Introduction

Transthyretin (TTR) is one of the three thyroid hormone-binding proteins found in the plasma of larger mammals. It was first found in human serum (0.1-0.4 mg/ml) and cerebrospinal fluid (CSF) (0.017 mg/ml) (Kabat *et al.*, 1942 a, b). The liver represents the main source of synthesis whereas the choroid plexuses and the retina produce small fractions of the protein. TTR is a homotetrameric protein (Blake *et al.*, 1978) and has a molecular mass of 55 kDa. Human TTR subunit consists of 127 amino acid residues (Kanda *et al.*, 1974). Crystallographic analysis reveals that each four-stranded has a β-sheet sandwich tertiary structure, one four-stranded β-sheet being stacked upon another four-stranded sheet. The subunits dimerize by intermolecular antiparallel β-sheet formation involving the H and H′ strands. Dimerization of these dimers is mediated by AB-GH loop interactions affording the tetramer. A large central channel with two sterically equivalent thyroid hormone binding sites, which differ in their relative binding affinity, is formed as the result of the tetrahedral arrangement of the AB-GH loop interface (Blake *et al.*, 1978). Although both binding sites are similar, they present different binding affinities for T4 because of negative co-operativity of binding. Normally TTR exists as an innocuous and soluble protein, however because of its extensive β-sheet structure making the molecule have a potential to amyloid formation. Mutations in the TTR sequence increase the susceptibility of its variant molecules to the extracellular deposition in tissues as amyloid through undefined mechanisms.

Amyloidoses is a complex group of diseases caused by the aberrrant self-assembly of some human proteins or a fragment(s) thereof into amyloid fibrils. All human amyloidogenic proteins found to date share no apparent sequence, structural, or functional homology, but they all form a similar cross-β-sheet quaternary structure (Jimenez, et.al., 1999; Blake, and Serpell, 1996). The amyloidogenic conformational intermediates that have been characterized either are rich in β-sheet structure or have the capacity to become rich in β-sheet structure before (Uversky and Fink, 2001) or upon oligomerization (Liu, et. al., 2000; Jackson, et. al., 1999). Several nonamyloidogenic proteins found form amyloid fibril under a severe denaturing stress (Guijarro, et. al., 1998; Konno, et. al., 1999). Familial amyloidotic polyneuropathy (FAP) and senile systemic amyloidosis (SSA), an amyloid deposition in old people,

are two forms of amyloidoses related to transthyretin (Costa, et. al., 1978; Westermark, et. al., 1990).

TTR amyloidosis is a systemic form of amyloidosis that is in the vast majority a hereditary disease. The typical manifestations of TTR-related amyloidosis are peripheral neuropathy, cardiomyopathy, carpal tunnel syndrome and vitreous opacities. In FAP, mutated TTR was shown to be the causative factors and over 70 different amyloidogenic mutants of TTR have been identified (for review see Saraiva, 2001). Majority of these TTR-associated amyloidoses is due to single amino-acid substitutions. Non-mutated or wild type TTR can also deposit as amyloid in the cardiac tissue of aged individuals leads to a wide spread geriatric disease termed senile systemic amyloidosis (Westermark *et al.*, 1990; Raraiva, 1995; Gustavsson et. al., 1995). To date, several amyloidogenic TTR variants have theirs crystal structure determined. While some of them do not show significant differences from the wild type protein. Others show a structural transformation of subunit, due to mutation, that lead to destabilize the quaternary structure of the protein (Terry *et al.*, 1993; Damas *et al.*, 1996; Hamilton *et al.*, 1996; Schormann *et al.*, 1998; Sebastião *et al.*, 1998). Increasing evidence supports the theory that a tetramer dissociation precedes amyloid formation (Colon and Kelly, 1992). Self-assembly properties of different TTR mutants have also been widely investigated by different approaches. Avoiding tetramer dissociation into monomeric and oligomeric intermediates, and disrupting amyloid fibrils are among possible avenues of theraputic agents development. However, since the factors influencing the phenotypic and genotypic variations remain unclear, further investigations on genetic and environmental modulators are essential.

Heterologous expression in *Pichia pastoris*, a methylotrophic yeast, has many of the advantages of eukaryotic expression (Cregg *et al.*, 1985; Cregg *et al.*, 1993; Higgins and Cregg, 1998b). More than 100 foreign proteins from bacteria, fungi, plants, invertebrates, vertebrates (includes humans) have been expressed in *P. pastoris*. These successful protein gene expression includes those of transthyretin from amphibian (Prapunpoj *et al.*, 2000) 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 that cannot be refolded from inclusion bodies or require post-translational modifications for proper folding or function (Wood and Komives, 1999). The yield

of expressed proteins from *P. pastoris* depends critically on growth conditions. The protein yield could be improved to 10 to 100 folds by fermentation process. When grown on methanol, foreign proteins secreted from *P. pastoris* can represent 80 % or more of the total protein in the culture medium (Tschopp *et al.*, 1987a).

This research project was first report for attempting to produce the recombinant human wild type TTR and its three amyloid variants in *Pichia pastoris* and obtain high or sufficient amount of the proteins for further studies. A non-amyloid variant, Gly6Ser (Jacobson *et al.* 1995), and two aggressive amyloid variants, Leu55Pro (Jacobson *et al.*, 1992), and Val30Gly (Petersen *et al.*, 1997), were chosen. The proteins were produced using the methanol-inducible expression vector, pPIC. The recombinant TTRs were found successfully extracellular expressed at moderate level with the TTR, itself, secretory signal, but not with that of the yeast α-factor protein. The recombinant human wild type TTR showed similar physico-chemical properties, as well as binding affinity to T4, as the native protein. The fibril formation capability of the recombinant human TTR variants also was determined.

Objectives

1. To generate recombinant human wild type TTR and its amyloid variants, Gly6Ser, Leu55Pro, and Val30Gly, using a heterologous protein expression system in *Pichia pastoris*.
2. To determine the physico-chemical properties and binding to thyroid hormones capacity of the recombinant human TTR.
3. To determine capability of the recombinant TTRs to form an amyloid fibril.
4. Scale up of the recombinant human TTRs synthesis to gain sufficient amount for further analysis.