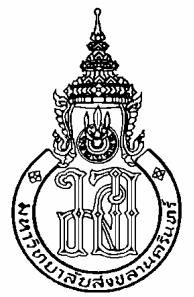

Influence of N-and C-terminal Structures on Function of Transthyretin

Jiraporn Khwanmunee

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science in Biochemistry

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Thesis Title	Influence of N-and C-terminal Structures on Function of Transthyretin
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ชื่อวิทยานิพนธ์ อิทธิพลของโครงสร้างปลายอะมิโนและปลายคาร์บอกซิลต่อหน้าที่ของ transthyretin
ผู้เขียน นางสาวจิราพร ขวัญมุณี
สาขาวิชา ชีวเคมี
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บทคัดย่อ

Transthyretin (TTR) เป็นโปรตีนที่ทำหน้าที่ขนส่งฮอร์โมนไธรอยด์และวิตามินเอ โดยจับกับ retinal binding protein (RBP) โปรตีนชนิดนี้พบในพลาสมาของสัตว์มีกระดูกสันหลังหลายชนิด ซึ่งในคน TTR มีแหล่งสร้างที่สำคัญ 2 แหล่งคือ ตับและ choroid plexus ในสมอง จากการศึกษาพบว่าในระหว่างวิวัฒนาการของสัตว์มีกระดูกสันหลังชั้นต่ำจนมาถึงสัตว์เลี้ยงลูกด้วยนมนั้น พบว่าความสามารถในการเข้าจับของ TTR กับ thyroxine (T4) เพิ่มขึ้น และในขณะเดียวกันจะจับกับ triiodothyronine (T3) ลดลง นอกจากนี้ปลายอะมิโนของ TTR จะสั้นลงและมีความชอบน้ำมากขึ้น และปลายคาร์บอกซิลของ TTR จะมีความชอบน้ำมากขึ้นเช่นกัน และยังพบอีกว่าปลายคาร์บอกซิลของ TTR ในสิ่งมีชีวิตบางชนิด มีความยาวของลำดับกรดอะมิโนมากกว่า TTR ในสิ่งมีชีวิตชนิดอื่น นอกจากนี้ปลายอะมิโนและปลายคาร์บอกซิลของ TTR มีตำแหน่งอยู่ใกล้กับบริเวณตำแหน่งจับสำหรับฮอร์โมนไธรอยด์ ดังนั้นจึงนำไปสู่สมมติฐานที่ว่าถ้ามีการเปลี่ยนแปลงโครงสร้างของปลายอะมิโนและ/หรือปลายคาร์บอกซิลของ TTR น่าจะมีผลต่อการเข้าจับของ TTR กับฮอร์โมนไธรอยด์และการจับกับ ligand อื่นๆ

การจะสามารถศึกษาถึงอิทธิพลของโครงสร้างปลายอะมิโนและปลายคาร์บอกซิลต่อหน้าที่ของ TTR นั้น จำเป็นต้องทำการสังเคราะห์ chimeric TTR ที่ถูกเปลี่ยนแปลงลำดับกรดอะมิโน ณ บริเวณที่ต้องการศึกษา (hu/croc/pig TTR) และเชื่อมต่อเข้ากับเวคเตอร์ *pPIC 9* จากนั้นนำเข้าสู่เซลล์ยีสต์สายพันธุ์ *Pichia pastoris* เพื่อให้ผลิต recombinant protein ออกมา ภายใต้การควบคุมของ AOX1 promoter ของยีสต์ จากการศึกษาพบว่า การสังเคราะห์ recombinant TTR ในเซลล์ *Pichia* สามารถหลั่งโปรตีนออกมานอกเซลล์สู่อาหารเลี้ยงเชื้อได้สูงสุดหลังจากกระตุ้นด้วยเมธานอลที่ความเข้มข้น 1% เป็นเวลา 3 วัน โดยจะมีปริมาณโปรตีนทั้งหมด 50 มิลลิกรัมต่อ1 ลิตรของอาหารเลี้ยงเชื้อ นอกจากนี้ recombinant TTR ที่ได้สามารถทำบริสุทธิ์ได้เพียงขั้นตอนเดียวโดยวิธี preparative native gel electrophoresis อย่างไรก็ตามปริมาณโปรตีนที่ได้จากการทำบริสุทธิ์ เท่ากับ 1 มิลลิกรัมต่อ 1 ลิตรของอาหารเลี้ยงเชื้อ และจากการศึกษาคุณสมบัติทางเคมีกายภาพของ recombinant TTR ที่ผลิตได้ พบว่ามีคุณสมบัติคล้าย

กับ TTR ในสิ่งมีชีวิตชนิดอื่น โดยที่ recombinant TTR ที่ได้มีคุณสมบัติในการเคลื่อนที่ในกระแสไฟฟ้าภายใต้สภาวะธรรมชาติ ได้เร็วกว่าอัลบูมินในพลาสมาของคน ในการวิเคราะห์หาน้ำหนักโมเลกุลโดยวิธี HPLC พบว่า recombinant TTR ที่ได้มีน้ำหนักโมเลกุลเท่ากับ 58,884 daltons ส่วนการหาน้ำหนักโมเลกุลของหน่วยย่อยของ recombinant TTR ที่ได้โดย SDS-PAGE พบว่ามีน้ำหนักเท่ากับ 15,488 daltons นอกจากนี้จากการศึกษาโดยวิธี western blotting พบว่า recombinant TTR สามารถจับอย่างจำเพาะกับแอนติบอดีต่อ *Crocodylus porosus* TTR และยังมีความสามารถในการจับอย่างจำเพาะกับ RBP จากการศึกษายังแสดงให้เห็นว่าการเปลี่ยนแปลงปลายอะมิโนและปลายคาร์บอกซิลของ TTR มีผลต่อการเพิ่ม molar ratio ของการจับกันระหว่าง TTR กับ RBP

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ABSTRACT

Transthyretin (TTR) is one of the major thyroid hormone binding proteins found in plasma of higher vertebrates and plays role in metabolism of retinol via its binding to the retinol binding protein (RBP). In human, TTR is mainly synthesized in liver and choroid plexus of the brain. During evolution of mammalian TTR from its ancestor, the binding affinity to T4 increased while that to T3 decreased, the N-terminal region of TTR is shorter and more hydrophilic, and C-terminal region is more hydrophilic and some are longer. Together with the fact that these N-and C-termini locate at the entrance of thyroid hormone binding site, it prompted to an assumption that changes in structure of N-terminal segment and/or C-terminal segment of the TTR subunit affect the accession of thyroid hormones and also other ligands to the binding site of the TTR molecule.

In order to examine the influence of the primary structure of the terminal regions on biological function of TTR, the cDNA coding for chimeric hu/croc/pigTTR was constructed and cloned into the pPIC9 expression vector. The cDNA was induced to express under the *AOX1* promoter in a methylotrophic yeast *P. pastoris*. The result showed that the recombinant hu/croc/pigTTR was successfully synthesized and extracellularly secreted into the yeast culture medium. The synthesis reached maximum after induction with 1% methanol for 3 days at 50 mg per liter of culture. The TTR can be purified in a single step by preparative native gel electrophoresis, however, at 1 mg per liter of culture. The purified TTR showed physicochemical properties similar to other TTRs found in nature. Under native condition, the TTR showed the electrophoresis mobility faster than human albumin. By HPLC, it revealed that TTR formed tetramer and molecular weight of the tetramer was

58,884 daltons, and by SDS-PAGE, the mass of the TTR subunit was 15,488 daltons. In addition, the recombinant TTR showed cross reactivity with the antibody against *C. porosus* TTR and had specific binding to human RBP. Changes in N-and C-terminal regions of the TTR increased the molar ratio of the binding to RBP.

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Jiraporn Khwanmunee

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LIST OF ABBREVIATIONS AND SYMBOLS

^{o}A	=	angstrom
AOX	=	alcohol oxidase
cDNA	=	complementary deoxyribonucleic acid
cm	=	centimeter
CSF	=	cerebrospinal fluid
^{o}C	=	degree Celcius
h	=	hour
HPLC	=	high performance liquid chromatography
kDa	=	kilodalton
kg	=	kilogram
kV	=	kilovolt
l	=	liter
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
nm	=	nanometer
OD.	=	optical density
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
pmol	=	picomole
RBP	=	retinol binding protein
rpm	=	revolutions per minute
s	=	second
SDS	=	sodium dodecyl sulfate
T3	=	triiodothyronine
T4	=	tetraiodothyronine
TTR	=	transthyretin
v/v	=	volume by volume

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

µF	=	micro Faraday
µg	=	microgram
µl	=	microliter
µM	=	micromolar

CHAPTER 1

INTRODUCTION

Introduction

Thyroid hormones have a wide range of biological effects on numerous vertebrate species. Their major roles involve in growth, development and regulation of metabolism of the vertebrates (Brent, 1994). There are several forms of thyroid hormones, but two major forms that are important for biological activity are L-3,5,3′,5′-tetraiodothyronine (L-thyroxine, T4) and L-3,5,3′-triiodothyronine (L-triiodothyronine, T3). T4 functions as prohormone and exists in blood circulation as a major product of thyroid grand, while, T3 is the biologically active form and its significant amount is obtained from the conversion of T4 by 5′-deiodinase in peripheral tissue (Surk *et al*., 1973; Samuels *et al*., 1989 and Greenspan, 1994). The action and metabolism of thyroid hormones occur in the intracellular compartment. According to highly hydrophobic phenyl structure of thyroid hormones, they are transported in blood stream as a complex with plasma proteins. This prevents the partitioning into lipid membranes of thyroid hormones and maintains the appropriate distribution of the hormones between extracellular aqueous and lipid environment (for review see Schreiber and Richardson, 1997).

Transthyretin (TTR) is one of the three major thyroid hormone binding proteins found in blood of larger mammalian. In human, it is mainly synthesized in liver and choroid plexus of the brain. TTR is a homotetrameric protein of which each subunit has a molecular mass of the 14 kDa (Blake *et al*., 1978). In human, the TTR subunit consists of 127 amino acid residues (Kanda *et al.*, 1974). The functional tetramer of TTR contains two binding sites that differ in relative binding affinity for thyroid hormones (Kanda *et al.*, 1974). However, under physiological conditions, only one binding site is found occupied by thyroid hormones (Pages *et al*., 1973 and Nilsson *et al*., 1975). Binding affinity of TTR to thyroid hormones varied among

vertebrate species. The affinity to T_4 increased while that to T_3 decreased during the evolution of mammalian TTR from its ancestor (Chang *et al*., 1999).

The structural analysis of TTR genes from numerous vertebrate species revealed that the amino acid sequences in the central channel with thyroid hormones binding site is highly conserved. Predominant changes occurred within first ten amino acids from N-terminal end and seven amino acids from C-terminal end. During evolution of vertebrates, N-terminal region of TTR is longer and more hydrophobic in avian, reptilian, amphibian and fish than in mammalian. Similar variation also occurred at C-terminal region of TTR, leading to more hydrophobicity of the region in amphibian and fish than in mammalian. In addition, the C-terminal segment of some TTRs, i.e. from pig, *Xenopus laevis* and bullfrog, is longer than that of TTRs from other animal species. Due to the changes in length and hydrophathy occurred at N-terminal and C-terminal regions, and due to the fact that these N-terminus and C-terminus of TTR locate at the entrance of thyroid hormone binding site, it prompted to an assumption that changes in structure of N-terminal segment and/or C-terminal segment of the TTR subunit affect the accession of thyroid hormones as well as other ligands to the binding site of the TTR molecule.

Apart from transporting of thyroid hormones, transportation of retinol binding protein (RBP) is another important function of TTR. Complex formation of TTR with RBP, the specific carrier of retinol (an alcohol form of vitamin A), has been suggested to prevent loss of vitamin A from glomerular filtration in kidney (Peterson, 1971). Binding of TTR and RBP molecule was shown involved with the hydrophobic interaction. The study by X-ray crystallography revealed that several amino acids are involved in the intermolecular contact of TTR-RBP molecule (Naylor and Newcomer., 1999), and amino acid substitution at some positions found in TTR variant, e.g. substitution of isoleucine to serine in TTR Ile84Ser, brought to reduction of binding affinity to RBP of TTR (Berni *et al.*, 1994).

Currently, interaction between TTR and amyloid proteins has been reported. Inhibition on fibril formation of amyloid β-protein as well as a proteolytic activity on apoA-I, a variant of apolipoprotein A (Liz *et al*., 2004), of TTR has been revealed in human. This finding led to an assumption on a possible relationship

between TTR and amyloid proteins in physiological as well as pathological conditions. Although proteolytic activity of TTR has been clarified as a serine protease (Liz *et al*., 2004), amino acid residues involved in catalysis are still unknown.

To elucidate the role of N and/ or C-terminal segment on the biological functions of TTR including the proteolytic property and ability in binding to ligands, the chimeric TTRs of which N- and/or C-terminal sequence is properly altered is necessary. In this thesis, cDNA of a chimeric *C. porosus* TTR was constructed, the recombinant chimeric TTR was synthesized using the heterologous protein expression system of *Pichia pastoris* and properties of the TTR were determined.

Review of Literatures

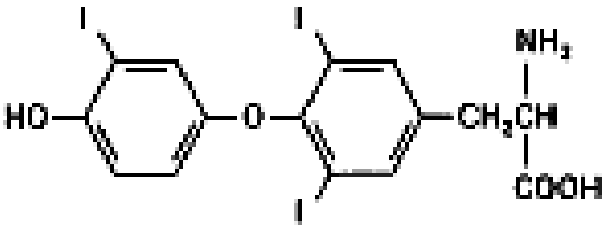
1. Thyroid hormone

1.1 Structure and synthesis site of thyroid hormone

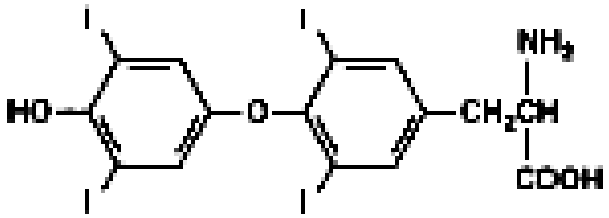
Thyroid hormones are involved in the regulation of growth, differentiation, and metabolism in vertebrate. The major forms of thyroid hormones synthesized and secreted by thyroid gland into the bloodstream of vertebrates with significant biological activity are L-3,5,3′,5′-tetraiodothyronine (L-thyroxine, T4) and L-3,5,3′-triiodothyronine (L-triiodothyronine, T3) (Samuels *et al*., 1988). The molecule of thyroid hormone can be divided into there regions: a hydrophobic 4′-hydroxyl group, two phenyl rings that are linked together by an ether bond with an angle of 120 degree, and the amino acid side chain on the tyrosine ring (Figure 1.1) (Ribeiro *et al*., 1998 and Samuels *et al*., 1988). The major form of thyroid hormone secreted by thyroid gland is thyroxine (T4). Within target cell, T4 is converted to T3 by outer ring deiodination (for reviews see Kohrle *et al*., 2002 and Richardson *et al*., 2005). T3 has higher affinity for thyroid hormone receptors (TR) than T4 (Sandler *et al*., 2004 and Richardson *et al*., 2005) and appears to be more potent in thyroid hormone action than T4. Thus, T3 can be considered to be the major active form of thyroid hormones in target tissues (Gross *et al*., 1952; Lerman, 1953; Surk and Oppenheimer, 1997).

1.2 Distribution of TH

Thyroid hormone action and metabolism occur in the intracellular compartment. In brain, thyroid hormones in the bloodstream must cross the blood brain barrier to reach the brain (Seibert and Nelson, 1942). However, because the thyroid hormone is a typical extracellular hydrophobic signaling molecule, to be able to mediate transferring of information from outside cells by permeation through membranes, the hormone requires an extracellular thyroid hormone binding protein system (Schreiber, 2002). In the bloodstream of vertebrates, there are three thyroid hormone distributors, thyroxine binding globulin (TBG), TTR and albumin (Larsson *et al.* 1985). In human TBG has the highest affinity for thyroid hormones, while TTR has intermediate affinity and albumin has the lowest affinity (Robbins and Edelhoch, 1986). In lower vertebrates such as reptile, amphibian and fish, albumin is proposed to be a major carrier for thyroid hormones in the blood stream (Richardson *et al*., 1994).



3, 5, 3′-triiodothyronine (T3)



3, 5, 3′, 5′-Tetraiodothyronine (thyroxine, T4)

Figure 1.1 Structure of thyroid hormones (from Yen, 2001)

1.3 Role of TH in vertebrate

Thyroid hormone is known playing an important role in development, metabolism and homeostasis in vertebrate (Morreale de Escobar *et al*., 1987; Tata, 1999; Silva, 2001; and Yen, 2001). It also has the effects on metamorphosis as well as development and differentiation of specific organs of reptilian (Chiu and Lam, 1994), fish (Miwa *et al*., 1988) and the parr-smolt transformation (smoltification) of migratory salmonids (Hoar, 1939). In human, various pathological stages and developmental abnormalities result from inadequate or excessive levels of circulating thyroid hormones. These include mental retardation, stunted growth, hearing loss and alterations in thermogenic homeostasis and heart rate (Norman and Lavin, 1989 and Lim *et al*., 2002). During amphibian metamorphosis, activation of thyroid gland resulted in elevating of circulating thyroid hormones have been observed (Etkin, 1965; Regadr *et al*., 1978; Suzuki and Suzuki, 1981). In many vertebrate species, the same raising of thyroid hormone levels in blood crucial stages of thyroid hormone-dependent development (Hulbert, 2000).

2. Transthyretin

2.1 Structure of transthyretin

Transthyretin (TTR) is homotetrameric protein with a molecular mass of ~55 kDa (Branch *et al*., 1971 and Blake *et al*., 1978). In human, each subunit of TTR is composed of 127 amino acids with mass of 13,745 daltons, leading to molecular mass of the entire molecule is 54,980 daltons (Kanda *et al*., 1974). The tetramer of TTR is organized from two dimers. A monomer is arranged into two of four-stranded β-sheets. Dimers of TTR are then formed by edge-to-edge interaction of two monomers resulting in a pair of twisted eight-stranded β-sheets. Association of two dimers by dimer-dimer contacts resulted in a tetramer structure with a large solvent channel passing through the molecule in which two sterically equivalent thyroid hormone binding sites exist (Naylor and Newcomer, 1999). Although a molecule of TTR provided two binding sites for thyroid hormone, only one site was occupied under physiological condition (Pages *et al*., 1973 and Nillsson *et al*., 1975).

The X-ray crystallography analysis revealed that human TTR has a very high β-sheet content with only a small length of a helix. Each TTR subunit consists two of four-β strands (DAGH and CBEF) that are antiparallel to each other. Nearly all residues are involved in the β-strands or the loops connecting them, or in the short helix. Only ten N-terminal and five C-terminal residues do not appear to be involved in the folding but merely exist as a 'head' and 'tail' (Blake *et al.,* 1978). The interaction between monomers that forms a stable dimer is predominantly involved with hydrogen bonding between the β-strands at the edges of two monomers (strands F and H) (Blake *et al*., 1978). To form the tetramer, two dimers interact through the hydrophobic contact involving residues that situated in AB and GH-loops. The thyroxine-binding sites are situated in one large hydrophobic channel that is formed between two dimers at the tetramer interface (Figure 1.2) (Wojtcak *et al*., 1996). The unusual high content of β-structure, the interaction between monomers to form dimers and dimers to form tetramer contribute to formation and make TTR to be the most stable globular protein (Branch *et al*., 1971 and 1972). However, in comparing, the TTR tetramer was less stable than the dimer (Blake *et al.,* 1978).

A large central channel with two sterically equivalent thyroid hormone binding sites that differ in their relative binding affinity is formed in consequence of tetrahedral arrangement of the TTR subunits (Blake *et al*., 1978). These two identical funnel-shaped thyroxine binding sites are found located deeply in the central pocket connected by a very narrow channel centered (Figure 1.3) (Green *et al*., 2003). The negative cooperativity of hormone binding implies that only one molecule of thyroxine binds TTR tetramer (Blake *et al*., 1978).

The TTR tetramer is less stable than the dimer (Blake *et al.,* 1978). However, the tetramer does not dissociate in strong acidic or alkaline or even in 0.1% sodium dodecyl sulfate (SDS) solution. Dissociation of the tetramer occurred slowly in 6 M guanidinium chloride at pH 5.9 (Branch *et al.,* 1972). In the presence of 1% SDS and 10 mM β-mercaptoethanol, at least 80% of human TTR tetramer was still intact after boiling for less than 20s (Bellovino *et al.,* 1998). Loses in binding activity to thyroid hormone of TTR evidenced when TTR was treated with 6 M urea, however, it was restored after removing out urea (Raz and Goodman, 1969).

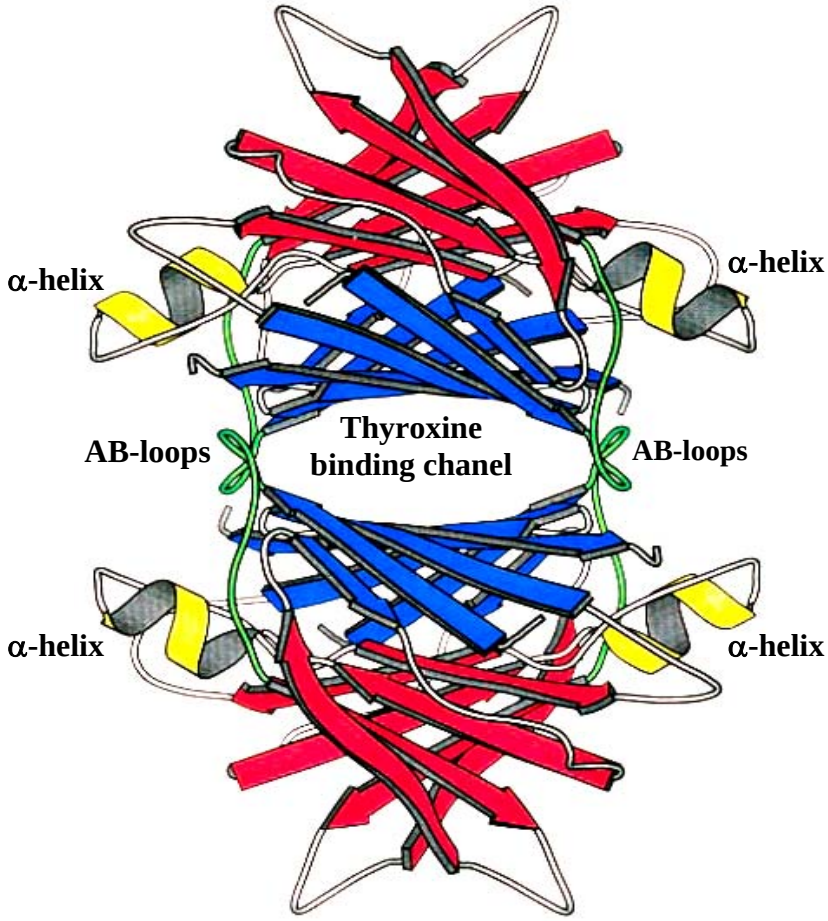


Figure 1.2 Structure of TTR tetramer

The extended inner β-sheet (DAGHH′G′A′D′) is shown in blue, the outer (CBEFF′E′B′C′) in red. The helix is yellow, and the loops, which contribute to the formation of TTR tetramer, are in green (modified from Hamilton and Benson, 2001)

2.2 Sites of TTR syntheis and secretion

Hepatocyte is the major site of TTR synthesis and its secretion into blood (Dickson *et al.,* 1982). The synthesis site in liver is confirmed by the presence of large amounts of TTR mRNA detected by *in situ* hybridization (Dickson *et al.,* 1985 and Jacobsson, 1988). In addition, TTR is the protein that found most abundantly synthesized by choroid plexus, which secreted the TTR into cerebrospinal fluid (CSF) (Palha *et al*., 2002). The concentration ratio of TTR compared to that of albumin and other plasma proteins is much higher in CSF than in the blood. High concentration of TTR in the CSF suggested to a major role for TTR in mediating T4 transfer across the blood-choroid-plexus-CSF barrier from blood into brain (Schreiber, 2002). In addition, epithelium of the choroid plexus (for review see Bartalena, 1990; Dickson *et al*., 1986 and Stauder *et al.,* 1986;) and ependymal cells of the subcommissural organ in brain (Montecinos *et al*., 2005) were also shown to be a strong synthesis site of TTR. Besides, TTR mRNA was also localized, though with much less extent, by immuno-reactivity and *in situ* hybridization in several other tissues, including endocrine cells of stomach, small intestine and colon (Gray *et al.,* 1985 and Liddle *et al.,* 1985), retinal pigment epithelium of eye (Martone *et al.,* 1988; Herbert *et al.,* 1986; Cavallaro *et al.,* 1990; Dwork *et al.,* 1990; Mizuno *et al.,* 1992; Ong *et al.,* 1994 and Jaworowski *et al.,* 1995), kidney (Katoh *et al*., 1982 and Kato *et al*., 1984), and visceral extraembryonic endoderm and foregut endoderm (Makover *et al*., 1989). In fetus, it demonstrated a synthesis site of TTR in placental tissue (McKinnon *et al*., 2005).

2.3 Function of TTR

2.3.1 Thyroid hormone distributor

The widely known main biological function of TTR is transport of thyroid hormones in both T4 and T3 forms (Robbins, 1996). The tetramer of TTR contains a central channel with two binding sites for thyroid hormones (Figure 1.3) (Blake *et al*. 1978). However, only one binding sites of TTR is occupied by thyroid hormone (Pages *et al*., 1973 and Nilsson *et al*., 1975), due to negative cooperativity (Wojtczak *et al*., 1996). In human, 10-15% of T4 in serum and up to 80% of the hormone in central nervous system are transported by TTR. The TTR that is

synthesized in the choroid plexus epithelial cells is secreted into CSF (for review see Schreiber *et al*., 1990), and in the CSF, the majority of T4 binds to TTR (Hagen and Solberg, 1974). However, some TTR in the CSF may not bind T4 at all near the choroid plexus and T4 is also likely to permeate through the blood–brain barrier away from the ventricular region. This TTR, therefore, participate in equilibration between free and TTR-bound T4 (for review see Schreiber, 2002).

The binding characteristic of TTR to thyroid hormones was changed during evolution of vertebrates (for reviews see Schieber and Richardson, 1997 and Scheiber *et al*., 1999). Binding of human TTR to T4 was stronger than T3 whereas TTR from fish, amphibians, reptiles and birds binds T3 better than T4 (Pages *et al*., 1973; Chang *et al*., 1999 and Prapunpoj *et al*., 2000a, 2002).

2.3.2 Carrier of retinol via binding to RBP

TTR is also known as a retinol or vitamin A carrier by which through interaction with the plasma RBP (Raz *et al*., 1970). The binding of TTR to RBP was suggested to prevent the loss of small RBP as well as vitamin A molecule from glomerular filtration in kidney (Peterson, 1971). It was also shown that the formation of RBP and TTR complex stabilized the binding between retinal and RBP (Goodman, 1974 and Raz *et al*., 1970). By crystallography experiment, it revealed that two molecules of RBP bind to one TTR tetramer (Figure 1.4) (Rask *et al*., 1971 and Hörnberg, 2004). In addition, binding of T4 and RBP to the same TTR molecule was independent and did not show any effect on each other.

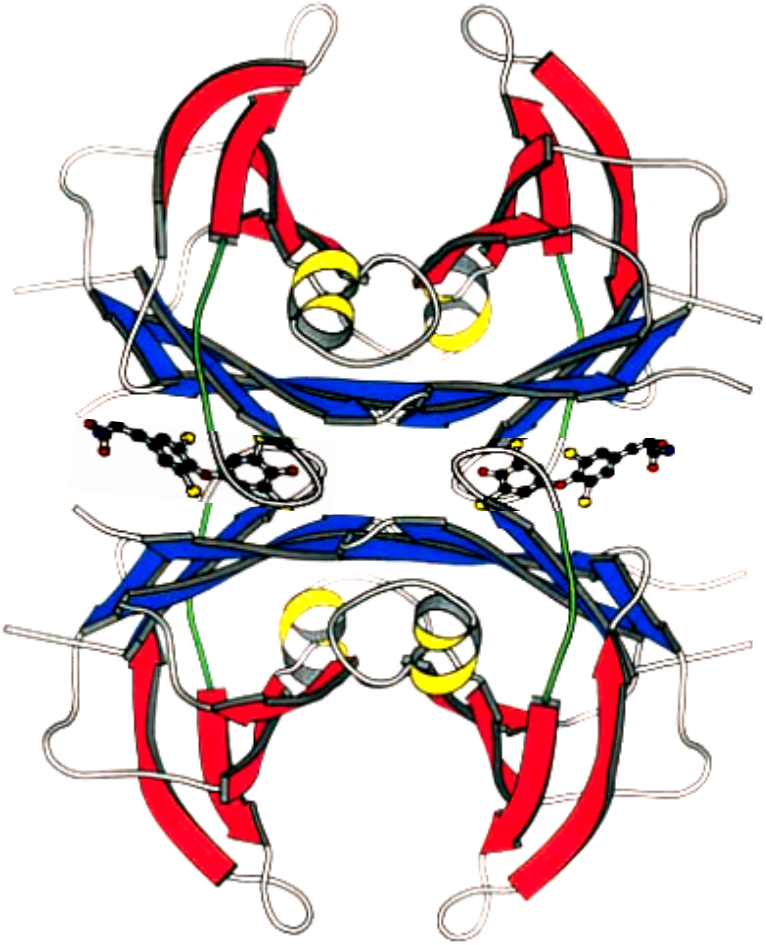


Figure 1.3 The thyroid hormone binding sites in TTR molecule

The thyroid hormone is shown in the two possible binding pockets between the dimers, although there is negative co-operativity of binding for the two positions (modified from Hamilton and Benson, 2001).

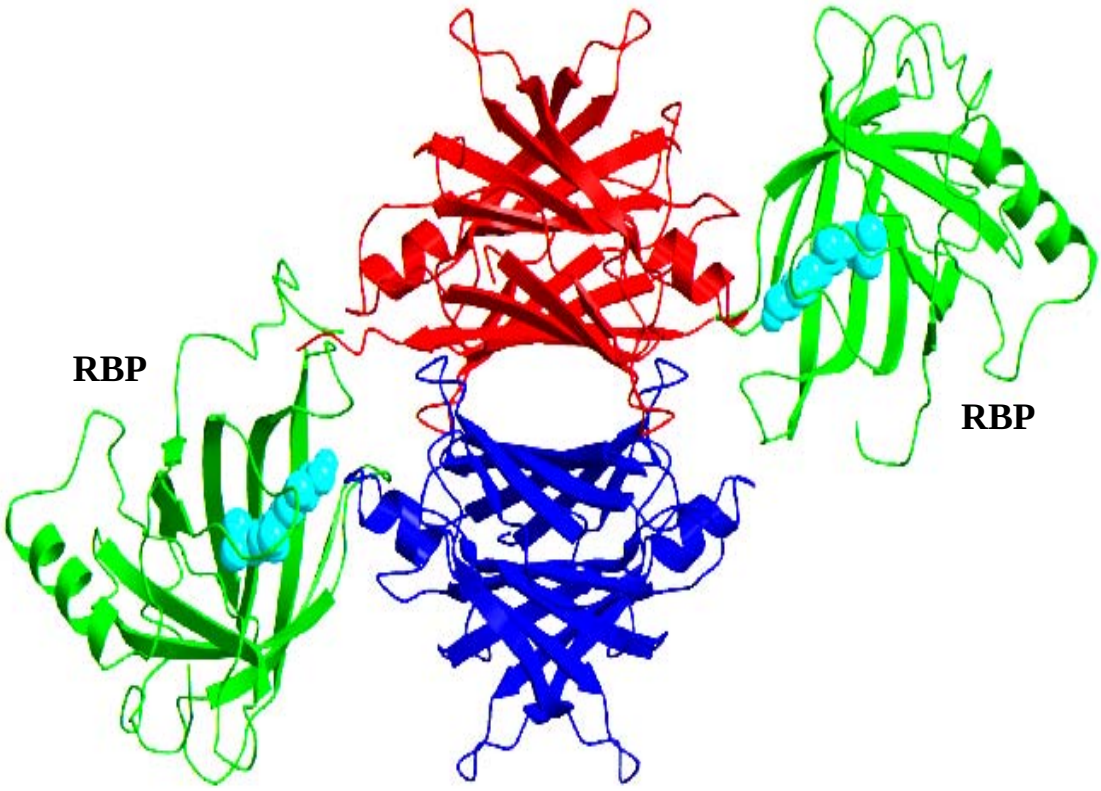


Figure 1.4 The TTR-RBP complex

Two molecules of RBP bind to the TTR tetramer (from Hörnberg, 2004).

2.4. Evolution of structure and function of TTR

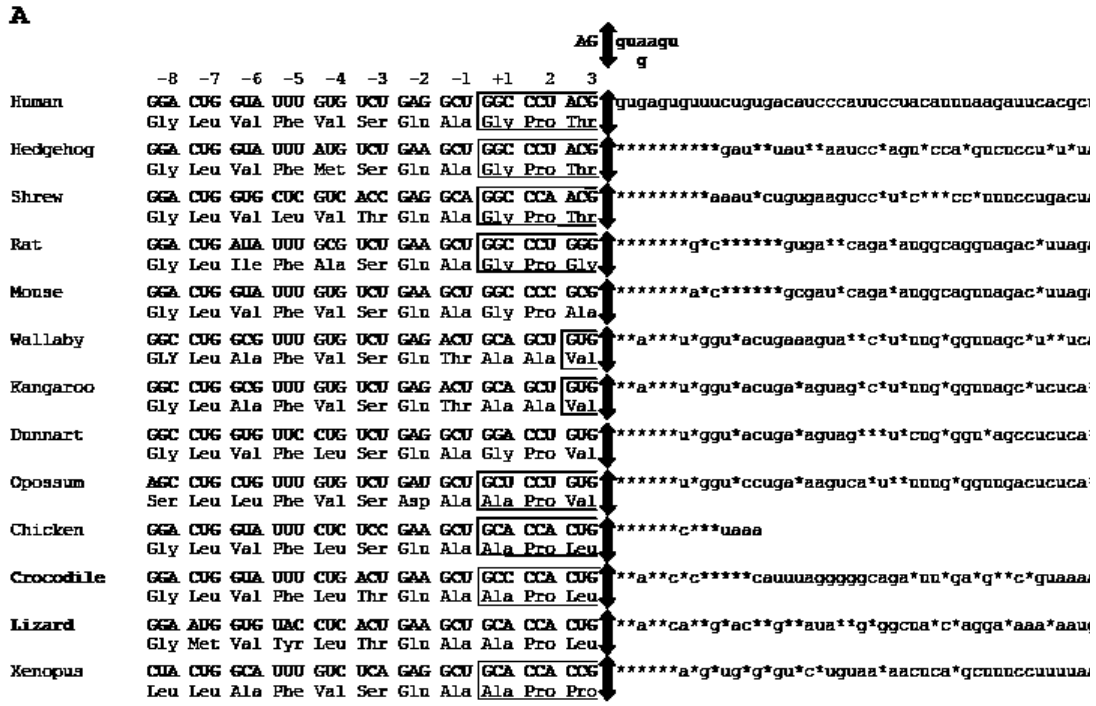
Comparison of the primary structure of TTR from several animal species revealed the conservation during evolution of vertebrates of amino acids in core of the subunit and in the central channel with the thyroid hormone binding sites of TTR (Chang *et al*., 1999; Schreiber *et al*., 1998; Hamilton *et al*., 1993; for reviews see Schreiber Richardson, 1997 and Schreiber *et al*., 1998). Alteration of the primary structure of the TTR N- terminus occurred predominantly with in the first ten amino acids that protrude out from the TTR molecule and are located near the entrance to the central channel of TTR (Schreiber *et al*., 1998 and Hamilton *et al*., 1993). During the evolution of mammalian TTRs from TTR ancestors, the affinity to T4 increased while the affinity to T3 decreased, i.e. TTR from fish, amphibians, reptiles and birds binds T3 with higher affinity, whereas TTR from mammals binds T4 with higher affinity (Chang *et al*., 1999; for review see Scheiber *et al*., 1999 and Prapunpoj *et al*., 2000a, 2002). The nucleotide triplets encoding for Val-Ser-His at position 4-6 from the N-terminus is lost in eutherians compared with birds and reptiles (Duan *et al*., 1991, 1995a and Achen *et al*., 1993) (Figure 1.5), leading to increase in hydrophilic of the N-terminal of the eutherian TTR subunit (Duan *et al*., 1995a). In other word, the N-terminal segment of the TTR subunit is longer and more hydrophobic in avian and reptilian than in eutherian. *X. laevis* TTR has an even longer and more hydrophobic N-terminal region than avian and reptilian TTRs (Figure 1.5) (Prapunpoj *et al*., 2000b). The stepwise shifted of mRNA splicing has been proposed to be a mechanism of the evolutionary change (Aldred *et al*., 1997; for reviews see Schreiber and Richardson, 1997 and Schreiber *et al*., 1998). Changes in the N-terminal region e.g. longer and more hydrophobic N-termini of TTR, have been suggested to affect the accessibility of the thyroxine-binding site in the central channel of TTR (Duan *et al.,* 1995a). Comparative analysis of thyroid hormones binding affinity of recombinant crocodile TTRs supported the influence of the N-terminal segment on binding affinity to T4 and T3 (Prapunpoj *et al*., 2002).

Variation in amino acid sequence of TTRs during evolution of vertebrates also observed at C-terminal segment. The C-termini of TTRs from pig and bullfrog have more amino acid residues than that from other vertebrates (Figure 1.6).

In addition, hydropathy of the C-terminal sequence was more hydrophobic in reptilian, amphibian and fish TTRs than in mammalian TTR. The X-ray crystallography analysis of TTR tetramer showed that not only N- but also C-terminal region locates at the entrance to the central channel that harbors the thyroid hormones binding site (Black *et al*., 1978). Therefore, changing the amino acid residues at N- and/or C-terminal regions possibly affect the accession of thyroid hormones and other ligands to the binding site that locates inside of the TTR molecule.

To date, 100 point mutations in TTR gene have been identified and up to 80 of these mutations were demonstrated related to amyloidosis, a group of protein folding disease in which normal or mutate form of proteins aggregate into insoluble fibril and deposit in extracellular space of organs/tissues leading to dysfunction and death of the organs and tissues. The mechanism of self-assembly to a fibrillar structure of these soluble proteins is still unclear. Proteolysis has been proposed to be a key regulator of the process. Several evidences have been reported to an involvement of proteolytic enzymes in both activation of amyloid fibril formation and clearance of the fibrils (Hemming and Selkoe, 2005; for reviews see Selkoe, 2004; Russo *et al*., 2005 and Higuchi *et al*., 2005). Co-localization of TTR with amyloidogenic apolipoprotein A-I (apoA-I) was first identified in kindred with apoA-I amyloidosis (Sousa *et al*., 2000). Thereafter, proteolytic cleavage of C-terminus of apoA-I by TTR was revealed as a cryptic nature and TTR triggering the fibril formation of apoA-I was proposed. However, the canonical catalytic triad could not be identified (Liz *et al*., 2004). The proteolytic activity of TTR can be inhibited by serine and chymotrypsin-like serine protease inhibitors, and it has a cleavage preference for a Phe residue on P1. Therefore, TTR was suggested to be a cryptic chymotrypsin-like serine protease (for review see Liz and Sousa, 2005). To understand the role of TTR proteolysis in amyloidoses, its catalytic site needed to be identified. Role of TTR in catabolism of amyloid β-protein (Aβ), which is a main component of amyloid plaques in Alzheimer's disease, has been suggested in many reports (Schwarzman *et al*., 1994; Mazur-Kolecka *et al.,* 1995 and Link, 1995). TTR was found formed complexes with Aβ and inhibited aggregation and fibril formation of the amyloid protein *in vitro* and *in vivo* (Golabek *et al*., 1995; Tsuzuki *et al*., 1997

and Mazur-Kolecka *et al*., 1997). However, relationships between TTR and this amyloid protein remain unclear. Studying pathogenic relationship between amyloidogenic processes could only suggest that amyloidogenic mutation in TTR might affect the interaction of TTR with Aβ in which lead to the inability of aggregated TTR proteins to prevent Aβ polymerization (Schwarzman *et al*., 2004).


A
AG guaagu
g
−8 −7 −6 −5 −4 −3 −2 −1 +1 2 3
Human
Hedgehog
Shrew
Rat
Mouse
Wallaby
Kangaroo
Dunnart
Opossum
Chicken
Crocodile
Lizard
Xenopus

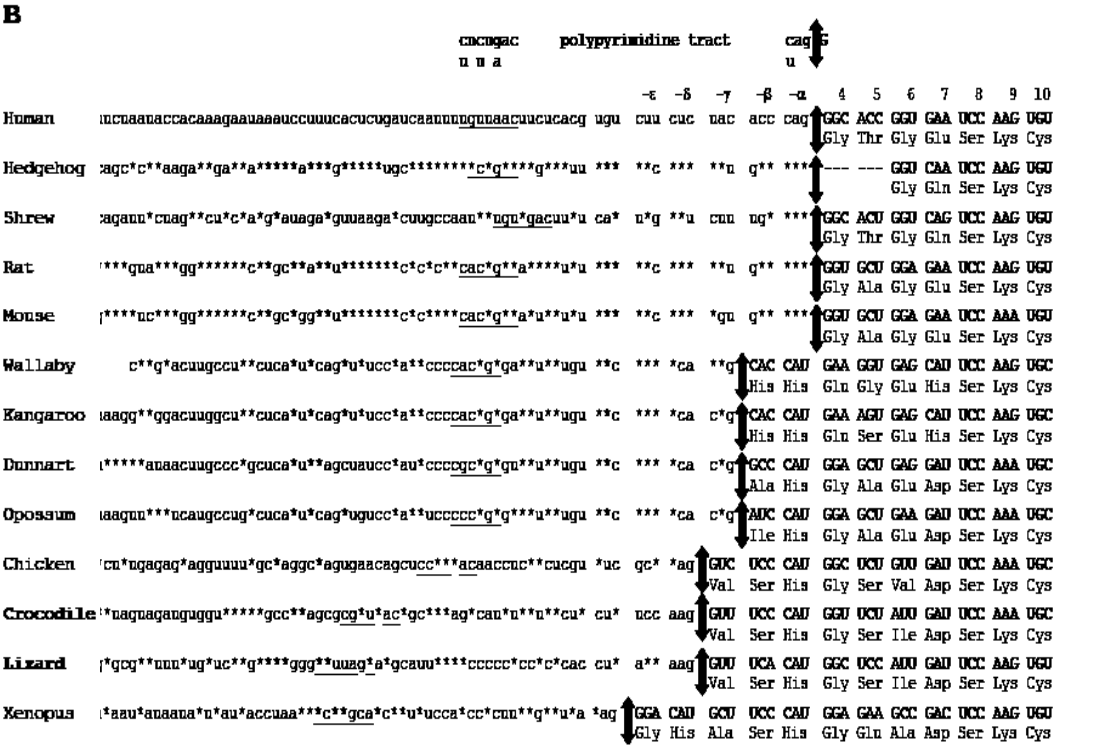

B
cucugac
u u a
polypyrimidine tract
cag G
u
−ε −δ −γ −β −α 4 5 6 7 8 9 10
Human
Hedgehog
Shrew
Rat
Mouse
Wallaby
Kangaroo
Dunnart
Opossum
Chicken
Crocodile
Lizard
Xenopus

Figure 1.5 Comparison of the nucleotide and derived amino acid sequences of TTR precursor at the exon 1/intron 1 (A) and intron 1/exon 2 (B) borders with those from other vertebrate species

The nucleotide sequences in exons are in bold upper case, while those in introns are in lower case. The deduced amino acid sequences are given beneath the nucleotide sequences. The consensus recognition sequences for splicing are indicated in bold above the positions of the splice sites in human TTR mRNA. The amino acid sequence of the N-terminus, determined by Edman degradation of the mature or the recombinant TTRs, are indicated by a box open at the right end (modified from Prapunpoj *et al*., 2002; for details see Prapunpoj *et al*., 2002).

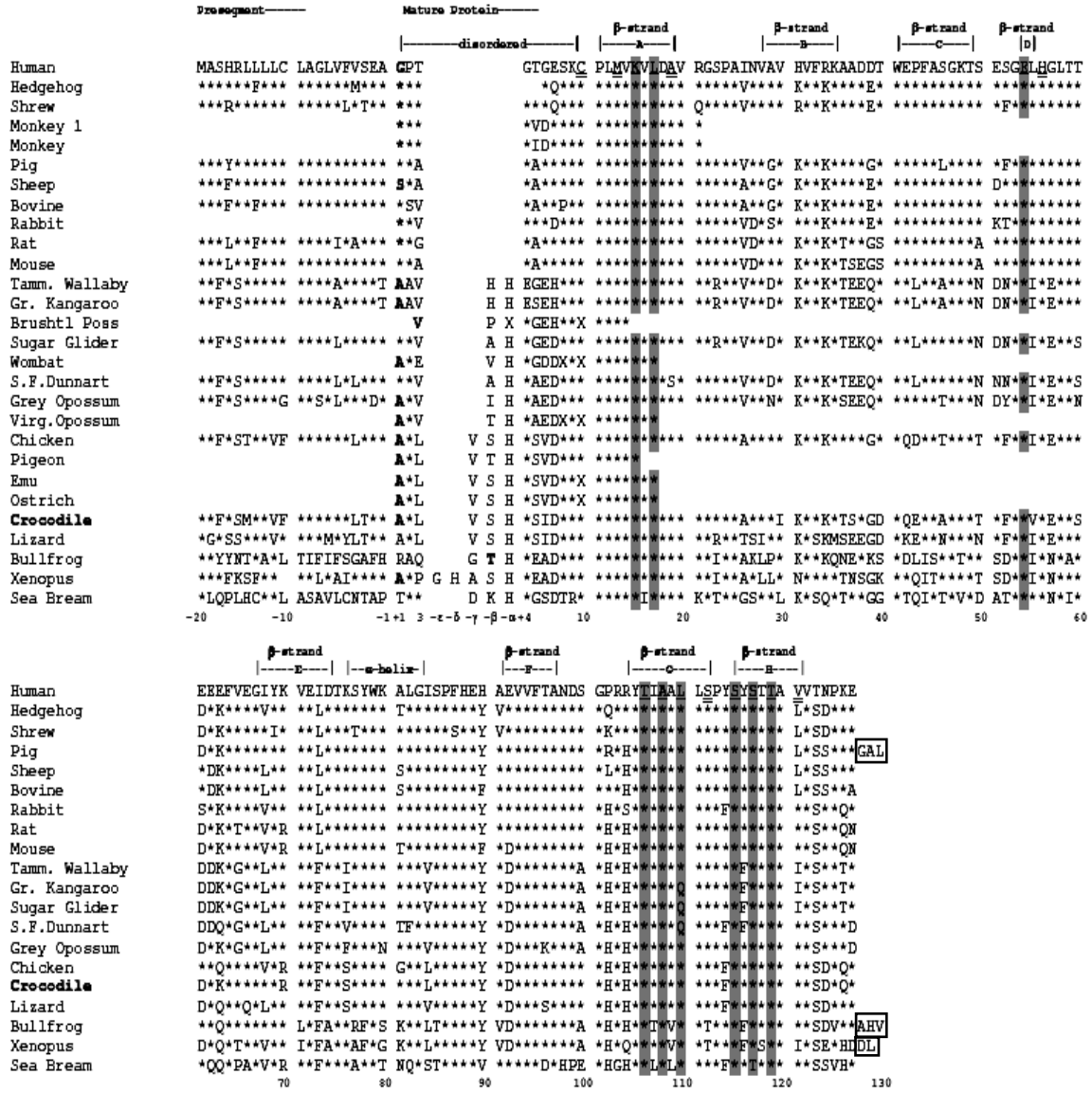

Presegment
Mature Protein
disordered
β-strand A
β-strand B
β-strand C
β-strand D
β-strand E
α-helix
β-strand F
β-strand G
β-strand H
Human
Hedgehog
Shrew
Monkey 1
Monkey
Pig
Sheep
Bovine
Rabbit
Rat
Mouse
Tamm. Wallaby
Gr. Kangaroo
Brushtl Poss
Sugar Glider
Wombat
S.F.Dunnart
Grey Opossum
Virg.Opossum
Chicken
Pigeon
Emu
Ostrich
Crocodile
Lizard
Bullfrog
Xenopus
Sea Bream

Figure 1.6 Comparative alignment of structure of vertebrate TTRs

Amino acid sequence or that derived from the cDNA sequence of vertebrate TTRs are aligned with the amino acid sequences of human TTR. Single-letter amino acid abbreviations are used. X indicates an amino acid could not be unambiguously identified by Edman degradation. Asterisks are residues identical to those in human TTR. Gaps were introduced for proper alignment. Features of secondary structure of human TTR are indicated above the sequences. Numbering of residues is based on that for human TTR. Negative numbers indicate the residues in the presegment; positive numbers represent residues of the mature protein. Negative Greek letters α, β, δ, γ, and ε were introduced to indicate positions of residues in non-eutherian species. Bold letters indicate the first amino acid of mature TTRs. Double underlining indicates amino acid residues located in the central channel. Gray shading indicates amino acid residues, in the central channel, that are involved in binding thyroid hormones (from Prapunpoj *et al*., 2002). Extra amino acid residues found in TTRs from pig, bullfrog and *X. laevis* are in boxes.

3. Recombinant protein synthesis systems

3.1 General

Two general categories of expression systems are prokaryotic and eukaryotic systems. In general, the prokaryotic systems are easier to handle and satisfactory for most purposes. However, there are serious limitations in using prokaryotic cells for the production of eukaryotic proteins. There is no universal expression system for heterologous proteins. All expression systems have some advantages as well as some disadvantages that should be considered in selecting which one is best for the target protein. Choosing the best one requires evaluating the options from yield to glycosylation and to proper folding (Rai and Padh, 2001). In comparison with bacteria, eukaryotes, in particular yeast, have a number of advantages as hosts for heterologous protein production. The eukaryotic cell system has an intrinsic ability in express, process and folds the protein correctly. Particular post-translational modifications such as glycosylation that may be required for the *in vivo* biological function of the protein (Dube *et al.,* 1988; Han and Martinage, 1992; Claffey *et al.,* 1995; Hooker and James, 1998), immunogenic properties, and other features of authentic eukaryotic proteins related to the three-dimensional structure can be obtained by the eukaryotic cell systems. Among eukaryotic cells, heterologous expression in *Pichia pastoris*, which is the methylotrophic yeast, has many of the advantages of eukaryotic expression (Cregg *et al.,* 1985; Cregg *et al.,* 1993; Higgins and Cregg, 1998). More than 100 foreign proteins form bacteria, fungi, plants, invertebrates, vertebrates (includes humans) have been expressed in *P. pastoris*. The yield of recombinant proteins from *P. pastoris* depends critically on growth conditions. Using high cell densities in fermentation has been shown to improve protein yields by 10 to 100 folds. When grown on methanol, the foreign proteins secreted from *P. pastoris* can represent 80% or more of the total protein in the culture medium (Tschopp *et al.,* 1987a). The recent developments of the *P. pastoris* expression system have had an impact on not only the expression levels can be achieved, but also the bioactivity of various heterologous proteins (Macauley *et al*., 2005).

Use of heterologous expression systems for synthesis in large amount of proteins has allowed to determine both biochemical and structural parameters of many different enzymes. However, because several different organisms have been adapted for the recombinant protein expression, the choice of which system to use will depend upon the characteristics of the protein itself, the yield required for, and subsequent ease of purification (Morton and Potter, 2000). For recombinant TTR, the synthesis in large amount has been reported using two expression systems i.e. *E. coli* and *P. pastoris*.

3.2 Bacterial expression system

The bacterial expression systems, especially in *E. coli*, is by far the most widely employed host for that post-translational modifications of the product are not essential. *E. coli* has high growth rates and can express high level of heterologous proteins. Furthermore, it can grow rapidly to high density in such simple and inexpensive media. Strains that are used for recombinant production have been genetically manipulated so that they are generally regarded as safe (GRAS) for large-scale fermentation. However, several disadvantages of bacterial expression systems have been reported. The system poses significant problems in post-translational modifications of proteins. Common bacterial expression systems in particular *E. coli* have no capacity to glycosylate proteins in either N- or O-linked conformation. Although other bacterial strains such as *Neisseria meningirulls* have recently been shown to O-glycosylate some of their endogenous proteins, the trisaccharide added is different from the O-linked sugars found in eukaryotes (Marston and Hartley, 1990). Synthesis of recombinant human TTR (both wild-type and variants) has been reported successfully by using *E. coli* system and with different expression vectors. Wild-type TTR was reported synthesized by using pCZ11, a thermo-inducible bacterial expression vector (Murrell *et al*., 1992), and pQE30 (Matsubara *et al*., 2003). By using the pQE30, TTR was expressed under control of a lipoprotein promoter. TTR Met30 variants were synthesized as a fusion protein by using the pIN-III-ompA-1 vector. The recombinant protein was co-translated with an *E. coli* outer membrane protein, proteinA (Furuya *et al*., 1989). Other TTR variants, i.e. Gly6Ser, Leu55His, Thr60Ala, Ile84Ser and Ala109Thr,

were also produced by using pCZ11 (Murrell *et al*., 1992). In all cases, the recombinant TTRs were reported forming a tetramer with molecular size similar to that of human TTR in nature and having ability in binding to thyroxine (Furuya *et al*., 1989; Murrell *et al*., 1992 and Mutsubara *et al*., 2003). In addition, the recombinant TTR variants showed the same characteristics in amyloid formation at acidic pH as the isolated TTR variants (Mutsubara *et al*., 2003).

3.3 Expression system of TTR in yeast

P. pastoris belongs to the methanol-assimilating yeasts (Lee and Komagata, 1980). Growth on methanol of *Pichia* induces expression of genes whose products are required for metabolism of the yeast. Three of enzymes involved in the methanol pathway are located in the organelle called peroxisome. As a result, both the enzymes that are involved in methanol pathway and the proteins that are involved in peroxisome biogenesis (*KEX* proteins) are induced in response to methanol. The most highly regulated among these genes is *AOX1* that coding for alcohol oxidase (AOX), which is a hydrogen peroxide-producing oxidase working in peroxisome and is the first enzyme of the methanol pathway (for review see Lin-Cereghino *et al*., 2006). The promoter of *AOX1* gene, which is induced by methanol but repressed by many carbon sources such as glucose, glycerol and ethanol (Lin-Cereghino *et al*., 2006), is widely used to drive the expression of the heterologous genes in the *Pichia* system. Accordingly, methanol is often used as the carbon source to induce the production of the heterologous proteins. Besides of induction, shifting of the carbon source to methanol was reported causes major structural and physiological changes within the cell. The enzymes for methanol metabolism are synthesized *de novo* and some of them are translocated into peroxisomes (Sauer *et al*., 2004). The methanol metabolism pathway in *P. pastoris*, similarly to that in other methylotrophic yeasts (Veenhuis *et al.,* 1983), begins with the oxidation of methanol to formaldehyde by the enzyme AOX. Because AOX has a poor affinity for oxygen, yeast synthesizes large amount of the enzyme to overcome the low specific activity of the enzyme. Biochemical and physiological researches have shown that under methanol induction, up to 30% of the cellular proteins is AOX (van Dijken *et al.,* 1976; Couderc and

Barratti, 1980; Veenhuis *et al.,* 1983 and Giuseppin *et al.,* 1988). There are two genes that code for AOX, *AOX1* and *AOX2*. However, in comparison, *AOX1* has stronger promoter and is responsible for the majority of the AOX activity in yeast cell (Cregg *et al.,* 1989). Expression of the *AOX1* gene is tightly regulated and induced by methanol at the transcriptional level (Cregg *et al.,* 1985; Ellis *et al.,* 1985 and Cregg *et al.,* 1989). Most genes of interest are placed and expressed under control of the *AOX1* promoter. The heterologous proteins expressed in *P. pastoris*, can remain intracellular after their synthesis or they can be secreted into the medium outside. However, secretion of foreign proteins is more popular because it facilitate for downstream processing in particular purification of the foreign protein product from proteins and other contaminants from the yeast cell. *Pichia* strain GS115 (*his4*) is the most commonly used as host for the heterologous proteins expression. This wild-type strain contains the *AOX1* and *AOX2* genes, however, it grows on methanol at wild-type rate (Mut^+) because its growth on methanol mainly relies on the presence of the *AOX1* gene.

More than 100 foreign proteins from bacteria, fungi, plants, invertebrates, vertebrates (includes humans) have been expressed in *P. pastoris*. These successful protein gene expressions include TTRs from human (Prapunpoj *et al*., 2006), amphibian (Prapunpoj *et al.*, 2000a) and reptile (Prapunpoj *et al.*, 2002). As in other yeast systems, the production of foreign proteins in *P. pastoris* occurs in simple minimal defined media. This makes the system a choice for NMR analysis of proteins.

Objectives

1. To construct cDNA of the chimeric TTR in which its N-and/or C-terminal region (s), and use the expression system of *P. pastoris* for synthesis of the recombinant chimeric protein.
2. To purify and determine general physicochemical properties of the recombinant chimeric TTR.
3. To examine the binding to RBP of the chimeric TTR.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Instruments

Instruments	Model	Company
Autoclave	ES-315	Tomy
Balance (4 digits)	AB204-S	Mettler
Balance (2 digits)	PG5002-S	Mettler
Centrifuge	Aanti J-30 I	Beckman
Centrifuge	J2-21	Beckman
Centrifuge	5804R	Eppendorf
Centrifuge	Harrier 18/8 (MSE)	SANYO
Fraction collector	2110	Bio-Rad
Gel Document (Labworks 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
pH meter	713	Metrohm
Power supply	ELITE 300 plus	Wealtec

Instrument	Model	Company
Power supply	PowerPac 3000	Bio-Rad
Preparative gel electrophoresis	PrepCell 491	Bio-Rad
Slab gel electrophoresis	AE-6450	ATTO
Slab gel electrophoresis	Mini Protean 3 cell	Bio-Rad
Slab gel electrophoresis	mini Protean-II	Bio-Rad
Spectrophotometer	8453	Hewlett-Packard
Spectrophotometer	G20	Thermo
Stirrer		Corning
Vortex-mixer	VX 100	Labnet
Water Bath	WB-710M	Optima

1.2 Chemicals

1.2.1 Analytical grade

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

Chemical	Company
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

1.2.2 Molecular biology grade

Chemial	Company
Agarose	GenePure
Ampicillin	Calbiochem
100 bp DNA ladder	New England Biolabs (NEB)
*Eco*RI	NEB
Ethidium bromide	Promega
pGEM-T Easy vector	Promega
*Xho*I	NEB

1.2.3 Reagent kits

Reagent	Company
QIA kit for plasmid purification	QIAGEN
QIA kit for PCR purification	QIAGEN
QIA kit for gel extraction	QIAGEN

1.3 Yeast and Bacterial cells

Pichia pastoris strain GS115 is a product of Invitrogen. *E. coli* DH5α is a gift from Professor Schreiber, Australia.

2. Methods

2.1 Construction of chimeric cDNA and cloning into pGEM Teasy vector

Crocodylus porosus TTR cDNA was used as a template to generate by PCR a cDNA coding for the chimeric *C. porosus* TTR (hu/croc/pig TTR) in which its N- and C-terminal regions are replaced by that of human TTR and pig TTR, respectively, and the compatible restriction ends (*Xho*I at 5′ end and *Eco*RI at 3′ end) were introduced to the cDNA for ligation into the *Pichia* expression vector, pPIC 9. The PCR amplification was performed as previous described (Prapunpoj *et al*., 2002). In brief, 50 ng of *C. porosus* TTR cDNA in an *E. coli* plasmid was amplified in the presence of 40 pmol of the specific primers (Table 1) in 100 μl of the reaction mixture. The amplification was started with an initial denaturation step at 94°C for 5 min. Then, it was followed by 25 cycles of denaturation at 94°C for 30s, annealing at 53 °C for 30s and extension at 72°C for 1 min. The final extension was carried out at 72°C for 5 min. The PCR product was checked on 1% agarose gel containing 0.4 μg/ml ethidium bromide for a single band with an expected size, and the reaction mixture was purified using the PCR purification kit (QIAGEN). The *in vivo* amplification of the chimeric TTR cDNA in bacterial cell was carried on by ligation of the purified cDNA into pGEM Teasy (Promega) with T4 DNA ligase. The ligation reaction mixture (10μl in total volume) was comprised of 20 ng of the DNA, 50 ng of pGEM Teasy vector, and 3 Weiss units of T4 DNA ligase.

2.2 Amplification and preparation of the expression vector for transformations into *Pichia pastoris*

The purified TTR cDNA inserted pGEM Teasy vector was transformed in to *Escherichia coli* strain DH5α with a standard protocol and the *E. coli* cells were grown overnight at 37°C. Thereafter, three to five single white colonies, which are expected containing the DNA inserted plasmid, were selected and colonies were grown in 2 ml of Luria-Bertani (LB) medium containing 100 μg/ml ampicillin for overnight at 37°C. The plasmid was purified using the plasmid purification kit (QIAGEN), and size of the DNA insert was checked by double

digestion of the plasmid with *Xho*I and *Eco*RI at 37°C for 2 h prior being purified by QIAquick Gel Extraction kit (QIAGEN) as described in 2.14. Ligation of the chimeric TTR cDNA into pPIC9, which was previously double digested with *Xho*I and *Eco*RI, was carried out with T4 DNA ligase at 4°C overnight. Thereafter, the hu/croc/pigTTR cDNA in pPIC9 was transformed into *E. coli* for an *in vivo* amplification of the plasmids. The DNA plasmid was purified, and the insertion direction of the DNA in the plasmid was determined by DNA sequencing, using 5′*AOX1* primer (5′-GACTGGTTCCAATTGACAAGC-3′). The TTR inserted plasmid was, then, linearlized by digestion with *Sal*I at 37°C for 2 h and purified using QIAquick PCR purification kit (QIAGEN) prior to being transformed into *Pichia* cells.

2.3 Transformation of the expression vector into *P. pastoris*

To prepare the 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^{o}C for overnight. One hundred microliters of the overnight culture was inoculated 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 ^{o}C and resuspended with 45 ml of ice-colded distilled water. Cells were collected and resuspended once more with 22.5 ml of ice-colded distilled water. Thereafter, cells were resuspended with 1.8 ml of ice-colded 1 M sorbitol and finally resuspended in 0.135 ml of ice-colded sorbitol give a final volume of approximately 0.2 ml.

Transformation of the *Sal*I linearized chimeric TTR cDNA plasmid into *Pichia* competent cells strain GS115 was carried out by an electroporation using Gene Pulser (Bio-Rad) and followed the protocol recommend by the company. In brief, an aliquot (80 μl) of the competent *Pichia* cells was mixed with 1.5 μg of the linearized plasmid, then transferred the cell mixture to an ice-cold 0.2 cm electroporation cuvette. The electroporation was carried out at 1.5 kV, 25 μF and 400 Ω, generating pulse lengths of ~9.4 milliseconds with a field strength ~1.37 kV/cm. Then, the cell suspension was flushed with 1 ml of ice-cold

1 M sorbitol, and all of the mixture was spreaded onto the minimal dextrose medium (MD) agar plate and incubated at 30^{o}C for 3 days.

2.4 Screening for His^{+}Mut^{+} transformant

Pichia transformants with phenotype His^{+}Mut^{+} (histidine synthesis and methanol utilization plus) were selected form His^{+}Mut^{s} (histidine synthesis and methanol utilization slow) by growing the transformants on two selective agar media, minimal dextrose medium (MD) and minimal methanol medium (MM). A single colony of His^{+} transformant from a the original MD plate was picked with a sterile toothpick, patched onto MM and subsequently onto MD plate. Plates were incubated at 30°C for 2 to 3 days. Growth of the His^{+}Mut^{+}, but not His^{+}Mut^{s}, is similar on both MM and MD. Screening was performed with 150 to 200 His^{+} transformants and up to 50 single colonies of the His^{+}Mut^{+} transformants were selected for synthesis of the recombinant protein.

2.5 Synthesis of recombinant TTR

2.5.1 Small scale synthesis

The *Pichia* transformants were grown and first induced for synthesis of the recombinant TTRs in small scale. In brief, a single colony of *Pichia* clone, is usually on yeast extract peptone dextrose (YPD) plate, was inoculated into 5 ml of medium containing glycerol (BMGY). Cells were grown at 30°C in an orbital-shaking incubator (200 rpm) until OD_{600} reaches 2 to 6. Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature and transferred to grow in 5 ml of the medium containing methanol (BMMY) to an OD_{600} around 1.0. Methanol was added every 24 h to maintain level of methanol in the culture to 0.5% for 3 days. After induction, the culture supernatant was collected by centrifugation at 5,000 rpm for 5 min and kept at -20°C until used. The yeast culture supernatant was analyzed for secreted proteins by SDS-PAGE and/or native-PAGE and the protein band was detected by silver staining.

2.5.1.1 Optimization of methanol concentration for the induction

The *Pichia* clone that provides the highest production of the chimeric TTR was selected, and optimization was performed for the synthesis of the clone in large scale. The optimization was attempted at 0.5% and 1% of methanol concentration.

2.5.1.2 Optimization of cell density in culture

Optimization of cell density, the induction with methanol was carried out at 1% of methanol with density of the *Pichia* cells in culture equivalent to 2 or 4 units of OD_{600}. The induction was carried on for 3 days, and the culture supernatant was analyzed by native-PAGE.

2.5.2 Large scale synthesis

Large scale preparation of the chimeric TTR was performed by using the shaking flask culture method and the culturing condition described for the small scale synthesis was adopted. A single colony of the *Pichia* clone from YPD plate was inoculated into 10 ml of BMGY and the yeast cell was grown at 30°C for 16-18 h. Five milliliters of the overnight culture was transferred into 300 ml of BMGY in a 1-liter flask, and cells were grown until OD_{600} reached 2 to 6. Then, cells were collected by centrifugation at 2,500 rpm for 5 min at room temperature, resuspended in 300 ml of BMMY in a 2-liter flask to a density of 4.0 (at OD_{600}). Cells were grown at 28^{o}C and induced with methanol for 3 days. Methanol was added to the culture every 24 h to maintain the final concentration to 1% thoroughly the induction period. Thereafter, the culture supernatant was collected by centrifugation at 5,000 rpm for 10 min at 4^{o}C. Level of synthesis and secretion into culture medium of the recombinant chimeric TTR was determined by native-PAGE and SDS-PAGE followed by silver staining.

2.6 Purification of the recombinant TTR

In the *Pichia* expression system, the recombinant chimeric TTR was synthesized and secreted into the medium, facilitating in downstream processing of the protein. The recombinant chimeric TTR was purified from the *Pichia* culture supernatant by preparative native-PAGE using Prep Cell 491 (Bio-Rad). The discontinuous gel (12% and 4% for resolving and stacking gels, respectively) was selected for the separation and the electrophoresis condition was performed according to the instruction manual of the company. The *Pichia* culture supernatant was concentrated by ultrafiltration, then the concentrated solution was centrifuged at 10,000 rpm for 10 min prior loading onto a gel tube. After separartion, protein bands were eluted with 50 mM Tris-HCl, pH 7.4 and collected at flow rate of 1 ml/min, 2 ml/fraction. Proteins in fractions were analyzed by native-PAGE and detected by silver staining. The fractions containing TTR were pooled, concentrated and stored at -20^{o}C for further analysis.

2.7 Determination of physicochemical properties of the recombinant TTR

General physicochemical properties of the recombinant chimeric TTR including molecular weight (indicating tetramer formation of the TTR), mass of the monomer, and electrophoretic mobility were examined to confirm the proper structure and folding of the synthesized TTR.

2.7.1 Determination of molecular weight

Molecular weight of the recombinant TTR was estimated by HPLC/gel-permeation chromatography on BioSil SEC 250 column (Bio-Rad), equilibrated in 0.2 M sodium phosphate buffer pH 6.0. Aliquot of the purified chimeric TTR (50 μg) was loaded onto the column and separation was conducted at flow rate of 0.4 ml/min. The chromatogram was followed up from the absorbance at 280 nm. The column was calibrated with blue dextran (2,000 kDa), aldolase (158 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kD).

2.7.2 Determination of the subunit mass

Subunit mass of the recombinant TTR was determined by SDS-PAGE according to the method of Laemmli and Favre (1973). The purified TTR was incubated with a solution containing 2% of sodium dodecyl sulphate (SDS) and 2.5% of β-mercaptoethanol at 100^{o}C for 30 min before loading on SDS-polyacrylamide gel **(**15% resolving gel and 4% stacking gel) and the electrophoresis separation was performed at constant voltage (100 volts). The protein band was detected by staining gel with Coomassie brilliant blue R-250.

2.8 Determination of binding to RBP

To determine the binding property to RBP, the purified recombinant chimeric TTR was mixed and incubated with purified human RBP at 4°C for 1 h. Thereafter, the reaction mixture was analyzed on 10% native-PAGE, electro-blot transferred to a nitrocellulose or PVDF membrane, and the TTR-RBP complex was immunochemically detected using the anti-crocodile TTR antibody.

2.9 Purification of human TTR from plasma

Human TTR that used as a control was purified from plasma in 3 steps, affinity chromatography on Cibacon blue column, electrophoresis in a preparative native polyacrylamide gel, and electrophoresis in urea gel.

In the first step, human plasma was centrifuged at 10,000 rpm for 20 min to remove any non-dissolved particles prior to loading onto the Cibacon blue column which 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 washed with PB. Thereafter, bound proteins were eluted with PBS. Both bound and unbound fractions were analyzed by native-PAGE. The fractions containing TTR were pooled and concentrated by ultrafiltration.

In the second step, the concentrated pooled TTR fraction from the first step was loaded onto a preparative gel column (12% resolving gel and 4% stacking gel). After separation, protein bands were eluted with 50 mM Tris-HCl, pH 7.4, and

the eluting fractions were collected at flow rate of 1 ml/min, 2 ml/fraction. Each fraction was analyzed for proteins by native-PAGE and the protein bands were visualized by silver staining. The fractions containing TTR were pooled and concentrated by ultrafiltration.

In the final step, the concentrated pooled TTR fraction from the second step was loaded onto a preparative urea gel (12% polyacrylamide gel containing 6M urea). The separation was carried out in Tris-Boric-EDTA buffer, pH 8.3 (TBE), using the Prep Cell model 491 (Bio-Rad). Proteins were separated and eluted at flow rate of 1 ml/min. TTR in fractions was analyzed by native-PAGE and detected by silver staining. The fractions containing TTR were pooled, concentrated, and stored at -20^{o}C (for use as a control).

2.10 Western blot analysis

Separation by SDS-PAGE or native-PAGE of protein was preformed in duplicate. Then, the protein was electro-transferred to nitrocellulose or PVDF membrane, using the transfer buffer containing 25 mM Tris, 192 mM glycine and 20% (v/v) methanol, pH 8.3, for 1 h at 4°C. Protein bands on the membrane were visualized by staining with 0.1% Amido black. Specific proteins were detected by immunochemistry, using enzymatic detection. Firstly, non specific binding sites on the membrane were blocked by incubation, with gentle shaking, in the blocking solution containing 5% skim milk, 25 mM Tris, pH 7.4, 0.15 M NaCl and 0.1% Tween 20 at room temperature for 1 h. Thereafter, the membrane was incubated with primary antibody (antiserum against *C. porosus* TTR raised in rabbit), diluted in blocking agent (1:500), for 1 h at room temperature. The membrane was, then, washed once for 15 min and three times for 5 min with 25 mM Tris buffer pH 7.4 containing 0.15 mM NaCl and 0.1% Tween 20 (TBS-T) at room temperature. After washing, the membrane was incubated with secondary antibody (horseradish peroxidase (HRP)-linkd anti-rabbit immunoglobulins, raised in donkey) at dilution 1:2,500 for 1 h at room temperature. Then, the membrane was washed once for 15 min and three times for 5 min with TBS-T at room temperature. To detect the TTR,

activity of the HRP was followed by ECL and the chemiluminescence's signal was detected and analyzed by the Bio-Chemi System (UVP).

2.11 Preparation of competent cell and transformation of DNA

2.11.1 *E. coli* competent cell

The competent cells were prepared from *E. coli* strain DH5α using calcium chloride as described by Cohen *et al.* (1992). A single colony from fresh LB agar plate was inoculated into 5 ml of LB broth in a 50-ml tube and grown overnight at 37°C in an orbital shaking incubator (150 rpm). Then, 50 µl of the overnight culture was transferred to 25 ml of LB broth and cells were grown until the OD_{600} reached 0.4 to 0.5 (took 2 to 3 h). Then, cells were cooled down to 0^oC by storing on ice for 10 min and collected by centrifugation at 2,500 rpm for 10 min at 4^oC. The supernatant was removed and tube was inverted for 1 to 2 min to drain any trace of medium. Cell pellets were resuspended in 4 ml of ice-cold 0.1 M calcium chloride and cooled down to 0°C. After centrifugation and draining, cells were resuspended in 0.8 ml of ice-cold 0.1 M calcium chloride. Thereafter, the competent cells were aliquot (100 µl) and 60% glycerol was added to the final concentration of 15.5% (v/v). Immediately, cells were frozen in liquid nitrogen and stored at -80°C as a glycerol stock.

To transform cDNA plasmid, 50 µl of the *E. coli* competent cell suspension was mixed with 5 µl of the cDNA plasmid in a polypropylene tube and stored on ice for 10 min. Then, the cell mixture was subjected to heat-shock for exactly 90 seconds at 42^oC without shaking and cooled down to 0^oC for 1 to 2 min. Thereafter, 100 µl of SOC was added and the mixture was incubated at 37^oC for 1 h prior to plating onto LB agar plate, in the presence of X-gal (250 µg/10 ml agar) and ampicillin (100 µg/ml agar). Cells were allowed to grow at 37°C for 16-18 h.

2.11.2 *Pichia* competent cell

To prepare the competent cell, a single colony of *P. pastoris* strain GS115 from yeast extract peptone dextrose (YPD) agar plate was inoculated in 5 ml of YPD broth at 30^oC, overnight. Then, 100 µl of the overnight culture was

transferred into 120 ml of fresh YPD medium in a 1-liter flask and continuously grown to an OD_{600} of 1 to 1.6. Cells were collected by centrifugation at 2,000 rpm for 5 min, at 4^{o}C, and the cell pellet was resuspended in 45 ml of ice-cold sterile distilled water. Cells were collected by centrifugation and resuspended once in 22.5 ml of the ice-cold distilled water. Thereafter, cells were resuspended in 1.8ml of ice-cold 1.0 M sorbitol, and finally resuspended in 0.135 ml of the ice-cold sorbitol. The competent cells were aliquot, kept at -80^{o}C or immediately used in transformation.

To transform the DNA of interest into the *Pichia* competent cells, an aliquot (80µl) of the *Pichia* cells was mixed with the linearized DNA and the cell mixture was transferred to a 0.2 cm electroporation cuvette. The cuvette containing the cells was incubated on ice for 5 to 10 min, then, the electro-transfer was carried out using GenePulser (Bio-Rad) at 1.5 kV, capacitance of 25 µF and resistance of 400 Ω, which generating pulse length of ~9.4 milliseconds with a field strength ~1.37 kV/cm. Immediately thereafter, 1 ml of ice-cold 1 M sorbitol was added into the cuvette. The cells were flushed, spread onto a minimal dextrose medium (MD) agar plate, and grown at 30°C (took 2 to 3 days).

2.12 Purification of PCR product

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

2.13 Purification of plasmid

2.13.1 by using the QIAprep Spin kit

The *E. coli* plasmid was prepared by the alkaline lysis described by Birnboim and Doly (1979), using a QIAprep spin kit. An overnight culture of the plasmid was prepared in the presence of antibiotic (50 μg ampicillin/ml of culture). The bacterial cells were collected by centrifugation at 10,000 rpm for 15 s, and resuspended in 250 μl of 50 mM Tris-HCl containing 10 mM EDTA, pH 8.0 and RNase A. Then, the bacterial membrane was disrupted with 250 μl of a lysis buffer containing NaOH for 5 min at room temperature. The cell lysate was neutralized and adjusted with guanidine hydrochloride, a chaotropic salt, to a concentration of 1.6 M. Any precipitates of the bacterial chromosomal DNA and cell debris were removed by centrifugation at 14,000 rpm for 10 min. Then, plasmid in the supernatant was purified by absorption onto the surface of silica filter in a spin column. Bound plasmid was then separated from salts, proteins and other cellular impurities by washing with 750 μl of 20 mM NaCl and 2 mM Tris-HCl, pH 7.5 in 80% methanol. The plasmid was finally eluted with 50 μl of 10mM Tris-HCl, pH 8.5, and stored at -20^{o}C until used.

2.13.2 by using the PCR purification kit

A plasmid in an enzymatic reaction was purified with the same method described for purification of PCR product in section 2.12, using a QIAquick PCR Purification Kit (QIAGEN).

2.14 Purification of PCR product from agarose

The DNA that was separated on agarose gel can be extracted and purified from gel by using the QIAquick Gel Extraction kit (QIAGEN). After analysis on 1% agarose containing 0.4 μg of ethidium bromide, gel band containing the DNA fragment of interest was excised with a sterile razor blade. The gel slice was then dissolved in a solution containing quanidine thiocyanate with the gel:solution ratio of 1:3 (v/v). Thereafter, 1 gel volume of isopropanol was added, and all of the mixture was applied onto a QIA spin column. The DNA was adsorbed onto membrane in the

column while other impurities were washed out with 750 µl of a buffer containing ethanol. The purified DNA was eluted out from the column with 30 µl of 10 mM Tris-HCl, pH 8.5 and stored at -20^{o}C until used.

2.15 Restriction analysis of DNA insert

The chimeric TTR cDNA was constructed so that containing *Xho*I site at the 5′end and the *Eco*RI site at the 3′end of the molecule. The existence of the TTR cDNA in a plasmid, therefore, can be determined by double digestion of the plasmid with *Xho*I and *Eco*RI, and analysis the DNA insert by electrophoresis on 1% agarose gel containing 0.4 µg/ml of ethidium bromide. The double digestion was performed by incubating 4 µg of plasmid with 10 units of *Xho*I (Biolabs) and *Eco*RI (Biolabs) in 20 µl of the reaction volume, and the reaction mixture was incubated at 37^{o}C for 2 h prior the analysis by electrophoresis was performed.

2.16 SDS-PAGE and non-denaturing PAGE

Analysis of proteins under denaturing condition was carried out by sodium dodecyl sulfate polyacrylamide gel electrphoresis (SDS-PAGE) using 12% polyacrylamide, pH 8.6 and 4% polyacrylamide, pH6.8 for resolving and stacking gels, respectively, with the discontinuous buffer system of Laemmli and Favre (1973). Protein samples were denatured by incubated with a solution containing SDS (69 mM) and β-mercaptoethanol (320 mM) at 100°C for 30 min prior the proteins were loaded onto the gel. After separation, the protein bands were detected by staining with silver nitrate (Morrissey, 1981) or Coomassie Brilliant blue R-250.

The electrophoresis separation of proteins under non-denaturing conditions was performed similar to that performed for SDS-PAGE, except no SDS was added and 10% and 4% acrylamide were chose for resolving and stacking gels, respectively. The protein bands were detected by staining gel with Coomassie Brilliant blue R-250 or silver nitrate.

2.17 Protein staining

The protein on polyacylamide gel, after separation by electrophoresis under denaturing or non-denaturing condition, was detected either by Coomassie blue or silver nitrate.

2.17.1 by Coomassie blue staining

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

2.17.2 by silver staining

Gel staining with silver nitrate was performed by the method of Morrissey (1981). After electrophoresis, gel was placed 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. Thereafter, gel was rinsed with distilled water for 3 to 4 changes prior to soaking in a solution of dithiothreitol (DTT) (500 μg/ml) for 30 min. The DTT solution was discarded and 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 (took ~2-5 min depending on amount of proteins). To stop the reaction, the solution of 50% acetic acid was added until a few air bubbling occurred. Gel was washed several times with distilled water, and stored at 4^{o}C. The protein pattern and band intensity were analyzed by gel document (with Labwork 4.0 program, Bio-Rad).

2.18 Determination of protein concentration

Concentration of proteins was determined by the method of Bradford (1976). The reaction mixture comprised of 0.1 ml of protein sample 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 protein-dye complex was allowed to perform at room temperature for 2 min to 1h prior the optical density at 595 nm of the mixture was measured. The standard curve was generated using bovine serum albumin (BSA) at amounts of 0, 5, 10, 15, 20, and 30 μg, and used to determine the protein concentration of the unknown proteins.

Table 1 **Oligonucleotide primers used to generate cDNA for hu/croc/pigTTR**

PCR step	Sequence 5′ → 3′	Direction
1	AACGGGCACTGGTGAATCCAAATGCC	Sense
1,2	ACGGAATTCTCAAAGAGCTCCCTCCTTGGG	Antisense
2	CTCGAGAAAAGAGAGGCTGAAGCTGGCCCA ACGGGCACTGG	Sense

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 Construction of cDNA and cloning into expression vector for synthesis of the recombinant chimeric TTR in *Pichia pastoris*

The primary structure of TTR subunit is well conserved among vertebrate species with the exception of the N-terminal region, which is located at the entrances to the central channel containing the thyroid hormone binding sites (for review see, Schreiber and Richardson, 1997). Since the last decades, TTRs from over 20 vertebrate species, including mammalian, avian, reptilian, and fish have been isolated and their amino acids or that derived from the cDNA clones were elucidated and compared (for reviews see Schreiber, 2002 and Richardson, 2007). The comparison revealed that the amino acid residues involved in the binding of TTR to thyroid hormones (Blake *et al*., 1978 and 1974) remained unchanged during evolution of the vertebrates. However, the most marked changes are concentrated in the N-terminal region of the TTR subunit. The N-terminal segment of TTR is longer and more hydrophobic during evolution from avian, reptilian, amphibian and fish to mammalian (for review see Hamilton *et al*., 1993 and 2001). Similar variation also occurred at C-terminal region of TTR, leading to more hydrophobicity of the region in TTR from amphibian and fish than in mammalian. In addition, the C-terminal segments of TTRs from pig, *Xenopus laevis* and bullfrog are longer than other animal species. Due to the changes in length and hydrophathy occurred at N-terminal and C-terminal regions, and due to the fact that these N-terminus and C-terminus of TTR locate at the entrance to the thyroid hormone binding site, it prompted to an assumption that changes in structure of N-terminal segment and/or C-terminal segment of TTR subunit affect the accession of thyroid hormones as well as other ligands to the binding site of the TTR molecule. In order to elucidate roles of the N-and C-terminal regions on biological function of TTR, a chimeric TTR in which its

N- and C-terminals segments are altered is most required. In this thesis, the recombinant hu/croc/pig TTR was thus constructed
and its properties were examined.

The *C. porosus* TTR cDNA in pPIC9 vector was used as the template for PCR with the specific primer sets to generate the crocTTR having N-terminal sequence of human TTR and C-terminal sequence of pig TTR. By PCR, the recognition sequences for *Xho*I and *Eco*RI were also generated at 5′ and 3′ends, respectively, of the DNA to facilitate in ligation with the correct insertion direction of the DNA in pPIC9 expression vector. After PCR, the amplified DNA was purified and ligated to pGEM Teasy as described in section 2.2. The correct size of the DNA insert was determined by double digestion the DNA plasmid with *Xho*I and *Eco*RI as described in section 2.1. Analysis on 1% agarose gel, a single band of the DNA insert with size (~400 bp) as expected for the hu/croc/pig TTR was detected (Figure 3.1) indicating correct direction of the insertion. The plasmid was transformed into *E. coli* strain DH5α to *in vivo* increase a number of the plasmid. To synthesize the hu/croc/pigTTR, *P. pastoris* strains GS115 was chose as a host and pPIC9 was selected as a carrier. The TTR gene was inserted into the pPIC9 at the multiple cloning sites, immediately after 3′end of the α-factor signal sequence (Figure 3.2). By nucleotide sequencing, it confirmed position and direction of insertion of the hu/croc/pig TTR gene in the pPIC9. To transform into *Pichia* cell, the recombinant pPIC9 was firstly linearized by *Sal*I as described in section 2.3. By electroporation, from 10^3 to 10^4 cells of the *Pichia* transformants were obtained per microgram of the plasmid similarly to that previously reported (Scorer *et al*., 1994). Within the *Pichia* cell, the expression “cassette”, consisting of α-factor signal sequence and hu/croc/pigTTR gene was integrated into the yeast genome at the *HIS4* gene locus. The expression of the hu/croc/pigTTR gene and secretion of the recombinant protein are mainly regulated by the *AOX1* promotor.

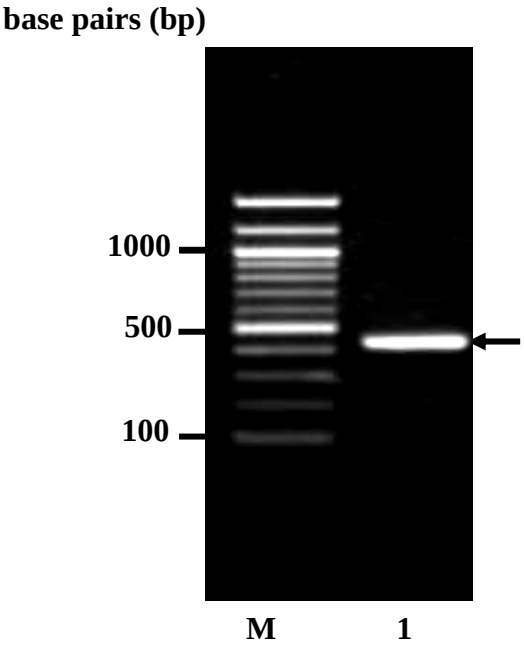


Figure 3.1 Generation and amplification of hu/croc/pigTTR cDNA by PCR

The hu/croc/pigTTR cDNA was generated and amplified by PCR from the *C. porosus* TTR cDNA. The PCR product (1) was analyzed on 1% agarose gel containing ethidium bromide along with the DNA markers (M). The DNA bands were visualized under UV. A single band of the hu/croc/pigTTR cDNA with 400 bp in size is indicated by arrow.

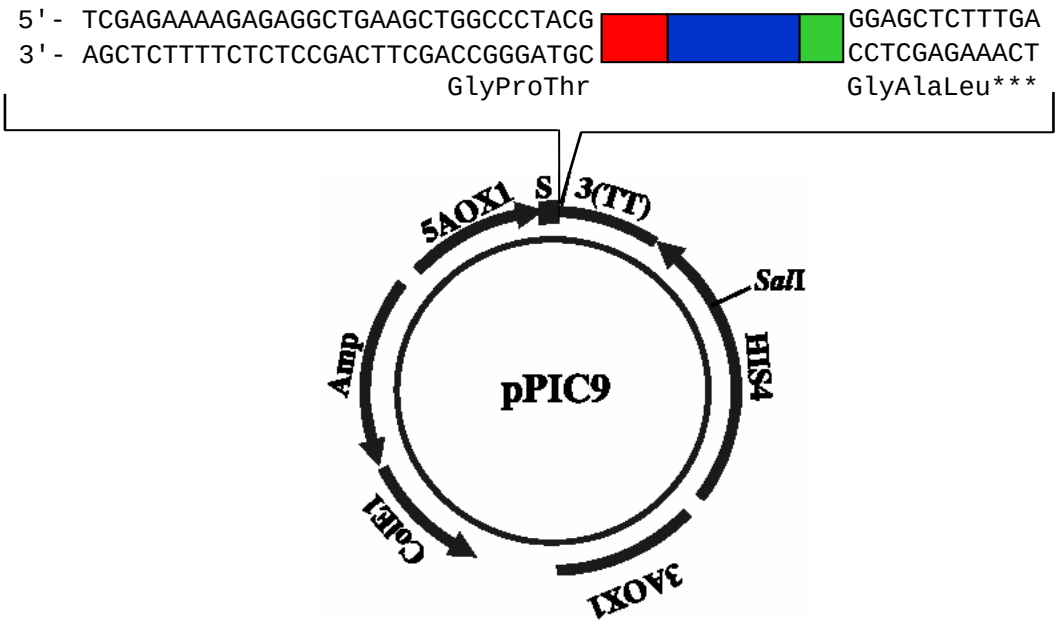


Figure 3.2 The *Pichia* expression vector pPIC9 for recombinant hu/croc/pigTTR

The cDNA of hu/croc/pigTTR was inserted into pPIC9 so that expression of the gene was under control of the AOX1 promoter and extracellular translocation and secretion was based on the α-factor signal sequence. Red, blue and green boxes are nucleotide sequences coding for N-terminal segment of human TTR, crocTTR without N-and C-termini and C-terminal segment of pig TTR, respectively. 5AOX1, promoter of *P. pastoris* alcohol oxidase 1 gene; 3(TT), native transcription termination and polyadenylation signal of alcohol oxidase 1 gene; 3AOX, sequence from the alcohol oxidase 1 gene that locates 3′ to the TT sequences; HIS4, histidinol dehydrogenase gene; Amp, ampicillin resistance gene, ColE1, *E. coli* origin of replication; *Sal*I, the *Sal*I restriction site for linearization of the vector.

3.2 Screening for $His^{+}Mut^{+}$ transformant

The pPIC9 expression vector containing the hu/croc/pigTTR cDNA was placed under the control of the native *AOX1* promotor at *Xho*I and *Eco*RI sites. An expression of the *AOX1* gene is controlled at the level of transcription. The inducer, methanol, is necessary for detectable levels of *AOX1* expression (Ellis *et al.*, 1985; Koutz *et al.*, 1989 and Tschopp *et al.*, 1987a). However, losses of the *AOX1* gene leading to loss of ability in producing the alcohol oxidase and resulted in the methanol utilization slow (Mut^{s}) phenotype of the yeast. The Mut^{S} transformants cannot efficiently metabolize methanol as a carbon source therefore grow poorly on the minimal methanol (MM) medium. This slow growth when methanol is used as the carbon source can be used to distinguish the His^{+} transformant in which the *AOX1* gene has been disrupted ($His^{+}Mut^{S}$) from the His^{+} transformant with an intact *AOX1* gene ($His^{+}Mut^{+}$). Up to 210 colonies of the hu/croc/pigTTR *Pichia* clones were screened for the $His^{+}Mut^{+}$ by comparing their growth on MM and MD plates as described in section 2.4. Growth pattern of the clones with $His^{+}Mut^{+}$ and $His^{+}Mut^{s}$ phenotypes is shown in Figure 3.3. Fifty two colonies of the putative $His^{+}Mut^{+}$ colonies were selected for the synthesis induction of recombinant hu/croc/pigTTR in small scale.

MM **MD**

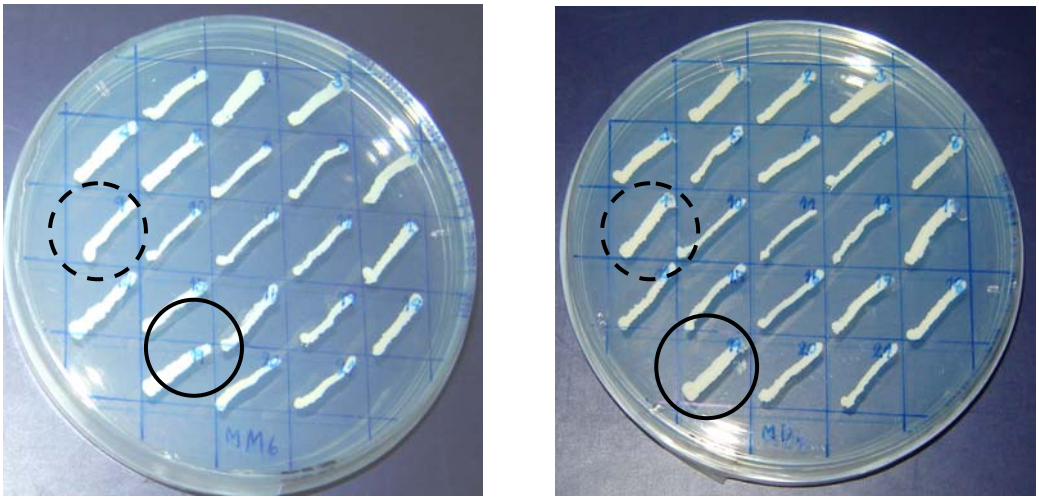


Figure 3.3 Selection for His^+Mut^+ transformants

The *Pichia* transformants with His^+Mut^+ or His^+Mut^S phenotype were identified by comparing their growth on the MM and MD. His^+Mut^+ shows similar growth rate on both media, whereas, His^+Mut^S has higher growth in MD than in MM. Close and broken circle indicate His^+Mut^+ and His^+Mut^S phenotype, respectively.

3.3 Expression of hu/croc/pigTTR in *Pichia pastoris*

P. pastoris has many advantages of eukaryotic expression systems such as protein processing, protein folding and posttranslational modification, while being easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. It is faster and easier to use and less expensive than other eukaryotic expression systems in particular the mammalian expression system. As a yeast, *Pichia* shares the advantages of molecular and genetic manipulations with *Saccharomyces*, however, provides 10- to 100-fold higher expression, making the *Pichia* to be very useful as the production system of eukaryotic proteins (Grinna and Tschopp, 1989 and Tschopp *et al*., 1987b). *P. pastoris* is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. In the first step of the methanol metabolism, oxidation of methanol to formaldehyde using molecular oxygen is catalyzed by the enzyme alcohol oxidase. This reaction generates both formaldehyde and hydrogen peroxide. To avoid toxicity of the hydrogen peroxide, metabolism of methanol takes place within a special organelle called peroxisome, which sequesters the toxic by products from the rest of the cell. Alcohol oxidase has a poor affinity for O_2, and *P. pastoris* compensates by generating large amounts of the enzyme. In the system of *P. pastoris*, high expression of the heterologous protein genes is regulated under the promoter of the alcohol oxidase (for review see Cregg *et al*., 2000).

3.3.1 Small scale synthesis

In small scale synthesis of the hu/croc/pigTTR, a single colony of the *Pichia* clone on yeast extract peptone dextrose (YPD) was induced with 0.5% methanol at 30^{o}C for 3 days. Analysis of the culture supernatant by native-PAGE (10% and 4% of resolving and stacking gels, respectively) followed by silver staining showed that the chimeric TTR was successfully synthesized and extracellularly secreted into the culture medium (Figure 3A). A protein band with mobility similar to that of the TTR in human serum was observed. However, each *Pichia* recombinant clones provided different level of the expression (as shown in Figure 3.4A). The mobility of the recombinant chimeric TTRs was greater than albumin in human serum similarly to that previous reported for most TTRs from

vertebrates including human (Seibert and Nelson, 1942) and bird (Chang *et al*., 1999), which migrates faster than albumin during electrophoresis at pH 8.6 (Farer *et al*., 1962 and Refetoff *et al*., 1970). Intensity of the TTR band obtained from each clone was measured by gel document to compare the production level. The result showed that the *Pichia* clone number 3 (as shown in Figure 3.4B) provided the highest production of the TTR. Therefore, this *Pichia* recombinant clone was selected for further optimization for synthesis of the recombinant TTR including methanol concentration and density of cells in the culture.

3.3.1.1 Optimization of the methanol concentration

Growth of cell is very important for obtaining the secreted proteins because concentration of the protein product in medium is roughly proportional to the concentration of cell in culture. This is clearly observed when growing the *P. pastoris* in fermentation. In addition, the level of transcription initiated from the *AOX1* promoter can be 3-5 times greater in cell that is fed with methanol at growth-limiting rates compared to that grows in an excess methanol (Cereghino and Cregg, 2000). Therefore, optimization of growth and induction conditions including concentration of methanol and cell density is necessary and is one of the positive factors leading to the highest expression of recombinant protein.

In optimizing, the *Pichia* clones were grown in 5 ml of BMGY at 30°C overnight prior being transferred to BMMY and cells were induced with 0.5% and 1% of methanol as described in section 2.5.1.1. Analysis of the culture supernatant by native-PAGE (10% resolving and 4% stacking gels) followed by silver staining revealed higher expression obtained when cells were fed with 1% methanol (Figure 3.5A). Intensity of the TTR produced from each clones was measured and analyzed by gel document and the result showed and confirmed that amount of the recombinant TTR obtaining when induced with 1% methanol was almost three times higher than when induced with 5% methanol (Figure 3.5B). Therefore, induction with 1% methanol was chosen for scaling up the synthesis.

3.3.1.2 Optimization of cell density in culture

To explore for the suitable cell density, the induction with methanol was carried out at 1% of methanol with density of the *Pichia* cells in culture equivalent to 2 or 4 units of OD_{600}. The induction was carried on for 3 days, and the culture supernatant was analyzed by native-PAGE. After staining gel with silver nitrate, a major protein band migrated with mobility corresponding to TTR was observed (Figure 3.6A). By analysis intensity of the TTR band from each *Pichia* clones with gel document, it revealed that production of the recombinant TTR increased from one to one and a half when density of cell in culture shifted from 2 to 4 units of OD_{600} (Figure 3.6B).

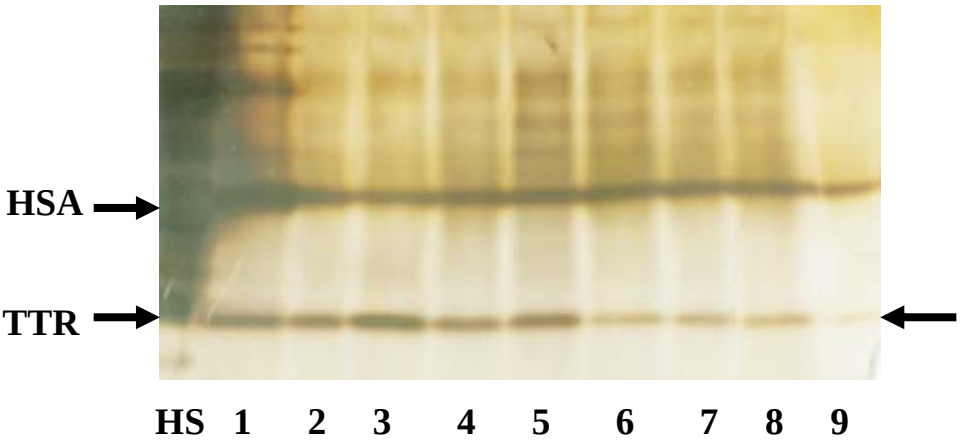
3.3.2 Large scale expression of recombinant TTR

To obtain large or sufficient amount of the recombinant chimeric TTR for further study, up scaling of the synthesis was attempted using the optimal conditions for the small scale synthesis in particular methanol concentration (1% methanol) and density of cell in culture (4 units of OD_{600}). During the induction period, aliquot of the culture medium was collected every 24 h. Analysis of the culture supernatant by native-PAGE (10% resolving and 4% stacking gels) after the synthesis induction was carried on for 3 days was shown in Figure 3.7. On the native gel, the protein that migrated with mobility similar to TTR first appeared after the induction with methanol for 24 h (Figure 3.7A). Then, production of the TTR increased and reached maximum after induction for 3 days. Moreover, intensity of the TTR band was not changed after induction for 4 days, which was confirmed by analysis by gel document (Figure 3.7B). This revealed no degradation by proteases and indicated to high stability of the TTR in the culture medium similar to that previously reported for the recombinant TTR from other vertebrates i.e. Shrew (Prapunpoj *et al*., 2000a), *X. laevis* (Prapunpoj *et al*., 2000b) and *C. porosus* (Prapunpoj *et al*., 2002). In average, production of the hu/croc/pigTTR by the expression system of *Pichia* was ~50 mg per liter of the 3 days culture, a little bit lower than that previously reported for TTRs from *X. laevis* (Prapunpoj *et al*., 2000b) and *C. porosus* (Prapunpoj *et al*., 2002). It should indicate to the possible variation

effect of nucleotide sequence of the gene, which is inserted into the expression cassette, on transcription and translation regulatory processes of the yeast.

Amount of the recombinant protein synthesized and secreted by *P. pastoris* was highly depended on composition of the medium for growth. Several media were reported for successfully in expression of recombinant proteins in *Pichia*. The *Pichia* cell grew slower in minimal medium than in other complex media. The amounts of secreted proteins increased when the buffered medium was used (Clare *et al*., 1991). Therefore, to obtain the maximum production, growing and induction with methanol for synthesis of the recombinant TTRs were performed in the buffered medium i.e. BMGY and BMMY. The presence of substrates such as peptide components of the yeast extract and peptone was demonstrated reduces in action of the pH-dependent extracellular proteases in *P. pastoris*, which consequently decreases the proteolytic degradation of the secreted recombinant proteins (Clare *et al.*, 1991). A time-course experiment for the production of the hu/croc/pigTTR showed that TTR was stable in the buffered medium throughout the induction period.

A


HSA
TTR
HS 1 2 3 4 5 6 7 8 9

B

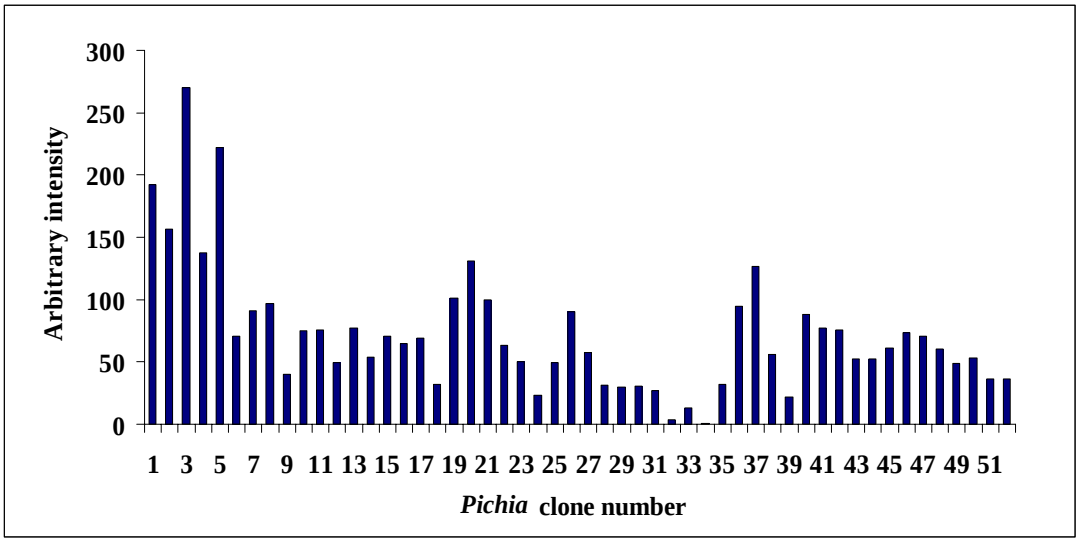
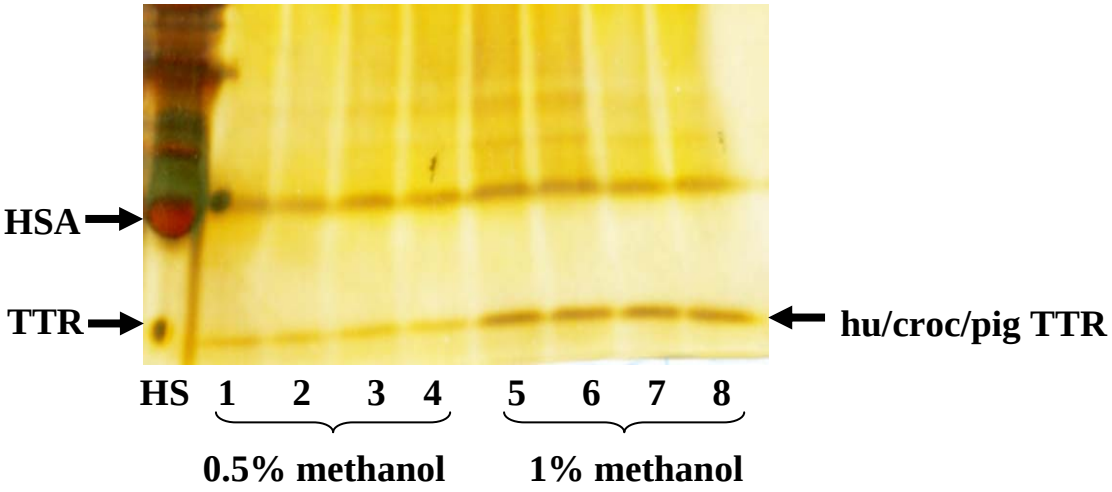

Arbitrary intensity
300
250
200
150
100
50
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51
Pichia clone number

Figure 3.4 Synthesis of the recombinant hu/croc/pigTTR in small scale

(A); The *Pichia* transformants were individually grown in BMMY and induced for synthesis with 0.5% methanol for 3 days. Thereafter, yeast cells were removed by centrifugation and aliquot (80 µl) of the culture supernatant was analyzed on native-PAGE (10% resolving and 4% stacking gels). Protein bands were detected by silver staining. HS, human serum that overloaded to show positions of albumin (HSA) and TTR (TTR) in serum; 1 to 9, individual *Pichia* clone. Position corresponding to the hu/croc/pigTTR band is indicated by arrow.

(B); Arbitrary intensity measurement by gel document of the hu/croc/pigTTR produced from 52 individual recombinant *Pichia* clones.

A


HSA
TTR
hu/croc/pig TTR
HS 1 2 3 4 5 6 7 8
0.5% methanol
1% methanol

B

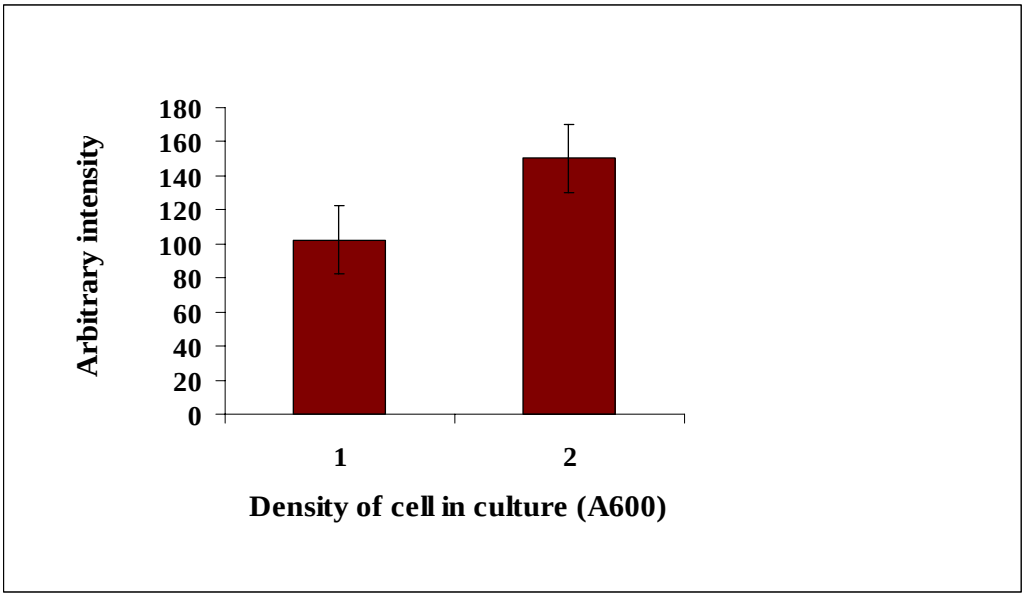
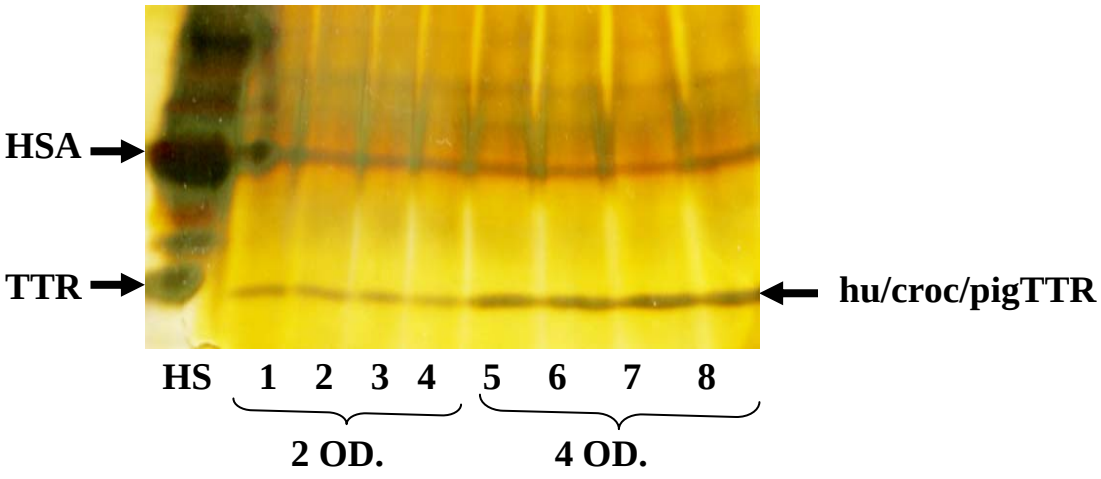

Arbitrary intensity
180
160
140
120
100
80
60
40
20
0
1
2
Density of cell in culture (A600)

Figure 3.5 Effect of methanol on production of the recombinant chimeric TTR

(A); The hu/croc/pigTTR clones were induced for synthesis with 0.5% or 1% methanol for 3 days. Aliqout (100 μl) of the culture supernatant was analyzed by native-PAGE (10% resolving gel and 4% stacking gels) and protein bands were detected by silver staining. HS, human serum that overloaded to show positions of albumin (HSA) and TTR (TTR); 1 to 8, the individual recombinant colonies of recombinant clone number 3. Position corresponding to the hu/croc/pigTTR band is indicated.

(B); Arbitrary intensity of the TTR produced by induction with 0.5% or 1% methanol was measured by gel document. Data are the mean of three separate experiments. The error bars are the standard error of the mean between the experiments.

A


HSA
TTR
hu/croc/pigTTR
HS 1 2 3 4 5 6 7 8
2 OD.
4 OD.

B

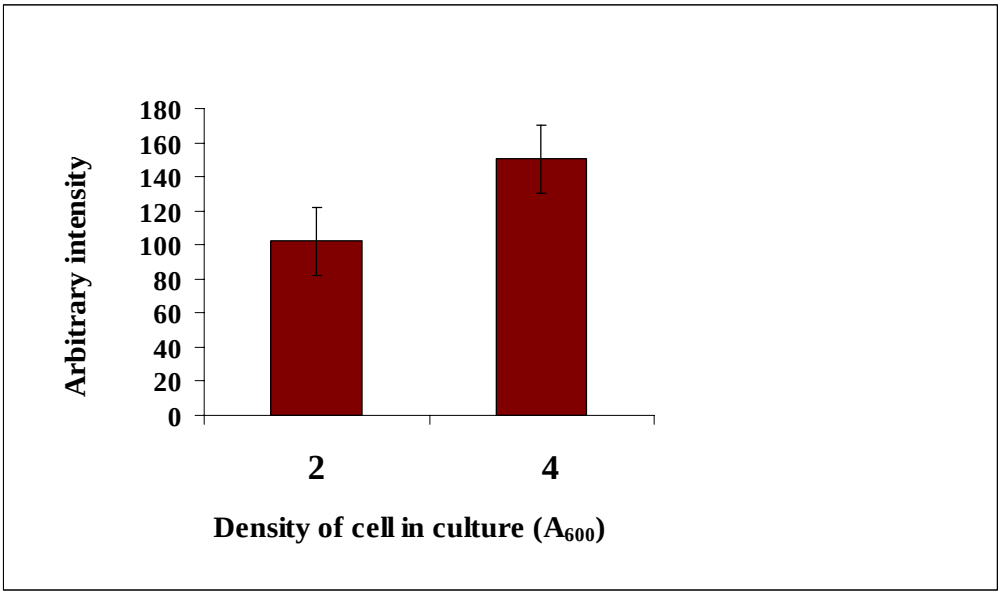
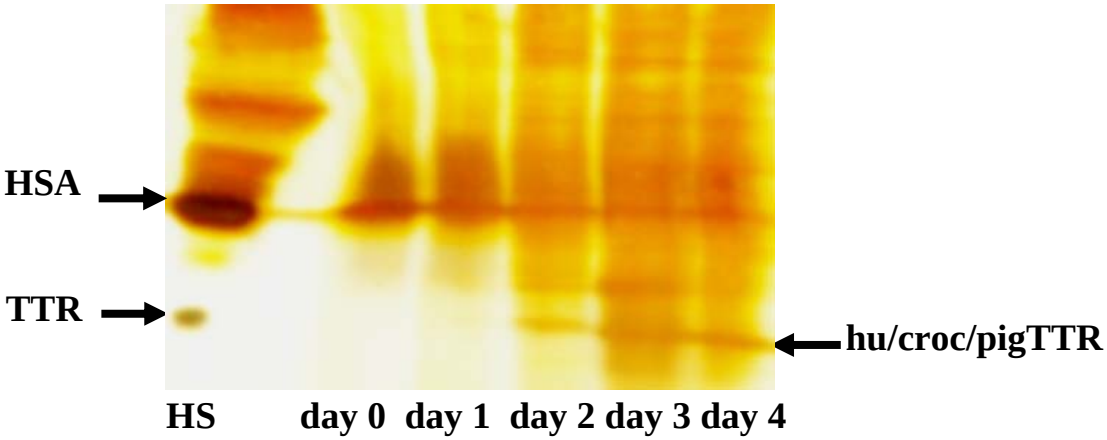

Arbitrary intensity
180
160
140
120
100
80
60
40
20
0
2
4
Density of cell in culture (A_{600})

Figure 3.6 Effect of density of *Pichia* cells in culture on production of the recombinant chimeric TTR

(A); The recombinant *Pichia* clone was grown and induced to synthesize TTR with the start density of the *Pichia* cells in culture equivalent to 2 and 4 units of OD_{600}. Aliqout (80 μl) of culture supernatant was analyzed by native-PAGE (10% resolving and 4% stacking gels) and protein bands were detected by silver staining. HS, human serum that was overloaded to show positions of albumin (HSA) and TTR (TTR); 1 to 8, the individual colonies recombinant clone number 3. Position corresponding to the hu/croc/pigTTR band is also indicated.

(B); Arbitrary intensity of the hu/croc/pigTTR protein was measured by geldocument. Data are the mean of three separate experiments. The error bars are the standard error of the mean between the experiments.

A


HSA
TTR
hu/croc/pigTTR
HS
day 0
day 1
day 2
day 3
day 4

B

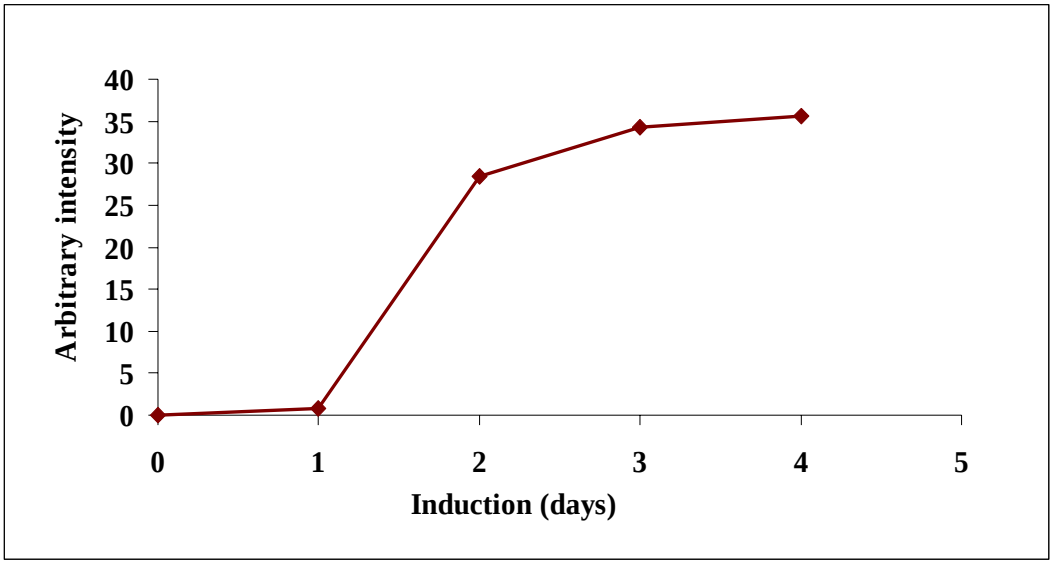

Arbitrary intensity
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
Induction (days)

Figure 3.7 Kinetic expression of the hu/croc/pigTTR

The *Pichia* transformant was induced with methanol for synthesis and secretion of the recombinant hu/croc/pigTTR expression for 4 days. The culture medium was daily sampling and aliquot (100 μl) of the culture supernatant was analyzed by native-PAGE.

(A); HS, human serum that overloaded to show positions of albumin (HSA) and TTR (TTR); 1 to 5 indicate day 0 to day 4 of induction with 0.5% methanol. Position corresponding to the hu/croc/pigTTR band is indicated.

(B); Arbitrary intensity of the TTR was measured by gel document. Position corresponding to the hu/croc/pigTTR band is indicated.

3.4 Purification of the recombinant chimeric TTR

In the *Pichia* expression system, the recombinant chimeric TTR was synthesized and secreted into medium, facilitating in downstream processing of the protein. Electrophoresis in a preparative polyacrylamide gel is useful technique for purification of the protein that migrates anodal ahead all other protein present. It is well known that most TTR from vertebrate species migrate anodal ahead other plasma proteins during non-denaturing polyacrylamide gel at pH 8.6 (Larsson *et al*., 1985; Duan *et al*., 1995b and Prapunpoj *et al*., 2002). Analysis by native-PAGE of the *Pichia* culture supernatant showed that the expected protein band of the hu/croc/pigTTR produced by *Pichia* cells migrated preceding the albumin in human serum (Figure 3.4-3.7). Therefore, this method was chosen to purify the recombinant hu/croc/pigTTR from other endogenous protein of *Pichia* that co-secreted into the culture medium.

The culture supernatant was concentrated prior loading onto the polyacrylamide gel tube. TTR in fractions can be determined by analysis on native-PAGE followed by silver staining of the protein band (Figure 3.8). As the recombinant chimeric TTR clearly migrated ahead other proteins, this facilitated in pooling out fractions containing the TTR. From several preparations, it showed that only ~1 mg of the recombinant hu/croc/pigTTR can be purified per 1 liter of culture, which is much less than that previously reported for the recombinant crocTTR (16 mg per liter of yeast culture) (Prapunpoj *et al*., 2002). It suggested that low efficiency in the culture concentration step was the major factor leading to this extraordinary low purification yield.

Facilitation in downstream processing of the recombinant protein of the *P. pastoris* expression system can be confirmed once in this thesis. The recombinant hu/croc/pigTTR that secreted out to the culture medium can be purified by the preparative native-PAGE. Although lower yield than expected was obtained but it is easy to perform and much less in time consuming as the purification of the TTR could be completed in only a single step. By other expression systems particularly of *E. coli*, several steps of purification including disruption the bacterial cell with some strong chemicals and detergents are required (Furuya *et al.*, 1989;

Murrell *et al.,* 1992; Berni *et al.,* 1994; Steinrauf *et al.,* 1993 and Rosen *et al.,* 1994). Moreover, additional amino acid tags were needed to incorporate at N-terminus of TTR to facilitate in purification (Matsubara *et al*., 2003).

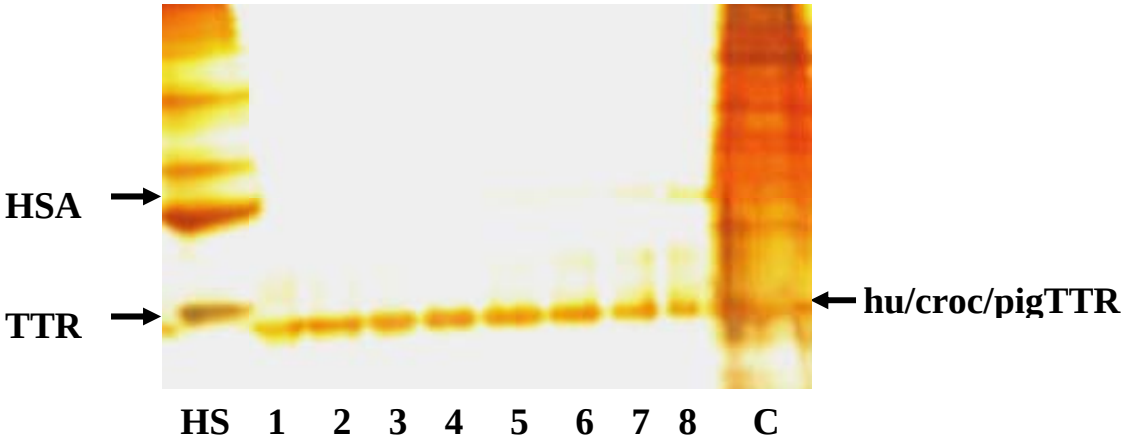


Figure 3.8 Analysis of the TTR eluting fraction from preparative native-PAGE
The 3-days induction *Pichia* culture was analyzed by preparative native-PAGE (10% resolving and 4% stacking gels) using the PrepCell model 495. The protein fractions were eluted at flow rate 60 ml/h and collected at 2 ml per fraction. HS, human serum that overloaded to show positions of albumin (HSA) and TTR (TTR); 1 to 8, the individual fractions eluted from the preparative gel tube; C, the concentrated culture supernatant that loaded to show the position of the recombinant TTR. Position of the recombinant hu/croc/pigTTR is also indicated.

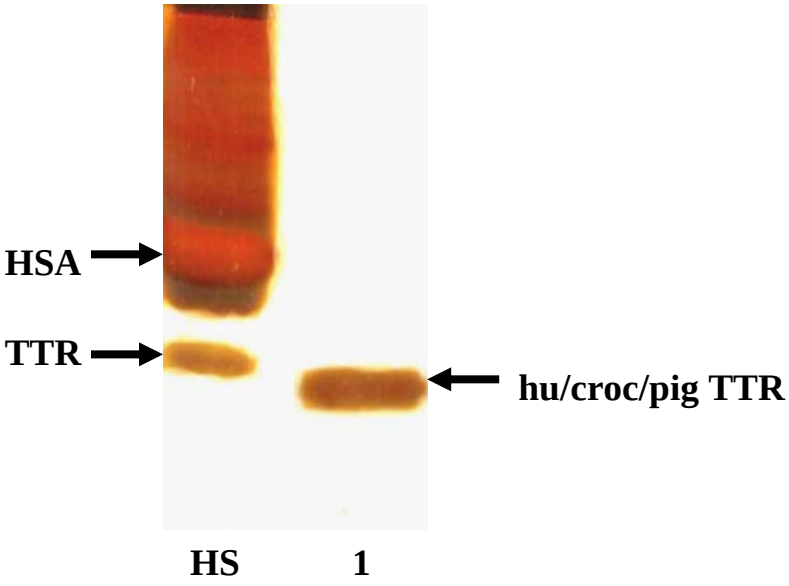


Figure 3.9 Mobility of the hu/croc/pigTTR in native-PAGE

The purified hu/croc/pigTTR (1) was separated in native-PAGE (10% resolving and 4% stacking gels). The protein bands were visualized by silver staining. Human serum (HS) was overloaded to show the position of TTR (TTR) and albumin (HSA).

3.5 Purification of native TTR from human plasma

Purification of native TTR from human plasma that use as a control for sufficient amount can be accomplished in 3 steps as described in section 2.9.

In the first step, proteins in human plasma was separated by affinity chromatography on a Cibacon blue column. Unbound proteins including TTR were collected. The analysis of the unbound fraction by native-PAGE showed that although TTR can not be isolated out from most other proteins but most of albumin, which is the most abundant protein in plasma, can be removed out from the fraction (Figure 3.10). This was confirmed from major existence of albumin in the bound fraction that eluted out of the column with high salt (data not shown). In the second step, the fractions containing TTR were pooled and concentrated by ultrafiltration prior to purification was carried on based on typical mobility under native condition of the TTR. The concentrated pooled fraction was subjected to preparative native-PAGE prior to the eluting fractions were analyzed. The result shown that, within the step of preparative native-PAGE, the human TTR can be isolated from other proteins in the plasma (Figure 3.11), and it migrated to the similar position as TTR in human serum. It was reported that human TTR in plasma is always in bound form, particularly, with RBP (van Jaarsveld, 1973). Therefore, to obtain the TTR in a free form, the third step of purification was incorporated. The fractions containing TTR from the preparative native-PAGE step were pooled and concentrated prior to the electrophoresis separation was performed on urea gel. Analysis of the eluting fractions on native-PAGE and SDS-PAGE (Figure 3.12) followed by silver staining revealed no RBP was detected in the TTR containing fraction. The RBP was observed only in fraction of TTR before processing on the urea gel. This indicated that RBP can be completely removed out from the TTR-RBP complex. The TTR fractions were then pooled, concentrated and determined for concentration by Bradford method (Bradford, 1976).

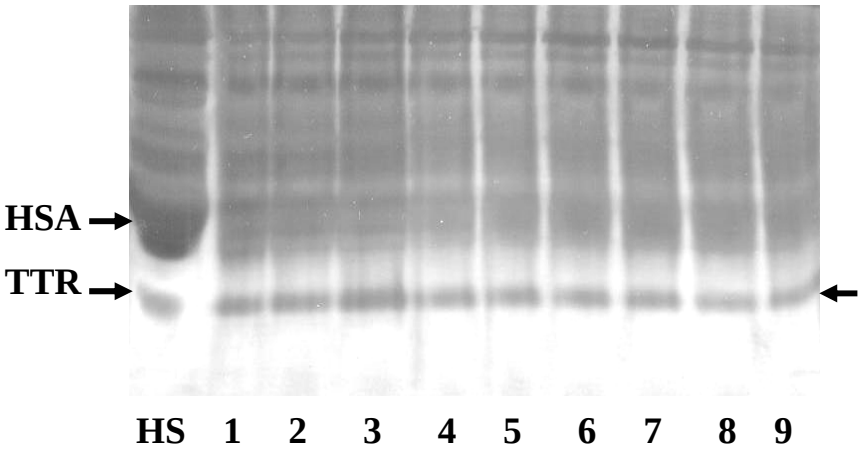


Figure 3.10 Purification of native human TTR from plasma by Cibacon blue column chromatography

Human plasma was loaded into the Cibacon blue column at a flow rate of 10 ml/h. Fractions of unbound protein were collected and aliquot (30μl) of each fraction was analyzed by native-PAGE. The protein bands were detected by silver staining. HS, human serum that overloaded to show positions of albumin (HSA) and TTR (TTR); 1 to 9, an individual unbound fraction eluted from the Cibacon blue column. Arrow indicates position of the expected human TTR band.

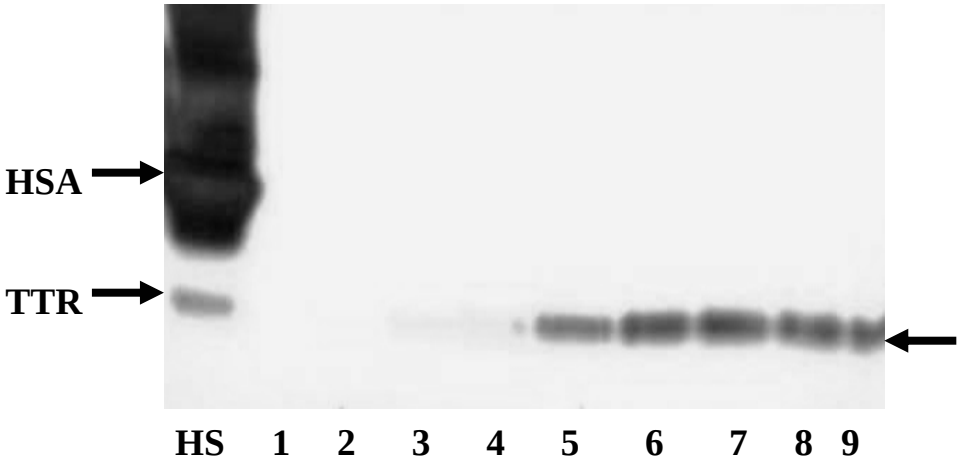
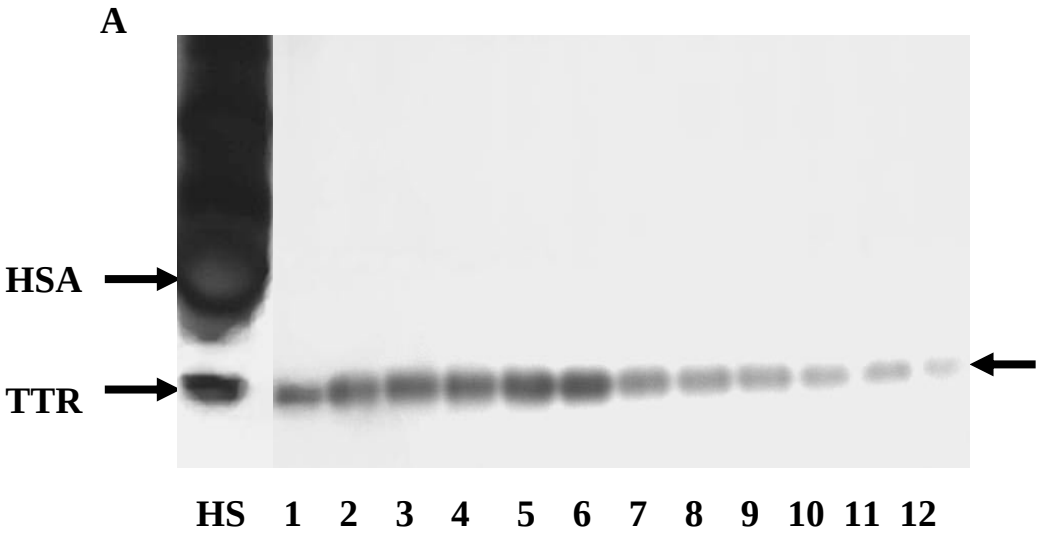


Figure 3.11 Elution pattern of native human TTR from the preparative native polyacrylamide gel

The concentrated pooled fraction containing human TTR from the chromatography on Cibacon blue column was applied onto a preparative native polyacrylamide gel (12% resolving and 4% stacking gels) and electrophoresis separation was performed using the Prep Cell model 491. Eluting fractions (2 ml/fraction) were collected, and aliquot (30 µl) of each fraction was analyzed by native-PAGE followed by silver staining. HS, human serum that was loaded to indicate TTR (TTR) and albumin (HSA) in serum; 1 to 9, the individual TTR containing fractions; arrow indicates the expected human TTR band.


A
HSA
TTR
HS 1 2 3 4 5 6 7 8 9 10 11 12

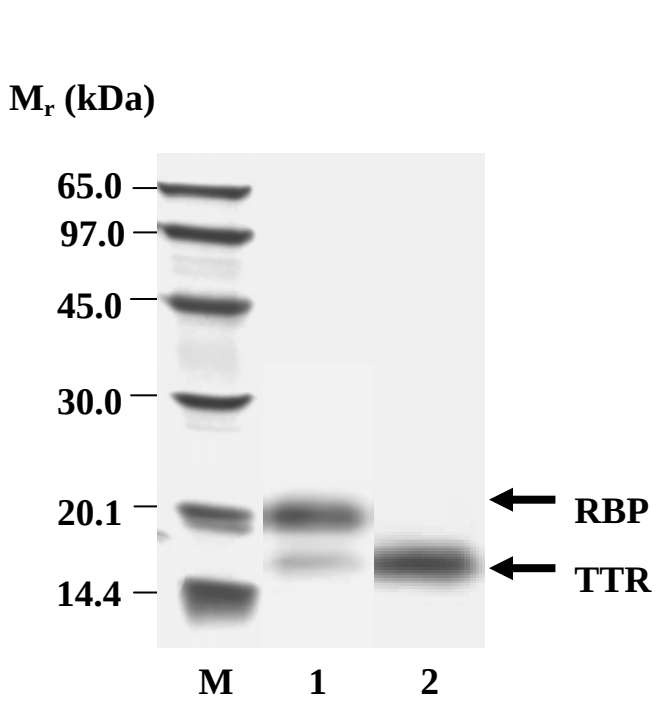

B
Mr (kDa)
65.0
97.0
45.0
30.0
20.1
14.4
RBP
TTR
M 1 2

Figure 3.12 Elution pattern of native human TTR from urea gel

Separation of

TTR from RBP was carried out on a preparative urea gel (12% acrylamide resolving gel containing 6M urea). The concentrated pooled fraction containing TTR was loaded on the gel column and separation was conducted as described for the preparative native-PAGE. Aliquots (30 µl) of the eluting fractions from the urea gel were analyzed by native-PAGE (A) and SDS-PAGE (B), and protein bands were detected by silver staining. HS, human serum; HSA, albumin in human serum; TTR, native TTR in human serum; M, protein standard marker; 1, TTR before the urea gel separation step; 2, TTR after applying onto the urea gel ; 1 to 12, the individual elution fractions.

3.6 Physicochemical properties of the recombinant TTR

Most of eukaryotic proteins require some or all of modifications in cells for their biological function (Dube *et al*., 1988). These include processing of signal peptide, folding, and O- and N-linked glycosylation (Cereghino and Cregg, 2000) of protein. Although the expression system of *P. pastoris* has capability in many post-translation modifications typically those presences in higher eukaryotes, the mechanism be hide the processing slightly differ from higher eukaryotes and possibly lead to alteration of properties and, thus, functions of the recombinant proteins. Therefore, physicochemical properties of the recombinant chimeric TTR including electrophoresis mobility under non-denaturing condition, molecular weight of molecule (indicates the tetramer forming), subunit mass and immuno-reactivity were determined.

3.6.1 Mobility in under non-denaturing condition

The electrophoresis mobility of TTR in several vertebrates such as mammal, diprotodont marsupial and avain (Duan *et al*., 1995b and Chang *et al*., 1999) in non-denaturing polyacrylamide gel, at pH 8.6, was greater than that of albumin. Only in some eutherian such as pig, TTR co-migrated with albumin in the non-denaturing gel (Farer *et al*., 1962 and Refetoff *et al*., 1970). In comparison, mobility of the purified recombinant hu/croc/pigTTR synthesized in *Pichia* was faster than albumin in human serum (Figure 3.13). Moreover, it was also clearly faster than human TTR that purified from human (Figure 3.13, lane 2). Incidentally, this mobility was similar to that previously reported for the chimeric *C. porosus* TTR in which the N-terminal region of the TTR was replaced by that of human TTR (Prapunpoj *et al*., 2006), indicating that only the N-terminal segment but not the C-terminal segment affected on the mobility under native condition of the chimeric TTR.

3.6.2 Molecular weight of the recombinant TTR

Molecular weight of the recombinant hu/croc/pigTTR was determined by HPLC/gel-permeation chromatography on BioSil SEC 250 column (Bio-Rad) equilibrated in 0.2 M sodium phosphate buffer pH 6.0 as described in section 2.7.1. The proteins with known molecular weight i. e., chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (158 kDa) and blue dextran (2,000 kDa) were used for calibration of the column. A standard curve was plotted between molecular weight in the logarithmic term (logMW) and K_{av}, which is calculated from the equation (Ve-Vo)/(Vt-Vo) where Ve is the elution volume of the protein, Vo is the exclusion volume of the column (equivalent to the elution volume of blue dextran 2000) and Vt is the total gel bead volume (equivalent to the elution volume of chymotrypsinogen A). The standard curve as shown in Figure 3.14 was used to determine the molecular weight of the recombinant chimeric TTR. Determination from the standard curve, molecular weight of the hu/croc/pigTTR tetramer was 58,884 daltons, which was similar to that reported for *C. porosus* TTR and it chimeric TTR (Prapunpoj *et al*., 2006), suggesting a proper tetramer forming of the recombinant chimeric TTR produced by *Pichia*.

3.6.3 Molecular mass of the subunit

To determine mass of the subunit, an aliquot of the purified recombinant hu/croc/pigTTR was boiled for 30 min in presence of 2% SDS and 2.5% β-mercaptoethanol, prior to performing SDS-PAGE (15% resolving and 4% stacking gels) and protein band was detected by staining with Coomassie brilliant blue as described in section 2.7.2. Migration of the TTR was showed in Figure 3.15A. The molecular mass of the subunit calculated from the standard curve (Figure 3.15B), which plotted between the relative mobilities (Rf) of proteins and molecular weight in the logarithemic term (logMW), was 15,488 daltons, similarly to that previously reported for TTRs from other vertebrate species (Prapunpoj *et al*., 2002 and Richardson *et al*., 1994). This should demonstrate there was no modification, in particular glycosylation, occurred.

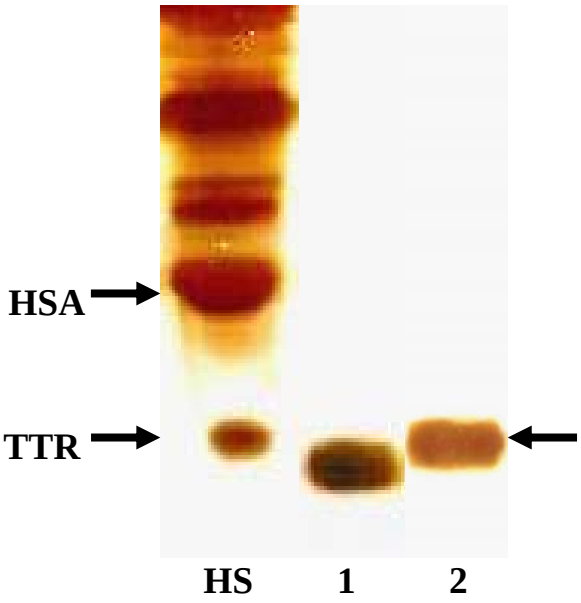


Figure 3.13 Mobility of purified recombinant hu/croc/pigTTR in native-PAGE

Purified recombinant hu/croc/pigTTR (1) and purified human TTR (2) were analyzed by native PAGE (10% resolving gel), and the protein band was stained by silver nitrate. HS is human serum. HSA and TTR are albumin and TTR detected in human serum, respectively.

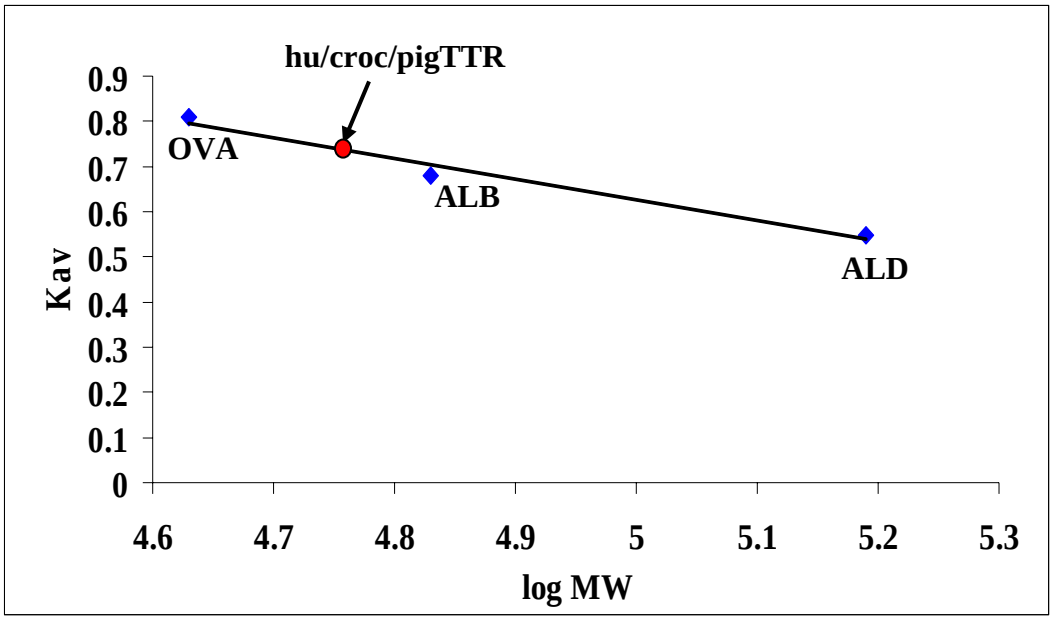
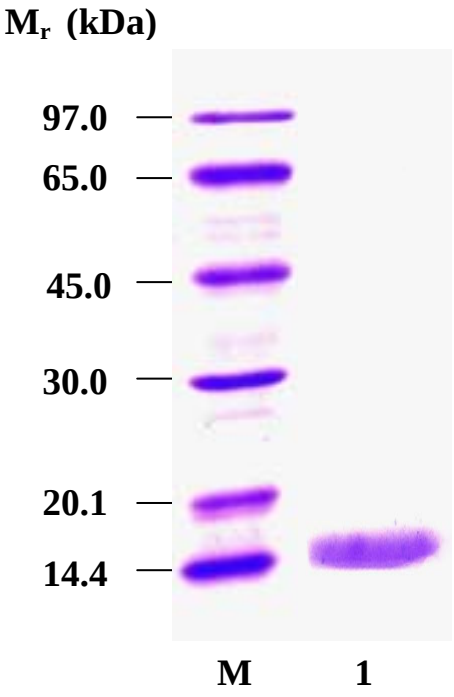


Figure 3.14 Determination of molecular weight of the recombinant hu/croc/pigTTR

Molecular weight of the TTR tetramer was determined by HPLC. The purified recombinant hu/croc/pig TTR (5 μg) was separated by gel filtration chromatography on Bio-Sil SEC 250 column at flow rate 0.4 ml/min. The optical density of protein in fractions was measured at 280 nm. The column was calibrated with proteins whose molecular weights are known including chymotrypsinogenA, (OVA; 43 kDa) albumin (ALB; 68 kDa), and aldolase (ALD; 158 kDa). A standard curve was plotted between K_{av} and log of molecular weight (log MW).

A


Mr (kDa)
97.0
65.0
45.0
30.0
20.1
14.4
M
1

B

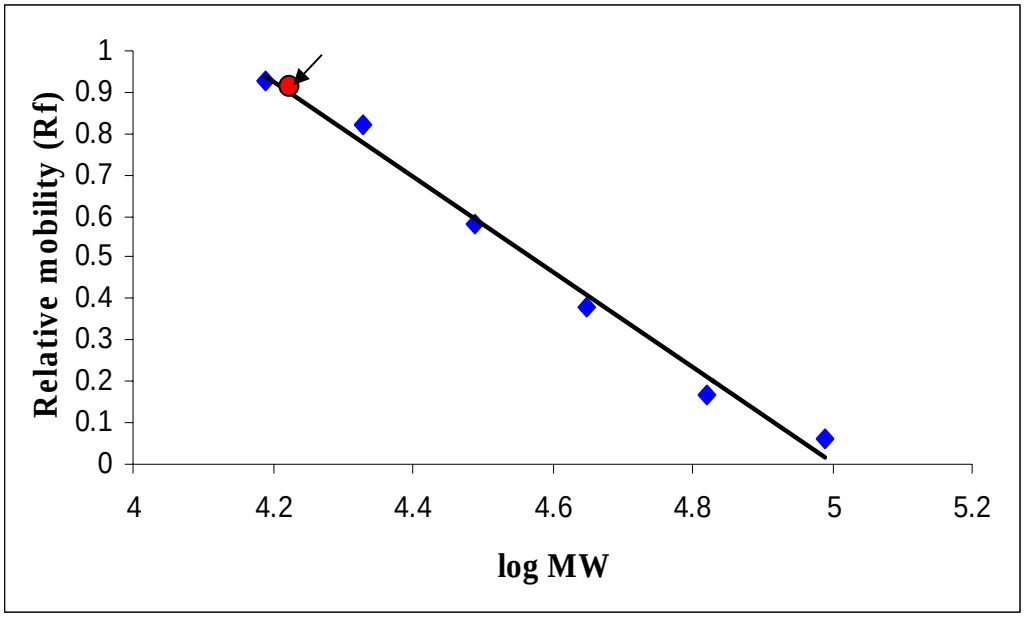

Relative mobility (Rf)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4
4.2
4.4
4.6
4.8
5
5.2
log MW

Figure 3.15 Determination of subunit mass of the recombinant hu/croc/pigTTR

(A) Purified recombinant hu/croc/pigTTR was boiled for 30 min in the presence of 2% SDS and 2.5% β-mercaptoethanol prior to analysis by SDS-PAGE (10% to 16% gradient resolving and 4% stacking gels) followed by staining with Coomassie brilliant blue. The molecular weight markers (M) are phosphorelase b (97.0 kDa), albumin (65.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa). The TTR subunit mass was calculated from the R_f of the TTR monomer and compared it to those of the protein markers (1).

(B) Plot of the R_f against log of molecular weight (log MW) of the protein marker.

3.7 Western analysis

The purified recombinant hu/croc/pigTTR (3 μg) and crocTTR (3 μg) (use as a control) were separated in duplicate on SDS-PAGE (15% resolving and 4% stacking gels) and then transferred onto PVDF membrane. The TTR bands were detected with Coomassie brilliant blue and identified by the immuno-reactivity with antiserum. Non-specific binding was eliminated by blocking with the skim milk proteins. Antiserum against TTR purified from *C. porosus*, raised in rabbit, was used as the primary antibody (1:500). The excess antibody was removed with a buffer containing Tween 20 as described in section 2.10. The specific binding of the *C. porosus* TTR antibody to the recombinant chimeric TTR was enhanced for detection by using the horseradish peroxidase-linked anti-rabbit IgG as a secondary antibody. Two bands showing the immuno-reactivity were observed (Figure 3.16). The first protein band, with much higher intensity, migrated in the same position as the TTR monomer detected by Coomassie blue. The second band, with much low intensity, migrated slower and had a molecular mass ~30 kDa, consisting with a dimer of TTR that always found when denaturation of the protein is not complete even with harsh condition (Dickson *et al.*, 1982; Furuya *et al.*, 1989; Prapunpoj *et al*., 2000b and 2002). This observation of the recombinant chimeric TTR dimer under the harsh denaturation should demonstrate to the strength of dimer-dimer interaction in the TTR. No the immuno-reactivity was detected in the protein markers, confirming the completion of the non-specific blocking.

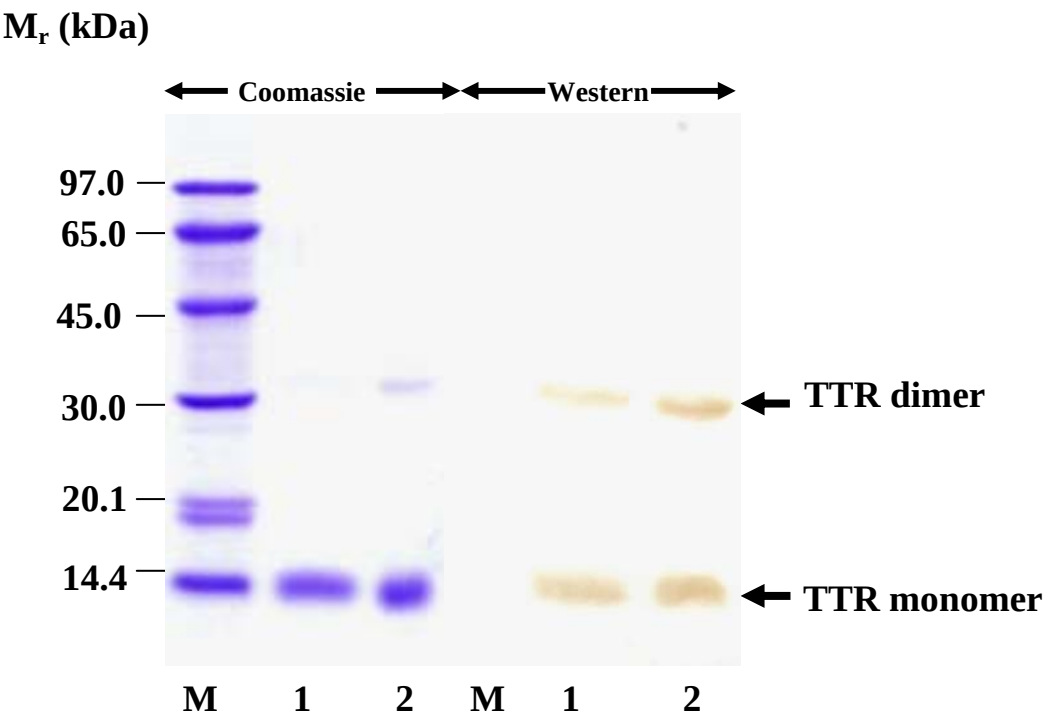


Figure 3.16 Characterization of recombinant hu/croc/pigTTR

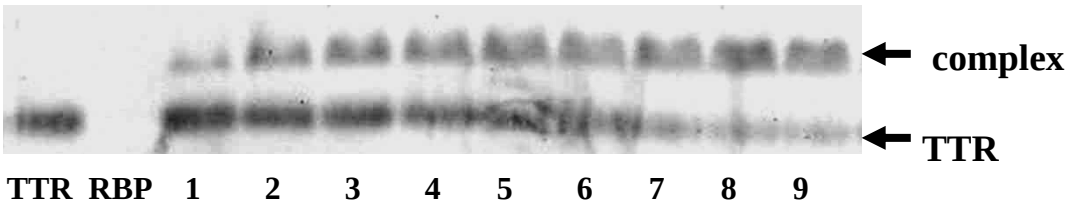
The purified recombinant hu/croc/pigTTR (1) and crocTTR (2) (as a control) were analyzed by SDS-PAGE and the proteins were electrophoretically transferred onto a PVDF membrane. The protein bands were stained with Coomassie blue R (Coomassie) and identified with antiserum (Western). The membrane was incubated with anti-*C. porosus* TTR raised in rabbit (1:500) followed by anti-(rabbit immunoglobulin) (1:2500) conjugated with horseradish-peroxidase. Detection was carried out by the enzymatic reaction using DAB as substrate. The molecular weight markers (M) included were phosphorylase b (97.0 kDa), albumin (65.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa). Position of the TTR monomer (monomer) and TTR dimer (dimer) are indicated.

3.8 Determination of the binding to RBP

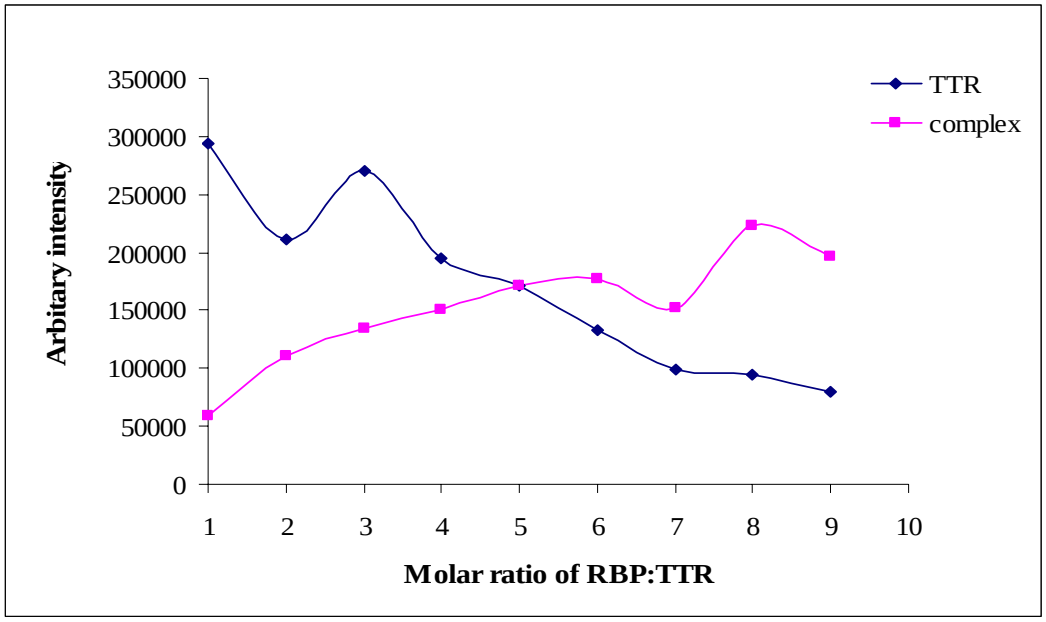
Another major function of TTR is binding to RBP, a single polypeptide chain with MW of 21 kDa that is responsible for the transport of the retinol (Monaco *et al*., 1995). Binding between TTR and RBP is thought prevents loss of the RBP from glomerular filtration in kidney (Nilsson and Peterson, 1971; Peterson and Rask, 1971; Kanai *et al*., 1968). Binding of TTR to RBP is specifically occurred at C-terminal region of the TTR (Monaco *et al*., 1995). In this thesis influence of N-and C-terminal regions of TTR on this property was studied. The binding between recombinant hu/croc/pig TTR to human RBP at difference molar ratio was investigated in comparison to that between the recombinant *C. porosus* TTR and human TTR. The binding reaction was carried out for 1 h at 4°C. Unbound TTR and that complexed with RBP were assessed. The results showed that changes in N- and C-terminal segments affected the complex formation of TTR with RBP (Figure 3.17). The complex forming between *C. porosus* TTR and RBP was detected from the molar ratio of 1:1 (Figure 3.17A), while that between the hu/croc/pigTTR was first detected at the molar ratio of 4:1, indicating that changes in either or both N- and C-termini decreased the formation rate of the complex. Chang in formation of the TTR-RBP complex was confirmed by the intensity plots of that unbound TTR and the complex with RBP (Figure 3.17), which revealed that the chimeric TTR bound to RBP at molar ratio of 7.5:1 (Figure 3.17D) while the *C. porosus* TTR bound to RBP at the molar ratio of 5:1 (Figure 3.17B). It was reported that the TTR-RBP complex is maintained through the hydrophobic interaction of the participated amino acid residues at the intermolecular contacts (Monaco *et al*., 1995). Therefore, longer and more hydrophilic of the hu/croc/pigTTR C-terminal region might be a major cause of less affinity in binding and, therefore, the binding rate of TTR to RBP as was observed in the experiment. Changes in length and hydropathy of the N-terminal region, on the other hand, may not directly effect on the binding between TTR and RBP. However, as it locates at the entrance to the central channel of the TTR molecular similarly to the C-terminal region, change in hydropathy environment due to change in amino acid sequence of the N-terminal region, may also effect on the hydrophobic interaction between hu/croc/pigTTR and RBP. However, the molar ratios of the dynamic binding observed in this thesis were higher than that previously

reported for the binding between human TTR and human RBP, which is 2:1 by X-ray crystallography (Monaco *et al*., 1995). Therefore, further experiments such as comparing the complex formation of other *C. porosus* TTR chimeric forms are required to confirm the postulation.

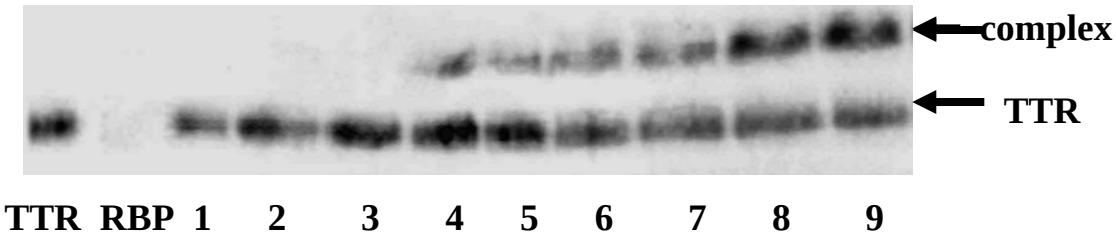
A


complex
TTR
TTR RBP 1 2 3 4 5 6 7 8 9

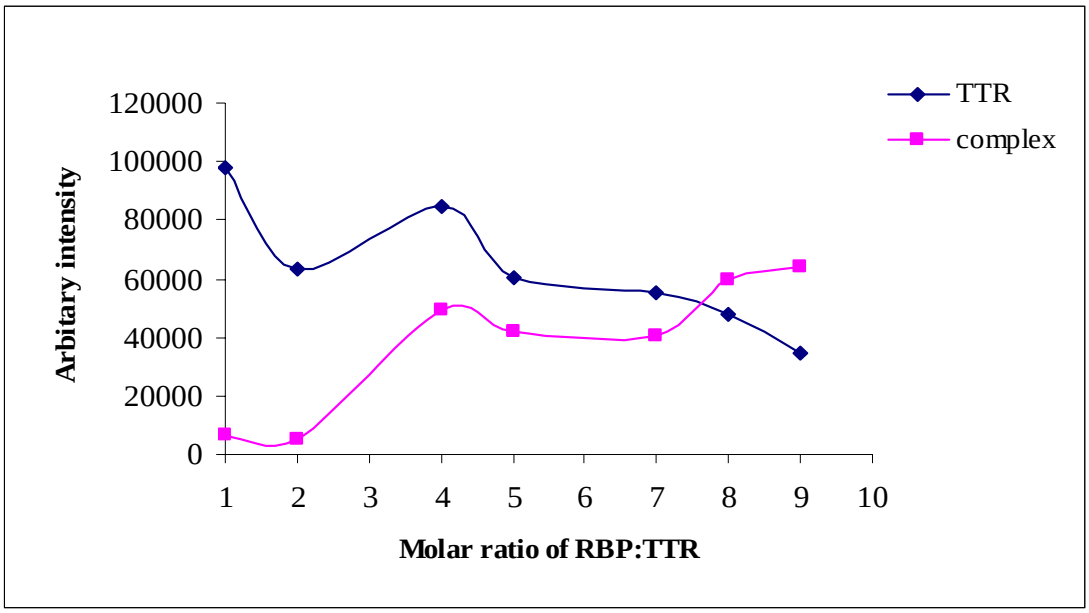
B


350000
300000
250000
200000
150000
100000
50000
0
Arbitary intensity
1 2 3 4 5 6 7 8 9 10
Molar ratio of RBP:TTR
TTR
complex

C


complex
TTR
TTR RBP 1 2 3 4 5 6 7 8 9

D


120000
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Arbitary intensity
1 2 3 4 5 6 7 8 9 10
Molar ratio of RBP:TTR
TTR
complex

Figure 3.17 Binding to RBP

Purified recombinant *C. porosus* TTR or hu/croc/pigTTR was incubated with purified human RBP at molar ratio of RBP:TTR ranging from 1:1 to 9:1, at 4^{o}C for 1h. Then, the reaction mixture was analyzed on native-PAGE (10% resolving and 4% stacking gels). The protein bands were transferred to nitrocellulose membrane and immuno-reactivity was detected using *C. porosus* TTR antibody (1:500) as the primary antibody and HRP-linked anti-rabbit IgG antibody (1:2,500) as the secondary antibody. Free TTR and TTR-RBP complex were detected by ECL.

(A); Protein band pattern of the *C. porosus* TTR and the complex with RBP, 1-9 indicate molar ratio of RBP:TTR ranging from 1:1 to 9:1

(B); Arbitrary intensity of the *C. porosus* TTR and the complex with RBP (n=3)

(C); Protein band pattern of the hu/croc/pigTTR and the complex with RBP, 1-9 indicate molar ratio of RBP:TTR ranging from 1:1 to 9:1

(D); Arbitrary intensity of the hu/croc/pigTTR and the complex with RBP (n=3)

CHAPTER 4

CONCLUSION

1. The cDNA for the chimeric hu/crco/pigTTR in which N- and C-terminal sequences of *C. porosus* TTR were replaced by the N- and C-terminal sequences of human TTR and pig TTR, respectively was successfully constructed in the pPIC9 expression vector of *P. pastoris*. The cDNA was expressed in *P. pastoris* and the chimeric TTR was synthesized and extracellularly secreted to culture medium.
2. The optimal conditions for synthesis in *P. pastoris* of the recombinant hu/croc/pigTTR were induction with 1% methanol and *P. pastoris* cell density in culture equivalent to 4 units of OD_{600}. At the optimal condition, the recombinant yeast produced ~50 mg of the chimeric TTR per liter of culture medium after induction with methanol for 3 days.
3. The recombinant chimeric TTR was purified by only a single step of preparative polyacrylamide gel electrophoresis under native condition with the purification yield of 1 mg per liter of culture.
4. The recombinant chimeric TTR had the electrophoresis mobility under native condition faster than albumin in human serum and the subunit mass determined by SDS-PAGE was 15,488 daltons.
5. By gel permeation HPLC, the recombinant TTR tetramer showed the molecular weight of 58,884 daltons.
6. By western analysis, the recombinant TTR had specific binding to the antibody against *C. porosus* TTR.
7. In comparison to *C. porosus* TTR, the chimeric TTR showed higher molar ratio of binding to RBP, suggesting the effect of changes in N- and C-terminal segments of the TTR on this binding property.

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