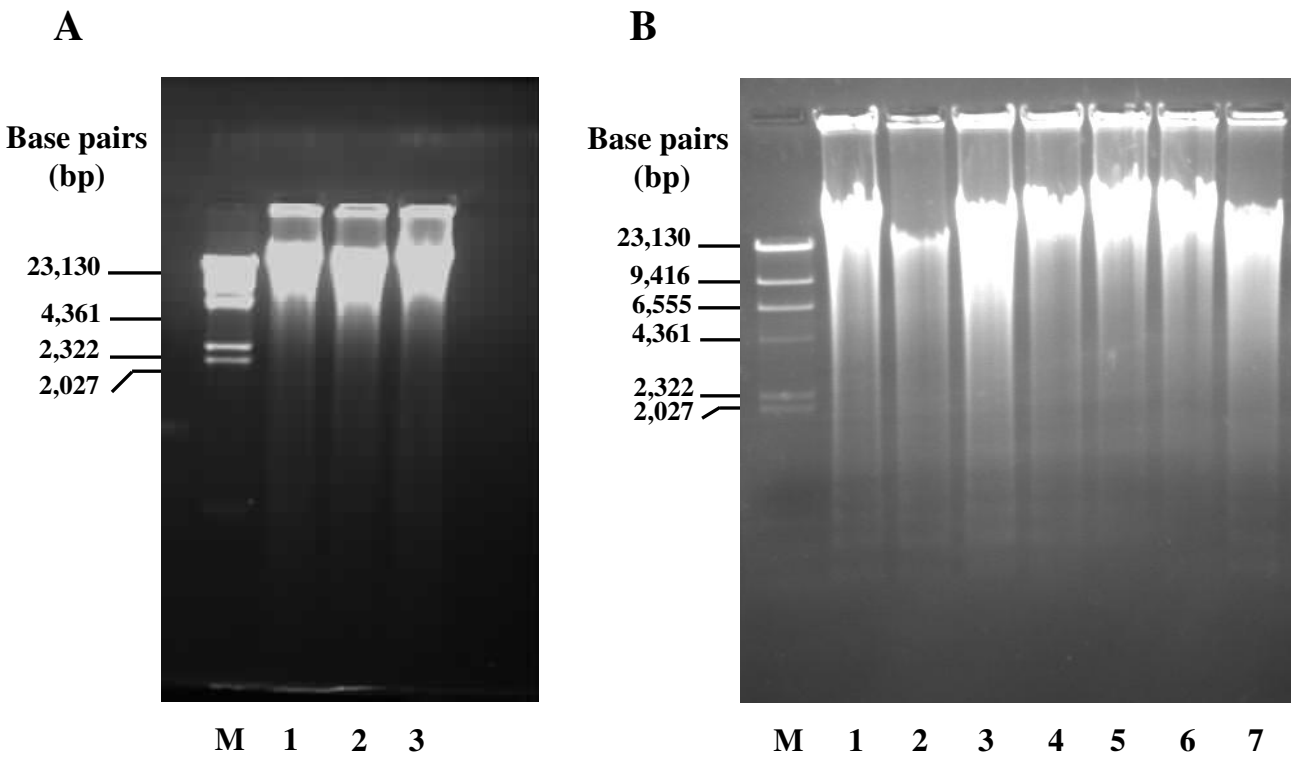
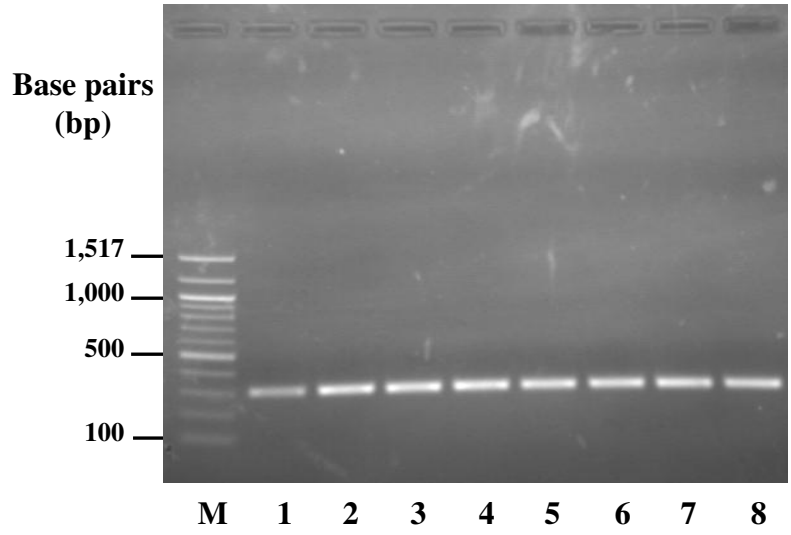


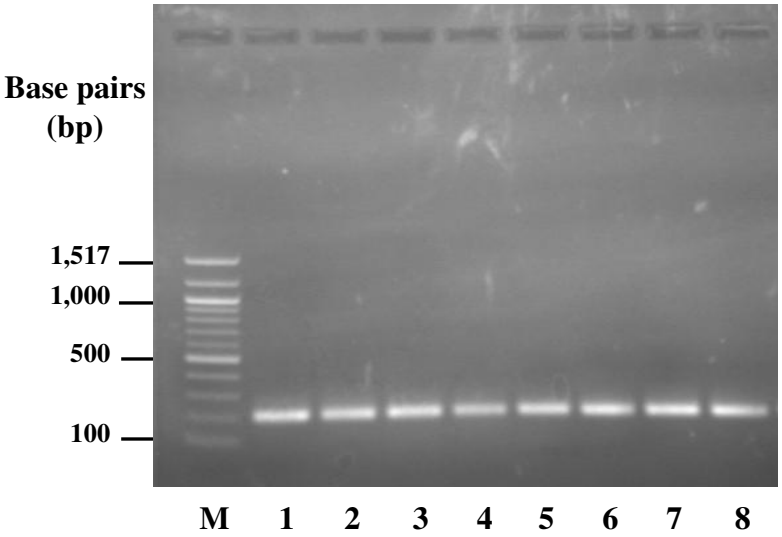
Figure 3.1 **Mobility of human total genomic DNA on agarose gel**

Total genomic DNA (5 μg) was analyzed on 1% (A) or 0.8% (B) agarose. M is a DNA marker (23 kbp lambda DNA/Hind III). Lane 1 to 3 in (A) or 1 to 7 in (B) are the DNA isolated from an individual blood samples.

A


Base pairs
(bp)
1,517
1,000
500
100
M 1 2 3 4 5 6 7 8

B


Base pairs
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M 1 2 3 4 5 6 7 8

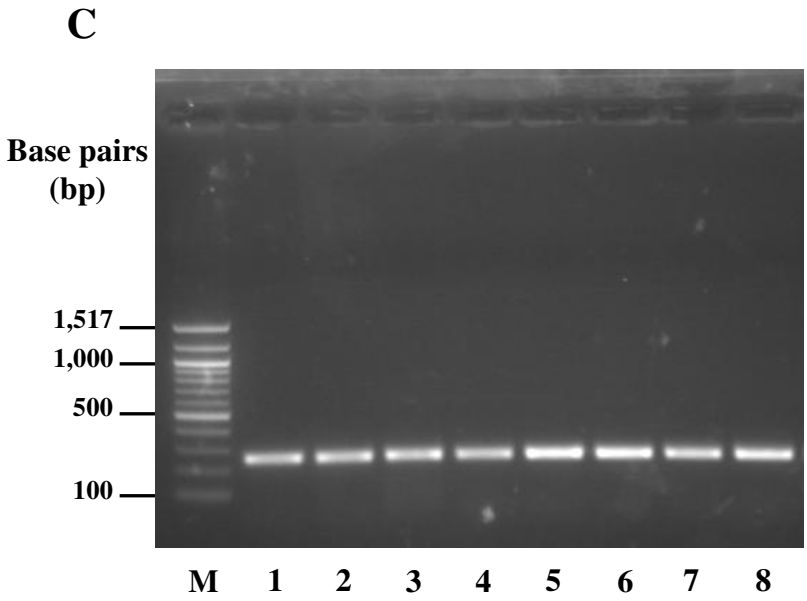


Figure 3.2 Amplification of TTR gene fragments by PCR

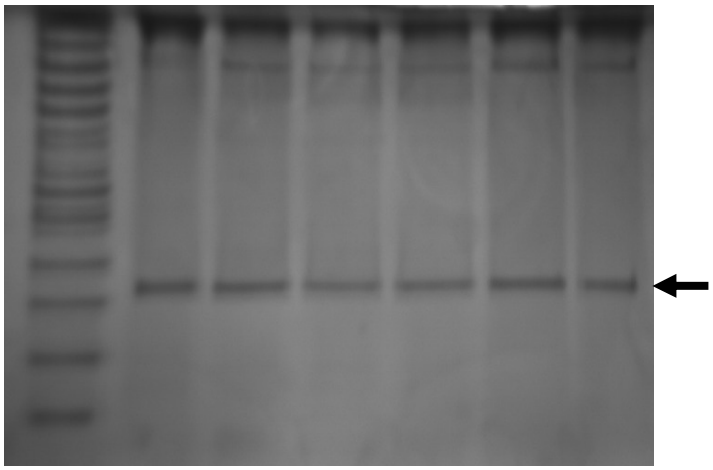
PCR was used to amplify TTR exon 1 (A), exon 2 (B) and exon 3 (C) by using human total genomic DNA as a template. The PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide, and the DNA bands were visualized under UV. M is a DNA marker (100 bp ladder). 1 to 8 is the PCR product amplified from an individual genomic DNA.

3. Single-stranded conformation polymorphism (SSCP)

Screening for the point mutation of TTR gene was performed by SSCP technique. SSCP is one of the techniques most widely used to identify a mutated sequence or a polymorphism of genes. By this technique, separation of single-stranded nucleic acids is based on subtle differences in sequence, which is often a single base pair, that results in a different secondary and a measurable difference in mobility through gel. Therefore, very small changes in sequence, even one changed nucleotide out of several hundreds, can affect the mobility of single strands. A single point mutation of TTR nucleotide sequence can be identified by this technique because of the relatively unstable nature of single-stranded DNA. In the absence of a complementary strand, the single strand may undergo intra-strand base paring, resulting in loops and folds that give the single strand a unique three dimension structure, regardless of its length.

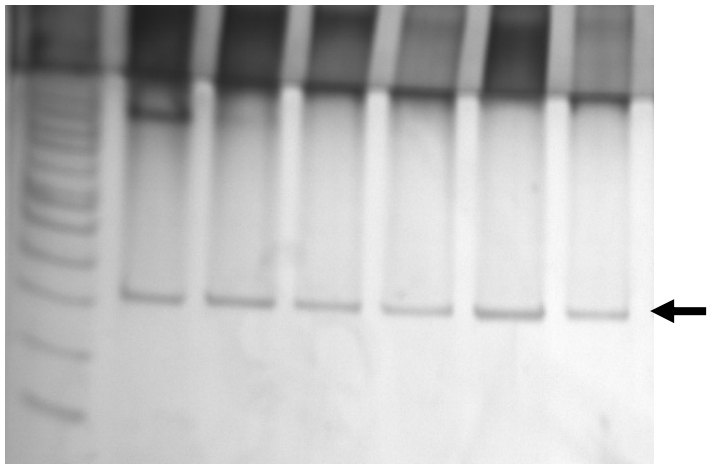
Single stranded TTR DNA fragments of exon 2, 3 and 4 were prepared by the alkaline denaturation as described in section 2.4. The denatured DNAs were, then, separated on 15% polyacrylamide gel containing 10% glycerol. The electrophoretic mobility of the TTR exon 2, exon 3 and exon 4 amplified from normal and mental retardation cases were shown in Figure 3.3. The mobilities of all TTR exon 2 and 3 fragments of the mental retardation cases were similar to those of the control (Figure 3.3A-D. Similarly, a single band of DNA was observed in TTR exon 4 amplified from the controls (Figure 3.3E). However, one of the case with mental retardation showed a change in mobility of the exon 4 (Figure 3.3F). Two bands rather than a single sharp band of DNA were detected on the agarose. These two bands had different mobilities, i.e. one moved with the same rate as that observed in the control while the other moved slower. This should indicate that there is a mutation on the exon 4 fragment of this case. The finding of two DNA bands in the exon 4 of the case is well agreed with the fact that TTR amyloidosis is an autosomal dominant. Both normal and mutated TTR are detected in plasma of the patient.

A



M 1 2 3 4 5 6

B

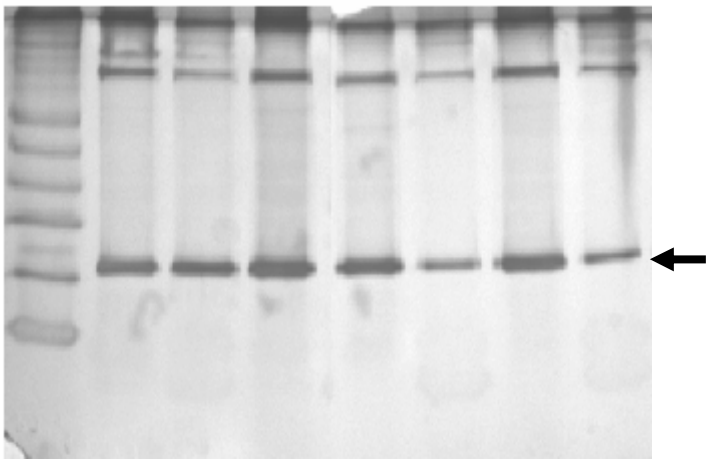


M 1 2 3 4 5 6

C

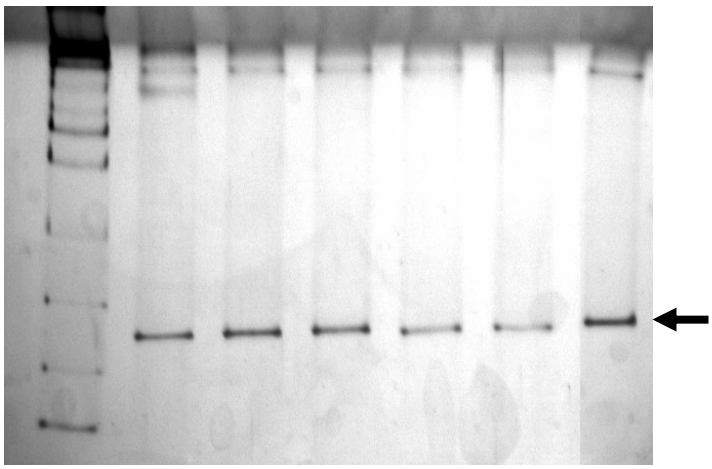
M 1 2 3 4 5 6

D



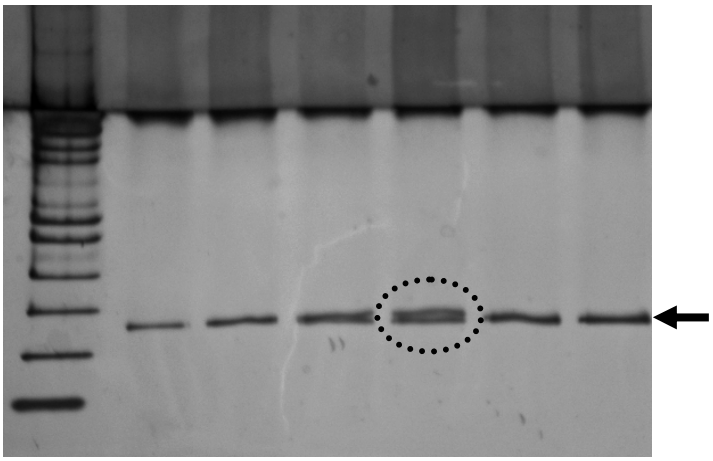
M 1 2 3 4 5 6 7

E



M 1 2 3 4 5 6

F



M 1 2 3 4 5 6

Figure 3.3 SSCP analysis of human TTR exon 2, 3 and 4

SSCP analysis was performed to determine the conformational mobility of human TTR exons. The DNA (100 ng) was denatured to a single-strand prior to analysis by native PAGE (15% acrylamide) electrophoresis and the DNA bands were visualized by silver staining as described in sections 2.4 and 2.19. M is a DNA marker (100 bp ladder). (A), (C) and (E) are TTR exon 2, 3 and 4 from healthy individual, respectively. (B), (D) and (F) are TTR exon 2, 3 and 4 from an individual with mental retardation, respectively. 1 to 6 or 1 to 7 indicates individual samples. The case with mental retardation that showed a change in mobility of the exon 4 is indicated by broken circle.

4. Nucleotide sequencing

In order to obtain nucleotide sequences of TTR exons, the PCR products were purified as described in section 2.8 and section 2.10 prior to insert into the *E.coli* cloning vectors, pGEM-T Easy or pDrive vector, at the EcoR I insert sites as shown in Figure 3.4. According to the insertion, the DNA fragment was placed after the promoter of enzyme β-galactosidase (or Lac Z gene). The recombinant plasmid was transformed into bacterial competent cell prepared as described in section 2.7.1. The recombinant clones could be identified by blue/white screening using 5-bromo-4-chloro-3-indolyl- β-D-galactoside (X-gal), a chromogenic substrate (Horwitz *et al*., 1964). The molecular mechanism of blue/white screening is based on the Lac operon. The insertion of TTR gene within Lac Z gene leads to disruption of the activity of the β-galactosidase when TTR is expressed. The hydrolysis of colorless X-gal by the β-galactosidase causes the characteristic blue color in the colonies, therefore, the colonies that contain unligated vector will become blue. White colonies indicate insertion of TTR gene into the Lac Z region, and loss of the ability of the *E. coli* cells to hydrolyze that substrate marker. However, a bacterial colony that contains no vector at all should also become white. These are suppressed by the presence of an antibiotic such as ampicillin on the plate and a resistance gene on the vector, which together inhibit the growth of the bacterial colonies with no vector.

Five to ten white colonies were selected for plasmid preparation. The plasmid was isolated from bacterial cells by an alkaline lysis as described in section 2.9.1. The isolation depends on a unique property of plasmid that is able to rapidly anneal following denaturation, thus allowing the plasmid DNA to be separated from chromosome of the *E. coli*. The TTR gene fragment inserted in the plasmid can be checked by size after the plasmid is digested with EcoR I. The purified plasmid (2 to 5 μg) was mixed with 10 units of EcoRI (Gibco, Biolabs) in the total reaction volume of 20 μl. The digestion was allowed at 37°C for 2 h before the reaction mixture was analyzed on 1% agarose gel containing ethidium bromide. The sizes of the inserted TTR exons were as expected (Figure 3.5), i.e. 311 bp, 205 bp and 258 bp for TTR exon2, 3 and 4, respectively.

The nucleotide sequence of the inserted DNA fragments was determined by the dye terminator sequencing method as described in section 2.6. In

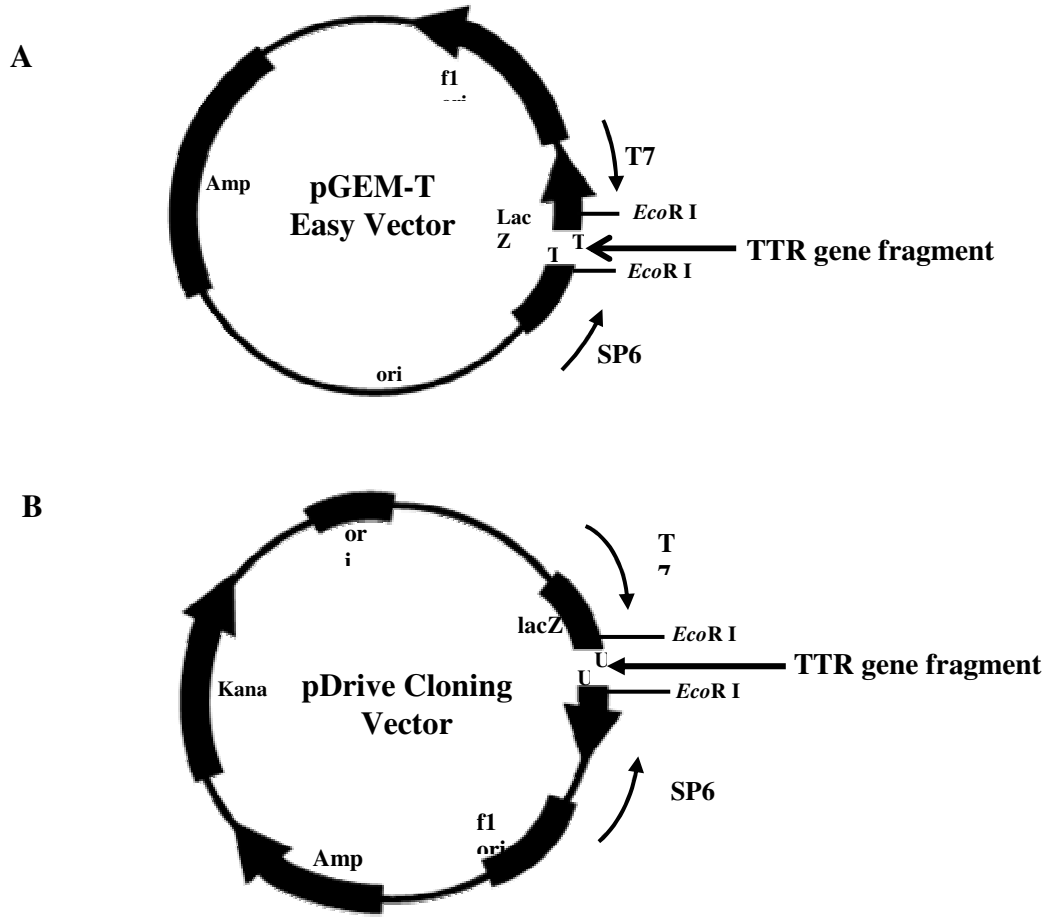


Figure 3.4 **Cloning of TTR gene fragments into vectors**

pGEM-T easy (A) and pDrive (B) are selected as vehicles of bringing TTR gene fragments into and being amplified in an *E. coli* cell. The vectors contain both origin of replication of the filamentous phage f1 and T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of β -galactosidase. The DNA fragments were inserted into the vectors between the EcoRI sites. Priming between the DNA of interest with the pGEM –T Easy or the pDrive vectors is ensured by a single 3′-T and 3′-U overhangs at the insert site, respectively. f1 origin represent phage f1 origin; Amp is ampicillin resistance gene; kanamycin is kanamycin resistance gene; lacZ is lacz α-peptide coding region used in selection of the blue/white colony screening.

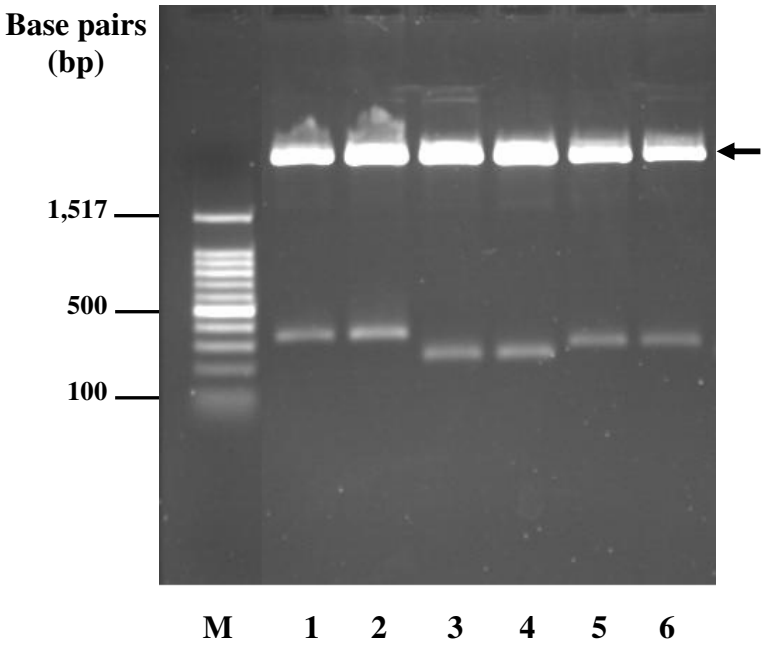


Figure 3.5 **Digestion of TTR DNA inserted plasmid by restriction endonuclease**

A recombinant vector (2.5 to 5.0 μg) containing TTR exon was digested with EcoR I (10 units) prior the digestion reaction was analyzed on 1% agarose. M is a DNA marker (100 bp ladder). Arrow indicates the plasmid after the DNA insert was removed. The DNA fragments of TTR exon 2 (lane 1 and 2), exon 3 (lane 3 and 4) and exon 4 (lane 5 and 6), which were released after the plasmid digestion

this method, in contrast to the chain-termination developed by Sanger (Sanger *et al*., 1977), the chain terminators, i.e. dideoxynucleotides (ddATP, ddGTP, ddCTP, and ddTTP), rather than primers were labeled. The major advantage of the dye terminator sequencing is that the nucleotide sequencing can be performed in a single reaction. In dye terminator sequencing, each of the four dideoxynucleotide chain terminators is labeled with a different fluorescent dye, which each fluorescing at a different wavelength. Along with high-throughput computer-controlled DNA sequence analyzer, the fluorescent signals generated from the incorporation of the dideoxynucleotides are collected and chromatogram of the sequence is created.

The nucleotide sequence of the TTR exons of the control and the case were aligned to the sequence of human TTR cDNA deposited in GenBank (accession no.NM 000371). All of exons amplified from the control and exon 2 and 3 amplified from the case had the same nucleotide sequence as that in GenBank. However, a single base substitution (T to C) was observed in TTR exon 4 of the case, which confirmed the abnormal mobility in SSCP of the DNA. This mutation leads to substitution of the amino acid at position 110 of the TTR monomer from leucine (CTG) to proline (CCG) as shown in Figure 3.6. No i*n vivo* mutation of TTR at leucine position 110 has been reported so far. Only two engineered TTR, Leu110Ala (substitution of leucine to alanine) and Leu110Met (substitution of leucine to methionine) (Redondo *et al*., 2000; Jiang *et al*., 2001) were prepared and showed no ability in forming amyloid fibril. Therefore, this Leu110Pro is a novel mutation of the TTR.

5. Construction of *Pichia* clone of TTR Leu110Pro

In general, there are two main desires for *in vitro* synthesis of protein. First is to produce in sufficient amount, for further study, of protein that its expression in some animal species is very low or occurs in only a short period of their lives. Second is to determine relationship between structure and function of the protein.

Since TTR amyloidosis is the most common form of autosomal dominant hereditary systemic amyloidosis. Both normal and mutated TTR are synthesized and secreted into the blood stream. Therefore, it is impossible to purify the mutated TTR from the normal in sufficient amount for further characterization.

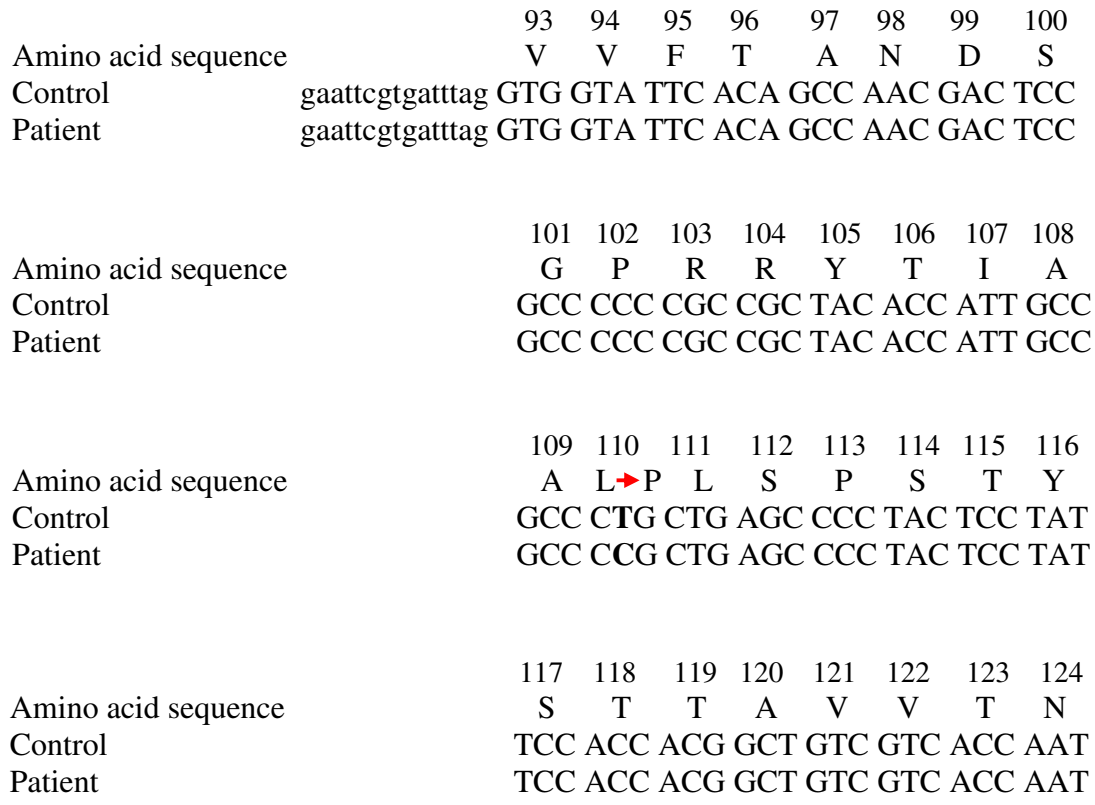


Figure 3.6 Comparison of nucleotide and derived amino acid sequences of TTR exon 4

Nucleotide sequence of TTR exon 4 purified from control (Control) was aligned with that of a patient with mental retardation (Patient). The derived amino acid sequence is indicated above the nucleotide sequence. Numbers that are on top the derived amino acid sequence indicates the positions on the amino acid sequence of TTR isolated from human serum (Mita *et al*., 1984). Replacement of thymine (T) with cytosine (C) in the nucleotide sequence of the patient TTR is shown in bold. The amino acid substitution of leucine (Leu) by proline (Pro) is shown on top of the mutant codon.

Recombinant of the wild-type and several of its variants have been successfully produced in the *E. coli* expression system, and they were used to reveal the molecular mechanism of amyloid fibril formation.

In this thesis, the Leu110Pro was constructed and *in vitro* synthesis with the aim to further characterization of the variant. A variety of DNA manipulations can be used to introduce mutations into cloned genes. A precise alteration in a gene sequence can be introduced by site-direct mutagenesis technique. To produce Leu110Pro DNA for the *in vitro* synthesis, a single nucleotide, i.e. T, at position 110 on native human TTR DNA has to be changed to C. This alteration was achieved by the site-direct mutagensis and a synthetic oligonucleotide was used to introduce a predetermined nucleotide alteration into the TTR gene.

The native human TTR DNA in a bacterial cloning plasmid was first subcloned into *Pichia* expression vector, pPIC3.5 as described in section 2.13.1. PCR and specific primers were used to generate the TTR DNA with BamH I and EcoR I ends for ligation into the vector, and the DNA was constructed so that BamHI site located at the 5' end and the EcoRI site located at the 3' of the molecule. Insertion of the TTR DNA was checked by double digestion of the plasmid with EcoRI and BamHI and analysis of the digested DNA by the electrophoresis. Figure 3.7 showed insertion of the TTR DNA and size of the insert can be estimated to ~ 400 bp. To construct the Leu110Pro, the human TTR DNA in pPIC 3.5 was used as template for the site-directed mutagensis, and the mutagenic oligonucleotide was used to create the specific mutation as described in section 2.13.2. The plasmid containing DNA of Leu110Pro was purified and its nucleotide sequence was performed to check the direction of the insertion and the desired mutation. The sequencing chromatogram confirmed the correct insertion and mutation at the amino acid position 110 (Figure 3.8).

In order to express in *Pichia*, the pPIC3.5 containing Leu110Pro DNA (0.56 µg) was linearlized by *Sal*I prior to introducing into the competent *Pichia* GS115 by electroporation as described in section 2.13.2. About 900 transformants were obtained from 0.56 µg DNA, or equal to ~1.6×10^3 of transformants per µg DNA. This was well agreed with the previous observation that between 10^3 and 10^4 transformants were obtained per µg DNA (Scorer *et al.,* 1994).

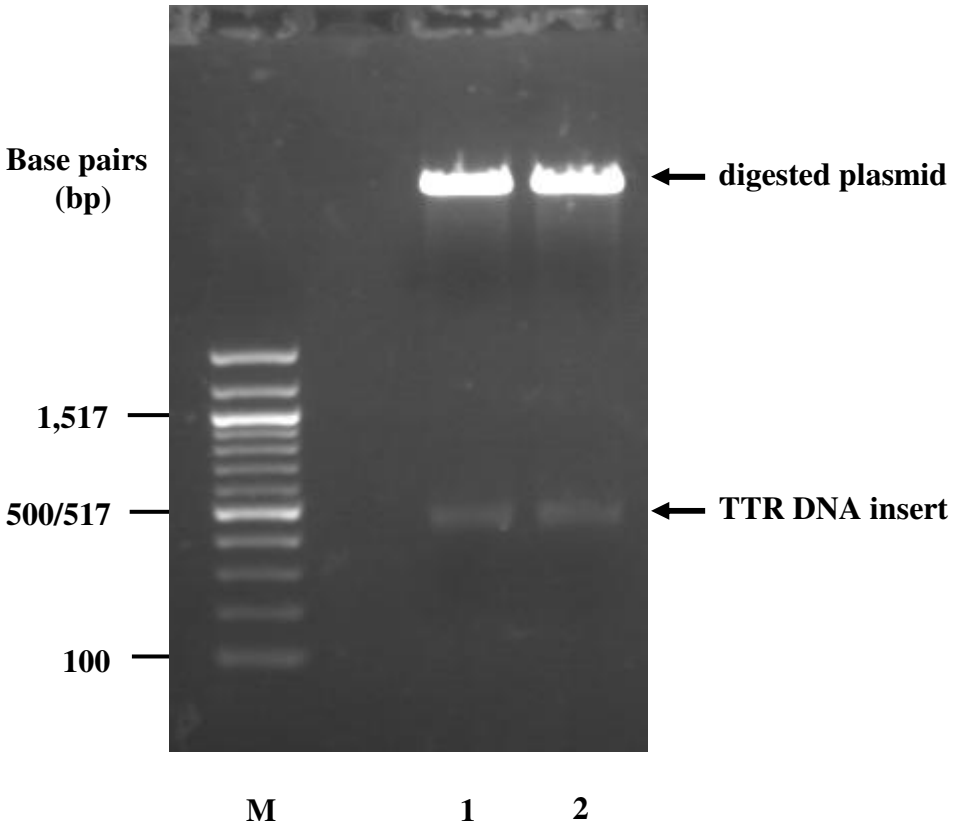


Figure 3.7 **Size determination of the human TTR DNA insert**

Aliquots (2.5 to 5.0 μg) of the plasmid purified from the recombinant clones were double digested with EcoRI and BamHI at 37°C for 2 h. Analysis of the DNA was carried out on 1% agarose gel. M is a 100 bp DNA marker. 1 and 2 are the digested plasmids purified from individual clones. Position of the digested plasmid and human TTR DNA insert are indicated.

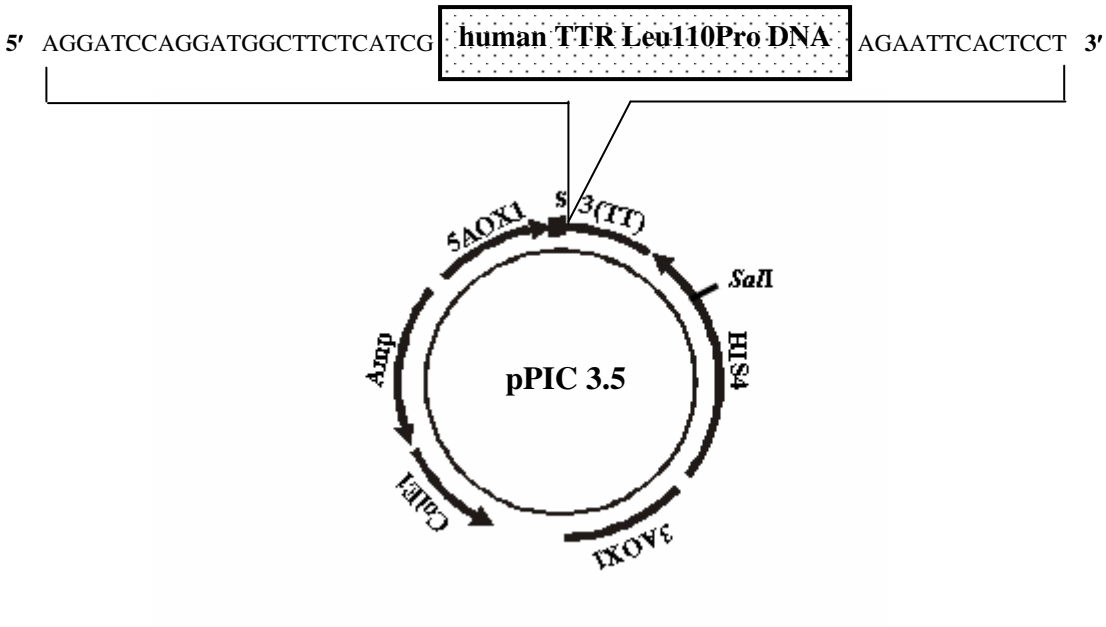


Figure 3.8 **Construction of vector for expression of Leu110Pro DNA in *Pichia***
The expression plasmid was constructed from pPIC3.5 using signal sequence of the Leu110Pro for gene expression and secretion of the recombinant Leu110Pro. The TTR cDNA was inserted into pPIC3.5 vector between BamHI and EcoRI sites. The figure shows the sequence at the 5′end and the 3′ end of the TTR gene after restriction sites were generated, and the region flanking the nucleotide sequence where the single point mutation was occurred. 5′AOX1 represents the promoter of the alcohol oxidase 1 gene of *P. pastoris*; 3(TT) is the native transcription termination and polyadenylation signal of alcohol oxidase 1 gene; 3′AOX1 is the sequence from the 3′ end of alcohol oxidase 1 gene to the TT sequences; S is the secretion signal of Leu110Pro; Ampicillin is ampicillin resistant gene; HIS4 is histidinol dehydrogenase gene using in selection of transformants; *Sal* I is *Sal* I restriction site for linearization of vector.

6. Screening for His^+Mut^+ tranformant

The expression and secretion of Leu110Pro in *Pichia* are mainly regulated by the *AOX1* promoter, which is transcriptional-activated by the inducer, methanol (Ellis *et al.*, 1985; Koutz *et al.*, 1989; Tschopp *et al.*, 1987). The expression "cassette", consisting of signal sequence plus human TTR gene, was inserted into the yeast genome at the *HIS4* locus. *P. pastoris* strain GS115 has a mutation in the histidine dehydrogenase gene (*HIS4*) (Cregg *et al.,* 1985), therefore, it can use methanol, but requires supplementation with histidine for growth (genotype *his4*, phenotype Mut^+/His^-). Crossing over event between the *HIS4* gene on the pPIC3.5 that contains Leu110Pro DNA and the *his4* locus of the *Pichia* chromosome (Cregg *et al.,* 1985; Cregg *et al.,* 1989), creates His^+/Mut^+ recombinants which synthesize histidine and utilize methanol. However, loss of the *AOX1* gene can lead to loss of the alcohol oxidase activity, which result in a slow methanol utilization (Mut^s) strain that exhibits poor growth on methanol media. Mut^+ transformants could be differentiated from the methanol utilization slow Mut^s, transformants by culturing in a minimal medium containing methanol. One hundred *Pichia* transformants were patched on MM and then MD plates as explained in section 2.15. Figure 3.9 shows the characteristic growth of the His^+Mut^+ transformants on both media.

7. Synthesis in *P. pastoris* of the recombinant humanTTR Leu110Pro

Twenty nine putative His^+Mut^+ colonies were selected for a small scale synthesis of the recombinant Leu110Pro. The induction of expression in 5 ml cultures was carried out at a final concentration of 0.5% methanol at 30°C for 7 days. Aliquots of culture supernatant (100 µl) were analyzed by SDS-PAGE. Silver staining of the gel revealed high expression of a secreted protein band that migrated with an approximate molecular mass of 15 kDa (Figure 3.10), which is consistent with the molecular mass of the subunit of human TTR. Thus, this recombinant clone was selected for large scale synthesis, therefore sufficient amount of the TTR can be obtained for further characterization.

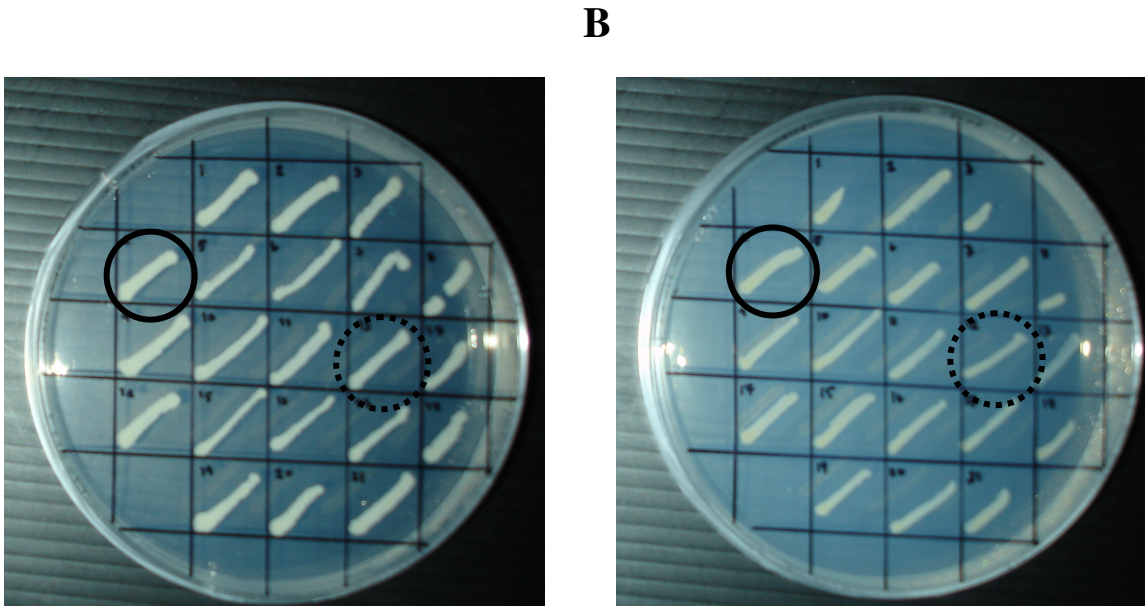
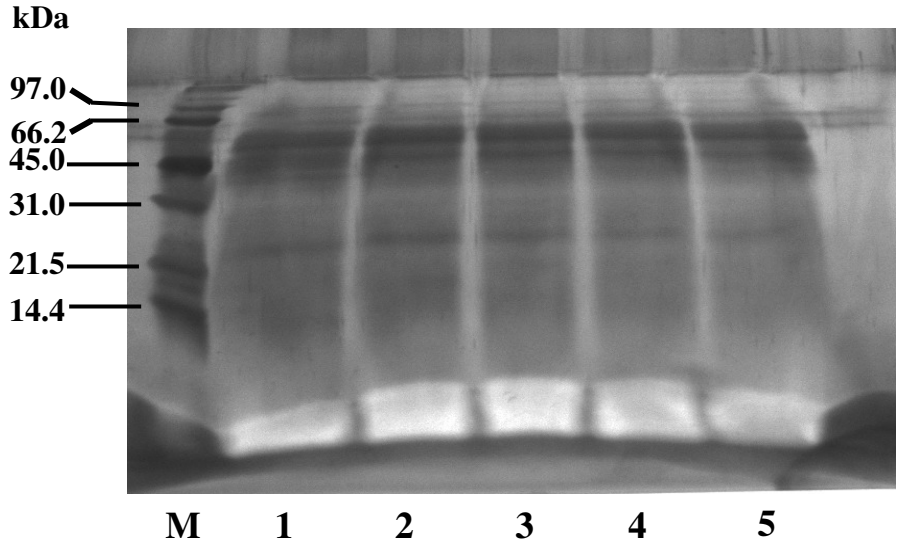


Figure 3.9 **Growth patterns of His^+Mut^+ transformants**

The *Pichia* transformants were selected by comparing their growth in minimal dextrose medium (MD) (A) and minimal methanol medium (MM) (B). His^+Mut^+ transformants grew on both media with similar rate. An example of His^+Mut^+ transformant is indicated by black circule, and that of His^+Mut^s transformant is indicated by broken circle.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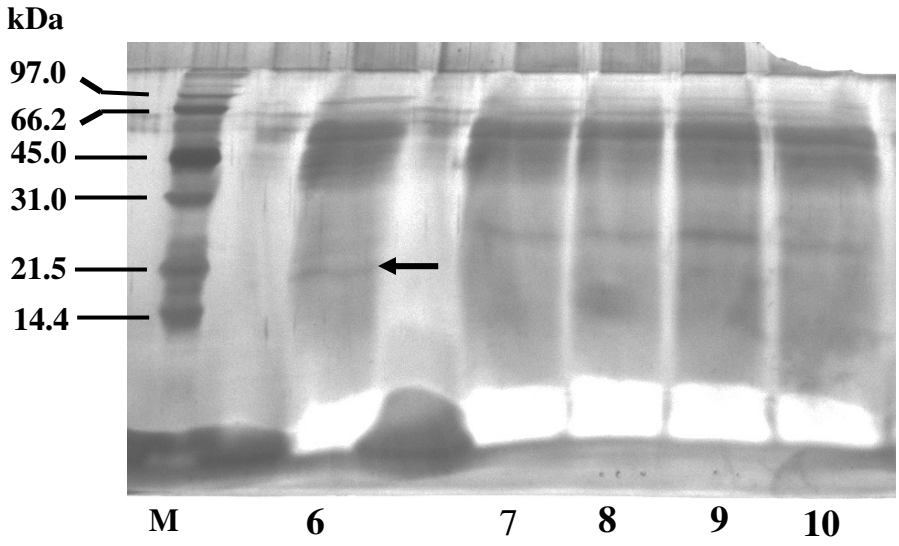
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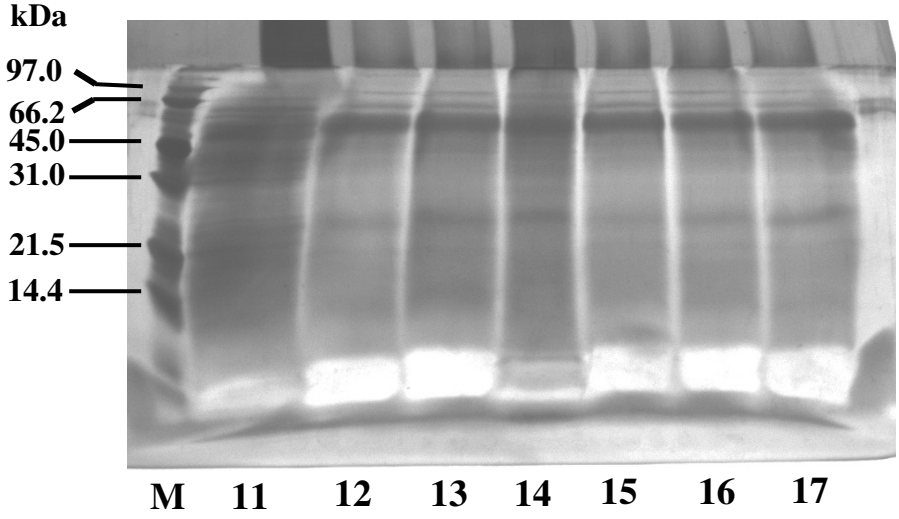
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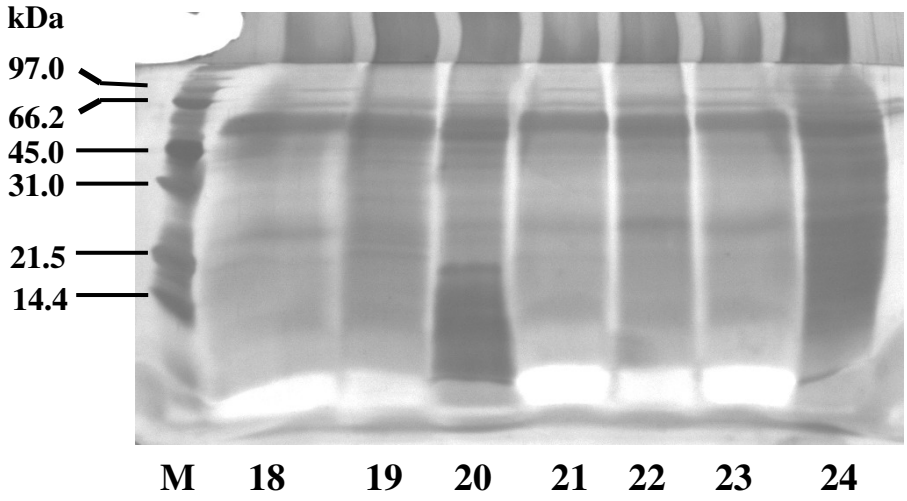
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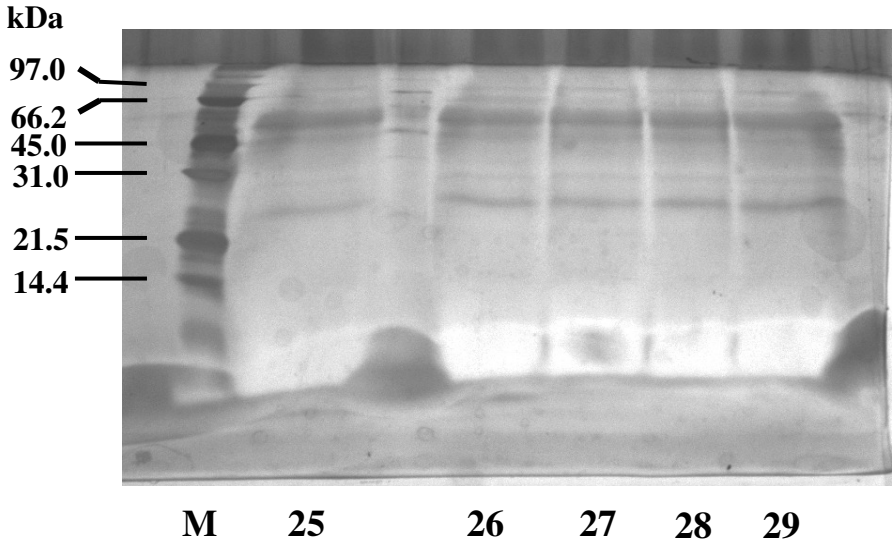
kDa
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26
27
28
29

Figure 3.10 Scaling up synthesis of the recombinant TTR Leu110Pro

Twenty-nine recombinant *Pichia* clones were grown and induced to synthesize human TTR Leu110Pro in 5 ml of medium as described in section 2.16.1. The cell culture was removed after induction with 0.5% methanol for 7 days and the culture supernatant was determined for secretion of the recombinant TTR by 12% SDS-PAGE. The protein band was detected after staining the gels with silver nitrate. The *Pichia* clone number 6 (as indicated by arrow) was selected for large scale production of the protein.

1 to 29 indicate an individual recombinant *Pichia* clones; M, protein markers (phosphorylase b (97 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydase (31 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

8. Large scale production of the recombinant Leu110Pro

To obtain sufficient amount of the Leu110Pro for further characterization, synthesis of the proteins was scaled up to larger volumes by using the same conditions as the small scale synthesis. The shaking flask method is very well adapted to this purpose because it requires simple equipment. However, other parameters in particular oxygenation efficiency in the medium, are also important during methanol induction for an efficient large scale expression in *Pichia*. In general when using a shake flask, the culture medium less than 10% to 30% of the total flask volume is recommended.

The Leu110Pro *Pichia* transformant was induced for synthesis in a 2-liter flask containing 300 ml of BMMY as described in section 2.16.2. Aliquot of the culture were collected every 24 h, and subjected to analysis by native-PAGE. The TTR band was detected by silver staining. The result showed that in the native PAGE, the recombinant Leu110Pro has similar mobility as the native TTR in plasma (Figure 3.11).

9. Purification of recombinant of TTR Leu110Pro

Single step purification by the affinity chromatography on a human retinol binding protein-Sepharose (RBP-Sepharose) was an efficient method to isolate recombinant TTR from the yeast culture medium (Prapunpoj *et al.*, 2000, 2002). The method was based on the fact that TTR can bind to retinol-binding protein (RBP) in plasma (Kanai et al, 1968; Peterson, 1971). However, there are limitations, particularly as no RBP resin is commercially available. In addition, the commercial RBP with enough purity for preparation of the affinity resin is expensive, e.g. 0.6 US$ per μg. The TTR in human plasma typically displays a characteristic migration during electrophoresis, i.e. it moves faster than the plasma albumin (Ingbar, 1958). Due to its electrophoretic mobility, the TTR could be isolated from other protein in the plasma by the preparative native gel electrophoresis (Richardson *et al.*, 1994). Since the native-PAGE (Figure 3.11) showed that the recombinant has the same mobility in an electric field as the TTR in human plasma. Therefore, purification of the Leu110Pro by the preparative gel electrophoresis was attempted. The *Pichia* culture supernatant

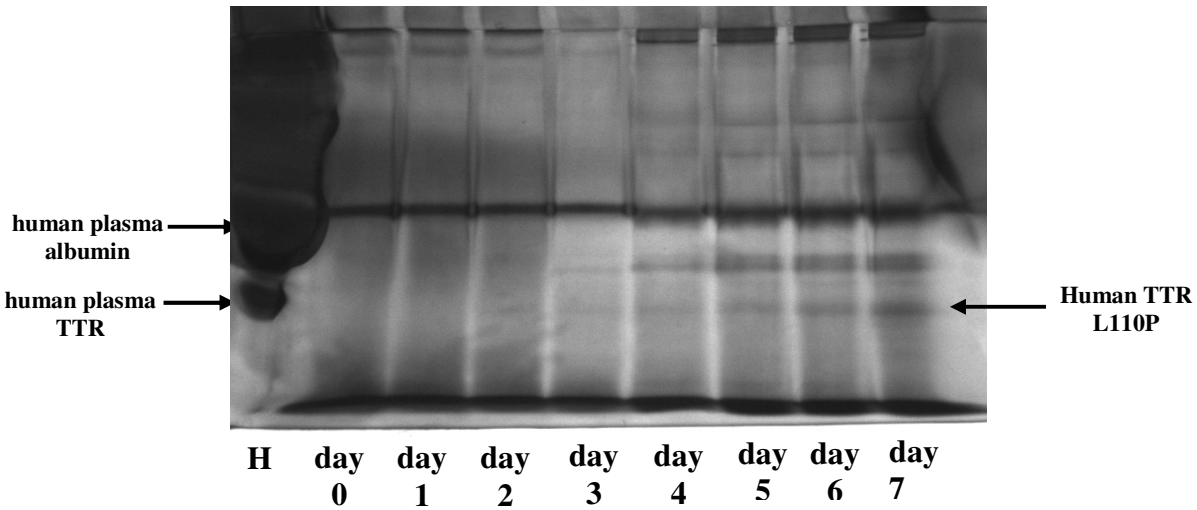


Figure 3.11 Kinetic secretion of recombinant human TTR Leu110Pro

The recombinant *Pichia* cell containing gene of human TTR Leu110Pro were grown in BMGY, the then transferred to BMMY as described section 2.16.2. The induction for protein synthesis was carried out with 0.5% methanol for 7 days. Aliquot of the cell culture was removed every 24 h from day 1 to day 7, and its supernatant was analyzed by native PAGE (10% resolving gel) before the protein bands were detected by silver staining. Day 0 is the cell culture in BMMY at the beginning of the induction with methanol. Human plasma was included to indicate the position of native TTR.

of Leu110Pro from the large scale preparation was concentrated by ultrafiltration prior to onto a polyacrylamide gel as described in section 2.17, and eluting fractions were collected. The presence of Leu110Pro in each fraction was detected by native gel electrophoresis (10% acrylamide resolving gel) followed by staining the gel with silver nitrate. The elution pattern of the TTR was shown in Figure 3.12. It illustrated a clearly separation of the TTR from other endogenous proteins of *Pichia*, which also secreted into the culture medium. The eluting fractions that contained TTR were pooled, concentrated and further characterized.

10. Characteristics of the recombinant Leu110Pro

The characteristics of recombinant Leu110Pro was interested since it is a novel TTR mutation, thus in this thesis the electrophoretic mobility in native-PAGE and subunit mass of recombinant TTR Leu110Pro were determined.

10.1. Mobility in native gel of recombinant TTR

Human TTR was noted to migrate faster than albumin during electrophoresis at pH 8.6 (Seibert and Nelson, 1942). The mobility of the purified recombinant Leu110Pro on non-denaturing polyacrylamide gel (10% resolving gel) was compared with albumin and TTR in human plasma. Figure 3.13 shows the electrophoretic pattern of the recombinant proteins, which revealed that the TTR had the same mobility as the TTR in human plasma and it moved faster than the plasma albumin.

10.2. Subunit mass of the Leu110Pro

The purified recombinant Leu110Pro was analyzed by SDS-PAGE (12 % resolving and 4 % stacking gel) and gel was stained with silver nitrate to visualize protein bands as described in section 2.19. Mobility on SDS-PAGE of the TTR was shown in Figure 3.14A. Single band of the TTR was located between carbonic anhydrase (31 kDa) and trypsin inhibitor (21.5 kDa), and molecular mass of the subunit was calculated from the calibration curve as shown in Figure 3.14B. The result demonstrated that the subunit mass of this recombinant TTR was 22.7 kDa,

which is slightly different from that reported for the subunit of TTR found in blood. Several factors might effect on this alteration including glycosylation of the protein subunit.

Figure 3.12 Elution pattern of TTR obtained from preparative native gel electrophoresis

Isolation of TTR Leu110Pro from the endogenous proteins of *Pichia* was carried out using preparative native polyacrylamide gel electrophoresis. 10 ml of concentrated *Pichia* culture supernatant was applied onto gel (12% acrylamide of resolving and 4% of stacking) and electrophoresis separation was performed using a Prep cell model 491. Eluting fractions (2 ml/ fraction) of proteins were collected, and aliquot (80 µl) of each fraction was analyzed by native PAGE (10% resolving gel). The protein bands were detected after silver staining. H, human plasma; lane 1 to 8 are an individual eluted fraction; lane C is concentrated supernatant of the *Pichia* culture.

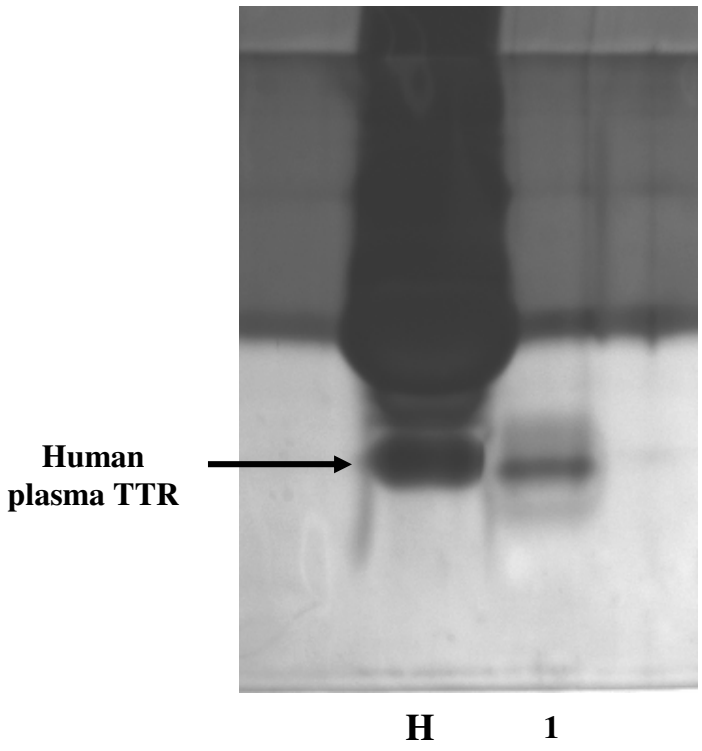
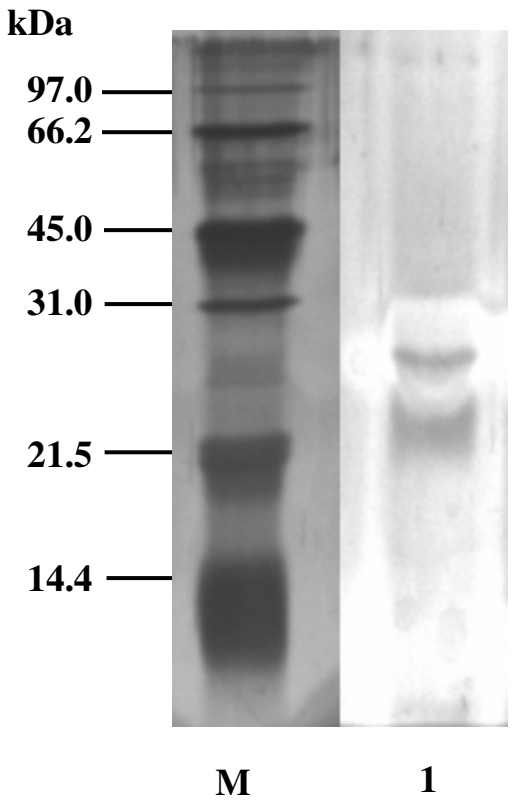


Figure 3.13 Native PAGE of purified recombinant TTR

Purified human TTR Leu110Pro lane 1 was analyzed by native PAGE (10% resolving gel), and the proteins were detected by silver staining. Human plasma (H) was also loaded to indicate the position of the native TTR in plasma.

A


kDa
97.0
66.2
45.0
31.0
21.5
14.4
M
1

B

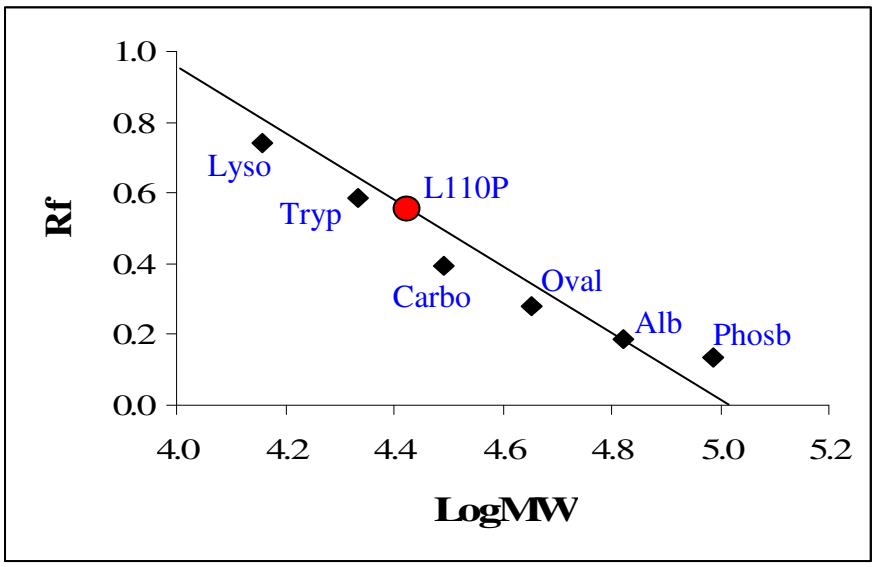

1.0
0.8
0.6
0.4
0.2
0.0
Rf
Lyso
Tryp
L110P
Carbo
Oval
Alb
Phosb
4.0
4.2
4.4
4.6
4.8
5.0
5.2
LogMW

Figure 3.14 Determination of subunit mass of the recombinant Leu110Pro

(A) Aliquots (80 μl) of purified human Leu110Pro (1) was heated for 30 min in the presence of 2% SDS and 2.5% β-mercaptoethanol prior analysis by SDS-PAGE (12% resolving gel). The protein bands were detected by silver staining. The protein markers (M) included phosphorylase b (97.4 kDa; Phosb), bovine serum albumin (66.2 kDa; Alb), ovalbumin (45 kDa; Oval), carbonic anhydrase (31 kDa; Carbo), trypsin inhibitor (21.5 kDa; Tryp), and lysozme (14.4 kDa; Lyso).

(B) The relative mobility (R_f) of each marker was logarithmic plotted against its molecular weight. The R_f value of the Leu110Pro subunit (L110P) was determined and mass of it was calculated.