รายงานวิจัยฉบับสมบูรณ์

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ABSTRACT

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To elucidate the catalytic site and role of N- and C-terminal sequences on the proteolysis ability of TTR, the cDNAs coding for four chimeric TTRs i.e. croc/huTTR, hu/crocTTR, pigC/crocTTR and truncated crocTTR, were constructed in pPIC9 under the alcohol oxidase 1 (*AOX1*) promoter. The TTRs were successfully synthesized in *Pichia pastoris* and extracellulary secreted using α-factor mating signal sequence. Sequencing of the N-terminal segments confirmed correct processing of the α-factor signal sequence in pPIC9. These recombinant TTRs had the subunit masses ~15 kDa to ~17 kDa in range, and molecular weights of the tetramers were 50 kDa to 60 kDa in range, which are ~4 times of the subunit mass. In addition, all of them reacted to the antibodies raised against native TTRs. The influence of N- and C-terminal regions on the binding to human retinol binding protein (hRBP) was examined and more influence of the amino acid sequence in the N-terminal region was observed than in the C-terminal region. The proteolysis ability of chimeric TTRs were studied on casein and apolipoprotein A-I (apoA-I). All these chimeric TTRs specifically cleaved substrates with different catalytic rates from the TTR wild type. Amongst, pigC/crocTTR showed 3 to 8 folds higher in the catalysis, on both casein and apoA-I, than the other types including wild type. These experimental results clearly revealed the effect of N- and C-

terminal regions on this ability of TTR. To demonstrate ability to inhibit the cytoxicity induced by amyliod β (Aβ), human fibroblast cells were treated with Aβ. Cell viability and membrane integrity were determine by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays, respectively. In the presence of chimeic TTR, cell viability and membrane integrity increased ~20% and 33%, respectively. These indicated to the ability of the chimeric TTR to protect cells from the toxicity induced by Aβ, and suggested to the possible use of the chimeric TTR as a therapeutic agent for AD in future

เพื่อให้ทราบถึงบริเวณ active site และบทบาทของโครงสร้างปลายอะมิโนและปลายคาร์บอกซิลที่มีต่อความสามารถของ transthyretin (TTR) ในการเร่งปฏิกิริยาการสลายโปรตีน ผู้วิจัยได้ทำการสร้าง cDNA สำหรับ chimeric TTR จำนวน 4 ชนิด คือ croc/huTTR, hu/crocTTR, pigC/crocTTR และ truncated crocTTR ขึ้นมา และนำเข้าสู่ เวคเตอร์ชนิด pPIC9 เพื่อให้เกิดการแสดงออกของยีนในเซลล์ *Pichia pastoris* ภายใต้การควบคุมของ alcohol oxidase1 (*AOX1*) promoter ผลการทดลองพบว่าสามารถสังเคราะห์ TTR ดังกล่าวได้สำเร็จ และ TTR ถูกหลั่งออกนอกเซลล์ยีสต์ได้ โดยอาศัย α-factor mating signal sequence การวิเคราะห์หาลำดับกรดอะมิโนที่ปลายอะมิโนของ TTR ที่ถูกสร้างขึ้นยืนยันว่าการตัดชิ้นส่วนของ α-factor signal peptide ออก เกิดขึ้นได้อย่างถูกต้อง Chimeric TTRs ที่สร้างขึ้นมานี้ มีน้ำหนักหน่วยย่อยอยู่ในช่วงประมาณ 15 ถึง 17 กิโลดาลตัน มีน้ำหนักโมเลกุลประมาณ 4 เท่าของน้ำหนักหน่วยย่อย กล่าวคือมีน้ำหนักอยู่ในช่วงประมาณ 50 ถึง 60 กิโลดาลตัน อีกทั้งสามารถเกิดปฏิกิริยาอย่างจำเพาะกับแอนติบอดี้ต่อ TTR ในธรรมชาติ จากการนำ chimeric TTRs มาใช้ในการศึกษาถึงอิทธิพลของโครงสร้างปลายทั้งสองของสายโพลีเปปไทด์หน่วยย่อย ที่มีต่อความสามารถในการเข้าจับกับ retinol binding protein (RBP) พบว่าโครงสร้างทางปลายอะมิโนส่งผลต่อความสามารถในการเข้าจับมากกว่าปลายคาร์บอกซิล นอกจากนี้การเปลี่ยนแปลงลำดับกรดอะมิโนที่ปลายทั้งสอง ยังส่งผลต่อความสามารถในการเร่งปฏิกิริยาการสลายโปรตีนของ TTR อีกด้วย โดย chimeric TTR ทุกชนิดนี้ สามารถสลายโมเลกุลของทั้ง casein และ apolipoprotein A-I (apoA-I) ได้อย่างจำเพาะ แต่มีอัตราการเร่งปฏิกิริยาที่แตกต่างไปจาก TTR ที่พบในธรรมชาติ และเมื่อเปรียบเทียบกันแล้ว พบว่า

pigC/crocTTR มีอัตราเร็วของการเร่งปฏิกิริยาสูงกว่า chimeric TTR ชนิดอื่นรวมทั้ง TTR ในธรรมชาติประมาณ 3 ถึง 8 เท่า อีกทั้งจากการศึกษาผลการยับยั้งความเป็นพิษของ amyloid β (Aβ) ต่อเซลล์ไฟโบรบลาส (fibroblast) โดยการ incubate เซลล์กับ Aβ โดยมี chimeric TTR อยู่ด้วย และตรวจหาอัตราการมีชีวิตรอดและความสมบูรณ์ของผนังเซลล์โดยวิธี MTT reduction และ LDH release ตามลำดับ พบว่าเซลล์มีอัตราการรอดมากกว่าเมื่อไม่มี TTR ถึงประมาณ 33% ชี้ให้เห็นถึงความสามารถของ chimeric TTR ในการป้องกันความเป็นพิษของ Aβ และประโยชน์การนำไปใช้เพื่อการรักษาในอนาคต

EXECUTIVE SUMMARY

Amylodosis is a group of heterogeneous diseases caused by the deposition of insoluble protein and peptide aggregates called ‘amyloid’. The accumulation of large amounts of amyloid fibrils found in various organs and tissues, and can lead to progressive and life-threatening organ disfunction. Up to date, more than 26 different proteins including amyloid β (Aβ), apolipoprotein A-I (apoA-I) and transthyretin (TTR) have been identified to form amyloid. These amyloids are associated with several disorders including Alzheimer’s disease (AD).

TTR is one of the three major thyroid hormone (TH) binding proteins found in plasma and cerebrospinal fluid (CSF) of human as a tetramer of four identical β-sheet rich subunits. TTR has been demonstrated as the most abundant protein component of amyloid fibrils. Currently, it was revealed as a cryptic protease. Aβ, apoA-I and neuropeptide Y are the natural substrates have been identified till now. The cleavage by TTR associates to fibrillation of its substrates and could result to either promote or inhibit the process. In Alzheimer’s disease (AD), the proteolysis by TTR inhibits and disrupts the fibrillation of Aβ with consequent reduction of the amyloid plaque and abrogation of toxicity to cells. In contrast to many other proteases involved in pathological mechanism, TTR lacks canonical structure which is necessary for protease, and its function apparently does not relate to proteolysis. The amino acid residues involved in the catalysis are still unknown. The structural analysis of TTR genes from numerous vertebrate species revealed highly conservation of the amino acid sequences in the central channel where THs binding site locates. These changes were demonstrated effecting on the binding affinities to THs. Together with other several research evidences, we proposed that the catalytic site of TTR locates at or nearby the C-terminal region of its subunit. In addition, both N- and C-terminal regions would have influence on the binding of TTR to its substrates. To elucidate, the chimeric TTR having the alteration in the amino acid sequence of N- and/or C-terminus is required.

In this research, four chimeric TTRs, i.e. croc/huTTR, hu/crocTTR, pigC/crocTTR and truncated crocTTR, were produced in *P. pastoris*. These TTRs had general properties including subunit mass, molecular weight of TTR tetramer and reactivity to the specific antibodies against TTR similar to the vertebrate TTRs found in nature. The influence of N- and C-terminal regions on binding to human retinol binding protein (hRBP) was examined and it showed more effect of the N-terminal segment on

the binding. All of these chimeras cleaved casein and apolipoprotein A-I (apoA-I) with different rates. Amongst, pigC/crocTTR had several folds higher rate than the wild type. The experimental results clearly demonstrated the effect of N- and C-terminal regions on the catalytic ability of TTR. To demonstrate ability to inhibit the cytoxicity induced by amyliod β (Aβ), human fibroblast cells were treated with Aβ in the presence of pigC/crocTTR. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and lactate dehydrogenase (LDH) release assays were performed to detect viability and membrane integrity of cells. Without TTR, cell viability measured by LDH release was 30% relative to control cells. In the presence of TTR, cell viability and membrane integrity increased ~20% and ~33%, respectively. These suggested to ability to protect cells from the toxicity induced by Aβ of the chimeric TTR.

In summary, changing in either N-terminal or C-terminal sequence of the subunit was demonstrated leading to changes in properties of TTR tetramer. Although the amino acid residues lining in the active site and those directly interact to substrate during the catalytic process of TTR can not be directly revealed, role of these end-terminal segments on properties and functions of TTR was clearly demonstrated. In addition, since the chimeric TTR we produced have ability to cleave and inhibit the cytoxicity induced by Aβ. The chimeric TTR may be useful as a therapeutic agent for AD in future.

INTRODUCTION

Amylodosis is a group of diseases caused by the deposition of proteins or protein fragments which lost their native solubility and transform into an insoluble amyloid fibril. The amyloid fibrils have been found accumulated in a variety of organs and tissues of the body including peripheral nerves, kidney, gastrointestinal tract, heart and thyroid gland (Kelly, 1996; Rochet and Lansburg, 2000). The accumulation of large amounts of amyloid fibrils can result in damaging of function and structure of organs. Up to date, more than 26 different proteins have been identified as the amyloidogenic in human (for review sees Westermark, 2005). These are associated with several disorders including Alzheimer's disease (AD) (Solkoe, 1996), Parkinson's disease (Conway *et al*., 1998), familial amyloidotic polyneuropathy (FAP) and familial amyloidotic cardiomyopathy (FAC) (Costal *et al*., 1978; Mc Cutchen *et al*., 1995), light chain amyloidosis (for review sees Buxbaum and Gallo, 1999), and dialysis-related amyloidosis (Drueke, 2000).

Transthyretin (TTR), previously referred to as prealbumin, is one of the three major thyroid hormone (TH) binding proteins found in plasma of larger mammals. It was found mainly in both serum and cerebrospinal fluid (CSF) of human (Kabat *et al*., 1942 a, b) as a tetramer of four identical β-sheet rich subunits of which comprises 127 amino acid residues (Blake *et al*., 1974, 1978; for review sees Hamilton and Benson, 2001). TTR has been demonstrated as the most abundant protein component of amyloid fibrils (Costa *et al*., 1978). Over 80 TTR genetic mutations resulted in a single amino acid substitutions of TTR subunit have been reported (for review, sees Schwarzman *et al*., 2004). Most of them are related to two clinical syndromes, familial amyloidotic polyneuropathy (FAP), which TTR amyloids found mainly deposit in peripheral nervous system, and familial amyloid cardiomyopathy (FAC).

Currently, the cryptic protease property of TTR was reported (Liz *et al*., 2004) and till now only 3 natural substrates have been identified, i.e. amyloid β (Aβ), apolipoprotein A-I (apoA-I) and neuropeptide Y (for review see Prapunpoj and Leelawatwattana, 2009). The cleavage by TTR could promote or inhibit fibrillation depending on substrate. To apoA-I, the cleavage by TTR trigger the fibril formation (Liz *et al*., 2004) and decrease ability to promote the cholesterol efflux of the cleaved apolipoprotein. Whereas, TTR was shown sequestering the fibrillation of Aβ, the main

component of amyloid plaques in Alzheimer's disease (AD) (Golabek *et al*., 1995; Schwarzman *et al*., 1994, 1996), both by inhibiting and disrupting Aβ fibrils, with consequent abrogation of toxicity. The proteolytically process was later demonstrated to be the protective mechanism of TTR in AD. Although the relationship between the catalytic activity of TTR and amyloidogenesis is still unclear, i.e. either activation or inhibition of the amyloid fibrils formation could obtain, both functions are depended on same proteolytic ability of TTR. Since TTR is one of the negative acute phase plasma proteins, the cleavage of the amyloid precursor proteins by TTR possibly is another function of TTR in both plasma and brain.

The proteolytic mechanism and specific substrates of several proteases are well known nowadays. These enzymes are conventionally classified base on their catalytic mechanisms, which can be divided into four canonical mechanistic classes i.e. the serine proteinases, cysteine proteinases, aspartic proteinases and metallo proteinases. All of which are involved in physiological and pathological mechanisms. TTR lacks canonical structure for protease and its function apparently does not relate to proteolysis. Even though the proteolytic activity of TTR has been clarified as a serine protease (Liz *et al*., 2004), the amino acid residues involved in catalysis and the underlying mechanism are still unknown. It was demonstrated that binding of retinol binding protein (RBP) interfered the proteolytic activity of TTR to apoA-I (Liz *et al*., 2004) suggesting that the substrate binding site for apoA-I may locate at or nearby the binding site for RBP. Our hypothesis is, thus, prompted to that the catalytic site of TTR locates at or nearby the C-terminal region of TTR. If this is true, N- and C-terminal regions should have influence on the binding of TTR to this protein. The structural analysis of TTR genes from numerous vertebrate species revealed that the amino acid sequences in the central channel with THs binding site is highly conserved. The predominant changes occurred within ten and seven amino acids from the N-terminal and C-terminal ends, respectively. These changes in particular the N-terminal region was demonstrated have influence on binding affinities to THs (Prapunpoj *et al*., 2002, 2006). The questions arise whether or not these regions involved or have influence on the catalytic activity of TTR and whether or not the catalytic activity of TTR can be increase by alteration the animo acid sequences of these terminal ends. To elucidate, the synthesis of a recombinant TTR in which amino acid sequence of N- or C-terminus is required.

Heterologous expression in *P. pastoris*, a methylotrophic yeast, has many of the advantages of eukaryotic expression (Cregg *et al.,* 1985; Cregg *et al.,* 1993; Higgins and

Cregg, 1998). Its growth can be raised up to a very high density in a simple defined medium. The yield of expressed proteins from *P. pastoris* depends critically on growth conditions. The protein yield could be improved to 10 to 100 folds by fermentation process (Wood and Komives, 1999). When grown on methanol, foreign proteins secreted from *P. pastoris* can represent 80 % or more of the total protein in the culture medium (Tschopp *et al.,* 1987). *P. pastoris* has a strong inducible promoter that can be used for protein production and is capable to generate post translational modifications, which are similar to those of higher eukaryotes. In addition, there are commercially available methods, host strains and expression vector for genetic manipulations (Invitrogen Corporation, Carlsbad, CA, USA) (Cregg *et al*., 1993; Higgin and Cregg, 1998; for review see Zhang *et al*., 2000). Many foreign proteins including TTRs from several vertebrate species (Prapunpoj *et al*., 2000, 2002, 2006) have been produced in *P. pastoris*. Therefore, in this research the hetreologous gene expression system of *P. pastoris* was chose as a tool for producing the recombinant chimeric TTRs.

OBJECTIVES

To study on the influence of changes in the N- and/or C-terminal sequences of TTR on its ability to inhibit amyloid fibril formation by:

1. Produce the chimeric TTRs in which N- and/or C-terminal sequence is altered.
2. Characterize general physicochemical properties of the chimeric TTRs
3. Compare proteolytic abilities of the chimeric TTRs
4. Determine the chimeric TTR-protective ability on cytotoxicity of amyloid protein

MATERIALS & METHODS

Cells and vectors

P. pastoris GS115 and *E. coli* DH5α were gifted from Professor Schreiber, University of Melbourne, Australia. *P. pastoris* SMD 1168 and pPIC9 expression vector were from Invitrogen, whereas, *E. coli* JM 109 and pGEM Teasy vector were products of Promega.

Amyloid β (Aβ) peptide

Aβ (1-42) was purchased from Millipore (USA). The peptide was dissolved in PBS at 200 μM, aliquot, and stored as stock at -80^{o}C until use.

Methods

1. Preparation of the recombinant chimeric TTR clones

1.1 Construction of chimeric TTR cDNAs and the expression vector to be expressed in P. pastoris

The cDNAs encoding for four chimeric TTRs were generated by PCR using *Crocodylus porosus* TTR cDNA as templates. The TTR cDNAs with compatible restriction ends for ligation into pPIC9 (*Xho*I at 5′ end and *Eco*RI at 3′ end) expression vector were amplified as previously described (Prapunpoj *et al*., 2002; 2006). In brief, the reaction mixture (100 μl) contained 50 ng of crocodile TTR cDNA and 40 pmol of the each appropriate primers (Table 1) was carried out with an initial denaturation step at 94^{o}C for 5 min followed by 25 cycles of denaturation at 94^{o}C for 30s, annealing at appropriate temperature for 30s and extension at 72^{o}C for 1 min. The final extension was carried out at 72^{o}C for 10 min. After amplification, the PCR product was analyzed on 1% agarose gel and DNA bands were visualized by staining with EtBr. The PCR product with an expected size was purified, ligated into pGEM Teasy cloning vector (Promega), and transformed into *E. coli* DH5α for *in vivo* amplification. Thereafter, the recombinant plasmid was purified from a single white colony. Double digestion with *Xho*I and *Eco*RI was performed to release the chimeric TTR cDNA insert prior the DNA insert was ligated into pPIC9. The DNA insertion direction in the vector was determined by nucleotide sequencing.

Table 1 Oligonucleotide primers used to generate cDNAs for chimeric TTRs

	PCR step	Sequence 5′ → 3′	Sense
hu/crocTTR	1	AACGGGCACCGGTGAATCCAAATGCC	Sense
		ACGGAATTCTTATTCTTGTGGATCACTG	Antisense
	2	CTCGAGAAAAGAGAGGCTGAAGCTGGCCCAACGGGCACCGG	Sense
		ACGGAATTCTTATTCTTGTGGATCACTG	Antisense
croc/huTTR	1	ACTGGTTTCCCATGGTTCTATTGATTCCAAGTGTCC	Sense
		AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
	2	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
		AGGAGTGAATTCTCATTCCTTGGGATTGG	Antisense
truncated crocTTR	1	CTCGAGAAAAGATCCAAATGCCCACTTATGG	Sense
		ACGGAATTCTTATTCTTGTGGATGACTG	Antisense
pigC/crocTTR	1	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
		TGGGGCTGCTGACGAGGGCTGTG	Antisense
	2	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
		AGCTCCCTCCTTGGGGCTGCTGAC	Antisense
	3	TCTCTCGAGAAAAGAGCCCCACTGGTTTCCC	Sense
		ACGGAATTCTCAAAGAGCTCCCTCCTTGGG	Antisense

1.2 Transformation into Pichia

To introduce into *Pichia* cells, the recombinant expression vector for chimeric TTR cDNA was digested with SalI prior electroporation was carried out using Gene Pulser (Bio-Rad) and the protocol recommended by the company. In brief, 80 µl of the *Pichia* cells were mixed with ~1.2 µg of the linearized plasmid. Then, the cell mixture was transferred to an ice-cold 0.2 cm electroporation cuvette. Cells were incubated on ice for 5-10 min and electroporation was carried out at 1.5 kV, 25 µF and 400 Ω, generating pulse lengths of ~7.68 milliseconds with a field strength ~1.37 kV/cm. Thereafter, the cell suspension was immediately 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 cells were allowed to grow at 30^{o}C for 3 days.

2. Screening for His^{+}Mut^{+} transformant

Pichia transformants with phenotype His^{+}Mut^{+} (histidine synthesis and methanol utilization plus) were identified and selected by growing on selective agar media, minimal dextrose medium (MD) and minimal methanol medium (MM). The screening was performed from 80 to 100 colonies of the transformants, and ~40 single colonies of the His^{+}Mut^{+} were subsequently induced for synthesis of the recombinant TTR.

3. Synthesis of recombinant chimeric TTRs

3.1 in small scale

A single colony of the recombinant *Pichia* clone was inoculated into 5 ml of the buffered medium containing glycerol (BMGY). Cells were grown at 30^{o}C in an orbital-shaking incubator (200 rpm) until OD_{600} reaches 2 to 6 (~18 h). Then, cells were collected by centrifugation at 2,500 rpm for 5 min prior being transferred to 5 ml of the buffered medium containing methanol (BMMY). The induction with methanol was conducted for 3 to 5 days. During this period, methanol was added every 24 h to maintain the concentration to 0.5%. After induction, the culture supernatant was collected by centrifugation and kept at -20°C until use. The secreted proteins in the yeast culture supernatant were analyzed by SDS-PAGE and/or native-PAGE and protein bands were detected by silver staining.

3.2 in large scale

In order to obtain in sufficient amount of the recombinant TTR, a large scale preparation was performed by using the shaking flask culture method. A single colony of the *Pichia* clone was inoculated into 10 ml of BMGY and grown at 30^{o}C for 16 18 h. Thereafter, 5 to 10 ml of the overnight culture was transferred into 300 ml of BMGY in a 1-liter flask and allowed to grown until OD_{600} reached 2 to 6. Cells were then collected by centrifugation and suspended in 300 ml of BMMY in a 2-liter flask to a density equivalent to OD_{600} 1.0. The induction for synthesis with methanol (0.5%) was performed for 3-5 days at 28^{o}C. Thereafter, the culture supernatant was collected by centrifugation and the levels of synthesis and secretion into culture medium of the recombinant TTRs were determined by native-PAGE and SDS-PAGE followed by silver staining.

4. Purification of recombinant chimeric TTRs

The recombinant chimeic TTRs were purified from the *Pichia* culture supernatant by preparative native-PAGE using Prep Cell 491 (Bio-Rad) and followed by affinity chromatography on human retinol binding protein (hRBP)-Sepharose column. The fractions containing TTR were pooled, concentrated and stored at -20^{o}C until use.

5. Determination of physicochemical properties of recombinant chimeric TTRs

To confirm the proper structure and folding of the recombinant chimeric TTRs, general physicochemical properties including the subunit mass, molecular weight, and the electrophoretic mobility under native condition were examined

5.1 Subunit mass

The subunit masses were determined by SDS-PAGE as described by Laemmli and Favre (1973). Purified TTR was boiled in the solution containing 2% sodium dodecyl sulphate (SDS) and 2.5% β-mercaptoethanol for 30 min prior loading onto SDS-polyacrylamide gel (12% resolving gel and 4% stacking gel). Protein bands were detected by staining gel with Coomassie brilliant blue R-250. The relative mobility of TTR subunit was determined and the subunit mass was calculated.

5.2 Molecular weight of TTR by gel filtration

The molecular weight of TTR tetramer was determined by HPLC/gel-permeation chromatography on BioSil SEC 250 column (Bio-Rad). The column was standardized

with blue dextran (2,000 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa) and chymotrypsinogen A (25 kD).

5.3 Mobility under native condition

The native TTRs from all vertebrates, except those from pig and cattle, have been known to migrate faster than albumin during electrophoresis at pH 8.6. To determine this property of the chimeric TTRs, non-denaturing PAGE was performed using 10% polyacrylamide gel and 0.05 M Tris-glycine, pH 8.6. After analysis, protein and DNA bands were detected by Coomassie blue or silver staining.

6. Binding ability to human retinol binding protein (hRBP)

To determine the binding to hRBP, the purified chimeric TTR (0.4 µg) was incubated at 4°C, for 1 h, with various amount of hRBP. Then, the reaction mixture was analyzed on a native-PAGE (10% resolving, 4% stacking) at 4^{o}C. Free TTR and TTR-RBP complex were immunochemically detected by western blotting using antibodies against human native TTR (for croc/huTTR) or against *C. porosus* TTR (for hu/crocTTR, pigC/crocTTR and truncated crocTTR) (Table 2). The dissociation constant (K_d) was analyzed by Scatchard plot (Scatchard, 1949).

Table 2 Antibodies used in Western blot analysis

TTR	Primary antibody and dilution	Secondary antibody and dilution
hu/crocTTR truncated crocTTR pigC/crocTTR	anti-crocTTR polyclonal antibody, raised in rabbit (dilution 1:500)	Rabbit IgG-HRP, raised in goat (dilution 1:2500)
croc/huTTR	anti-human TTR polyclonal antibody, raised in sheep (dilution 1:2500)	Sheep IgG-HRP, raised in rabbit (dilution 1:2500)

7. Western blot analysis

Proteins were separated by SDS-PAGE or native-PAGE in duplicate. One of the gels was stained with Coomassie blue to detect for proteins. Proteins in the other gel 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 (for SDS-gel) or at 25 volts for 30-40 min (for native gel), with a cooling system. The buffer used was 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3. The protein bands those were transferred to the membrane were visualized by staining with 0.1% Amido black. Specific proteins were detected by immunochemistry, using enzymatic detection. To block the non-specific binding sites, the membrane was incubated in a 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 the blocking solution at room temperature for 1 h. The excess antibody was removed by washing with TBS-T at room temperature. Thereafter, the membrane was incubated with a secondary antibody linked with horseradish peroxidase (HRP) at room temperature for 1 h. The specific protein was detected by enhance chemiluminescence (ECL, GE Healthcare) and the fluorescence signal was detected by Bio-Chemi System (UVP).

8. Proteolytic activity of TTRs

The proteolytic activity of chimeric TTRs was quantified by using casein and apoA-I as substrates as follows.

The caseinolytic activity was analyzed as described by Twining (1984) with modifications. Two micrograms of purified TTR was incubated in a solution (50 µl) containing 0.2% fluorescein isothiocyanate (FITC)-casein (Sigma), 0.2 M NaCl and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH 8.8 at 37^{o}C for 12 h in the dark. The reaction was terminated by 5% trichloroacetic acid (TCA; 120 µl). The insoluble pellet was removed by centrifugation at 12,000 rpm for 10 min. The supernatant was neutralized with Tris-HCl, pH 8.5 prior the fluorescence intensity was determined at the excitation and emission wavelengths of 490 and 525 nm, respectively.

By using apoA-I as substrate, 2 µg of purified TTR and 1 µg of apoA-I were incubated in 50 mM Tris, pH 7.5 at 37^{o}C. Cleaved and uncleaved apoA-I were

separated by SDS-PAGE (20% resolving gel) and their amount was determined after Coomassie blue staining by Gel document using Lab Works version 4.6 (Bio-Imagine System, UVP).

9. Cell culture

Skin biopsy of 3 mm punch was obtained from healthy volunteer. Explants were performed and plated in 25-cm^2 flasks. The cells were grown in DMEM, supplemented with 20% FBS, at 37^oC in 5% CO_2 atmosphere. Cells were harvested at confluence in T-25 flasks, 7 days after previous subculture.

10. Amyloid cytoxicity and anti-toxicity assays

The toxicity of Aβ was assessed by LDH release and MTT reduction assays. Fibroblasts were plated on 96-well plates in 100 µl of fresh medium. Before reaching confluence (~60-70% confluence), the cells were incubated overnight with 100 µl of fresh media without serum in the presence of Aβ 1-42 at various concentrations. The fibroblast culture media were then transferred into a 96-well plate to determine LDH release by using Cytotoxicity Detection Kit LDH (Roche Diagnostics). The absorbance at 490 nm was recorded and the amount of LDH release was calculated as a percentage of the maximum LDH release (cells with lysis buffer) after subtracting the spontaneous LDH release (cells alones) as described by the company. The results were expressed as percentage of cell membrane integrity (100 - %LDH release).

By MTT reduction assay, 100 µl of fresh medium and 10 µl MTT labelling reagent was immediately added. After incubation at 37^oC for 4 h, 100 µl of cell lyses buffer (20% SDS, 50% N,N-dimethylformamide, pH 4.7) was added to each well and the samples were incubated overnight at 37 ^oC. Absorbance values of blue formazan were determined at 570 nm with an automatic plate reader.

To determine the ability of chimeric TTR to inhibit toxicity of Aβ, fibroblast cells were incubated at 37^oC in the presence of both Aβ 1-42 and chimeric TTR.

11. Purification of PCR product

The DNA fragment amplified by PCR was purified by using PCR Purification Kit or by separation on an agarose gel followed by extraction with Gel Extraction kit, using the methods described by the company.

12. Purification of plasmid

An overnight culture of *E. coli* containing plasmid was prepared in the presence of antibiotic (50 µg ampicillin/ml of culture). Thereafter, the plasmid was extracted by the alkaline lysis as described by Birnboim and Doly (1979), and purified using a plasmid purification kit.

13. Protein staining

13.1 Coomassie blue staining

After electrophoresis, gel was immersed in 0.2% Coomassie brilliant blue R-250, dissolved in 50% methanol and 10% acetic acid, for 1 h. Excess dye was removed by rinsing the gel in a solution of 50% methanol and 7.5% acetic acid until background was clear. For storage, gel was kept in a solution of 5% methanol and 7.5% acetic acid.

13.2 Silver staining

Gel staining was performed as described by Morrissey (1981). After electrophoresis, gel was immersed in the solution of 45% methanol and 10% acetic acid for 30 min and then transferred to the solution of 7.5% acetic acid and 5% methanol for 30 min. Gel was then rinsed with distilled water for 3 to 4 changes prior soaking with dithiothreitol (DTT) (500 µg/ml) for 30 min. Thereafter, gel was soaked in 0.1% silver nitrate for 20 min. To develop, gel was immersed in a solution of 3% (v/v) sodium carbonate and 0.02% (v/v) formaldehyde until protein bands appeared (~2-5 min). Then, the reaction was stop with 50% acetic acid. Gel was washed with distilled water, prior the protein band intensity was analyzed by gel document with LabWork 4.6 (UVP).

14. Sequencing of N-terminal region

The N-terminal amino acid sequences of the recombinant chimeric TTRs were determined by automatic Edman degradation at Department of Biochemistry, La Trobe University, Melbourne, Australia, or at Scientific Equipment Center, Prince of Songkla University, Thailand, or at Bioservice, NSTDA, Thailand.

15. Determination of protein concentration

Protein content was determined by Bradford protein assay (Bradford, 1976). The assay solution (1 ml) comprising 0.085 mg/ml Coomassie blue G-250, 5% methanol and 5.06% H_3PO_4. The protein-dye complex formation was carried out at room temperature

for 2 min prior the optical density of the reaction mixture was determined at 595 nm. Bovine serum albumin (BSA) was used as standard.

RESULTS & DISCUSSION

1. Construction of *Pichia* clone of recombinant chimeric TTRs

Up to date, the primary structure of TTRs, either partial or full length, has been analyzed. The alignment of the vertebrate TTR amino acid sequences revealed that all residues in the central channel and those involved in the binding interaction with THs were conserved (Blake and Oatley, 1977; Blake, 1981; Wojtczak *et al*., 1996; Wojtczak, 1997). In the contrary, the predominant changes concentrated at the N-terminal region and lesser on the C-terminal region of the TTR subunit. The change of length and hydropathy of these regions during evolution are correlated to the change in binding affinities to ligands in particular thyroid hormones (Chang *et al*., 1999; Prapunpoj *et al*., 2002). Since both N-and C-termini locate at the entrance to the central channel of the molecule where the catalytic site possibly exist, the cDNAs for chimeric TTRs were, thus, constructed by replacing the N- and/or C-terminal sequence of one TTR with that/those of other TTRs which has different THs binding preferences.

In this research, four chimeric TTR cDNAs were constructed and amplified by PCR. These are the croc/huTTR cDNA that would code for residues Ala1 to Cys10 of *C. porosus* TTR and residues Pro11 to Glu127 of human TTR, hu/crocTTR cDNA that would code for residue Gly1 to Cys10 of human TTR and residues Pro11 to Glu127 of *C. porosus* TTR, truncated crocTTR cDNA that would code for residue Ser8 to Glu127 of *C. porosus* TTR, and the pigC/crocTTR cDNA that would code for residue Ala1 to Ala120 of *C. porosus* TTR and residue Leu121 to Leu130 of pigTTR.

The *Pichia* recombinant clones were constructed in pPIC9 expression vectors. The cDNAs were amplified and generated the compatible restriction ends for ligation into the expression vectors by PCR using the primer sets. The cDNA coding for croc/huTTR was constructed and amplified by PCR using human TTR cDNA as template. Whereas, the cDNAs coding for hu/crocTTR, truncated crocTTR and pigC/crocTTR were generated using *C. porosus* TTR cDNA as template. Pairs of the specific primers were used to alter the N- or C-terminal sequence and generate the recognition sequences for *Xho*I at 5' and *Eco*RI at 3' ends in the TTR template (Table 1). The DNA product with an expected size of ~400 bp could be obtained by PCR (Figure 1). The PCR product was purified and double digested with the appropriate restriction enzymes prior to being ligated into the expression vector that was digested with the same enzymes. In pPIC9, the TTR cDNA with the compatible restriction sites

for *Xho*I and *Eco*RI was placed behind the yeast α-factor signal sequence (Figure 2). The α-factor signal sequence was used in the gene expression and removed within Golgi apparatus by KEX2, the diabasic amino acid sequence recognizing protease (Julius *et al*., 1984a and 1984b). It was shown that the sequence Glu-Lys-Arg is necessary for the release process of α-factor peptide from the gene product by KEX2, and the cleavage by KEX2 occurs between Arg and Gln in the sequence Glu-Lys-Arg-Glu-Ala-Glu-Ala, and the Glu-Ala-Glu-Ala sequence is necessary for the correct cleavage and will be removed during translocation of the recombinant protein. Therefore, the chimeric TTR cDNAs were immediately placed in-frame with the coding portion of Glu-Ala-Glu-Ala.

The nucleotide sequence of the recombinant expression plasmid and the insertion direction of the TTR cDNA in the vector were confirmed by DNA sequencing prior transformation of the DNA into *P. pastoris* cell was carried on. The DNA sequencing results confirmed the nucleotide sequence and correct position and direction in vector of the cDNAs (data not shown). Thereafter, the *Pichia* recombinant expression vectors were linearlized by *Sal*I prior they were introduced into *P. pastoris* by electroporation. Since histidinal dehydrogenase gene (*HIS*4) of the *Pichia* strain was mutated (Cregg *et al*., 1985), so that cell can use methanol but requires histidine supplementation for growth (genotype his4, phenotype $Mut^{+}His^{-}$). In order to create the His^{+}/Mut^{+} recombinant that can synthesize histidine and utilize methanol, crossing over between the *HIS*4 gene on the plasmid and the *his*4 locus of the yeast chromosome is required (Cregg *et al*., 1985; Cregg *et al*., 1989). The *Pichia* cell that obtains an expression vector therefore would posses a His^{+} phenotype. By electroporation, approximate $2x10^{3}$ to $5x10^{3}$ colonies of the *Pichia* transformants with His^{+} phenotype were obtained per microgram of the recombinant plasmid. This transformation efficiency was similar to that previously reported by Scorer *et al*., 1994 in which 10^{3} to 10^{4} colonies of His^{+} were obtained per microgram of DNA.

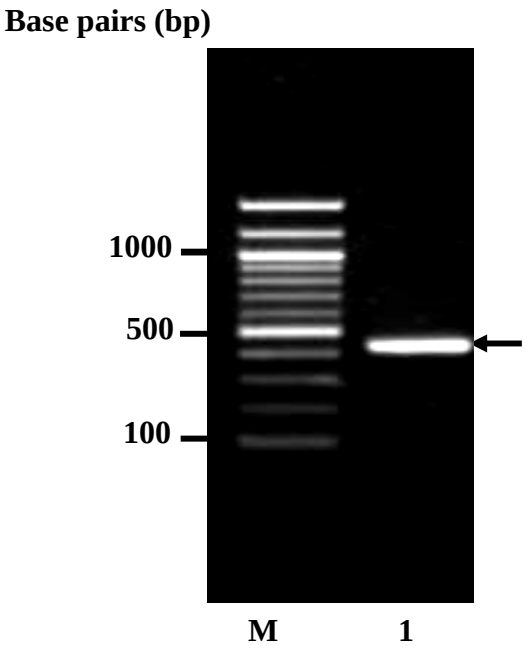

Figure 1 **Generation and amplification of TTR cDNA by PCR**

The cDNA coding for pigC/crocTTR was generated and amplified by PCR using *C. porosus* TTR cDNA as template. The PCR product was analyzed on agarose gel (1%) in the presence of ethidium bromide. DNA bands were visualized under UV. A single band of the DNA with approximate 400 bp was indicated by arrow. M is 100 bp DNA markers.

A

```
5'- TCTCTCGAGAAAAGAGCCCCACTG          CCCAAGGAATGAG -3'
3'-     AGCTCTTTTCTCGGGGTGAC          GGGTTCCTTACTC -5'
                    AlaProLeu         ProLysGlu***
                     1   2   3        125 126 127
```

B

```
5′- TCGAGAAAAGAGAGGCTGAAGCTGGCCCTACG          CCACAAGAATAA -3′
3′-     TCTTTTCTCTCCGACTTCGACCGGGATGC          GGTGTTCTTATTC -5′
                    GlyProThr                  ProLysGlu***
                     1   2   3                 125 126 127
```

C

```
5′- TCGAGAAAAGATCCAAATGCCCACTTATG          CCAACCGAATAA -3′
3′-     TCTTTTCTAGGTTTACGGGTGAATAC          GGTGTTCTTATTC-5′
                SerLysCysProLeuMet          ProLysGlu***
                 8   9  10  11  12  13      125 126 127
```

D

```
5′- TCTCTCGAGAAAAGAGCCCCACTGGTTTCC          GGAGCTCTTTGA -3′
3′- AGAGAGCTCTTTTCTCGGGGTGACCAAAGG          CCTCGAGAAACT -5′
                AlaProLeuValSer             GlyAlaLeu***
                 1   2   3   4   5          125 126 127
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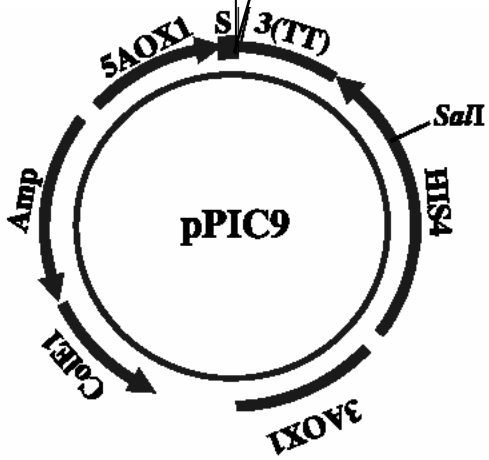


Figure 2 **The recombinant expression vectors for chimeric TTRs**

The recombinant expression plasmids for (A) croc/huTTR, (B) hu/crocTTR (C) trunctaded crocTTR and (D) pigC/crocTTR were constructed in pPIC9. Pink box, fragment of human TTR cDNA; grey box, fragment of *C. porosus* TTR cDNA; yellow box, fragment of pig TTR cDNA; 5AOX, promoter of alcoholoxidase1 gene in *P. pastoris*; 3(TT), native transcription termination and polyadenylation signal of alcohol oxidase1 gene; 3AOX1, sequence from the alcohol oxidase1 gene, 3 to the TT sequences; HIS4, histydinol dehydrogenase gene; Amp, ampicilin resistance gene; ColE1, *E. coli* origin of replication; S, α-factor secretion signal (269 bases); *Sal*I, Sal I restriction site for linearization of the vector. Numbering of the amino acid residues was based on that for human TTR (Aldred *et al*., 1997). The cDNA fragment of TTR from human, *C. porosus* and pig are represented by pink, grey and yellow boxes, respectively.

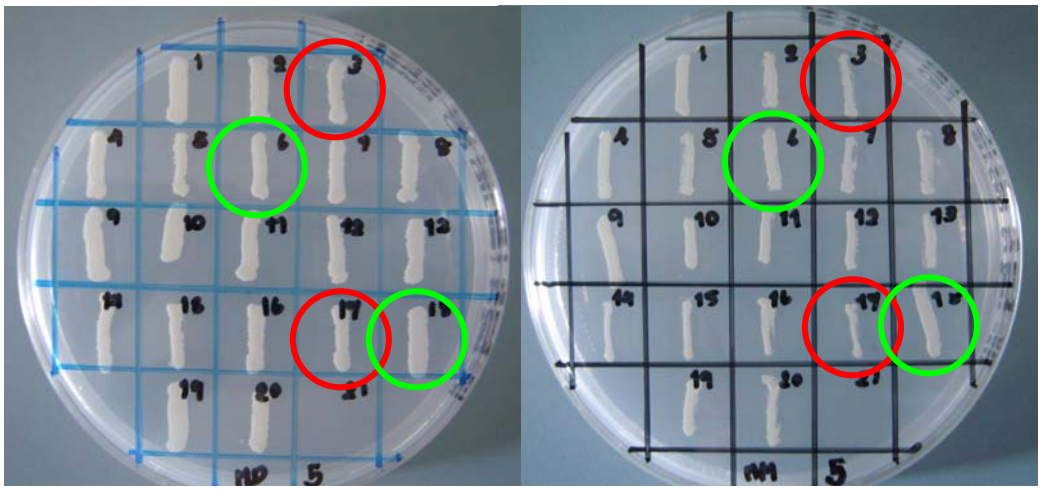
2. Screening for His^+Mut^+

The heterologous protein expression in *P. pastoris* uses the *AOX*1 promoter to drive an expression of the TTR genes. However, there is a possibility that cell would lose the *AOX*1 gene while transformation leading to the strain methanol utilization slow or Mut^S that exhibit poor growth on methanol media because of absence of the alcohol oxidase activity. The wild type (Mut^+) transformants that have ability to metabolize methanol could be differentiated from the Mut^S transformants by comparing their growth on the minimal methanol medium (MM) and the minimum dextrose medium (MD). Screening for the His^+Mut^+ clones was performed with 80 to 100 single colonies of His^+ transformants. The result showed that more than 90% of the *Pichia* His^+ transformants obtained had His^+Mut^+ phenotype (Figure 3). Approximate 40 single colonies of each His^+Mut^+ transformants were, thus, selected for the induction synthesis of the recombinant chimeric TTRs.

3. Production of the recombinant TTRs

3.1 Small scale production

The production of the chimeric TTRs was attempted in a small scale so that the transformant that provided the highest amount of TTR could be selected and optimization of the synthesis condition could be pursued. The *Pichia* His^+Mut^+ clones were induced with 0.5% methanol for synthesis in 5 ml of culture medium at 30°C for 3 to 5 days. Aliquots of the culture supernatant were analyzed by SDS-PAGE. By silver staining, it revealed the production of all chimeric TTRs was quite high. The approximate subunit mass determined by SDS-PAGE was 17 kDa for croc/huTTR and 14 kDa for hu/crocTTR, truncated crocTTR and pigC/crocTTR. Only one of the highest TTR producting clones from each construct was selected for the synthesis in larger scale.



MD **MM**

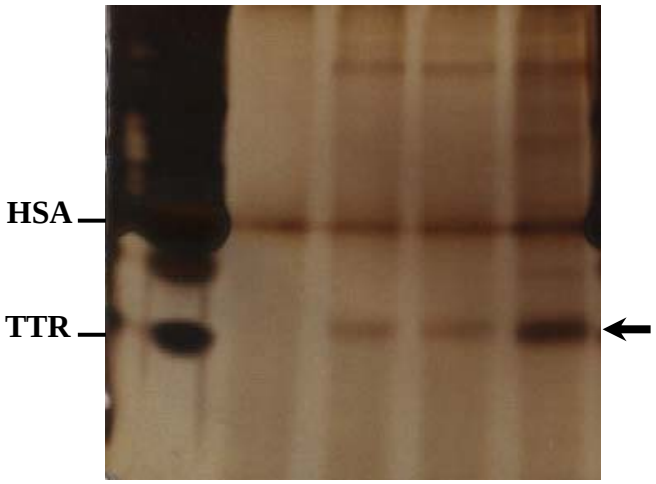
Figure 3 **Screening for His^+Mut^+ transformants**

The phenotype His^+Mut^+ can be differentiated from His^+Mut^s by comparing growth of the *Pichia* His^+ transformant between on MM and MD plates. In comparison, His^+Mut^+ clones grow better on these two media. Samples of His^+Mut^s and His^+Mut^+ clones are circled in red and green, respectively.

3.2 Large scale production

In order to obtained sufficient amount of the recombinant chimeric TTRs for further studies, the TTR clones were subjected to the protein synthesis in large scale. The shaking flask method was chosen for the production. In this method, the synthesis was carried out at 300 ml of BMMY using 2000 ml-flask. Aliquots of the culture were collected every 24 h and subjected to analysis by native-PAGE (10% resolving and 4% stacking gels). The secreted proteins were detected by silver staining. Kinetic of the TTR production and secretion from each type of the recombinant clone was shown in Figure 4. All recombinant chimeric TTRs first appeared after induction with 0.5% methanol for 24 h and reach maximum after 72 h of the induction. Very small or no degradation of the TTRs was detected after induction for 72 h. This indicated the stability of the recombinant chimeric TTRs produced by *P. pastoris*, similarly to that previously reported for other recombinant TTRs including that from *X. laevis* (Prapunpoj *et al*., 2000) and *C. porosus* (Prapunpoj *et al*., 2002). The production yield quite varied as shown in Table 3. Sixty to two hundreds milligrams of the recombinant TTRs could be synthesized and secreted in 1 liter of culture medium.

A



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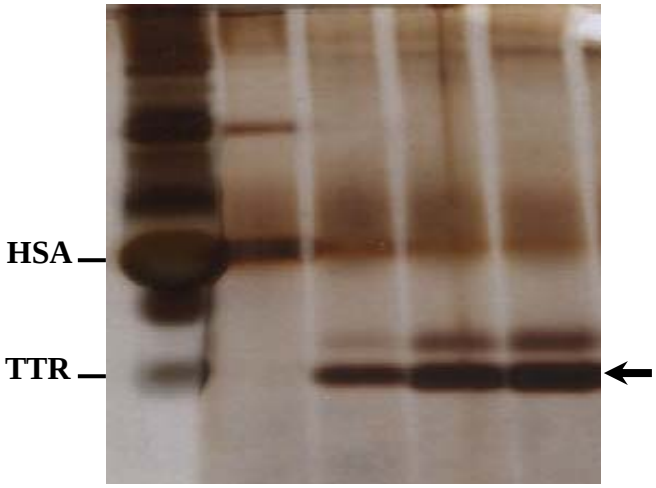

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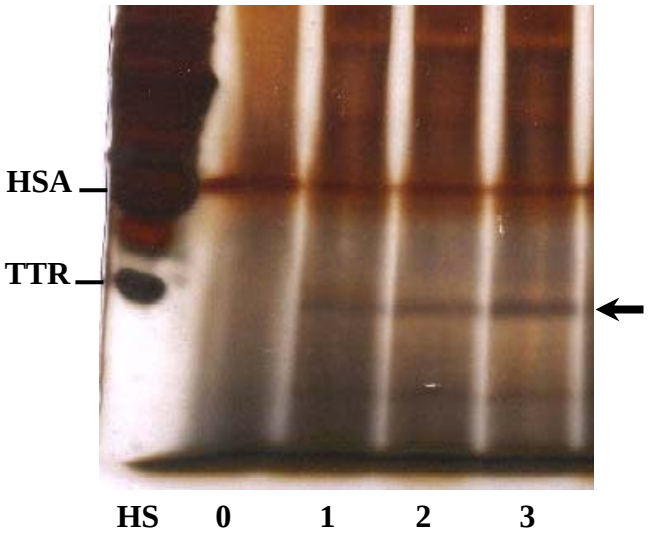

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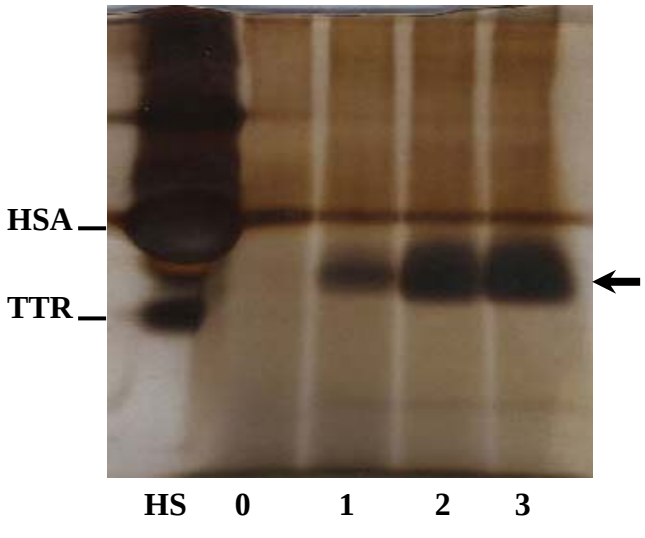


Figure 4 **Kinetic expression of the recombinant chimeric TTRs in *P. pasrotis***

The *Pichia* recombinant clones i.e. croc/huTTR (A); hu/crocTTR (B); truncated crocTTR (C); pigC/crocTTR (D) were induced with methanol for protein synthesis in large scale for 3 days. The culture medium was collected every 24 h and aliquot of the culture supernatant was analyzed. HS is human serum, which was overloaded to show positions of albumin (HSA) and TTR. Position of the expected TTR band from each clone was indicated by arrow. 0 to 3 is day(s) of the induction with methanol.

Table 3 Induction condition for large scale synthesis and production of the recombinant chimeric TTRs

Recombinant TTR	Methanol concentration (%)	Induction period (days)	Yield of TTR (mg/l of culture)
croc/huTTR	0.5	3	147
hu/crocTTR	0.5	3	220
pigC/crocTTR	0.5	3	60
truncated crocTTR	0.5	3	200

4. Purification of the recombinant chimeric TTRs

In comparison to *E. coli*, *P. pastoris* provide more advantages in production of eukaryotic proteins; in particular, *Pichia* extracellular secretes the synthesized foreign proteins together with only small amount of its endogenous proteins. This facilitates the purification process. The recombinant chimeric TTRs could be purified from the culture medium in a single step by affinity chromatography on a human RBP-Separose 4B column or by preparative discontinuous native-PAGE using the Bio-Rad Prep Cell. The purification by hRBP-Sepharose column is an efficient method to purify TTR from yeast culture medium (Prapunpoj *et al*., 2000, 2002) based on the fact that TTR and RBP specifically bind to each other to form a complex (Kanei *et al*., 1968, Peterson, 1971). However, large amount of RBP is required for preparing the column. Although the commercial hRBP is available, it is too expensive to set up a preparative column. Therefore, nn alternative effective purification method is needed. It has been demonstrated that TTR in plasma from all vertebrate species except those from pig and cattles migrated faster than albumin and other proteins in plasma under non-denaturing electrophoresis (Ingbar, 1958). This typical characteristic enabled to purify the protein by the preparative polyacrylamide gel electrophoresis under native condition (Richardson *et al*., 1994). Since all the recombinant chimeric TTRs showed the similar electrophoretic mobility to that observed in TTR from human plasma, the purification of the TTRs were mainly carried out by the preparative native-PAGE. The eluting

fractions collected and analyzed by native-PAGE showed a single protein with migration corresponding to TTR (Figure 5), indicating an effective of this purification method. However, the truncated crocTTR was not be effectively purified by the preparative native-PAGE because quite large amount of other proteins co-secreted into the culture medium, the next step of purification by affinity chromatography on the RBP-Sepharose was performed. A single protein peak corresponded to the truncated crocTTR was eluted out (Figure 6).

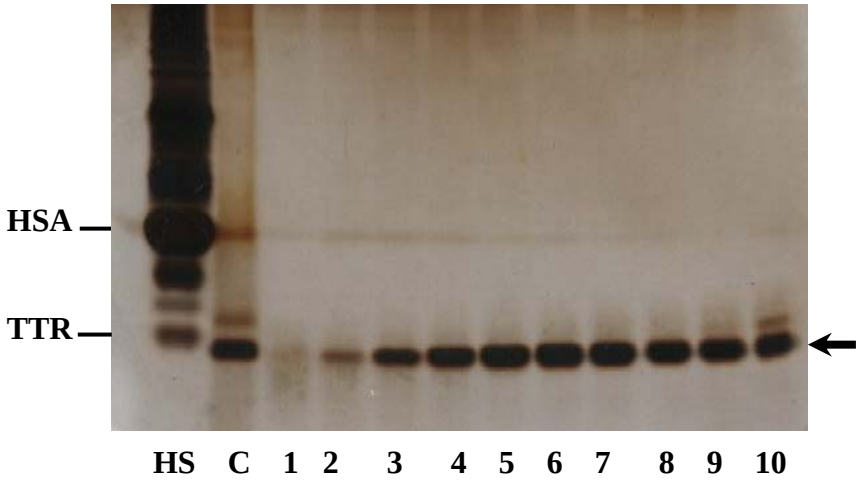


Figure 5 **The elution pattern of recombinant TTR separated by the preparative native-PAGE**

The concentrated yeast culture supernatant was applied onto a cylindrical polyacrylamide gel column (12% resolving and 4%stacking gels; 55 ml of gel). Eluting fractions were collected (2 ml/fraction) at flow rate 60 ml/h. To determine the presence of TTR in fractions, an aliquot (10 µl) of each fraction was analyzed by native-PAGE (10% resolving and 4%stacking gels). The protein bands were detected by silver staining. HS, human serum; C, concentrate culture supernatant; 1 to 10, an individual eluting fractions; HSA, albumin in human serum; TTR, TTR in human serum. The expected TTR band in each fraction was indicated by arrow.

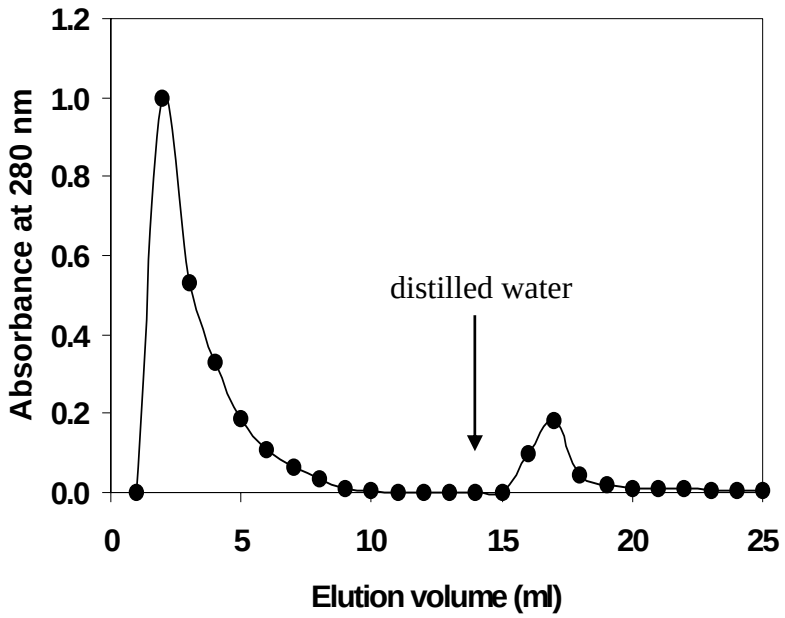


Figure 6 **The affinity chromatogram of truncated crocTTR on hRBP-Sepharose**

The induction synthesis was carried out with 0.5% methanol for 3 days. The culture supernatant was applied onto a column of hRBP-Sepharose (1 ml of gel) equilibrated in 0.05 M Tris/HCl, pH7.4 buffer containing 0.5 M NaCl. The column was washed and the bound protein was eluted with distilled water. The separation was carried out at room temperature with flow rate 10 ml/h, and eluting fractions were collected at 4 ml/fraction.

5. Physicochemical properties of recombinant chimeric TTRs

Although heterologous protein expression system of *P. pastoris* has many of post-translational modifications similar to those presence in higher eukaryotes, some of them slightly differ from those in mammals. For example, the carbohydrate moieties those incorporated into the secreted proteins produced by *P. pastoris* are predominantly composed of mannose residues and the hyperglycosylation was often detected in proteins (Cereghino and Cregg, 2000). These differences in post-translational modifications can lead to the alterations of properties and, thus, functions of the proteins produced by *Pichia*. To ascertain the possible unwanted post-translational modifications, the N-terminal sequence and the physicochemical properties including the electrophoresis mobility under non-denaturing condition, molecular mass of the monomer, molecular weight of the tetramer, and the immunochemical reactivity of the recombinant TTRs were determined.

5.1 N-teminal sequence

To ascertain whether the chimeric TTRs were synthesized and the α-factor presegment cleavage was correctly processed or not, the N-terminal sequence of the proteins was determined by animo acid sequencing. The analysis revealed that the first four or eight amino acid residues in the N-terminal sequences of croc/huTTR, truncated crocTTR and pigC/crocTTR were as expected, except only two additional amino acid residues, E and A, were detected at the N-terminal sequence of hu/crocTTR resulted to **E A G P** rather than **G P T G** are the first four amino acid residues at the N-terminal sequence of this TTR. This indicated that cleavage of the α-factor signal sequence by KEX2 protease occurred highly accurate.

5.2 Mobility in non-denaturing gel

At pH 8.6, TTRs from most vertebrates including human and birds (Chang *et al*., 1999) electrophoretically migrate faster than albumin. Only some eutherians i.e. pig and cattles, TTRs were found co-migrate with albumin in non-denaturing gel (Farer *et al*., 1962; Refetoff *et al*., 1970). From the analysis by native-PAGE, the mobilities of croc/huTTR and hu/crocTTR in tetrameric form were faster than albumin in human serum (Figure 7), while truncated crocTTR and pigC/crocTTR migrated a little bit slightly slower, however faster than albumin. The slower migration rate may be resulted from the difference in the overall charges of the TTRs. The migration under native condition of all these synthesized chimeric TTRs, which is faster than albumin, is

correlated to the typical characteristic of most vertebrate TTRs found in nature (Richardson *et al*., 1994). This confirmed that the recombinant chimeric TTRs produced by the synthesis system of *P. pastoris* have the same proper physiological structure and molecular folding as TTRs in nature.

5.3 Molecular mass of the TTR monomer

To determine the subunit masses, SDS-PAGE (12% resolving and 4% stacking gel) was adopted and the protein bands were visualized by staining gel with Coomassie blue. The migration of the chimeric TTRs was shown in Figure 8. The TTRs showed slightly differences in mobility comparing to each others. Besides a major protein band corresponding to TTR monomer was detected, an additional band corresponding to TTR dimer was also observed with a slower migration than the monomer. The dimer of TTR usually occurs when denaturing of the protein is not complete even with hash condition (Dickson *et al*., 1982; Furuya *et al*., 1989; Prapunpoj *et al*., 200b and 2002), indicating to strength of dimer-dimer interaction in the TTR molecule. The subunit masses of the recombinant chimeric TTRs were ~15 kDa to ~17 kDa in range (Table 4), which are similar to that of TTR purified from human plasma (16.1 kDa). In comparison to those directly calculated from the derived amino acid sequences, the subunit masses obtained from SDS-PAGE were a little bit higher (Table 4).

5.4 Molecular weight of chimeric TTR tetramers

Molecular weights of all the chimeric TTRs synthesized in *P. pastoris* were determined by HPLC using the Bio Sil-SEC column. It showed that the tetrameric masses of all chimeric TTRs produced in *Pichia* were 50 kDa to 60 kDa in range (Table 5), which are ~4 times of the subunit mass. This indicated that the recombinant TTRs could be successfully formed tetramer during synthesis in *P. pastoris*.

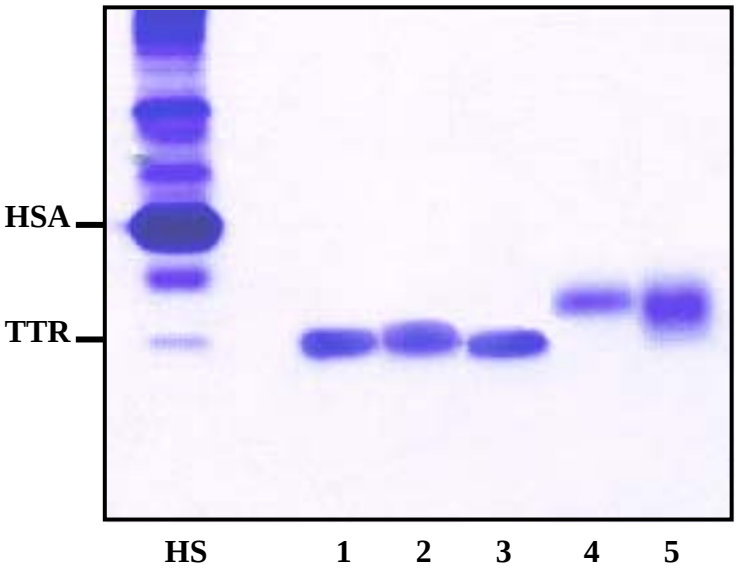


Figure 7 **The mobility in native-PAGE of recombinant chimeric TTRs**

The purified plasma human TTR (1), croc/huTTR (2), hu/crocTTR (3), pigC/crocTTR (4) and truncated crocTTR (5) were separated by native-PAGE (10% resolving and 4% stacking gels) and the protein bands were visualized by Coomassie blue staining. Human serum (HS) was overloaded to indicate the TTR (TTR) and albumin (HSA) in serum.

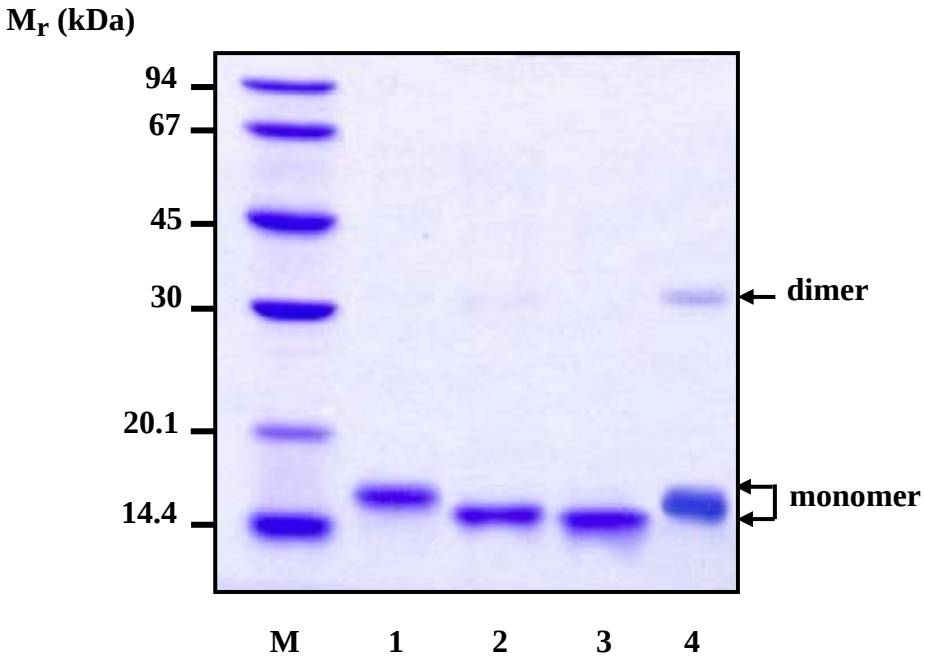


Figure 8 **Analysis by SDS-PAGE of the recombinant TTRs**

The purified recombinant hu/crocTTR (1), pigC/crocTTR (2), truncated crocTTR (3), and croc/huTTR (4) were boiled for 30 min in the presence of 2.5% 2-mercaptoethanol and 2% SDS prior the protein separation was carried out by 12% SDS-PAGE. Protein bands were stained with Coomassie blue. M was standard protein markers. Positions of TTR monomer and dimer were indicated.

Table 4 **The subunit masses of recombinant chimeric TTRs determined by SDS-PAGE and by directly calculated from the deduced amino acid sequences**

Type	Determined by SDS-PAGE (kDa)	Determined from deduced amino acid sequence (kDa)
croc/huTTR	15.3	14.1
hu/crocTTR	16.3	14.8
pigC/crocTTR	15.1	13.7
truncated crocTTR	14.8	14.1

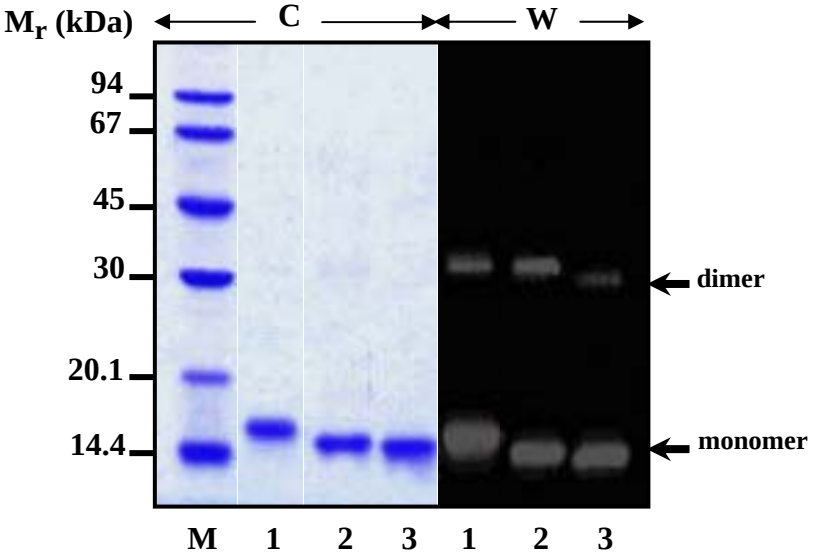
Table 5 **Molecular weight of recombinant chimeric TTR tetramers**

Type	Molecular weight (kDa)
croc/huTTR	63
hu/crocTTR	63
pigC/crocTTR	54
truncated crocTTR	51

5.5 Immunochemical cross-reactivity of the recombinant chimeric TTRs

The immunochemical reactivity to a specific antibody is one of the effective strategies to identify or confirm the presence of the protein of interest. The purified chimeric TTRs were resolved on SDS-PAGE prior they were electrophoretically transferred onto nitrocellulose membranes. The antiserum against human TTR purified from serum or against recombinant crocTTR was used as a primary antibody. After the peroxidase activity was detected by ECL, two bands with immuno-reactivity signal were observed for all recombinant chimeric TTRs (Figure 9). The first specific band, with very high intensity, migrated to the same position corresponded to the TTR subunit detected by Coomassie blue staining. In addition, the TTR dimer could also be detected with a migration corresponding to mass ~30 kDa. These results confirmed the recombinant chimeric TTRs were successfully produced in *Pichia* cell.

A


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

B

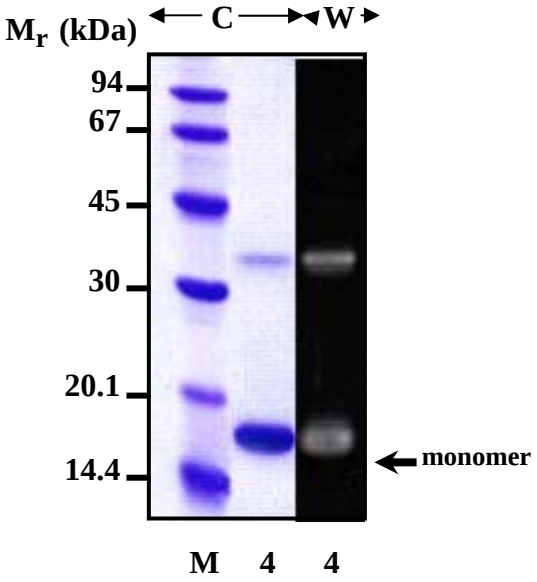

Mr (kDa)
C
W
94
67
45
30
20.1
14.4
monomer
M
4
4

Figure 9 **Western analysis of recombinant chimeric TTRs**

The chimeric TTRs were separated on SDS-PAGE and then subjected to Western analysis. The protein band were stained with Coomassie blue (Coomassie) while Western analysis was carried out using rabbit antiserum raised against crocTTR (1:500) (A) or sheep antiserum against serum huTTR (1:2500) (B) as the primary antibody. The horseradish peroxidase (HRP)-linked rabbit IgG antibody and the HRP-linked sheep IgG antibody at 1:2500 dilution were used as the secondary antibody for crocTTR and serum huTTR, respectively. M, standard low molecular weight protein markers; 1, hu/crocTTR; 2, pigC/crocTTR; 3, truncated crocTTR; 4, croc/huTTR. Detection was performed using Enhance Chemiluminescence kit (Amersham). The position of TTR monomer and dimer were indicated.

6. The effect of change in N- and C-terminal sequences on binding to hRBP

One of the main functions of TTR is to transport of vitamin A via binding to RBP. The binding of TTR has believed to prevent loss of RBP through glomerular filtration in kidney (Kanai *et al.*, 1968; Raz and Goodman, 1969; Goodman, 1974; Noy *et al.*, 1992). Binding affinity of these two proteins from various animal species varied from 10^{-6} to 10^{-7} M depending on the techniques used (Noy *et al.*, 1992; Kopelman *et al.*, 1976). One to up to 2 molecules of RBP per TTR tetramer was demonstrated participating in the binding (for review sees Monocot *et al.*, 2000). Both TTR and RBP contribute each 21 amino acids to the interaction of the complex, and most of these involved residues locate at C-terminal regions of the proteins (Naylor and Newcomer, 1999). There was no report mentioned to the involvement or influence of the TTR N-terminus on this interaction. However, since this region remarkably changes during evolution of vertebrates and locates closely to the C-terminal region at the entrance to the central channel of the TTR tetramer, the question arises whether changes in amino acid sequence of the N-terminal region involves with the binding between TTR and RBP.

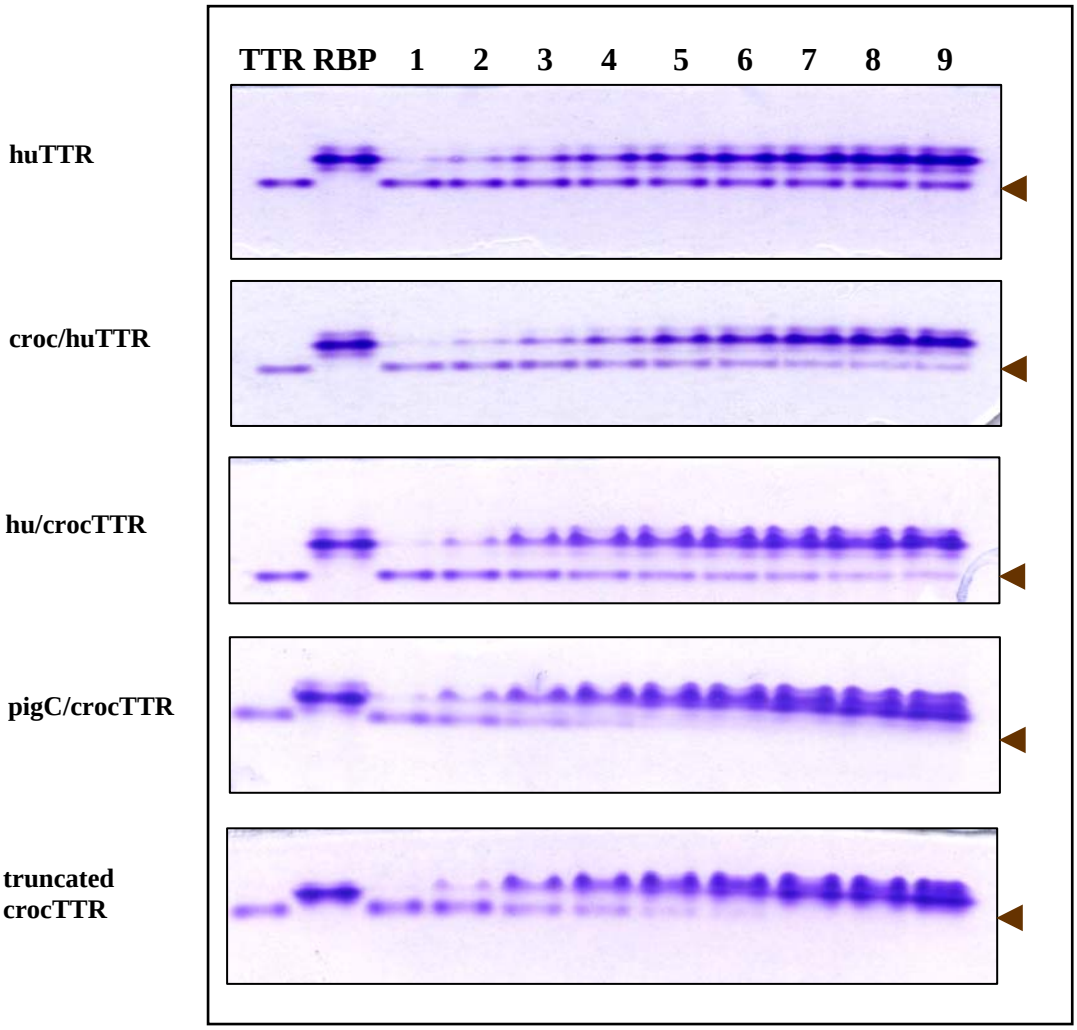
To reveal, the binding interaction between chimeric TTRs and hRBP were performed and it was compared to wild type TTRs. TTR and hRBP at different molar ratios were allowed to bind at 4°C for 1 h prior free and bound forms of TTR and RBP were separated by electrophoresis and quantitatively analyzed by western analysis using a specific TTR antiserum. The kinetic of the binding could be directly observed in gel (Figure 10A), and it was confirmed by probing with the specific antibodies to TTR (Figure 10B). In comparison, free hu/crocTTR and croc/huTTR decreased when higher amount of RBP was added, similary to that observed in human TTR purified from plasma, however, at slower rate than other tested TTRs. These should imply to an effect of N-terminal sequence changing on binding affinity to hRBP of these chimeric TTRs.

To determine Kd of the binding, the concentrations of bound and free RBP are required. Although free and bound RBP could be detected by using a specific antibody raised against RBP, their amount could not be determined because of the overlapping of these two protein bands. In contrast, the protein bands of free and bound TTRs could be clearly separated from each other (Figure 10A), and the fluorescent signals of these two bands could be accurately determined. Therefore, the concentration of bound RBP was indirectly determined from the concentration of bound TTR with the assumption that

TTR binds to RBP at 1:1 molar ratio. Free RBP was directly obtained by subtraction of the bound from total RBP.

By Scatchard analysis, all chimeric TTRs, particularly croc/huTTR and hu/crocTTR, showed significant change in Kd in comparison to the wild-type (Figure 11). This should demonstrate the influence of N- and C-terminal sequences on binding of TTR to RBP. Interesting, it seems likely that changing in the N-terminus rather than the C-terminus had more effect on the binding affinity to hRBP. In addition, based on hydropathy of these two region sequences, the results primary suggested that changing the N-terminal sequence to more hydrophilic decrease the binding affinity of TTR to hRBP. Although most of the amino acids from TTR that are involved in the TTR-RBP complex formation locate at the C-terminal region of the TTR subunit (Naylor and Newcomer, 1999), alteration in K_d of pigC/crocTTR from its wild type was not observed (Table 6). This should confirm the strong influence of the N-terminal segment on the binding and complex formation between TTR and RBP.

A


TTR RBP 1 2 3 4 5 6 7 8 9
huTTR
croc/huTTR
hu/crocTTR
pigC/crocTTR
truncated crocTTR

B

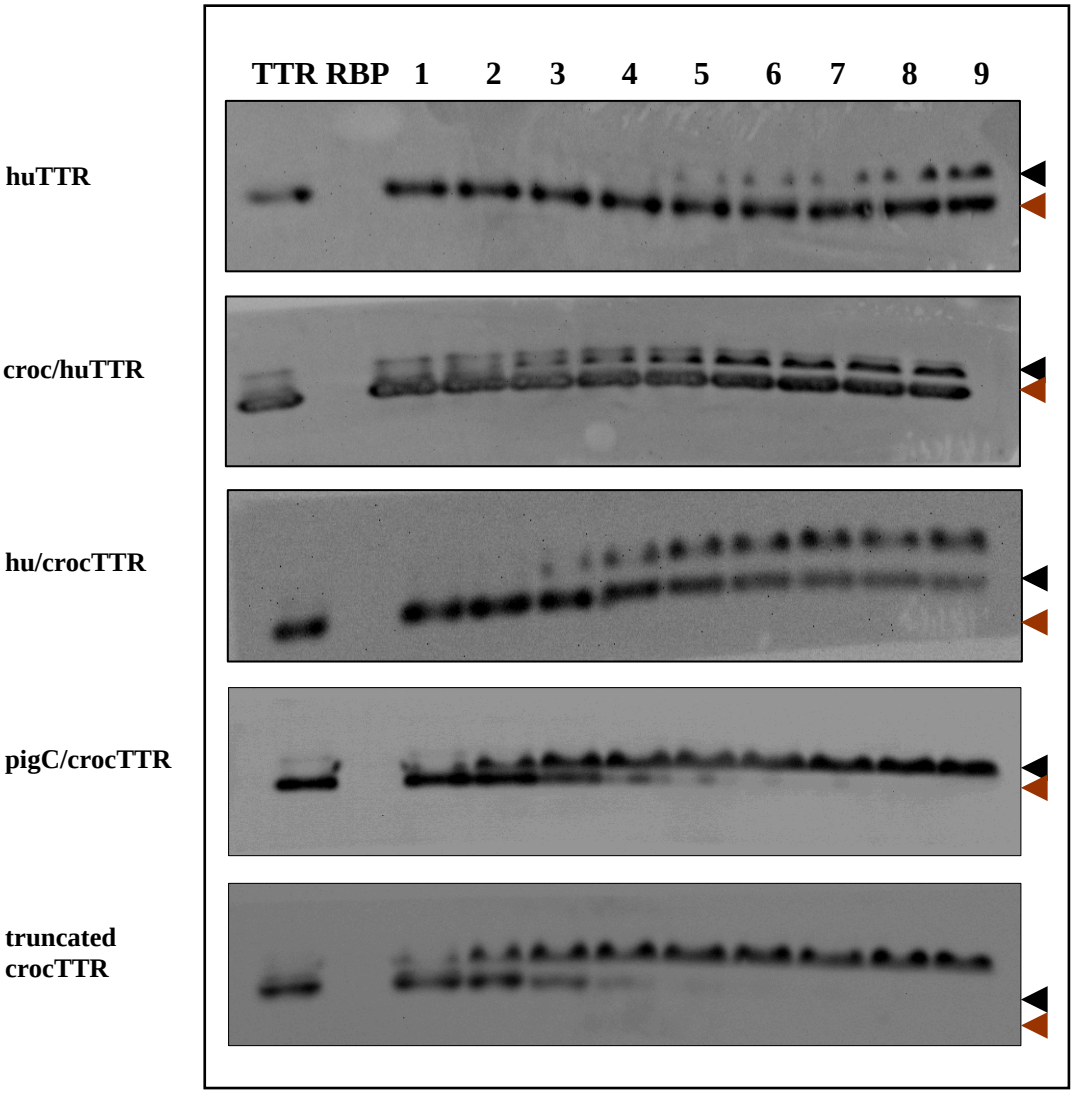

TTR RBP 1 2 3 4 5 6 7 8 9
huTTR
croc/huTTR
hu/crocTTR
pigC/crocTTR
truncated crocTTR

Figure 10 **Analysis of the binding of chimeric TTRs to RBP**

Purified recombinant chimeric TTR (0.4 μg) was incubated with various amount of purified hRBP (0 to 0.4 μM), at 4°C for 1 h prior the reaction mixture was analyzed by native-PAGE (10% resolving and 4% stacking gels). Proteins were identified by staining with Coomassie blue (A). Bound and free TTRs were identified by western blot analysis followed by ECL using anti-human TTR antibody (for huTTR and croc/huTTR) or anti-crocTTR (for hu/crocTTR, pigC/crocTTR and truncated crocTTR) as the primary antibody (B). Purified TTR (TTR) or hRBP (RBP) alone was added as controls. 1 to 9 was the reaction mixture contained RBP:TTR to a final molar ratio of 0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1 and 8:1, respectively. The bound and free TTRs were indicated by black and brown arrowhead, respectively.

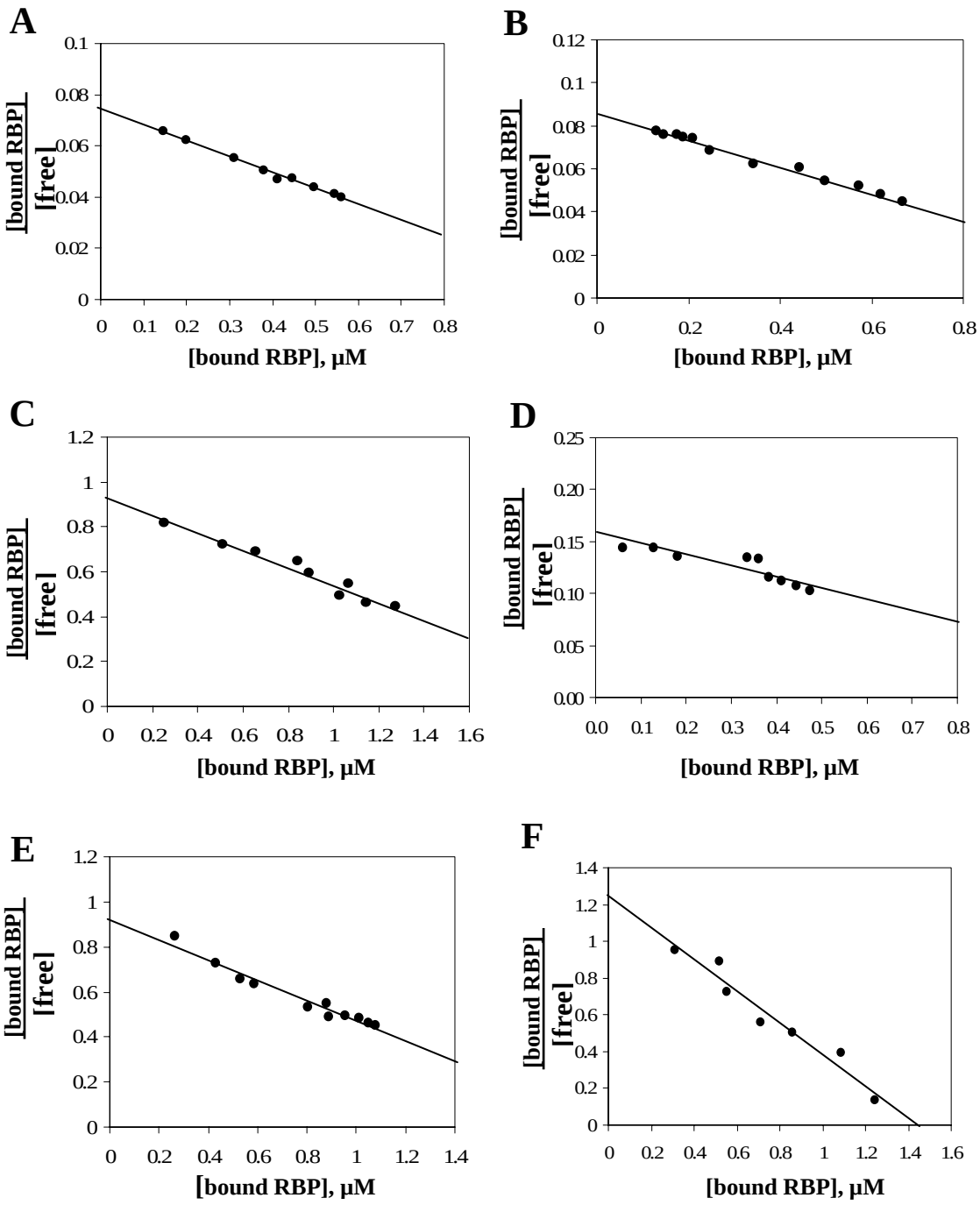

A
[bound RBP] / [free]
0.1
0.08
0.06
0.04
0.02
0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
[bound RBP], µM
B
[bound RBP] / [free]
0.12
0.1
0.08
0.06
0.04
0.02
0
0 0.2 0.4 0.6 0.8
[bound RBP], µM
C
[bound RBP] / [free]
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6
[bound RBP], µM
D
[bound RBP] / [free]
0.25
0.20
0.15
0.10
0.05
0.00
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
[bound RBP], µM
E
[bound RBP] / [free]
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1 1.2 1.4
[bound RBP], µM
F
[bound RBP] / [free]
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1 1.2 1.4 1.6
[bound RBP], µM

Figure 11 Scatchard plots of the binding between TTRs and hRBP

TTRs and hRBP with different molar ratio were incubated at 4 °C for 1 h prior the Western blot analysis was performed. Bound and free hRBP were determined, and dissociation constant (K_d) of the binding was determined. At least four independent replications were carried out in each experiment. A, human TTR purified from plasma; B, croc/huTTR; C, crocTTR; D, hu/crocTTR; E, pigC/crocTTR; F, truncated crocTTR.

Table 6 The K_d of the binding between TTRs and hRBP

TTR	K_d (μM)
plasma huTTR	17.08 ± 3.25
croc/huTTR	22.52 ± 5.84
crocTTR	2.54 ± 0.23
hu/crocTTR	11.31 ± 0.07
pigC/crocTTR	2.34 ± 0.45
truncated crocTTR	1.19 ± 0.19

7. The effect of change in N- and C-terminal sequences on proteolytic activity TTR

In nature many proteases are involved in physiology and pathology. The proteolysis mechanism and the specific substrate for most these enzymes well defined. The proteolytic activity of TTR was recently demonstrated and it was classified as a cryptic enzyme involving in Alzheimer's disease (AD) (Liz *et al.*, 2004). The cryptic nature of TTR was defined based on its lacking of the canonical structure for protease determinants. In addition, its physiological function is apparently unrelated to normal proteolysis (for review sees Liz and Sousa, 2005) since only three substrates have been revealed so far i.e. apoA-I, amyloid β peptide and neuropeptide Y (NPY). To reveal the effect of N- and C-terminal segments on the catalytic activity of TTR *in vitro*, cleavage of two substrates, i.e. casein and apoA-I, by the chimeric TTRs originated from crocTTR were performed.

7.1 using casein-FITC as substrate

The caseinolytic activity was determined following the method described by Twining (1984). The uncleaved casein was precipitated out by TCA and the supernatant was determined for the cleaved product by fluorospectrophotometer. The FITC-casein controls with and without precipitation by TCA, were included to determine autolysis. The result showed that the autolysis of FITC-casein at 37^{o}C was ~30-40% over 12 h (data not shown). This was not significantly effect on the assay preformed here. The catalytic rates of each chimeric TTR were summarized in Table 7. The result revealed

that all chimeric TTRs except truncated crocTTR can cleave the substrate, however, at higher rates than the wild type TTR, indicating that the proteolytic activity of TTR changed when its N- or C-terminal sequence was changed. This suggested that some amino acid residues that locate in these two regions are directly participated in the catalytic site of TTR or, if not, possibly facilitate the accession to the active site of substrate. In comparison to pigC/crocTTR, hu/crocTTR had much slower rate, suggesting to more effect of the residues in the C-terminus than in the N-terminus. However, since truncated crocTTR did not show activity even at high amount of the TTR (data not shown). The possibility that active site locates at the N-terminus could not be ruled out.

Table 7 Proteolytic activity of TTRs using FITC-casein as substrate

TTR	Activity(nM/min)*	Relative activity to crocTTR (folds)
crocTTR	0.64 ± 0.03	1
hu/crocTTR	0.98 ± 0.12	1.53
pigC/crocTTR	5.16 ± 0.12	8.06
truncated crocTTR	0	0

* Results were shown as mean ± SD from 3 experiments.

7.2 using apoA-I as substrate

TTR was allowed to digest apoA-I at 37°C prior the cleaved and uncleaved products were analyzed by SDS-PAGE. The result showed that two fragments corresponding to uncleaved and cleaved (with faster mobility) apoA-I were observed and these were clearly separated from each other on 20% polyacrylamide gel (Figure 12). After longer incubation, intensity of the cleaved fragment became faint and other smaller fragments were observed (data not shown). However, there was no cleavage or degradation of apoA-I could be observed in the reaction without TTR even incubation was performed longer than 3 h (data not shown). This suggested that apoA-I was first

specifically cleaved into a shorter fragment which was more susceptible to later digestion, however, with less specificity to many small fragments. To determine the catalytic rates, the cleavage products at various incubation times were performed. Intensity of cleaved and uncleaved apoA-I were analyzed. The catalytic activity of each TTR was shown in Table 8. In comparison to crocTTR, pigC/crocTTR showed ~4 folds higher catalytic rate on apoA-I. In contrast, hu/crocTTR and trancated crocTTR had slower rate than the wild type. Similar to the experiment on casein, the catalysis to apoA-I suggested the contribution of amino acid residues in N- and C-terminal regions on proteolytic activity of TTR which could lead to the possible involvement of these two terminal segments in novel function of TTR as a protease.

8. The protective ability of chimeric TTR on toxicity of Aβ

8.1 Cleavage of Aβ

The native human TTR has been demonstrated having a protective role on Aβ toxicity via cleaving Aβ monomer (Liz *et al*., 2004; Costa *et al*., 2008). Among the chimeric TTRs produced in this project, pigC/crocTTR showed the highest catalytic activity either to casein or apoA-I. Therefore, this chimeric TTR was chose to determine the ability to inhibit the cytoxicity induced by Aβ. By analysis on SDS-PAGE of the reaction mixture between pigC/crocTTR and Aβ, the band corresponding to the Aβ (1-42) monomer was lesser intense in comparison to the Aβ incubated alone (Figure 13). In addition, the kinetic study showed that the Aβ monomer band was fainter with increasing incubation time. In comparison to TTR isolated from human serum, the cleavage by the chimeric TTR was faster (Figure 13). The wild type crocTTR did not cleave Aβ monomer within the same incubation period (data not shown). This confirmed specificity and higher proteolytic activity on Aβ of this constructed TTR.

8.2 Inhibition of Aβ-induced cytotoxicity

The ability of pigC/crocTTR to inhibit the toxicity induced by Aβ on fibroblast cells was performed. Aβ (2 μM) and the purified chimeric TTR (1 μM) was added simultaneously to the cell culture or they pre-incubated prior the addition. To determine the intrinsic toxicity, Aβ or TTR alone was also added to the cells. After 72 h of incubation, MTT reduction and LDH release assay were determined as described. By MTT, It showed that exposure of the cells to Aβ resulted in reduction of the cellular

metabolic activity (Figure 14A). Only ~27% formazan was detected, indicating that under the condition the cells were metabolically inactive. However, in the presence of pigC/crocTTR, the fibroblast cells were more inactive, the formazan production increased up to ~33% compared to control. This indicated that the toxicity of Aβ decreased in the presence of the TTR. The protective effect of the chimeric TTR was confirmed by LDH release assay. The cell viability of 30% relative to control cells, as measured by LDH release (Figure 14B). In the presence of TTR, cell viability and membrane integrity increased to ~22% and ~33%, respectively (Figure 14). Moreover, pre-incubation between Aβ and TTR before adding to cells gave higher membrane integrity of cells in comparison to without pre-incubation. Together with MTT, these results suggested that TTR could protect cells from death by inhibit the effect of Aβ on both metabolic activity and the plasma membrane integrity of cells.

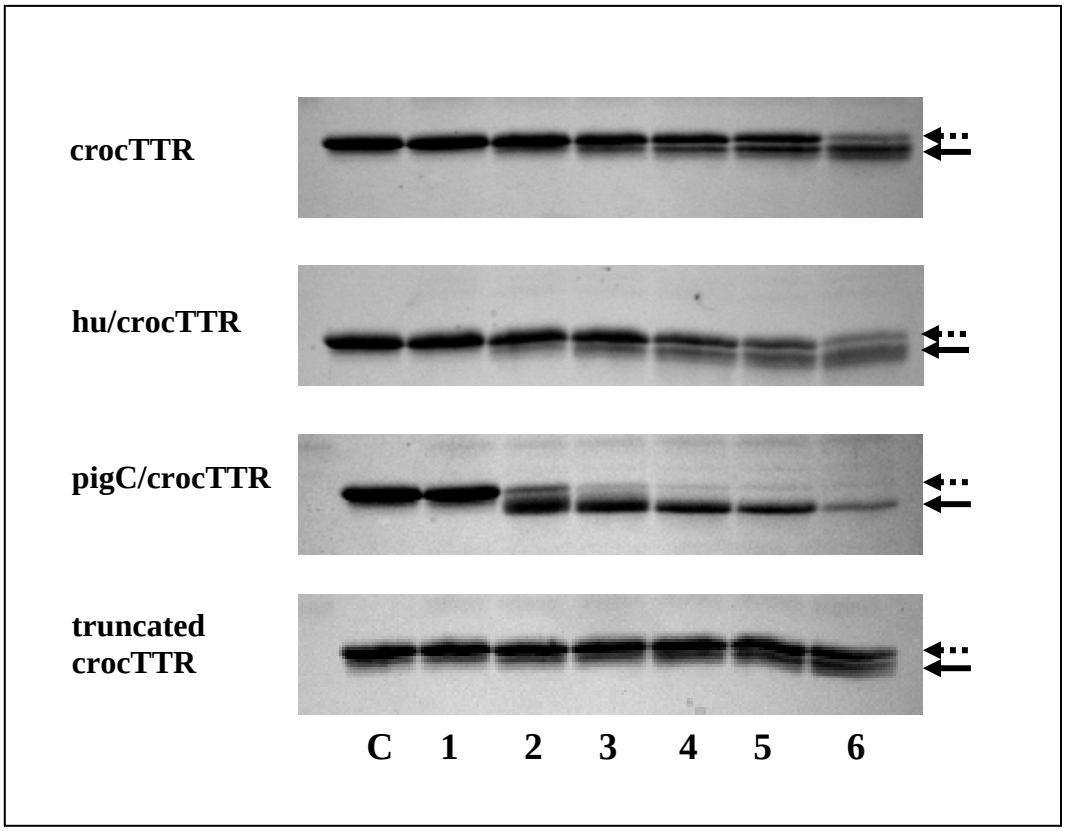


Figure 12 **The ApoA-I cleavage by chimeric TTRs**

Aliquots of ApoA-I (1 μg) and TTR (2 μg) were incubated at 37°C for 6 h. The reaction was then terminated and analyzed by SDS-PAGE on 20% polyacrylamide gel, and protein bands were detected by staining gel with Coomassie blue. C, ApoA-I alone; 1 to 6, the reaction mixture after incubation for 0, 30, 60, 120, 180 and 360 min, respectively. Positions of uncleaved and cleaved ApoA-I were indicated by dot arrow and solid arrow, respectively.

Table 8 The apoA-I cleavage activity of chimeric TTRs

TTR	Activity * (ng /min)	Relative activity to crocTTR (folds)
crocTTR	7.15 ± 1.15	1
hu/crocTTR	5.55 ± 0.62	0.78
pigC/crocTTR	26.17 ± 0.98	3.66
truncated crocTTR	2.16 ± 0.91	0.3

* Results were shown as mean ± SD from 3 experiments.

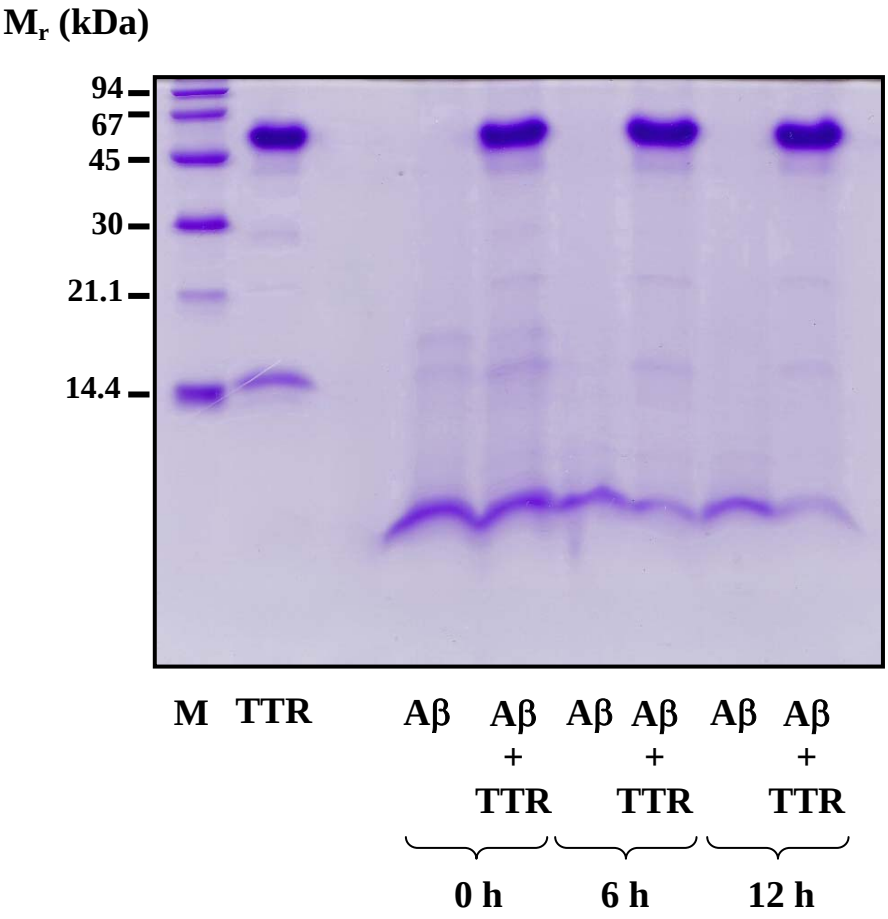
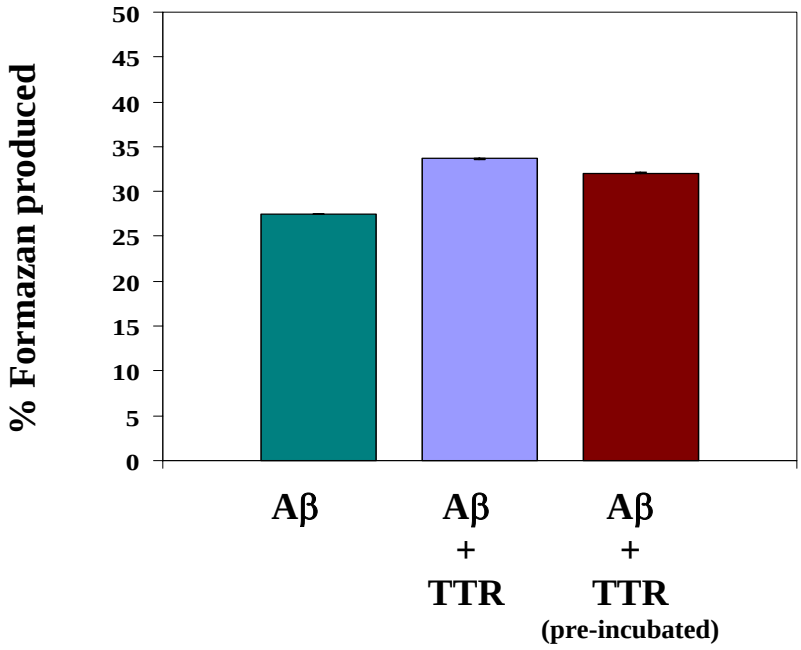


Figure 13 **SDS-PAGE patterns of Aβ cleavage by chimeric TTR**

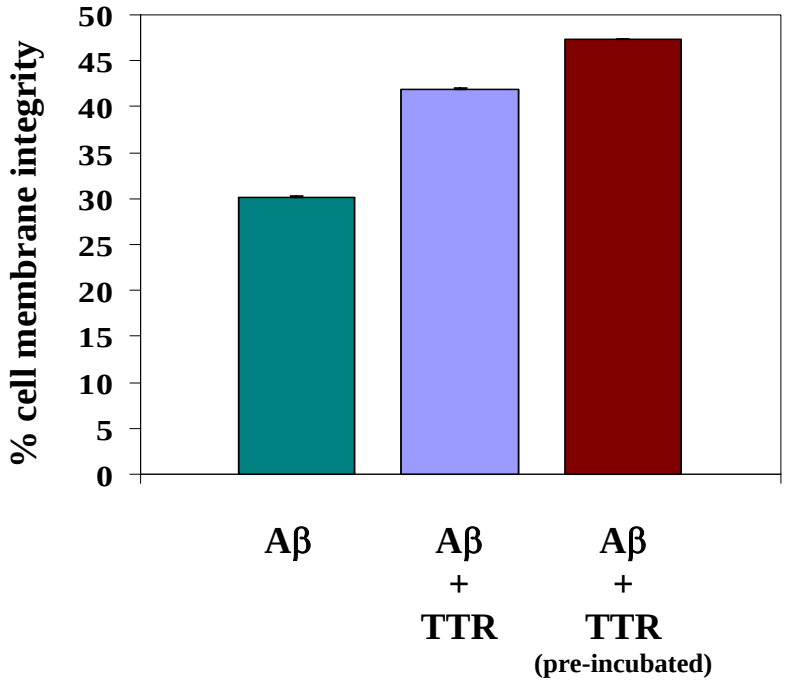
Aβ (1-42) (5 µg) and pigC/crocTTR (5 µg) were incubated at 37^{o}C for 0, 6 and 12 h. The reaction was stopped by immediately frozen the reaction mixture in liquid nitrogen. Then, all reaction mixtures were analyzed on 16.5% tricine SDS-PAGE (non-reducing condition). The protein bands were visualized by staining with Coomassie blue. Arrow indicates the position of Aβ (1-42).

A



	% Formazan produced
Aβ alone	27.46 ± 0.01
Aβ + TTR	33.65 ± 0.01
Aβ + TTR (pre-incubated)	32.07 ± 0.01

B



	% cell membrane integrity
Aβ alone	30.19 ± 0.05
Aβ + TTR	41.96 ± 0.03
Aβ + TTR (pre-incubated)	41.34 ± 0.02

Figure 14 Protective ability of chimeric TTR on amyloid cytotoxicity

Fibroblast cells were treated with Aβ alone or in the presence of pigC/crocTTR for 72 h prior the protective effect of the chimeric TTR on the cytotoxicity of Aβ was measured by MTT reduction (A) and LDH release (B) assays. The concentrations of Aβ and the chimeric TTR were 2 μM and 1 μM, respectively. Aβ and TTR were added to cells at the same time or they were pre-incubated for 12 h before the addition. The MTT result was expressed as percentage of formazan produced in treated cells compared to cells treated with medium only. Whereas, the LDH release quantitative was expressed as percentage of cell membrane integrity in treated cells compared to cells treated with medium only (100% value), with the zero value corresponding to cells that were lysed with detergent prior LDH release measurement as described in Materials and methods. All experiments were done in triplicate.

CONCLUSION

In this research, the cDNAs coding for chimeric TTRs were successfully constructed in pPIC9. By using the heterologous gene expression system of *P. pastoris*, the cDNAs were expressed; the proteins were synthesized and extracellularly secreted into culture medium outside yeast cell. All these chimeras contain physicochemical properties including subunit masses, tetramer formation and immuno-reactivity similarly to those found in nature. Functions of TTR that require, either directly or indirectly, the participation of the amino acid residues in these two regions including binding to RBP and proteolytic activity were examined. Changing in either N-terminal or C-terminal sequence of the subunit was demonstrated leading to changes in properties of TTR tetramer. Although the amino acid residues lining in the active site and those directly interact to substrate during the catalytic process of TTR can not be directly revealed, role of these terminal segments on the binding to RBP and the proteolytic activity of casein and apoA-I was clearly demonstrated. In addition, since the chimeric TTR had ability to cleave and inhibit the cytoxicity induced by Aβ which is one of the main causes of AD. The chimeric TTR may be useful as a therapeutic agent for AD in future.

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