Biochimica et Biophysica Acta 1840 (2014) 416–427

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Single amino acid substitutions in recombinant plant-derived human α_1-proteinase inhibitor confer enhanced stability and functional efficacy

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ARTICLE INFO

Article history:
Received 8 February 2013
Received in revised form 22 September 2013
Accepted 23 September 2013
Available online 30 September 2013

Keywords:
Human α_1-proteinase inhibitor
Recombinant therapeutic protein
Transgenic plant
Single amino acid substitution
Protein stability
Oxidation resistance

ABSTRACT

Background: Human α_1-proteinase inhibitor (α_1-PI) is the most abundant serine protease inhibitor in the blood and the heterologous expression of recombinant α_1-PI has great potential for possible therapeutic applications. However, stability and functional efficacy of the recombinant protein expressed in alternate hosts are of major concern.

Methods: Five variants of plant-expressed recombinant α_1-PI protein were developed by incorporating single amino acid substitutions at specific sites, namely F51C, F51L, A70G, M358V and M374I. Purified recombinant α_1-PI variants were analyzed for their expression, biological activity, oxidation-resistance, conformational and thermal stability by DAC-ELISA, porcine pancreatic elastase (PPE) inhibition assays, transverse urea gradient (TUG) gel electrophoresis, fluorescence spectroscopy and far-UV CD spectroscopy.

Results: Urea-induced unfolding of recombinant α_1-PI variants revealed that the F51C mutation shifted the mid-point of transition from 1.4 M to 4.3 M, thus increasing the conformational stability close to the human plasma form, followed by F51L, A70G and M374I variants. The variants also exhibited enhanced stability for heat denaturation, and the size-reducing substitution at Phe51 slowed down the deactivation rate ~5-fold at 54 °C. The M358V mutation at the active site of the protein did not significantly affect the conformational or thermal stability of the recombinant α_1-PI but provided enhanced resistance to oxidative inactivation.

Conclusions: Our results suggest that single amino acid substitutions resulted in improved stability and oxidation-resistance of the plant-derived recombinant α_1-PI protein, without inflicting the inhibitory activity of the protein.

General significance: Our results demonstrate the significance of engineered modifications in plant-derived recombinant α_1-PI protein molecule for further therapeutic development.

1. Introduction

Human α_1-proteinase inhibitor (α_1-PI), also known as α_1-antitrypsin is the most abundant circulatory protease inhibitor of 52 kDa belonging to the serpin super-family. It is a major component of human plasma and inhibits a broad spectrum of serine proteases including trypsin, chymotrypsin, cathepsin G, plasmin, thrombin, tissue kallikrein and plasminogen, while its key physiological function is inhibition of neutrophil elastase [1]. Mutations in the *SERPINA1* (*PI*) gene can cause loss or deficiency in the circulating α_1-PI levels. This leads to severe tissue damages due to uncontrolled elastase activity in the lungs or accumulation of misfolded or aggregated protein in the liver, causing potentially lethal hereditary diseases like pulmonary emphysema and cirrhosis [2,3]. Intravenous augmentation of purified α_1-PI from human serum is the only available clinical treatment that suffers from the risk of blood-borne pathogen contamination and limited supply [4]. The purified product from serum can be pasteurized, but this process includes heat treatment at 60–70 °C for ≥10 h which results into heat-induced aggregation and inactivation of the α_1-PI. Two other methods being used for elimination and inactivation of human pathogens and viruses are treatment with solvents or detergents and nanofiltration of plasma fractions, as used for preparation of Prolastin-C, recently approved for therapeutic usage. Despite effective viral inactivation steps in the manufacturing of plasma proteins, the risk of contamination with transmitting human pathogens like viruses and prions for the Creutzfeldt–Jakob disease (CJD) and other emerging or unknown pathogens may still exist [5,6].

Overexpression of recombinant α_1-PI in diverse alternative hosts including microbial expression systems and transgenic animals has been considered over the period, however, none of them could fulfill

Abbreviations: α_1-PI, α_1-proteinase inhibitor; CD, circular dichroism; C_m, mid-point of transition; DAC-ELISA, direct antigen coating-enzyme linked immunosorbent assay; PPE, porcine pancreatic elastase; RCL, reactive center loop; TSP, total soluble protein; TUG, transverse urea gradient gel electrophoresis

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http://dx.doi.org/10.1016/j.bbagen.2013.09.034

the requirement of clinically safe, biologically active and cost-effective production of recombinant α_1-PI for therapeutic applications [7]. The majority of previous studies on α_1-PI mutants have employed expression of recombinant α_1-PI protein in *Escherichia coli* or yeast, and the protein remained unglycosylated or aberrantly glycosylated. Glycosylation seems to play a very important role for intrinsic stabilization of protein structure, modulating intermolecular interactions, preventing aggregation and increasing the lifetime of circulating proteins by conferring resistance to proteolytic degradation [8]. Plants provide a versatile system for tissue and organ-specific accumulation of proteins that allow protection against proteolysis, post-translational modifications including complex type of glycosylation similar to animal system and safe storage for long duration [9]. Heterologous expression of biologically active recombinant human α_1-PI has been demonstrated previously in transgenic tomato plants [10,11], rice and tobacco cell suspension cultures [12,13], and more recently in seeds of transgenic rice and chickpea [14,15]. However, the stability and functional efficacy of recombinant α_1-PI were low and inadequate for possible therapeutic use. In the present study, we have characterized five site-specific mutations in the recombinant α_1-PI expressed in transgenic tomato plants for enhanced conformational and thermal stability, resistance to oxidative inactivation and inhibitory activity against elastase. Characterization of stable variants can also help in defining the relationship between intra-molecular strain and the tertiary structure of the native α_1-PI. Such mutants may be of practical use because the thermal stability of recombinant α_1-PI is shown to be related to the biological turnover rate of the protein [16].

The tertiary structure of α_1-PI shares a common structure with other serpins and is composed of three β-sheets (designated as sheets A, B and C) and nine α-helices (hA–hI) that connect the strands into the sheets [17]. Inhibitory serpins have a metastable native strained (S) conformation, in which the molecule is intact and the reactive center loop (RCL) is exposed for proteolytic cleavage. The presence of such a mobile RCL is presumably critical for inhibitory function of serpins. The inhibitory mechanism involves a large irreversible conformational change known as S→R (stressed→relaxed) transition. The major structural changes occur in RCL around the scissile bond (Met358–Ser359). In the stable cleaved-R state, the newly created N-terminal portion of the cleaved loop is completely inserted with formation of an inhibitory complex with the protease, and forms an additional strand within the major β-sheet (A-sheet) [18–20]. Enhancement in stability appears to be mainly due to the insertion of the cleaved loop into the sheet A with concomitant increase in the number of strands in the sheet and buried surface area [21,22]. Two other sites vital for this conformational change from S→R transition were identified as shutter [23] and breach [20], which in turn proved to be very important for the stability of α_1-PI protein. The breach, located at the top of sheet-A, is the region where the RCL first inserts. The shutter region is located in the middle of the serpin and controls the opening of the sheet-A. Both regions contain a number of highly conserved residues and several positions at which specific mutations resulted into hyperstability, without affecting the inhibitory activity [24].

Enzymatic degradation of lung connective tissues is accelerated by cigarette smoking, due to inactivation of α_1-PI by oxidizing agents in the smoke and conversion of the reactive site methionine into its sulfoxide derivatives [25,26]. An oxidation-resistant variant of α_1-PI might make it possible to reduce the large doses of α_1-PI required to provide treatment for acute inflammatory respiratory conditions. Since neutrophil elastase cleaves peptide bonds preferentially after valyl residues [27], a mutated form of α_1-PI with valine at the P_1 reactive site position would be a good potential choice as an inhibitor with therapeutic value. In this study, we have developed an active, oxidation-resistant variant of α_1-PI containing Met358 to Val substitution at the reactive center, together with other stabilizing single amino acid substitutions in recombinant α_1-PI molecule. These mutant variants of recombinant α_1-PI have been expressed and purified from transgenic tomato plants and further characterized for enhanced stability and functional efficacy. The results obtained in this study provide valuable information for engineering clinically important inhibitory serpins including recombinant α_1-PI for possible therapeutic development.

2. Materials and methods

2.1. Plant expression vectors and development of transgenic plants

The full-length 1182 bp cDNA sequence of modified α_1-PI gene (GenBank accession no. EF638826) was designed for high-level expression in dicot plants, codon-optimized and synthesized by PCR-based gene assembly approach as described earlier [6]. Five variants of the modified α_1-PI gene encoding for single amino acid substitutions at specific sites, such as Phe51 to Cys (FC, GenBank accession no. KF156767), Phe51 to Leu (FL, GenBank accession no. KF156768), Ala70 to Gly (AG, GenBank accession no. KF156769), Met358 to Val (MV, GenBank accession no. KF156770) and Met374 to Ile (MI, GenBank accession no. KF156771) were developed by site-directed mutagenesis for the respective codons (Fig. 1a, c). The modified α_1-PI gene and its single amino acid substituted variants were sub-cloned into plant transformation vector pBI101 (Clontech, USA) at *Bam*HI and *Sac*I sites along with *CaMV35S* duplicated enhancer promoter and 38 bp Alfalfa mosaic virus (AMV) 5′-UTR for optimum expression in plants. A multiple cloning site (MCS) and optimal translation initiation context (TIC) sequence were also incorporated upstream of the α_1-PI gene (Fig. 1b). The 90 bp codon optimized PR1a signal peptide (GenBank accession no. EF638827) was used at N-terminus in conjunction with C-terminal KDEL motif for retention of the recombinant protein into endoplasmic reticulum. *Agrobacterium tumefaciens* strain LBA4404 was transformed with these vectors separately and used for nuclear transformation of tomato (*Solanum lycopersium* L. var. PED) using leaf-disk method with some specific modifications [28]. The kanamycin-resistant T_0 transgenic tomato plants were developed under culture room conditions and then transferred to contained glasshouse for further growth, development and seed setting as described earlier [11].

2.2. Molecular characterization of transgenic tomato plants

Integration and expression of the modified α_1-PI transgene and its site-specific mutants were examined by PCR and RT-PCR using standard protocols [29]. Total RNA from transgenic and untransformed control tomato plants was isolated from 100 mg of young leaf tissue using TRI-Reagent according to the manufacturer's instructions (Sigma, USA). The first-strand of cDNA was synthesized with Enhanced Avian HS RT-PCR kit (Sigma, USA) and subsequently amplified using a set of α_1-PI gene specific primers: forward (PI-F) 5′-GAAGATCCTCAAGGA GATGCTGC-3′ and reverse (PI-R) 5′-CTTCTGAGTAGGGTTAACCACCTT-3′ respectively. Semi-quantitative RT-PCR analysis was performed using tomato *β-actin* gene (Tom 52, GenBank accession no. U60482) as endogenous control using the forward primer 5′-GCTGGATTTGCT GGAGATGATGC-3′ and reverse primer 5′-TCCATGTCATCCCAATTGCTA AC-3′ respectively.

2.3. Qualitative and quantitative detection of recombinant α_1-PI protein and its variants

Leaf tissues from 12-week old transgenic tomato plants were homogenized in liquid nitrogen, resuspended in ice-cold protein extraction buffer (200 mM Tris–HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 14 mM β-mercaptoethanol, 10 mM EDTA and 0.05% w/v Tween-20) and cell-free extracts were prepared by centrifugation for 10 min at 4 °C. The total soluble protein (TSP) in the crude extracts was determined by dye-binding procedure using Bradford reagent (Sigma, USA) with bovine serum albumin as a standard [30]. The quantification of recombinant α_1-PI protein was performed by direct antigen coating-

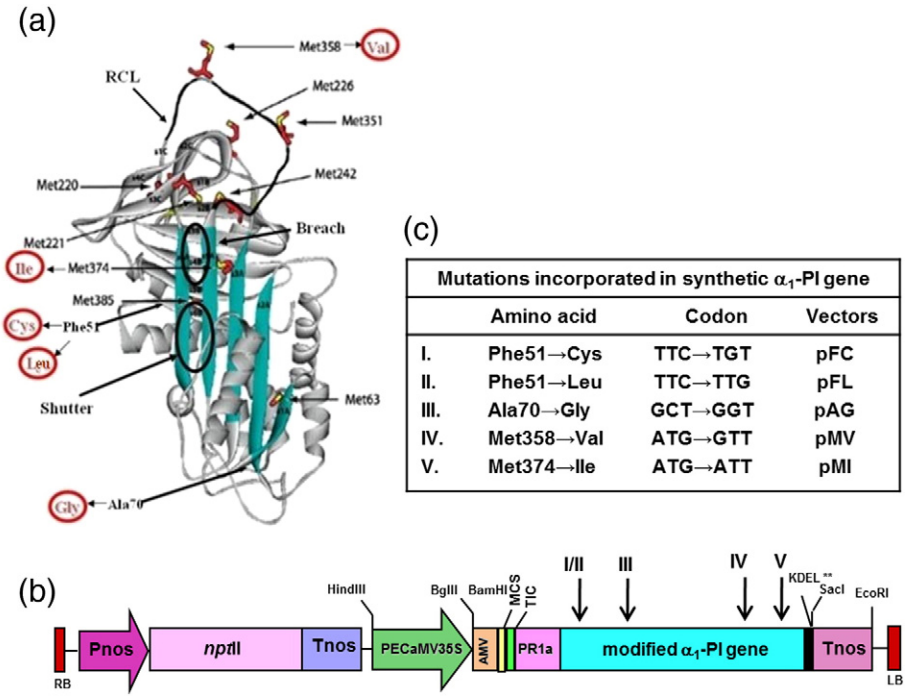

Fig. 1. Tertiary structure of human α_1-PI protein, site-specific mutations in the modified α_1-PI gene and chimeric gene vectors. (a) Ribbon model of human α_1-PI protein molecule (PDB ID: 1hp7) highlighting the reactive center loop (RCL), other critical domains such as breach and shutter and positions of incorporated single amino acid substitutions (encircled) to generate five variants for increased stability and biological activity in recombinant α_1-PI. (b) T-DNA region of plant expression vector harboring the modified synthetic α_1-PI coding sequence. Five independent expression vectors for α_1-PI variants generated with site-specific mutations are shown with arrows and designated as (I) pFC, (II) pFL, (III) pAG, (IV) pMV and (V) pMI respectively. (c) Table showing five incorporated site-specific mutations and corresponding codon replacements in the modified synthetic α_1-PI gene and plasmid vectors.

enzyme linked immunosorbent assay (DAC-ELISA) using the anti-human α_1-PI antibody, as described earlier [11]. Expression levels were quantified on a linear standard curve plotted with pure human serum α_1-PI (Sigma, USA). For qualitative detection of the recombinant α_1-PI protein, a 50 μg of cell-free extracts or 100 ng of purified recombinant protein samples was electrophoresed on 12% SDS-PAGE [31] and transferred onto immunoblot polyvinylidene difluoride (PVDF) membrane for Western immunoblotting using commercial anti-human α_1-PI antibodies as described earlier [11].

2.4. Purification of recombinant α_1-PI variants

Plant expressed recombinant α_1-PI variants were purified from leaves of transgenic tomato plants using ammonium sulfate precipitation followed by immunoaffinity chromatography as described earlier [11]. 100 g batches of fresh leaf tissue from different sets of transgenic plants were homogenized and purified using rabbit anti-α_1-PI antibody coupled to CNBr-activated Sepharose 4B matrix (Sigma, USA). The eluted fractions containing α_1-PI protein were pooled, dialyzed, concentrated and analyzed by DAC-ELISA, SDS-PAGE, Western immunoblotting and residual porcine pancreatic elastase (PPE) activity assay for determination of yield, purity, homogeneity and specific activity.

2.5. Determination of biological activity for protease inhibition

The biological activity of recombinant α_1-PI and its variants towards inhibition of elastase enzyme in cell-free extracts and purified samples was determined by residual porcine pancreatic elastase (PPE) activity assay using the chromogenic substrate N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma, USA), as described earlier [11]. The residual PPE activity was determined by measuring the release of *p*-nitroaniline from chromogenic substrate at 405 nm. Pure human α_1-PI was used as the standard to quantify the biologically active recombinant α_1-PI.

2.6. Mobility shift assay

The ability of recombinant α_1-PI and variants to form irreversible covalent complex with elastase was monitored by shift in the mobility of bands on SDS-PAGE. Purified native and recombinant α_1-PI protein samples were incubated with 100 pM of PPE in equimolar ratio for 20 min at 37 °C. Aliquots of the samples were then electrophoresed on 12% SDS-PAGE and shift in mobility of protein complex was observed after staining.

2.7. Determination of stoichiometry of inhibition and rates of association

The stoichiometry of inhibition (SI) of elastase enzyme (E) by α_1-PI inhibitor (I) was determined by incubating 100 pM of PPE with varying amounts of purified native or recombinant α_1-PI variants (I/E molar ratio = 0, 0.5, 1.0, 1.5) in the standard assay buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.01% Tween-80) at 25 °C and the residual enzyme activity was measured at periodic intervals as described above. The fraction of inhibited enzyme in each sample was plotted against the ratio of [α_1-PI]/[PPE], results were fitted with linear regression and the SI value was determined as the lowest value of [I]/[E] showing 100% inhibition of elastase. The association rate constants (k_{ass}) for the interaction of porcine pancreatic elastase with human serum α_1-PI and recombinant variants of α_1-PI inhibitor were determined in a similar manner under second-order reaction conditions, using equimolar concentrations of enzyme and inhibitor; followed by periodic withdrawal of aliquots and measuring the residual enzyme activity at various time periods.

2.8. Analysis of conformational stability

2.8.1. Transverse urea gradient (TUG) gel electrophoresis

Conformational stability of recombinant α_1-PI protein variants was analyzed on transverse urea gradient (TUG) gels, prepared with a gradient of urea (0–8 M) perpendicular to the direction of electrophoresis with an opposing gradient of acrylamide from 15 to 11% [32]. The

purified protein samples of native human serum and recombinant α_1-PI variants (20 μg in 100 μl tracking dye containing 50% glycerol, 0.01% BPB and 1× Tris–acetate buffer) were applied on the top of the TUG gels. The gels were run at a constant current of 6 mA for 3 h at 20 °C in electrode buffer (50 mM Tris–acetate, 1 mM EDTA, pH 7.5). The protein bands were visualized by staining with Coomassie brilliant blue.

2.8.2. Fluorescence spectroscopy

The conformational stability of native human serum and recombinant α_1-PI protein variants was further analyzed as a function of urea-induced equilibrium unfolding of the protein, monitored by fluorescence spectroscopy. Protein samples (4.0 μM) were allowed to equilibrate in the buffer (10 mM potassium phosphate, pH 6.5, 50 mM NaCl, 1 mM EDTA, 1 mM β-ME) containing increasing concentrations of urea (0–8 M) for 8 h at 25 °C, before recording the measurements. The changes in intrinsic tryptophan fluorescence were measured for each sample using Perkin Elmer LS-55 luminescence spectrometer at 25 °C in a 96-well microplate (Nunc, Denmark), with an excitation at 280 nm and emission at 360 nm (slit width = 5 nm for both). Experimental data were fitted to a two-state unfolding model, as described earlier [33]. The free energy of unfolding for each data point (ΔG) was calculated according to the following equation: $\Delta G = -RT \ln$; where R is the gas constant (1.987 cal mol^{-1} K^{-1}), T is the absolute temperature (298 K) and K is the equilibrium constant, calculated from: $K = f_U/f_F$; where f_U is fraction unfolded, and f_F is fraction folded, $f_U = y - y_F/y_U - y_F$ and $f_F = 1 - f_U$. In this equation, y is the observed fluorescence of the sample at different concentrations of urea, and y_F and y_U are the fluorescence for the folded and unfolded conformational states of the protein samples respectively at the same wavelength. The ΔG values were plotted against urea concentration to determine the free energy for stabilization in the absence of denaturant, ΔG_{H2O}, which is referred as the conformational stability of the protein, and determined by linear extrapolation of the ΔG values to zero denaturant concentration [34].

2.8.3. Circular dichroism (CD) spectroscopy

The unfolding transition assays were monitored by CD spectroscopy in samples (3.0 μM) incubated with different urea concentrations as described above, and far-UV CD spectra were recorded using JASCO J-800 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate at 25 °C. Spectra were recorded by continuous wavelength scans from 200 to 250 nm with scan speed of 20 nm/min, using 0.1 cm path length cell and average of two scans was calculated for each sample. Results were expressed as relative CD ellipticity (θ) in mill degrees (mdeg). The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of urea under similar conditions.

2.9. Thermal stability analysis

Thermal stability of recombinant α_1-PI variants and native human serum α_1-PI was measured by following the kinetics of inactivation at a designated temperature (54 °C). Purified protein samples (5 μg ml^{-1}) were incubated at 54 °C for 60 min, aliquots were withdrawn periodically and the remaining inhibitory activity against elastase was measured by residual PPE activity assay as described above. Half-life of thermal denaturation was calculated from fitting the experimental data into a single exponential decay.

2.10. Analysis of oxidation resistance

In order to determine the effect of methionine oxidation on the inhibitory activity of α_1-PI variants against elastase enzyme, the purified recombinant and native serum α_1-PI protein samples (5 μg ml^{-1}) were incubated with or without oxidizing agents. Resistance to methionine oxidation was determined after the treatment of protein samples with 10 mM N-chlorosuccinimide (Merck, Germany) and 0–100 mM hydrogen peroxide (Qualigens, India) in 100 μl assay buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl) at room temperature. Aliquots were withdrawn at periodic intervals followed by measurement of the remaining inhibitory activity by residual PPE activity assay.

3. Results

3.1. Development of α_1-PI constructs with site-specific mutations for plant transformation

Five variants of recombinant α_1-PI protein were designed and developed by the substitution of specific amino acids at the critical regions of the molecule, as shown in the ribbon model of native human α_1-PI protein (Fig. 1a). The phenylalanine at position 51 was replaced with either cysteine or leucine (F51C, F51L) and the methionine at 374 was replaced with isoleucine (M374I), whereas alanine at position 70 was substituted with glycine (A70G) to enhance the stability of the protein. An oxidation-resistant mutant of α_1-PI was constructed by replacing methionine at position 358 with valine (M358V). The corresponding nucleotide substitutions were incorporated in different overlapping oligos containing preferred codons for amino acids based on the codon table prepared for highly expressed genes in tomato [10]. The variants were synthesized by PCR based gene synthesis and assembly approach and the clones were characterized by nucleotide sequencing. The modified α_1-PI gene having site-specific mutation(s) was subcloned into binary vector pBI101 to generate the pFC, pFL, pAG, pMV and pMI plant expression vectors, respectively (Fig. 1b, c). These were used for *Agrobacterium*-mediated transformation of tomato leaf disks along with the vector pPAK harboring non-mutated wild type α_1-PI gene [11]. Transgenic tomato plants were developed for expression of recombinant α_1-PI variants in the ER lumen, under the control of *CaMV35S* double enhancer constitutive promoter along with AMV 5′-UTR sequence (Fig. 1b).

3.2. Single amino acid substitutions do not inflict the expression and biological activity of recombinant α_1-PI

Several independent primary transformants of tomato developed with different vectors harboring the respective modified α_1-PI gene variants were screened and verified for integration and expression of the transgene by PCR, Southern hybridization and RT-PCR (data not shown). The transcript level of modified α_1-PI gene mutants in the promising transgenic plants was analyzed by semi-quantitative RT-PCR using tomato *β-actin* gene as an endogenous control. Results showed amplification of similar amounts of α_1-PI transcripts in reference to that of the endogenous control (Fig. 2a), which indicates the presence of steady-state levels of α_1-PI mRNA in all the T_0 transgenic plants developed with different vectors. Western immunoblot analysis of crude protein extracts from different sets of T_0 transgenic plants showed hybridization of single prominent band of ~50 kDa with α_1-PI specific antibodies, revealing the integrity of recombinant α_1-PI protein variants similar to non-mutated wild-type recombinant α_1-PI expressed in plants (Fig. 2b). Pure human serum α_1-PI showed an apparent molecular weight of ~52 kDa, whereas no such band was detected in untransformed control tomato plants, which reflect the specificity of polyclonal antibody to the α_1-PI protein only.

The promising T_0 transgenic tomato plants were analyzed by DAC-ELISA for quantitation of recombinant α_1-PI protein expression. A total of 28, 24, 21, 15, 19 and 23 numbers of T_0 transgenic plants developed with pPAK, pFC, pFL, pAG, pMV and pMI vectors, respectively were assayed for the expression of variant proteins and compared with recombinant wild-type (non-mutated) α_1-PI. The average accumulation of α_1-PI protein variants expressed in T_0 transgenic population ranged from 1.02 ± 0.49% of TSP for F51C, 0.84 ± 0.37% of TSP for F51L, 0.6 ± 0.25% of TSP for A70G, 0.99 ± 0.43% of TSP for M358V and

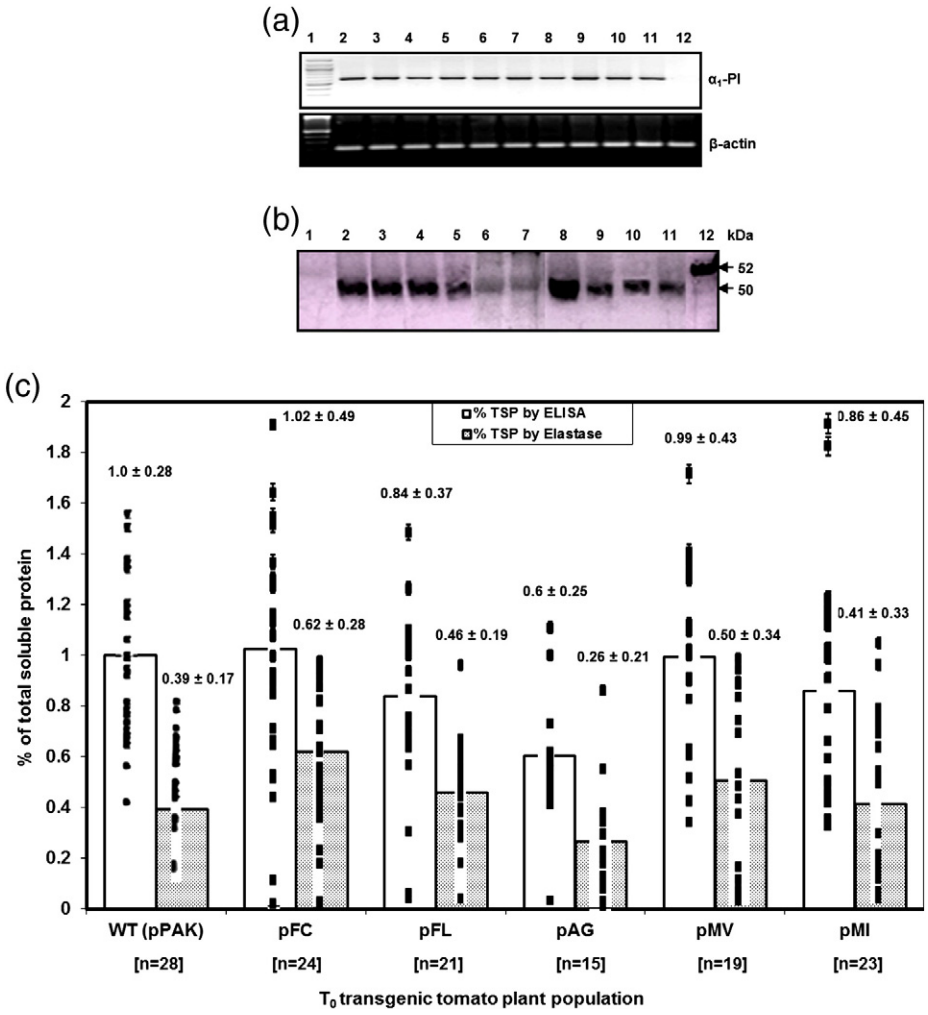

Fig. 2. Molecular and immunological characterization of promising T_0 transgenic tomato plants expressing recombinant α_1-PI protein with different single amino acid substitutions. (a) Transcript analysis by semi-quantitative RT-PCR; amplification of α_1-PI in reference to endogenous control of tomato *β-actin* gene; lane 1, 1 kb DNA ladder; lane 2–11, transgenic plants; and lane 12, untransformed control plant. (b) Western immunoblot analysis with crude protein extracts; lane 1, untransformed control plant; lane 2–11, transgenic plants expressing recombinant α_1-PI protein variants having F51C (lane 2–3), F51L (lane 4–5), A70G (lane 6–7), M358V (lane 8–9) and M374I (lane 10–11); and lane 12, purified human serum α_1-PI. (c) Quantitative DAC-ELISA and corresponding residual PPE activity for recombinant α_1-PI protein expressed in T_0 transgenic population developed with pPAK (wild-type), pFC, pFL, pAG, pMV and pMI vectors, respectively. Average expression of total recombinant α_1-PI protein and its corresponding biological activity is shown as % of TSP ± standard deviation on top of histogram bars while 'n' represents the number of T_0 transgenic plants.

0.86 ± 0.45% of TSP for M374I substitutions respectively, as compared to 1.0 ± 0.28% of TSP for wild-type recombinant α_1-PI (Fig. 2c). These results demonstrated no significant effect of site-specific mutations on the quantitative expression of recombinant α_1-PI in transgenic tomato plants, except for A70G mutation that reduced the expression to 1.7-fold as compared to wild-type. The biologically active recombinant α_1-PI protein with F5IC, FSIL, A70G, M358V and M374I substitutions was found to be 0.6 ± 0.28%, 0.46 ± 0.19%, 0.26 ± 0.21%, 0.50 ± 0.34% and 0.41 ± 0.33% of TSP respectively, in comparison to 0.39 ± 0.17% of TSP for wild-type recombinant α_1-PI (Fig. 2c), as determined by residual PPE activity assay. These results clearly demonstrate that stabilizing substitutions incorporated in recombinant α_1-PI have not inflicted the inhibitory activity of the protein and the variant proteins were as functional as wild-type. Indeed, specific activities of the purified α_1-PI protein variants were found to be increased as compared to wild-type, as described in the following section.

3.3. Recombinant α_1-PI variants were purified to high homogeneity from transgenic plants

Recombinant α_1-PI protein and its variants were purified from leaves of transgenic tomato plants commencing from 50 to 90% fraction of ammonium sulfate precipitation followed by immunoaffinity chromatography using rabbit anti-α_1-PI antibody. The purified preparations of recombinant α_1-PI proteins were analyzed by SDS-PAGE and Western immunoblotting to confirm the size, integrity and purity of the protein. A single band of ~50 kDa for all the purified variants was observed on SDS-PAGE and on the corresponding immunoblot developed with α_1-PI specific antibody (Fig. 3a, b). Recombinant α_1-PI protein variants were purified to homogeneity with high yield ranging from 14 to 17% and found to be biologically active as determined by DAC-ELISA and residual PPE activity assay, respectively (Table 1). The specific activities of all the variants of recombinant α_1-PI, especially for M358V variant, were significantly enhanced in comparison to wild-type non-mutated recombinant α_1-PI, which suggests the stabilizing effect of incorporated mutations in the expressed α_1-PI protein (Table 1).

3.4. Recombinant α_1-PI protein variants inhibit elastase by forming a stable complex

The ability of plant-derived recombinant α_1-PI and its variants to bind and form a stable SDS-resistant complex with elastase was demonstrated by band-shift assay. Results showed a shift in mobility to higher molecular weight on SDS-PAGE, due to formation of irreversible

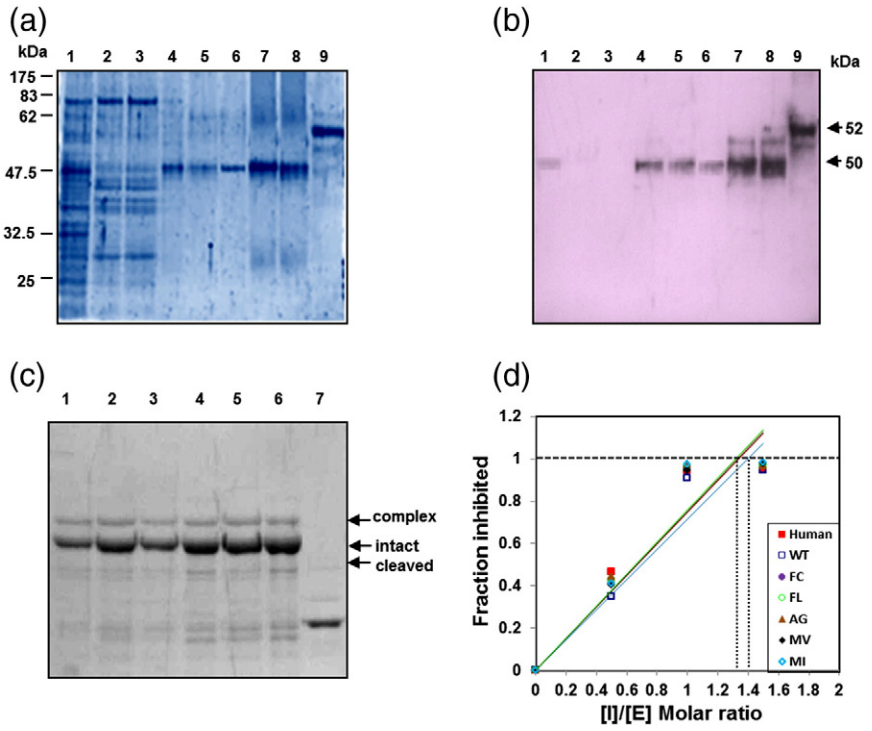

Fig. 3. Purification and inhibitory properties of recombinant α_1-PI protein variants with different single amino acid substitutions. (a) SDS-PAGE analysis of recombinant α_1-PI variants purified from the leaves of transgenic tomato plants by immunoaffinity chromatography; lane 1, whole cell-free extract; lane 2, flow through (unbound); lane 3, wash; lane 4–8, pooled eluted fractions of purified recombinant α_1-PI with F51C, F51L, A70G, M358V and M374I substitutions, respectively; and lane 9, pure human serum α_1-PI protein as positive control. (b) Western immunoblot of the gel (a) developed with α_1-PI specific antibody. (c) Mobility shift assay of purified recombinant α_1-PI protein and its variants. Plant-purified recombinant α_1-PI and its variants incubated with PPE in equimolar ratios; lane 1, recombinant wild-type (WT) α_1-PI; lane 2, recombinant α_1-PI with F51C; lane 3, F51L; lane 4, A70G; lane 5, M358V; lane 6, M374I mutations; and lane 7, pure PPE. Migration positions of the α_1-PI — PPE covalent complex (≈76–78 kDa), intact native or recombinant α_1-PI (≈50–52 kDa) and the RCL-cleaved α_1-PI (≈48–50 kDa) are indicated. (d) Stoichiometry of inhibition of elastase activity by human serum α_1-PI, recombinant wild-type and its variants. The lowest value of [α_1-PI]/[PPE] giving 100% inhibition is determined as the SI value.

enzyme-inhibitor complex after incubation of the protein variants with 100 pM of PPE in equimolar ratio (Fig. 3c). Wild-type recombinant α_1-PI protein and its variants were also used as substrate by elastase (suicidal mechanism) and resulted into cleavage product of lower molecular weight in all the PPE treated samples (Fig. 3c). Similar results were obtained with native human serum α_1-PI protein.

3.5. Recombinant α_1-PI protein variants inhibit elastase in equimolar ratio with similar rates of association

Kinetics of elastase inhibition by the purified recombinant wild-type α_1-PI and variants exhibited complete inhibition (>99%) of enzyme activity within 10 min at equimolar ratio of inhibitor to protease. Studies on the inhibition of porcine pancreatic elastase by the various purified variants of recombinant α_1-PI indicated essentially the same second-order association rate constants, k_{ass} for all the variants, except for slightly lower values than native human serum inhibitor (Table 2). The α_1-PI protein variants were analyzed to determine the number of moles of inhibitor required for complete inhibition of 1 mol of elastase enzyme (stoichiometry of inhibition, SI), by incubating the α_1-PI protein variants with constant concentration of PPE (100 pM) in different molar ratios of inhibitor to enzyme. The inhibited fractions of PPE versus [α_1-PI]/[PPE] molar ratios were plotted and the lowest value of [I]/[E], which gives 100% inhibition, was considered as the SI value, which was obtained by fitting the data with a straight line using linear regression ($p < 0.01$). The observed SI value for all the α_1-PI protein variants

Table 1
Purification and recovery of recombinant α_1-PI variants from 100 g leaves of transgenic tomato plants.[a]

Plant expression vector	Recombinant α_1-PI variant	Protein content (mg)			Yield (%)[b]	Specific activity[c]
		Total soluble protein[d]	Recombinant α_1-PI[e]	Biologically active α_1-PI[f]		
pPAK	WT	2.93	2.61 ± 0.21	1.72 ± 0.12	17.0	0.59
pFC	F51C	3.3	2.99 ± 0.11	2.57 ± 0.08	17.3	0.78
pFL	F51L	2.5	2.27 ± 0.09	1.68 ± 0.15	16.04	0.67
pAG	A70G	3.1	2.54 ± 0.08	2.02 ± 0.13	14.2	0.65
pMV	M358V	3.4	3.13 ± 0.10	3.10 ± 0.07	16.9	0.91
pMI	M374I	2.7	2.46 ± 0.12	1.73 ± 0.18	16.08	0.64

[a] Determined for recombinant α_1-PI protein variants after final purification step (immunoaffinity chromatography).
[b] Recovery of recombinant α_1-PI protein after final purification step, considering 100% yield in the crude extract, as determined by DAC-ELISA.
[c] Calculated by taking ratio of biologically active α_1-PI protein to total soluble protein and defined as the amount of α_1-PI that inhibits PPE per mg of total protein under standard assay conditions.
[d] Estimated by Bradford assay.
[e] Estimated by DAC-ELISA using human anti-α_1-PI antibody.
[f] Estimated by residual PPE activity assay.

Table 2
Characteristic profile of wild-type and single amino acid substituted variants of recombinant α_1-PI protein purified from transgenic tomato plants.

α_1-PI variants	Site of mutation	k_{ass} (M^{-1} s^{-1})[a]	C_m in urea (M)[b]		ΔΔG[c] (kcal mol^{-1})	Half-life (min)	
			TUG	Trp fluorescence		Thermal denaturation[d]	Methionine oxidation[e]
Recombinant WT	–	$1.4 \pm 0.1 \times 10^6$	1.1	1.4	–	11	23
F51C	s6B	$1.6 \pm 0.3 \times 10^6$	4.4	4.3	3.6	58	52
F51L	s6B	$1.3 \pm 0.2 \times 10^6$	2.5	2.8	1.76	50	40
A70G	hC	$1.2 \pm 0.2 \times 10^6$	2.1	2.5	1.39	42	41
M358V	RCL	$1.5 \pm 0.1 \times 10^6$	1.6	1.9	0.63	15	>120
M374I	s4B	$1.3 \pm 0.2 \times 10^6$	2.2	2.4	1.26	35	50
Human Serum	–	$1.1 \pm 0.2 \times 10^7$	5.0	4.7	4.15	>60	66

[a] Second-order association rate constants for the inhibition of porcine pancreatic elastase by native human serum and recombinant α_1-PI variants.
[b] C_m is the midpoint of transition, determined by TUG gel electrophoresis and fluorescence spectroscopy.
[c] Changes in the free energy of stabilization, calculated from $\Delta C_m \times 1.26$ (wild-type m value). ΔC_m is the difference between the transition midpoints of the variants and that of the recombinant wild-type α_1-PI in urea-induced equilibrium unfolding, and m is the measure of dependence of ΔG on urea concentration for wild-type. Data were fitted to a two-state unfolding model.
[d] Calculated from fitting the experimental data of thermal denaturation at 54 °C into a single exponential decay.
[e] Determined by incubating the protein with 10 mM N-chlorosuccinimide for 120 min.

and native human serum α_1-PI was found to be 1.3, whereas relatively higher SI value (~1.4) was observed for wild-type recombinant α_1-PI (Fig. 3d).

3.6. Single amino acid substitutions enhance the conformational stability of recombinant α_1-PI

The conformational stability of recombinant α_1-PI and its variants were qualitatively analyzed by transverse urea gradient (TUG) gel electrophoresis. The recombinant wild-type as well as variant α_1-PI proteins exhibited two-state unfolding transitions with gradual increase in urea concentration from 0 to 8 M (Fig. 4a). The variants showed moderate to significantly enhanced conformational stability in comparison to wild type recombinant α_1-PI, as the unfolding transition mid-points (C_m) of variant proteins was shifted to higher urea concentrations. The C_m values for the F51C, F51L, M374I, A70G and M358V mutations were found to be increased by ~4, 2.3, 2, 1.9 and 1.4 folds, respectively (Table 2). It is evident from the results that F51C variant

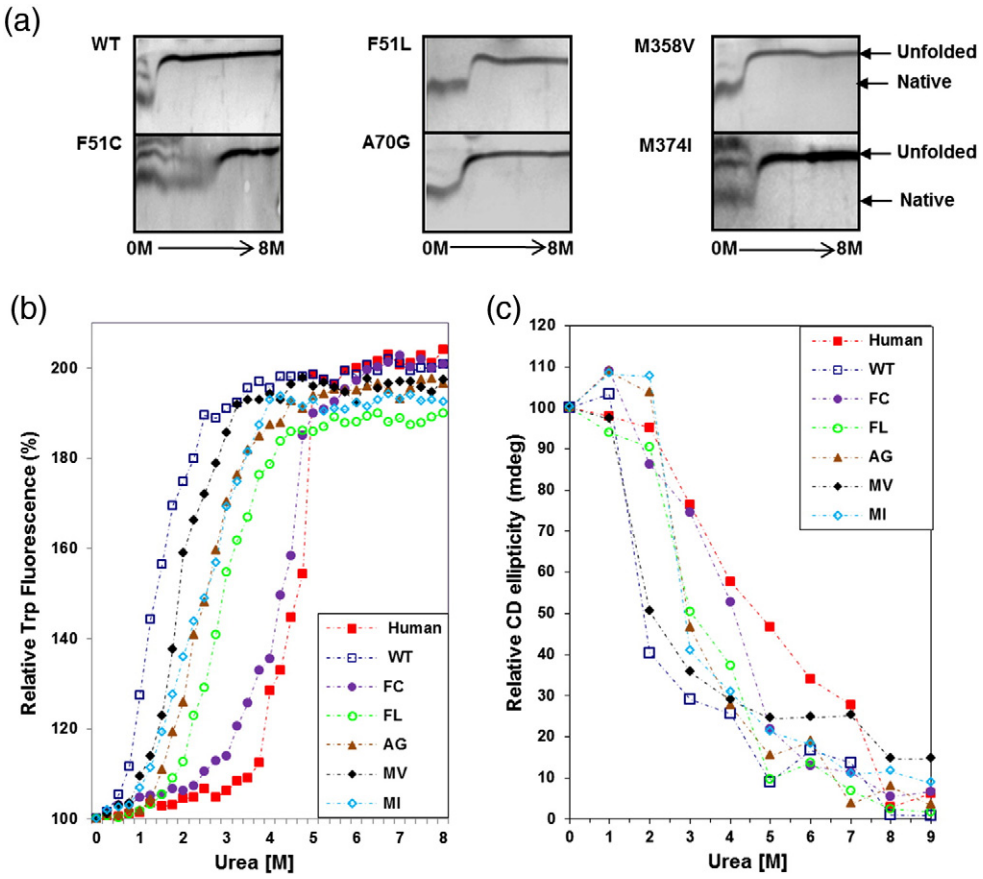

Fig. 4. Analysis of conformational stability of purified recombinant α_1-PI protein and variants. (a) Transverse urea gradient (TUG) gel electrophoresis of purified protein samples on a gradient of 0–8 M urea perpendicular to the direction of electrophoresis. Positions of the native (folded) and unfolded forms are indicated. (b) Urea-induced equilibrium unfolding transitions of the human serum α_1-PI, recombinant wild-type and the five site-specific α_1-PI variants, monitored by increase in Trp fluorescence emission intensity at 360 nm (λ_{ex} = 280 nm, excitation and emission slit widths = 5 nm for both) on treatment with increasing concentration of urea. The protein concentration was 4.0 μM for each sample. The data are represented as the relative percentage of fluorescence, taking fluorescence of native folded protein as 100%. (c) Changes in CD ellipticity at 222 nm obtained from the far-UV CD curves at increasing concentration of urea. The data are represented as the percentage of ellipticity at 222 nm, taking the values observed for native protein as 100%. The protein concentration was 5.0 μM for each sample.

of recombinant α_1-PI has acquired significant conformational stability close to its serum counterpart.

To further quantify the extent of enhancement in conformational stability due to different mutations in recombinant α_1-PI, the equilibrium unfolding of protein variants was monitored by the changes in intrinsic tryptophan fluorescence intensity as a function of urea concentration (Fig. 4b). Most of the recombinant α_1-PI variants shifted the C_m values towards higher urea concentrations as compared to wild-type, reflecting an increase in the conformational stability (Table 2). The C_m values obtained by fluorescence spectroscopy were in good agreement with the results obtained with TUG gel electrophoresis. The fractions of unfolded molecules (f_U) at the various urea concentrations were determined by fitting the experimental data onto a two-state unfolding model. Urea denaturation curves were used to determine the free energy of stabilization in the absence of denaturant (ΔG_{H2O}). The changes in the free energy of stabilization ($\Delta\Delta G$) by these single amino acid substitutions are summarized in Table 2, which clearly demonstrate that F51C mutation has shifted the transition mid-point by 3 M, increasing the free energy of stabilization ($\Delta\Delta G$) by 3.6 kcal mol^{-1}, followed by F51L, A70G and M374I mutations, while no significant increase was observed for M358V substitution.

Far-UV CD studies on urea-induced equilibrium unfolding were carried out to demonstrate the effect of urea on the secondary structure and stability of recombination α_1-PI and its site-specific variants. The CD spectra of native human α_1-PI as well as recombinant wild-type and mutant variants of α_1-PI showed the presence of substantial α-helical conformation in the far-UV region (spectra not shown). The data summarized in Fig. 4c shows the effect of increasing urea concentrations on the CD ellipticity at 222 nm for both wild-type α_1-PI and its variants. Although the transitions monitored by the fluorescence signal were fitted well with the two-state unfolding model, the transitions monitored by the CD signal indicated that unfolding of the α_1-PI is multi-phasic phenomena and undergoes three-state unfolding reactions with the formation of one stable intermediate. The mid-points of transition showed a shift towards higher urea concentrations due to mutations, similar to the results obtained by fluorescence spectroscopy, reflecting the increased physical stability of the α_1-PI variants.

3.7. Single amino acid substitutions enhance the thermal stability of recombinant α_1-PI

Thermal stability analyses of the purified wild-type recombinant α_1-PI and its variants were performed at 54 °C. The α_1-PI protein variants with increased conformational stability also showed significantly enhanced stability towards thermal deactivation, as compared to the wild-type α_1-PI, which had completely lost its biological activity after 30 min of treatment (Fig. 5a). Especially, the size-reducing substitution at Phe51 by Cys or Leu showed ~5-fold increase in thermal stability and maximum protection to heat inactivation, with a shift in the half-life of heat denaturation from 11 min for wild-type, to 58 min and 50 min for F51C and F51L variants, respectively. Other substitutions also exhibited increase in thermal stability of recombinant α_1-PI to ~1.4 to 4 fold as shown in Table 2. The human serum α_1-PI lost only 20% of its biological activity after 60 min of treatment at 54 °C as shown in Fig. 5a.

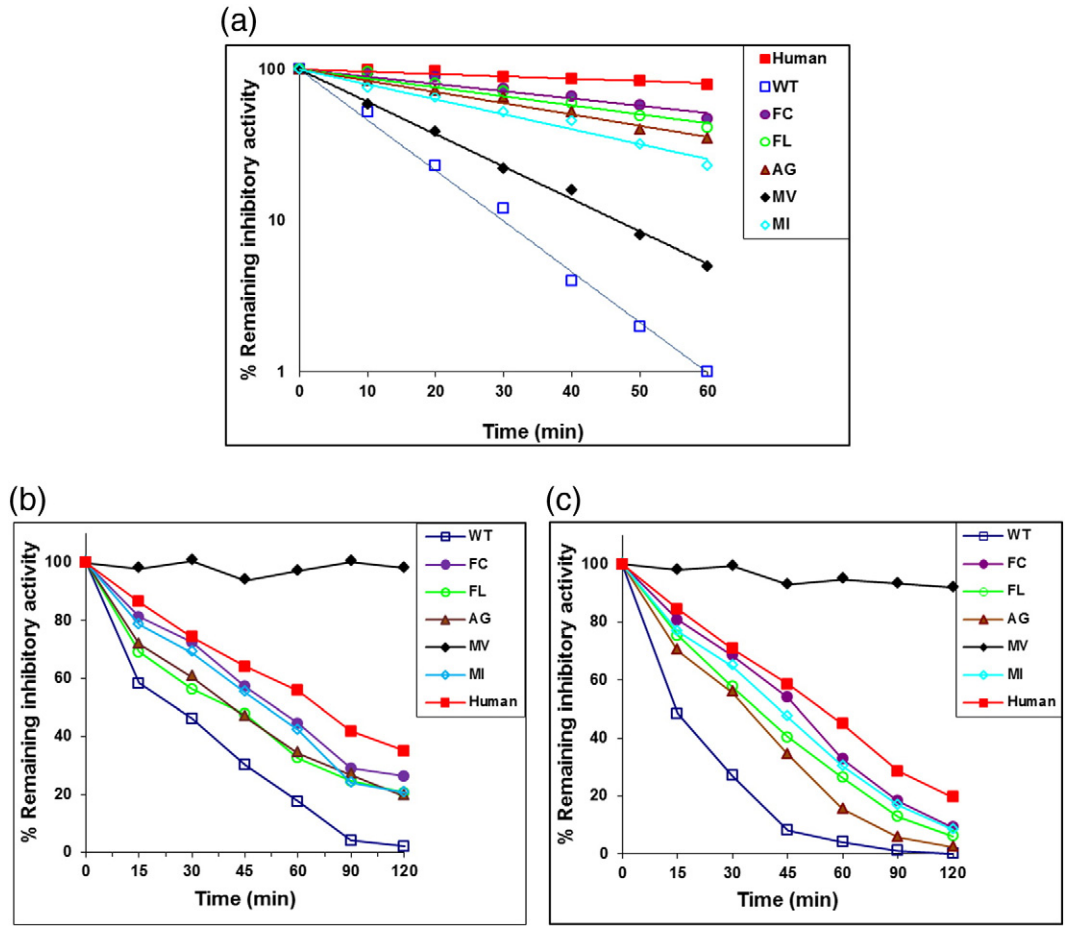

Fig. 5. Analysis of thermal stability and oxidation resistance of human serum α_1-PI, recombinant wild-type and its site-specific variants. (a) Stability towards heat deactivation was determined at 54 °C. Aliquots were taken at different time intervals and the remaining inhibitory activity against elastase was measured by residual PPE activity assay. Lines were drawn by fitting experimental data to a single exponential decay. (b–c) Effect of methionine oxidation on the inhibitory activity of α_1-PI variants, as determined by kinetic studies of inactivation of α_1-PI upon incubation with 10 mM N-chlorosuccinimide (b) and 50 mM H_2O_2 (c) for 120 min. Protein concentration in each case was 5 μg ml^{-1}.

3.8. Engineering oxidation-resistance in recombinant α_1-PI

Met358 residue located at the active site (RCL) of α_1-PI is susceptible to oxidation that results in the loss of inhibitory activity towards elastase. This methionine residue was mutated to valine (M358V) to engineer the oxidation-resistance in the recombinant α_1-PI molecule. The purified α_1-PI protein variants along with wild-type and human serum α_1-PI were treated with oxidizing agents like N-chlorosuccinimide (NCS) and hydrogen peroxide (H_2O_2), followed by measurement of biological activity for elastase inhibition. Our results showed that except for M358V variant, oxidation of methionine residue occurs readily in the native human serum α_1-PI as well as in wild-type recombinant α_1-PI and its variants, showing drastic reduction in anti-elastase activity under standard assay conditions (Fig. 5b, c). The recombinant α_1-PI having site-specific mutation for M358V remained resistant to oxidative inactivation and showed maximum half-life and retention of almost 100% inhibitory activity even after treatment with higher concentration of oxidizing agents for 120 min (Table 2).

4. Discussion

Apart from the high expression of recombinant therapeutic proteins, including α_1-PI in transgenic plants, several other issues such as stability, structural integrity and functional efficacy of the protein in heterologous environment of alternate host are of major concern and are important to decipher the mechanism of proteinase inhibition, folding, misfolding and aggregation for developing the therapeutic strategies [35]. The tertiary architecture of α_1-PI highlights the importance of different domains like RCL, breach and shutter for the stability and inhibitory activity towards elastase. The native form of human α_1-PI and other serpins exists in metastable strained state which is very critical for inhibitory function; however, it is not the thermodynamically stable conformation [19,20]. The RCL of α_1-PI is exposed in the native strained conformation, but gets inserted into the major β-sheet A after the complex formation between inhibitor and protease, with specific changes in the shutter and breach domain of the protein [17,20,36]. However, this strain could be relieved by the incorporation of different single amino acid substitutions at specific sites for more stable conformation of serpins [37]. Previous studies have reported that various unfavorable interactions, like over-packing of side chains, cavities and polar–nonpolar interactions are the structural basis of the native strain distributed throughout the α_1-PI molecule [38,39]. However, the activity regulating strain appears to be highly localized to the regions that are presumably mobilized during the complex formation with the target enzyme [38]. Stabilizing amino acid substitutions at those regions, such as β-sheet A and near the RCL, concomitantly decreased the inhibitory activity [37]. Interestingly, stabilizing substitutions at most other sites including the hydrophobic core did not affect the inhibitory activity despite a large increase in stability [33,39]. This suggests that only local strain at critical sites is utilized for functional regulation of serpins and stabilizing mutations at other regions can be introduced without hampering the biological activity. This has been explored in the current study for developing stable plant-derived recombinant α_1-PI protein for further therapeutic consideration.

We have previously expressed the human serum α_1-PI protein in transgenic tomato plants from the codon-optimized modified gene, and targeted the recombinant protein to different subcellular locations for determining the impact of subcellular environment on final yield, stability and biological activity of the protein [11]. Maximum accumulation of biologically active recombinant α_1-PI was achieved by retention in the ER lumen. However, a significant difference was found between the physical and *in vivo* stability of recombinant α_1-PI and human serum α_1-PI despite having similar amino acid sequence, which can be attributed to differences in glycosylation profile in the two forms [11]. ER-expressed recombinant α_1-PI protein exhibited high mannose type glycan structure in contrast to bi/triantennary complex type of glycans in human serum α_1-PI protein, which is responsible for the faster mobility of recombinant protein on SDS-PAGE in comparison to plasma-derived α_1-PI. In a previous study, recombinant α_1-PI forms harboring different types of oligosaccharide moieties showed different conformational and thermal stabilities [40,41]. Glycosylation confers the increase in stability of α_1-PI and the oligomannose sugars produce a less stable molecule than the complex type sugars in the plasma form. Therefore, it appears that the nature of glycosylation in α_1-PI determines the extent of stability. Glycosylation increases the conformational stability by lowering the energy level of the native folded state of α_1-PI. Mature monomeric α_1-PI contains three N-linked bi- or triantennary carbohydrate moieties attached to asparagine (Asn) residues 46, 83 and 247. The carbohydrate side chains attached to Asn 247 residue are located in the turn joining strands 3B and 2B; and extend in the vicinity of the opening of the β-barrel structure where one of the two tryptophan residues (Trp 238) is situated. It is therefore possible that the observed differences in the conformational stability of the two glycoforms of recombinant α_1-PI result from different degrees of interaction of glycosyl side chains from Asn 247 with Trp 238 residue.

In addition, the carbohydrate moieties may also contribute to the kinetic stability of α_1-PI towards non-specific aggregation by shielding the surface hydrophobic patches on the protein. Structural examination revealed that Asn 46 is close to the surface hydrophobic pocket composed of Leu 260, Pro 369, and Val 389 residues [42]. It is possible that different glycan moieties attached to Asn 46 in the two glycoforms exert differential shielding effect, thereby causing difference in stability. Moreover, glycosylated proteins are usually much more soluble than their unglycosylated counterparts. The increase in solubility may be due to the influence of carbohydrates on the protein surface chemistry. The oligosaccharides located on the protein surface can influence the interaction with solvent and the structured surface solvent somehow interferes with the aggregation process. Thus the nature of glycosylation (high mannose or complex type) may decide the degree of interaction with the solvent, thereby affecting the aggregation process and stability of the protein. Our earlier studies showed that the human plasma form was the most resistant towards thermal and urea denaturation, followed by plant-expressed glycosylated variants of the recombinant protein, while *E. coli* expressed non-glycosylated α_1-PI was found to be least stable among them [11,43].

In the present study, we have attempted to increase the stability of the ER-targeted glycosylated form of recombinant α_1-PI protein by introducing five individual single amino acid substitutions at specific sites, based on the mechanism of inhibition and results of earlier studies [37,44]. The recombinant α_1-PI variants were expressed in transgenic tomato plants, purified to homogeneity and evaluated for different parameters in comparison to non-mutated wild-type recombinant α_1-PI and native human serum α_1-PI. We have optimized the elution conditions at high pH using sodium carbonate (pH 11.2) to prevent the antibody leaching from immunoaffinity column which commonly occurs under highly acidic conditions. The purified protein preparation was characterized by silver staining [11] and Western immunoblotting using the same α_1-PI specific antibody used for preparation of immunoaffinity column (Fig. 3b). Results of both experiments showed one single band of ~50 kDa representing pure α_1-PI protein with no additional bands. The smear like diffuse staining around ~60 kDa and ~30 kDa on SDS-PAGE (Fig. 3a) may be attributed to non-specific background of the gel due to high protein concentration.

The yield of recombinant protein purified from transgenic plants is lower than obtained from *E. coli*, however, expression in plants provides additional advantages like glycosylation of the recombinant protein. Bacterial expression system has its own limitations, and lacks the cellular machinery needed for post-translational modifications (PTMs). Plants enable the specific and controlled modification of recombinant protein that cannot be exploited in bacterial or yeast expression systems, and are very critical for stability of the protein. In addition, plant expression system demonstrates a high degree of tolerance to changes in PTM pathways,

allowing the production of a humanized recombinant protein with modifications functionally similar or identical to the native protein [45]. This is the first report describing the production of stable glycosylated variants of recombinant α_1-PI using dicot plant platform, resulting in bioactive and biosimilar plant-made biopharmaceutical protein.

Our results have demonstrated that substitution of single amino acid at specific site significantly enhances the conformational and thermal stability of recombinant α_1-PI without altering the expression level and biological activity of the protein. Indeed, the specific activity of the purified variant proteins was found to be increased as compared to wild-type recombinant α_1-PI (Table 1), perhaps due to their increased physical stability. None of these mutations have altered the inhibitory activity and partitioning between inhibitor and substrate pathways, as evident by the formation of SDS-resistant proteinase-inhibitor complex that was as stable as the wild-type complex. The rates of association of recombinant α_1-PI variants with elastase and stoichiometry of inhibition were also not affected, which demonstrates enhanced stability of the protein without altering the biological activity. Interestingly, all the five site-specific variants of recombinant α_1-PI were found to be non-allergenic, as evaluated by online immunogenicity prediction softwares, EVALLER (http://www.slv.se/en-gb/Group1/Food-Safety/e-Testing-of-protein-allergenicity/) and AllerTOP (http://www.pharmfac.net/allertop/) 1.0. In our previous study, we have shown that KDEL-mediated endoplasmic reticulum (ER) retention of the recombinant α_1-PI protein resulted into addition of high-mannose core glycan [11]. The addition of H/KDEL tag restricts entry of the protein into the Golgi, thereby the complex modification of the glycans is significantly reduced. This prevents the addition of immunogenic N-glycan residues, such as $\beta(1,2)$-xylose and $\alpha(1,3)$-fucose to the protein, which are responsible for inducing allergic reactions in therapeutic setting. All variants of recombinant α_1-PI protein described in the present study have been targeted to ER, thereby reducing the risk of inducing possible immunogenicity.

The conformational stability of different variants of recombinant α_1-PI was measured by urea-induced equilibrium unfolding, monitored by TUG gel electrophoresis, tryptophan fluorescence and optical CD spectroscopy. In urea gradient gels, electrophoretic mobility is determined by the hydrodynamic volume of different conformational states of polypeptides induced by urea denaturation [37]. Among various spectroscopic methods developed to study protein folding, intrinsic Trp fluorescence is a convenient tool to detect conformational changes in the protein. Previous studies have shown that Trp194 residue is the major contributor for the fluorescence change during the unfolding of α_1-PI, and is likely to be a sensitive probe for the opening of major β-sheet A because it is located at the top of the strand 3 of the A-sheet and is completely buried [46]. Our results of fluorescence spectroscopy showed that F51C mutation has shifted the transition mid-point by 2.9 M, increasing the free energy of stabilization ($\Delta\Delta G$) by 3.6 kcal mol^{-1}, followed by F51L, A70G and M374I mutations. The transition mid-points of variants obtained by the fluorescence spectroscopy were comparable with those determined on TUG gels. These results confirmed that site-specific mutations in the modified α_1-PI gene have conferred enhancement in conformational stability of the recombinant variant proteins.

The unfolding transition of α_1-PI molecules, measured by fluorescence spectroscopy, reflects only the initial unfolding step that fits with the two-state model. However, unfolding transition probed by far-UV CD spectroscopy shows that equilibrium unfolding of α_1-PI is multiphasic, involving at least two slow steps, with the formation of one stable intermediate. The folding intermediate detected in our study is likely to be an open-state of the A-sheet, slightly less compact than the native form, with Trp fluorescence being fully dequenched, but reflecting lesser CD signal than the native folded form of α_1-PI. The stabilizing mutations of α_1-PI have specifically influenced the initial unfolding without affecting later steps and shifted the first unfolding transition to a higher urea concentration. The calculated energy difference, therefore, is between the native and partially unfolded form. Earlier kinetic studies have also shown that mutations in α_1-PI increase the conformational stability by lowering the energy level of the native folded state, rather than by increasing the energy level of the unfolded state [44].

Enhancement of A-sheet closure from the hydrophobic core is one of the strategies to increase the stability of native serpin molecule. It is clear that the decrease in the volume of side chains at the hydrophobic core of α_1-PI confers increased stability. Phe51 and Met374 residues lie in the breach domain between strands of sheet-B, at hydrophobic core of the α_1-PI protein molecule. Substitution of these residues with small linear aliphatic residues like Cys, Leu and Ileu would decrease the size of chains around hydrophobic core of α_1-PI and allows more freedom and improved tertiary packing, preventing opening of sheet-A and RCL insertion. This would eventually increase the stability of native α_1-PI protein by releasing the native strain caused by over-packed side chains. The mutation at Phe51 may also regulate the opening–closing of the A-sheet through interaction with residues in strand 5 of A-sheet [33]. Thus, change in the A-sheet opening is one of the physical changes caused by these mutations. In addition, native human α_1-PI protein contains single unpaired cysteine at position 232, and introduction of another cysteine at position 51 in place of phenylalanine may result in formation of disulfide bond between two thiol groups, which may result in improved tertiary packing, preventing opening of sheet-A and RCL insertion, thus increasing the stability of α_1-PI protein conformation.

Increased backbone freedom is another structural basis for enhancing stability of inhibitory serpins. Ala70 is located at the beginning of C-helix, at the turn between helix B and helix C, and its substitution with small flexible residue like Gly would result in better packing of proximal residues, thereby increasing conformational stability of α_1-PI by releasing the energy constraint associated with Ala70 [37]. Our results indicate that A-sheet closure due to size-reducing mutations in α_1-PI has increased the stability without affecting the complex formation with target proteinase.

Most of the substitutions engineered in recombinant α_1-PI to improve its conformational stability have also shown enhanced thermal stability of the protein. The stable mutant variants of recombinant α_1-PI were relatively more resistant to thermal deactivation than the wild-type, but still less stable than the native plasma form. Our results are in good agreement with the earlier reports and suggest significance of these mutations at specific sites to improve the physical stability of α_1-PI protein which persists *in planta* as well. Earlier studies have shown that heat-induced deactivation of α_1-PI is a concentration-dependent aggregation process [33,47]. Lack of glycosylation also leads to an increase in aggregation of the *E. coli* expressed α_1-PI upon heat deactivation [41,43] or incubation with denaturant solution [46]. The results described herein suggest that lower conformational stability of wild-type recombinant α_1-PI may contribute substantially for an increased tendency to aggregate. Therefore, equilibrium stability of α_1-PI is related to the kinetic stability towards aggregation. Loop-sheet polymerization is the proposed mechanism for *in vivo* aggregation of α_1-PI, caused by genetic disorders in humans where the RCL of one molecule is inserted into the A-sheet of another molecule [22,47]. It has also been suggested that similar specific interactions are involved in denaturant-induced aggregation of α_1-PI [22,48]. Earlier studies have indicated that unfolding of native α_1-PI reflects the opening of central β-sheet A [23,44,49]. Significantly, stable mutations at the hydrophobic core of α_1-PI, which retarded the opening of the A-sheet, also retarded aggregation of the molecule. Thus lower equilibrium stability of wild-type recombinant α_1-PI, with a higher tendency for opening of the A-sheet, might lead to intermolecular aggregation and vulnerability to heat deactivation.

The M358V mutation at the reactive site has not significantly affected the conformational or thermal stability of the recombinant α_1-PI but shown increased functional efficacy of the protein in oxidizing environment. Oxidation of Met358 residue located at the P_1 position of RCL domain results into dramatic decrease in inhibitory activity towards elastase that abolishes the protective function of α_1-PI. There are several

reports that Met358 oxidizes to sulfoxide derivatives when α_1-PI is exposed to oxidizing agents such as myeloperoxidase/H_2O_2 [50,51] or N-chlorosuccinimide [25]. The oxidative inactivation of α_1-PI due to smoking is probably the major cause of lung damage by elastase. The site-specific mutation in yeast-expressed α_1-PI for Met358 to valine substitution has shown enhanced resistance to oxidative inactivation of the protein [16,52,53]. Interestingly, a similar approach used in our study resulted into an oxidation-resistant derivative of plant-expressed recombinant α_1-PI with a single Met to Val substitution at residue 358. This substitution at the reactive center of the molecule was based on the observation that a synthetic peptide with Val substitution showed enhanced affinity for elastase [27]. The recombinant α_1-PI with M358V substitution could circumvent the problem of oxidative inactivation, which is common in an inflammatory milieu, by maintaining high anti-protease activity even under an oxidant-rich environment of human lungs.

In our previous study, pharmacokinetic evaluation of recombinant plant derived (ER-targeted) α_1-PI did not reveal any adverse affect on the feeding regimen and behavioral response in the treated rats compared to non-administered control rats, nor any allergic reaction or mortality was noticed in treated rats after 3 days post-administration [11]. However, the recombinant protein showed faster plasma clearance and lower area under curve (AUC) compared to human serum α_1-PI in rat model system, which may be attributed to association of sialic acid to the glycan termini in the serum derived glycosylated α_1-PI [41], in comparison to the plant expressed glycosylated recombinant α_1-PI lacking this moiety. However, it may not be an issue depending on the intended use. There is no need to maintain higher levels of recombinant protein in blood longer than its required action. Moreover, other routes for administration of recombinant α_1-PI can be considered, such as aerosol therapy for the treatment of lung emphysema, and topical application for various skin diseases. It is generally assumed that products directly delivered into the lungs may not require the same degree of stability as α_1-PI administered intravenously. In addition, only approximately 2–3% of the infused α_1-PI actually reaches the lungs after intravenous augmentation, therefore inhalation of aerosolized α_1-PI has been suggested as a less invasive and more efficient way to deliver large amounts of α_1-PI directly to the lungs where it is most required. Thus, despite the shorter *in vivo* half-life of plant-derived recombinant α_1-PI, it can be considered for therapeutic use by employing alternate routes of administration.

In conclusion, our results have demonstrated the significance of engineered modifications in recombinant α_1-PI protein that were designed to improve the physical stability and functional efficacy of the plant-expressed recombinant α_1-PI, crucial for its therapeutic applications. Moreover, the construction of novel α_1-PI derivatives would provide a better understanding of the regulation and inhibitory mechanism of this important broad spectrum protease inhibitor. However, it is important to analyze the impact of these site-specific mutations on pharmacokinetic behavior of plant-expressed recombinant α_1-PI, including the *in vivo* half-life and blood clearance rate in animal model system, for further therapeutic consideration.

Acknowledgements

We are grateful to Dr. C.S. Nautiyal, Director, CSIR-NBRI, Lucknow for providing infrastructural support and to the late Dr. Vinod Bhakuni, Chief Scientist, CSIR-CDRI, Lucknow for providing CD spectroscopy facility and valuable suggestions. We acknowledge Dr. Saurabh Agarwal for providing help in joint development of constructs for α_1-PI mutants. We thankfully acknowledge the Council of Scientific and Industrial Research (CSIR), India for providing funds and senior research fellowships to SJ. This work was carried out under the In-House Project OLP 0031.

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