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Expression, purification and characterization of recombinant Z α_1-Antitrypsin—The most common cause of α_1-Antitrypsin deficiency

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ABSTRACT

α_1-Antitrypsin (α_1AT), the most abundant proteinase inhibitor circulating in the blood, protects extracellular matrix proteins of the lung against proteolytic destruction by neutrophil elastase. α_1AT deficiency predisposes patients to emphysema, juvenile cirrhosis and hepatocellular carcinoma. Over 90% of clinical cases of severe α_1AT deficiency are caused by the Z variant (E342K) of α_1AT. The presence of the Z mutation results in misfolding and polymerization of α_1AT. Due to its inherent propensity to polymerize there are no reported cases of recombinant Z α_1AT production. This has created a major impediment to studying the effect of the Z mutation on α_1AT. Here we report our attempts to produce recombinant Z α_1AT using both *Escherichia coli* and *Pichia pastoris* as host systems. Using a range of expression vectors in *E. coli* we were unable to produce soluble active Z α_1AT. Cytosolic expression of the Z α_1AT gene in *P. pastoris* was successful. Monomeric and active recombinant Z α_1AT was purified from the yeast cytosol using affinity chromatography and anion exchange chromatography. Biochemical analyses demonstrated that the recombinant Z α_1AT has identical properties to its native counterpart purified from plasma of patients homozygous for the Z allele. A recombinant source of pathological Z α_1AT will increase the chances of elucidating the mechanism of its polymerization and thus the development of therapeutic strategies.

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Introduction

Recombinant protein production is a major challenge for many fields of study including biotechnology and biomedicine. Without the supply of correctly folded and active protein structural, biochemical and drug design projects are difficult to complete. Without doubt, one of the most challenging groups of targets for recombinant protein production are proteins involved in the conformational diseases [1,2]. The conformational diseases are a large class of progressive diseases such as Alzheimer disease, Huntington disease, and the serpinopathies in which proteins misfold and aggregate. Understanding how these proteins function, fold and misfold is critical for the development of therapeutic strategies.

The serpinopathies comprise diseases such as emphysema, liver cirrhosis and dementia, caused by the misfolding of serine proteinase inhibitors (serpins) [3,4]. α_1-Antitrypsin (α_1AT)[1] is the most abundant circulating proteinase inhibitor and the archetypal member of the serpin superfamily. It is expressed in hepatocytes and released into circulation where its primary function is to protect the lower respiratory tract from proteolytic destruction by elastase secreted by human neutrophils at sites of inflammation [5–8]. Most individuals have two M α_1AT alleles but approximately 1 in 2000 is homozygous for the Z allele [9,10]. The presence of the Z mutation results in the accumulation and retention of the polymerized α_1AT within the endoplasmic reticulum of hepatocytes [11,12]. The deposition of polymerized Z α_1AT within hepatocytes predisposes homozygotes to neonatal hepatitis, juvenile cirrhosis and hepatocellular carcinoma. In addition, the drastically decreased plasma levels of active α_1AT leads to a higher risk of developing early onset pulmonary emphysema [7].

There has always been a high demand for the recombinant form of α_1AT which would enable extensive studies of its mechanisms of proteinase inhibition, folding, misfolding and aggregation [13]. The first recombinant α_1AT (M allele) was purified from yeast in 1985 [14]. Since then, a number of expression systems have been utilized to successfully produce M α_1AT. Using the expression host *Escherichia coli*, a non-glycosylated, N-terminally truncated version of M α_1AT has been produced in inclusion bodies and successfully refolded [15,16]. Recently, soluble expression of full-length M α_1AT has been achieved using a different *E. coli* expression vector [17]. Glycosylated M α_1AT has also been produced from yeast (bakers and methylotrophic) [18] and plant systems [19–21]. However, even though many expression systems are now available for recombinant

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[1] *Abbreviations used:* α_1AT, α_1-Antitrypsin; DTT, 1,4-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GST, glutathione-S-transferase; HNE, human neutrophil elastase; HRP, horseradish peroxidase; MBP, maltose binding protein; SI, stoichiometry of inhibition; TEV, tobacco etch virus; YPD, yeast peptone dextrose.

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M α_1AT production, there are to our knowledge no published reports for the expression and purification of recombinant Z α_1AT.

In this current study we detail our ultimately successful attempts to produce recombinant Z α_1AT. Initially, we used *E. coli* as the expression host, however, despite extensive screening of expression space we were unable to successfully produce monomeric protein. We next attempted expression in the cytosol of the methylotrophic yeast *Pichia pastoris* and were able to purify monomeric and active Z α_1AT. Biochemical characterization of our recombinant Z α_1AT confirmed it possesses the same properties as Z α_1AT purified from patients [22–24]. The availability of recombinant Z α_1AT will be of major benefit for α_1AT deficiency research as it will allow large-scale production and thus the design and screening of therapeutic agents to commence, which otherwise would have been difficult with the limited supply of blood plasma from Z α_1AT patients.

Materials and methods

Materials

All media components were purchased from Merck and Sigma. Enzymes for DNA manipulations were from Promega. Oligonucleotides synthesized by Geneworks (Australia). Complete EDTA-free tablets from Roche were used for protease inhibition during purification. Bovine chymotrypsin and HNE were purchased from Merck and Sigma.

Cloning and mutagenesis of M and Z α_1AT

The M α_1AT gene was cloned into the pLIC-His and pLIC-MBP expression vectors using Ligation Independent Cloning as previously described [25]. The Z mutation was introduced into each expression vector using the Quickchange mutagenesis kit (Stratagene). All constructs were verified by sequencing prior to expression trials. The constructs, vectors and N-terminal fusion tags are listed in Table 1.

Small-scale expression screening of M and Z α_1AT

The expression plasmids were transformed into *E. coli* BL21 (DE3) and plated on 2xTY plates containing 0.1 mg/mL ampicillin. Single colonies were used to inoculate 1 mL cultures of Overnight Express Media (Novagen) with ampicillin (0.1 mg/mL) and incubated shaking (250 rpm) at 30 °C. After 18 h the cells were harvested by centrifugation (10,000*g* for 5 min at 4 °C). Solubility of the α_1AT fusion protein products was assessed using SDS–PAGE on samples from the supernatant and the whole cell lysate (Fig. 1A and B).

Large-scale expression and purification of M and Z MBP-α_1AT fusion proteins

Five-hundred milliliters of Overnight Express Media (Novagen) with ampicillin (0.1 mg/mL) was inoculated with a fresh colony of *E. coli* BL21 (DE3) cells carrying the pLIC-MBP-α_1AT plasmid and incubated shaking (250 rpm) at 30 °C. After 18 h the cells were harvested by centrifugation (10,000*g* for 5 min at 4 °C). For purification of M and Z MBP-α_1AT the cell paste (4 g of wet weight cells) was re-suspended in 30 mL lysis buffer (25 mM NaH_2PO_4, 0.5 M NaCl, 2 mM β-mercaptoethanol, 25 mM imidazole, pH 8.0). The cell suspension was lysed using sonication in the presence of 1 mg/mL lysozyme. The cell debris was removed by centrifugation (48,000*g* for 20 min at 4 °C). The supernatant was then filtered through 0.45 μm filter before loading onto a 1-mL HisTrap HP column (GE Healthcare) equilibrated with lysis buffer. The column was washed with lysis buffer containing 0.5% (v/v) Triton X-100 followed by lysis buffer with 150 mM NaCl. Subsequently, the protein was eluted with 25 mM NaH_2PO_4, 0.15 M NaCl, 2 mM β-mercaptoethanol, 300 mM imidazole, pH 8.0. Fractions containing either M or Z MBP-α_1AT as determined by SDS–PAGE were pooled.

The MBP-α_1AT fusion proteins (at about 2 mg/mL) were subjected to tobacco etch virus (TEV) protease cleavage to separate α_1AT from its fusion solubility tag (MBP). The cleavage was performed overnight at 4 °C in elution buffer containing 0.5 mM EDTA and 1 mM DTT and a final TEV concentration of 0.12 mg/mL [26]. Following incubation the protein sample was diluted with 25 mM NaH_2PO_4, 0.15 M NaCl, pH 8.0, to decrease the imidazole concentration to 25 mM and the solution was loaded onto a 5-mL HiTrap HP column (GE Healthcare) pre-equilibrated with five column volumes of 25 mM NaH_2PO_4, 0.5 M NaCl, 2 mM β-mercaptoethanol, 25 mM imidazole, pH 8.0, for "subtraction" his-tag purification. In this step, the uncleaved MBP-α_1AT, the cleaved MBP and the TEV protease (all three proteins are his-tagged) bind to

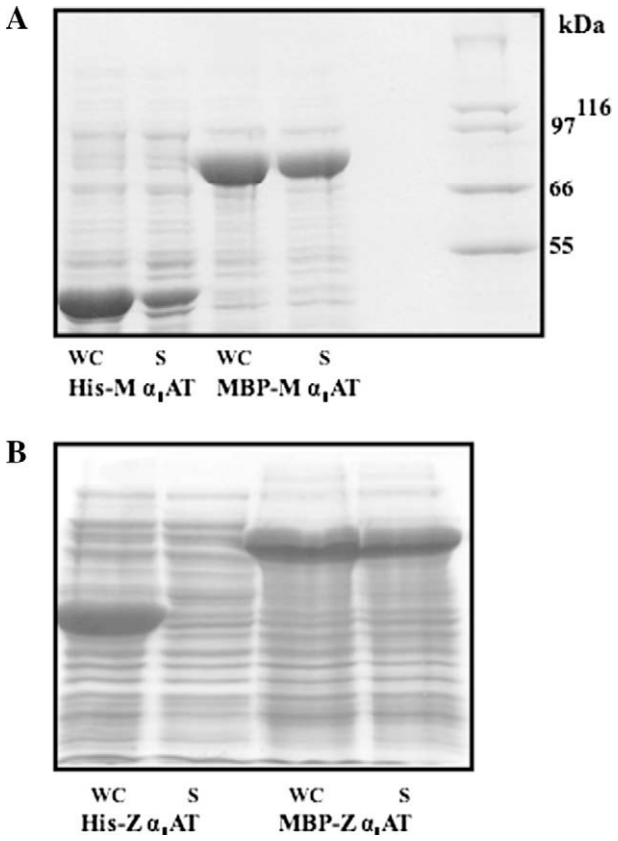

Fig. 1. SDS–PAGE analysis of M and Z α_1AT expression in *E. coli*. M (A) and Z (B) α_1AT and its fusion partner derivatives were expressed as described in Materials and methods. Proteins were analyzed using a 12% polyacrylamide gel and stained with Coomassie blue. Both whole cells (WC) and supernatants (S), following lysis and centrifugation, are shown.

Table 1
Escherichia coli expression constructs used to identify soluble expression of M and Z α_1AT.

	N-terminal tag	Expression	Purification	Solubility following TEV cleavage
M α_1AT	6xHis	S	Excellent	Excellent
	6xHis-MBP	S	Excellent	Excellent
Z α_1AT	6xHis	I	n/a	n/a
	6xHis-MBP	WS	Poor	No

I, insoluble; S, soluble; WS, weakly soluble.

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