4. DISCUSSION

Construction of Pichia clone

In order to determine the influence of the N-terminal region of TTR on the affinity of binding to thyroid hormones, structure and function analyses of chimeric TTR containing an altered N-terminal segment are most required. In addition, a system to synthesize such chimeric recombinant TTR with an authentic N-terminal sequence and in sufficient amount for determining the binding affinity is essential. The comparison of the amino acid sequence of TTRs from several vertebrates showed that Xenopus TTR had five extra amino acids at N-terminus that was not present in human TTR (Figure 1.9). In addition, study of evolution of the TTR function revealed that human TTR has binding affinity for T₄ higher than for T₃, which is opposite to Xenopus TTR that prefer T₃ rather than T₄ (Prapunpoj et al., 2000b). Thus, in this thesis, the chimeric human TTR, with the N-terminal segment replaced by that of the Xenopus TTR (Xenopus/humanTTR), was constructed, and the TTR protein was synthesized using the heterologous protein expression system of Pichia pastoris of which the GS115 was selected as a host. The TTR cDNA of Xenopus/humanTTR was generated by PCR using the specific pairs of oligonucleotide primer and native human TTR cDNA as the template. The expression of Xenopus/humanTTR was attempted by using the yeast expression vector, pPIC9, in which the mature TTR coding sequence was ligated to the α-factor signal sequence from yeast. In wild type strain of GS115, the expression and secretion of the TTR are mainly regulated by the AOX1 promoter. The expression “cassette”, consisting of the α-factor signal sequence plus Xenopus/humanTTR gene, was inserted into the yeast genome at the HIS4 locus. The integration was achieved by digesting the vector with XhoI and EcoRI, prior to transformation. The transformation was conducted by an electroporation, which the efficiency obtained in this thesis was 1800 transformants per 1 µg of DNA similarly to that previously reported (Scorer et al., 1994). This suggested a higher efficiency of the competent cells used in transformation and the presence of plasmid as an autonomous element in the yeast cells. Other methods of transformation could be used in particular that using chemicals such as polyethylene glycol and lithium chloride. However,
although it is convenient for the laboratory that has no electroporator and its procedures are not complicate (Geitz, 1996). They provide ten-fold less ($10^2$-$10^3$ transformants per µg of DNA) of efficiency than electroporation. Almost all of the transformants obtained were His’Mut’ as expected. The cultures were induced by transferring the exponentially growing cells to the medium containing methanol. The newly synthesized TTR was found secreted into the culture medium. This suggested that the α-factor signal sequence in the constructed vector was very efficient for the insertion of the chimeric TTR into the endoplasmic reticulum membrane of the yeast.

**Synthesis of recombinant TTRs in Pichia pastoris**

The amount of recombinant protein secreted by *P. pastoris* was highly dependent on the composition of the growth medium. The *Pichia* cells grew more slowly in minimal medium compared with other more complex media, and increases in the amounts of secreted protein obtained when the buffered medium was used (Clare *et al.*, 1991). Thus, in this thesis, yeast cell growth and induction with methanol was performed in the buffered medium, i.e. BMGY and BMMY, for the maximum production.

To gain the maximal recombinant protein synthesis, not only the type of medium but also condition optimization is required. The maximized condition should provide the maximal amount of the TTR. However, yeast also can synthesize and secrete other proteins into the culture medium. This high amount of the contaminated proteins would bring to a difficulty in purification and characterization of the TTR. Thus, to reach the optimal condition, amount of the TTR synthesized daily and the amount ratio of the TTR to other proteins synthesized by yeast were determined and used as the indicators. In this thesis, five distinct *Pichia* clones that contain the cDNA of human native TTR, human TTR with the N-terminus of crocodile TTR (croc/humanTTR), human TTR with N-terminus of *Xenopus* TTR (*Xenopus*/humanTTR), crocodile TTR with the N-terminus of human TTR (human/crocTTR) and crocodile TTR with truncated N-terminus (truncated croc TTR), were optimized for the protein synthesis. All of examined factors namely the methanol concentration, period of induction, and the starting density of the yeast cells in the induction with methanol, showed effects on the production of TTRs, although,
with different degree to each *Pichia* clones. The kinetic secretion of the five recombinant TTRs, in both small and large scale preparation, was different. This implied that distinct nature characteristic of the constructed sequences reflected the speed of growth and methanol utilization, which resulted in the TTR production of each clones. Particularly, the yeast clones that contained the nucleotide sequences encoding for a part of amino acid sequence of crocodile TTR, i.e. croc/humanTTR, human/crocTTR and truncate crocTTR, produced larger amount of the protein than that found in other clones. The nucleotide sequence of the crocodile TTR was probably highly matched to the gene promoter present in the *Pichia* expression vector, thus, high preferable synthesis of the protein in the yeast cell was obtained. Multiple gene insertion events at a single locus in *Pichia* could occur spontaneously with a low frequency, 1% to 10%, of all selected His\(^+\) transformants. This yeast transformants provided much more secreted recombinant protein than those contained a single-copy (Clare *et al.*, 1991). Therefore, the high expression clones containing part of the crocodile TTR might also receive multiple copies of the TTR genes. However, to confirm this event, quantitative dot blot analysis, Southern blot analysis, differential hybridization or the genomic analysis by PCR should be performed.

The period of the methanol induction for the maximal synthesis in small and large scales of all clones was not different, except the human native TTR clone. It was reported that oxygenation of the media was different when *Pichia* was grown in the container with different type and shape (Villate *et al.*, 2001). Thus, it might indicate high sensitivity to the oxygen concentration in the culture of medium of the human native TTR clone. Therefore, for all constructed *Pichia* clones, the condition optimization for small and large scale synthesis is necessary. In comparison, all examined *Pichia* clones cultivated in a shake flask (with 200 ml of medium) secreted larger amount of TTRs than that cultivated in a 30-ml McCartney bottle (at 5 ml of medium). It is suggested that scaling up the cultivation using a shake flask with no baffle was still efficient for the recombinant TTR production. In addition, the cultivation can be scaled up to 500 ml of the culture medium without any effect on the TTR production. As only slightly more TTR was obtained in the 300-ml cultivation (1.276 mg of TTR) comparing to the 200-ml cultivation (1.125 mg of TTR). It suggested that culturing the *Pichia* clones in several shake flasks at the 200-ml scale
of each rather than in one flask with larger than 200 ml of the medium should be more efficient in obtaining large amount of the TTRs.

**Purification of recombinant proteins**

One advantage of using *Pichia* expression system to produce the recombinant proteins is that only small amount of endogenous proteins were secreted. This facilitates the purification of the protein of interest. The TTRs were able to be isolated from the *Pichia* culture medium by using a single step of affinity chromatography on human retinol binding protein (RBP)-Sepharose or by preparative gel electrophoresis. The purified chimeric TTRs obtained from this thesis was approximately 2 to 5 mg per 1 liter of yeast culture, which was less than that reported for the crocodile native TTR that provided up to 16 mg per 1 liter of the yeast culture (Prapunpoj *et al.*, 2002) and by using the polyhistidine-tagged fusion system that provided 130 mg of the pure protein (Matsubara *et al.*, 2003). This confirmed the dependence of TTR synthesis in *Pichia* on the characteristic of the secreted protein, and preference pathway of the yeast cell for synthesis of the protein.

In *E. coli*, most of recombinant proteins were produced intracellularly, and required several steps in purification, including lysis of the bacterial cells with several detergents that resulted in denaturation and loss of function of the protein. Refolding of the recombinant proteins were possible but with complicate procedure. Moreover, there is significantly less chance of the eukaryotic protein to recover to its full biological activity. Binding of the recombinant TTRs produced from yeast to RBP was confirmed and it suggested that the proteins were synthesized and secreted with a fully biological function. This led to high efficiency in purification of the recombinant TTRs from the *Pichia* culture by affinity chromatography on the RBP-resin column. However, as RBP-resin was not commercially available and large amount of RBP with highly purified form is required to prepare the affinity resin. In addition, RBP is very difficult to isolate and that of commercially available is very expensive. Thus, it is quite difficult for small laboratories to generate the affinity column for their own use. Therefore, based on the fact that TTR migrates fastest in native electrophoretic gel, the preparative gel electrophoresis was thus attempted in the purification of TTR from the yeast culture media with the aim to use as a cheaper alternative procedure.
The results revealed that the preparative gel electrophoresis method was convenient. For TTRs with high secretion in the culture supernatant, the proteins can be easily purified in a single step. Moreover, for the TTRs with low expression i.e. human native TTR, *Xenopus* /humanTTR and truncated crocTTR, purification also can be carried out in a single step by affinity chromatography, thus reduced the loss of the proteins in purification process. One factor should be considered in using the preparative gel electrophoresis, too concentrated yeast culture can give poor isolation. Not more than 10-folds concentration of the yeast culture medium is recommended.

**Physicochemical properties of the recombinant TTRs**

Purified recombinant TTRs were analyzed for their mobility in native and denaturing polyacrylamide gel. The observation showed that all recombinant TTRs moved faster than albumin in native gel similar to human TTR in blood plasma. Among the five recombinant TTRs, slightly different in mobility was observed in *Xenopus* /human TTR and truncated croc TTR, which migrated faster than the others. This might because of difference in the overall charges of the chimeric TTRs. Relative mobility of the TTR subunits was performed in SDS-PAGE. It showed a very little difference of the relative mobility among all of the recombinant TTRs, suggesting that changes in the N-terminal region of the TTR molecule had little effect on the molecular mass of the subunits, and all of them had a similar molecular mass.

The subunit mass of some recombinant TTR in particular the human native TTR that produced in this thesis was slightly different from that reported, i.e. ~15 kDa, as well as that obtained by calculation from the deduced amino acid sequence. The mass of the subunit of recombinant human native TTR was 18 kDa. In addition, the truncated crocTTR showed three major bands in the position corresponding to the TTR subunit. These demonstrated that recognition and cleavage of the *S. cerevisiae*-derived α-factor prepro segment might occur incorrectly leading to longer segment at the N-terminus. However, the amino acid sequencing of the protein must be done to confirm.

Tetramer formation of the recombinant TTRs including human native TTR was confirmed by HPLC on gel-permeation column. The molecular weight of the chimeric TTRs was in the same range as reported for TTRs of many vertebrates.
An interesting result was that the recombinant truncated crocTTR showed a single peak with a molecular weight corresponding to the TTR tetramer, although three discrete bands in the position corresponding to TTR subunit were observed in SDS-PAGE. It is conceived that the N-terminal segment of the TTR subunit might not involved in the tetrameric formation of the protein. The HPLC analysis revealed that in addition to the main peak corresponding to the TTR tetramer, a protein peak with higher molecular weight than aldolase (158 kDa) was also detected in human native TTR, *Xenopus/human TTR* and truncated crocTTR (data not shown).

Amyloid fibril formation of many mutate human TTR in denaturation stress condition was reported (for review see Damas et al., 2000). Acidic condition facilitated the TTR tetramer dissociation and the tertiary structural change, allowed the TTR to self-assembly into amyloid fibril leading to a medical disorder called amyloidosis (Lai et al., 1996). Native TTR was also reported to easily form the amyloid-like fibril *in vitro* (Gustavsson et al., 1991). Thus, it is conceivable that the protein peaks with high molecular weight were the aggregate form of the TTRs. The recombinant TTRs produced in yeast had different N-terminal structure from that in nature, so possibly aggregate or form a fibril under the conditions performed in the experiments. All of the recombinant TTRs was completely dissociated after boiling for 30 min in the presence of SDS and β-mercaptoethanol confirming the same strong binding interaction of subunits of the recombinant TTRs as that detected in TTRs in nature.

The investigation in this thesis provided some important data involving the conditions that maximized the production of the recombinant TTRs by *Pichia pastoris* as well as the purification methods. Some physicochemical properties were also examined to show the biological function of the recombinant TTRs obtained. However, more studies, particularly binding affinity to thyroid hormones, are still required in order to confirm their proper structure and full biological function.