High-level production and isolation of human recombinant α_1-proteinase inhibitor in yeast

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The cDNA coding for mature human α_1-proteinase inhibitor (α_1-PI) has been inserted into a variety of yeast expression vectors. Yeast cells transformed with these plasmids were then assayed for the production of mature, unglycosylated α_1-PI. The production level is optimal when the recombinant plasmid carries the TDH promoter, the complete 2μ and the leu2D selection marker. Biologically active recombinant α_1-PI can be purified either analytically, by affinity chromatography using a monoclonal antibody, or on a large scale, by a procedure involving precipitation of high-M_r yeast material with polyethylene glycol 3300 followed by successive chromatography on DEAE-agarose, Zn-chelate agarose, κ-chain agarose, heparin-agarose and aminohexyl-agarose.

α_1-Proteinase *Enzyme inhibitor* *α_1-Antitrypsin isolation* *Elastase inhibitor substitution* *Emphysema* *(Yeast)* *Expression vector* *Affinity chromatography*

1. INTRODUCTION

Severe α_1-PI hereditary deficiency is associated with reduced neutrophil elastase inhibition and hence with lung tissue degradation which results in the development of emphysema [1,2]. Replacement therapy with α_1-PI to restore plasma anti-elastase activity requires large amounts of the protein [3]. In this context, human α_1-PI has been cloned and expressed in *E. coli* [4,5] and yeast [6,7]. The recombinant α_1-PI has been purified to homogeneity and, although non-glycosylated, it exhibits similar physico-chemical and biological properties to the human plasma derivative [8–10].

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Abbreviations: α_1-PI, α_1-proteinase inhibitor, α_1-antitrypsin; r-α_1-PI, recombinant α_1-PI; PEG, polyethylene glycol; TDH3, glyceraldehyde-3-phosphate dehydrogenase; TNB, 4-nitrophenylsulfide 3,3′-dicarbonic acid

In order to find therapeutic application, large quantities of active and highly pure α_1-PI will have to be provided regularly [11]. In a continuing effort to optimize the production of r-α_1-PI in yeast, we report here the construction of a recombinant plasmid, pRIT12655, which directs the expression of the protein to high levels. In addition, we describe a protocol for the large-scale purification of r-α_1-PI from yeast cells carrying the new plasmid.

2. MATERIALS AND METHODS

2.1. *Products and procedures*

Restriction endonucleases and other DNA-modifying enzymes were used as recommended by the manufacturer (Boehringer-Mannheim). General cloning techniques were performed as described [12].

Plasmids used in this report, pBR327, pRIT10787 and YIp5, have been described

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[6,13,14]. The genotype of yeast strains 10S44C and TCY1 is shown in table 1.

Transformation of yeast strains, conditions for yeast cultivation, and preparation of extracts have been described [6,11].

Reagents and buffers were as in [10,11], Sepharose products were purchased from Pharmacia (Sweden), and $\varkappa$-chain agarose was synthesized as described [15]. Monoclonal antibody against α_1-PI, AATY6, has been described previously [16].

2.2. *Isolation of r-α_1-PI*

Small volumes (5 ml) of crude yeast extracts [6] containing 1 mM PMSF, 20 mM β-mercaptoethanol and 5 mM EDTA in 50 mM Tris-HCl buffer, pH 8.0, were centrifuged for 20 min at 16000 $\times$ g to remove membrane fragments. This supernatant was supplemented with Triton WR1339, final concentration 0.1%, and applied to a column of immobilized monoclonal antibody raised against α_1-PI (5 mg AATY6 coupled to 5 ml Sepharose 4B). The column was washed with 10 mM sodium phosphate buffer, pH 7.5, containing 150 mM NaCl and 0.1% Triton WR1339, and α_1-PI was eluted stepwise using the same buffer to which 1 M NaSCN had been added.

Large volumes, up to 2.5 l, of crude yeast extracts adjusted to pH 6.5 with solid Tris powder or 10% acetic acid were incubated at 4°C with a final concentration of 6.5% PEG 3300 for 2 h and then centrifuged at 16000 $\times$ g for 1 h. The resulting clear extract was loaded onto a 2 l column of DEAE-Sepharose FF equilibrated in 20 mM sodium phosphate buffer, pH 6.5. After washing, α_1-PI was eluted using 150 mM NaCl in the same buffer. The eluate was adjusted to pH 7.5 and loaded onto a 1 l Zn-chelate column equilibrated in 25 mM sodium phosphate buffer, pH 7.5, containing 200 mM NaCl. The α_1-PI fraction was recovered after elution with 50 mM phosphate buffer, pH 5.5, 200 mM NaCl, 10% ethylene glycol. EDTA was added to 20 mM, and the pH was raised to 8.5 by addition of solid Tris powder. This pool was then applied to a 0.5 l $\varkappa$-chain agarose column, and α_1-PI was eluted with an excess of TNB^- in the starting buffer [11,15]. Pooled fractions were dialysed against 20 mM phosphate buffer, pH 7.5, and loaded onto a heparin-agarose column, serially connected to a final AH-Sepharose column equilibrated in 20 mM phosphate buffer, pH 7.5. The AH-Sepharose was then washed with 50 mM imidazole buffer, pH 5.5, and α_1-PI was eluted by a linear NaCl gradient (25–300 mM) in this buffer.

3. RESULTS AND DISCUSSION

3.1. *Construction and description of recombinant plasmids for expression of human α_1-PI in yeasts*

Expression cassettes for synthesis of mature human α_1-PI contain the mature structural gene sequence fused via a *Bam*HI site overlapping the second codon to *Bam*HI sites created by in vitro manipulation at the ATG initiation codons of ARG3 or TDH3 promoter fragments [6]. The α_1-PI coding sequence is fused 3′ to a 1150 bp fragment carrying the transcription termination signal for the yeast ARG3 gene. Cassettes can be excised as 3870 bp (ARG3 promoter) or 3450 bp (TDH3 promoter) *Hin*dIII fragments for insertion into yeast shuttle vectors. Three types of vectors were used in this study (table 1). Plasmid pRIT12348 was obtained from pRIT10787 [6] by replacement of the ARG3 with a TDH3 promoter fragment. The vector pRIT12645 comprises the complete sequence of yeast 2μ DNA inserted at the *Eco*RI site of pBR327, a 2200 bp yeast LEU2 gene fragment as selector inserted at the *Sal*I site of pBR327, and the ARG3-α_1-PI expression cassette inserted by blunt end cloning at the *Bam*HI site of the plasmid. The parent vector is described elsewhere [17]. Expression vectors pRIT12653 and pRIT12655 carry the ARG3-α_1-PI and TDH3-α_1-PI expression cassettes, respectively, inserted by blunt end cloning at the *Bam*HI site of recipient vector pRIT12495. This vector plasmid consists of YIp5 (URA3 yeast gene inserted on pBR322) together with the full 2μ DNA sequence and the leu2D gene from JDB219 [18]. The feature of this vector is the double selection available in appropriate yeast strains enabling variation in copy number from moderate (URA3 selection) to high (leu2D selection) depending on the selective medium. The structure of the most efficient vector (pRIT12655) for the expression of human α_1-PI cDNA is shown in fig.1. The plasmids were introduced into recipient strains 10S44C or TCY1 (table 1) and assayed for expression.

Table 1

α_1-PI expression vectors and strains

Transformed yeast strain	Recipient strain	Vector	α_1-PI expression cassette		2μ sequences	Selection
			Promoter	Terminator		
Y157	10S44C	pRIT 12348	TDH3	ARG3	partial	LEU2
Y398	TCY1	pRIT 12645	ARG3	ARG3	complete	LEU2
Y521	TCY1	pRIT 12653	ARG3	ARG3	complete	leu2D,URA3
Y497	TCY1	pRIT 12655	TDH3	ARG3	complete	leu2D,URA3

Strain 10S44C has the leu2-3, leu2-112, pep4-3 genotype. Strain TCY1 is a cir° derivative of 10S44C

3.2. *Expression levels of α_1-PI in yeast*

Yeast strains, Y398 and Y157 (table 1), were grown to an A_{650} of 0.5 and total protein composition was analyzed on SDS-polyacrylamide gels. As seen in fig.2A, a minor band with respect to the total protein assortment appears at M_r 43000 corresponding to α_1-PI, as confirmed by immunoblotting (not shown), this band being more prominent in strain Y157 than in Y398. On the other hand, a similar analysis on proteins derived from strains Y521 and Y497 shows that α_1-PI is one of the major protein constituents, the optimal expression level again being obtained when the vector carries the pTDH3-α_1-PI-tARG3 expression cassette (table 1, fig.2B). In leucine-deprived medium, transformed strains carrying the leu2D selection marker grow more slowly (generation time, 6 h) than those having the normal LEU2 selector

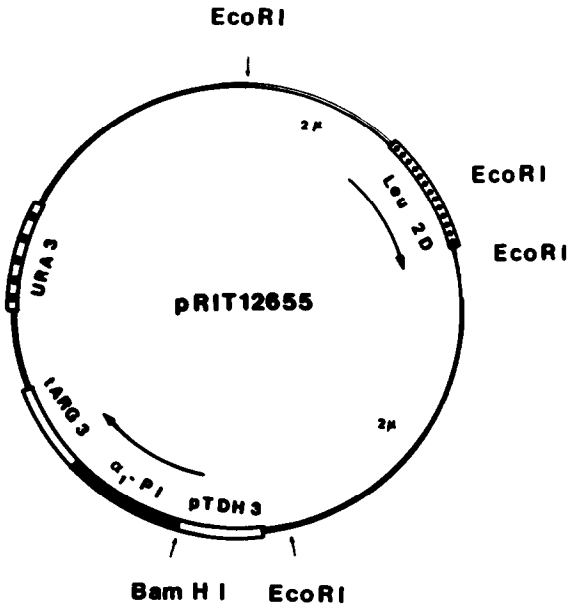

Fig.1. Schematic representation of yeast plasmid pRIT12655 (see section 3).

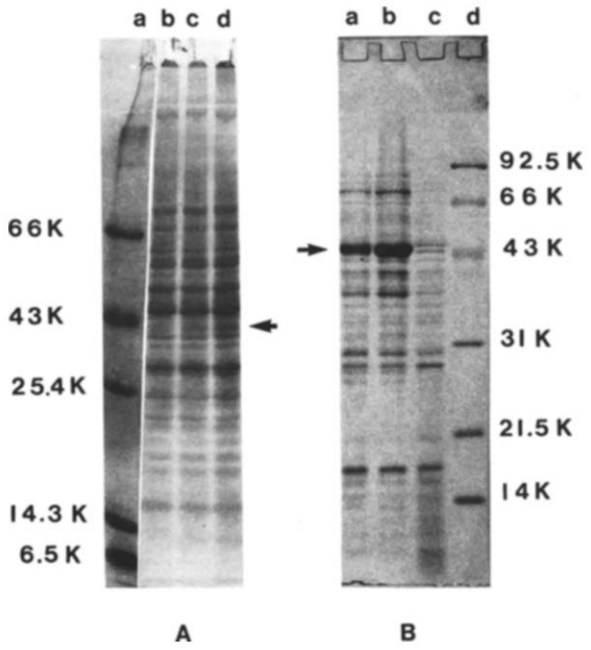

Fig.2. SDS-polyacrylamide gel electrophoresis of total yeast proteins. Ac,d, strain Y398 (pARG3) and strain Y157 (pTDH3); Ba,b, strain Y521 (pARG3) and strain Y497 (pTDH3); Ab,Bc, control strains carrying recipient vectors pRIT12377 [17] and pRIT12495, respectively; Aa,Bd, molecular mass markers.

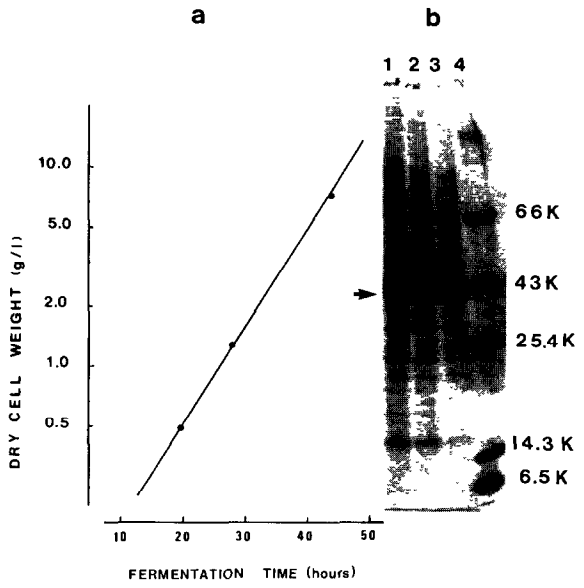

Fig.3. (a) Weight of yeast cells recovered during large-scale fermentation of yeast strain Y497. (b) Protein composition of the final extract as revealed by SDS-polyacrylamide gel electrophoresis. Lane 1, 2 and 3, 150, 100 and 50 μg proteins respectively; lane 4, molecular mass markers.

(generation time, 3 h), a result observed for small- and large-scale fermentations (fig.3a). Despite its slow growth, strain Y497 produces unaltered α_1-PI as the major protein (5–6% of soluble proteins) even during fermentation for 2 days up to 35 g wet yeast per l medium (fig.3b).

3.3. *Purification of r-α_1-PI derived from yeast strain Y497*

It is relatively easy to isolate r-α_1-PI with a purity of at least 80% using a monoclonal antibody immobilized on Sepharose 4B. In this respect the antibody AATY6 is particularly suited for the analytical purification of α_1-PI, due to its moderate affinity for the antigen ($K_d = 5 \times 10^{-9}$ M). Recovery of active α_1-PI from the affinity column can therefore be achieved under rather mild conditions (1 M NaSCN). However, extending this procedure to large-scale purification of α_1-PI would require prohibitive amounts of monoclonal antibodies. We therefore chose to develop an alternative isolation scheme, suited for treatment of kilogram quantities of yeast involving (i) DEAE chromatography (fig.4b,c) which yields a clear protein fraction slightly enriched in α_1-PI

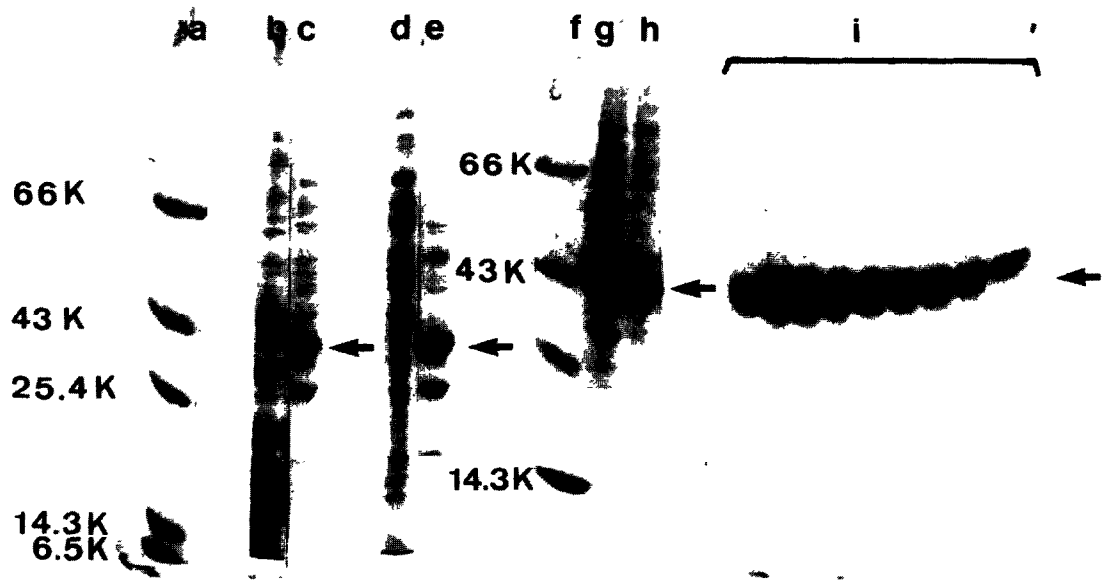

Fig.4. Large-scale isolation of α_1-PI from yeast strain Y497. DEAE-chromatography: flow-through (b) and stepwise elution (c); Zn-chelate chromatography: flow-through (d) and stepwise elution (e); $\varkappa$-chain chromatography: flow-through (g) and stepwise elution (h); complete NaCl gradient elution from AH-Sepharose (i); molecular mass markers (a,f).

(10–11%) but essentially free from all added reagents; (ii) a Zn-chelate column from which α_1-PI elutes about 25% pure (fig.4d,e); (iii) κ-chain-agarose chromatography which increases α_1-PI purity up to 70% (fig.4g,h); and (iv) linked heparin- and AH-Sepharose columns which yield a highly purified inhibitor (fig.4i). Omission of the preliminary clarification step (PEG precipitation) drastically reduced the resolution and yield in the subsequent DEAE chromatography.

The combination of four successive affinity chromatographies, following the clarification step, is required to obtain a highly purified product (>95%).

Recombinant α_1-PI, purified as described, was recovered with an overall yield of 40% and displayed an inhibitory activity (not shown) equivalent to that of its plasma counterpart in the inactivation of bovine trypsin and porcine elastase, measurements which have been shown previously [11].

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