Results

1. Construction of expression vectors for the synthesis of recombinant TTRs

The promoter of the *AOXI* gene has been used as a tool for heterologeous in *Pichia pastoris*. In order to obtain the expression of a secreted protein, the native secretion signal and secretion signals of proteins in *Pichia*, α-factor and PHO 1 could be used. However between these two secretion signals of the yeast proteins, it has been reported that more successful expression was obtained with the α-factor signal sequence. Thus, in this experiment, plasmids for expression of normal crocodile TTR and crocodile chimeric TTRs were constructed using pPIC 3.5 (using the native secretion signal) and pPIC 9 (using secretion signal for α-factor), respectively, as vectors (Figure 1). cDNAs of crocodile TTRs with native N-termini and modified N-termini were amplifid by PCR using selected primer pairs (Table 1) to generate the compatible restriction ends for ligation into the pPIC vectors. TTR in the recombinant plasmids which were constructed by ligating the cDNA to the α-factor signal sequence will be co-expressed with α-factor protein and the latter will be removed within the Golgi by the dibasic amino acid sequences recognizing KEK2 protease (Julius *et al.*, 1984 a and 1984 b). *Pichia* vectors were linearized by *SalI* prior to being introduced into *P. pastoris* strain SMD 1168 by electroporation. More than 10^3 transformants were obtained per μg DNA. *Pichia pastoris* strain SMD1168 has mutation in the histidinol dehydrogenase (*HIS*4) and proteinase A genes which prevent yeast from synthesizing histidine and proteinase A, a major protease secreted by yeast cell. Because of the absence of *HIS4* gene, this *Pichia* strain can use methanol, but requires supplementation with histidine for growth (genotype his4, phenotype Mut^s / His^-). The crossing over event between the *HIS*4 gene on the plasmid and the his4 locus of yeast chromosome (Cregg *et al.*, 1985; Cregg *et al.*, 1989), creates His^+/Mut^+ recombinants that synthesize histidine and utilize methanol. Mut^+ transformants could be differentiated by culturing in a minimal medium containing methonol. 20 to 40 putative *Pichia* recombinants of each expression vectors were selected. The induction of expression in a small scale cultures (5 ml) was carried out at final concentration of 0.5 % methanol at 30 ° C for 72 h. Aliquotes of culture supernatant (90 μl) were analyzed by SDS-PAGE , with 15 % resolving and 4 % stacking gels. Silver staining of the gel revealed similar high-level expression of a secreted protein that migrated with an approximate subunit molecular mass of 15 kDa (Figure 2). This is consistent with the molecular mass of the subunit of TTR in other vertebrate species. One recombinant clone of each plasmid constructs was selected for further characterization.

Table 1 **Oligonucleotide primers used for generation of crocodile TTRs, native and those with *Xenopus* N-terminus and human N-terminus, cDNA fragments with compatible restriction ends for ligation into *Pichia* expression vectors**

Crocodile TTR	*Pichia* expression vector	Annealing temperature (°C)	Restriction end generated	Primer sequence (5′→3′)
with native N-terminus	pPIC 3.5		*Eco*RI	ACGGAATTCTTATTCTTGTGGATCACTG
		50	*Bam*HI	AAGGATCCAGAATGGCTTTTC
with *Xenopus* N-terminus	pPIC 9		*Eco*RI	Same as above
		50	*Xho*I	TCCCATGGAGAAGCCGATTCC
		50		ACCGGGACATGCTTCCCATGG
		56		CTCGAGAAAAGAGCACCACCGGGACATGC
with human N-terminus	pPIC 9		*Eco*RI	Same as above
		56	*Xho*I	AACGGGCACTGGTGAATCCAAATGCC
		56		CTCGAGAAAAGAGAGGCTGAAGCTGGCCCAACGGGCACTGG

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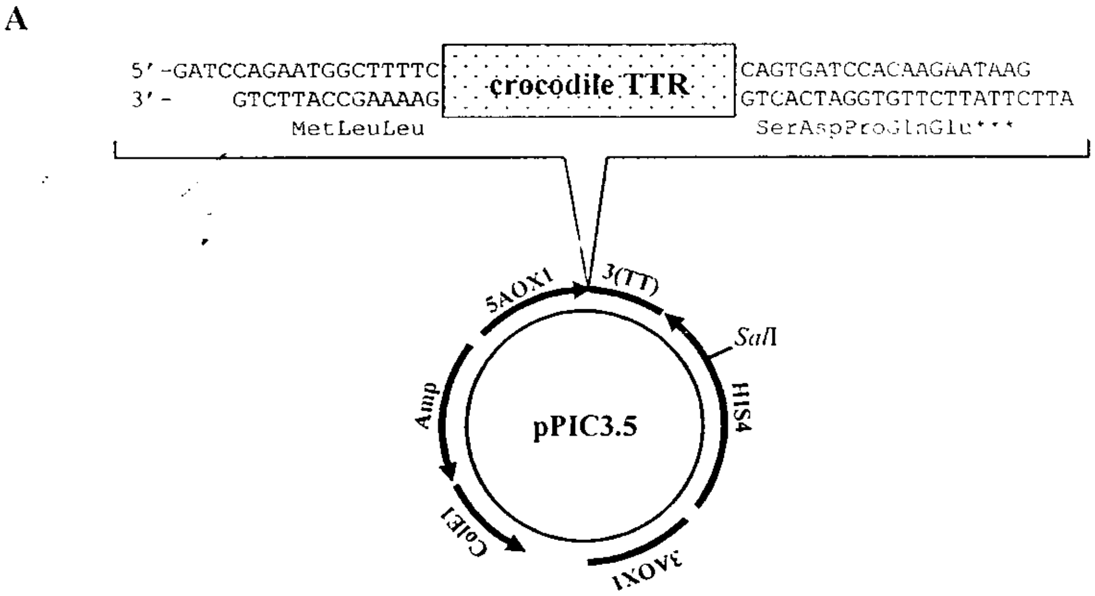

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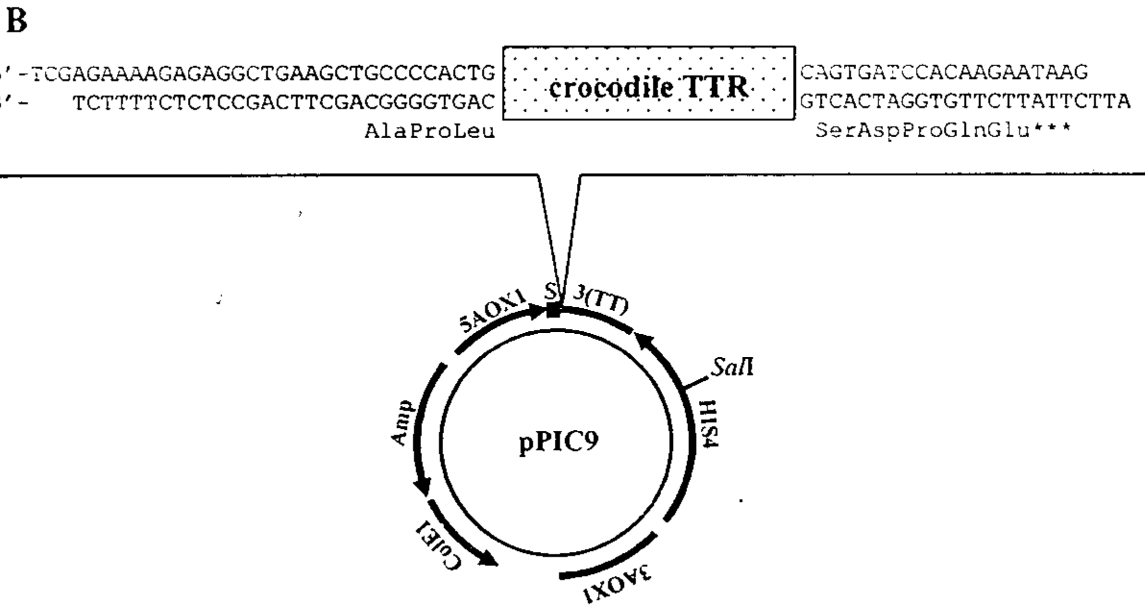

Figure 1 Construction of vectors for crocodile TTR expression

Expression plasmids were constructed from pPIC 3.5 (A) using the crocodile TTR presegment, and from pPIC 9 (B) using α-factor signal sequence, for expression of the crocodil TTR gene and secretion of the TTR. The figures show the sequence at the 5′ end and the 3′ end o crocodile TTR gene after restriction sites were introduced by polymerase chain reaction. 5AOX1 promotor of *Pichia pastoris* alcohol oxidase 1 gene; (3)TT, native transcription termination an polyadenylation signal of alcohol oxidase 1 gene; 3AOX1, sequence from the alcohol oxidase 1 gene, 3′ to the TT sequences; HIS4; histidinol dehydrogenase gene; Amp, ampicillin resistanc gene, ColE1, *E. coli* origin of replication; S, α-factor secretion signal (269 bases); *Sal*I, *Sal* restriction site for linearization of the vector.

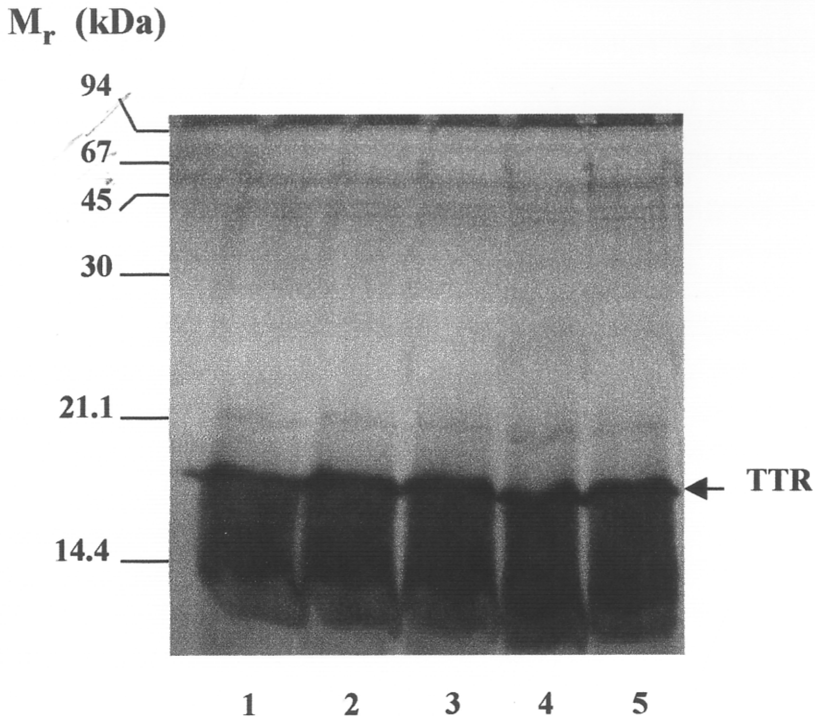

Figure 2 **Recombinant TTR expression pattern from *Pichia* transformant clones**

Transformed *Pichia pastoris* clones were grown and induced with methanol for 3 days. Supernatant of the yeast culture were collected, and aliquotes of 90 μl were analyzed by SDS-PAGE (15% gel) and silver stained. Positions of molecular weight marker proteins and TTR were indicated. The number under each lane indicates the individual clones.

2. Media selection

Optimal conditions for the successful synthesis of recombinant protein in *Pichia pastoris* depend on the characteristics of the protein. Buffered media such as BMG and BMM, and BMGY and BMMY are usually used for the expression of secreted proteins whose structure and activity are sensitive to the pH of the medium. MGY and MM are used for some protein specifically susceptible to proteases that are active at neutral pH. To determine which medium provided maximum expression level of crocodile TTR and its chimeras in *Pichia*, crocodile TTR clones were grown overnight at 30 ° C in media with difference compositions, as indicated in Table 2. Amount of TTR secreted by *Pichia* grown in different media was determined, after induction with methanol, for 72 h by SDS-PAGE and silver staining as shown in Figure 3. *Pichia* grown in BMMY produced the highest amount of secreted recombinant crocodile TTR.

3. Kinetics of secretion of crocodile TTR synthesized in recombinant *Pichia*

The TTR expressing recombinant clones were grown overnight in BMGY at 30°C until reaching log phase. Then cell pellets were resuspended to a density of 1.0 at A600 in 50 ml of methanol containing medium and incubated in 250 ml baffled flask at 30 °C for 72 h. The concentration of methanol in the medium was maintained at 0.5% throughout induction. Aliquotes (0.5 ml) of culture were withdrawn and 90 μl of supernatant was analyzed by SDS-PAGE (15% resolving and 4% stacking gels) with silver staining to monitor protein expression and secretion. The result of SDS-PAGE analysis is shown in Figure 4. The amount of secreted TTR was maximum at 72 h. No decrease of the intensity of the protein band corresponding to crocodile TTR was found, though, after prolonged induction for 72 h.

4. Purification of recombinant TTR

4.1 by affinity chromatography on a human retinol-binding protein Sepharose 4B column

The capability of TTR to bind to retinol-binding protein with high affinity was used for the purification of the recombinant TTR from other proteins in the yeast culture supernatant. TTR could be purified in a single step on a human retinol-binding-protein-Sepharose 4B column. Supernatant from the culture was collected after induction with methanol for 72 h. Yeast cells were removed by centrifugation at 1500 x *g* for 5 min. The supernatant was centrifuged once at 12000 x *g* for 20 min at 4 ° C to remove cell debris, and was added to a final concentration of NaCl of 0.5 M, prior to apply to the column which previously equilibrated

Table 2 **Media and their compositions used in synthesis and secretion of crocodile transthyretin and its chimeras in *Pichia pastoris***

Composition of medium	with glycerol	with methanol
100 mM potassium phosphate pH 6.0, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol or 0.5 % methanol	MGY	MMY
1 % yeast extract, 2 % peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol or 0.5 % methanol	BMGY	BMMY
1 % yeast extract, 2 % peptone, 2 % dextrose or 0.5 % methanol	YPD	YPM
1 % yeast extract, 2 % peptone, 1.34 % YNB, 4×10^{-5} % biotin, 1 % glycerol or 0.5 % methanol	MMGY	MMMY

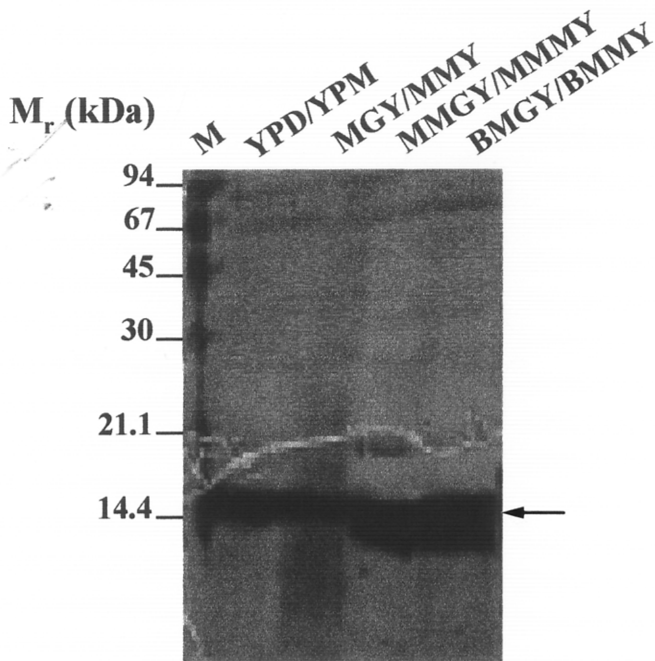

Figure 3 Secretion of crocodile TTR by *Pichia pastoris* in various media

Transformed *Pichia pastoris* cells contain crocodile native TTR were inoculated in glycerol or dextrose containing media, then transferred to methanol containing media as described in Materials and Methods. Cultures were induced with methanol for 3 days. Supernatant (90 μl) of cultures was analyzed by SDS-PAGE (15% acrylamide) and gels were stained with silver stain. Position of protein band which corresponds to TTR is indicated by an arrow. Composition of media used were as described in Table 2. Type of medium is given on the top of the gel. Molecular weight markers (M) were indicated.

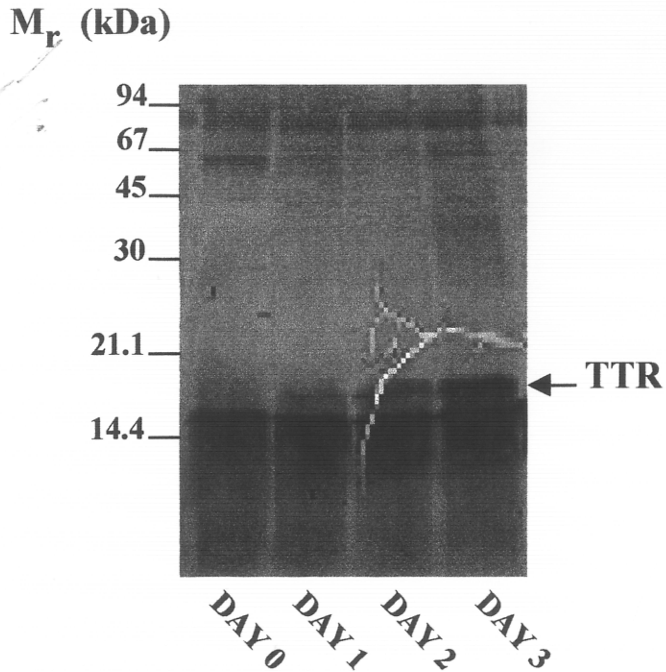

Figure 4 Kinetics of secretion of crocodile TTR by recombinant *Pichia pastoris*

Culture of transformed *Pichia* cell contains normal crocodile TTR was induced in BMMY and aliquots of culture were removed after induction with methanol for 0, 1, 2, and 3 days. An aliquot (90 µl) of supernatant was analyzed by SDS-PAGE (15% resolving gel). Gel was stained with silver staining to determine protein bands. Secreted protein band in the culture corresponded to crocodile TTR is indicated by an arrow.

with 40 mM Tris-HCl, pH 7.4, and 0.5 M NaCl. TTR binds to the retinol-binding protein and can be eluted from the column by decreasing hydrophilicity (Larsson *et al.*, 1985). Crocodile TTR bound strongly to the human RBP-Sepharose and could be eluted from the column with distilled water (Figure 5A). Approximately 16 mg of the recombinant TTR was obtained from 1 liter of 72 h-culture.

4.2 by preparative native-polyacrylamide gel elctrophoresis (preparative native PAGE)

The electrophoresis in a preparative polyacrylamide gel is one of a useful technique for purification of a protein which its properties, in particular the mobility in an electric field, is well characterized. TTR from most vertebrate species migrates with the same mobility as, or faster than, albumin during electrophoresis at pH 8.6 (Siebert and Nelson, 1942; Larsson *et al.*, 1985; Duan *et al.*, 1995; Prapunpoj *et al.*, 2000a). With the typical mobility characteristic of TTR, the preparative native PAGE was successfully applicable for purification of the recombinant TTR from other proteins in the yeast culture. 16 ml of 4 times concentrated of the 72 h yeast culture supernatant could be loaded into a gel tube without loss of resolution. Eluting fractions could be checked for TTR by silver stained (Figure 6). Approximately 14 mg of the recombinant TTR was obtained from 1 liter of 72 h culture.

5. Physicochemical properties of the recombinant crocodile TTRs

In the bloodstream of vertebrates, TTR exists as a tetramer of identical subunits. Each subunit has molecular mass of ~15 kDa which is typical for all studied TTRs in vertebrates. To reveal whether the expression and secretion of crocodile TTR in *Pichia pastoris* using the native TTR signal sequence or a native *Pichia* secretion signal lead to different post-translational modifications, the properties of purified recombinant crocodile TTRs produced by *Pichia* were determined. These properties include the N-terminal sequence, subunit molecular mass, tetramer formation and immunochemical cross-reactivity with antisera for TTRs from other vertebrates.

5.1 Mobility in SDS-PAGE

Purified recombinant normal crocodile TTR and its chimeras were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. The TTRs showed the same relative mobility as TTRs from other vertebrate species (Figure 7). The molecular mass determined from the calibration curve were 15.5 kDa for all recombinant crocodile TTRs, i.e. the TTR with native N-terminus, with N-terminal segment of *Xenopus* TTR and with N-terminal segment of human TTR. This subunit mass of crocodile TTR is similar to those for TTR subunits from other vertebrates (Richardson *et al.*, 1994).

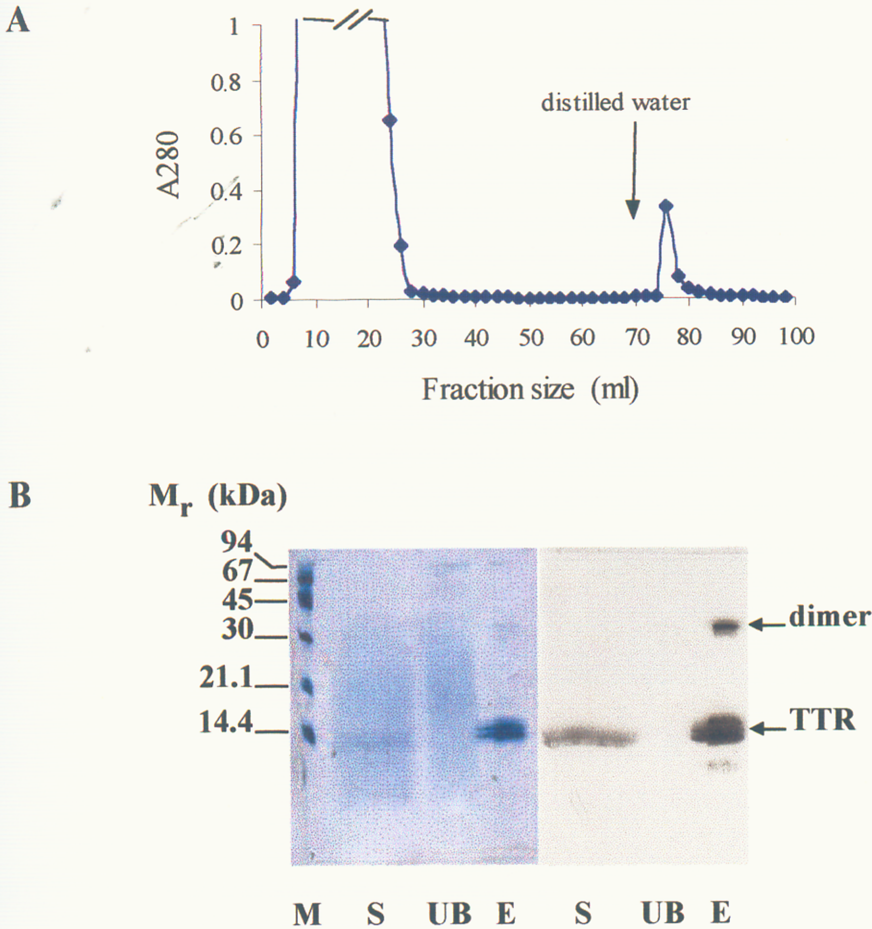

Figure 5 **Chromatographic profile of recombinant crocodile TTR from a human RBP-Sepharose 4B affinity column**

A : *Pichia* culture from the BMMY incubation was collected after induction for 3 days. Chromatographic separation was carried out at 4 °C. 40 ml of the supernatant was loaded on a column of human RBP-Sepharose (10 ml of gel bead) equilibrated in 0.04 M Tris-HCl, pH 7.4, containing 0.5 M NaCl. Bound protein was eluted with distilled water. 2 ml fractions were collected, and absorbance of the fractions were monitored at 280 nm.

B : Supernatant of yeast culture (S) (20 µl), concentrated pool of unbound protein (UB) and that of eluted protein (E) were subjected to Western analysis as described in Materials and Methods. Proteins bands were stained with Amido black and identified by reaction with antiserum against a mixture of purified TTRs from human, wallaby and chicken. The positions of the molecular weight markers (M), TTR subunit and its dimer were indicated.

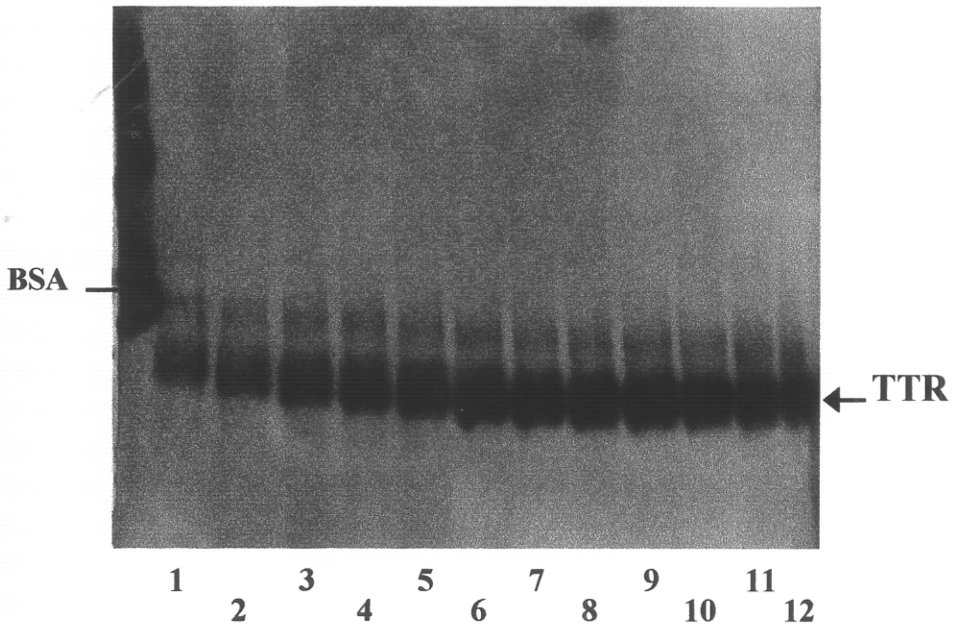

Figure 6 **Non-denaturing gel analysis of recombinant crocodile TTR from preparative native gel**

The 3-days culture from the BMMY incubation was concentrated and chromatographic separated by preparative native polyacrylamide gel electrophoresis (PAGE) (10% resolving gel and 3% stacking gel) in the Model 491 Prep Cell (Bio-Rad). 2 ml fractions were collected and 20 µl of each was analyzed by native PAGE gel (10% gel) and silver stained. Positions of bovine serum albumin (BSA) and TTR were indicated.

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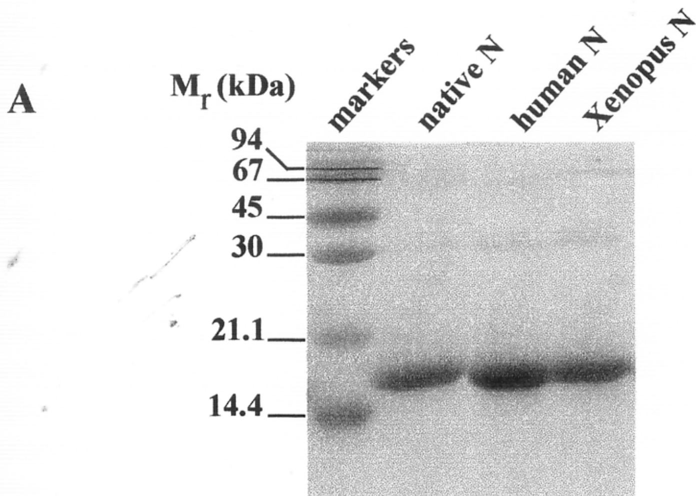

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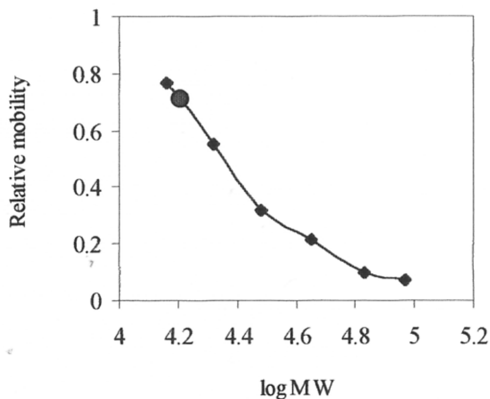


Figure 7 **Analysis by SDS-PAGE and determination of the size of the subunit of recombinant crocodile TTRs**

Aliquots of purified recombinant crocodile TTRs with native (native N), *Xenopus* (Xenopus N), or human (human N) N-terminus were boiled for 20 min in the presence of 2.5% β-mercaptoethanol and 2% SDS, prior to analysis by SDS-PAGE (A). The gels were stained with Coomassie blue R 250 to determine protein bands. Positions of molecular weight protein markers were indicated. The size of the TTRs subunit (red dot) was obtained by comparison of its electrophoretic mobility with those of molecular weight markers, plotting the relative mobilities against the logarithmic values of molecular weight of the markers (B).

5.2 Mobility in non-denaturing gel

Whilst, most of TTRs from vertebrates including human TTR (Siebert and Nelson, 1942), avian TTRs (Chang *et al.*, 1999) and *Xenopus* TTR synthesized in yeast (Prapunpoj *et al.*, 2000a) were reported to migrate faster than albumin during electrophoresis at pH 8.6. The comparative mobility of TTRs to albumin from other vertebrates in non-denaturing gel is differed (Farer *et al.*, 1962; Refetoff *et al.*, 1970). Some eutherian species, such as pigs and cattle, TTR co-migrate with albumin in non-denaturing gels. The mobility of normal crocodile TTR and its chimeric TTRs synthesized in yeast were compared with albumin (Figure 8). Recombinant crocodile TTRs migrated faster than albumin, similar to human TTR and TTRs from most vertebrates.

5.3 Immunochemical cross-reactivity of the recombinant crocodile TTRs with antibodies against human, wallaby and chicken TTRs.

Immunochemical cross-reactivity among TTRs from several vertebrates is well known (see Richardson *et al.*, 1996; Prapunpoj *et al.*, 2000a). To determine the immunochemical cross-reactivity of the recombinant crocodile TTRs with antiserum against TTRs from other vertebrates, the purified crocodile TTRs were analyzed in duplicate by SDS-PAGE, followed by transfer of the proteins onto nitrocellulose membrane. The position of the protein band were detected by staining with Coomassie Brillant Blue (Figure 9). Rabbit antiserum against human, wallaby and chicken TTRs was used as the primary antibody for immunochemical detection. Antibodies against human, wallaby and chicken TTRs were detected by enhanced chemiluminescence with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin as the second antibody. Membranes were exposed to X-ray film for 10 min and the film was immediately developed after exposure. Two bands were observed for each recombinant crocodile TTRs (Figure 9). One of the protein bands migrated in the same position as the subunit of recombinant crocodile TTRs detected by Coomassie blue. Another band, less intensity, migrated more slowly had a molecular mass of about 30 kDa, consistent with being a dimer of TTR. Dimers of TTR subunits were always found when denaturation of the protein was not complete (Prapunpoj *et al.*, 2000a). Cross-reactive material could not be detected in the lane of standard molecular weight markers.

5.4 Molecular mass of the recombinant crocodile TTR tetramers

To determine whether the formation of tetramers from the recombinant TTR subunits produced by *Pichia* occurred efficiently, TTRs purified from culture supernatant were analyzed by fast protein liquid chromatography on a Superose 12 (HR 10/30, Pharmacia) column in 50 mM potassium phosphate buffer saline, pH 7.4. Proteins with known molecular masses were

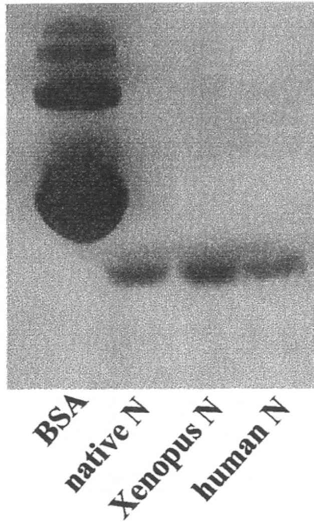
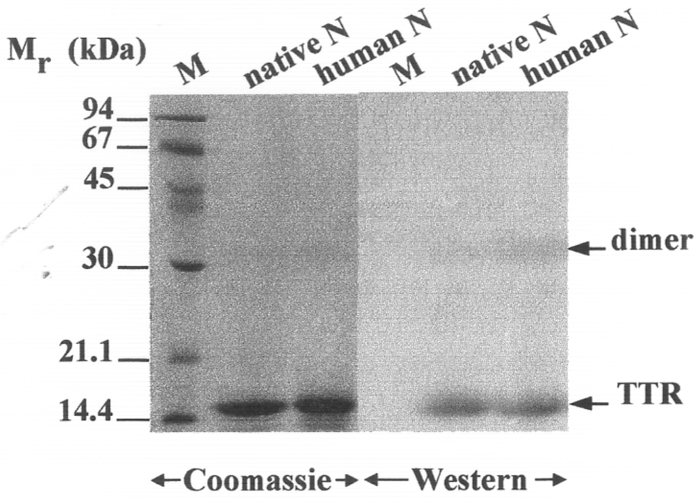


Figure 8 Electrophoretic movement of recombinant crocodile TTRs in non-denaturing polyacrylamide gel

Aliquots of purified recombinant crocodile TTRs with native (native N), or *Xenopus* (Xenopus N), or human N-terminus (human N) were analyzed by non-denaturing polyacrylamide gel (10% polyacrylamide) at pH 8.6. For comparison, purified bovine serum albumin (BSA) was included in the analysis. After electrophoresis, the gel was stained with Coomassie brilliant blue to determine the position of proteins.



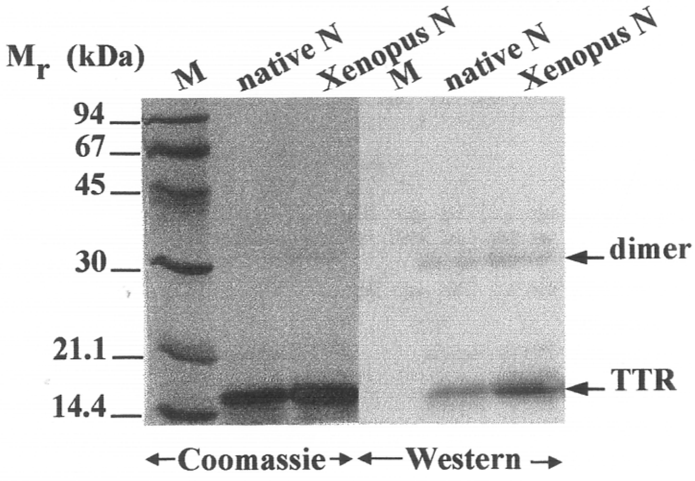


Figure 9 Western analysis of recombinant crocodile TTRs

10 μg of purified crocodile TTR with native (native N), or *Xenopus* (Xenopus N), or human N-terminus (human N) was subjected to Western analysis as described in Materials and Methods. Protein bands were stained with Coomassie brilliant blue (Coomassie) and identified by reaction with antiserum (Western). The primary antiserum (1:5000) used had been generated against a mixture of purified TTRs from human, wallaby and chicken. The secondary antibody (1:10000) was horseradish-peroxidase linked anti-rabbit immunoglobulin, raised in sheep. Detection was performed using an Enhanced Chemiluminescence kit (Amersharn) and X-ray film. The position of the TTR subunit and its dimer are indicated.

used to calibrate the column. The log of the relative molecular mass was plotted against K_{av}, where $K_{av} = (V_e - V_o)/(V_t - V_o)$. V_e is the elution volume of the protein, V_o is the exclusion volume of the column (elution volume of Blue dextran 2000) and V_t is the total gel bead volume (in this case 24 ml). The obtained curve is shown in Figure 10. Purified normal TTR was found to have a molecular mass of 56 kDa, whereas the molecular masses of the TTR with N-terminal segment of *Xenopus* TTR and that with N-terminus of human TTR were 50 kDa and 48 kDa, respectively. These molecular masses were four times the mass of each TTR subunits. This strongly suggested a tetrameric structure for all of three crocodile TTRs produced by the *Pichia*.

6. Binding affinities between recombinant crocodile TTRs and thyroid hormones

The dissociation constants of the complex of the recombinant TTRs with thyroid hormones were determined using the highly reproducible, rapid and sensitive method developed by Chang *et al.* (1999). Chicken TTR was included as controls for thyroxine and triiodothyronine binding affinities. Three individual experiments were performed for each hormone. The binding curve was plotted following the general equation according to Scatchard, 1949:

$$r/[u] = K_a n - K_a r = -1/K_d(r-n)$$

where r is the fraction of available binding sites on a macromolecule, which is occupied by a ligand, [u] is the free ligand concentration, n is the number of binding sites per macromolecule, K_a is the intrinsic association constant, and K_d is the dissociation constant.

For analysis, least squares linear regression lines with 95 % confidence were calculated using the statistical package in Microsoft Excel and the K_d values were derived from their slopes. The binding capacity to thyroid hormones was derived from the intercept with the abscissa in the Scatchard plot.

The K_d values of chicken TTR derived from the Scatchard analysis and plots, both for thyroxine (T4) (35.98 ± 2.22 nM; Figure 11 B) and for triiodothyronine (T3) (7.67 ± 1.09 nM; Figure 11 A) were quite different from those reported by Chang *et al.* (1999) (K_d for T3 was 12.3 ± 0.8 nM and K_d for T4 was 28.8 ± 2.6 nM), and gave the K_dT3/ K_dT4 ratio of 0.21. Possibly, the system set up in this report was more sensitive for T3 than T4. However, the values obtained still agreed well with the previous reported, i.e. chicken TTR binds T3 better than T4. Recombinant normal TTR from crocodile showed higher affinity for T3 than for T4.

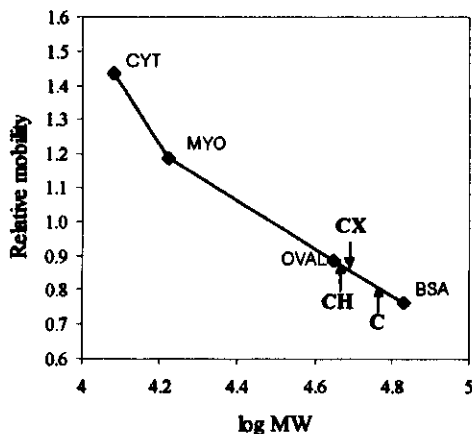
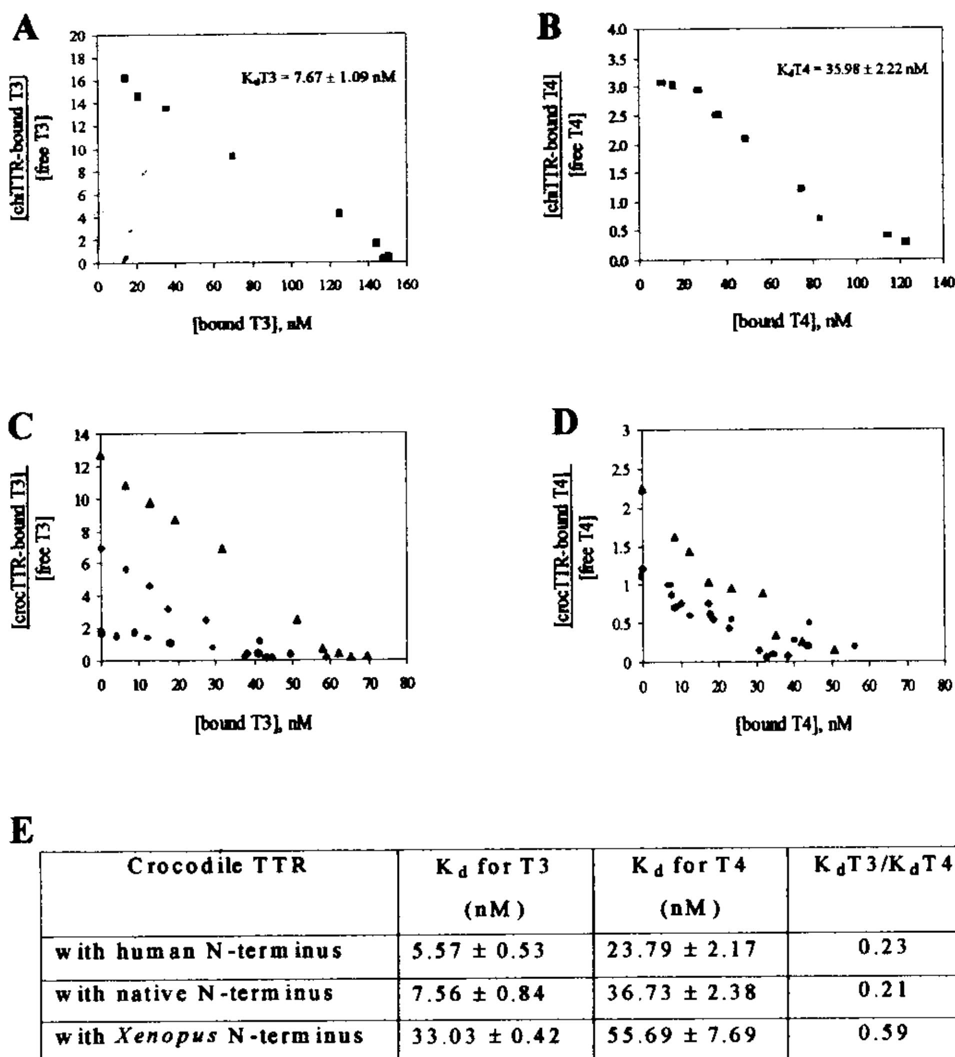


Figure 10 Determination of mass of crocodile TTRs by gel filtration

50 µl (~2 mg / ml) of each purified recombinant normal crocodile TTR and its chimeras was separated by gel filtration on a Superose-12 column and the absorbance at 280 nm of fractions was measured as described in Materials and Methods. Proteins with known molecular masses included bovine serum albumin (BSA) (68 kDa), ovalbumin (OVAL) (45 kDa), horse myoglobin (MYO) (18.7 kDa) and horse heart cytochrome *c* (CYT) (12kDa) were also chromatographed to calibrate the column. A standard curve was plotted of relative mobility (K_{av}) against log of the molecular mass (log MW). K_{av} of the crocodile TTR with the N-terminus of native TTR (C), with the N-terminus of *Xenopus* TTR (CX), and with the N-terminus of human TTR (CH), are indicated.



Crocodile TTR	K_d for T3 (nM)	K_d for T4 (nM)	K_dT3/K_dT4
with human N-terminus	5.57 ± 0.53	23.79 ± 2.17	0.23
with native N-terminus	7.56 ± 0.84	36.73 ± 2.38	0.21
with *Xenopus* N-terminus	33.03 ± 0.42	55.69 ± 7.69	0.59

Figure 11 Analysis of binding of recombinant crocodile TTRs to thyroid hormones : Scatchard plot for T3 and T4 bindings

100 nM of recombinant crocodile TTRs (crocTTR) was incubated with ^{125}I-L-T3 or ^{125}I-L-T4 in the presence of various concentrations of unlabeled hormone at 4° C, overnight. Free hormone was separated from the TTR-bound hormone by filtering the incubation mixture through a layer of methyl cellulose-coated charcoal. All corrections including those for non-specific binding were applied before performing the Scatchard analysis. Purified chicken TTRs (chiTTR) were included as controls for T3 (A) and T4 (B) binding affinities. The plots for the affinities (K_d) for T3 (C) and T4 (D) of crocodile TTRs were performed, as well as the ratio of K_d for T3 to K_d for T4 were summarized (E). ◆, ●, and ▲ represented the binding affinities of the recombinant crocodile TTR with N-terminus of the native, *Xenopus*, and human TTR, respectively. ■ indicates the binding affinity of purified chicken TTR.

This feature is similar to that of avian, amphibian and fish, but opposite to that of mammalian TTRs (Chang *et al.*, 1999; Prapunpoj *et al.*, 2000a). The K_d value obtained for T3 of crocodile normal TTR was 7.56 ± 0.84 (Figure 11 C,E) while that for T4 was 36.73 ± 2.38 (Figure 11 D,E), provided a K_d T3/ K_d T4 ratio of 0.21 which was the same ratio as that obtained from chicken TTR. The binding capacity of the recombinant crocodile TTR derived from the abscissa intercepts for both T3 and T4 suggested a capacity of 2 molecules of thyroid hormones binding per molecule of TTR.

In comparison to their normal TTR, the recombinant crocodile chimeric TTRs, whose N-terminal region was changed, showed alterations in the binding affinities to T3 and T4, as well as the K_d T3/ K_d T4 ratio. The crocodile TTR with N-terminus of *Xenopus* TTR had K_d values of 33.03 ± 0.42 nM and 55.69 ± 7.69 nM for T3 and T4, respectively. The binding affinities of this chimeric TTR to both thyroid hormones were moderately higher than those of the normal TTR, while a dramatic differences in both T3 and T4 binding affinities was found when compared with those in *Xenopus* TTR (K_d for T3 was 247.8 ± 19.3 nM and K_d for T4 was 508 ± 33.8 nM) (Prapunpoj *et al.*, 2000a). However, K_d T3/ K_d T4 ratio (0.60) (Figure 11 E) was more similar to that of the *Xenopus* TTR (0.49; Prapunpoj *et al.*, 2000a) than that of the normal crocodile TTR. On the other hand, recombinant crocodile TTR with N-terminus of human TTR showed K_d of 5.57 ± 0.53 nM for T3 and 23.79 ± 2.17 nM for T4 (Figure 11 C, D, E). Moreover, the K_d T3/ K_d T4 ratio (0.23) of this chimera was slightly higher than that of its normal TTR. These revealed an increasing in preference of the crocodile TTR to T4 when its N-terminus was changed to that of human TTR.

Discussion

In vitro synthesis of TTR is desirable for two main reasons. First, the expression of TTR gene in some animal species occurs in only a specific organ, or in only a short period of their lives. For example, the TTR gene is expressed in only a choriod plexus of crocodile, and in the liver of frog and toad tadpoles only during premetamorphic stages but not in the adults. To obtain sufficient amount of TTR for further study, a heterologous gene expression system is a need. Second, to determine the influence of the N-terminal region of TTR on the affinity binding to thyroid hormones, the construction of chimeric recombinant TTRs containing variable sequences of the N-termini is required. The functional properties (thyroid hormones binding) of such chimeric TTRs thus could then be analyzed for providing insight into the relationship between structure and function.

In wild type *P. pastoris* strain SMD 1168, the expression and secretion of crocodile TTR are mainly regulated by the *AOX1* promoter. The expression "cassette", consisting of signal sequence plus crocodile TTR gene, was inserted into the yeast genome at the *HIS4* locus. The integration was achieved by digesting the vector pPIC 3.5 with *Bam*HI and *Eco*RI, or the vector pPIC 9 with *Xho*I and *Eco*RI, prior to transformation. Most of the obtained recombinant clones were single-copy transformants, though spontaneous multiple gene insertion at a single locus is likely to occur with a frequency of 1% to 10 % (Sreekrishna *et al*., 1989; Romanos *et al*., 1998). The cultures were induced by transferring exponentially growing cells to the medium. The newly synthesized TTR was found to be secreted into the culture medium. In this report, the expression of crocodile TTR and its chimeras was attempted using two yeast expression vectors, pPIC 3.5 and pPIC 9. The two vectors were modified in two ways. First, the nucleotide sequence coding for normal TTR plus its signal sequence was introduced. Second, the TTR coding sequence with an altered N-terminal segment was ligated to the α-factor signal sequence from yeast. TTRs were synthesized and secreted efficiently by both vectors used, suggested that the signal sequences in the constructed vectors have the same efficiency for the insertion of the protein into the endoplasmic reticulum membrane of the yeast.

The amount of crocodile TTRs secreted by recombinant yeasts were dependent on the composition of the growth medium. The *Pichia* cells grew more slowly and secreted less amount of protein in the minimal medium compared with other more complex media (data not shown). This result is consistent with previously reported increases in the amounts of secreted protein when buffered medium was used (Clare *et al*., 1991). The presence of substrates such as

the peptide components of yeast extract and peptone reduced the action of pH-dependent extracellular proteases in *Pichia pastoris*, decreasing the proteolytic degradation of secreted recombinant proteins. A time-course experiment for normal crocodile TTR production showed that the protein was stable in the buffered medium through 3 days of induction (see Figure 4).

One of the advantages of using *Pichia* for the expression of heterologous genes is the very small amount of secreted endogenous proteins. The secreted heterologous protein (TTR in this case), thus, can comprise the majority of total protein in the medium. This facilitates purification of the foreign protein from other proteins. The expressed crocodile TTR could be purified to homogenity by a single step of affinity chromatography on human RBP-Sepharose column, or by a preparation native-gel electrophoresis. The yield of secreted crocodile TTR obtained from the *Pichia pastoris* system (14 to 16 mg per liter medium, depended on the purification method used) was higher than the expression of TTR in *E.coli* expression system using *ompA* signal peptide (5 mg per liter medium for human TTR) (Furuya *et al.*, 1991) but comparable to that was obtained in the intracellular expression when pCZ11 vector was used (20 mg per liter medium for human TTR) (Murrell *et al.*, 1992). However, as in the intracellular expression system the TTR was produced and accumulated in bacterial cells, several steps of purification were required (Furuya *et al.*, 1991; Murrell *et al.*, 1992; Berni *et al.*, 1994; Steinrauf *et al.*, 1993; Rosen *et al.*, 1994). Using the same heterologous genes expression system, *Xenopus* TTR was synthesized at 4 mg per liter medium (Prapunpoj *et al.*, 2000a). This expression level is ~4 times lower than that of crocodile TTR. It is conceivable that gene sequence of crocodile TTR was more preferable for the protein synthesis in *Pichia*.

Purified normal crocodile TTR, as well as its chimeras, migrates on SDS-PAGE as a single band with an approximated molecular weight of 15500 daltons, similarly to the molecular weight of the TTR subunits from other vertebrate species (Figure 7). Immumnochemical cross reactively with antibody against other TTRs confirmed that the band of 15000 daltons was corresponded to TTR. The electrophoretic mobility of recombinant crocodile TTRs in non-denaturing polyacrylamide gel at pH 8.6 was greater than that of albumin, which is a typical character of TTR of most vertebrates.

The N-terminal sequence of the subunits of secreted *Xenopus* and shrew TTRs purified from *Pichia* culture, were reported to identical to mature TTRs as predicted from alignment of their cDNA structure with human TTR (Prapunpoj *et al.*, 2000a,b). This demonstrated that the signal sequences of TTRs were correctly recognized and the leader sequence was accurately and efficiently cleaved in *Pichia pastoris*. Based on the same principle, alanine was predicted, from alignment with human TTR, to be the first amino acid at

the N-terminus of crocodile TTR subunit. However, the amino acid sequencing of secreted recombinant normal crocodile TTR synthesized in *Pichia* showed that leucine is the first amino acid. This revealed that crocodile TTR is 2 and 4 amino acid longer than those of marsupials and eutherian TTRs, respectively. Because of the facts that the recombinant normal crocodile TTR was synthesized using the TTR secretory signal sequence, and the SMD 1168 is the proteinase deficient *Pichia* strain, it should be conceivable that the N-terminus of crocodile TTR with shorter sequence than that expected was not a result of proteolytic degradation. The recombinant chimeric crocodile TTRs with N-terminus of TTR from *Xenopus* or human contained no extra amino acid, confirmed accurately recognition and efficiently cleavage of the *Xenopus* and human TTRs leader sequences in *Pichia pastoris*.

Native TTR in plasma exists in the tetrameric form. Part of it can remain as a dimer after denaturation by SDS (Dickson *et al*., 1982; Furuya *et al*., 1991; Prapunpoj *et al*., 2000a). This feature was also found in the recombinant crocodile TTRs produced by *Pichia*. Recombinant crocodile TTR migrated as a band in the position corresponding to the dimer in SDS-PAGE after boiling for 20 min in the presence of 2% SDS and 2.5% β-mercaptoethanol, demonstrating the strength of dimer-dimer interaction in TTR. This dimer band was confirmed to be TTR by the interaction with antibody against a mixture of TTRs (Figure 9). The mass of the recombinant proteins determined by gel filtration analysis was about four times the mass of the subunit determined by SDS-PAGE, indicating that the recombinant crocodile TTRs existed in the form of a tetramer similar to native TTR. Analysis of binding to retinol-binding protein and thyroid hormones showed that the recombinant TTRs could function as retinol-binding as well as thyroid hormone-binding proteins.

Binding of thyroid hormones is one of the main functions of TTR. This binding of thyroid hormones to TTR is functionally integrated with that of the other two plasma proteins, i.e. albumin and thyroxine-binding globulin, in a kind of network system to ensure the appropriate extracellular and intracellular distribution of thyroid hormones. The existence of the network system explains why TTR "knockout" mice possess a normal phenotype (Episkopou *et al*., 1993). Apparently, in the "network" of thyroxine-binding proteins, a deficiency in one component can be compensated for by the other components. The dissociation constant, K_d, quantitatively characterized the binding strength of TTR to thyroid hormones. Using a method that minimizes non-specific binding of thyroid hormones to surfaces (Chang *et al*., 1999), K_d values for T4 and T3 with only little variations of measurement within species were reported for TTRs from eutherians (human, rats and sheeps), marsupials (wallabies, wombats, and possums), avians (pigeons, chickens, emus) (Chang *et al*., 1999), and amphibians (Prapunpoj *et*

al., 2000a). TTRs from mammalians bound T4 better than T3, contrary to TTR from avian and amphibian that bound T3 with higher affinity than T4. In this report, K_d values for T4 and T3 of the recombinant TTR from crocodile were determined with the method described by Chang *et al.*, 1999 (Figure 11). It is the first report of binding affinity to thyroid hormones of TTR from reptilian. Moreover, it is the first evidence that function of the brain TTR from reptile has been studied. The crocodile TTR bound T3 with higher affinity than T4. This feature of higher binding affinity for T3 was similar to those reported for TTR from avian, amphibian (Chang *et al.*, 1999; Prapunpoj *et al.*, 2000a), and fish (Santos and Power, 1999). It confirmed that affinities for T3 decreased while the affinities of T4 increased during the evolution of TTR from fish and amphibian-like ancestors to mammals. The ratio of affinity of TTR for T3 over that of T4 is at least ten times lower in avian (Chang *et al.*, 1999), reptilian, (this report) and amphibian (Prapunpoj *et al.*, 2000a) than mammalian species.

In the evolution of the structure of TTR, two types of mutation patterns can be distinguished. The first type consists of random mutations found throughout the polypeptide chain of the human TTR subunit. Many of these cause medical disorders called amyloidosis. The second type consists of mutations occurring near the N-terminal region of the TTR subunit. These mutations lead to change in length and hydropathy of the N-terminal regions during evolution of TTR. What could have been the functional selection pressure operating in evolution of the TTR structure? Comparison of the primary structure of TTR from various vertebrates including mammal, avian, reptilian, amphibian, and fish shows that residues in all positions in the central channel of TTR which harbor the binding sites for thyroid hormones are conserved. These amino acid sequences have not been altered since, at least, 400 million years ago. In contrast, pronounced changes in the primary structure of TTR occurred during evolution within the first 10 amino acids from the N-terminal. The N-terminal segment is longer and more hydrophobic in avian, reptilian and amphibian TTR than in mammalian TTR. Although, these segments located near the entrance to the central channel that harbors the binding sites and may not involve directly with the binding of thyroid hormones. Their random-coil structure (Hamilton *et al.*, 1992), combined with high conformational flexibility within this region (Wilce *et al.*, 1995) may play a role in the binding of thyroid hormones, perhaps, by interfering with the access of thyroid hormones to the binding sites in the interior of the TTR molecule.

Comparative analysis of binding affinities to thyroid hormones of the recombinant normal crocodile TTR and those with alteration at N-terminal segments in this report clearly demonstrated that changes of the N-termini of TTR subunits involved in accessibility of the hormones to the binding sites in the central channel of TTR. Both strength (K_d values for T3

and T4) and preference (K_d T3/ K_d T4 ratio) in binding to thyroid hormones altered. Longer and more hydrophobicity of the N-terminal region led to less in binding affinities to both T3 and T4. However, degree of changing was different between chimeric TTRs. Decreasing of K_d for T3 was twice that of K_d for T4 when it was changed from *Xenopus* TTR N-terminus to crocodile N-terminus. On the other hand, decreasing of K_d for T4 was almost 4 times of that occurred in K_d for T3 when the N-terminal segment of normal crocodile TTR was altered to that of human TTR. This could suggested that not only the length and hydropathy, but also the nature, i.e. amino acid composition and sequence, of the N-terminus play role in binding of TTR to thyroid hormones.