Characteristics and fibrillation of transthyretin variants detected in Thai people

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ชื่อวิทยานิพนธ์ คุณลักษณะและกระบวนการเกิดเป็น fibril ของ transthyretin variants ที่พบในคนไทย

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บทคัดย่อ

TTR เป็นโปรตีนที่ทำหน้าที่หลักในการขนส่งฮอร์โมนไธรอยด์และวิตามินเอในกระแสเลือดของมนุษย์ โดยมีแหล่งสร้างที่สำคัญคือ ตับและ choroids plexus ในสมอง TTR เป็นโปรตีนชนิดหนึ่งที่สามารถ ก่อเกิดเป็น amyloid ได้ TTR ที่เกิดมิวเตชั่น (mutation) เป็นสาเหตุหลักของโรค Familial amyloidotic polyneuropathy (FAP) และ cardiomyopathy (FAC) จากการศึกษาพบมิวเตชั่นของ TTR เกิดขึ้นมากกว่า 100 ตำแหน่ง และมีมากถึง 80 ตำแหน่งที่สัมพันธ์กับโรค FAP โดยเมื่อไม่นานนี้ ได้มีการค้บพบ TTR ชนิด Val122Asp (V122D) ซึ่งเป็นมิวเตชั่นชนิดใหม่ ในผู้ป่วย คนไทย นั้น ได้แก่ Val122Asp (V122D) เนื่องจากความถี่และลักษณะทางคลีนิค ของโรคมีความผันแปรในหมู่ประชากร ความเข้าใจในคุณลักษณะของ TTR จึงมีความสำคัญทางคลีนิค งานวิจัยวิทยานิพนธ์นี้ ได้ทำการสังเคราะห์ cDNA ของ V122D และ TTR ที่เกิดมิเตชั่นอีก 2 ชนิด คือ Val30Met (V30M) และ Leu55Pro (L55P) โดยวิธีการ site-directed muatagenesis จากแม่แบบ (template) ชนิด wild type TTR แล้วเชื่อมต่อเข้าสู่เวคเตอร์ pPIC3.5 โปรตีนรีคอมบิแนนท์ (recombinant protein) ดังกล่าวถูกสังเคราะห์ขึ้นโดยอาศัยระบบการสังเคราะห์ของ *Pichia pastoris* ผลการทดลองปรากฏว่ายีสต์สามารถสังเคราะห์และหลั่งรีคอมบิแนนท์โปรตีน ออกสู่อาหารเลี้ยงภายนอกเซลล์ได้ และโปรตีนเหล่านี้ถูกแยกให้บริสุทธิ์จากโปรตีนชนิดอื่นได้ โดยผ่านการแยกเพียงขั้นตอนเดียวโดย เทคนิค preparative native-PAGE ซึ่ง TTR ทุกชนิดที่ทำการสังเคราะห์มีคุณสมบัติเคมีกายภาพเช่นเดียวกับ TTR ในธรรมชาติ กล่าวคือมีการเคลื่อนที่ในสนามไฟฟ้าภายใต้สภาพธรรมชาติเร็วกว่าอัลบูมินในพลาสมาของคน แสดงให้เห็น การพับทบของโครงสร้างที่สมบูรณ์ น้ำหนักหน่วยย่อยของ V122D V30M และ L55P ที่ได้จากาการวิเคราะห์โดย SDS-PAGE มีค่าเท่ากับ 16.9, 15.9 และ 15.5 กิโลดาลตัน ตามลำดับ ผลจากการวิเคราะห์โดยใช้ ThT พบว่า V122D สามารถเกิดเป็นโปรตีนเส้นใย (fibril) ได้เร็วกว่า wild type TTR และ L55P แต่เกิดได้ช้ากว่า V30M โดยอัตราการเกิดเป็น fibrils ของ wild type TTR, V30M, L55P และ V122D เท่ากับ 9.9%, 38.6%, 8.4% และ 10.8% ตามลำดับ ซึ่งเมื่อเปรียบเทียบการเกิดเป็น fibril ของ wild type TTR พบว่า V122D, V30M และ L55P เกิดได้เป็น 1.2, 4.3 และ 0.9 เท่า ตามลำดับ ได้ทำการศึกษาคุณสมบัติการ

เร่งปฏิกิริยาย่อยสลา ยโปรตีน (proteolytic activity) ของ V122D โดยใช้เคซีนที่เชื่อมต่อกับ fluorescence isothiocyanate (casein-FITC) เป็นสับสเตรท และเปรียบเทียบผลที่ได้กับ TTR ชนิดอื่น พบว่าอัตราการเร่งปฏิกิริยาโดย V30M, L55P, V122D และ human native TTR มีค่าเท่ากับ 0.97, 0.51, 0.40 และ 0.26 นาโนโมลาร์ต่อนาที ตามลำดับ อย่างไรก็ตาม specific activity สำหรับ V122D มีค่าเท่ากับ 8038.67 นาโนโมลาร์ต่อนาทีต่อมิลลิกรัมโปรตีน ในขณะที่ V30M, L55P และ wild type TTR มีค่าดังกล่าวเท่ากับ 3887.81, 2936.10 และ 1024.56 นาโนโมลาร์ต่อนาทีต่อมิลลิกรัมโปรตีน ตามลำดับ

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ABTRACT

TTR is one of the three major thyroid hormone (TH) distributors and a vitamin A transporter in bloodstream of human. It is mainly synthesized in liver and choroids plexus of the brain. TTR is one of the precursor proteins those are known to form amyloid fibril. Familial amyloidotic polyneuropathy (FAP) and cardiomyopathy (FAC) are the systematic amyloidoses that have mutated TTR as major cause. Up to date, more than 100 point mutations of TTR have been identified and up to 80 were revealed associate with FAP. A novel mutated TTR, Val122Asp (V122D) was

recently identified in a Thai patient. Since frequency and clinical manifestations of the disease are varied among populations. Insight into TTR characteristics is clinically importance. In this thesis, cDNA of V122D and that of another two variants, Val30Met (V30M) and Leu55Pro (L55P) were constructed from wild type TTR cDNA template by site-directed mutagenesis, and ligated into pPIC3.5 expression vector. The recombinant proteins were synthesized by using the heterologous gene expression system of *Pichia pastoris*. The result showed that all recombinant TTRs were successfully synthesized and extracellularly secreted into the yeast culture medium. They were efficiently isolated from other proteins in the culture supernatant with a single step by preparative native-PAGE. All of these TTRs had similar physicochemical properties to native TTR. Their electrophoretic mobilities in polyacrylamide under non-denaturing condition were faster than albumin in human plasma indicating to a proper structural folding of the TTRs. The subunit masses determined by SDS-PAGE were 16.9 kDa, 15.9 kDa and 15.5 kDa for V122D, V30M and L55P, respectively. Based on ThT assay, V122D formed fibril faster than human wild type TTR and L55P, but slower than V30M. Percentages of the fibril forming were 9.9, 38.6, 8.4 and 10.8 for human wild type TTR, V30M, L55P, and V122D, respectively. In addition, the relative fibril formation compared to human wild type TTR were 1.2, 4.3, and 0.9 folds for V122D, V30M and L55P, respectively. The proteolytic activity of V122D was examined using fluorescein isothiocyanate linked casein (casein-FITC) as substrate, and compared to other TTRs. The catalytic activities of V30M, L55P, V122D and human native TTR were 0.97, 0.51, 0.40 and 0.26 nM/min, respectively. However, the specific activity of V122D was 8038.67 nM/min/mg protein, whereas, those of V30M, L55P, and wild type TTR were 3887.81, 2936.10 and 1024.56 nM/min/mg protein, respectively.

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LIST OF ABBREVIATIONS AND SYMBOLS

AOX	=	alcohol oxidase
β	=	beta
BMGY	=	buffer medium containing glycerol
BMMY	=	buffer medium containing methanol
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
°C	=	degree Celcius
DNA	=	deoxyribonucleotide triphisphate
DTT	=	dithiothreitol
h	=	hour
kDa	=	kilodalton

kV	=	kilovolt
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimolar
OD	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
rpm	=	revolutions per minute
s	=	second
SDS	=	sodium dodecyl sulfate
TTR	=	transthyretin
v/v	=	volume by volume
YPD	=	yeast exteact peptone dextrose
µF	=	micro Faraday
µl	=	microliter
µM	=	micromolar

CHAPTER 1

INTRODUCTION

Amyloidosis refers to a group of protein deposition diseases in which normal or variant forms of protein aggregate to form insoluble fibrils called 'amyloids'. Most of the amyloid precursor proteins have or rich in β–structure. Once it forms to amyloid, the protein becomes to resist to proteolysis and preferably accumulates in the cellular interstitial. Deposition of amyloid proteins associates to several types of amyloidosis including hereditary/familial amyloidosis and systemic amyloidosis. Usually, the target organs/tissues of the deposition are heart, kidneys and neurons. Up to date, more than 20 amyloid precursor proteins including transthyretin (TTR) have been identified.

TTR is one of the three major thyroid hormone (TH) distributors and a vitamin A transporter in bloodstream of human. It is mainly synthesized in liver and choroids plexus of the brain. TTR is one of the precursor proteins those are known to form amyloid fibril (Damas and Saraiva, 2000). The TTR amyloidosis including familial amyloidotic polyneuropathy (FAP) and cardiomyopathy (FAC) are caused by mutation of the TTR gene. Up to date, more than 100 point mutations of TTR have been identified and most of them were revealed associated with FAP. The frequency and clinical manifestations including age of onset, sex ratio and duration of the disease of the TTR amyloidosis are varied among populations. For examples, V30M is the most frequent variant (Saraiva, 1984, 2001), vastly found in Portuguese and Swedish. Whereas, incidences of Leu55Pro (L55P) and V122I on FAC were reported in many other populations including Taiwanese and African Americans (Jacobson *et al*., 1992; Yamamoto *et al*., 1994), and other 20 different amino acid substitutions have been reported among Japanese (Andersson, 1970; Araki, 1984; Ikeda *et al*., 2002). In Thai people, a few novel TTR variants were identified (Tayeh, 2007; Srinuan, 2008). This includes Val122Asp (V122). Based on the mutation position, V122D is expected to associate with FAC. To reveal, its characteristics in particular fibril formation and toxicity are necessary.

Many mechanisms have been proposed for the conformational change of the precursor protein that leads to the fibril formation including point mutation, overproduction of the precursor protein, change in local pH of membrane, and proteolytic cleavage. For TTR, the mechanisms behind the self-assembly of native and mutated TTRs into amyloid deposits remain unclear, even though it is known that the β-sheet-rich secondary structure of the TTR monomers facilitates the fibrillation and has been suggested involving in the structural changes required for the amyloid formation. To reveal the mechanism of fibril formation and amyloidogenesis processes, numerous techniques were developed (Clements *et al*., 1993; LeVine, 1993). Amongst, ThT and Congo red are commonly used (for reviews see Nilsson, 2009; Groenning, 2009).

Review of literature

1. Amyloidosis and Alzheimer disease (AD)

Amyloidosis is a group of diseases in which a normal protein, with or without proteolytic cleavage, forms insoluble amyloid fibrils and extracellular deposits leading to diffusely or focally affects any organ. It is usually fatal, and be clinically classified to primary systemic (56%), secondary systemic (8%), localized (9%), myeloma-associated (26%) and hereditary-familial (1%) (Simpson *et al*., 1984; for review see Kokong *et al*., 2007).

Changes in conformation of amyloid precursor protein (Table 1) as a result of gene mutation or by unknown mechanisms turn the soluble native proteins to aggregate as insoluble fibrils called “amyloid” which prefer to deposit in organs, leading to functional and structural damage of the organs (Obici *et al*., 2005). Amyloids are rigid non-branching structures and highly consist of β-pleated sheet conformation (Cunnane and Whitehead, 1999). Amyloid aggregates are detected in all different forms of diseases including Alzheimer’s disease (AD), which is the most common amyloidosis and dementia in human (Wisniowaki *et al*., 1997).

Table 1.1 Amyloid fibril proteins and their precursors in humans.*

Amyloid protein	Precursor	Systemic (S) or localized, organ restricted (L)	Syndrome or involved tissues
AL	Immunoglobulin light chain	S, L	Primary Myeloma-associated
AH	Immunoglobulin heavy chain	S, L	Primary Myeloma-associated
Aβ_2M	β_2-microglobulin	S	Hemodialysis-associated
		L?	Joints
ATTR	Transthyretin	S	Familial Senile systemic
		L?	Tenosynovium
AA	(Apo)serum AA	S	Secondary, reactive
AApoAI	Apolipoprotein AI	S	Familial
		L	Aorta, meniscus
AApoAII	Apolipoprotein AII	S	Familial
AApoAIV	Apolipoprotein AIV	S	Sporadic, associated with aging
AGel	Gelsolin	S	Familial (Finnish)
ALys	Lysozyme	S	Familial
AFib	Fibrinogen α-chain	S	Familial
ACys	Cystatin C	S	Familial
ABri	ABriPP	S	Familial dementia, British
ALect2	Leukocyte chemotactic factor 2	S	Mainly kidney
ADan*	ADanPP	L	Familial dementia, Danish

Table 1.1 (con't)

Amyloid protein	Precursor	Systemic (S) or localized, organ restricted (L)	Syndrome or involved tissues
Aβ	Aβ protein precursor (AβPP)	L	Alzheimer's disease, aging
APrP	Prion protein	L	Spongioform encephalopathies
ACal	(Pro)calcitonin	L	C-cell thyroid tumors
AIAPP	Islet amyloid polypeptide**	L	Islets of Langerhans Insulinomas
AANF	Atrial natriuretic factor	L	Cardiac atria
APro	Prolactin	L	Aging pituitary Prolactinomas
AIns	Insulin	L	Iatrogenic
AMed	Lactadherin	L	Senile aortic, media
AKer	Kerato-epithelin	L	Cornea, familial
ALac	Lactoferrin	L	Cornea
AOaap	Odontogenic ameloblast-associated protein	L	Odontogenic tumors
ASemI	Semenogelin I	L	Vesicula seminalis

(Source: Jean *et al*., 2010)

AD is characterized by progressive loss of memory and other cognitive functions. It is a degenerative disorder of the nervous system that most affects in elderly. The prevalence of the disease increases to an estimated 19% in individuals aged 75-84, and is 30-35% for those older than 85 years. It may be caused by complex interactions among multiple genetic, epigenetic, and environmental factors. The development of dementia involves a decline in short-term memory, impairment of judgment, and a loss of emotional control. These symptoms develop slowly over a period of years, and finally complete breakdown of the mental function. Amyloid-β peptides (Aβ) are the main constituent of senile plaques which are a pathological hallmark in AD. In the past ~100 years, the fibrillar "amyloid hypothesis" has been hypothesized, suggesting that Aβ plaques are the cause of neuronal loss. Recently attention has become focused on pre-fibrillar monomeric Aβ and small oligomers of Aβ, which believed to induce the deleterious cascade(s) involved in the pathophysiology of AD (Lorenzo and Yankner, 1996; Yankner, 1996; Walsh *et al*., 1997). Aβ, typically a 40- or 42-amino acid peptide, forms in excess in AD brain by processing of the amyloid precursor protein (APP) (Sridhar *et al*., 2001). It has been shown that Aβ kills neurons in cell culture and in the brain *in vivo* (Yankner *et al*., 1989; Lambert *et al*., 1998; Lesne *et al*., 2006; Walsh *et al*., 2002; Yankner *et al*., 1990). However, the nature and physiological relevance of Aβ toxicity still be required to further observe. The neurotoxic fragment Aβ (25–35) is a membrane active peptide representing the membrane spanning domain of the amyloid precursor protein (APP) and of the full-length peptide Aβ with 39–42 amino acids, which occurs in Alzheimer affected brains with 39–42 amino acids. It is thought to represent the main responsible region for the neurotoxic effect of Aβ (1–42) even though only a part of the mechanisms driving the toxicological properties is active in the truncated peptide, but it causes an increased and faster oxidative damage than the full-length peptide Aβ (1–42) (Alexandra *et al*., 2010, Sridhar *et al*., 2001). Moreover, neurofibrillary tangle of hyperphosphorylated tau protein aggregation into nerve cell bodies also associated with the amyloid plaques (for review see De Strooper, 2010).

2. Transthyretin

2.1 General characteristics

Transthyretin (TTR) is one of the three major thyroid hormone (TH) distributor proteins (THDPs) (Richardson, 2007), for both 5′,3′,5,3-L-tetraiodothyronine (thyroxine, T4) and 3′,5,3-L-triiodothyronine (triiodothyronine, T3), in the extracellular fluid of vertebrates. It is a non-glycosylated protein with a MW of ~55 kDa, composing of four identical subunits (Figure 2.1). In human, each subunit comprises 127 amino acid residues with a MW of ~15 kDa. The tetrameric form of TTR contains a central channel where two binding sites for THs exist however, only one binding site is occupied by TH because of the negative cooperativity. In human, the binding site for TH of TTR binds T4 with higher affinity than binds to T3. Beside of binding to THs, TTR also binds to retinol-binding protein (RBP) which plays role in metabolism of vitamin A (for reviews see Hamilton and Benson, 2001; Palha, 2002). In human, liver and choroid plexus of the brain are the main synthesis sites of TTR (Figure 2.2).

2.2 Structure and chemical properties of TTR

Based on the X-ray crystallography, the secondary structure of the TTR subunit is an anti-parallel β-sheet-rich conformation (Blake *et al*., 1978; Power *et al*., 2000) which arranged into eight stranded β-sheet structure and only 5% of α-helix (Sorgjerd *et al*., 2008) (Figure 2.2). Dimer of TTR is composed of a pair of twisted eight-stranded β-sheets. The interactions between two monomers, to form a dimer, are predominantly involved with hydrogen bonding between β-strands (Blake *et al.,* 1978). Association of two dimers results in a tetrameric structure with two pairs of eight-stranded β-sheets. A large central channel of about 8 Å in diameter and 50 Å long is formed as a consequence of the tetrahedral arrangement of the subunits (Blake *et al.,* 1978; Morgado *et al*., 2008).

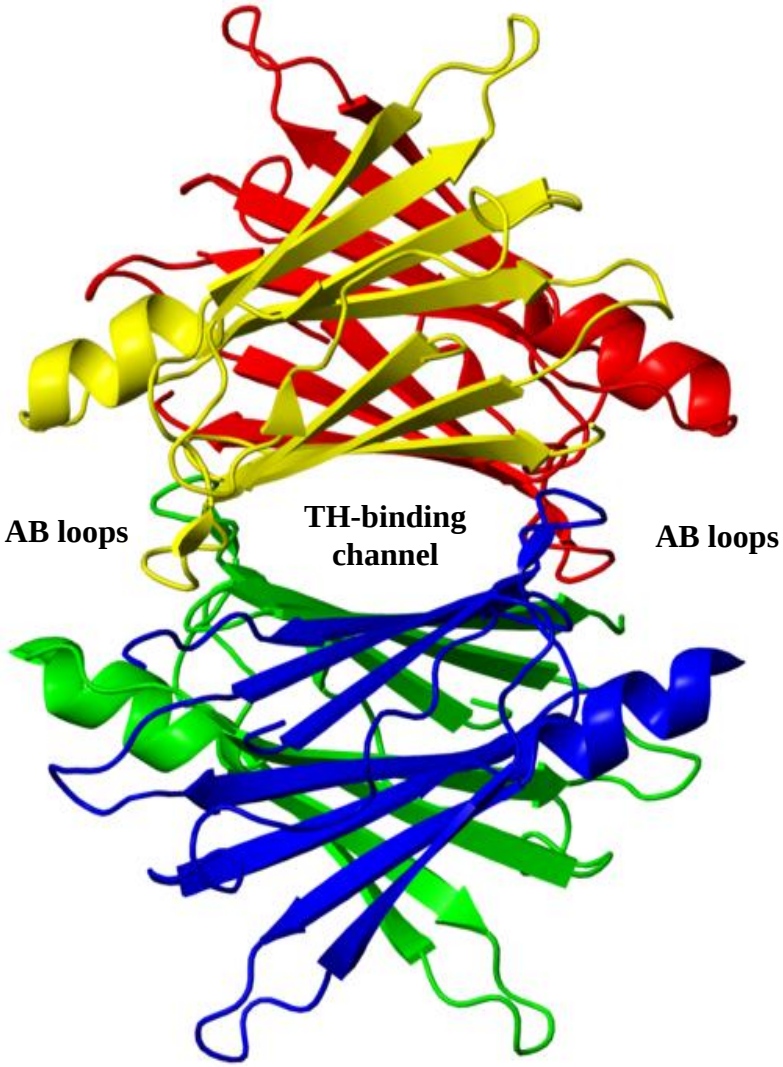


Figure 1.1 The structure of TTR tetramer

Four monomer of TTR are in different color. Two dimmers are linked with AB loops to form the tetramer and a central channel for binding with THs. Ribbon with arrow head represents β-pleated sheet structure, whereas spiral ribbon represents α-helix structure of TTR subunit. TH binding sites in the central channel, and AB loops are indicated. (Source: http://en.wikipedia.org/wiki/File:Transthyretin.png)

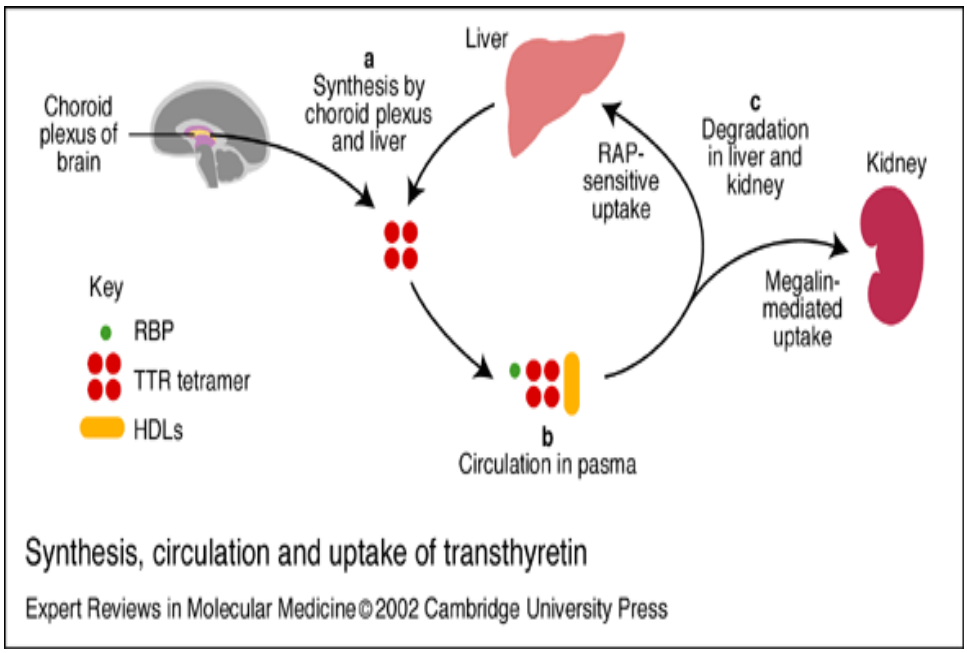


Figure 1.2 Synthesis, circulation and uptake of TTR

(Source: Maria, 2002)

The unusual high content of β-structure, the interaction between the monomers to form a dimer and that between dimers to form the tetramer contributes to the stability of TTR (Branch *et al*., 1971, 1972). In comparison, the TTR tetramer is less stable than the dimer (Blake *et al*., 1978). The dissociation of TTR tetramer occurred slowly in 6 M guanidine-HCl solution, but did not in strong acidic or alkaline or 0.1 % sodium dodecyl sulfate (SDS) solution (Branch *et al.,* 1972). The tetramer dissociation of TTR has been suggested to occur at pH 7.0 or at nearly physiological ionic strength upon dilution in the submolecular range (Quintas *et al*., 1999). In the presence of 1% SDS and 10 mM β-mercaptoethanol, at least 80 % of human TTR tetramer was intact after boiling for less than 20s (Bellovino *et al.,* 1998).

2.3 TTR amyloidosis

2.3.1 General characteristics of TTR amyloidosis

TTR amyloidosis is a systemic amyloidosis which is a vast autosomal dominant hereditary disease. The mutation leads to change in conformation and extracellular deposition of the TTR subunits in tissue as amyloid (Jacobson *et al*., 1997). It is believed that TTR fibrillation is a multi-steps process which primarily involves the dissociation of the tetramer into mis-folding monomers that converted into an insoluble amyloid (Morgado *et al*., 2008). TTR amyloid is a predominantly cause of senile systemic amyloidosis (SSA), familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC). SSA is a disease of the elderly in which mainly found in approximately 25% of the people with an over 80 years old (Sorgjerd *et al*., 2008).

Up to date, over 80 TTR mutants have been identified related to FAP and FAC (Table 2), and deposition of the amyloid fibrils found in various tissues including connective tissue, peripheral nervous system, heart, and vitreous body of the eye (Sorgjerd *et al*., 2008). In FAP, TTR amyloid mostly deposits in peripheral nerve and the heart resulted in neuropathy and cardiomyopathy, respectively (Lindgren *et al*., 2005). TTR amyloidosis is an autosomal dominant and adult onset resulting to death within 10 years on average after the first symptoms (Said and Plante-Bordeneuve, 2009).

The frequency and clinical manifestations including age of onset, sex ratio and duration of the disease of the TTR amyloidosis are varied among populations. V30M is the most frequent variant (Saraiva, 1984, 2001), vastly found in Portuguese and Swedish. L55P and V122I are major causes of FAC found in Taiwanese and African Americans (Jacobson *et al*., 1992; Yamamoto *et al*., 1994). In addition, other 20 different mutated TTRs have been reported in Japanese (Andersson, 1970; Araki, 1984; Ikeda et al., 2002) and German (Maria *et al*., 1991). Among these mutated TTRs, L55P is highly susceptible to amyloid formation (Maria *et al*., 1996).

2.3.2 TTR variants detected in Thai patients

Recently, a few novel TTR variants have been identified in Thai people. These include L110P, Y116N and V122D (Tayeh, 2007; Srinuan, 2008). Based on the mutation positions have been reported previously (Misrahi *et al*., 1998; Saraiva *et al*., 1990; Uemichi *et al*., 1995; Munar Qués *et al*., 2000; Theberge *et al*., 1999), L110P and Y116N have been proposed to associate with FAP, whereas, V122D was expected relating to FAC. To reveal its amyloidogenicity and to explore a specific treatment, their fibrillation, cytotoxicity, pathogenesis, and underlying mechanism of these variants are need to be elucidated. Since TTR amyloid is an autosomal trait, both normal TTR and its variant genes are simultaneously expressed. Therefore, it is difficult to obtain TTR variant directly from serum or plasma.

Table 1.2 Transthyretin mutations in amyloidoses

Mutation	Codon change		Predominant Clinical feature	Origin
Cys10Arg	TGT	CGT	PN, AN, Eye	Hungary
Leu12Pro	CTG	CCG	LM, PN, AN	UK
Asp18Glu	GAT	GAG	PN, AN	Columbia
Asp18Gly	GAT	GGT	LM	Hungary
Val20Ile	GAT	ATC	Heart	Germany
Ser23Asn	AGT	AAT	Heart	Portugal
Pro24Ser	CCT	TCT	Heart, CTS, PN	USA
Val28Met	GTG	ATG	PN, AN	Portugal
Val30Met	GTG	ATG	PN, AN, Eye	Several
Val30Ala	GTG	GCG	Heart, AN	Germany
Val30Leu	GTG	CTG	PN, AN	Japan
Val30Gly	GTG	GGG	LM, Eye	France
Phe33Ile	TTC	ATC	PN, Eye	Poland
Phe33Leu	TTC	CTC	PN, AN	Poland
Phe33Val	TTC	GTC	PN, AN	UK
Arg34Thr	AGA	ACA	PN, Heart	Italy
Lys35Asn	AAG	AAC	PN, AN, Heart	France
Ala36Pro	GCT	CCT	PN, Eye	Greece
Asp38Ala	GAT	GCT	PN, Heart	Japan
Glu42Gly	GAG	GGG	PN, AN	Japan
Glu42Asp	GAG	GAT	Heart	France
Phe44Ser	TTT	TCT	PN, AN, Heart	Ireland
Ala45Asp	GCC	GAC	Heart	Italy
Ala45Ser	GCC	UCC	Heart	Sweden
Ala45Thr	GCC	ACC	Heart	Italy
Gly47Arg	GGG	CGG	PN, AN	Japan
Gly47Ala	GGG	GCG	Heart, PN, AN	Italy
Gly47Glu	GGG	GAG	PN	Germany
Thr49Ala	ACC	GCC	Heart, PN	Italy

Table 1.2 (continued)

Mutation	Codon change		Predominant Clinical feature	Origin
Thr49Ile	ACC	ATC	PN, Heart	Japan
Ser50Arg	AGT	AGG	PN, AN	Japan
Ser50Ile	AGT	ATT	Heart, PN, AN	Japan
Glu51Gly	GAG	GGG	Heart	USA
Ser52Pro	TCT	CCT	PN, AN, Heart	UK
Gly53Glu	GGA	GAA	LM, Heart	France
Glu54Gly	GAG	GGG	PN, AN	UK
Glu54Lys	GAG	GAA	PN, AN, Heart	Japan
Leu55Arg	CTG	CGG	LM, PN	Germany
Leu55Pro	CTG	CCG	PN, heart, AN	Taiwan
His56Arg	CAT	CGT	Heart	USA
Leu58His	CTC	CAC	CTS, Heart	Germany
Leu58Arg	CTC	CGC	CTS, AN, Eye	Japan
Thr59Lys	ACA	AAA	Heart, PN	Italy
Thr60Ala	ACT	GCT	Heart, CTS	Ireland
Glu61Lys	GAG	AAG	PN	Japan
Phe64Leu	TTT	CTT	PN, CTS, Heart	Italy
Phe64Ser	TTT	TCT	LM, PN, Eye	Italy
Ile68Leu	ATA	TTA	Heart	Germany
Tyr69His	TAC	CAC	Eye	Scotland
Lys70Asn	AAA	AAC	CTS, PN, Eye	Germany
Val71Ala	GTG	GCG	PN, Eye	spain
Ile73Val	ATA	GTA	PN, AN	Bangladesh
Ser77Phe	TCT	TTT	PN	France
Ser77Tyr	TCT	TAT	PN	Germany
Ile84Asn	ATC	AAC	Eye, Heart	Italy
Ile84Thr	ATC	ACC	Heart, PN, AN	Germany

Table 1.2 (continued)

Mutation	Codon change		Predominant Clinical feature	Origin
Glu89Gln	GAG	CAG	PN, Heart	Italy
Gku89Lys	GAG	AAG	PN, Heart	USA
Ala91Ser	GCA	TCA	PN, CTS, Heart	France
Ala97Gly	GCC	GGC	Heart, PN	Japan
Ala97Ser	GCC	TCC	PN, Heart	France
Ile107Val	ATT	GTT	Heart, CTS, PN	Germany
Ile107Met	ATT	ATG	PN, Heart	Germany
Ala109Ser	GCC	TCC	PN	Japan
Leu111Met	CTG	ATG	Heart	Denmark
Ser112Ile	AGC	ATC	PN, Heart	Italy
Tyr114Cys	TAC	TGC	PN, AN, Eye	Japan
Tyr114His	TAC	CAT	CTS	Japan
Tyr116Ser	TAT	TCT	PN, CTS	France
Ala120Ser	GCT	TCT	Heart, PN, AN	Africa
Val122Ile	GTC	ATC	Heart	Africa
Val122del	GTC	Loss	Heart, PN, CTS	Equador/spain
Val122Ala	GTC	GCC	Heart, Eye, PN	UK

AN, autonomic neuropathy; CTS, carpal tunnel syndrome; Eye, vitreous deposition; PN, peripheral neuropathy; LM, leptomenigeal amyloid; Heart; cardiomyopathy. (Source: Saraiva, 2001)

2.4 Mechanism of TTR amyloid formation

The formation and deposition of protein fibrils including TTR is a common feature of amyloid diseases. The basic mechanism of the fibril assembly is of significant interest. Several pathways have been hypothesized for the TTR amyloid formation. Nevertheless, the exact underlying mechanism is still unclear. Dissociation and reassembly of TTR subunits are proposed highly important in the fibril forming process. The amyloidogenic potential of TTR has been proposed associated with an extensive β-sheet structure of its subunit (Blake *et al*., 1994) as TTR found in the amyloid fibril contained a large extensive of β-structure conformation (Damas *et al*., 1995). The X-ray diffraction of the crystal structure of amyloidogenic Leu55Pro revealed monomers as the building blocks of the oligomer of the amyloid TTR (Sebastiao, 1998). The amyloidogenic potential is also related to decrease in the tetrameric stability of TTR. It has been proposed that triggering of the amyloid fibril formation of TTR mutants by dissociation of the tetramer to a compact non-native monomer with low conformational stability, which resulted in the unfolded monomers that self-assembly to an originate non-fibrillar TTR aggregates, then protofibrils and finally mature amyloid fibrils (Quintas *et al*., 2001) (Figure 2.3).

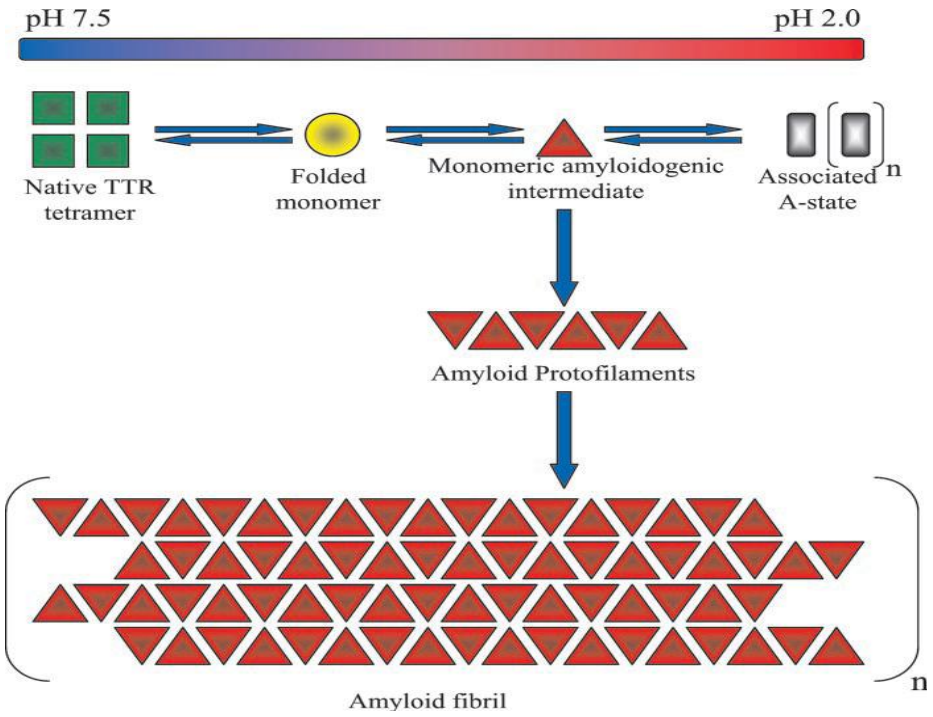


Figure 1.3 Acid-induced denaturation fibrillogenesis pathway of TTR

(Source: Avijit, 2001)

3. Analysis of the fibril formation

According to the fact that aggregation and deposition of amyloid proteins are the main cause of diseases such as Alzheimer's disease (AD) and TTR amyloidosis (see for review Sousa and Sariana, 2003). Understanding in the amyloid formation process is essential for both prognosis and therapeutics of the diseases. However, protein aggregation and amyloid formation *in vivo* and *in vitro* require specific techniques to obtain correct information so that development of diagnostic tools and assays for drug screening could be carried on. Several techniques such as fluorescence spectroscopy (Lindgren *et al*., 2005) have been developed. Among these developed techniques, the histological dyes in particular Congo red and Thioflavin T (ThT) (Bolder *et al*., 2007) are commonly used. These dyes are effective and always have been used as indicators for the presence of amyloid in tissues (Eisert *et al*., 2006; for review see Reinke and Gestwicki, 2011).

3.1 Thioflavin T based assay

ThT is a cationic benzothiazole dye that shows enhanced fluorescence upon binding to amyloid fibrils particular in tissue sections (Vassar and Culling, 1959; Khurana *et al*., 2005). It is wildly used to investigate the presence and evaluate kinetics of amyloid. ThT interacts equally with amyloids either in β-sheet-rich or non-β-sheet (Groenning *et al*., 2007), however, do not interact with proteins either in native form or completely unfolded or partially folded denatured state of the melted globule or amorphous aggregates (Levine, 1995; Vorapai *et al*., 2003; Groenning *et al*., 2007). Although it is known that common structural characters of amyloid fibrils are important for the ThT binding, the precise mechanism of the binding has not completely understood (for review see Reinke and Gestwicki, 2011).

The determination under confocal microscopy, the dipole excitation axis of ThT was found lied parallel to its long molecular axis, thus, binding of the dye was proposed occur in the channel that run on β-sheet of the amyloid fibrils (Krebs *et al*., 2005). The micelles of ThT that formed in an aqueous solution was claimed to have a hydrogen bonding to amyloid fibrils, leading to enhancement of fluorescence emission of ThT (Khurana *et al*., 2005).

3.2 Congo red based assay

Congo red (CR) is a histological dye that displays green birefringence under polarized light when bound to amyloid in tissue sections (Divry, 1927; Khurana *et al*., 2001; for review see Reinke and Gestwicki, 2011). In the spectral shift assay, bound CR showed the maximum absorbance shift to the red wavelength, e.g. from 488 nm to 514 nm when bound to Aβ(1-40) (Klunk *et al*., 1999). It was proposed that β-sheet structure of amyloid fibrils participate in changes of the tinctorial and polarization properties of CR (Glenner *et al*., 1972).

Several mechanisms of the CR binding to amyloid fibrils have been hypothesized. With cellulose, CR bound to hydroxyl group of the linear polysaccharide component of amyloid fibril via hydrogen bonding (Puchtler *et al*., 1962). On the contrary, it was entrapped by non-specific close-rang forces in channels of the beta-pleated sheet of Aβ fibrils (Cooper, 1974; Klunk *et al*., 1989). It was also hypothesized that CR intercalates two molecules of protein and to bring to oligomerization of the proteins (Khurana *et al*., 2001).

4. Heterologous gene expression system of *Pichia pastoris*

P. pastoris can be easily growth to a very high cell density using defined minimal medium and is able to introduce eukaryotic posttranslational modifications, which are similar of higher eukaryotes. *P. pastoris* has a strong inducible promoter that can be used for protein production. Distribution and chain length of N-linked oligosaccharides are different to those of *S. cerevisiae*, namely the chain length is significantly shorter, making it an interesting alternative for the extracellular expression of human proteins (for review see Boettner *et al*., 2002). More than 100 foreign proteins from bacteria, fungi, plants, invertebrates and vertebrates includes humans have been expressed in *P. pastoris*. These heterologous gene expressions include TTRs from human and vertebrates (Prapunpoj *et al*., 2002; 2006).

P. pastoris is capable of metabolizing methanol; however, the methanol concentration in the culture must be kept within a relatively narrow range. Accumulation of methanol can lead to cytotoxic effects and the induction of the promoter is highest when the methanol level is kept to growth at limiting rates

(Boettner et al., 2002). It can grow in many carbon sources such as glucose and glycerol. To metabolite methanol, the production of alcohol oxidase (AOX) is induced. In the first step of methanol utilization is an oxidation of methanol to formaldehyde and hydrogen peroxide which is catalyzed by enzyme AOX in peroxisome. The hydrogen peroxide that is generated will then be degraded to by enzyme catalase. AOX has poor affinity to oxygen, thus yeast need to synthesize for a large amount of the enzyme to overcome the low specific activity of the enzyme (Couderc and Barratti,1980; Giuseppin *et al*., 1988). The promoter for AOX gene is used a strong inducible promoter in the expression system of *P. pastoris* and codes for two alcohol oxidase genes, *AOX1* and *AOX2*. In comparison, *AOX1* gene is responsible for 85% of AOX activity in the yeast cell and is the promoter that has been used to drive heterologous gene expression in *P. pastoris*. The expression of the *AOX1* gene is highly regulated by the *AOX1* promoter (Liu *et al*., 2006). The regulation of *AOX1* gene appears to involve with two mechanisms, the repression and de-repression mechanism and the induction mechanism. Most genes of interest is an expressed under control of the *AOX1* promoter. The heterologous gene expressed in *P. pastoris* can remain intracellular after their synthesis or they can be secreted into the medium outside. However, secretion of foreign protein is more popular because it facilitate for downstream processing in particular purification of the foreign protein product from other proteins and contaminants from the yeast cell. *Pichia* strain GS115 (*his4*) is the most commonly used as host for the heterologous gene expression. This wild-type strain contains the *AOX1* and *AOX2* gene, however, it grows on methanol at wild-type rate (Mut^+) because its growth on methanol mainly relies on the presence of the *AOX1* gene.

Objectives

To elucidate the association of the novel TTR variants detected in Thai people to amyloidosis by characterize and reveal the mechanism underlying the fibrillation of these TTRs.

CHAPTER 2

MATERIALS AND METHODS

1 Meterials

1.1 Instruments

Instruments	Model	Company
Autoclave	ES-315	Tomy
Balance (4 digits)	AB204-S	Mettler
Balance (2 digits)	PG5002-S	Mettler
Centrifuge	Anti J-30 I	Beckman
Centrifuge	J2-21	Beckman
Centrifuge	5804R	Eppendorf
Centrifuge	Harrier 18/8 (MSE)	SANYO
Digital Incubator	WIG-32	WiseCube
Fraction collector	2110	Bio-Rad
Fraction collector	Frac-920	HiTrap
Gel Document (Lab works 4.0)	C-80	UVP
Horizontal Electrophoresis	B1	Owl Scientific
Incubator		Memmert
Microcentrifuge	260D	DENVILLE
Micropipette		Gilson, Labnet, Labmate, Nichipet EX
Oven	240 litre	Binder
Orbital shaking incubator		GallenKamp SANYO
Orbital shaker	SH 30	FINEPCR
Orbital shaker	MS-OR	Major Science

Instruments	Model	Company
Orbital shaker	MS-OR	Major Science
pH meter	713	Metrohm
Power supply	ELITE 300 plus	Wealtec
Power supply	MP-300N	Major science
	MP-300V	
	MP-500V	
Power supply	PowerPac 3000	Bio-Rad
Precise shacking incubator	WLS-10R	WiseCube
Preparative gel electrophoresis	PrepCell 491	Bio-Rad
Slab gel electrophoresis	AE-6450	ATTO
	Mini Protean 3 cell	Bio-Rad
	Mini Protean-II	Bio-Rad
Spectrofluorometer	RF-1501	Shimadzu
Spectrophotometer	8453	Hewlett-Packard
Spectrophotometer	G20	Thermo
Stirrer	MS-20A	Wise stir
Vortex-mixer	VX 100	Labnet
Water bath	WB-710M	Optima

1.1 Chemicals

1.1.1 Analytical grade

Chemical	Company
Absolute ethanol	Normapur
Acetic acid	Lab Scan
Acrylamide	Fluka
Agar	Merck

Chemical	Company
Argento nitrate (Silver Nitrate)	Merck
Biotin	Fluka
Bis-acrylamide	Fluka
Boric acid	Merck
Bovine serum albumin	Sigma
Calcium chloride	Merck
Coomassie brilliant blue G-250	Bio-Rad
Coomassie brilliant blue R-250	Bio-Rad
D-glucose	Univar
Di-potassium hydrogen phosphate	J.T. Baker
Dithiothreitol	Bio-Rad
Ethylene diamine tetraacetic acid (EDTA)	Carlo
Glycerol	Univar
Glycine	Fisher
Methanol	Lab Scan
Peptone	Merck
Potassium dihydrogen phosphate	Fisher
Sodium carbonate	Merck
Sodium chloride	Lab Scan
Sodium dodecyl sulfate (SDS)	Finechem
Sorbitol	Sigma
Tris (Hydoxymethyl)- methylamine	USB
Tryptone	Merck
Yeast extract	Merck
Yeast nitrogen base	Difco

1.2 Yeast and Bacterial cells

Pichia pastoris stain GS115 and *E.coli* DH5α cells were gifted from Professor Schreiber, Australia. *E.coli* stain JM109 is a product of Invitrogen

2. Methods

2.1 Transformation of an expression plasmid for TTR variant into *P. pastoris*

To prepare a yeast competent cell for electroporation, a single colony of *Pichia* GS115 from fresh yeast extract peptone dextrose (YPD) agar plate was grown in 5 ml of YPD medium in a 50 ml-tube at 30°C, overnight. One hundred microliters of the overnight culture was incubated in 120 ml of YPD medium in a 1 liter-flask and grown overnight at 30°C to an OD_{600} of 1 to 1.6. Then, cells were collected by centrifugation at 2,000 rpm for 5 min at 4 °C and suspended in 45 ml of ice-cold distilled water. Cells were removed from the suspension by centrifugation, and suspended once in 22.5 ml of ice-cold distilled water. Thereafter, cells were collected and suspended in 1.8 ml of ice-cold 1 M sorbitol and finally in 0.135 ml of ice-cold sorbitol to give a final volume of approximately 0.2 ml.

Transformation of available *Sal*I linearized TTR cDNA plasmid into *Pichia* competent cells strain GS115 was carried out by an electroporation using Gene Pulser (Bio-Rad), following the protocol recommend by company. In brief, an aliquot (80 µl) of the competent *Pichia* cells was mixed with 1.5 µg of linearized plasmid, then the cell mixture was transferred to an ice-cold 0.2 cm electroporation cuvette. The electroporation was carried out at 1.5kV, 25 µF and 400 Ω, generating pulse lengths ~9.4 milliseconds with a field strength ~1.37 kV/cm. Then, cell suspension was flushed with 1 ml of ice-cold 1 M sorbitol, and all of the mixture was spread onto the minimal dextrose medium (MD) agar plate and incubated at 30°C for 3 days.

2.1.1 Screening for Mut^+ transformants

Pichia transformants with phenotype His^+Mut^+ (histidine synthesis and methanol utilization plus) were separated from His^+Mut^s (histidine synthesis and methanol utilization slow) by growing on minimal dextrose medium (MD) and minimal methanol medium (MM) agar plates. A colony of His^+ transformant from a MD plate was picked with a sterile toothpick and patch onto MM, then onto MD plate. Colony screening was performed with 50 to 100 His^+ transformants. Plate was incubated at 28°C for 3 days. His^+Mut^+ transformants were identified as those having a similar growth on both MD and MM plates. Mut^s

transformants, on the other hand, grow on MD plate much faster than on MM plate. Twenty to fifty colonies of His^+Mut^+ transformants were selected for TTR synthesis in small scale.

2.2 Induction for synthesis of TTR variants

2.2.1 Small scale synthesis

The synthesis of a TTR variant was first carried out in a small scale. A single colony of a *Pichia* clone on yeast extract peptone dextrose (YPD) plate was transferred into 5 ml of a buffered medium containing glycerol (BMGY). Cells were grown at 30°C in an orbital-shaking incubator (180 rpm) until OD_{600} reaches 2 to 6 (~18 h). Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature, and transferred to 5 ml of a buffered medium containing methanol (BMMY) to an OD_{600} ~1.0. To induce for the synthesis, methanol was added every 24 h to maintain its concentration in the culture to 0.5%. Induction with methanol was carried out for 3 to 5 days. Thereafter, the culture supernatant was collected by centrifugation at 5,000 rpm for 5 min and kept at -20°C until used. Proteins those secreted in the yeast culture supernatant were, later, analyzed by native-PAGE followed by silver staining.

2.2.2 Large scale synthesis

The available *P. pastoris* clone containing cDNA of TTR variant was induced to synthesize and extracellular secrete the recombinant TTR into culture medium according to the method described (Invitrogen) with modifications. In brief, a single colony of *Pichia* clone was picked from a YDP plate and inoculated into 10 ml of BMGY, then grown overnight. Thereafter, 10 ml of the overnight culture was transferred into 150 ml of BMGY in a 1-liter flask, and cells were further grown until OD_{600} reached 3 to 4. Cells were harvested by centrifugation at 2,500 rpm for 5 min at room temperature and suspended in 200 ml of BMMY in a 2-liter flask to density of 1 $OD_{600.}$ The induction with methanol was carried out at 28°C for 1 day. After the induction, the culture supernatant was collected by centrifugation at 5,000 rpm for 5 min, then was analyzed for secreted proteins by native-PAGE followed by silver staining. The culture supernatant was kept at -20°C until purification.

2.3 Purification of recombinant TTR from *Pichia* culture supernatant

Recombinant TTR was purified from the culture supernatant by preparative native-PAGE using Prep Cell model 491 (Bio-Rad) with discontinuous gel (12% of resolving gel and 4% of stacking gel). The culture supernatant was firstly concentrated by ultrafiltration, and then any pellet occurred was removed by centrifugation at 10,000 rpm for 10 min. Aliquot of clear culture supernatant was loaded onto the polyacrylamide gel tube, and proteins were electrophoresis separated at constant power (15 Watts). Protein bands were eluted with 50 nM Tris-HCl, pH 7.4 at a constant flow rate of 1 ml/min. The eluted fractions were collected and TTR in the fractions were detected by native-PAGE. TTR containing fractions were pooled, concentrated by ultrafiltration, and stored at -20°C for analysis.

2.4 Purification of human TTR from plasma

Human TTR that used as a control was purified from plasma in 2 steps, i.e. by affinity chromatography on Cibacron blue column and electrophoresis in a preparative native polyacrylamide gel tube.

In the first step, human plasma was centrifuged at 10,000 rpm for 20 min to remove any non-dissolved particles prior loading onto a Cibacron blue column, previously equilibrated with 50 mM phosphate buffer, pH 7.4 (PB), at flow rate of 10 ml/h. Unbound proteins were collected and column was recovered with 0.5 M NaCl containing PB to elute bound proteins in particular albumin. The TTR fractions were pooled and concentrated by ultrafiltration.

In the second step,the concentrated TTR fraction, eluted from Cibacron column, was loaded onto a preparative gel tube (12% resolving gel and 4% stacking gel). During the separation, protein bands were eluted with 50 mM Tris-HCl, pH 7.4, and eluting fractions were collected at flow rate of 1 ml/min (2 ml/fraction). The eluted fraction was analyzed by native-PAGE followed by silver staining. The fractions containing TTR were pooled, concentrated by ultrafiltration, and stored at -20^{o}C until use.

2.5 Determination of general properties of recombinant TTR

2.5.1 Subunit mass

Mass of TTR monomer was determined by SDS-PAGE with discontinuous buffer system of Laemmli and Favre (1973). Aliquot of purified TTR was mixed with a solution containing 2% SDS and 2.5% of β-mercaptoethanol. Then, mixture was boiled for 30 min, and analyzed by gradient (7-15%) SDS-PAGE. Protein bands were visualized by staining with Coomassie brilliant blue R-250. The relative mobilities of TTR subunits were calculated and mass of subunit was determined from a standard curve plotted between R_f and log molecular weights of standard proteins.

2.5.2 The electrophoresis mobility under native condition

The electrophoresis mobility of purified TTR was determined by native-PAGE (10% resolving gel, 4% stacking gel). After the separation, protein bands were detected by Coomassie brilliant blue R250 or by silver staining. Recombinant TTR was expected to move faster than albumin similarly to native TTR purified from human plasma.

2.6 Amyloid fibril formation

Purified TTR was diluted in 50 mM sodium acetate, pH 4.2, containing 0.4 mM KCl and 0.4 mM EDTA, to a final concentration of ~50.9 μM. The solution mixture was incubated at 37°C in darkness for 5 days. Then, the formation of TTR fibril was determined by ThT assay.

2.6.1 ThT assay

An aliquot (5 μl) of protein solution was added with 10 mM sodium phosphate buffer, pH 7. Then, 150 mM NaCl and 0.15 mM ThT solution to final concentration of 2 nM. The fluorescence spectrum was immediately measured by fluorospectrometer (Shimadzu AF 1501) using a 1.0×0.4 cm path length fluorescence cuvette. Excitation was performed at wavelength 440 nm (Lashuel et al., 2002), and emission spectrum or value was recorded.

2.7 Proteolytic activity assay

Proteolytic activity of recombinant TTR was examined using casein which is a universal substrate for protease as substrate. The reaction assay was performed following the method described by Twining (1984) with some modifications. All reagents and the reaction mixture were filter-sterilized to protect the possible protease contamination from microorganisms. Aliquot (50 μl) of a reaction mixture comprising 0.4 μg of purified TTR, 1.3% fluorescein isothiocyanate (FITC)-casein (Sigma), 0.2 M NaCl and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.8, was incubated at 37^{o}C for 12 h in the dark. Then, the reaction was terminated by adding 30 μl of 5% trichloroacetic acid (TCA). Then, it was incubated at 37°C for 30 min. An insoluble protein pellet occurred was removed by centrifugation at 10,000 rpm for 30 min. The supernatant was collected and neutralized with 500 μl of Tris-HCl, pH 8.5. The fluorescence intensity was determined using fluorospectrometer (Shimazu) at an excitation and emission wavelengths of 485 nm and 535 nm, respectively. The proteolytic activity of TTR was calculated using quinine hydrochloride as standard. One relative fluorescence unit is defined as the fluorescence generates from 6 nM of quinine hydrochloride at excitation and emission wavelengths of 350 nm and 450 nm, respectively.

2.8 Detection of protein in polyacrylamide gel

2.8.1 by Coomassie blue staining

After electrophoresis, gel was placed for 2 h in a solution of 0.2% Coomassie brilliant blue R-250, 50% methanol and 10% acetic acid. Then, excess dye was removed by destaining with a solution of 50% methanol and 7.5% acetic acid. For storage, gel was kept in a solution of 5% methanol and 7.5% acetic acid.

2.8.2 by silver staining

Staining of protein with silver nitrate was performed as described by Morrissey, 1981. After electrophoresis, gel was placed in a solution of 10% acetic acid and 45% methanol for 30 min, and then transferred to a solution of 7.5% acetic acid and 5% methanol for 30 min. Thereafter, gel was rinsed off with distilled water for 3-4 changes prior soaking gel in a solution of DTT (500 μg/ml) for 30 min, and

then in 0.1% silver nitrate for 20 min. To develop color, gel was soaked in a solution of 3% (v/v) sodium carbonate and 0.02% (v/v) formaldehyde. The color development was terminated with 50% acetic acid.

2.9 Determination of protein concentration

Concentration of protein was determined by Bradford assay (Bradford, 1976). The reaction mixture comprised 0.1 ml of protein solution and 1.0 ml of the assay reagent (0.085 mg/ml Coomassie blue G-250, 5% methanol, and 5.06% H_3PO_4). The complex between protein and dye was allowed to form at room temperature for 2 min prior the optical density at 595 nm of the mixture was measured. Bovine serum albumin (BSA) at 0, 5, 10, 15, 20, and 30 µg were included as standard. A standard curve was plotted and used to determine concentrations of unknown proteins.

2.10 Western blot analysis

Proteins were separated by SDS-PAGE in duplicate. Then, proteins were electrophoretically transferred to a nitrocellulose membrane (Hybond-ECL,Amersham Phamacia Biotech) using a vertical tank electrophoresis blotting system (Bio-Rad) at 100 volts for 1 h, with cooling system. The transferred buffer was 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. Specific proteins were detected by immunochemistry (Kuno and Kihara, 1967) using enhanced chemiluminescence detection. The membrane was first incubated in blocking solution containing 5% skim milk, 25 mM Tris, pH 7.4, 0.15 M NaCl and 0.1% Tween 20 (TBS-T) at room temperature for 1 h with gentle shaking. Thereafter, the membrane was incubated with a primary antibody at an appropriate dilution in blocking solution at room temperature for 1 h. Excess antibody was removed by washing with TBS-T at room temperature for 15 min followed by 5 min for another three times. Thereafter, the membrane was incubated with a secondary antibody at room temperature for 1 h prior washing with TBS-T, 15 min once, and then 5 min for three times. The specific protein was detected by enhance chemiluminescence (ECL, GE Healthcare) and the fluorescence signal was detected by Bio-Chemi System (UVP).

CHAPTER 3

RESULTS

3.1 Construction of a plasmid of TTR V122D for expression in *P. pastoris*

To prepare a *P. pastoris* clone for TTR V122D, the TTR cDNA was firstly constructed by site-direct mutagenesis using the cDNA of human wild type TTR in pPIC 3.5 as template as described in Materials and Methods. The specific mutagenic primer that used to generate cDNA of the TTR variants was 5'ACGGTCGTCGACACCAATCCC3' The mutant strand was synthesized and two ends of strand were joined together with DNA ligase prior transformation of the hetero-duplex DNA strands into the *E. coli* BMH 71-18. Then, the target plasmid was purified and transformed into *E.coli* DH5α. The recombinant plasmid of V122D was isolated and digested with BamHI and EcoRI. A single band of the DNA fragment migrated on agarose gel to the position corresponding to approximate 400 base pairs was observed as shown in Figure 3.1. This is the same size as the fragment of TTR cDNA reported previously (Kaewmeechai, 2007). It confirmed that the insertion of the cDNA fragment into the plasmid occurred at the correct positions, and there was no mutation to the recognition sequences for BamHI and EcoRI on the plasmid.

3.2 Screening for Mut^+ transformants

The *P. pastoris* GS115 that used as host strain for the heterologous expression of V122D gene contains *AOX*1 promoter for driveing the expression of the target protein. This yeast strain was mutated in the histidine dehydrogenase gene (*HIS4*), therefore, it can use methanol but requires supplementation with histidine for growth (genotype *his4*, phenotype Mut^+/His^-) (Cregg *et al.*, 1985). By transformation, however, the yeast strain with methanol utilization slow or Mut^s phenotype that exhibit poor growth on methanol media due to the absence of alcohol oxidase activity can also generated. This mutated transformant can be differentiate from the wild type (Mut^+ or methanol untilization plus) transformant by comparing their growth on minimal methanol medium (MM) and minimum dextrose medium (MD). The

His^+Mut^+ clones of V122D were screened from ~115 His^+ transformants. The different growth pattern on MD and MM of those His^+Mut^+ and His^+Mut^s was shown in Figure 3.2. Approximate 35% of the screened His^+ transformants were Mut^+. Up to fifty colonies of His^+Mut^+ transformants were selected for the synthesis of V122D in small scale.

3.3 Induction for synthesis of TTR V122D

3.3.1 Small scale synthesis

The synthesis of a TTR V122D was first carried out in a small scale. A single colony of the *Pichia* clone was collected from a yeast extract peptone dextrose (YPD) plate (Figure 3.3), and inoculated into 5 ml of BMGY. Cells were grown at 30°C, overnight. Then, cells were removed from the culture medium by centrifugation and transferred to 5 ml of BMMY. The induction for synthesis was carried out with methanol at the final concentration of 0.5%, for 4 days. The analysis of the culture supernatant by native-PAGE followed by silver staining showed a single protein band migrated on the polyacrylamide gel faster than albumin in human plasma (Figure 3.4). Similar pattern was observed in every selected His^+Mut^+ clones. In addition, intensity of the protein band was not significantly different among clones. Therefore, only one clone was selected for further synthesis in large scale to obtain sufficient amount of the TTR for other analysis.

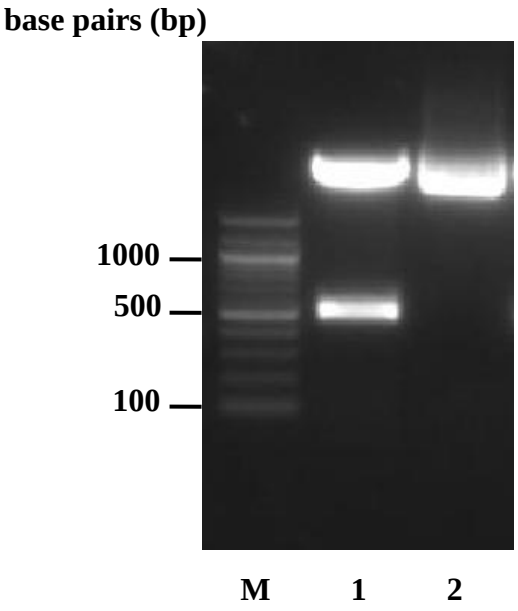


Figure 3.1 Analysis of the V122D TTR cDNA in a plasmid

The recombinant plasmid containing V122D TTR cDNA was digested with *Bam*HI and *Eco*RI prior separation on 1% agarose gel containing ethidium bromide along with the DNA markers (M). DNA bands were visualized under UV. 1, the plasmid after digested with *Bam*HI and *Eco*RI; 2, uncut plasmid. The V122D TTR cDNA with 400 bp in size is indicated by arrow.

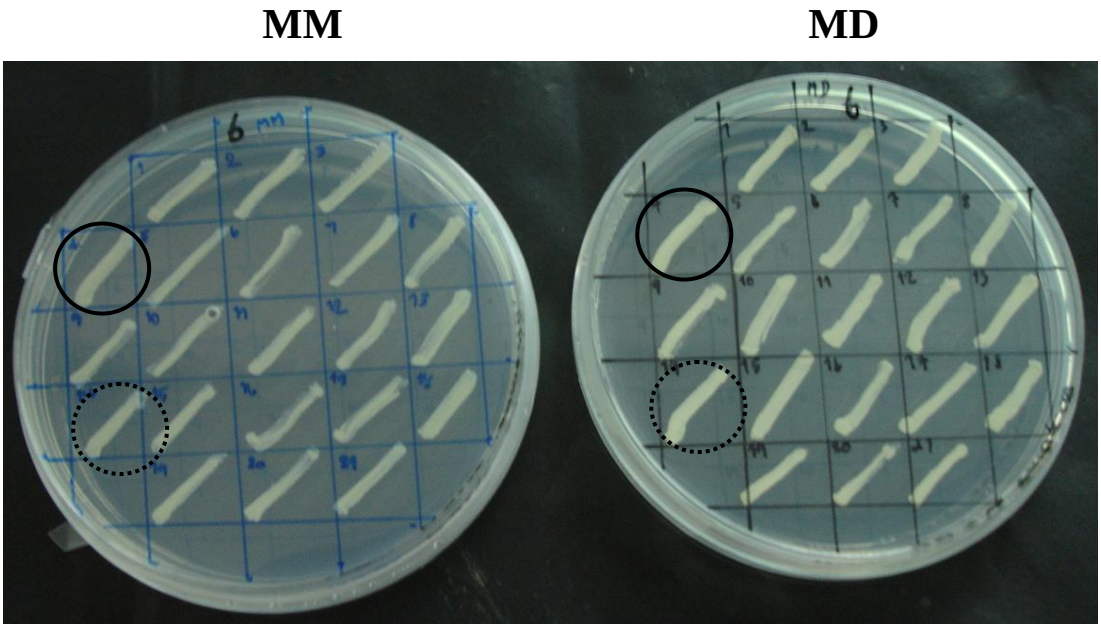


Figure 3.2 **The screening for His$^+$ Mut$^+$ transformants on MD and MM plates**
The *Pichia* transformants with His$^+$ Mut$^+$ or His$^+$ Mut^s phenotype were identified by compared their growth on the MM and MD. His$^+$ Mut$^+$ shows similar growth on both media, whereas, His$^+$ Mut^s has more growth in MD than in MM. Close and broken circle indicate His$^+$ Mut$^+$ and His$^+$ Mut^s phenotype, respectively.

3.3.2 Large scale synthesis

To produce for higher amount, the synthesis of V122D clone was performed using the shake-flask system as described in Materials and Methods. All conditions including temperature, percentage of methanol and speed of shaking were carried out as same as in small scale. Aliquot of the culture supernatant was collected every 24 h. Only a single protein band still was observed when the culture supernatant was analyzed by native-PAGE (Figure 3.5). The intensity of this increased with increase of the induction period. The production of V122D after the induction for 2 days was not significantly different from the induction for 3 days, indicated to the maximum production at two days induction. Since the production after 1 and 2 days of the induction was not much different, to prevent any degradation might occur, 1 day induction was later performed. The result also showed that no degradation of the synthesized proteins was significantly observed. This should indicate to high stability of the tetrameric structure of V122D to the temperature at 30^{o}C.

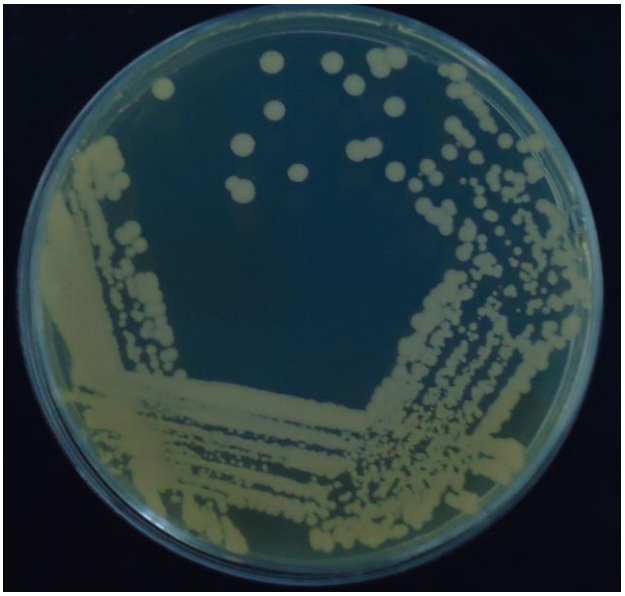


Figure 3.3 The isolated colonies of *P. pastoris* on YPD plate

The *Pichia* clone was grown on YPD agar. The isolated single colonies were obtained after growing at 30°C for 3 days.

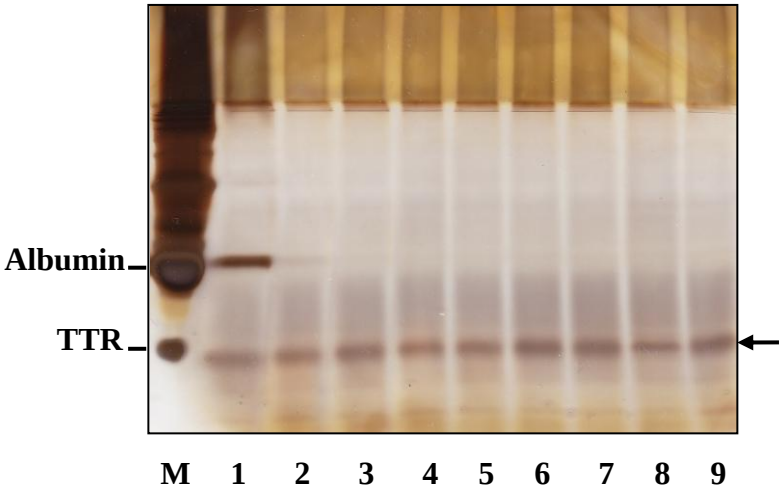


Figure 3.4 The expression of *Pichia* transformant

The *Pichia* clone was induced to synthesize V122D with 0.5% for 4 days in 5 ml of BMMY. An aliquot of the culture supernatant was analyzed by native-PAGE, followed by silver staining. M, human plasma (3 µl); 1 to 9, individual *Pichia* transformants of V122D; arrow, position of TTR in the culture. Positions of albumin and TTR in plasma were indicated.

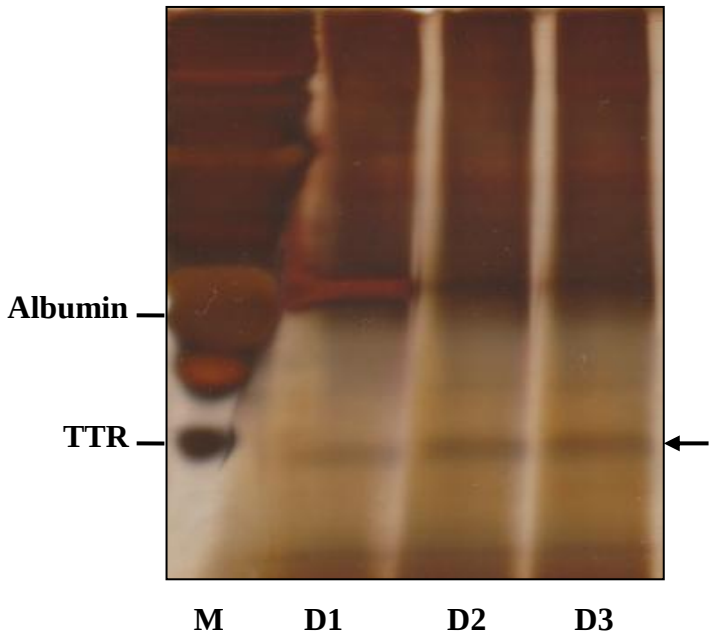


Figure 3.5 Kinetic expression of *Pichia* transformant of V122D

The *Pichia* transformant was grown and induced with 0.5% methanol in BMMY for 3 days. Aliquot of the culture was collected and cells were removed out by centrifugation, and clear supernatant (50 µl) was analyzed by native-PAGE on 10% resolving gel and 4%stacking gel. The protein bands were visualized by silver staining. M, human plasma (3 µl) that overloaded to indicate the positions of albumin and TTR in plasma; arrow, position of TTR in the culture; D1 to D3, day of the induction with methanol.

3.4 Purification of recombinant TTR from *Pichia* culture supernatant

Recombinant TTR was purified from the culture supernatant by preparative native-PAGE using Prep Cell model 491 (Bio-Rad) with discontinuous gel (12% of resolving gel and 4% of stacking gel). The culture supernatant was firstly concentrated by ultrafiltration, then, any pellet occurred was removed by centrifugation at 10,000 rpm for 10 min prior separation by electrophoresis. Proteins were eluted with 50 nM Tris-HCl, pH 7.4 with a constant flow rate at 1 ml/min. The analysis of the eluted fractions by native-PAGE, followed by staining gel with silver nitrate, showed a single intense protein band which moved on the polyacrylamide gel faster than albumin to the similar position to TTR in human plasma (Figure 3.6). This indicated to an effective separation method of the preparative native-PAGE for isolation of recombinant TTR, and indicated there was no unfavorable modification in the recombinant TTR synthesized by *P. pastoris*.

3.5 Purification of human TTR from plasma

Human TTR that used as a control in the experiments was purified from plasma in 2 steps by affinity chromatography on Cibacron blue column and electrophoresis in a preparative native polyacrylamide gel tube. In the first step, albumin which a major protein in plasma bound to the Cibacron resin, while other proteins including TTR passed through the column and came out in unbound fractions (data not shown). In the second step, TTR was isolated from other plasma proteins according to its typical electrophoresis characteristic, i.e. migrates fastest to the anode. The analysis by native-PAGE revealed a single protein band moved faster than albumin to the same position as TTR in human plasma (Figure 3.7). This confirmed the efficacy of the preparative native-PAGE method for purifying TTR in human plasma.

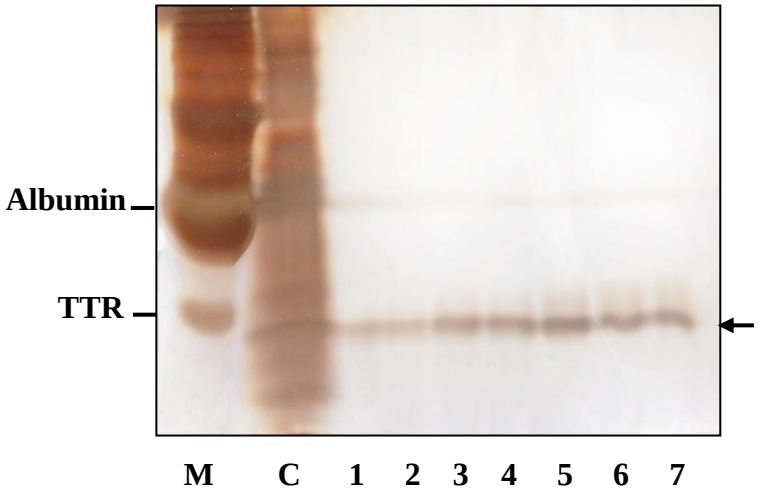


Figure 3.6 **The elution profile of recombinant V122D from preparative native-PAGE**

The *Pichia* clone was induced for the synthesis of V122D for 3 days. Then, the culture supernatant was concentrated and loaded onto a polyacrylamide gel tube (12% resolving gel, 4% stacking gel), and the protein was separated by electrophoresis. The eluting fractions (1 ml/fraction) were collected and 50 µl of each fraction was analyzed by native-PAGE followed by silver staining. M, human plasma (3 µl); C, concentrated culture supernatant; 1 to 7, individual eluting fractions.

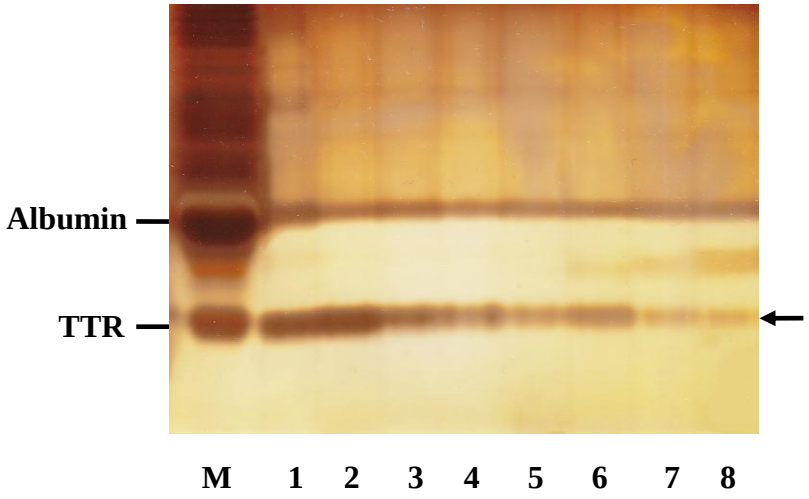


Figure 3.7 The eluting pattern of human TTR from the preparative native-PAGE

The human TTR fractions eluted from the Cibacron blue column were pooled and concentrated prior to load onto a native polyacrylamide gel (12% resolving gel and 4% stacking gel). The electrophoresis was performed using a Prep Cell model 491. Eluting fractions (2 ml/fraction) were collected, and 30 µl of each were subjected to native-PAGE followed by silver staining. M, human plasma (3 µl); 1 to 8, individual eluting fractions.

3.6 Determination of the physicochemical properties of recombinant TTR

The heterologous gene expression system of *P. pastoris* has more advantages than prokaryotic expression system as it has most post-translational modifications particularly glycosylation that are necessary for proper folding of the proteins from higher eukaryotes. To confirm that the recombinant TTR produced by *Pichia* system has a proper tertiary structure and folding, in addition, forming into a tetramer, its physicochemical properties were examined.

3.6.1 The electrophoresis mobility in SDS-PAGE

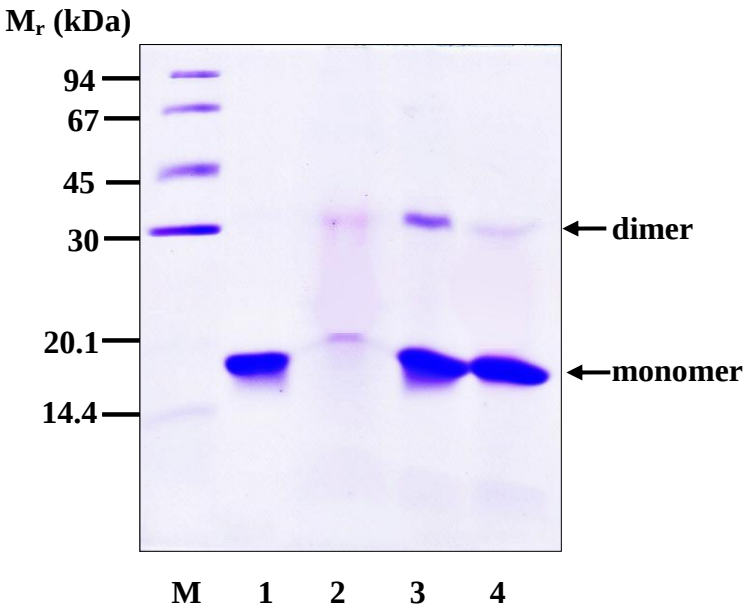
Mass of the TTR monomer was determined by SDS-PAGE with discontinuous buffer system. The protein mixture was boiled for 30 min prior analysis on a gradient 12% SDS-polyacrylamide gel. Protein bands were visualized by staining with Coomassie brilliant blue R-250 as described in the section 2.5.1. The mobility on gel of each TTR subunit was shown in Figure 3.8A. A major protein band moved to position corresponded to TTR monomer was detected. An additional band that less intensity and migrated slower to the position that corresponded to MW ~30 kDa, the same as TTR dimer, was also observed. The dimer of TTR usually occurs when denaturing of the protein is not complete even with hash condition (Furuya *et al*., 1989 and Prapunpoj *et al*., 2002). The subunit masses of human TTR, V122D, V30M and L55P determined from the calibration curve that plotted between R_f and log molecular weights of standard proteins, were 16.2, 16.9, 15.9 and 15.7 kDa, respectively (Figure 3.8B). These were similar to that previous reported for TTR purified from human plasma.

3.6.2 The electrophoresis mobility under native condition

It reported that human TTR in serum and plasma migrated faster albumin under native condition, at pH ~8. In this thesis, the electrophoresis mobilities of the purified TTRs under non-denaturing condition were determined and compared. After the separation, protein bands were detected by Coomassie brilliant blue R250. All TTR variants showed a single band on gel (Figure 3.9). In comparison to albumin in plasma, these TTRs moved anodal ahead. However, they were at the similar position to the human native TTR, except V122D migrated slightly slower The

obtained result indicated that the recombinant TTRs produced by *P. pastoris* had the similar molecular folding to human TTR in plasma.

A


Mr (kDa)
94
67
45
30
20.1
14.4
dimer
monomer
M
1
2
3
4

B

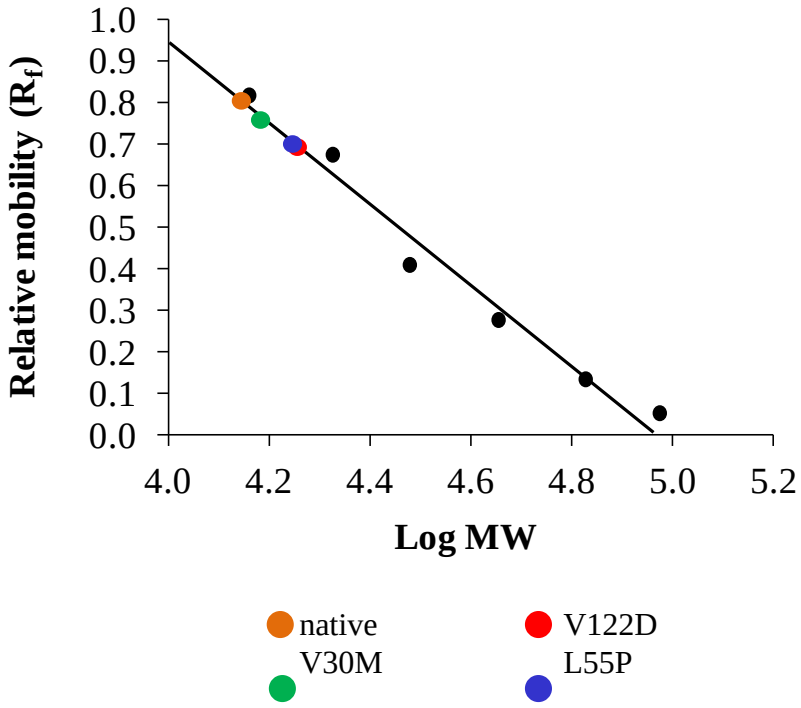

Relative mobility (Rf)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
4.0
4.2
4.4
4.6
4.8
5.0
5.2
Log MW
native
V30M
V122D
L55P

Figure 3.8 Determination of the subunit weight of V122D by SDS-PAGE

(A) The migration of purified V122D on SDS-polyacrylamide under denaturing condition (2) in comparing with that of human TTR (1), V30M (3) and L55P (4). Aliquot (0.2 μg) of purified V122D was loaded onto the gel, and separation was carried out at a constant voltage. The proteins were visualized by staining with Coomassie blue. Positions of monomer and dimer of TTRs are indicated. M, standard protein markers.

(B) A plot between the relative mobility (Rf) and log of molecular weight (log MW) of the protein markers was performed. This calibration curve was used in determining the subunit weight of TTRs. The relative mobilities of human TTR, V122D, V30M and L55P were indicated.

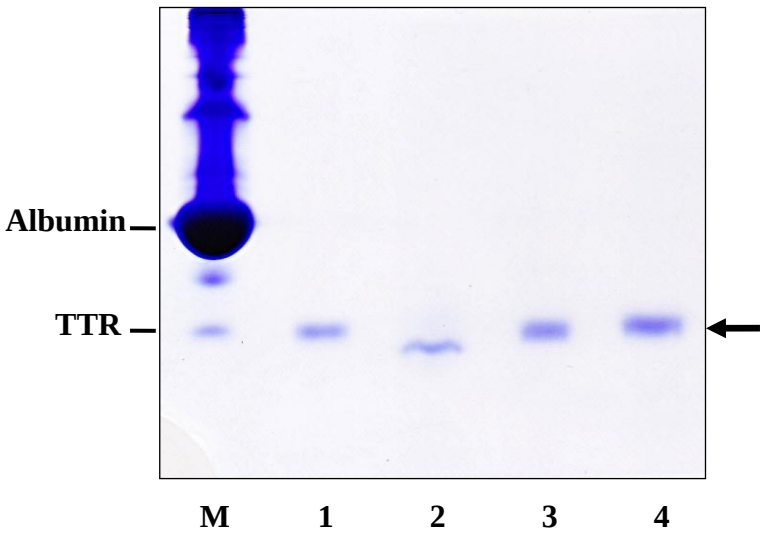


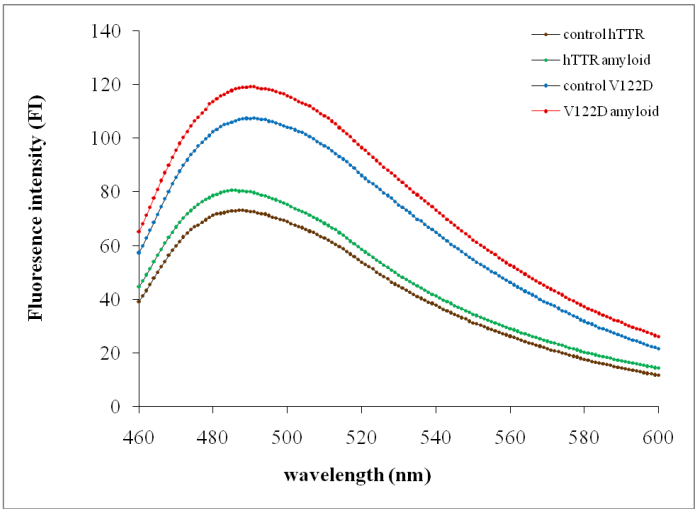
Figure 3.9 **The mobilities of recombinant TTRs on native-polyacrylamide gel**

Human TTR purified from plasma (1), recombinant V122D (2), V30M (3), and L55P (4) were separated by PAGE under non-denaturing condition on 10% resolving and 4% stacking gels. Protein bands were visualized by Coomassie blue staining. M, human plasma that was overloaded to indicate the positions of TTR (TTR) and albumin in plasma.

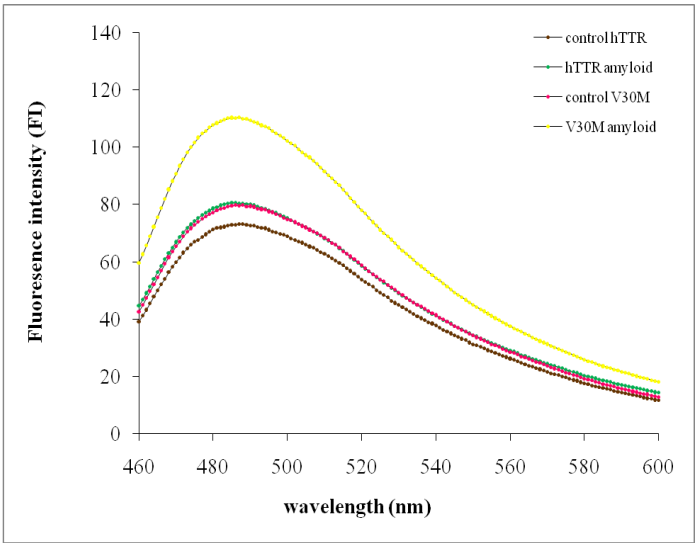
3.7 Amyloid fibril formation

Purified TTR at the final concentration of 6.35 μM was induced to form fibril by an acidic condition as described in section 2.6. The fibrillar induction was carried out at 37°C in darkness. Then, TTR fibril was determined by ThT assay. The reaction mixture was excited at 440 nm, and fluorescence intensity was record from 460-600 nm. The fluorescence spectra of V122D, V30M, and L55P were shown in Figure 3.10 in compared with that of human TTR. It showed that the emission spectra of TTR variants had higher intensity, and percentage of the fibrillation of human TTR, V30M, L55P, and V122D were 9.9, 38.6, 8.4 and 10.8, respectively (Table 3.1). In comparison, the fibril formation of V122D, V30M and L55P were 1.2, 4.3, and 0.9 folds to that of human TTR.

A


control hTTR
hTTR amyloid
control V122D
V122D amyloid
Fluoresence intensity (FI)
wavelength (nm)
0
20
40
60
80
100
120
140
460
480
500
520
540
560
580
600

B


control hTTR
hTTR amyloid
control V30M
V30M amyloid
Fluoresence intensity (FI)
wavelength (nm)
0
20
40
60
80
100
120
140
460
480
500
520
540
560
580
600

C

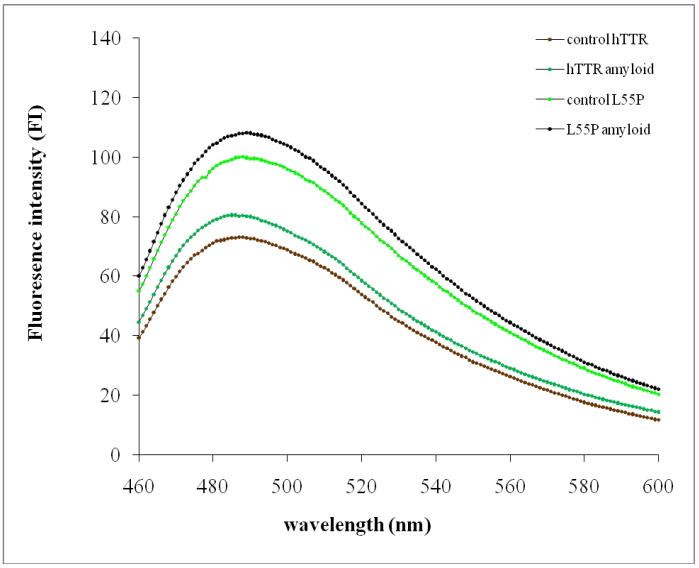

control hTTR
hTTR amyloid
control L55P
L55P amyloid
Fluoresence intensity (FI)
wavelength (nm)
0
20
40
60
80
100
120
140
460
480
500
520
540
560
580
600

Figure 3.10 The comparison of the fibril formation of TTR variants to human native TTR

(A) The fibril formation of recombinant V122D

(B) The fibril formation of recombinant V30M

(C) The fibril formation of recombinant L55P

Table 3.1 Summary of the fibrillation of human native TTR and variants determined by ThT assay

human TTR	Fibril formation (%)	Relative fibril formation to human TTR (folds)
native	9.9	1.0
V122D	10.8	1.2
V30M	38.6	4.3
L55P	8.4	0.9

3.8 The proteolytic activity of recombinant TTRs

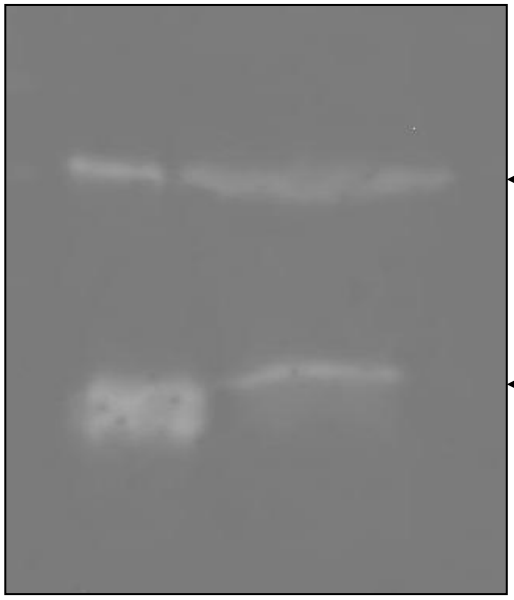
The proteolytic activity of TTR was examined by using fluorescein isothiocyanate linked casein (casein-FITC) which is a universal substrate for serine protease as a substrate. The reaction assay was set up and performed as described section 2.7. All reagents and the reaction mixture were filtered sterile to protect a possible contamination by the protease from microorganisms. The reaction was terminated by precipitation with trichloroacetic acid (TCA), and the fluorescence intensity was measured by fluorospectrometer (Shimazu). The proteolytic activity of TTR was determined, and quinine hydrochloride was used as standard for converting the intensity to amount and concentration. The reaction containing casein-FITC alone was included to determine the autolysis of the casein. The result showed that there was no autolysis of FITC-casein observed at 37^{o}C for 12 h in the dark (data not shown). The catalytic activity of V30M, L55P, V122D and human native TTR was 0.97, 0.51, 0.40 and 0.26 nM/min, respectively (Table 3.2). In addition, the specific activities for casein degradation of V30M, L55P, V122D, and human native TTR were 3887.81, 2936.10, 8038.67 and 1024.56 nM/min/mg protein, respectively.

Table 3.2 Summary of the proteolytic activity of TTRs using casein-FITC as substrate

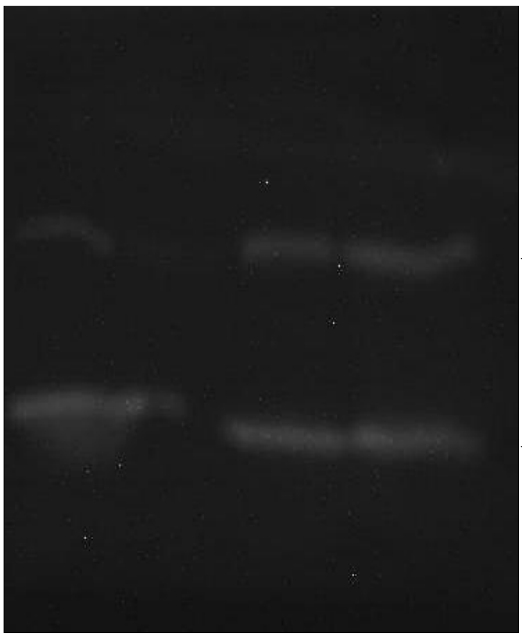
human TTR	Activity (nM/min)	Specific activity (nM/min/mg)
Native	0.26	1024.56
V122D	0.40	8038.67
V30M	0.97	3887.81
L55P	0.51	2936.10

3.9 Immunological reactivity with antibodies specific to TTR

The immunochemical reactivity assay with a specific antibody is one of the effective methods to identify or confirm the protein of interest. To examine the immunological reactivity of TTRs prepared in this thesis, the proteins were separated by SDS-PAGE, then electrophoretically transferred onto a PVDF membrane. Thereafter, the membrane was probed with the polyclonal antibody specific to TTR purified from human serum, as the primary antibody. Based on ECL detection, there were two bands cross-reacted with the specific antibody (Figure 3.11). The major band had position corresponded to TTR monomer, whereas another appeared with much less intensity at the position corresponded to TTR dimer. This feature was observed in all of the examined TTRs. These results suggested and confirmed that TTRs were produced and isolated from the *Pichia* transformants.



Native **V122D**



Native **V30M** **L55P**

Figure 3.11 Western analysis of recombinant V122D

Purified human native TTR, recombinant V122D, V30M and L55P were subjected to SDS-PAGE (12% resolving gel) prior transfer the protein bands onto a nitrocellulose membrane. The Western analysis was carried out using sheep antiserum against human TTR purified from serum (1:1,000) and horseradish peroxidase (HRP)-linked rabbit IgG antibody (1:2500) as primary and secondary antibodies, respectively. Detection was performed by ECL. The positions of TTR monomer and dimer were indicated.

CHAPTER 4

DISCUSSION

1. Synthesis of recombinant TTR variants

The *P. pastoris* clones for TTR V122D, V30M and L55P were constructed in our laboratory, by site-directed mutagenesis using wild type TTR cDNA as template (unpublished data). The TTR variants were later synthesized using the heterologous gene expression system of *P. pastoris* using *Pichia* strain GS115 as a host. In the expression vector pPIC3.5, the TTR gene was placed so that the TTR gene was under the regulation of *AOX*1 promoter which is responsible for the activity of alcohol oxidase in the yeast cell (Cregg *et al*., 1989). The expression "cassette" was inserted into the yeast genome at the *HIS4* locus. The integration was success by digesting the expression vectors with specific restriction enzymes so that were in linear forms prior transformation by electroporation into the *Pichia* cells. High percentage of the $His^{+}Mut^{+}$ transformants of V122D was generated and this indicated to high efficiency of the selected transformation method. Other chemically transformations such as using polyethylene glycol and lithium chloride are available. Although these methods are convenient but it provides ten folds less of the transformation efficiency (Gietz and Woods, 2002). By induction with methanol, these three types of TTR variant were synthesized and successfully secreted outside the yeast cells into the culture medium, though with different production levels. Since in the pPIC3.5, the protein itself presegment was used to mediate co-translational translocation of the entire polypeptide across membrane into the ER of *Pichia* (Paifer *et al*., 1994). The secretion of the recombinant TTRs could demonstrate to the effective of the TTR presegments in direct the proteins into the secretory pathway of the heterologous protein expression system of *Pichia*.

The synthesis and secretion of recombinant proteins highly depend on the composition of the growth medium. The *Pichia* recombinant clones grew more slowly in minimal medium compared with other more complex media (Clare *et al*., 1991). In addition, with the presence of substrates such as peptide components of

yeast extract and peptone, the action of pH-dependent extracellular protease in *Pichia* reduced. As a consequence, the proteolytic degradation of secreted recombinant proteins decreased (Clare *et al*., 1991). Therefore, to increase the amount of secreted recombinant TTRs and to protect the proteins from any possible degradation, the buffered medium, i.e. BMGY and BMMY were selected.

2. General properties of recombinant TTRs

Nowadays, the use of *P. pastoris* as a cellular host for the expression of recombinant proteins has been more popular. Not only *Pichia* is easier to genetically manipulate and culture than mammalian cells, but it also provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the posttranslational modifications, including glycosylation, proline cis/trans isomerization, disulphide isomerization, lipidation, sulphation and phosphorylation, required for functions (White *et al*., 1994; Lueking *et al*., 2000). Although glycosylation in *P. pastoris* expression systems do not generally hyper-glycosylate as in *S. cerevisiae* systems, there are some other proteins expressed that have been found to be similarly hyper-glycosylated in both *P. pastoris* and *S. cerevisiae* (Hitzeman *et al*., 1990). Based on the physicochemical properties of the recombinant TTRs, it indicated that all TTRs were synthesized and undergone properly posttranslational modifications as that exist in the system of human. Particularly, the TTRs were not hyper-glycosylate as their subunits had masses similar to that reported for TTR purified from human plasma.

3. Fibril formation of TTR variants

Many mutated TTRs from fibrils *in vitro* under an acidic condition (Lai *et al*., 1996). The mutations in the TTR molecule have been proposed affect the tetramer–dimer–monomer equilibrium due to protein dissociation and consequently result in the formation of an amyloidogenic intermediate, which associates in the fibrils. It showed that the tetramer among TTR variants is very sensitive to acidic condition and readily dissociating to form the monomer amyloidogenic intermediate. Tetramer dissociation and monomer unfolding was previously examined. Under urea

denaturation, L55P was demonstrated less stable than wild type TTR and V30M (Babbes et al., 2008). However, under an acidic condition, V30M formed fibril better but less toxic than L55P. Less fibril forming ability of L55P compared to V30M was confirmed in our results. In comparison, V122D was slightly more sensitive to the acidic condition than L55P, but less sensitive when compared to V30M as it formed fibril better that L55P but less than V30M. Since, TTR protofibril was reported more toxic to cells than the mature fibril. Less prefer to form fibril of V122D possibly indicates to its highly toxic potency, however, additional experiments must be carried out to prove this.

4. Proteolytic activity of TTRs

Proteases are one of the most abundant classes of enzymes that involved in a wide range of biological processes. Many proteases found in nature are deeply involved in physiology and pathology. Seven distinct classes of proteases have been grouped according to the amino acid or ion that catalyzes peptide bond cleavage These are aspartate proteinases, cysteine proteinases, glutamate proteinases, metalloproteinases, serine proteinases, threonine proteinases and asparagine peptide lyases (for review see Deu *et al*., 2012). The proteolysis mechanism and specific substrate are well defined for most of these enzymes. Recently, TTR was shown having a protease activity and new function that the activity may be involved has been postulated (Liz *et al*., 2004). In the past, it was proposed as a serine protease cleavages a few substrates i.e. apoA-I, Aβ, and amidated neuropeptide Y. Based on metal chelators abolished TTR activity Very recent, it was suggested as a metallopeptidase based on the findings that its proteolytic activity depended on metal ions and was inhibited by metal chelators (Liz *et al*., 2012). The Zn^{2+} chelating sequence, HXHXE, was found and conserved in TTRs of human and primates. Upon assayed using casein as substrate, the specific proteolytic activity of V122D was revealed much higher than the wild type and the two variants. Since, the TTR binding site for apoA-I was believed locate at its C-terminal region (Liz *et al*., 2007), and HXHXE sequence locate at position 88 to 90 on TTR polypeptide chain. It could conceive that the mutation of TTR polypeptide at position 122 (in V122D) affect to

the catalytic activity of TTR than that at other positions such as position 30 (in V30M) and position 55 (in L55P).

CHAPTER 5

CONCLUSIONS

1. The recombinant V122D that detected in Thai people, as well as V30M and L55P were successfully synthesized by using the heterologous protein expression system of *Pichia pastoris* and they were efficiently extracellular secreted to culture medium.

2. The recombinant protein can be purified in a single step by preparative native-PAGE. These recombinant TTRs showed similar physicochemical properties similar to human native TTR in particular electrophoretic migrate under native condition faster than albumin.
3. Subunit mass of recombinant V122D was 16,906 daltons which is similar to the subunit mass of human native TTR. In addition, it showed cross-reactivity to the antibody that is specific to human TTR purified from plasma.
4. According to ThT assay, V122D formed fibril faster than human native TTR and L55P, but slower than V30M.
5. Based on caseinolysis assay, V122D had higher proteoltytic activity than human wild type TTR, V30M and L55P.

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