Amino Acids (2006) 30: 317–332
DOI 10.1007/s00726-005-0324-4

Amino Acids
Printed in Austria

Recombinant human alpha-1 proteinase inhibitor: towards therapeutic use

Review Article

E. Karnaukhova, Y. Ophir, and **B. Golding**

Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, Maryland, U.S.A.

Received December 12, 2005
Accepted January 31, 2006
Published online May 26, 2006;

Summary. Human alpha-1-proteinase inhibitor is a well-characterized protease inhibitor with a wide spectrum of anti-protease activity. Its major physiological role is inhibition of neutrophil elastase in the lungs, and its deficiency is associated with progressive ultimately fatal emphysema. Currently in the US, only plasma-derived human alpha-1-proteinase inhibitor is available for augmentation therapy, which appears to be insufficient to meet the anticipated clinical demand. Moreover, despite effective viral clearance steps in the manufacturing process, the potential risk of contamination with new and unknown pathogens still exists. In response, multiple efforts to develop recombinant versions of human alpha-1-proteinase inhibitor, as an alternative to the plasma-derived protein, have been reported. Over the last two decades, various systems have been used to express the human gene for alpha-1-proteinase inhibitor. This paper reviews the recombinant versions of human alpha-1-proteinase inhibitor produced in various hosts, considers current major safety and efficacy issues regarding recombinant glycoproteins as potential therapeutics, and the factors that are impeding progress in this area[1].

Keywords: Alpha-1-proteinase inhibitor – Antitrypsin – Emphysema – Glycosylation – Recombinant

Introduction

Human alpha-1-proteinase inhibitor (α_1-PI), also known as alpha-1-antitrypsin[2], is the most abundant serine protease inhibitor in human plasma. α_1-PI is synthesized mainly in hepatocytes, but is also produced in small amounts by alveolar macrophages, neutrophils, and some other cells (White et al., 1981; Paakko et al., 1996). Concentration of α_1-PI in blood normally varies from 20 μM to 53 μM (1.04–2.76 g/l) (Brantly et al., 1988, 1991) with a half-life in the circulation of about 4–5 days (Crystal, 1989; Archibald et al., 1990). While α_1-PI inhibits a wide range of serine proteases, its main physiological role is inhibition of polymorphonuclear leukocyte (neutrophil) elastase (NE) in the lungs (Travis, 1988). A hereditary α_1-PI deficiency (with levels of α_1-PI in blood below 11 μM, insufficient for inhibition of NE) is classically associated with development of premature, ultimately fatal, pulmonary emphysema (Crystal, 1991; Snider, 1992). Over 100 alleles of α_1-PI have been identified with approximately 35 of them being associated with α_1-PI deficiency, including Z-allele, the most common cause of the deficiency when inherited in a homozygous fashion (Brantly, 1996). Due to a single mutation in the mobile domain (Glu342Lys), an α_1-PI mutant (Z-mutant) undergoes aberrant conformational transitions, which allow the protein to aggregate. This results in retention of polymerized α_1-PI Z mutant within hepatocytes, thus causing α_1-PI deficiency in the circulation (Lomas, 2005). The epidemiology of α_1-PI deficiency, its clinical manifestations including lung disease and liver disease, and natural history have been described in detail (see recent reviews by Luisetti and Seersholm, 2004; Needham and Stockley, 2004; Lomas, 2005; Crystal, 1996).

[1] The opinions expressed in this paper reflect the authors' personal views, based on published data and the information available from the public domains, and have no relation to the official statements, if any, held by the US FDA, National Institutes of Health, or the US Department of Health and Human Services. FDA official recommendations for plasma protein therapeutics and recombinant proteins regarding safety, purity, and potency of new drugs and biologics produced by recombinant DNA technology are referred below as the US FDA Guidances.

[2] Serum was known to inhibit trypsin almost a century ago, therefore the inhibitor isolated from alpha-1-globulin fraction of human serum in 1962 was named alpha-1-antitrypsin. Later, this historical name was changed to alpha-1-proteinase inhibitor due to its capability to inhibit a number of serine proteases other than trypsin.

Structure and function of α_1-PI

α_1-PI is a 52 kDa glycoprotein belonging to the serine protease inhibitor (serpin) superfamily, which in addition to α_1-PI also includes α_1-antichymotrypsin, antithrombin, plasminogen activator inhibitor, C1 esterase inhibitor, and many others (Stein and Carrell, 1995; Silverman et al., 2001). α_1-PI is encoded by a 12.2 kb gene located on the long arm of chromosome 14 (Long et al., 1984; Rabin et al., 1986). A single polypeptide chain of α_1-PI is comprised of 394 amino acid residues, including one cysteine, 2 tryptophanes, and 9 methionine residues (Johnson and Travis, 1979; Carp et al., 1982). Oxidation of Met358 has been shown to cause a loss of anti-elastase activity (Beatty et al., 1980), and a similar effect was reported for Met351 (Taggart et al., 2000). A detailed analysis of oxidation of the methionine residues in α_1-PI has been performed by Griffith and Cooney (2002a, b, c).

Carbohydrates represent $\sim$12% of α_1-PI by molecular weight. 3 N-linked glycans, which are almost exclusively bi-antennary and tri-antennary structures, are attached to asparagine residues 46, 83, and 247 (Mega et al., 1980a, b; Carrell et al., 1981, 1982). Human α_1-PI has a typical complex glycosylation which is grounded on a tri-mannose fork core containing N-acetyl glucosamine, galactose, and terminal negatively-charged sialic (N-acetylneuraminic) acid (Mega et al., 1980b; Travis and Salvesen, 1983). Like the majority of other native glycoproteins, human α_1-PI is intrinsically a highly heterogeneous mixture, mainly due to variations in 2- and 3-antennary carbohydrates and missing of N-terminal pentapeptide (Vaughan et al., 1982; Hercz, 1985; Krasnewich et al., 1995; Lupi et al., 2000).

The tertiary structure of the protein features 9 α-helices, 3 β-sheets (denoted as A, B, and C), and a mobile 15-residue reactive center loop (RCL), which is exposed for interaction with the target serine protease (Johnson and Travis, 1977; Lomas, 2005). Protease attack of the RCL results in cleavage at Met358-Ser359, formation of a covalent α_1-PI-protease complex with the amino-terminal polypeptide inserted into the A β-sheet, and an overall dramatic conformational change (Wilczynska et al., 1997; Stratikos and Gettins, 1997, 1998, 1999; Huntington et al., 2000; Ludeman et al., 2001). The crystal structures of wild-type native (Elliott et al., 1996) and RCL-cleaved (Loebermann et al., 1984) α_1-PI are available at the Protein Data Bank (www.rcsb.org).

Unlike the majority of proteins, α_1-PI is naturally folded in a metastable structure. Thermodynamically this is not the most stable form, and therefore, the protein is prone to conformational modifications and aggregation (Lomas, 1993, 2005; Lomas et al., 1995).

Similar to other serpins, α_1-PI can convert intramolecularly into a more stable latent form, which is inactive, although the biological activity can be restored via denaturation and refolding (Lomas et al., 1995; Silverman et al., 2001).

In addition to its inhibitory antiprotease function, α_1-PI seems to have a broader spectrum of activities (Brantly, 2002; Janciauskiene et al., 2004; Nita et al., 2005). Due to nine methionine residues per α_1-PI molecule, its plausible role as a putative antioxidant has been suggested (e.g., Taggart et al., 2000; Levine et al., 1999, 2000). Anti-inflammatory properties and some other activities indicate that α_1-PI possesses a broader function than just as an antiprotease (Brantly, 2002; Stockley et al., 2002; Janciauskiene et al., 2004; Nita et al., 2005).

Treatment of α_1-PI deficiency

Currently-licensed treatment of pulmonary emphysema involves intravenous infusion of the plasma-derived α_1-PI preparation, with the recommended dose of 60 mg of active α_1-PI per kg of body weight administered once weekly. To maintain a threshold level of α_1-PI (11 μM), α_1-PI deficient-patients should receive augmentation therapy for the duration of their lives, to slow the progression of emphysema. This nadir level has been derived from α_1-PI levels observed in the plasma of individuals who are heterozygous for Z-mutant α_1-PI and who do not develop emphysema. Evaluation of the efficacy of α_1-PI products used in clinical studies is based on surrogate markers: the infusion of α_1-PI must elevate the circulating serum level of α_1-PI above an epidemiologically established 'protective threshold' and the protein must be detectable in bronchoalveolar lavage fluid (Sandhaus, 2004; Stoller et al., 2002; Juvelekian and Stoller, 2004). However, the ability of α_1-PI augmentation therapy to reduce the progression of emphysema still remains to be proven (Hutchison and Hughes, 1997; Stockley 2000; Abboud et al., 2005).

Efficiency of intravenous administration of α_1-PI has been evaluated in several studies (e.g., Seersholm et al., 1997; Wencker et al., 1998). According to Hubbard and Crystal (1990), approximately only 2–3% of the infused α_1-PI actually reach the lungs. Therefore, alternative routes of administration, such as inhalation of nebulized α_1-PI powder or aerosolized α_1-PI solution (Hubbard et al., 1989; Hubbard and Crystal, 1990; Sandhaus, 2004; Taylor and Gumbleton, 2004), and gene therapy as an alternative

treatment approach (Flotte, 2002; Stecenko and Brigham, 2003; Sandhaus, 2004) have been under development.

The estimate of approximately 60,000–100,000 severely deficient individuals in the US defines the α_1-PI deficiency as a rare disease. Yet, recent publications indicate that α_1-PI deficiency is widely under- and misdiagnosed (de Serres, 2002, 2003). As reported by the World Health Organization (WHO 1996), only 4% of individuals with α_1-PI deficiency cases are identified, and only a portion of them are receiving treatment.

Plasma-derived α_1-PI products

Currently there are three commercial plasma-derived α_1-PI (pd-α_1-PI) products licensed by the US FDA for intravenous treatment of patients with hereditary α_1-PI deficiency[3]. α_1-PI products are manufactured as part of a complex plasma fractionation scheme originally developed for large-scale production of albumin, but which now also yields many other plasma therapeutics.

Commercial plasma-derived α_1-PI products differ in terms of their purity, specific activity, some modifications, as observed by iso-electric focusing, and final product specifications (e.g., Lomas et al., 1997; Cowden et al., 2005). Their human origin ensures their tolerability.

However, the plasma supply itself is a limited source and appears to be insufficient to meet anticipated clinical demand, whereas, production of recombinant protein therapeutics will enhance availability. Moreover, despite effective viral clearance in the manufacturing of plasma proteins (e.g., review by Cai et al., 2005), the risk of contamination with new and unknown pathogens may still exist. As a result, recombinant technology as an alternative approach for the production of α_1-PI has been under comprehensive investigation, as reviewed below.

Recombinant α_1-PI

According to the available literature, both from academic research and industry, the human gene for α_1-PI has been expressed in virtually all available hosts (*E. coli*, various yeasts, insect cells, CHO cells, and produced in transgenic plants and animals). Nevertheless, no recombinant α_1-PI (r-α_1-PI) is available as a licensed therapeutic.

The essential criteria for the development of therapeutics for human use are safety, optimal clinical efficacy, and maximum cost-effectiveness. Thus, before any recombinant protein can be used in humans, certain factors must be evaluated and appropriately addressed: (a) the ability of the host to provide post-translational processing, if it is essential; (b) feasibility of large-scale production, i.e., ensuring that all parameters are under control, and that the lots can be produced in a reproducible manner; (c) development time from gene to purified final product; (d) cost of production, including expenses associated with clinical trials and (d) compliance with regulatory requirements for the approval and marketing.

Regarding α_1-PI, the expression of α_1-PI as cDNA from a human liver library, was pioneered in 1983 in *E. coli* (Bollen et al., 1983). Almost simultaneously it was performed in yeast (Cabezon et al., 1984; Rosenberg et al., 1984), followed by further multiple experiments in the both hosts for various research purposes (e.g., Straus et al., 1985; Courtney et al., 1984, 1985; Travis et al., 1985; Sutiphong et al., 1987; Johansen et al., 1987; Casolaro et al., 1987). From low levels of expression and undetectable biological activity of the initial experiments (Bollen et al., 1983, 1984) the research progressed to production of active recombinant α_1-PI with relatively high yields.

Table 1 lists the examples of expression of human gene of α_1-PI in various hosts[4]. This table is not intended to be a complete listing of all available work, but an assessment of potential therapeutic suitability of various expression systems by comparing the published data. The aim of collecting these data was mainly to identify the hosts used, the strains (when many), and to evaluate the yields of r-α_1-PI's and biological activity. Although the expression systems for production of r-α_1-PI (Table 1) have been mainly used at laboratory scale and depending on the problem to be solved, the available data illustrate how these systems meet common requirements for a therapeutic protein and how the properties of recombinant protein compares with that of pd-α_1-PI.

E. coli

The human gene of α_1-PI has been expressed in this host by many research groups. *E. coli* offers superior genetic

[3] Prolastin (registered trade name) has been available starting 1987 from Bayer Corporation (acquired by Talecris Biotherapeutics by April 1, 2005, www.talecris.com); Aralast (registered trademark of Alpha Therapeutic Corporation since 2002 (www.alphather.com) is now manufactured under the direction of Baxter Healthcare Corporation Baxter (www.baxter.com); and Zemaira (registered trade name of Aventis Behring since 2003) is now produced by ZLB Behring (www.zlbbehring.com). The US FDA product approval information is available at www.fda.gov/cber/sba/alpha1p.

[4] The way the systems are presented here greatly depends on the availability of the data; the authors do not promote any particular expression system over another.

Table 1. Examples of expression of human α_1-PI in various hosts*

Host	Strain line	Protein location	Activity[a]	Yield, mg/l[b]	Reference
E.coli	K-12	intracellular	inactive	3.4 mg/l	Bollen et al. (1983, 1984)
	TGE 900	intracellular	active	n.a.[c]	Courtney et al. (1984)
	AR68, AR120	intracellular	active and inactive	20–30% of total protein	Johansen et al. (1987), Sutiphong et al. (1987)
	TGE 7213	intracellular	1	20 mg/l[d]	Bischoff et al. (1991)
	BL21(DE3)	intracellular	>0.9	~0.8 mg/g[e]	Hopkins et al. (1993)
	BL21(DE3)	intracellular inclusion body	1	40% of total protein[f]	Kwon et al. (1995)
	BL21(DE3)	intracellular inclusion body	>0.9	5 mg/l	Bottomley and Stone (1998)
	BL21(DE3) *Rosetta-gami*	intracellular	active[g]	20 mg/l 38 mg/l	Karnaukhova et al. (2004)
Yeasts	*S. cerevisiae*	intracellular	active[h]	1–1.2%[h]	Cabezon et al. (1984)
	S. cerevisiae[i]	intracellular	up to 1.0	up to 3.5%[i]	Rosenberg et al. (1984)
	S. cerevisiae	intracellular	up to 0.8	2 mg/g[j]	Travis et al. (1985)
	S. cerevisiae	intracellular	active[k]	n.a.	Casolaro et al. (1987)
	S. diastaticus	secreted	active	1.1 mg/l[l]	Kwon et al. (1995)
	S. cerevisiae	secreted and intracellular	active	17.9 mg/l[m]	Chung et al. (1998)
	S. cerevisiae *H. polymorpha* *P. pastoris*	secreted	active	75 mg/l[n]	Kang et al. (1996, 1998)
	S. cerevisiae	intracellular	active	1230 mg/l	Tamer and Chisti (2001)
Fungi	*A. niger*	secreted	active	~50 mg/l	Karnaukhova et al. (2005)
Insect cells	*Baculovirus*	secreted	active[o]	n.a.	Sandoval et al. (2002)
Mammalian cells	CHO	secreted	n.a.	44–100 mg/l	Paterson et al. (1994)

[a] Activity is shown as reported in the original papers; numbers are related to inhibitory activity as compared to plasma α_1-PI (1)
[b] If not in mg/l, the yields are shown in the units reported in the original papers
[c] n.a. Means ''not available'' (not done or not reported in the publication referred)
[d] As reported, 300 mg of purified inhibitor from fermentation volume 15 l (overall as indicated for α_1-PI mutants with Leu or Arg at position 357 and double mutants with Ala-357, Arg-358)
[e] As reported, ~0.8 mg of purified α_1-PI per gram of wet cell paste (overall for wild type α_1-PI and α_1-PI mutants)
[f] ~40% of total cellular protein, as evaluated by SDS-PAGE and western blot
[g] The yields are shown for soluble His-tagged r-α_1-PI's from fermentations at 30 °C, as evaluated by SDS-PAGE and western blot, and quantified by ELISA; the inhibitory activity (at least 35–40% of the standard) was declining in time due to a rapid polymerization (as monitored by size-exclusion HPLC)
[h] *S. cerevisiae* strains 1c1697d and 10S44c were used; the yield of mature r-α_1-PI was ~1–1.2% of total soluble protein; activity, as measured for crude yeast extracts, was evaluated as at least 10% of the standard activity for r- α_1-PI from 1c1697d, and at least 30% for 10S44c
[i] Up to 3.5% of soluble protein cell extract as evaluated by activity assay for wild type and Met358Val mutant
[j] Reported as 28 mg of protein from 14 g (wet) cell biomass
[k] After infusion into rhesus monkeys, a clearance from the blood was 50 times faster than that of human pd-α_1-PI
[l] ~70% of r-α_1-PI was secreted, 1.1 mg obtained from 1 l of culture media yielded 0.18 mg of purified protein
[m] As reported, the final titer of the secreted α_1-PI was 17.9 μg/ml (24.7% of secretion efficiency)
[n] 75 mg/l as reported for *S. cerevisiae*
[o] Chimera protein containing α_1-PI fused to C-terminus of a human insulin-like growth factor analog was obtained
* This table provides examples but is not comprehensive

flexibility over other expression systems (Baneyx, 1999; Swartz, 2001; Bird et al., 2004, reviews). Moreover, some protein therapeutics like human insulin (Eli Lilly) and bovine growth hormone (Monsanto) have been successfully produced in *E. coli*, even though both proteins require some post-translational modifications. However, *E. coli* is not suitable for more complex heterologous proteins where more sophisticated processing such as glycosylation and the correct protein folding is required. Like all other recombinant proteins from prokaryotes, r-α_1-PI produced in *E. coli* (44 kDa) is not glycosylated. Lack of glycosylation affects the folding of the protein; the misfolded recombinant protein aggregates more easily, thus resulting in loss of activity.

Although the initial activity of non-glycosylated r-α_1-PI from *E. coli* is not directly affected by the absence of

carbohydrates, the protein's reduced stability in vitro and altered pharmacokinetic (PK) properties, i.e., rapid clearance from the blood after intravenous infusion, render it inferior to the plasma derived inhibitor and impractical as a therapeutic. Therefore, although expression of human gene for α_1-PI in *E. coli* has been intensively explored and did result in active protein (e.g., Bischoff et al., 1991; Hopkins et al., 1993; Kwon et al., 1995; Bottomley and Stone, 1998; Cantin et al., 2002a), it has mainly been used for research purposes only. The prolonging of half-life of non-glycosylated r-α_1-PI by thiol-specific conjugation with polyethylene glycol (below) seemes to open a new chapter in the development of r-α_1-PI for therapeutic needs (Cantin et al., 2002b).

Yeast[5]

Yeast eukaryotic expression systems are widely utilized for production of heterologous proteins (Bretthauer and Castellino, 1999; Pemberton and Bird, 2004; Macauley-Patrick et al., 2005). Yeasts are genetically well-characterized, relatively easy to manipulate, and grow fast; production is scalable, and costs are relatively low. In addition, some protease-deficient strains are now available. Unlike most bacteria, yeasts do not produce endotoxins, which fact significantly simplifies the purification of proteins intended for therapeutic purposes. However, products produced in yeast may cause allergic reactions due to contaminants derived from the host.

Expression in yeast offers the choice of intracellular protein production or protein secretion. However, the targeting of a protein for secretion usually results in a significantly lower expression level than that of intracellular protein production (Pemberton and Bird, 2004).

As a lower eukaryote, yeast can provide some post-translational modifications of protein, including glycosylation. Yeasts share the early steps of the mammalian N-glycosylation pathway in the endoplasmic reticulum (ER). However, further steps of glycan biosynthesis in the Golgi apparatus differ. In human Golgi, the biosynthesis of complex oligosaccharides is served by specific glycan-processing enzymes, and glycan maturation is completed by terminal sialylation. By contrast, in yeasts the elongation of carbohydrates proceeds to structures of high mannose content referred to as ''hypermannosylation'' (Cregg et al., 2000; Fukuda, 2000). Therefore, heterologous expression of a human gene in yeasts results in secreted mature recombinant protein with incorrect glycosylation patterns, moreover, hypermannosylation causes a much higher degree of glycoprotein heterogeneity.

Yeasts, mainly *Saccaromyces cerevisiae*, the most established yeast system, have been extensively utilized for expression of human α_1-PI both at laboratory levels and a larger scale production (Cabezon et al., 1984; Casolaro et al., 1987; Kwon et al., 1995; Chung et al., 1998; Kang et al., 1996, 1998; Tamer and Chisti, 2001). The highest yield reported for production of α_1-PI in *Saccaromyces cerevisiae* (Table 1) was 1.23 g/l in fed-batch culture (Tamer and Chisti, 2001).

Absence of glycans, or aberrant glycosylation, represents a major problem for the recombinant proteins intended for intravenous therapy, since they are rapidly cleared from the blood, thus compromising efficacy. Another serious problem and potential safety issue is the immune response that can be induced by recombinant glycoproteins with non-human glycans.

The in vivo half-life of nonglycosylated r-α_1-PI from *S. cerevisiae* is known to be significantly shorter than that of the native plasma-derived protein (Casolaro et al., 1987). Pharmacokinetic studies in primates have demonstrated that human r-α_1-PI has significantly different PK characteristics than human pd-α_1-PI. After infusion in rhesus monkeys, r-α_1-PI disappeared from the blood rapidly and was almost undetectable by 24 hours, while pd-α_1-PI remained in the bloodstream for more than 4 days. Further, 38% of r-α_1-PI was excreted in the urine within 3 hours, while pd-α_1-PI was not detectable in the urine at all (Casolaro et al., 1987).

Filamentous fungi

The attractiveness of these systems is based on recent reports that filamentous fungi may efficiently express heterologous eukaryotic proteins, mainly due to providing glycosylation patterns more similar to those of mammals (Maras et al., 1999; Ward et al., 2004; Gerngross, 2004; Nevalainen et al., 2005). At present, only a very few human genes have been expressed in filamentous fungi, including human cytokine interleukin-6 and manganese peroxidase (reviewed by Punt et al., 2002) and immunoglobulin IgG1 antibodies (Ward et al., 2004).

Upon reviewing different expression systems that could be used for production of human r-α_1-PI, we realized that filamentous fungi probably represent the only system in which α_1-PI has not been expressed yet. It is worthwhile to mention that although filamentous fungi have been used for commercial production of enzymes, such as

[5] Yeasts and filamentous fungi are considered separately as expression systems of different levels of development.

glucoamylase, for years, their genome is not yet completely known, and the molecular biology techniques for expression of human genes are under development and optimization (Gouka et al., 1997; Withers et al., 1998; Conesa et al., 2001).

Although very little information is available on glycosylation of recombinant proteins secreted by filamentous fungi, this system seems to have overcome hypermannosylation problems known for yeast (Maras et al., 1999; Punt et al., 2004). N-linked glycosylation of recombinant proteins from filamentous fungi features a mammalian-type core, no non-human sugars added and absence of terminal sialic acid (Nevalainen et al., 2005). However, it has been pointed out that fungi are capable of synthesis of sialic acid as part of their cell wall surface, thus indicating a possibility of pathway engineering to approach human-type of glycosylation (Alviano et al., 1999; Wasylnka et al., 2001; Nevalainen et al., 2005).

Recently we have successfully expressed α_1-PI in filamentous fungi and obtained r-α_1-PI as a secreted (glycosylated) protein in biologically active form with a relatively high yield, up to 50 mg/l (Karnaukhova et al., 2005). However, more investigation is necessary in order to better utilize a potential, which this system possesses.

Insect cells

Insect cells are used mainly as the hosts for baculovirus expression vectors system (BES). They are often used for heterologous gene expression due to their capability to perform many post-translational modifications, such as N- and O-glycosylation, phosphorylation, acylation with fatty acids, disulfide bond formation, even a possibility of simultaneous expression of several subunits in the same system for assemblying of multimeric proteins (reviews by Luckow, 1993; Altmann et al., 1999; Beljelarskaya, 2002).

A common technique is the expression of the foreign gene(s) under the transcriptional control of the viral polyhedrin (or p10) gene promoter. The enhanced expression under this exceptionally strong promoter can result in very high yields, as much as ~25–50% of the total protein (e.g., Beljelarskaya, 2002). The BES was used to produce recombinant HIV envelope protein to be used in vaccine clinical trials (e.g., Goebel et al., 1999). However, this protein did not function normally unlike native gp160.

Strategies and methods to achieve production of serpins using BES have been well described (e.g., O'Reilly et al., 1994; Jayakumar et al., 2004). Regarding α_1-PI, to our knowledge, there is no data that would allow us to compare expression of human α_1-PI in this system with other recombinant versions of α_1-PI from the hosts considered above. But the baculovirus system was successfully utilized for production of chimeric α_1-PI (Sandoval et al., 2002). The authors expressed the human gene for α_1-PI as a fusion protein linked to the C-terminus of a human insulin-like growth factor analog, an element known to be properly folded and secreted in insect cells (Sandoval et al, 2002). A similar construct was obtained for more stable Met351Glu variant of α_1-PI. Both fusion chimera proteins, i.e., containing normal type of α_1-PI and the Met351Glu analog, were found to be active in inhibition of human NE, moreover, the Met351Glu variant has been demonstrated to have an improved anti-elastase activity.

Much remains unknown regarding glycosylation of the heterologous proteins obtained from insect cells. According to a recent review (Nevalainen et al., 2005), the heterologous proteins produced in the insect cells have a complex glycosylation with the absence of sialic acid and the adding of non-human sugars. However, available literature does not provide a clear answer about sialylation in this system (reviewed by Marchal et al., 2001). Although most research data testify to the inability of insect cells to perform terminal capping of glycoproteins with sialic acid, other results suggest that sialylation may occur (e.g., Hollister et al., 2002).

Transgenic plants

Plant cell cultures have been suggested as a suitable alternative to the expression systems currently used for the production of therapeutic proteins. Major advantages of transgenic plants over bacterial and yeast expression systems and the achievements in the control of post-translational modifications are considered in recent reviews (Hellwig et al., 2004; Gomond and Faye, 2004; Chen et al., 2005).

The expression of human α_1-PI performed in rice (Terashima et al., 1999, 2000; Huang et al., 2001) may serve as an illustration of the differences in glycosylation pattern between mammalian and plant systems. In work reported by Terashima and coworkers, the rice callus tissues were transformed with the expression vector p3D-ATT containing cDNA for human α_1-PI (Terashima et al., 1999, 2000). Regulated expression and secretion of α_1-PI was achieved using a promoter, signal peptide, and terminator from rice. The glycosylation pattern of the secreted α_1-PI differed from that of the plasma-derived protein. As with yeast, initial glycosylation steps in the rice ER are similar to the mammalian type, but further maturation steps in the Golgi apparatus diverge, in particular, due to the inability of the plant Golgi apparatus to add galactose

and terminal sialic acid (Chrispeels and Loic, 1996; Chen et al., 2005). As reported by Huang and coworkers, plant-derived human r-α_1-PI was as *active* in vitro as the protein derived from human plasma; however, it showed drastically different pharmacokinetic behavior (Huang et al., 2001). An in vivo study in rats demonstrated rapid clearance of plant-derived r-α_1-PI compared to human plasma-derived. The initial half-life of human pd-α_1-PI in rat blood was about 180 min; moreover, it showed a long second phase with a half-life $\sim$11.6 hour, while the half-life of the plant-derived r-α_1-PI in the rat blood was shorter than 20 min, and no second phase was observed (Huang et al., 2001).

The main concern regarding r-α_1-PI from those hosts, which are capable of glycosylation, is that non-human glycosylation patterns may induce an immune response when administered to humans; and only long-term clinical trials in humans can clear up this issue.

In view of the importance of human type N-glycosylation for r-α_1-PI and other recombinant glycoproteins intended for therapeutic use, it is reasonable to turn to mammalian cells and transgenic animals, the systems which are generally assumed to provide N-linked glycans closely resembling those of humans. Production of glycosylated physiologically ''normal'' human α_1-PI in mouse fibroblasts (Garver et al., 1987) and expression of human α_1-PI by lymphoid cell lines derived from transgenic mice (Pavirani et al., 1989), despite small amounts produced, provided a scientific basis for future applications.

Mammalian cells

Mammalian host cells, mainly Chinese hamster ovary (CHO) and baby hamster kidney cells (BHK-21) have been widely used in the past decade (e.g., reviews by Grabenhorst et al., 1999; Chu and Robinson, 2001). Many glycoproteins produced in mammalian cells have been approved by the US FDA for therapeutic use in humans, including interferon β-1a (Avonex), coagulation factor IX (Benefix) and antihemophilic factor (ReFacto) (Chu and Robinson, 2001).

In regards to human α_1-PI, since CHO cells do not synthesize α_1-PI mRNA, they have been extensively used in model studies of intracellular retention and degradation of α_1-PI Z-mutant (e.g., Ciccarelli et al., 1993; Novoradovskaya et al., 1998).

Capability of CHO cells to produce quantitative amounts and the approaches to maximize stable expression of human α_1-PI in transformed CHO cells were investigated by Paterson and coworkers. The authors described combination of modifications (using the human cytomegalovirus immediate early promoter/enhancer, the incorporation of 3'RNA processing signals from the simplex virus, etc.) that allowed secretion up to 44 μg of α_1-PI per ml per day by cell lines growing in serum-rich medium (Paterson et al., 1994). They showed that chemical induction by using propionate, butyrate, or hexamethylene bisacetamide increases the yield of the secreted α_1-PI up to 100 μg/ml per day; those cell lines that were adapted to growth in protein-free medium produced lower levels of α_1-PI (up to 14 μg/ml per day upon chemical induction).

For recombinant glycoproteins intended for therapeutic use, the mammalian cell culture systems meet most of the requirements. Yet, when choosing CHO cells as an expression system for human genes, relatively high costs, uncertainties related to mammalian cell lines (e.g., lower than desired reproducibility), and a potential risk of contamination by adventitious agents have to be taken into account. There are no data available that would allow us to evaluate the glycosylation pattern and PK of α_1-PI expressed in CHO cells. Although they are expected to be similar to human type glycosylation, as has been shown for other glycoproteins, addition of non-human sugars is observed (Nevalainen et al., 2005). Glycosylation type and patterns depend on the CHO cell lines used and may vary significantly (Fenouillet et al., 1996). In addition, as it was observed for tissue-type plasminogen activator produced in CHO cells, N-glycosylation is dependent on multiple cell culture factors showing different glycosylation patterns (Andersen et al., 2000). The glycosylation pathway in mammalian host cells, and future engineering perspectives towards therapeutic glycoproteins with novel/improved *in vivo* properties have been considered elsewhere (e.g., Grabenhorst et al., 1999).

Transgenic animals

Since the 1980s, genetic modification of animals to achieve production of human proteins has progressed greatly (Colman, 1996, 1999; Dalrymple and Garner, 1998; Lubon and Palmer, 2000). Various techniques for gene transfer have first been worked out in mice and then adapted to other species.

Human α_1-PI from transgenic animals (t-α_1-PI) illustrates the achievements and the problems of transforming of the proteins produced in transgenic animals into the therapeutics for human use, which is especially important since there are still no therapeutic products (licensed) on the market that are derived from transgenic animal sources.

Besides mice (Simons et al., 1987; Sifers et al., 1987; Carlson et al., 1988; Archibald et al., 1990), in which studies have been performed mainly as a proof of concept, α_1-PI has been expressed in rats (Tsymbalenko et al., 1995), rabbits (Massoud et al., 1991), sheep (Wright et al., 1991; Carver et al., 1992, 1993) and goats (Ziomek, 1998).

Large scale production of human α_1-PI has been performed in dairy animals: in sheep (by PPL Therapeutics, Scotland, UK in partnership with Bayer Biologicals, CT, USA (Wright et al., 1991; Dalrymple and Garner, 1998), and in goats (by Genzyme Transgenics Corporation, MA, USA (Ziomek, 1998)).

Human α_1-PI derived from transgenic sheep milk can be obtained with high degrees of purity (Harris et al., 1997). In Phase II clinical trials in cystic fibrosis patients, t-α_1-PI from sheep milk has been used in aerosolized form (nebulized doses from 100 to 500 mg) in 12 patients, and the study has been reported to be successful with no adverse effects (Tebbutt, 2000).

However, a recent publication by Spencer and coworkers reports that in t-α_1-PI (human) derived from milk of transgenic sheep, the trace amounts of native sheep α_1-PI and sheep α_1-antichymotrypsin, may induce a systemic antibody response (Spencer et al., 2005). Two sequential clinical studies were performed to evaluate the safety and immunogenicity of aerosolized transgenic human α_1-PI given in daily doses of 250 mg to 41 patients with α_1-PI deficiency for eight weeks. This transgenic α_1-PI (Wright et al., 1991) recovered from sheep milk was purified to 99.9% purity. Even so, sheep native α_1-PI and sheep α_1-antichymotrypsin were major impurities, at 6.7–18.7 mg/l and 60.3–75.8 parts per million respectively. None of the subjects had an antibody response to human t-α_1-PI; however, antibody responses were observed to sheep α_1-PI ($n = 10$) and to sheep α_1-antichymotrypsin ($n = 32$). Four patients withdrew from the second study due to the development of dyspnea and a decline in lung function. Although in view of the small number of subjects and the absence of control group treated with placebo, these results cannot be considered definitive, the immune responses observed in this study indicate that a major hurdle exists in the development of transgenic human proteins.

Glycosylation, stability and biological activity of r-α_1-PI

The general regulatory requirements for biologicals intended for therapeutic use, including r-α_1-PI, are purity, safety, and efficacy. In order to be effective, therapeutic proteins have to be stable *in vivo* and *in vitro*. As indicated by the data reviewed so far, stability has been a major issue.

The available data indicate that glycosylation is not a stringent requirement for α_1-PI protease inhibitory activity (Travis et al., 1985; Luisetti and Travis, 1996). However, since glycosylation is important for the protein to maintain its correct conformation, the absence of carbohydrates (or aberrant glycosylation) leads to protein misfolding and aggregation, with loss of inhibitory activity. Non-glycosylated r-α_1-PI is known to be less stable than plasma derived protein (e.g., Travis et al., 1985; Yu et al., 1988; Vemuri et al., 1993). Absence of glycans (or non-human types of glycosylation) is considered to be a cause of rapid clearance from the circulation (Casolaro et al., 1987; Cantin et al., 2002a). Thus, stability of recombinant glycoproteins is a serious issue in the entire process of protein isolation, purification, storage (shelf-life stability), administration, PK, and ultimately efficacy of the glycoprotein therapeutics.

To improve stability of recombinant proteins various approaches are considered.

Table 2. Examples of production of human α_1-PI in transgenic plants and animals*

	Species	Location	Activity	Yield	Reference
plants	rice	secreted	active	4.6–5.7 mg/g[a]	Terashima et al. (1999, 2000)
animals	mice	milk	active	0.5 to 7 mg/ml	Archibald et al. (1990)
	mice	urine	active	up to 65 mg/l	Zbikowska et al. (2002)
	rabbit	milk	active	4.0 g/l[b]	Massoud et al. (1991)
	sheep	milk	fully active	1 to 35 g/l[c]	Wright et al. (1991), Carver et al. (1993)
	goat	milk	active	20 g/l[b]	Ziomek (1998)

[a] Reported as 4.6–5.7 mg per g of dry cells
[b] As shown by Colman (1999) (referring Genzyme Transgenic Corporation literature)
[c] ~35 g/l as lactation progressed
* This table provides examples but is not comprehensive

Humanized systems

Stability of r-α_1-PI's varies significantly from non-glycosylated inhibitor obtained from *E. coli* to glycosylated versions derived from transgenic sources. An overall comparison of the available expression systems in regards to N-linked glycosylation supports observations that even those systems that do provide complex glycans (e.g., insect cells, plant cells) usually add non-human sugars and do not perform terminal capping with sialic acid (see review by Nevalainen et al., 2005). It is worthwhile to emphasize that carbohydrate analysis is tedious work due to intrinsic structural diversity of the oligosaccharide, heterogeneity typical for glycoproteins and the complexity of carbohydrate structure determination (see review by Marchal et al., 2001).

A few promising results reported recently support a challenging idea to engineer yeasts and fungal expression systems that may acquire ability to provide human-like glycosylation pathway (reviewed by Bretthauer, 2003; Gerngross, 2004; Nevalainen et al., 2005). Advances in engineering of new humanized expression systems via elimination of yeast genes engaged in yeast-specific glycosylation pathway and/or introduction of human genes involved in human glycosylation could solve these problems in the near future (Chiba et al., 1998; Gerngross, 2004; Wild and Gerngross, 2005). For instance, the use of combinatorial genetic libraries to alter the N-glycosylation pathway in *P. pastoris* to yield N-linked oligosaccharides with hybrid structures that are the same as the intermediates of mammalian-protein N-glycosylation has been described by Choi et al. (2003). Although the manipulations with yeast genome could be detrimental for the viability of the yeast cells, some results look rather positive, indicating that yeast N-glycosylation pathways may be extensively re-engineered (Choi et al., 2003; Vervecken et al., 2004; Bobrowicz et al., 2004). Yet, the lack of the terminal sialic acid may remain problematic causing rapid clearance of protein from the bloodstream. An alternative approach can be *in vitro* partial (sialylation) or full glycosylation (Macmillan and Bertozzi, 2000; Ryckaert et al., 2005).

Amino acid mutations

The question whether recombinant glycoprotein with lack of glycans or aberrant glycosylation may be stable (*in vitro*/*in vivo*) and biologically active could be approached by improvement of the stability of r-α_1-PI via amino acid substitution(s) that would enhance stability without affecting r-α_1-PI biological activity.

Since decreasing of α_1-PI's inhibitory activity is mainly caused by oxidation of methionine 358, a majority of earlier studies focused on improving the activity of α_1-PI via construction of oxidation-resistant mutants (reviewed by Luisetti and Travis, 1996).

Those mutations, which may prevent polymerization of α_1-PI, represent another direction (e.g., Sidhar et al., 1995). A great deal of effort has been targeted at altering the structure of r-α_1-PI by various mutations to evaluate the impact of such structural changes on stability and biological activity of the protein. Part of the challenge in this research relates to the evidence that the metastable structure is important for inhibitory serpins to execute their function (e.g., Cho et al., 2005). The residues that contribute to stability may not be optimal for function and *vice versa*. For example, Im and coworkers investigated some stabilizing mutations of α_1-PI in a region presumably involved in complex formation with a protease (Im et al., 1999). The authors found various unfavorable interactions and observed a concomitant decrease in the inhibitory activity for several stabilizing mutations. The relationships between serpin conformation, stability and biological activity are considered in a few recent reviewes (e.g., Cabrita and Bottomley, 2004) and many research publications (e.g., Im et al., 2002, 2004; Lee et al., 2000). Noteworthy, Lee and coworkers reported an α_1-PI mutant as stable as ovalbumin. This was achieved by a combination of seven stabilizing single amino acid substitutions (Lee et al., 1996). That mutant (multi-7) has been shown to form a stable complex with a target elastase with the same kinetic parameters and stoichiometry of inhibition as the wild type α_1-PI.

The data on α_1-PI mutants from early research (Rosenberg et al., 1984) to further systematic studies (e.g., Kwon et al., 1994; Kim 1995; Elliott et al., 1996; Ryu et al., 1996; Im et al., 2004) will be considered separately.

Conjugation with polyethylene glycol

Conjugation of recombinant proteins with polyethylene glycol (PEG) is an efficient and relatively simple procedure used to increase half-life of therapeutically relevant proteins (Monfardini and Veronese, 1998; Chapman et al., 1999). In addition to improving of protein half-life, PEGylation has been shown to decrease the immunogenecity of therapeutic proteins (e.g., Harris et al., 2001).

In regards to r-α_1-PI, the preparation and properties of PEGylated r-α_1-PI's have been reported by two groups. In 1990, Mast and coworkers demonstrated that PEG-r-α_1-PI conjugates show longer retention times in plasma,

and therefore, have an improved therapeutic potential, as compared with non-derivatized r-α_1-PI (Mast et al., 1990a, b). Unfortunately, the conjugation of r-α_1-PI with PEG activated with 1,1'-carbonyl diimidazole required presence of dioxane and long incubation ($\sim$120 h), during which time a significant inactivation of the protein occurred.

Later on, Chapman and coworkers developed a procedure that allows a site-specific covalent linkage of a polyethylene glycol activated with maleimide to the thiol group of cysteine residue (Chapman et al., 1999). The α_1-PI molecule is a good candidate for site-specific conjugation with PEG since it has a single Cys232 with a free thiol group exposed at the surface (Elliott et al., 2000). Recently, Cantin and coworkers described preparation of PEGylated non-glycosylated r-α_1-PI (Cantin et al., 2002b). The reaction took only 2 h and resulted in a fully active protein with a rate of association with NE similar to that of pd-α_1-PI. The authors demonstrated that conjugation with PEG (20 kDa or 40 kDa) markedly improves the *in-vivo* half-life of non-glycosylated r-α_1-PI both in plasma and in lung. Although additional work is needed, the prolonging of the half-life of r-α_1-PI's is beneficial for intravenous administration and for airway delivery. This could also be applied to pd-α_1-PI.

Alternative routes of administration

In view of the inconvenience of life-time intravenous augmentation therapy and low levels of α_1-PI reaching lungs, the 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 needed (Hubbard et al., 1989; McElvaney et al., 1991; Cockett, 1999; Morrow, 2004). Although strategies for aerosol therapy of α_1-PI deficiency started development more than a decade ago (e.g., Hubbard et al., 1989; Hubbard and Crystal, 1990), there is still no α_1-PI aerosolized treatment available for α_1-PI deficient patients. Several studies examined efficiency of the α_1-PI inhalation therapy in animals (e.g., Smith et al., 1989) and in humans (e.g., Vogelmeier et al., 1997; Kropp et al., 2001). For example, by using ^{123}I-labeled α_1-PI, Kropp and coworkers determined that significantly more α_1-PI was deposited in the lungs through inhabation rather than intravenous infusion (14.6% vs. 2%) (Kropp et al., 2001).

With regards to recombinant versions of α_1-PI, it has been generally assumed that product directly delivered to the lungs may not require the same degree of stability as α_1-PI given intravenously. However, as mentioned above, human studies using recombinant α_1-PI from transgenic sheep, were associated with adverse reactions due to impurities derived from the host (Spenser et al., 2005). Thus, higher levels of purification and more clinical studies are required.

Immunogenicity issues

Unfortunately, very little is known about the immunogenicity of r-α_1-PI's. Immunogenicity issues related to recombinant glycoproteins have to be a primary concern regarding recombinant (human) glycoproteins themselves, as well as contaminating impurities. Features that may cause r-α_1-PI to be immunogenic are altered glycans, aggregation, oxidation of $-$SH and/or methionine residues. Immunogenicity of r-α_1-PI should be studied in a comprehensive manner (see reviews Chamberlain, 2002a, 2000b; Chamberlain and Mire-Sluis, 2003).

In summary, reviewing the work performed over the last two decades to produce stable and biologically active recombinant/transgenic α_1-PI in various hosts, one can see basically two major factors impeding the progress in the area: (1) impurities that may induce antibody responses and cause adverse reactions in patients, and (2) lower stability than that of plasma-derived, mainly caused by the lack of glycosylation or non-human type of glycosylation; the latter also may induce immune responses.

Although the first reason seems to be purely technical, removal of trace amounts of non-human native proteins derived from the host, e.g., sheep α_1-PI, from the human r-α_1-PI to exclude further adverse reactions, requires a much higher level of purification and thus, may not be economically feasible.

As for the second reason, the impact of glycans adopted from the host glycosylation pathway on recombinant protein folding, stability and therefore, biological activity poses a great scientific challenge by itself apparently requires more research, and hopefully will be successfully addressed through a variety of approaches.

It is worthwhile to mention that, apart from scientific, there are some other issues that are specific for rare diseases. First of all, in case of rare diseases, such as α_1-PI deficiency, large clinical trials are difficult to perform since α_1-PI deficient patients represent a small geographically dispersed population. Second, a limited population means a limited market, which is less attractive for large investments. No doubt, these reasons markedly slow down the development of r-α_1-PI.

Conclusions

For the development of recombinant/transgenic protein therapeutics, there is a significant time gap between research achievements and final gaining approval for use in humans. The hurdles in the way of production of recombinant protein therapeutics in general are illustrated by the prolonged efforts to develop viable recombinant versions of α_1-PI, difficulties of designing and performing of appropriate clinical trials, and certain economic factors that influence processing of r-α_1-PI from the bench to the biopharmaceutical market. Although, with three plasma-derived α_1-PI products licensed in the US, there is no current shortage for therapeutic needs, the anticipated future demand will likely not be satisfied by α_1-PI from plasma sources only. Development of viable recombinant α_1-PI product(s) promises to enhance supply of protein and will have the added advantage of being free from emerging infectious agents that may contaminate plasma. Nevertheless, a review of more than 20 years of r-α_1-PI development reveals critical scientific issues that are unresolved and are important for development of other recombinant glycoproteins.

Recent advances in re-engineering of protein-expression hosts to manipulate N-glycosylation pathways indicate that the restrictions caused by non-human glycosylation can be overcome in the future, bringing a variety of mammalian glycoproteins to therapeutic use in humans. In addition, stabilizing recombinant glycoproteins via strategically pointed mutations may lead to a significant improvement of recombinant protein stability. Thiol-specific conjugation with polyethylene glycol shows that it is possible to markedly improve the *in vivo* stability of r-α_1-PI.

These approaches challenge our current knowledge regarding α_1-PI and other therapeutically relevant glycoproteins and prompt further research towards better understanding of protein structure-function relationships.

Acknowledgements

EK is truly indebted to Dr. Mahmood Farshid and Douglas Frazier for their support and help with the manuscript. We thank Drs. Andrew Shrake, Abdu Alayash, and Roman Drews for their helpful remarks and valuable discussion.

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Authors' address: Dr. Elena Karnaukhova, Ph.D., Division of Hematology, Center for Biologics Evaluation and Research, Food and Drug Administration, 8800 Rockville Pike, National Institutes of Health Building 29, Bethesda, MD 20892, U.S.A.,
Fax: +1-301-402-2780, E-mail: elena.karnaukhova@fda.hhs.gov