Results and Discussion

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

There are two genes in *P. pastoris* that encode for an alcohol oxidase, AOX1 and AOX2. The *AOXI* is responsible for a vast majority of alcohol oxidase activity in the cell (Tschopp *et al.*, 1987; Ellis *et al.*, 1985; Cregg *et al.*, 1989). So, this promoter was used as a tool for expression of recombinant human TTR in *P. 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, it has been reported that more successful expression was obtained with the α-factor signal sequence. Thus, in this experiment, plasmids for recombinant protein expression were first trial constructed in pPIC 3.5 (using the native secretion signal) and pPIC 9 (using secretion signal for α-factor), for human wild type TTR (Figure 1). cDNA of the human wild type TTR was amplified by PCR using primer pairs (Table 1) to generate the compatible restriction ends for ligation into the pPIC vectors. Using the pPIC 9, the TTR will be co-expressed with α-factor protein and the latter is expected to 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* SMD 1168, a protease deficient strain (Brierley, 1998). Approximately, 10^3 transformants from 1 μg of DNA was obtained using an electroporator. Less, but sufficient, efficiency obtained with the transformation method using whole yeast cell, in the presence of polyethylene glycol (PEG) 1000.

Because of the absence of *HIS4* gene, this *Pichia* strain can use methanol, but requires histidine as a supplement 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 methanol. 20 putative *Pichia* recombinants from each expression vector 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

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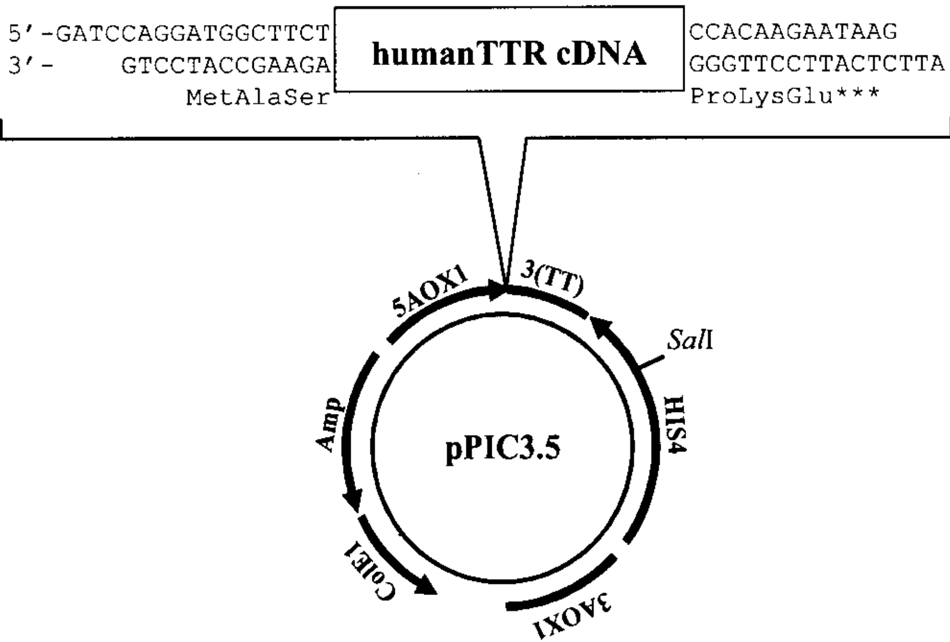

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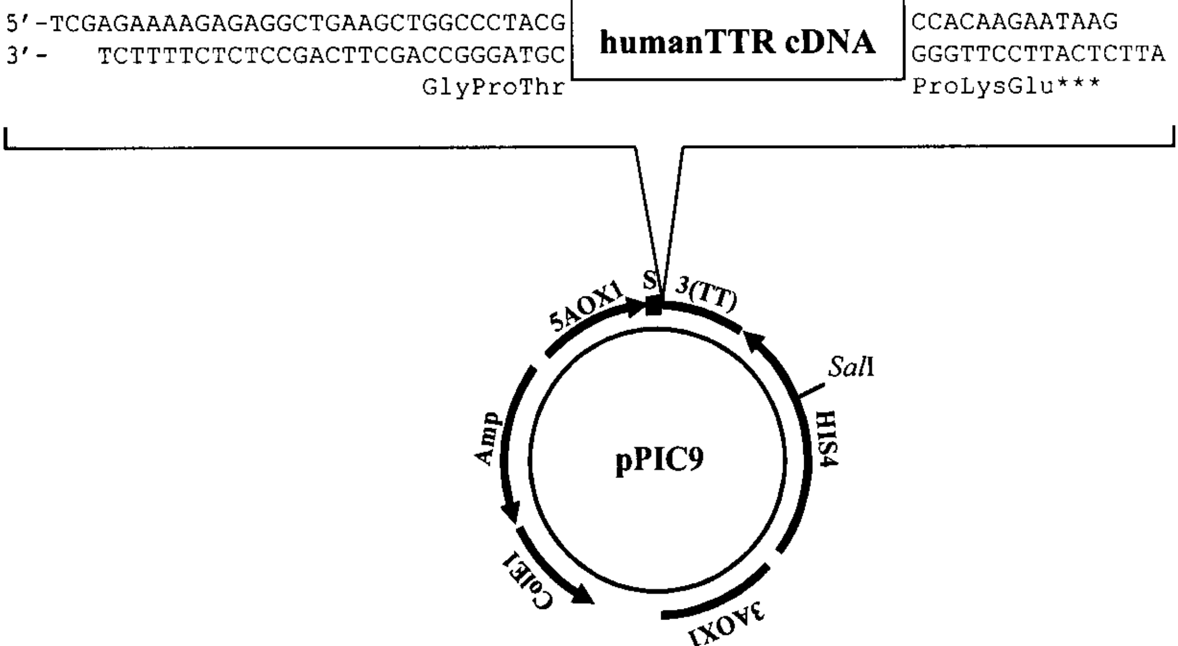


Figure 1 **Construction of expression vectors for human TTR gene**

For the synthesis and secretion of recombinant human TTR, the expression plasmid was constructed from pPIC 3.5 (A), using the human TTR presegment, and pPIC 9 (B), using the α-factor protein promoter of *Pichia*. Figures show the sequence at the 5′ end and the 3′ end of the TTR genes after restriction sites were introduced by polymerase chain reaction. 5AOX1, promotor of *Pichia pastoris* alcohol oxidase 1 gene; (3)TT, native transcription termination and 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 resistance gene, ColE1, *E. coli* origin of replication; *Sal*I, *Sal*I restriction site for linearization of the vector.

by SDS-PAGE, with 15 % resolving and 4 % stacking gels. Silver staining of the gel revealed an 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 native human TTR. However, only recombinant clones generated from expression vector pPIC 3.5, but not those from vector pPIC 9, showed the TTR protein expression and extracellular secretion of the protein (Figure 2). As the consequence of the gene expression in *Pichia* cell results obtained for recombinant human wild type TTR, the expression vectors for recombinant human TTR variants were constructed in the pPIC 3.5 only.

2. Optimization conditions for the recombinant TTR synthesis

2.1 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 protease those active at the neutral pH. To determine which medium provided maximum expression level of human wild type TTR and its amyloid variants in *Pichia*, recombinant human wild type 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. *Pichia* grown in BMMY produced the highest amount of secreted recombinant human TTR (data not shown). The similar characteristic also observed in other vertebrate TTRs (Prapunpoj *et al.*, 2000; Prapunpoj *et al.*, 2002). Thus, BMGY/BMMY is the media system was selected for synthesis of recombinant human wild type TTR and its variants through the rest of experiments.

2.2 Kinetics of secretion of recombinant human TTR synthesized in Pichia

The TTR expressing recombinant clones were grown overnight in BMGY at 30°C until reaching log phase. Cell pellet was then resuspended to a density of 1.0 at A_{600} in 5 ml of BMMY and incubated in 25 ml McCartney bottle at 30 °C for 96 h. Aliquotes (0.1 ml) of culture were withdrawn at every 24 h, and 20 μl of supernatant

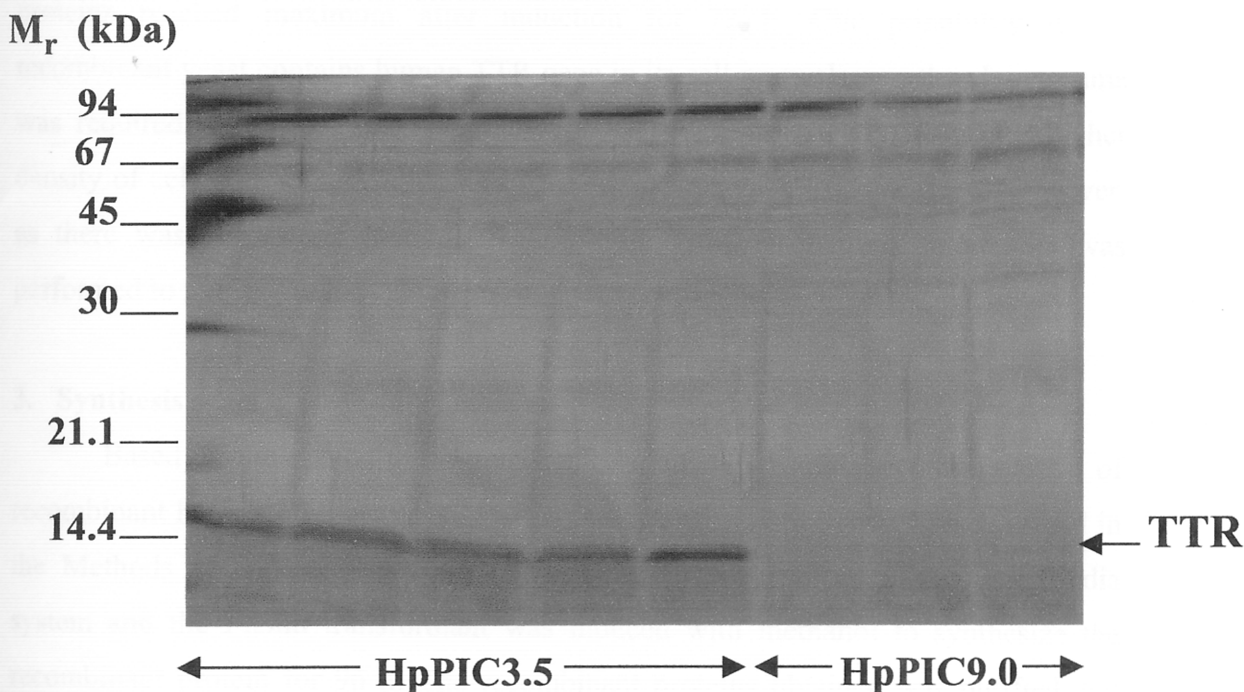


Figure 2 Recombinant TTR expression pattern from *Pichia* transformant clones

Transformed *Pichia* clones, by pPIC 3.5 (HpPIC3.5) or pPIC 9 (HpPIC9) expression vector, were grown and induced with methanol for 4 days. Supernatant of the yeast culture was collected, and aliquotes of 90 μl were analyzed by SDS-PAGE (15% resolving gel) and silver stained. Positions of protein markers and TTR were indicated. The number under each lane indicated an individual clone.

was analyzed by SDS-PAGE (15% resolving and 4% stacking gels) with silver staining to monitor protein expression and secretion. The amount of secreted TTR was found reach to maximum after methanol induction for 96 h (Figure 3). This feature is different from the *Xenopus* (Prapunpoj *et al.*, 2000) and crocodile (Prapunpoj *et al.*, 2002) TTRs experience. The secretion of these two recombinant proteins reached maximum after induction for 72 h. One possibility is the recombinant yeast contains human TTR gene in its cell grew slower, thus longer time was required in producing the protein. To overcome the slowly synthesis, higher density of cell used at the beginning of methanol induction could be tried. However, as there was no protein degradation occurred during induction, no attempt was performed to reduce the period length of the protein synthesis.

3. Synthesis scale up

Based on the results of varying media compositions and secretion pattern of recombinant human TTR obtained, synthesis scale up was performed as described in the Methods section with selected conditions. That is, in BMGY/BMMY media system and the *Pichia* transformant was induced with methanol to synthesize the recombinant protein for 96 h. The recombinant proteins obtained was purified and further analyzed for physico-chemical properties, as well as binding affinities to thyroid hormones and Congo red assay.

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 (human RBP-Sepahrose) column. Supernatant from the culture was collected after induction with methanol for 96 h. Yeast cells were removed by centrifugation at 1500 x *g* for 5 min. The supernatant was centrifuged again at 12000 x *g* for 20 min at 4 $^{\circ}$ C. NaCl was added to a final concentration of 0.5 M, prior to apply the supernatant to the column which previously equilibrated 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 of the resin-protein

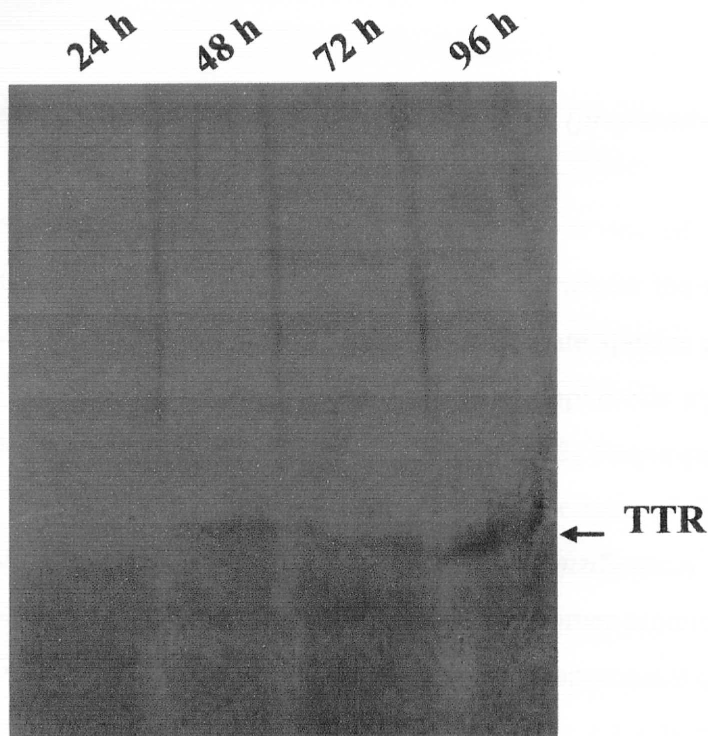


Figure 3 **Kinetics of recombinant human TTR synthesis**

The human TTR recombinant clone was grown overnight in BMGY and induced with methanol, at 30°C, for 96 h. Aliquotes of culture were withdrawn and supernatant (30 μl) was analyzed by SDS-PAGE (15% resolving and 4% stacking gels) with silver staining. The maximum amount of secreted TTR reached at 96 h. A period of withdrawal and the protein band corresponded to TTR was indicated.

environment (Larsson *et al.*, 1985). The recombinant human TTR found bind strongly to the human RBP-Sepharose and could be eluted from the column with distilled water (Figure 4). Approximately 1-2 mg of the recombinant TTR was obtained from 1 liter of 96 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.*, 2000; Prapunpoj *et al.*, 2002). With the typical mobility characteristic of TTR, the preparative native PAGE was successfully applicable for purification of the recombinant human wild type TTR and its amyloid variants from other proteins in the yeast culture. 16 ml of 6X concentrated of the 96 h yeast culture supernatant could be loaded into a preparative gel tube without loss of resolution. Eluting fractions could be checked for TTR by silver stained (Figure 5). However, as protein lost occurred during concentration step, approximately 20% less of the recombinant TTR was usually obtained comparing to that obtained by using the human RBP-Sepharose column.

With the reason that only small amount of the effective human RBP-Sepharose resin is available. To obtain the purified human RBP for generating a new affinity resin is an expensive and long time consume step. Though commercial human RBP is available but the protein costs very expensive (300-500 $US per μg) as purification is very difficult. So, in this research project, both the affinity chromatography using human RBP-Sepharose column and the electrophoretic separation in the preparative polyacrylamind gel were performed to obtained sufficient amount of the recombinant human wild type TTR and its variants for their properties analysis.

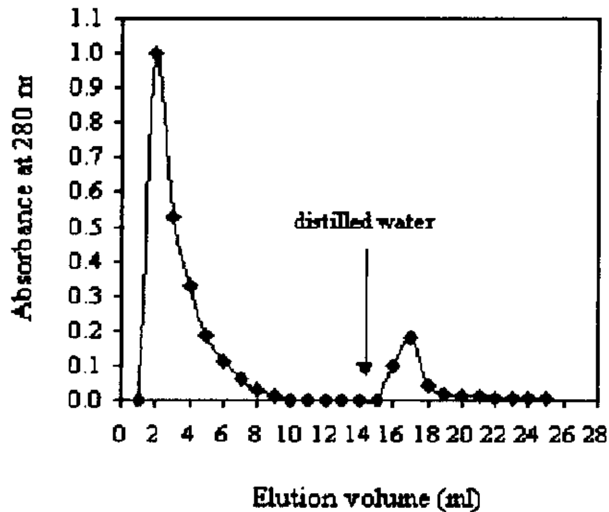


Figure 4 **Human RBP-Sepharose affinity chromatographic profile of recombinant human TTR**

Pichia culture of recombinant human TTR wild type was collected after induction for 4 days. 2 ml of the supernatant was loaded on a column of human RBP-Sepharose (1 ml of gel bead) equilibrated in 0.04 M Tris-HCl, pH 7.4 buffer contains 0.5 M NaCl. Bound protein was eluted with distilled water. Chromatographic separation was carried out at 4 °C.

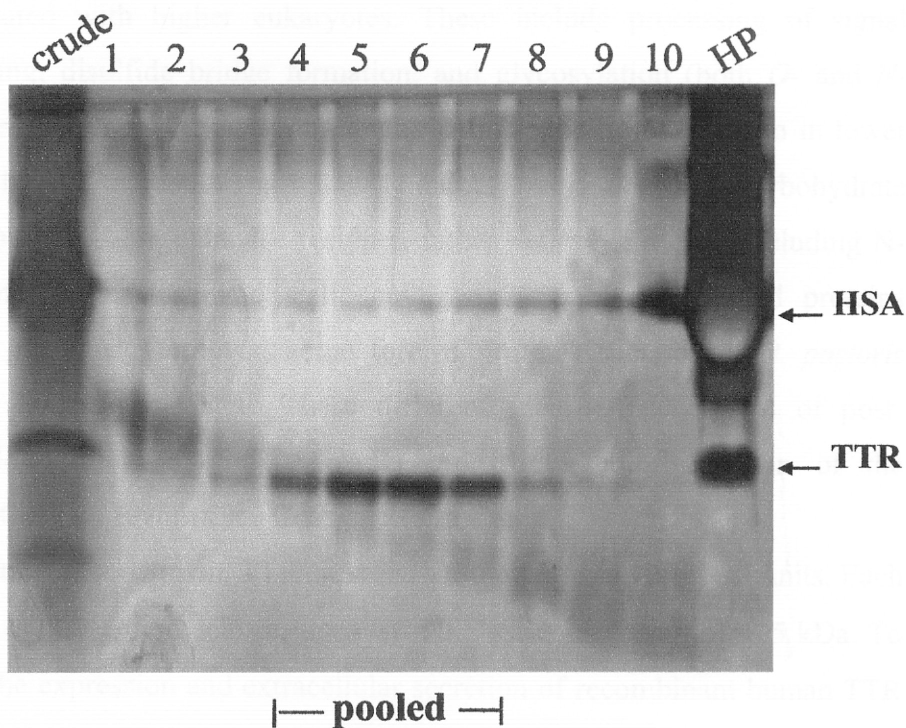


Figure 5 **Non-denaturing gel pattern of eluting fractions from preparative native gel of yeast culture**

The 4-days culture recombinant human TTR variants was concentrated and chromatographic separated by preparative native polyacrylamide gel electrophoresis (PAGE) (10% resolving gel and 3% stacking gel) using the Prep Cell, Model 491of Bio-Rad. 2-ml fractions were collected, and 20 µl of each was analyzed by native PAGE slab-gel (10% gel) and silver stained. Positions of human serum albumin (HSA) and TTR, as well as fractions pooled for further analysis (pooled) indicated.

5. Physicochemical properties of the recombinant human TTRs

P. pastoris expression system has one major advantage over that of bacteria. The yeats has the potential to perform many of the post-translational modifications typically associated with higher eukaryotes. These include processing of signal sequences, folding, disulfide bridge formation, and glycosylation (both *O*- and *N*-linked). However, some post-translational modification specific-mechanism in lower eukaryotes is slightly different from that found in mammals. The carbohydrate moieties with solely or high mannose residues, rather variety of sugars including N-acetylgalactosamine, galactose and sialic acid, were added to secreted proteins produced in *P. pastoris*. Moreover, some foreign proteins secreted in *P. pastoris* appeared to be hyperglycosylated. These different specific-mechanisms of post-translation could lead to alterations in its properties and/or function of the recombinant protein (for reviews see Cereghino and Cregg, 2000).

In the human bloodstream, TTR exists as a tetramer of identical subunits. Each subunit consists of 127 amino acid residues with the molecular mass of ~15 kDa. To reveal whether the expression and extracellular secretion of recombinant human TTR in *Pichia pastoris* using the native TTR signal sequence has different post-translational modifications from the native one or not, the properties of the purified human wild type TTR were determined. These properties include subunit molecular mass, tetramer formation, electrophoretic mobility property, and immunochemical cross-reactivity with antisera for native human TTR and TTRs from other vertebrates.

5.1 Mobility in SDS-PAGE

Purified recombinant human wild type TTR and its variants were analyzed by SDS-PAGE, and the protein bands were visualized by silver staining. The recombinant TTRs showed the same relative mobility as reported for native human TTR. The molecular masses of the TTRs, determined by comparing their mobility with the protein markers, all were 15 kDa. The result indicated that there was no post-translational modification occurred with these recombinant TTRs which produced in *Pichia* cell.

5.2 Mobility in non-denaturing gel

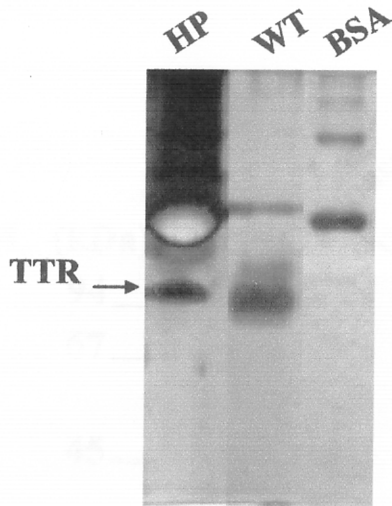
Though in some eutherian species, such as pigs and cattle, TTR co-migrate with albumin in non-denaturing gels (Farer *et al.*, 1962; Refetoff *et al.*, 1970). Most of TTRs from vertebrates including human TTR (Siebert and Nelson, 1942), avian TTRs (Chang *et al.*, 1999), as well as recombinant *Xenopus* and crocodile TTRs which were synthesized in yeast (Prapunpoj *et al.*, 2000; Prapunpoj *et al.*, 2002) migrate faster than albumin during electrophoresis at pH 8.6. This similar feature also observed in this research project. The recombinant human wild type TTR and its amyloid variants migrated faster than albumin (Figure 6), similarly to the native TTR in human plasma.

5.3 Immunochemical cross-reactivity of the recombinant human TTR 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.*, 2000; Prapunpoj *et al.*, 2002). To determine the immunochemical cross-reactivity of the recombinant TTR with antiserum against TTRs from other vertebrates, the purified recombinant human wild type TTR was analyzed in duplicate by SDS-PAGE. The proteins were capillary transferred onto nitrocellulose membrane, and the position of the protein bands was detected by staining with Coomassie Brillant Blue (Figure 7). 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. The membrane was exposed to X-ray film for 10 min and the film was immediately developed after exposure. One, with high intensity, band was observed (Figure 7). The band migrated in the same position as the subunit of recombinant human wild type TTR detected by Coomassie blue. This confirmed TTR is the recombinant protein synthesized by *P. pastoris*. Moreover, if occurred, the post-translational modification, in particular glycosylation, did not alter the immunological property of the protein.

5.4 Molecular mass of the recombinant human TTR tetramer

To determine whether the tetrameric formation of the recombinant human TTR occurred efficiently, purified TTR from culture supernatant was analyzed by fast protein liquid chromatography on a Superose 12, in 50 mM potassium phosphate



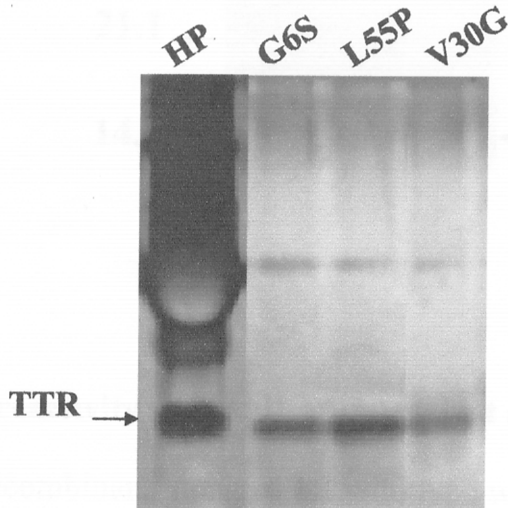


Figure 6 Electrophoretic mobility pattern of recombinant human TTR

Purified (~1-2 µg) recombinant human TTR wild type (WT) and its amyloid variants, Gly6Ser (G6S), Leu55Pro (L55P), and Val30Gly (V30G), were separated in a native polyacrylamind gel (10% resolving, 4% stacking) and protein bands was visualized by silver staining. Human plasma (HP) was overloaded to identify the native human TTR (TTR) position. Bovine serum albumin (BSA) was also loaded to identify the position of albumin.

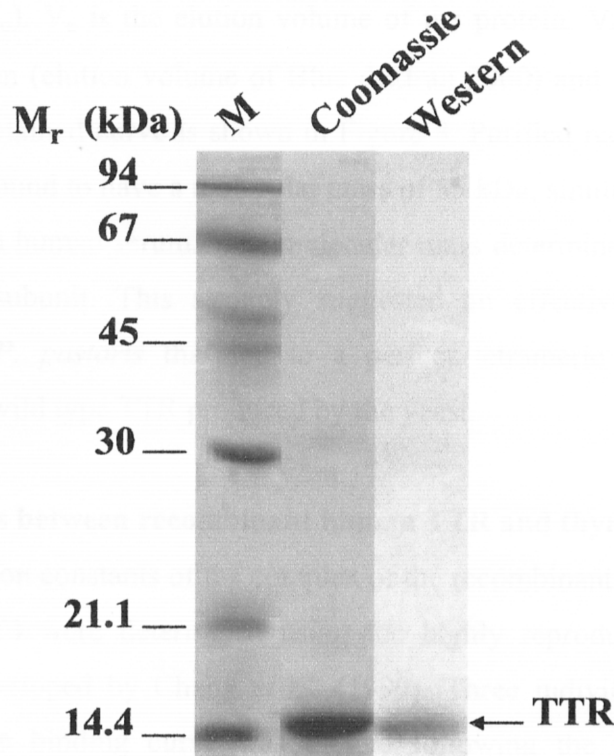


Figure 7 Cross-reactivity with antiserum against a mixture of transthyretins

The purified recombinant human TTR wild type was analyzed by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The protein bands were stained with Coomassie (Coomassie) and identified by reaction with antiserum (Western). The membrane filter was incubated with rabbit anti-mixture of human, wallaby and chicken transthyretins antiserum (1:5000) followed by horseradish-peroxidase-conjugated anti-(rabbit immunoglobulin) (1:10000). Detection was carried out by enhanced chemiluminescence. The position of the TTR monomer is indicated.

buffer saline, pH 7.4. Proteins with known molecular masses were 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. The obtained curve is shown in Figure 8. Purified recombinant human wild type TTR was found to have a molecular mass of 55 kDa, similar to that reported in TTR purified from human serum. The molecular mass determined was four times the mass of TTR subunit. This strongly suggested an effective protein-folding mechanism of the *P. pastoris* that led to a perfect tetrameric structure of the recombinant human wild type TTR produced by the yeast.

6. Binding affinities between recombinant human TTR and thyroid hormones

The dissociation constants of the complex of the recombinant human wild type TTR with T3 and T4 were determined using the highly reproducible, rapid and sensitive method developed by Chang *et al.* (1999). Three individual experiments were performed. 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 K_d values of recombinant human wild type TTR derived from the Scatchard analysis and plots for triiodothyronine (T3) and thyroxine (T4) were 23.79 ± 2.17 nM and 5.57 ± 0.53 nM, respectively (Figure 9). The values obtained in this research project was slightly different from those reported by Chang *et al.* (1999), they still agreed well with the previous reported.

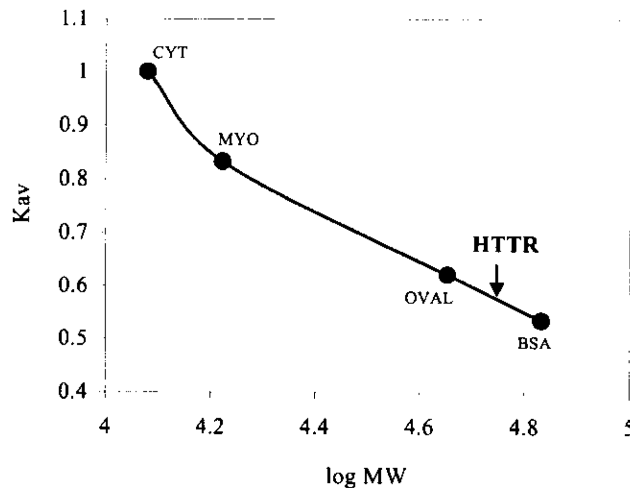
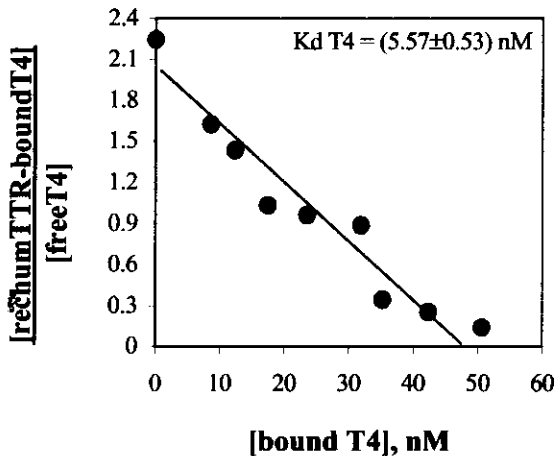


Figure 8 Tetrameric mass of recombinant human TTR

Purified recombinant human TTR wild type (~100 μg) was separated by gel filtration on a Superose-12 column. Standard proteins with known molecular mass include bovine serum albumin (BSA, 68 kDa), ovalbumin (OVAL, 45 kDa), horse myoglobin (MYO, 18.7 kDa) and horse heart cytochrome *c* (CYT, 12 kDa) were used to calibrate the column. A standard curve was plotted of K_{av} against log of the relative molecular mass (log MW). The K_{av} of recombinant human TTR wild type (HTTR) was indicated.



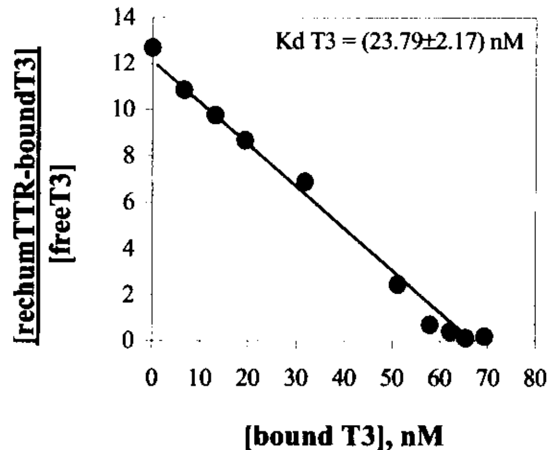


Figure 9 Analysis of binding of recombinant human TTR to thyroid hormones

100 nM of recombinant human TTR wild type 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. The affinity (K_d), for T3 and T4 of the protein were calculated.

7. Congo red binding to recombinant human TTR variants

In order to identify amyloid fibril formation of recombinant human TTR variants, shift in wavelength of Congo red binding to these variant proteins was investigated by spectrophotometry. The dye binding to non-amyloid TTR variant type, G6P, at acidic pH showed a specific wavelength-shifted similarly to that of its binding to amyloid proteins with β-structure (Klunk *et al.*, 1989) as well as partially denatured wild type TTR (Jiang *et al.*, 2001). The specifically binding of the Congo red to the protein was as shown in Figure 10B. The shift of wavelength from pink to bright red, as well as the maximal spectral difference at 540 nm occurred upon binding of congo red to amyloid were evident as reported with other amyloid protein (West *et al.*, 1999; Klunk *et al.*, 1989). Less, but visible, shift in wavelength found in the dye binding to L55P (Figure 10C). However, no significant difference in shift of wavelength observed in V30G (Figure 10A). Seemingly, by some mechanisms occurred during the protein synthesis led to an increasing the stability of the TTR tetramer, thus, changed the amyloidogenic property of the variant. An example of changes in structural stability caused by glycosylation that occurred during the post-translational modification in *P. pastoris* was reported (Jiang *et al.*, 2002).

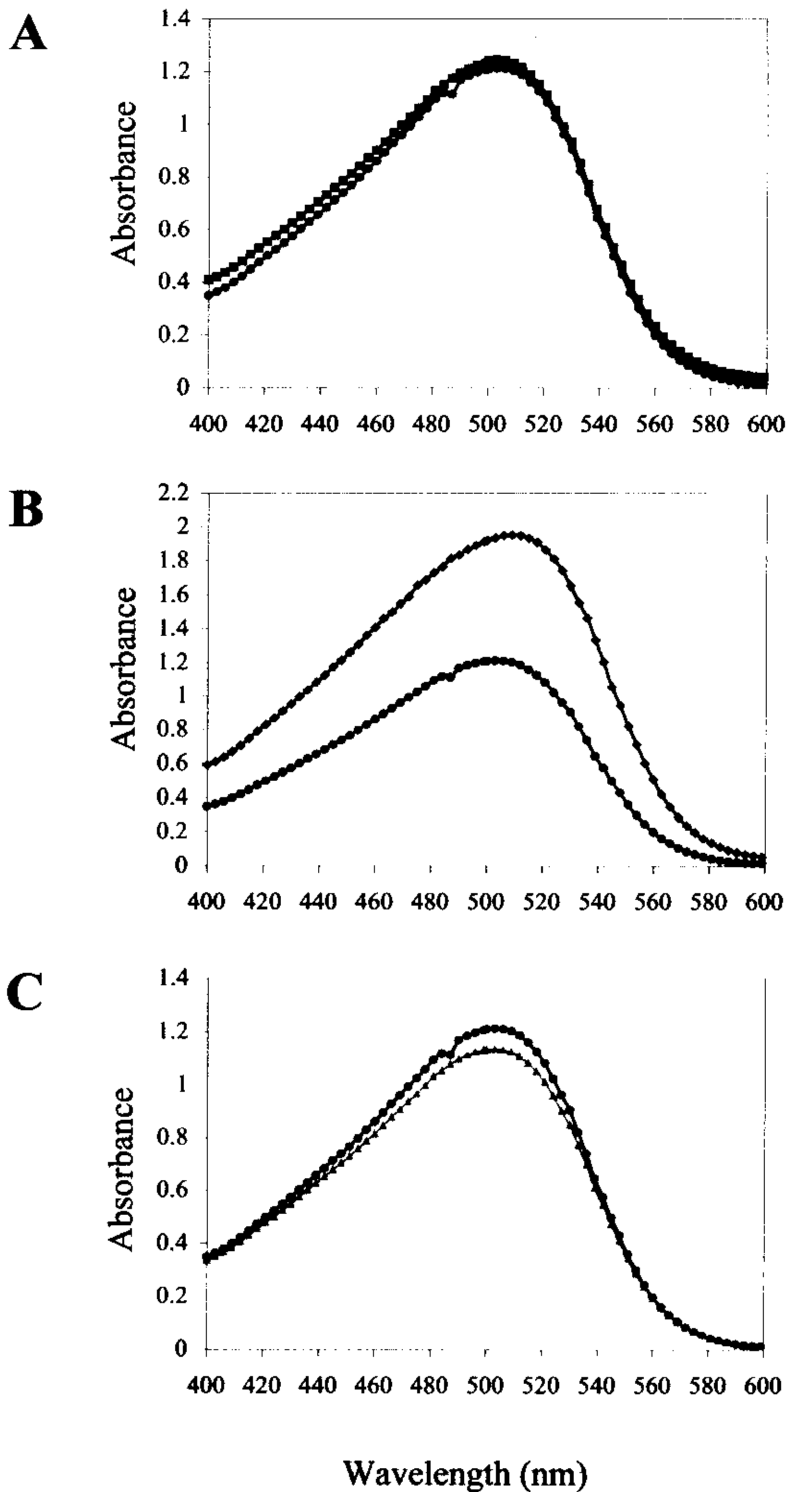


Figure 10 Binding to Congo red spectrum of recombinant human TTR variants

Each panel compares the spectrum of Congo red (blue) alone to that of the dye in the presence of human TTR variants, V30G (A, pink), G6S (B, red) and L55P (C, green).

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Conclusion

Taken together, the results obtained from this research project showed that recombinant human TTR, both wild type and its amyloid variants, could be efficiently synthesized using the heterologous protein expression system in *Pichia pastoris*. The protein could be successfully synthesized and secreted outside the yeast with the expression vector pPIC 3.5, but not with the pPIC 9. Synthesis scale up with yield of 1-2 mg per liter of the 96-h culture was obtained with BMGY/BMMY media system. The recombinant TTRs could be purified with only single step by affinity chromatography using the human RBP-Sepharose column. However, an electrophoretic separation in native preparative polyacrylamind gel was another choice with little yield less. The recombinant TTRs produced from *Pichia* contained properties, as studied, similarly to those observed in the native protein. Based on the Congo red binding, only the G6S and L55P, but not V30G, TTR variants showed the absorbance shift to the longer wavelength after the proteins were induced for amyloid fibril at pH 3.6. More studies should be conducted to reveal the reason behind.