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Construction of an engineered alpha 1-antitrypsin with inhibitory activity based on theoretical studies

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Abstract

Background: The elastase inhibitor α-1-antitrypsin (AAT), is a member of the serpin superfamily of protease inhibitors. AAT has a characteristic secondary structure of three-β-sheets, nine-α-helices and a reactive central loop (RCL). This protein inhibits target proteases by forming a stable complex in which the cleaved RCL is inserted into β-sheet-A of the serpin, leading to a conformational change in the AAT protein. Spontaneous polymerization and instability of AAT are challenges with regard to producing drugs against AAT-deficient diseases. Therefore, the purpose of many investigations currently is to produce drugs with lower degrees of polymerization and higher stabilities. In order to investigate the effect of the N-terminal segment (residues 1-43) on AAT structure, molecular dynamic (MD) simulation was used to study structural properties including Root-mean-square deviation (RMSD), internal motions, intramolecular non-bonded interactions and the total accessible surface area (ASA) of native and reduced AAT. These properties were compared in native and truncated AAT. **Results:** Theoretical studies showed no noticeable differences in the dynamic and structural properties of the two structures. These findings provided the basis for the experimental phase of the study in which sequences from the two AAT constructs were inserted into the expression vector pGAPZ and transformed into *Pichia pastoris*. Results showed no differences in the activities and polymerization of the two AAT constructs. **Conclusions:** As small-scale medicines are preferred by lung drug delivery systems, in this study AAT was designed and constructed by decreasing the number of amino acids at the N-terminal region.

Keywords: alpha1-antitrypsin, molecular dynamic simulation, *Pichia pastoris*

INTRODUCTION

α-1 antitrypsin (AAT) belongs to the serpin superfamily of proteins, and is one of the major protective substances against proteolytic digestion in the respiratory system by the human neutrophil elastase (Snyder et al. 2006). This 52 kDa protein consists of 394 amino acids and has three carbohydrate chains which contribute to 12% of its weight. The three β-sheets, nine α-helices and a reactive central loop (RCL) at the C-terminal region are characteristics of AAT and necessary for its inhibitory activity (Lomas, 2005; Ljujic et al. 2010).

Major structural properties which contribute to AAT inhibitory activity and structural stability are located in the middle and C-terminal region. These include the RCL, protease cleavage site (between Met258-

Ser259), salt bridges (between Glu342-Lys290 and Glu264-Lys387) involved in tertiary structure conservation and liver secretion, and Cys232 which attaches to other plasma proteins (IgA). However, the first 150 amino acids in AAT preferably form a helix structure and seem to play a less critical role when compared to the C-terminal sequence (Frazier et al. 1990; Parfrey et al. 2003). Three carbohydrate chains are located at positions Asp46, 83 and 247. The amount of AAT synthesized by the liver is approximately 32 mg per kg body weight, thus culminating in a plasma concentration of 1.5-3.5 g/L with a half-life of 4-5 days (Schlade-Bartusiak and Cox, 2006; Fregonese and Stolk, 2008).

The AAT gene, located on chromosome 14q32.1, is 12.2 kb in length and consists of three non-coding (IA, IB, IC) and four coding (II, III, IV, V) exons; exon V contains the sequence coding for the RCL (Wood and Stockley, 2007). This protein has many different genetic variations and its gene is highly polymorphic. So far, 125 single nucleotide polymorphisms (SNPs) have been reported, where each variant has been found to affect AAT serum concentration or function. Generally each AAT variant is identified on the basis of its electrophoretic mobility and the most common forms include: F (fast), M (medium), S (slow) and Z (very slow) (Jain et al. 2009). Furthermore, the AAT variants are divided into four groups based on serum levels and function. These include normal variants with serum levels within the normal range (150-350 mg/dL), deficient variants with reduced serum levels (having normal or deficient function), null variants with complete absence of the serum AAT, and nonfunctional variants with normal serum levels but deficient function (*e.g.* F). Most of the AAT diseases are caused by deficient or null alleles (Schlade-Bartusiak and Cox, 2006; Mclean et al. 2009). In general, it can be assumed that differences in the AAT variants which lead to functional or non functional types, and the ability or inability of the liver to secrete AAT are also associated with amino acids located at the C-terminal region.

Because AAT includes more than 90% of the anti-elastase activity in the lung, every disruption in the AAT serum levels and intensity of its function can lead to lung tissue damage (Crystal, 1990; Perlmutter et al. 2007). Different mutations arising from at least five different mechanisms cause function or AAT deficiency. These mechanisms include: gene depletion, AAT-unstable mRNA degradation, intracellular accumulation of new synthesized AAT, intracellular degradation and insufficient function of mature secreted protein (Crystal, 1990).

Serpins have 5 structural common forms which include native, cleaved, latent, delta and polymer structures. Due to the fact that serpins are mainly different with regard to the RCL structure (Irving et al. 2000), the residues that are directly within the RCL, and the amino acids which are effective in the optimum formation and function of, are mostly located at the C-terminal region of ATT. Any mutation in these mentioned regions can lead to AAT deficiency. Besides, most amino acids in the N-terminal region of the serpin superfamily of proteins are highly conserved.

In 1987, the American Food and Drug Administration (FDA) approved replacement therapy in emphysema patients using blood-derived AAT. The amount of AAT required for the injection of emphysema patients consisted of 60 mg/kg weekly doses, 120 mg/kg every two weeks and 180 mg/kg every three weeks. This treatment method reduces death, improves lung function and decreases lung contaminations in AAT-deficient patients with emphysema. The main problem concerning this therapy method is its high cost and inaccessibility of patients to purified AAT of blood origin (Blanco et al. 2005).

Studies have shown that only 2-3% of the injected AAT reach lung tissue. Therefore, other routes such as aerosolized AAT particles or solution can be considered as new treatment strategies. Usage of the aerosol form is more suitable because higher doses can reach the main area of action, the lung, in a shorter time without being diluted in the circulation. Consequently, aerosolized AAT has become the center of attention with regard to research and development in recent years (Sandhaus, 2004; Jha et al. 2010).

All infusion products of AAT including prolastin, zemeira and Aralast NP, are of blood origin, hence not only their high costs and limitations with respect to production, but also virus contamination can be challenging. Thus, production of recombinant AAT from microorganisms such as yeast has been regarded of vital importance (Tamer and Chisti, 2001; Karnaukhova et al. 2006). So, the extensive production of AAT at low cost in yeast and optimization of aerosolized delivery of AAT are two new strategies in AAT replacement therapy. In this research, an attempt will be made to produce small-scale AAT and investigate the effect of the N-terminal region on structure, function and stability of AAT,

based on theoretical studies (Kołoczek et al. 1996; Jezierski and Pasenkiewicz-Gierula, 2001). Reduction in size of AAT particles will lead to optimized drug packaging and delivery to the lung, thus resulting in a more effective and efficient treatment of AAT deficiency.

MATERIALS AND METHODS

Molecular dynamics simulation

Molecular dynamic (MD) simulation was performed using the AMBER9 program (Case et al. 2004). The Ptraj program was used to extract the information regarding root-mean-square deviations (RMSD) and root-mean-square fluctuations (RMSF) data from trajectories. In all of the calculations the protonated form of the ionizable amino acid at pH 7 was used. In order to neutralize the surface charge, positive and negative ions were added. In the case of native and truncated AAT ten and eleven sodium ions (Na^+) were added, respectively. With regard to the calculations, the ff03 force model was used (Duan et al. 2003) and for calculation of electrostatic forces, the Particle-Mesh Ewald method was applied (Darden et al. 1993). In all simulations, the molecule was placed in a water environment by using the TIP3P water model as solvent (Jorgensen et al. 1983) with a minimum solvent shell thickness of 8Å.

The initial coordinate of alpha-1 antitrypsin was obtained from the Protein Data Bank (PDB) with entry code 1QLP. The simulations began with 5000 iterations of energy minimization using the steepest descent method. Simulations were performed for 100 ps under NVT (constant number of particles or moles, volume, and temperature) conditions and the temperature increased from 200 K to 300 K. Subsequently, the simulations proceeded for another 100 ps under NPT (constant number of particles or moles, pressure, and temperature) conditions so that the system reached equilibration at constant pressure (the criteria for equilibration of the system under NPT conditions included total energy and density) and continued for 10 ns under NPT conditions where a time step of 2 fs was considered. In all simulations, the SHAKE algorithm was used to fix hydrogen bond trembling with other atoms. Finally, one structure was saved every 10 ps (in each simulation, 1000 structures were saved for analysis procedure).

Experimental methods

The AAT DNA sequence was obtained from previous work (Hasannia et al. 2006). With regard to the proliferation of both full-length and truncated AAT, the following PCR protocol was employed: reaction mixture containing 12.5 µl of the master mix (Biorad), 1 µl of the upstream primer (for-AAT), 1 µl of the downstream primer (rev-AAT) (Table 1), 1 µl of the DNA template and 9.5 µl of double-distilled H_2O was subjected to a 30-cycle PCR program consisting of 20 sec of denaturation at 95ºC, 30 sec of annealing at 56.1ºC and 50 sec of extension at 72ºC. The amplified fragment was electrophoresed on a 1% agarose gel and then isolated from the gel using the Gel Extraction Kit (Fermentas). Both the purified fragment (native and truncated AAT DNA) and the vector pGAPZαa, were digested with FastDigest *XhoI* and Not *I* enzymes (Fermentas) and then ligated using T4 DNA ligase (Fermentas). The resulting recombinant plasmids were linearized by digestion with *BspH I* (Fermentas). Transformation into *P. pastoris* was performed using the electroporation procedures (Biorad). Zeocin-resistant *Pichia* transformants were then analyzed for the presence of inserts by PCR using the pGAP forward and AOX1 primers (Table 1). In order to express the native and truncated AAT, cells were cultured in yeast extract peptone dextrose (YPD) medium. At different time intervals, culture samples were collected to evaluate expression levels and determine the optimal time for harvesting the culture. Analysis of expression was carried out by 12.5% SDS-PAGE. For sample preparation, the cells were removed and the supernatant was precipitated by using Aceton. After drying, the pellet was resuspended in loading buffer containing β-mercaptoethanol, heated for 10 min, in boiling water and electrophoresed at 12% SDS-PAGE/100 V along with molecular weight marker (Vivantis). For demonstrating our specific protein, samples that were to be used in SDS-PAGE were fractionated by SDS-PAGE and each fraction was subjected to the immunoblotting procedure. Protein samples were electro-blotted to Polyvinylidene Difluoride (PVDF) membrane (Millipore) in transferring buffer (0.025 M Tris, 0.19 M glycine, and 20% (v/v) methanol) overnight at 20 V/4ºC. The membrane was treated with PBS-T-BSA (PBS, 0.1% (v/v) Tween 20, 1% (w/v) BSA) for 2 hrs to block binding sites. After washing step, membrane was reacted with 1000-fold diluted goat anti-human alpha-1 antitrypsin polyclonal antibody, conjugated with HRP (Abcam) for 3 hrs. To eliminate non-specific reactions, a supernatant of

non-recombinant X-33 culture treated side by side accordingly as negative control. Subsequently, protein bands reacted positively, were visualized at the presence of 4-chloro1-naphtol substrate in PBS. The native and truncated AAT activities were investigated through the determination of elastase inhibitory capacity (EIC). Elastase activity was measured by Elastase Assay Kit (EnzChek®, Molecular Probes, Inc.) according to the manufacturer's recommendations. The EnzChek kit contains DQ™ elastin soluble bovine neck ligament elastin that has been labelled with BODIPY® FL dye such that the conjugate's fluorescence is quenched. The non-fluorescent substrate can be digested by elastase or other proteases to yield highly fluorescent fragments. The presence of an inhibitor such as AAT blocks the substrate digestion and lead to a subsequent fluorescent emission. The resulting change in fluorescence level was monitored using a fluorescence spectrophotometer (Varian Cary Eclipse, Australia) with a maximum absorption at 505 nm and a maximum fluorescence emission at 515 nm. Commercial human AAT was used as a positive control and the elution buffer and the supernatant from non-recombinant *P. pastoris* (X-33 strain) culture as negative controls.

Table 1. Primer sequences.

Name	Primer sequence	Mer
For native	5' AAACTCGAGAARAGRGGAGAGGATCCCCAGGGAGATGC 3'	37
Rev native	5' TTTGCGGCCGCTTATTTTTGGGTGGATTCACCAC 3'	34
For truncated	5' AAACTCGAGAARAGRCAGTCCAACAGCACCAATATC 3'	36
Rev truncated	5' TTTGCGGCCGCTTATTTTTGGGTGGATTCACCAC 3'	34
pGAP forward	5' GTCCCTATTTCAATCAATCAATTGAA 3'	22
AOX1	5' GCAAATGGCATTCTGACATCC 3'	21

RESULTS

Molecular dynamics simulation

The root-mean-square deviation (RMSD) and atom distance map. One of the most frequently used methods for assessing the stability of an MD simulation is the RMSD between the first coordinate and the generated structures in the trajectory. Therefore, RMSD values for the main chain atoms of the native and truncated AATs during a 10 ns simulation time were calculated. As revealed in Figure 1, simulation of the two structures during this time period showed good stability and no significant deviation from the previous structures. Hence, the structures reached their final conformations in each simulation. Furthermore, atom distance map of native and truncated AAT was calculated. Comparison of atoms distance between native and truncated structures showed no considerable deviation in overall structure and C-terminal region specially (Figure 2).

Total energy changes. Total energy changes during 100 ps of simulation time at the equilibrium phase under NPT conditions were carried out for the certitude of simulation stability (Figure 3). As illustrated, the system's total energy at equilibrium, under fixed pressure conditions after approximately 30 ps, remained constant for all simulations.

Temperature and density. The temperature for native and truncated AAT was 300 K and the density for both native and truncated AAT were 1/02 gr/cm^3, which ultimately became fixed and stable, representing further proof of simulation stability.

Structural fluctuations. Structural mobility of the interacting protein molecules, especially fluctuation of the sequence involved in the interaction is important with regard to the reaction and efficiency of protein function. Following the deletion of 43 amino acids from the N-terminal region, structural fluctuations at the C-terminal region that are involved in protease interaction were not affected considerably (Figure 4). Of course amino acids that are not involved in RCL in the C-terminal region of truncated AAT showed a decrease in mobility compared with the native structure.

Hydrogen bonds, salt bridges and hydrophobic accessible surface area. The comparison of hydrogen bonds, the number of salt bridges and hydrophobic accessible surface areasshowed a good compatibility in the native and truncated AAT. Results revealed few changes in the number of hydrogen bonds and salt bridges and therefore, it seems that total structural stability is not changed considerably. Only a very slight increase in the hydrophobic accessible surface was observed which does not affect protein activity (Table 2).

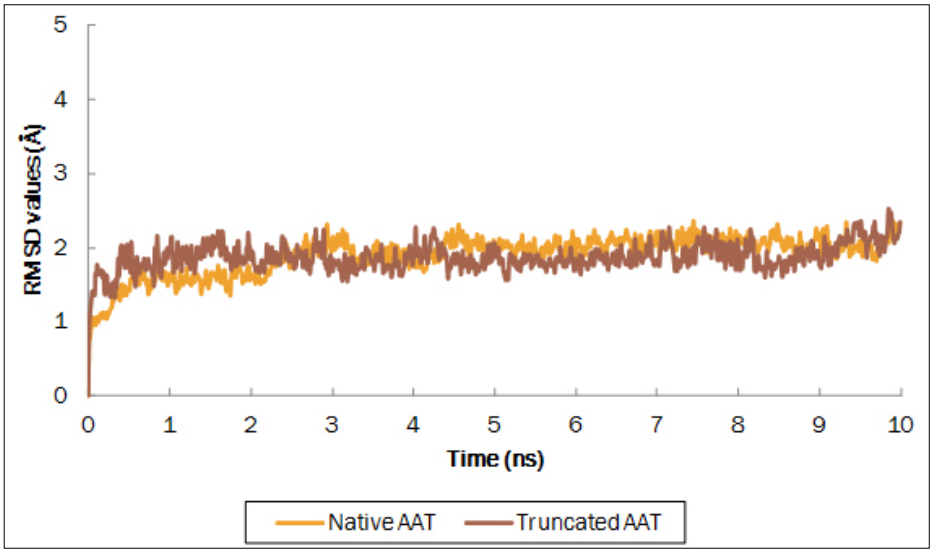

Fig. 1 Changes in RMSD of native and truncated AAT.

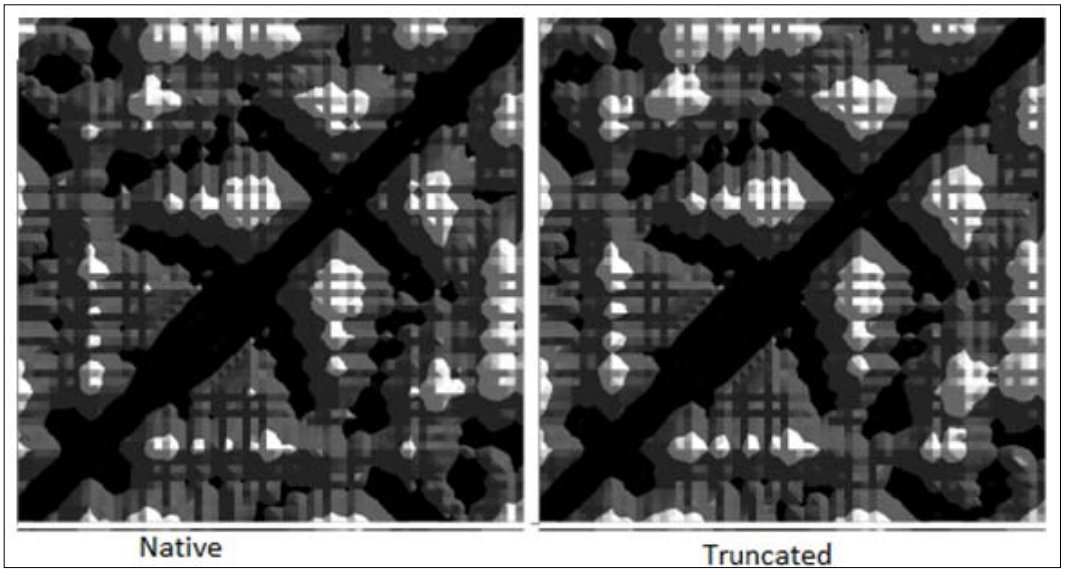

Fig. 2 Atom distance map of native and truncated AAT. Black, gray and white colours were indicating distance ranges 0-5, 5-10 and 10-15 Å, respectively.

Experimental studies

SDS page. The culture expressing native and truncated AAT was harvested at the following time intervals: 0, 12, 24, 48, 60, 72 and 96 hrs. The supernatant from each culture sample was examined using SDS-PAGE in order to determine the native and truncated AAT expression levels. The 72 hrs time period represented the best time for harvesting in comparison to non-recombinant X-33 culture (Figure 5).

Western blot. Western blotting using goat polyclonal to alpha-1 antitrypsin for both native and truncated AAT is shown in Figure 5, where both the native and truncated proteins are detected in both Western blots.

Elastase inhibitory capacity (EIC). The inhibitory function of native and truncated AAT showed no significant difference and both AATs were found to be functional (Figure 6).

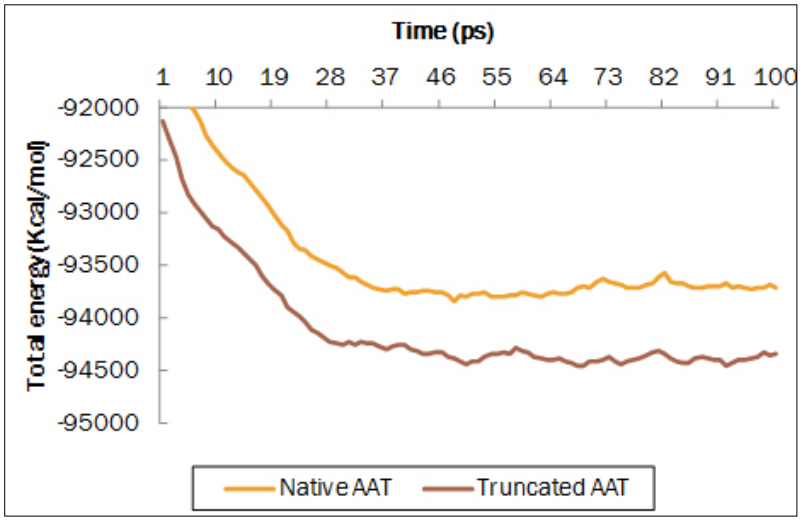

Fig. 3 Total energy changes of native and truncated AAT.

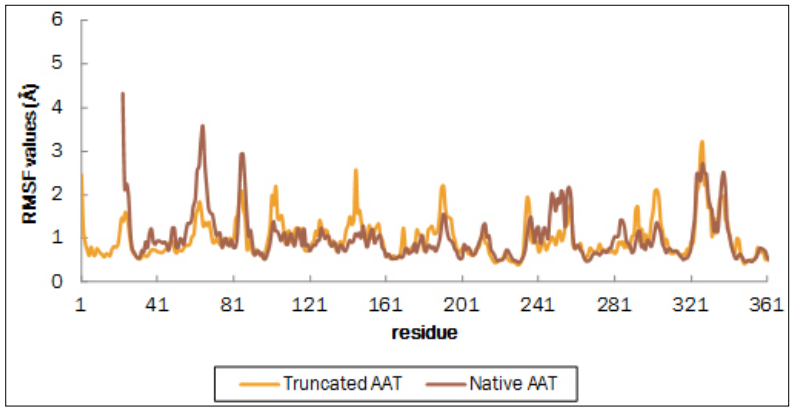

Fig. 4 Changes in structural fluctuations of the native and truncated AAT.

Table 2. Comparison of hydrogen bonds, salt bridges and hydrophobic accessible surface area in native and truncated AAT.

	Truncated AAT	Native AAT
Hydrogen bond/residue	1.4	1.56
Salt bridge/residue	0.56	0.32
Hydrophobic accessible surface area (ASA) (%)	21.72	20.3

DISCUSSION

Alpha-1 antitrypsin (AAT) is a major elastase inhibitor within the lung which protects this organ, and belongs to the serpin (serine proteinase inhibitor) family of inhibitors. There are over 100 genetic variants of the AAT allele. The protein phenotype is classified as 'Pi' with the most frequent AAT-deficient variants, Z and S. These variants are associated with AAT polymerization in the liver that mainly lead to liver and pulmonary diseases (Lomas, 2005; Granell et al. 2008; Mclean et al. 2009). Intravenous weekly infusion of plasma derived-AAT (60 mg/ml) is the common treatment for the human emphysema condition. Three drugs used for augmentation therapy-Prolastin, Zemaria, Aralastin-are currently the only FDA approved plasma-derived AAT products. However, it has been shown that only 2-3%t of the injected form of AAT reaches the lungs, therefore the efficiency of this treatment is very low. Furthermore, there are other problems, such as high costs of the production process and the potential risk of unknown viral or pathogen contaminations. The limited plasma-derived source is insufficient to meet patients' demands. Hence, the necessity of a replacement method for AAT production in an easy and cost-effective way, using a different method of treatment such as aerosol therapy has significant importance (Seersholm et al. 1997; Taylor and Gumbleton, 2004; Jha et al. 2010).

Hosts for recombinant protein expression include both prokaryotic and eukaryotic systems. Because of the importance of the glycosylation pattern in AAT stability, eukaryotic hosts are mainly used. Amongst eukaryotic hosts, yeasts are more applicable and popular with regard to recombinant protein production because of the ability to produce heterologous proteins at high levels, grow to high cell densities and perform glycosylation modification. Furthermore, the absence of endotoxins in yeasts is also another reason for their popularity (Pemberton and Bird, 2004). In spite of the possibility of glycosylation in yeasts, significant differences exist between human and yeast cells with regard to the length and types of oligosaccharides. These differences limit the usage of glycoproteins which are expressed in yeast as infusion medicines because of a decrease in stability, increase of clearance in blood and possible immune response in patients (Kamnaukhova et al. 2006).

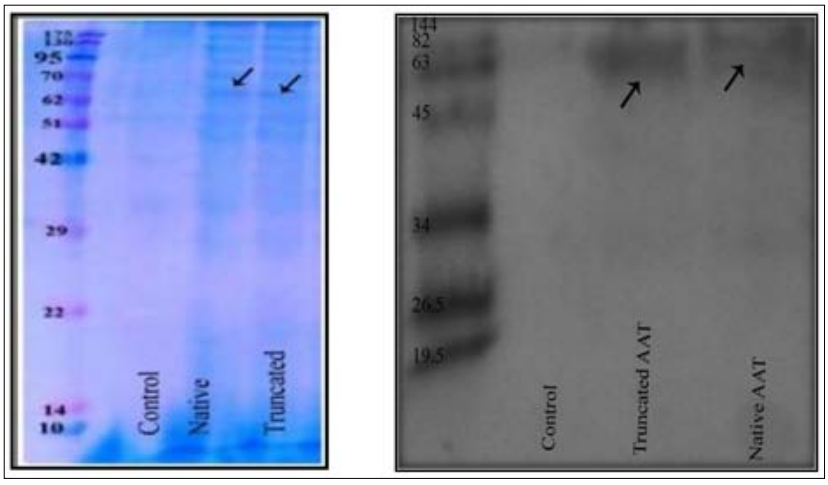

Fig. 5 SDS-page and western blot of native and truncated AAT expression media.

Another alternative to intravenous infusion is aerosol therapy which not only has a direct local effect on the lung but also remains at high concentrations in the lung. In fact little amounts of the drug enter the systemic circulation, so immunogenic effects which are the problem associated with all other recombinant proteins are reduced significantly.

During drug delivery, especially protein-based ones, the size of the protein has the major role with regard to its packaging and delivery. In other words, the smaller the protein size, its packaging, stability and delivery becomes more convenient. For this reason and because the N-terminal regions of AAT do not affect formation of its structure, in this project, attempts were made to reduce AAT size whilst maintaining its inhibitory activity and stability via a deletion of 43 amino acids from the N-terminal region of the protein.

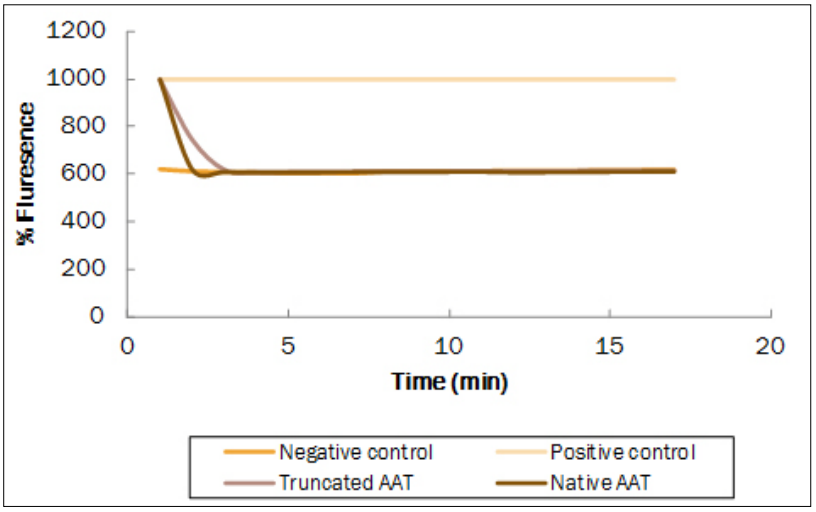

Fig. 6 Elastase inhibitory assay of truncated AAT.

The reason for not selecting the C-terminal region for this purpose was that this region, upon mutations, shows significant changes in activity and stability. Furthermore, RCL that is the interaction site with elastase is positioned at the C-terminal region. It has been shown that many mutations in the C-terminal region and β-sheets of AAT, lead to a decrease in stability and increase in polymerization. In fact other mutations, in spite of causing an increase in stability lead to a decrease in the inhibitory activity of the protein. Besides, most of the amino acids which are conserved in more than 70% of serpins are located at the C-terminal region and become motive during complex formation with the target protease. The high level of conservation shows the importance of these amino acids in serpins' function and structure. Based on previous studies, most of the activity-affecting mutations reside either near the RCL or at the loop insertion site on A sheet, in the other word mutations in the α-helix A does not affect protein inhibitory activity and stability (Elliott et al. 1998; Seo et al. 2000; Silverman and Lomas, 2007). Therefore, α-helix A was eliminated from the protein structure. An attachment loop between helix A and S6B (the sixth strand from the β-sheet) exists in the AAT structure which has a glycosylation site (amino acid 46). Previous studies have shown that glycosylation affects protein half life, and with elimination of this site, glycosylation pattern changes and thus the recombinant protein produced by *P. pastoris* may be less stable than the native AAT (Samandari and Brown, 1993; Solá and Griebenow, 2009; Sarkar and Wintrode, 2011). Therefore, the attachment loop, which is the site of interaction with the elastase, remained in the protein structure. Mutations in two critical amino acids, Phe52 and Ser53, which are located in S6B, lead to the formation of AAT deficient alleles. However, in some studies, it has been shown that with point mutations, a recombinant non-glycosylated protein can be produced which is stable (non-glycosylated stable AAT) (Karnaukhova et al. 2006). In this case, the loop between helix A and S6B can be eliminated too. But, the purpose of this study was reducing the size of the protein and investigating the effect of size reduction on AAT stability and function. No point mutations were performed and the first glycosylation site was not removed. Before entering the experimental phase of studies, the effect of eliminating the first 43 amino acids from the N-terminal

region, on protein structure was investigated using MD simulation. The native and small-scale structures were modelled and the results of the theoretical studies showed no meaningful differences between the structural properties such as conformational changes (with RMSD difference 0.035 Å) and overall structural mobility (with RMSF difference 0.022 Å) (Table 3). Furthermore, comparison of distance atom contact map in the native and truncated AAT showed no considerable deviation in C-terminal region between native and truncated structures. Thus, experimental studies were carried out to produce recombinant proteins. The level of expression and the functions of the native and truncated proteins were compared. Protein inhibitory activity was investigated through EIC (elastase inhibitory capacity) and the truncated protein was found to have appropriate inhibitory activity. However, the activity of the truncated protein was slightly similar to that of the native one. The results obtained from the theoretical studies and data showing an increase in salt bridge (with effect of 3-5 Kcal/mol for each bridge in structure) and partial reduction in hydrogen bonds (with effect of 1-3 Kcal/mol for each bond in structure) in the recombinant protein; suggest that the recombinant protein constructed in this study has good structural stability.

Table 3. Comparison of RMSD, RMSF in average for native and truncated AAT.

	Native AAT	Truncated AAT	Difference
RMSD	1.910374	1.875064	0.03531
RMSF	1.006042	1.028292	0.02225

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