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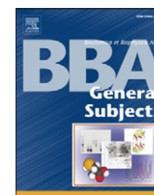

Single amino acid substitutions in recombinant plant-derived human α_1-proteinase inhibitor confer enhanced stability and functional efficacy

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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.

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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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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-

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