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High-level secretion of biologically active aprotinin from the yeast *Pichia pastoris*

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SUMMARY

A synthetic gene encoding aprotinin (bovine pancreatic trypsin inhibitor) was fused to the *Saccharomyces cerevisiae* prepro alpha mating factor leader sequence at the dibasic amino acid processing site. *Pichia pastoris* strains were developed to express one or multiple copies of a methanol-inducible expression cassette containing the gene fusion. *P. pastoris* containing a single copy of the vector secreted approximately 150 mg/l of immunoreactive protein. A construct bearing five copies of the expression cassette secreted 930 mg/l of aprotinin. The purified aprotinin molecule was equipotent with the native molecule in a trypsin inhibition assay. Protein sequence analysis showed that the alpha factor-aprotinin fusion was not processed at the basic amino acid residues Lys-Arg. Instead, recombinant aprotinin had additional N-terminal amino acids derived from prepro alpha factor. The N-terminal extension was variably 11 or 4 amino acids. Inclusion of the spacer DNA sequence encoding Glu and Ala between aprotinin and the Lys-Arg processing site led to the secretion of a biologically active aprotinin containing only a Glu-Ala N-terminal extension.

INTRODUCTION

Aprotinin is a 58-amino acid peptide which has trypsin inhibitory activity. The molecule has a molecular weight of 6512 Da and contains three disulfide bonds. Aprotinin is a trypsin-specific member of the Kunitz-type protease inhibitor family [12]. It has been purified from bovine lung, spleen, pancreas and other organs. As a protease inhibitor, aprotinin forms a tight complex ($K_i = 6 \times 10^{-14}$) with the active site of trypsin. A lysine residue at position 15 is implicated in providing the trypsin-like specificity [16].

Aprotinin is used as a human therapeutic, marketed under the name Trasylol™, for treatment of hemorrhagic shock. Although of bovine origin, aprotinin is well-tolerated in humans, presumably because of its identity with human Kunitz-type inhibitors [5]. This protease inhibitor is also used in mammalian cell culture media to stabilize the desired product and also to potentiate media growth factor activity by diminishing proteolysis, and as a reagent in the recovery and purification of proteins produced in cell culture [7].

This paper describes the expression of aprotinin in the yeast *Pichia pastoris*, a yeast developed for industrial scale production of proteins via recombinant DNA technology. The high cell density fermentations and methanol-regulated expression cassettes that characterize the *Pichia* technology have been described previously [2]. This expression system is characterized by high productivity and excellent reproducibility both at laboratory and pilot plant scale [2,4]. *Pichia* has been used to express both intracellular and secretory proteins [2,4].

In these experiments, we characterize the efficacy of the *Saccharomyces cerevisiae* prepro alpha factor secretion signal in the secretion of aprotinin. Prepro alpha factor is an 85-amino acid peptide that, in many cases, can facilitate the secretion of peptides fused directly to the paired basic amino acids at the C-terminus of the leader sequence. A membrane-bound endopeptidase can then cleave the mature peptide from the precursor allowing efficient secretion [6]. This work defines some aspects of the limitations of the prepro alpha factor leader sequence. Spatial relationships between the mature aprotinin and the dibasic amino acid processing site play a key role in the efficiency of processing.

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MATERIALS AND METHODS

Vectors, recombinant strain construction and growth

The aprotinin DNA sequence was generated from a back-translation of the protein sequence using codons from highly expressed *Pichia* genes [11]. Oligonucleotides were annealed, ligated together, and products were separated via agarose gel electrophoresis. A band of the correct size was eluted from the gel and subcloned into M13mp18. The sequence was verified by dideoxy sequencing [14].

The aprotinin coding sequence was fused to the prepro alpha factor sequence by primer-directed mutagenesis creating pAPR104 [17]. The prepro alpha factor-aprotinin fusion was then subcloned as an *Eco*RI fragment into pAO815, a *Pichia pastoris* integrative expression vector, to generate pAPR205 (Fig. 1A). This plasmid contains the aprotinin fusion gene under the control of the methanol responsive *AOX1* promotor and transcription terminator. The wild-type *Pichia HIS4* gene is also present for selection in yeast. A vector containing five copies of the aprotinin expression cassette was generated by an in vitro amplification scheme [1] and is called pAPR501 (Fig. 1A). The vector pAPR904, containing the Glu-Ala residues immediately upstream of the aprotinin, was generated by primer-directed mutagenesis of a subclone of pAPR205 in M13mp19 (pAPR305). This mutagenized gene was subsequently subcloned into pAO815 to create pAPR894.

Pichia pastoris strain GS115 (*his4*) [2] was separately transformed with plasmids pAPR205, pAPR501 and pAPR894 using the lithium chloride method [9]. Transformants were analysed via Southern blot analysis to ascertain both expression cassette copy number and locus of integration. The *Pichia* strains used in these experiments are listed in Table I.

Pichia pastoris strains were grown in 1-l fermentors essentially as described by Digan et al. [4]. The fermentation protocol for foreign protein expression in *Pichia* contained three steps. Cell mass was accumulated (in the absence of aprotinin expression) by growth on excess glycerol. A limited glycerol feed was used to increase cell mass and derepress the *AOX1* promoter followed by growth in a methanol fed-batch mode to fully induce expression.

Measurement of aprotinin activity

The biological activity of aprotinin was measured in a trypsin inhibition assay using benzoyl L-arginine *p*-nitroanilide as substrate. A 1-ml reaction contained 50 mM Tris · HCl pH 8.0, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 20 enzyme units of trypsin and either from 0 to 22.4 μg/ml standard (Sigma Chemical Co., St. Louis, MO) or an unknown concentration of recombinant aprotinin. The reaction was incubated for 30 min at ambient temperature. A 50-μl aliquot of the enzyme-inhibitor complex was then added to 20 mM substrate in a 1-ml total volume reaction at pH 8.0 and the change in absorbance at 410 nm was recorded for 5 min. A standard curve was generated by measuring residual trypsin activity for different quantities of aprotinin standard and trypsin-inhibitory aprotinin activity of the recombinant material was determined.

Radioimmunoassay

Aprotinin was iodinated using the chloramine T procedure [13]. Rabbit antisera was raised by injection of aprotinin with Freund's adjuvant. Reaction products were separated on a Bond-elute column, followed by HPLC fractionation on a C18 (Vydac) column, using a 0–100% CH_3CN gradient in 0.1% trifluoroacetic acid. A 1 : 20 000 dilution of antisera, incubated 20 h at 4 °C in the presence of 10^4 cpm of ^{125}I-aprotinin and various concentrations of unlabelled aprotinin in 50 mM sodium phosphate pH 7.4, 25 mM EDTA, 0.1 M NaCl, 0.1% BSA, 0.1% Triton X-100, and 0.1% sodium azide, was subsequently precipitated with Pansorbin (1/40). After addition of 2.0 ml of wash buffer (0.9% NaCl, 5 mM EDTA, 2% Triton X-100) the tubes were centrifuged, decanted, and ^{125}I-aprotinin was measured in a gamma counter. A dilution series using unlabelled aprotinin gave an ED_{50} of approximately 0.5 ng, with a linear range of 0.1 to 1.0 ng.

Protein sequencing

To determine the amino terminal peptide sequence of recombinant aprotinin, clarified broth samples were separated by SDS-PAGE in a tricine gel system [15]. Subsequently, proteins in the gel were transferred electrophoretically onto Immobilon, a polyvinylidene difluoride membrane, at 200 mA constant current for 60 min in 10 mM CAPS buffer, pH 11, containing 10% methanol. After the electrotransfer was complete, the membrane was stained with Coomassie Blue (0.1% Coomassie Blue 250, 50% methanol). The aprotinin band was excised from the blot and subjected to sequential Edman degradation on the Applied Biosystems 470 gas phase sequencer.

RESULTS

The strains listed in Table 1 were analysed by Southern blotting to determine expression cassette copy number and locus of integration. Single copy vectors, all integrated at the *AOX1* locus, were chosen for study. One strain, G + APR205S10, had a fortuitous double integration of the plasmid at *AOX1*, and is consequently, a two-expression cassette copy strain. The five-copy strain G + APR501S3 was integrated at the *HIS4* locus. Sub-

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TABLE 1

Aprotinin expression recombinant strains

Gene	Recombinant strain designation	Expression cassette copy number	Locus of integration
Aprotinin	G + APR205S5	1	*AOX1*
Aprotinin	G + APR501S3	5	*HIS4*
EA-Aprotinin	G + APR894S12	1	*AOX1*
Aprotinin	G + APR205S10	2	*AOX1*

sequently, these strains were grown in 1-l fermentors to characterize aprotinin expression. Aprotinin levels were measured in cell-free broth by RIA at the end of the fermentation (Table 2). Single-copy strains consistently produced approx. 150 mg of aprotinin/l, whereas a five-copy strain synthesized almost 1 g/l. Each of these fermentations was characterized by robust cell growth with final cell yields between 300 and 400 g/l (wet weight). These results suggest that aprotinin production was not toxic to *Pichia pastoris*. The increase in expression level seen between the one- and five-copy strains also suggested that cells were tolerant of aprotinin at high levels (around 1 g/l). The expression levels measured by RIA correlated well with the values measured by the enzyme inhibition assay, indicating that recombinant aprotinin is correctly folded and fully active (Table 2).

Biochemical characterization of the aprotinin peptide using high-resolution Tricine gels (followed by silver staining) indicated that aprotinin produced by the single-copy strain G + APR205S5 was slightly larger than the authentic aprotinin standard. The molecule produced by the two-copy strain G + APR205S10 was considerably larger (data not shown), and no authentic aprotinin was detected in the broth. These results were verified by protein sequence analysis (Table 3).

G + APR205S5, a single-copy strain, produced a peptide containing four amino acids of prepro alpha factor

TABLE 2

Aprotinin yields from four recombinant strains

Recombinant strain designation	Yeast cells (g/l wet weight)	Immunoreactive aprotinin (mg/l)	Active aprotinin (mg/l)
G + APR205S5	320	143	143
G + APR501S3	441	930	825
G + APR894S12	302	143	83
G + APR205S10	304	320	324

TABLE 3

N-terminal sequence of aprotinin produced by various recombinant strains

Recombinant strain designation	N-terminal sequence
G + APR205S5	LDKRRPDFC
G + APR205S10	AKEEGVSLDKRRPDFC
G + APR984S12	EARPDFC

appended to the N-terminus of aprotinin. The strain G + APR205S10, a two-copy strain, contained an 11-amino acid extension of aprotinin. In both cases, aprotinin was not processed at the paired basic amino acid residues at the C-terminus of prepro alpha factor (Fig. 1B). Time course analysis of aprotinin expression in strain G + APR205S5 revealed that the 11-amino acid extension was the first aprotinin species to appear in the broth. The molecule was subsequently degraded to the four-amino acid extension presumably by media proteases. There was no evidence of unprocessed material in the fermentation broth.

To further analyse the expression of this improperly processed aprotinin, we inserted the coding sequence for the dipeptide Glu-Ala between the Lys-Arg processing sites and the first amino acid of aprotinin. It was hypothesized that this modification would serve to separate the basic amino acids from the target peptide and introduce a potential exopeptidase processing site analogous to the dipeptidyl amino peptidase activity encoded by the *Saccharomyces cerevisiae STE13* gene [10]. The aprotinin analog gene was inserted into an expression vector, which was used to create a *Pichia* transformant, G + APR894S12.

Protein sequence analysis of the fermentor broth indicated that a single species was present which contained the N-terminal sequence Glu-Ala-Arg-Pro-Asp, i.e., the unprocessed Glu-Ala spacer followed by mature aprotinin. There was no indication that the authentic sequence was present.

DISCUSSION

We have demonstrated that aprotinin-like peptides, bearing various N-terminal extensions can be produced in *Pichia pastoris* as secreted products. High level of expression is related to three factors: high-level transcription of the *AOX1* promoter [3], efficient translation of the heterologous mRNA [2,4], and efficient processing and secretion of the aprotinin peptide.

The proportional increase in aprotinin expression seen

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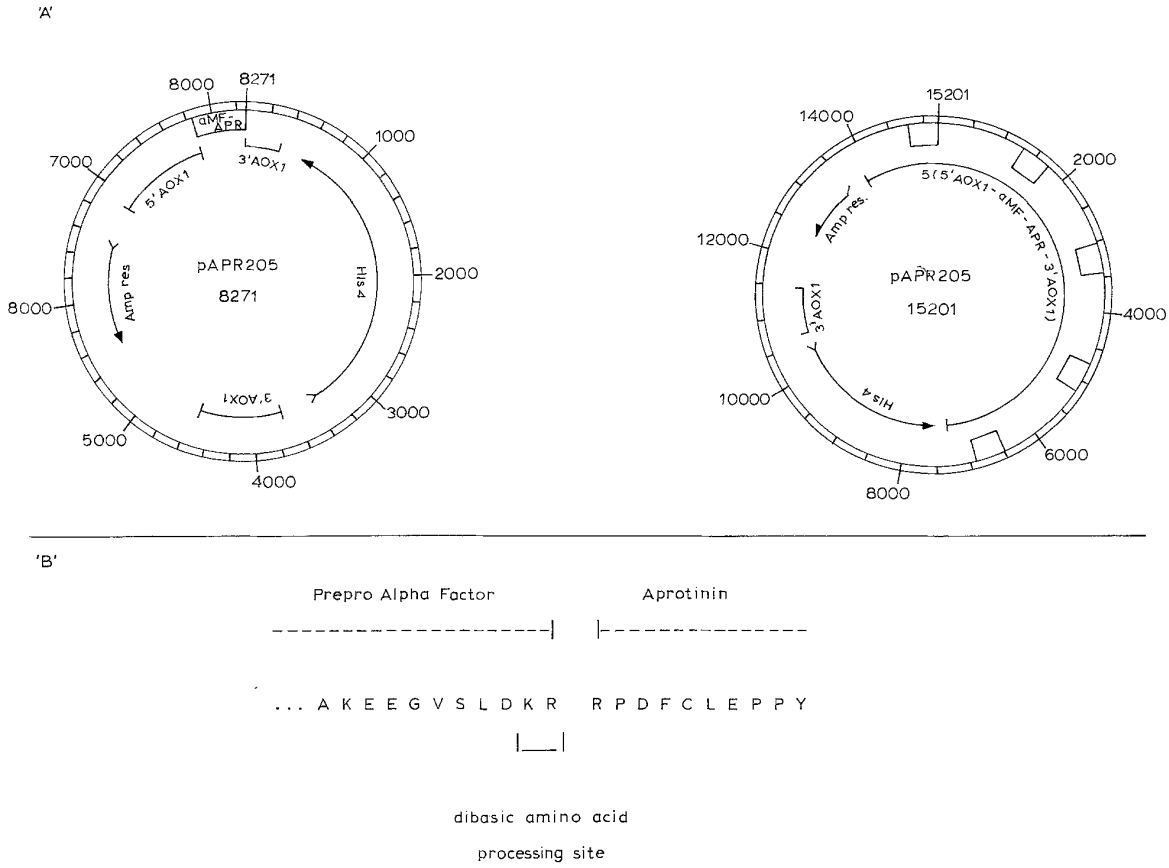

Fig. 1. **A.** Plasmid maps of the single-copy vector pAPR205 and multi-copy vector pAPR501. *AOX1* 5′ and 3′ regulatory regions are highlighted. The boxed areas are coding sequences flanked by the regulatory regions. The selectable marker, the *Pichia HIS4* gene is delineated by an arrow. **B.** Amino acid sequence at the junction of the prepro alpha factor leader sequence and the aprotinin peptide.

by increasing the copy number five-fold, was consistent with efficient secretion, since increased synthesis of the alpha factor-aprotinin fusion resulted in a direct increase in secreted aprotinin. The strain bearing five expression cassettes results in levels approaching 1 g/l. We anticipate that further increases in copy number would result in even higher levels of aprotinin.

One unexpected result of this research was the inability of the dibasic amino acid processing enzyme to cleave the precursor at the appropriate Lys-Arg residues. Previous studies have demonstrated the existence of this processing enzyme in *Pichia*, e.g., accurate processing of epidermal growth factor (EGF) from an alpha factor EGF fusion (R. Siegel, in preparation). Therefore, the resulting 11-amino acid extension seen in this work probably arises from another endoproteolytic activity located in the secretory pathway. The +11 form was the first aprotinin species that was detectable in broth. In some cases, it was degraded to a four-amino acid extension (+4 form) by proteases present in the fermentor broth. The lack of proteolysis at the Lys-Arg processing site was remedied by placing a Glu-Ala encoding spacer in the gene between Lys-Arg and the mature aprotinin. In this gene construct, complete cleavage was seen at Lys-Arg, implying that steric factors derived from the aprotinin portion of the fusion to alpha factor were responsible for inhibiting processing. By moving the basic amino acids away from the N-terminus of aprotinin, efficient proteolysis at Lys-Arg was achieved.

In the case of G + APR894S12, the Glu-Ala extension, there was no removal of the Glu-Ala residues (Table 3). Strains containing two and three Glu-Ala pairs at the N-terminus showed no evidence of Glu-Ala removal (data not shown). One possible conclusions is that a membrane-

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bound or secretory dipeptidyl amino peptidase is not present in *Pichia*. Alternatively, these enzymes, which are serine proteases, may be inhibited by aprotinin, resulting in the inability to remove the N-terminal Glu-Ala dipeptide [8].

We observed full anti-trypsin activity with the *Pichia*-derived aprotinin present in the crude broth and also with purified recombinant aprotinin in both G+APR205S5 and G+APR894S12. Apparently the +11, +4, and Glu-Ala extensions of aprotinin did not interfere with biological activity. Although these aprotinin analogs may not be acceptable for all human pharmaceutical applications they may find an excellent niche in industrial use. Further, high yields and simplicity of production may result in considerable cost reduction in mammalian cell culture applications.

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