HIGH-LEVEL EXPRESSION AND PURIFICATION OF A THERAPEUTIC RECOMBINANT SERINE PROTEASE INHIBITOR FROM TRANSGENIC TOMATO PLANTS

Shweta Jha[1], Indraneel Sanyal[2] and Devindra Vijay Amla[3]

[1] Centre of Advanced Studies in Botany, J. N. Vyas University, Jodhpur-342001, (India)
[2,3] CSIR- National Botanical Research Institute, Lucknow-226001, (India)

ABSTRACT

Human α_1-proteinase inhibitor (α_1-PI) is the most abundant serine protease inhibitor in blood and heterologous expression of recombinant α_1-PI has great potential for therapeutic applications. Present study is focused to express and purify functional recombinant α_1-PI protein from transgenic tomato plants. Human α_1-PI gene was designed and codon-optimized according to dicot plant preferences and the significance of flanking regulatory sequences was documented for higher expression in plants. In addition, response of protein accumulation site on yield, biological activity and in planta stability was analyzed via protein targeting to different subcellular locations. Modified gene encoding recombinant α_1-PI was employed for Agrobacterium-mediated transformation of tomato. Maximum accumulation of recombinant α_1-PI was achieved from 1.5 to 3.2% of TSP by retention in ER lumen with highest biological activity for elastase inhibition. Recombinant α_1-PI was purified from transgenic tomato plants with high yield, homogeneity and biological activity by immunoaffinity chromatography. The purified protein appeared as a single band of ~50 kDa on SDS-PAGE. Results of mass spectrometry revealed the identity and structural integrity of the purified protein comparable to native serum α_1-PI. Our data suggested significance of transgenic plants to use as bioreactors for the production of stable and biologically active recombinant therapeutic proteins.

Keywords :Human α_1-proteinase Inhibitor; Protein Purification; Recombinant Protein Expression; Serine Protease Inhibitor; Transgenic Tomato Plants

I INTRODUCTION

Human α_1-proteinase inhibitor (α_1-PI), also known as α_1-antitrypsin (AAT), is the most abundant serine protease inhibitor (SERPIN) in human plasma. While α_1-PI inhibits a wide range of serine proteases, its main physiological role is to control the proteolytic activity of neutrophil elastase and maintain protease-antiprotease homeostasis in the lungs [1]. Its deficiency is either due to genetic disorders or heavy smoking, which results into development of various diseases, including pulmonary emphysema, cystic fibrosis, hepatic carcinoma, rheumatoid arthritis, psoriasis and dermatitis [2]. Currently, plasma-derived human α_1-PI is the only available option for treatment of pulmonary

emphysema by augmentation therapy, which appears to be insufficient to meet the anticipated clinical demand, and also carries the potential risk of contamination with blood-borne pathogens. In this context, efforts to develop recombinant α_1-PI, as an alternative to the plasma-derived protein, have been reported [3, 4, 5, 6, 7]. Large-scale production of safe and biologically active recombinant α_1-PI has been exploited in several alternative hosts like *E. coli*, yeast, insect cells, CHO cells, transgenic animals, rice and tobacco cell suspension cultures for therapeutic applications [3, 8, 9, 10]. The recombinant α_1-PI protein expressed in *E. coli* and yeast cells was either non-glycosylated or aberrantly glycosylated resulting in significantly decreased stability and biological activity [11, 12, 13, 14], whereas recombinant human α_1-PI expressed in milk of transgenic mice, goat and sheep were found to be associated with animal native α_1-PI and α_1-antichymotrypsin as major immunogenic impurities, and contamination by animal-borne pathogens [15]. Plants provide an attractive expression platform for overexpression of recombinant proteins due to advantages of simple growth requirements, product safety, unlimited scalability, cost-efficacy and complete post-translational modifications [16]. The potential of 'molecular pharming', using transgenic plants as 'bioreactors' to produce therapeutic proteins has been demonstrated [17]. Expression and purification of functional recombinant human α_1-PI from rice cell cultures using *Amy3D* sugar-regulated gene cassette [8, 18], or from leaves of transgenic tobacco [19, 20] or tobacco suspension cultures [9, 10] using chemically-inducible virus amplicon system, and from transgenic tobacco chloroplasts [21] have been demonstrated. However, yield and quality of recombinant α_1-PI protein was low and poor or the protein was unglycosylated that severely restricted its therapeutic application.

Considering the importance of recombinant α_1-PI as possible alternative to serum-derived α_1-PI protein for therapeutic application, the present work has been developed with the objective of production ofstable and biologically active recombinant α_1-PI protein in transgenic tomato (*Solanum lycopersicum* var. PED) plants for possible use in therapeutic applications.

II MATERIALS AND METHODS

2.1 Plant expression vectors and transformation of tomato plants

The 1,182 bp sequence of α_1-PI gene (GenBank accession no. **EF638826**) was codon-optimized and designed for optimum expression in dicot plants. The native transit peptide sequence of human α_1-PI was substituted with 90 bp modified PR1a signal peptide sequence of tobacco in the pPWK vector for targeting of the recombinant protein to apoplast, while PR1a in conjunction with KDEL motif at 3' end was used to develop pPAK vector for ER retention of the protein (Fig. 1, 2a). The 114 bp modified transit peptide sequence of sweet potato sporamine with N-terminus propeptide (SPS-NTPP) without 3' KDEL motif was used to develop pSWK vector for vacuolar targeting, while modified gene without any flanking sequences either on 5' or 3' end was used to develop pWSP vector for cytosolic accumulation of the protein (Fig. 2a). *Agrobacterium tumefaciens* strain LBA4404 was transformed with these four vectors independently and used for nuclear transformation of tomato (*Solanum lycopersicum* var. PED) using leaf-disc method with some specific modifications [22]. The kanamycin-resistant T_0 plantlets were developed under culture room conditions and then transferred to glasshouse for growth, flower development and seed setting.

2.2 Molecular characterization of transgenic tomato plants

Genomic DNA was isolated from 100 mg of young leaf tissues of transgenic tomato plants using GenElute plant genomic DNA miniprep kit according to manufacturer's instructions (Sigma, USA). PCR analysis was performed using α_1-PI gene-specific primers, α_1-PI(F) 5'-GAAGATCCTCAAGGAGATGCTGC-3' and α_1-PI(R) 5'-CTTCTGAGTAGGGTTAACCACCTT-3' and Southern hybridization was performed using α [^{32}P] dCTP labelled α_1-PI gene probe for detection of α_1-PI gene [23]. Total RNA was isolated from 100 mg fresh leaves of transgenic tomato plants in 1 ml TRI-reagent for analysis of α_1-PI transcript. The first strand of cDNA was synthesized with enhanced Avian HS RT-PCR kit according to manufacturer's instructions (Sigma, USA). The α_1-PI transcript was detected by RT-PCR using 100 ng of cDNA template and α_1-PI gene-specific primers.The tomato β-actin gene (Tom 52: GenBank accession no. **U60482**) was used as endogenous control and 194 bp fragment was amplified using the forward primer 5'-GCTGGATTTGCTGGAGATGATGC-3' and reverse primer 5'-TCCATGTCATCCCAATTGCTAAC-3'.

2.3 Quantitative estimation of recombinant α_1-PI protein

Cell free plant extracts were prepared from leaves of 12-week old transgenic tomato plants and quantification of recombinant α_1-PI protein in the crude extracts or purified protein samples was performed by direct antigen coating-enzyme linked immunosorbent assay (DAC-ELISA) using the commercial anti-human α_1-PI antibody (Sigma, USA) as described earlier [24]. Expression levels were quantified on a linear standard curve plotted with pure human serum α_1-PI protein (Sigma, USA).

2.4 Western immunoblotting

A 50 µg of cell free plant extract or 100 ng of purified recombinant α_1-PI protein sample was fractionated on 12% SDS-PAGE followed by staining with silver salts according to the standard procedure [23]. Electrophoresed protein samples were transferred onto Immobilon polyvinylidenedifluoride (PVDF) membrane (Millipore, USA) for Western blotting using the commercial anti-human α_1-PI antibodies (Sigma, USA).

2.5 Biological activity of recombinant α_1-PI protein for elastase inhibition

The biological activity of recombinant α_1-PI in cell free plant extracts and of purified protein samples was determined by residual porcine pancreatic elastase (PPE) inhibition activity assay using N-succinyl-Ala-Ala-Ala-*p*-nitroanilide (Sigma, USA) as chromogenic substrate, as described earlier [7]. Pure human serum α_1-PI was used as a standard to quantify the biologically active recombinant α_1-PI.

2.6 Purification of recombinant α_1-PI from transgenic tomato plants

A 100 g fresh leaf tissue from the promising transgenic tomato plants developed with different vectors and showing maximum expression was homogenized in liquid nitrogen and resuspended in five volumes of homogenization

buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 14 mM β-ME and 0.05% Triton X-100). The crude homogenate was filtered through nylon mesh, centrifuged at 12,000 rpm for 20 min and soluble proteins in the supernatant were precipitated by adding increasing amounts of ammonium sulphate upto 50% saturation. The remaining proteins in the supernatant were precipitated with 50-95% saturation of ammonium sulphate, resuspended, dialyzed against equilibration buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl) and filtered through 0.22 µm syringe filter (Whatman, CA) prior to loading onto immunoaffinity column prepared by coupling of rabbit anti α_1-PI antibody to CNBr-activated Sepharose 4B matrix (Sigma, USA). The column was pre-equilibrated and the clarified protein samples were loaded onto the antibody column at a flow rate of 0.5 ml min^{-1}. The column was washed with equilibration buffer until A_{280} of the effluent reached to zero. The bound antigen was eluted with 0.1 M Na_2CO_3, pH 11.2, 0.5 M NaCl and immediately neutralized with 1 M Tris-HCl, pH 6.8. The eluted fractions containing α_1-PI protein were pooled, dialyzed against storage buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl), concentrated and used for further biochemical investigations.

2.7 Mass spectrometric analysis

Protein spots were excised from SDS-PAGE gel and 'in-gel' digested with trypsin [25]. Identification and characterization of purified protein sample was performed with 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, USA) in reflector positive ion mode respectively, as described earlier [14].

III RESULTS

3.1 Construction of plant expression vectors and α_1-PI gene cloning

The cDNA sequence of human α_1-PI gene (GenBank accession no. X01683) was designed *in silico* to display codon usage patterns of abundantly expressed dicot plant genes to achieve high-level expression of recombinant α_1-PI protein in dicot plants (Fig. 1a). In modified α_1-PI gene, 52% of the native human protein codons (205 out of total 394) were replaced with dicot-preferred codons according to CUTG (Codon Usages Tabulated from Gene Bank) website (http://www.kazusa.or.jp/codon/) with substitution of about 281 favoured nucleotides. The final G+C content in the modified α_1-PI gene was decreased to 45.8% in contrast to 51.2% in native human α_1-PI gene, to complement with the overall G+C content of dicot plant genes. Several potential factors responsible for lowexpression level of protein were identified and eliminated, such as polyadenylation signals, mRNA instability sequences, RNA polymerase II termination signals and potential splicing sites in the coding region of the α_1-PI gene. A 5' untranslated region (UTR) of Alfalfa mosaic virus (AMV) and optimum translation initiation context (TAAACAATGG) was also incorporated upstream of the signal peptide sequences for proper initiation of translation (Fig. 1b).Different signal peptide sequences like signal sequence of tobaccopathogenesis related protein (PR1a) gene and sweet potato sporamine-A (SPS-NTPP) gene were incorporated at 5'end of the modified α_1-PI gene for apoplast and vacuolar targeting respectively. For accumulation of the mature α_1-PI protein into the endoplasmic reticulum (ER) of the plant cell, a 12 nucleotide sequence (AAAGATGAACTG) for KDEL (Lys-Asp-Glu-Leu)

amino acids as ER-retention signal was incorporated at 3' end of the modified α_1-PI gene (Fig. 1b, 2a). The *in silico*designed and modified α_1-PI gene was synthesized in three parts by PCR-based gene synthesis (PGS) approach using 24 to 55 bp long overlapping oligonucleotides (Fig. 1a).

The modified α_1-PI gene was expressed under the control of CaMV35S double enhancer (PECaMV35S) constitutive promoter along with 38 bp AMV 5'-UTR and various 5' and 3' flanking regulatory sequences. The gene constructs were subsequently cloned into backbone of binary vector pBIN19, mobilized into competent *Agrobacterium tumefaciens* strain LBA4404 *via* electroporation, and used for nuclear transformation of tomato for development of stable transgenic lines. The various binary vectors harbouring the modified α_1-PI gene were characterized by restriction digestion of recombinant vector DNA with *Bam*HI and *Eco*RI, followed by Southern hybridization with radiolabeled *Xho*I/*Kpn*I fragment of α_1-PI gene as the probe, which showed the expected DNA fragment of 1.5 kb (Fig. 2b). PCR amplification with specific primers for modified α_1-PI gene and *npt*II gene showed amplified fragment of 1,182 bp full-length modified α_1-PI gene and 678 bp internal fragment of *npt*II gene, respectively (Fig. 2b).

3.2 Genetic transformation of tomato

Agrobacterium tumefaciens strain LBA4404 harbouring different chimeric plasmid constructs was used for genetic transformation of tomato (*Solanum lycopersicum*) variety Pusa Early Dwarf (PED) using leaf-disc method. Vegetative leaf discs of tomato were used as explants for co-cultivation with *Agrobacterium*, resulting in overall transformation frequency between 12-14%. Antibiotic selection on kanamycin based on *npt*II gene was used for screening of putative transformed plantlets. Different stages of tomato regeneration following transformation and selection of plantlets on kanamycin supplemented medium are shown in Fig. 3. The putative T_0 transformants were developed under culture room condition and then transferred to contained transgenic house for growth to maturity and seed setting. Constitutive expression of heterologous recombinant α_1-PI in transgenic tomato plants did not show any morphological alternations. All the transgenic plants were phenotypically normal, healthy and fertile.

3.3 Molecular characterization of stable transgenic tomato plants

Several independent primary transformants of tomato were screened and verified for integration and expression of recombinant α_1-PI gene following kanamycin selection. The integration of α_1-PI transgene in plant genomic DNA was confirmed by PCR and Southern hybridization. Results showed the amplification of anticipated fragment of 1,182 bp for α_1-PI gene and 678 bp for *npt*II gene with specific set of primers (Fig. 4a and b);however, no such amplification was observed in untransformed control plant under identical assay conditions. Copy number of the transgene was detected by Southern hybridization with radiolabeled *Xho*I/*Kpn*I fragment of α_1-PI gene used as the probe. Most of the transgenic plants showed the presence of single copy of α_1-PI transgene (Fig. 4c). The size of DNA fragments showing hybridization to radiolabeled probe were variable and quite larger than positive control of 1,326 bp of full-length modified α_1-PI gene, reflecting random integration of α_1-PI transgene in the genome of

transgenic plants.The presence of full-length stable transcripts for α_1-PI gene in transgenic plants was demonstrated by RT-PCR with α_1-PI gene specific primers, which revealed amplification of expected fragments of 1,182 bp similar to positive control (Fig. 4d).

3.4 Analysis of inheritance pattern of recombinant α_1-PI gene in transgenic population

Independent T_0 transgenic plants developed with different vectors expressing high-level of recombinant α_1-PI protein were self-pollinated for detailed investigation of inheritance and segregation of transgene. T_1 seeds of highly expressing transgenic tomato plants were germinated on antibiotic-supplemented medium (Fig. 5a) and observed for segregation pattern of *npt*II gene. The results of chi-square analysis based on kanamycin resistance trait showed that most of the plants reflected typical 3:1 Mendelian segregation ratio in T_1 generation (Table 1). The chi-square values (χ^2) for T_1 progeny were found to be statistically significant at 5% level of significance and 1 degree of freedom, except for few plants like PAK 25, SWK 8, and PWK 6, which showed higher χ^2 values (Table 1). On the basis of segregation pattern and performance of T_0 plants, four stable promising transgenic lines expressing higher levels of α_1-PI viz., PAK 31, SWK 1, PWK 26 and WSP 14 were selected and grown further for purification and biochemical characterization of the recombinant α_1-PI protein.

3.5 Expression level and biological activity of plant-derived recombinant α_1-PI protein

Qualitative and quantitative estimations of the recombinant α_1-PI protein expressed in T_1 transgenic plants were monitored by DAC-ELISA, residual PPE activity assay for elastase inhibition and Western immunoblotting. Significant variation in the final yield and accumulation of recombinant α_1-PI protein targeted to different subcellular location was observed in leaves of transgenic tomato plants developed with various gene constructs. Maximum yield of recombinant α_1-PI protein upto 3.05±0.89% of TSP was documented in ER-targeted transgenic line followed by vacuole and apoplast targeted lines which showed average expression level upto 1.89 ± 0.65 % and 1.40 ± 0.48 %, while cytosol targeted protein showed very low expression level (Fig. 5b). The biologically active recombinant α_1-PI protein was in correspondence with the level of total α_1-PI protein in all the four transgenic lines. The composite data is shown in fig. 5b. Cell-free extracts of untransformed control plants did not show any inhibition for elastase activity. Western immunoblot analysis of the protein in crude extract showed the expression of ~50 kDa recombinant α_1-PI protein in transgenic lines as compared to ~52 kDa of purified human serum α_1-PI protein (Fig. 5c). Differences in molecular weights between human serum α_1-PI and plant expressed recombinant α_1-PI protein might be due to differential glycosylation pattern in these two systems.

3.6 Purification of recombinant α_1-PI from transgenic tomato plants

T_1 Transgenic plants expressing high levels of recombinant α_1-PI protein were selected and crude cell free extracts of total protein were prepared from 100 g leaf tissues by homogenization in extraction buffer with liquid nitrogen. The soluble proteins in the crude extract were precipitated by adding increasing amounts of ammonium sulphate.

Precipitated fractions were re-suspended and analyzed by DAC-ELISA for quantitative estimation of α_1-PI protein. Results showed maximum precipitation of recombinant α_1-PI between 50-95% saturated fractions of ammonium sulphate (Fig. 6a). High quality purification of recombinant α_1-PI from transgenic tomato plants was achieved by subsequent immunoaffinity chromatography using anti-α_1-PI antibody affinity column. Positive fractions from ammonium sulphate precipitation were pooled, dialyzed and loaded onto pre-equilibrated CNBr-activated Sepharose 4B column having immobilized polyclonal rabbit anti-α_1-PI antibodies. The bound protein was eluted at high pH with 0.1 M sodium carbonate (pH 11.2) and eluted fractions were immediately neutralized with Tris.HCl (pH 6.8) to prevent the loss of biological activity. The fractions containing recombinant α_1-PI were pooled, dialyzed, concentrated and analyzed by SDS-PAGE and Western immunoblotting to confirm the molecular mass, integrity and purity of the eluted protein (Fig. 6b and c). The results had demonstrated effective purification of recombinant α_1-PI to ~99% homogeneity with high average yield and biological activity, as evident by a single band of ~50kDa on SDS-PAGE (Fig. 6b).

3.7 Recombinant α_1-PI protein analysis by MALDI-TOF/TOF

Analysis of peptide fragments generated by 'in-gel' trypsin digestion of purified recombinant α_1-PI protein by MALDI-TOF MS (peptide mass fingerprinting) and MS/MS (peptide sequencing) in reflector positive ion mode showed high resemblance with native human α_1-PI (Fig. 6d). The resulting spectrum was searched using Mascot search engine and the purified protein was significantly identified as α_1-PI with a MOWSE score of 212 at p <0.05 with high sequence coverage. These results confirmed the identity of the purified product as recombinant α_1-PI.

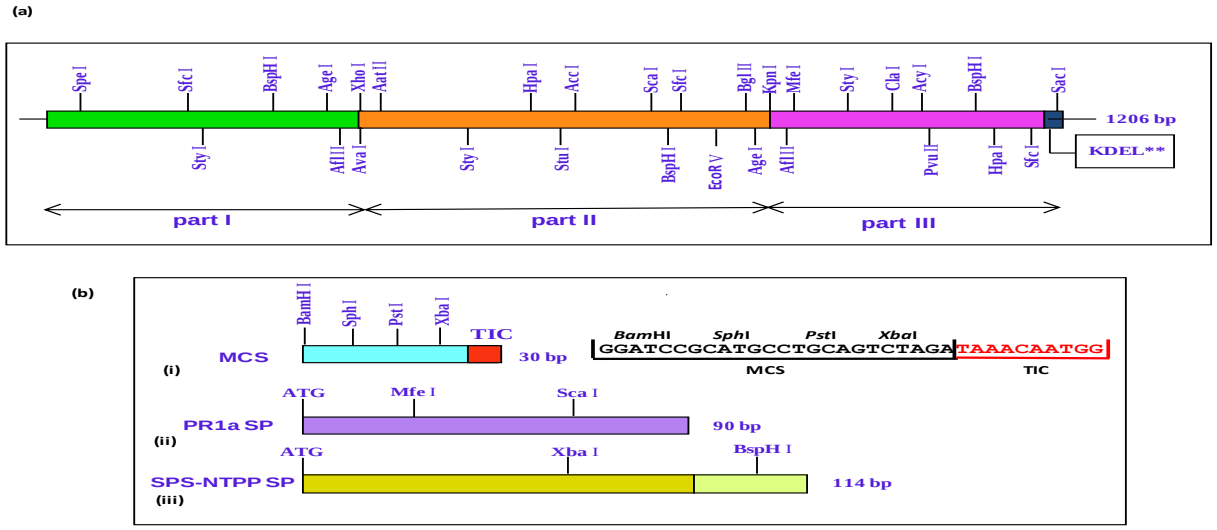

Fig. 1. Restriction architect of modified α_1-PI gene and signal peptide sequences. (a) Modified α_1-PI gene of 1206 bp divided into part I, II and III (shown with different colours) containing 25 unique restriction sites at distance of about every 50 bases.(b) (i) multiple cloning site (MCS) of 4 restriction sites with translation initiation context (TIC); (ii) 90 bp PR1a signal peptide sequence of

tobacco;(iii) 114 bpsporamineA signal peptide sequence with N-terminal propeptide of sweet potato (SPS-NTPP).KDEL- ER retention signal followed by two stop codons, SP-signal peptide.**

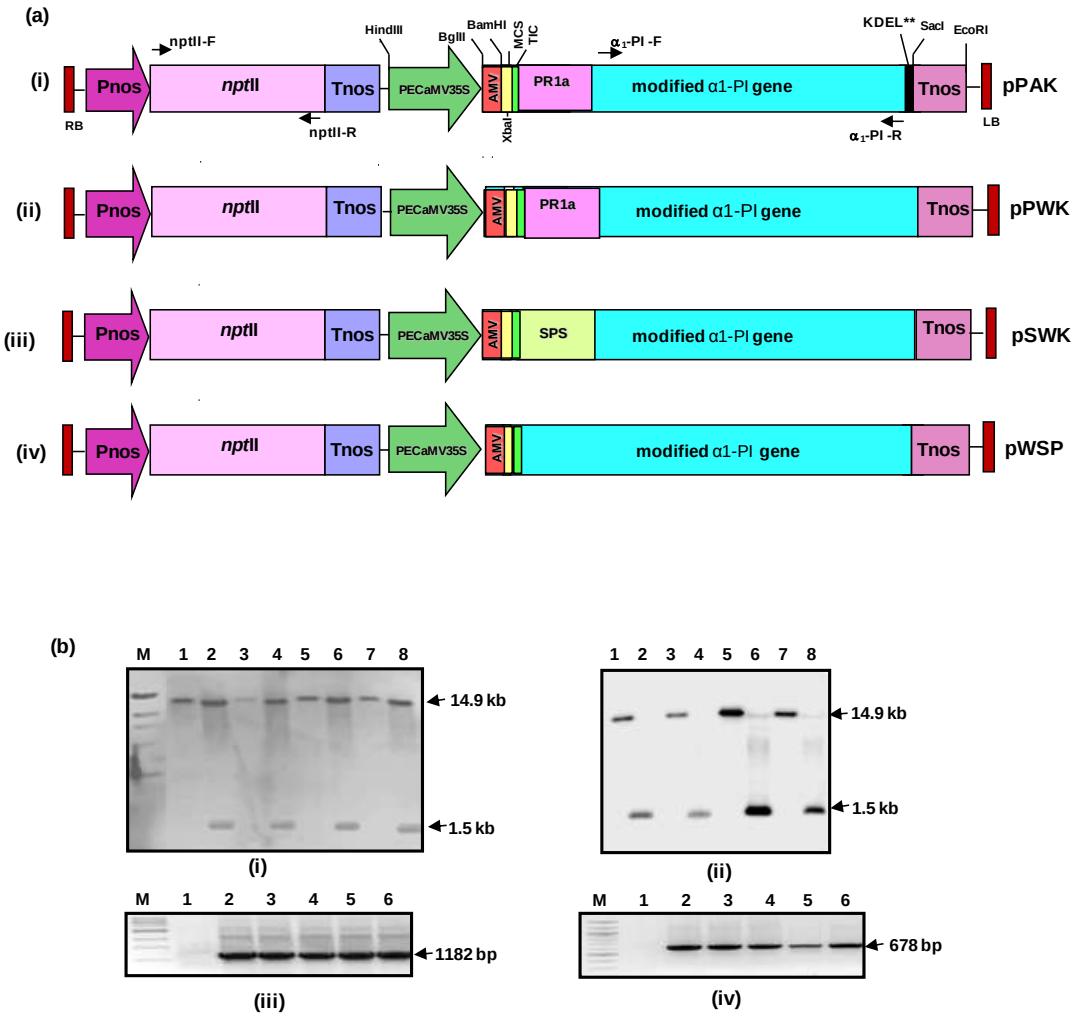

Fig. 2. Binary vector constructs of modified α_1-PI gene. (a) Schematic representation of the T-DNA region of modified α_1-PI gene constructs for sorting of protein to different subcellular locations; (i) endoplasmic reticulum (pPAK); (ii) apoplast (pPWK); (iii) vacuole (pSWK) and (iv) cytosol (pWSP). (b) Molecular characterization of α_1-PI gene constructs by (i) restriction digestion; lane M, λ-*Hin*dIII marker; lane 1, 3, 5, 7, different binary vectors of modified α_1-PI gene digested with *Bam*HI; lane 2, 4, 6, 8, with *Bam*HI/*Eco*RI; (ii) Southern blot of gel (i) hybridized with *Xho*I/*Kpn*I digested 500bp α_1-PI fragment as radiolabeled probe; (iii) PCR amplification with α_1-PI specific primers; lane M, 1 kb ladder; lane 1, negative control; lane 2-5, clones of binary vectors; lane 8,

pUC19 cloning vector as positive control; (iv) PCR amplification with *npt*II specific primers; lane M, 100 bp ladder; lane 1-6, as in (iii).

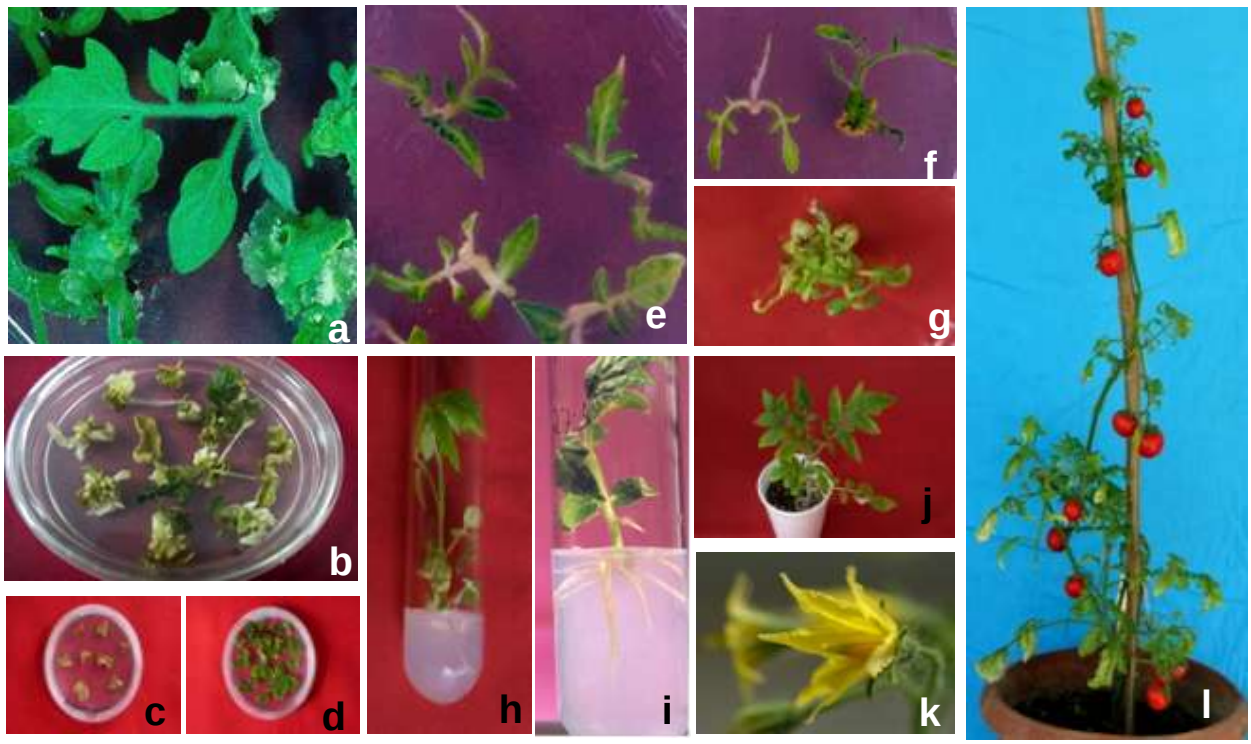

Fig. 3. *Agrobacterium*-mediated transformation of tomato and selection of transformants. (a) Vegetative leaf explants. (b) Shoot regeneration 3 weeks after culture on shoot induction medium (SIM) with 50 mg/l kanamycin. (c-d) Selection on kanamycin supplemented medium; (c) untransformed explants and (d) *Agrobacterium*-cocultivated transformed explants. (e-g) Stages during selection and screening showing non-transformed escapes, chimeric and transformed regenerating shoots. (h) Elongation of *in vitro* regenerated shoot. (i) Rooting. (j) Hardening. (k) Flower development in tissue culture developed plants in glasshouse; (l) Fertile transgenic tomato plant in glasshouse.

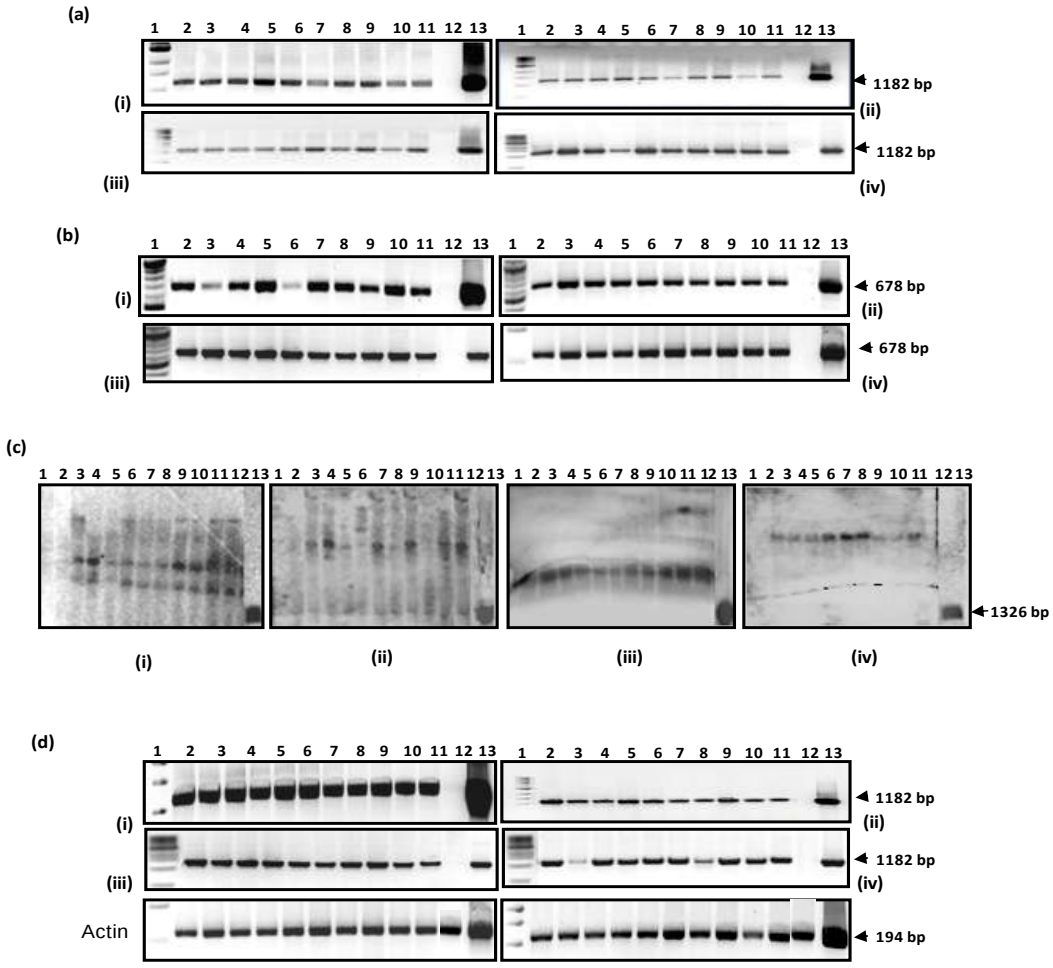

Fig. 4. Molecular characterization of T_0 transgenic plants of tomato. Confirmation of transgene integration by PCR and Southern hybridization with genomic DNA isolated from plants developed with constructs (i) pPAK, (ii) pPWK, (iii)pSWKand (iv) pWSP. PCR amplification of (a) 1182 bpα_1-PI gene and (b) 678 bp fragment of *npt*II gene;lane 1, 1 kb DNA ladder (a) or 100 bp ladder (b); lane 2-11, T_0 transgenic plants; lane 12, negative control- untransformed control plant; lane 13, positive control- corresponding binary vector. (c) Southern blot hybridized with α_1-PI specific radiolabeled probe; lane 1, λ-*Hin*dIII marker; lane 2, negative control- untransformed control plant; lane 3-12, transgenic plans; lane 13, positive control- full length modified α_1-PI gene.(d) Detection and quantitation of α_1-PI transcript in T_0 transgenic plants of tomato by RT-PCR amplification with RNA isolated from plants developed with constructs (i) pPAK, (ii) pPWK, (iii) pSWKand (iv) pWSP; lane 1, 1 kb DNA ladder; lane 2-11, T_0 transgenic plants; lane 12, negative control- untransformed control plant; lane 13, positive control. Tomato β-actin gene (lower panel) was taken as endogenous control.

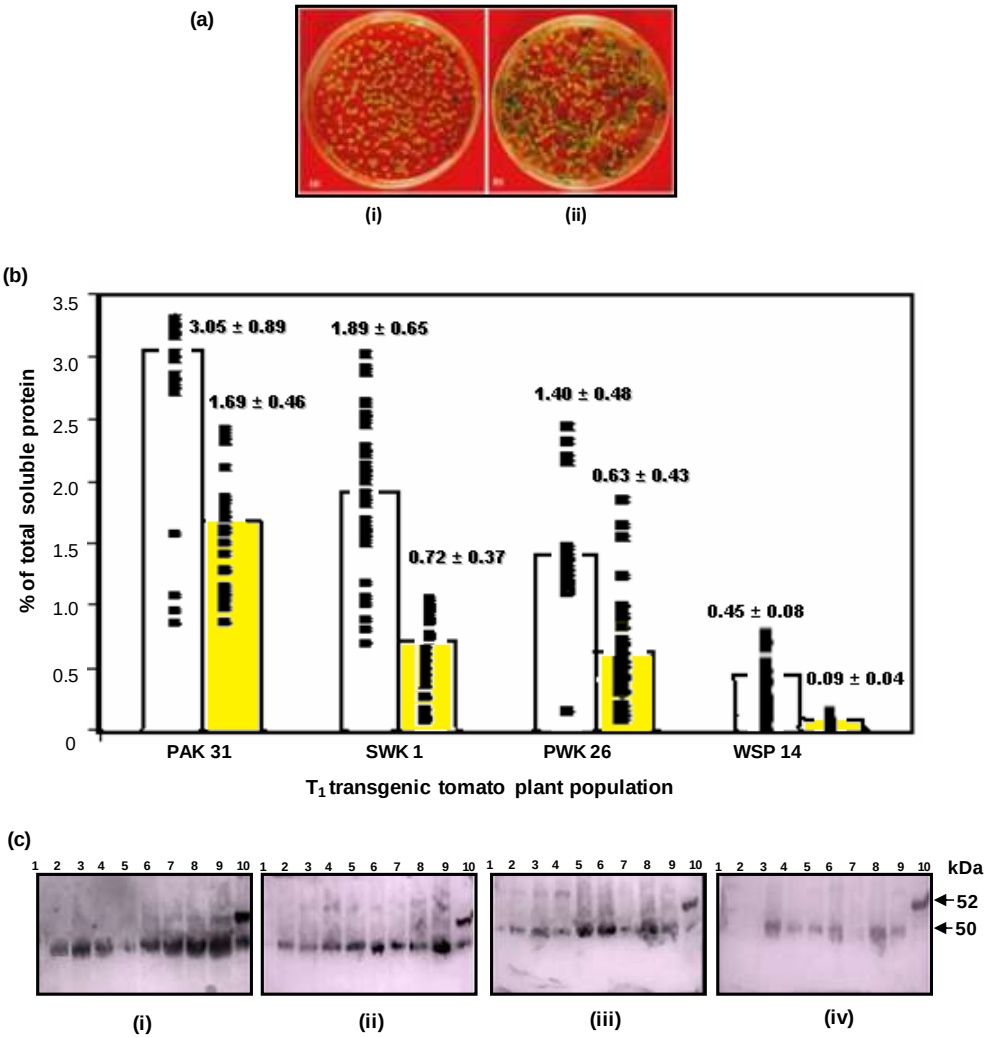

Fig. 5. (a) Germination of seeds on MS medium supplemented with kanamycin (100 mg l^{-1}); (i) Seeds from untransformed control plants; (ii) T_1 seeds obtained from T_0 transgenic plants. (b) Accumulation of recombinant α_1-PI protein in T_1 population of transgenic plants by quantitative DAC-ELISA (▭) and corresponding residual PPE activity assay (▭) for recombinant α_1-PI protein expressed in T_1 population of highly expressing transgenic lines PAK 31 (ER), SWK 1 (vacuole), PWK 26 (apoplast) and WSP 14 (cytosol) respectively. Average quantity of total recombinant α_1-PI protein and its corresponding biological activity is shown as % of TSP ± standard deviation on top of histogram bars. (c) Western immunoblot analysis with crude protein extract; lane 1, untransformed control plant; lane 2-9, transgenic plants expressing recombinant α_1-PI protein targeted to ER (i), vacuole (ii), apoplast (iii) and cytosol (iv); lane 10, purified human serum α_1-PI. The numbers on the right indicate the size of the α_1-PI protein.

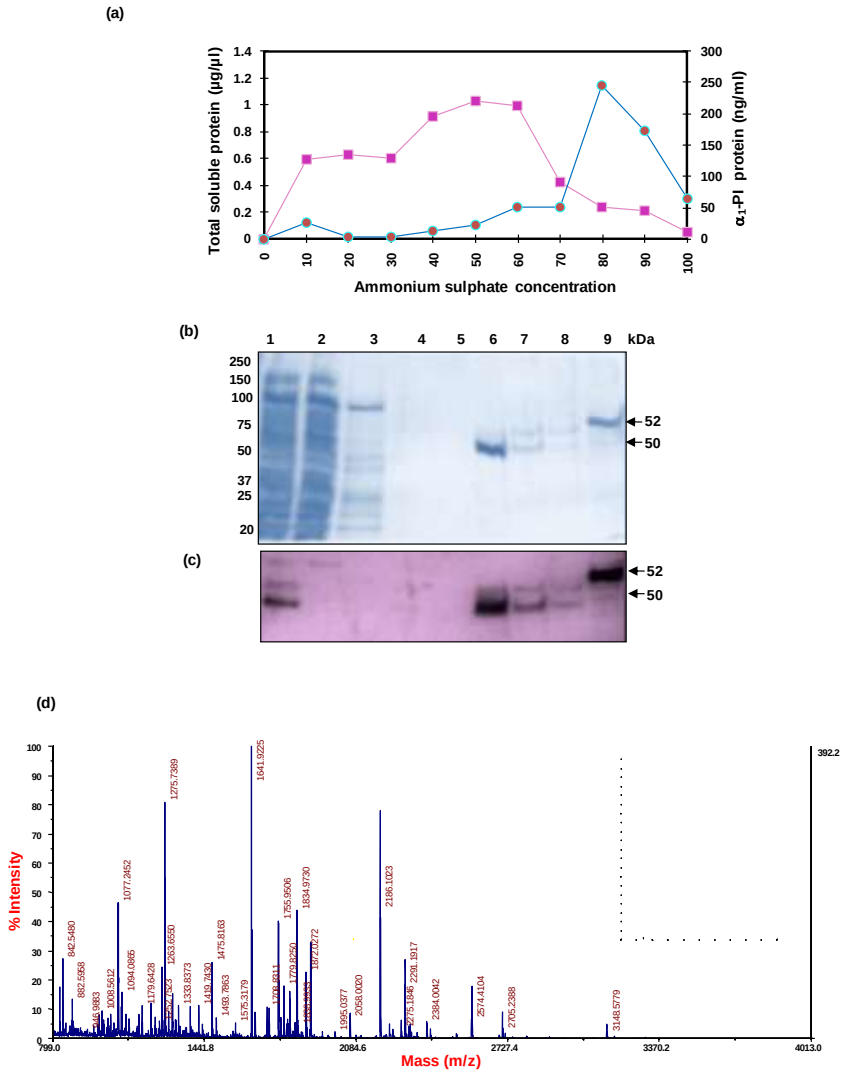

Fig. 6. Purification of recombinant α_1-PI protein from transgenic tomato plants by immunoaffinity chromatography. (a) Ammonium sulphate precipitation of crude protein extract showing amounts of precipitated total soluble protein (■) and recombinant α_1-PI protein (●) at different salt saturations. (b) SDS-PAGE analysis; lane 1, protein sample loaded onto anti-α_1-PI antibody column after ammonium sulphate fractionation at 50-95% saturation; lane 2, flow through; lane 3, 4, 5, wash; lane 6, 7,8, pooled elution fractions eluted with high pH sodium carbonate (pH 11.2); lane 9, pure human serum α_1-PI protein as positive control. (c) Western immunoblotting of the gel (b) with α_1-PI specific antibodies. (d) Identification and characterization of purifiedrecombinant α_1-PI protein by MALDI-TOF/TOF. The observed MS spectrum (peptide mass fingerprint) of the tryptic digests of recombinant α_1-PI protein was obtained in reflector positive ion mode.

Table 1. Segregation analysis of kanamycin resistance gene (*npt*II) in T_1 progeny of transgenic tomato plants.

T_0 transgenic plant ID	Response of seeds on kanamycin selection medium			χ^2 value[a]
	Total	Kan^r	Kan^s	
PAK 13	58	38	20	2.782
PAK 15	28	20	8	0.191
PAK 24	63	46	17	0.132
PAK 25	23	13	10	4.188
PAK 27	41	32	9	0.203
PAK 31	33	24	9	0.091
SWK 1	56	41	15	0.095
SWK 8	46	25	21	10.46
SWK 12	56	36	20	3.43
SWK 23	16	12	4	0.000
SWK 30	38	28	10	0.035
PWK 6	48	29	19	5.44
PWK 16	83	57	26	1.77
PWK 26	32	21	11	1.5
PWK 29	68	45	23	2.816
PWK 34	23	20	3	1.753
WSP 3	18	10	8	3.6
WSP 14	35	27	8	0.086
WSP 29	91	70	21	0.180
WSP 33	39	28	11	0.214
Negative Control	50	0	50	-

[a] $\chi 2$ value at 5 % level of significance = 3.84

IV DISCUSSION

Plant cell cultures and whole plants are currently being investigated as an alternative to microbial, mammalian cell cultures and animals for production of recombinant proteins, including human therapeutic molecules [17, 26]. The major advantages of transgenic plant cells as compared to bacteria, yeast and insect cell lines are post-translational modifications of recombinant proteins, including their glycosylation being similar to those in mammalian cells, agricultural-scale sustainable production and minimal risk of contamination by mammalian pathogens. Stable transgenic plants may offer an alternative approach for the production of safe, stable and biologically active recombinant α_1-PI protein. Low expression of heterologous foreign genes in plants results from codon bias [27], mRNA instability, premature polyadenylation [28] and instability of the expressed recombinant protein encoded by

the native transgene.The level of expression and stability of recombinant protein in plants can be influenced by several other factors like transgene copy number, chromosomal location, *cis*-regulatory elements and final location for accumulation of the protein in plant cells or tissues [29, 30]. Several strategies have been applied to increase yield of recombinant protein in plants including modified flanking sequences, use of specific subcellular targeting signals and development of downstream processing techniques [16, 31, 32]. Protein targeting to specific subcellular compartments is a key factor determining the in-planta stability and yield of the recombinant proteins owing to the biochemical environments of the compartments, available space for protein storage and their protease complement [33]. The sorting of foreign proteins into different subcellular organelles such as ER, apoplast, vacuole or cytosol using the appropriate signal peptides has shown strong impact on protein accumulation and final yield [34, 35, 36, 37]. In this study, recombinant α_1-PI was targeted to cytosol, ER, apoplast and vacuoles to evaluate accumulation, biological activity and stability of the protein in each compartment. The average yield of recombinant α_1-PI protein in leaves of transgenic lines developed with different vectors ranged between 1.55 to 3.2% of TSP except for cytosol targeted protein where significantly lower yield was obtained. This may be attributed to the negative redox potential of cytosolic milieu, which is unfavorable for disulfide bond formation and correct folding, absence of proper glycosylation and the action of ubiquitin-proteasome proteolytic pathway [38]. Maximum sequestration and accumulation of active α_1-PI protein was achieved with substitution of PR1a signal sequence and KDEL as reported earlier for other heterologous proteins in plants [39, 40, 41]. ER lumen provides a large space for accumulation of foreign proteins, relatively protective oxidizing environment favorable for disulfide bond formation, molecular chaperones for correct protein folding and low abundance of proteolytic enzymes. In order to enhance translational efficiency, a viral leader sequence in tandem combination of translation initiation context was introduced at the upstream of the modified gene [14]. The lytic vacuoles in leaves have high proteolytic content and acidic environment; therefore, they are not considered as a suitable destination for recombinant proteins [42].

It is well known that plasma derived α_1-PI inhibits neutrophil elastase with the formation of stable complex in an equimolar ratio [43]. Results from porcine pancreatic elastase inhibitory activity assay clearly demonstrated the formation of stable complex of recombinant α_1-PI with elastase and inhibition of elastase activity similar to that of plasma-derived α_1-PI. The genetic analysis of T_1 population of transgenic plants has shown inheritance and segregation of transgene in a typical Mendelian pattern, except for some T_0 plants. This may be due to random integration and some possible rearrangement of the transgene during the integration in the primary transformants.

Earlier expression and secretion of recombinant α_1-PI protein was demonstrated in genetically transformed rice cell suspension cultures [8, 18] but the C-terminal region of the protein was truncated that resulted in a lower biological activity and yield. Although recombinant α_1-PI was expressed with very high expression levels in transgenic tobacco chloroplasts [21], the protein was unglycosylated and less stable. Plesha *et al*. [20] and Huang *et al*. [10] had also transiently expressed the recombinant α_1-PI in tobacco leaves or suspension using chemically-inducible cucumber mosaic virus amplicon system, but observed micro-heterogeneity in the expressed protein. The recombinant α_1-PI protein expressed in rice and tobacco suspension cultures showed several bands of different molecular weights that reacted with rabbit anti- α_1-PI antibody and the amount of active α_1-PI was only 10–20% of

the total α_1-PI protein expressed [10]. Such micro-heterogeneity in the size of the recombinant α_1-PI protein expressed in tomato plants was not observed and only a single band of around ~50 kDa was cross-reacted with anti-α_1-PI antibody. This advocates the use of stable transgenic plants for sustainable expression of human therapeutic proteins, including α_1-PI, in preference to production by plant suspension culture or transient systems where expression is significantly affected by several factors and needs extensive optimization for competitive cost effective production [44].

Isolation and purification of recombinant protein from plants is a difficult task, owing to the complexity of the plant system compared to bacterial or yeast systems. The purification of recombinant α_1-PI from transgenic rice cell cultures had been described earlier by [8], but they could not achieve pure homogenous preparation of the protein. Their procedure involved a series of complex and cumbersome chromatography steps and several columns resulting in loss of the target protein in each step beside the increased manufacturing cost. In the present study, immunoaffinity chromatography in combination with ammonium sulphate precipitation was employed to obtain the purified recombinant α_1-PI from leaves of transgenic tomato plants. It is well established that affinity chromatography is a powerful purification method, which takes advantage of highly specific binding affinity of the target protein with an immobilized ligand that reduces the number of purification steps and increases the purity of the isolated product [45]. This technique allowed the purification of recombinant α_1-PI protein in one simple, rapid, convenient and cost-effective step unlike those employed earlier [8]. The eluted protein showed the electrophoretic mobility and other properties similar to those of the reference human serum α_1-PI, suggesting no major physicochemical changes occurred during the purification procedure. Moreover, the α_1-PI protein purified from immunoaffinity column showed higher yield, purity, homogeneity and specific activity as evident from SDS-PAGE,Western immunoblotting and mass spectrometric analysis.The results showed a single band of anticipated molecular mass (~50 kDa) for plant-expressed α_1-PI in comparison to serum purified α_1-PI (~52 kDa) reflecting differential patterns of glycosylation in both systems, particularly in the terminal galactosylation and sialylation [46]. Taken together, this method has the potential to be scaled up for obtaining purified homogenous preparations of recombinant α_1-PI protein from transgenic plants.

V CONCLUSION

This study has shown the feasibility to express and purifya clinically important human serine protease inhibitor, α_1-PI in transgenic tomato plants from the modified and codon-optimized gene. The significance of different 5' and 3' regulatory sequences flanking the modified gene, and protein sorting to different subcellular compartments for higher yield and stability have also demonstrated. Studies are now required to assess the *in vivo* stability and pharmacokinetic behaviour of the recombinant α_1-PI expressed in plants, and possibility of engineering the protein for humanized glycosylation for therapeutic applications [47].

VI ACKNOWLEDGEMENTS

We are grateful to Director, CSIR-NBRI, Lucknow for providing infrastructural support. We thankfully acknowledge Council of Scientific and Industrial Research (C.S.I.R.), New Delhi, India for providing funds and senior research fellowships to SJ. This work was carried out under the CSIR-NBRI In-house Project OLP 0031.

REFERENCES

[1] J. Travis, Structure, function, and control of neutrophil proteinases, *American Journal of Medicine, 84, 1988, 37-42.*

[2] C.A. Blank, and M. Brantley, Clinical features and molecular characteristics of α-1-antitrypsin deficiency, *Annals of Allergy, 72, 1994, 105-121.*

[3] E. Karnaukhova, Y. Ophir, and B. Golding, Recombinant human alpha-1 proteinase inhibitor: towards therapeutic use, *Amino Acids, 30, 2006, 317-332.*

[4] S. Jha, S. Agarwal, I. Sanyal, V.Jain, and D.V. Amla, Over-expression of human serum alpha-1-protease inhibitor (α_1-PI) in alternate hosts for therapeutic applications, *Journal of Surgical Sciences, 1(1), 2010, 31-37.*

[5] S. Jha, S. Agarwal, I. Sanyal, and D.V. Amla, Differential subcellular targeting of recombinant human α_1-proteinase inhibitor influences yield, biological activity and *in planta* stability of the protein in transgenic tomato plants, *Plant Science 196, 2012, 53-66.*

[6] S. Mishra, S. Jha, R. Singh, S. Chaudhary, I. Sanyal, and D.V. Amla, Transgenic chickpea expressing recombinant human α_1-proteinase inhibitor (α_1-PI) driven by a seed-specific promoter from the common bean *Phaseolus vulgaris* (L.), *Plant Cell Tissue & Organ Culture, 115, 2013, 23-33.*

[7] S. Jha, I. Sanyal, and D.V. Amla, Single amino acid substitutions in recombinant plant-derived human α_1-proteinase inhibitor confer enhanced stability and functional efficacy, *Biochimica et Biophysica Acta, 1840, 2014, 416-427.*

[8] J. Huang, T.D. Sutliff, L. Wu, S. Nandi, K. Benge, M. Terashima, A.H. Ralston, W. Drohan, N. Huang, and R.L. Rodriguez, Expression and purification of functional human α-1-antitrypsin from cultured plant cells, *Biotechnology Progress 17, 2001, 126-133.*

[9] T.-K. Huang, M.A. Plesha, B.W. Falk, A.M. Dandekar, and K.A. McDonald, Bioreactor strategies for improving production yield and functionally of a recombinant human protein in transgenic tobacco cell cultures, *Biotechnology and Bioengineering, 102, 2009, 508-520.*

[10] T.-K. Huang, M.A. Plesha, K.A. McDonald, Semicontinuous bioreactor production of a recombinant human therapeutic protein using a chemically inducible viral amplicon expression system in transgenic plant cell suspension cultures, *Biotechnology and Bioengineering, 106, 2010, 408-421.*

[11] R. Bischoff, D. Speck, P. Lepage, L. Delatre, C. Ledoux, S.W. Brown, and C. Roitsch, Purification and biochemical characterization of recombinant α-1-antitrypsin variants expressed in *Escherichia coli*, *Biochemistry, 30, 1991, 3464-3472.*

[12] K.-S. Kwon, M. Song, and M.-H. Yu, Purification and characterization of α-1-antitrypsin secreted by

recombinant yeast *Saccharomyces diastaticus, Journal of Biotechnology, 42, 1995, 191-195.*

[13] I.M. Tamer, and Y. Chisti, Production and recovery of recombinant protease inhibitor α-l-antitrypsin, *Enzyme Microbial Technology, 29, 2001, 611-620.*

[14] S. Agarwal, S. Jha, I. Sanyal, D.V. Amla, Expression and purification of recombinant human α_1-proteinase inhibitor and its single amino acid substituted variants in *Escherichia coli* for enhanced stability and biological activity, *Journal of Biotechnology, 147, 2010, 64-72.*

[15] L.T. Spencer, J.E. Humphries, and M.L. Brantly, Antibody response to aerosolized transgenic human alpha1-antitrypsin, *The New England Journal of Medicine, 352, 2005, 2030-2031*.

[16] S.J. Streatfield, Approaches to achieve high-level heterologous protein production in plants, *Plant Biotechnology Journal, 5, 2007, 2-15*.

[17] S.R. Karg, and P.T. Kallio, The production of biopharmaceuticals in plant systems, *Biotechnology Advances, 27, 2009, 879-894.*

[18] M. Terashima, Y. Murai, M. Kawanura, S. Nakanishi, T. Stoltz, L. Chen, W. Drohan, R.L. Rodriguez, and S. Katoh, Production of functional human α-1-antitrypsin by plant cell culture, *Applied Microbiology and Biotechnology, 52, 1999, 516-523*.

[19] M.R. Sudarshana, M.A. Plesha, S.L. Uratsu, B.W. Falk, A.M. Dandekar, T.-K. Huang, and K.A. McDonald, A chemically inducible cucumber mosaic virus amplicon system for expression of heterologous proteins in plant tissues, *Plant Biotechnology Journal, 4, 2006, 551-559*.

[20] M.A. Plesha, T.-K. Huang, A.M. Dandekar, B.W. Falk, and K.A. McDonald, High-level transient production of a heterologous protein in plants by optimizing induction of a chemically inducible viral amplicon expression system, *Biotechnology Progress, 23, 2007, 1277-1285*.

[21] M. Nadai, J. Bally, M. Vitel, C. Job, G. Tissot, J. Botterman, and M. Dubald, High-level expression of active human alpha-1-antitrypsin in transgenic tobacco chloroplasts, *Transgenic Research, 18, 2009, 173-178*.

[22] S. McCormick, J. Niedermeyer, A. Branson, H. Horsch, R. Fraley, Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens, Plant Cell Report, 5, 1986, 81-84.*

[23] J. Sambrook, and D.W. Russell, *Molecular cloning: a laboratory manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001).

[24] S. Agarwal, S. Jha, I. Sanyal, D.V. Amla, Effect of point mutations in translation initiation context on the expression of recombinant human α_1-proteinase inhibitor in transgenic tomato plants, *Plant Cell Report, 28, 2009, 1791-1798.*

[25] A. Shevchenko, H. Tomas, J. Havlis, J.V. Olsen, M. Mann, In-gel digestion for mass spectrometric characterization of proteins and proteomes, *Nature Protocol, 1, 2006, 2856-2860.*

[26] J.K. Ma, P.M.W. Drake, and P. Christou, The production of recombinant pharmaceutical proteins in plants, *Nature Reviews Genetics, 4, 2003, 794-805*.

[27] C. Gustafsson, S. Govindarajan, and J. Minshull, Codon bias and heterologous protein expression, *Trends in Biotechnology, 22, 2004, 346-353*.

[28] P. Jarvis, F. Belzile, and C. Dean, Insufficient and incorrect processing of the Ac transposase transcript in iae1 and wild-type *Arabidopsis thaliana, Plant Journal, 11, 1997, 921-931*.

[29] R.M. Twyman, E. Stoger, S. Schillberg, P. Christou, and R. Fischer, Molecular farming in plants: host systems and expression technology, *Trends in Biotechnology, 21, 570-578, 2003*.

[30] P.M. Doran, Foreign protein degradation and instability in plants and tissue cultures, *Trends in Biotechnology, 24, 2006, 426-432*.

[31] T.J. Menkhaus, Y. Bai, C. Zhang, Z.L. Nikolv, and C.E. Glatz, Considerations for the recovery of recombinant proteins from plants, *Biotechnology Progress, 20, 2004, 1001-1014*.

[32] M. Benchabane, C. Goulet, D. Rivard, L. Faye, V. Gomord, and D. Michaud, Preventing unintended proteolysis in plant protein biofactories, *Plant Biotechnology Journal 6, 2008, 633-648*.

[33] J. Callis, Regulation of protein degradation, *Plant Cell, 7, 1995, 845-857*.

[34] N.S. Outchkourov, B. Rogelj, B. Strukelj, and M.A. Jongsma, Expression of sea anemone equistatin in potato: effect of plant proteases on heterologous protein production, *Plant Physiology, 133, 2003, 379-390*.

[35] M. Gils, R. Kandzia, S. Marillonnet, V. Klimyuk, and Y. Gleba, High-yield production of authentic human growth hormone using a plant virus-based expression system, *Plant Biotechnology Journal, 3, 2005, 613-620*.

[36] J. Yang, L.A. Barr, S.R. Fahnestock, and Z.B. Liu, High yield recombinant silk-like protein production in transgenic plants through protein targeting, *Transgenic Research, 14, 2005, 313-324*.

[37] S. Petruccelli, M.S. Otegui, F. Lareu, O.T. Dinh, A.C. Fitchette, A. Circosta, A KDEL-tagged monoclonal antibody is efficiently retained in the endoplasmic reticulum in leaves, but is both partially secreted and stored to protein storage vacuoles in seed, *Plant Biotechnology Journal, 4, 2006, 511-527*.

[38] R.D. Vierstra, The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins, *Trends in Plant Science, 2003, 135-142*.

[39] A. Schouten, J. Roosien, F.A. van Engelen, G.A. de Jong, A.W. Borst-Vrenssen, J.F. Zilverentant, D. Bosch, W.J. Stiekema, F.J. Gommers, A. Schots, and J. Bakker, The C-terminal KDEL sequence increases the expression level of a single-chain antibody designed to be targeted to both the cytosol and the secretory pathway in transgenic tobacco, *Plant Molecular Biology, 30, 1996, 781-793*.

[40] T.-J. Kang, N.-H. Loc, M.-O. Jang, and M.-S. Yang, Modification in cholera toxin B subunit coding sequence to enhance expression in plants, *Molecular Breeding, 13, 2004, 143-153*.

[41] D. Li, , J.O. Leary, Y. Huang, N.P.A. Huner, A.M. Jevnikar, and S. Ma, Expression of cholera toxin B subunit and the B chain of human insulin as a fusion protein in transgenic tobacco plants, *Plant Cell Report, 25, 2006, 417-424*.

[42] C. Goulet, and D. Michaud, *Degradation and stabilization of recombinant proteins in plants* (In: Floriculture, Ornamental and Plant Biotechnology, Vol. IV (Taxeira de Silva, J., ed.), 35-40. London: Global Science Books, 2006).

[43] J. Travis, M. Owen, P. George, R. Carrell, S. Rosenberg, R.A. Hallewell, and P.J. Barr, Isolation and properties of recombinant DNA produced variants of human α-l-proteinase inhibitor, *Journal of Biological Chemistry, 260, 1985, 4384-4389*.

[44] A.K. Sharma, and M.K. Sharma, Plants as bioreactors: recent developments and emerging opportunities, *Biotechnology Advances, 27, 2009, 811-832*.

[45] M. Sugiura, S. Hayakawa, T. Adachi, Y. Ito, K. Hirano, and S. Sawaki, A simple one-step purification of human α_1-proteinase inhibitor by immunoadsorbent column chromatography, *Journal of Biochemical and Biophysical Methods, 5, 1981, 243-249.*

[46] E. Stoger, J.K. Ma, R. Fischer, and P. Christou, Sowing the seeds of success: pharmaceutical proteins from plants, *Current Opinion in Biotechnology, 16, 2005, 167-173*.

[47] A. Castilho, R. Strasser, J. Stadlmann, J. Grass, J. Jez, P. Gattinger, R. Kunert, H. Quendler, M. Pabst, R. Leonard, F. Altmann, and H. Steinkellner, *In planta* protein sialylation through overexpression of the respective mammalian pathway, *Journal of Biological Chemistry, 285, 2010, 15923-15930.*