OVER-EXPRESSION OF α-LYTIC PROTEASE AND ITS MUTANTS
BY RECOMBINANT ESCHERICHIA COLI

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SUMMARY

We report simple fermentation conditions for high-level extracellular production of α-lytic protease and its mutants by recombinant E. coli. Although transcription was controlled by the lac promoter, addition of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG) was generally found to reduce the expression of active protease. We also describe the effects of other factors on the levels of enzyme found in the culture medium.

INTRODUCTION

The enzyme α-lytic protease is a chymotrypsin homologue that is expressed extracellularly by the soil bacterium Lysobacter enzymogenes. Like elastase, this protease cleaves preferentially at small uncharged residues such as Ala (Kaplan et al., 1970; Bauer et al., 1981). During the past 30 years, the protease has been the subject of extensive study, including many NMR and X-ray analyses of its structure (e.g. Hunkapiller et al., 1973; Fujinaga et al., 1985). Protein engineering has shown that amino acid residues 190 and 213 are particularly influential in determining the size and shape of the primary substrate binding pocket. Replacement of either of these Met residues with Ala results in mutant proteases (the Ala190 and Ala213 enzymes) that prefer to cleave at large hydrophobic residues such as Met and Phe (Bone et al., 1989). We have described elsewhere many mutants of α-lytic protease derived from the Ala190 enzyme (Graham et al., 1993).

Efficient recombinant expression of α-lytic protease would greatly facilitate further engineering and characterisation of this important enzyme. Work in other laboratories has shown that active α-lytic protease can be expressed extracellularly by E. coli, although its production is temperature-dependent because maturation of the proenzyme is impaired above 30°C (Silen et al., 1989; Fujishige et al., 1992). In this paper we investigate the expression of α-lytic protease and some of its mutants by recombinant E. coli. and describe fermentation conditions that give high levels of active enzymes at low cost.
**MATERIALS AND METHODS**

*Bacterial strains*  E. coli TG1 and JM109 are common laboratory strains (Sambrook *et al.*, 1989), while AC2522 is an *E. coli* B strain (Carter *et al.*, 1985) that we obtained from BioExcellence (Colchester, U.K.).

*Expression constructs*  The endogenous pre-peptide of α-lytic protease was replaced with the *pelB* leader peptide, which directs gene products to the periplasm, and the resulting cassette was placed under the control of the *E. coli* lac promoter in pBS(+) (Stratagene). Construction of the expression plasmid and generation of the protease mutants was described by Graham *et al.* (1993).

*Fermentation conditions*  Terrific Broth was made according to Tartof and Hobbs (1987) and Sambrook *et al.* (1989) but without glycerol (unless otherwise stated). All media contained 50 µg/ml ampicillin to prevent loss of the expression construct in host cells, and all liquid cultures were grown in Erlenmeyer flasks at 24-25°C on an orbital shaker (300 r.p.m.). Cells from frozen stocks were streaked onto LB agar (Sambrook *et al.*, 1989), pH 7.5, containing 2% (w/v) glucose. After incubation overnight at 37°C the resulting colonies were used to inoculate Terrific Broth (50ml) containing 0.7% (w/v) glucose. These starter cultures were grown in 250ml Erlenmeyer flasks for 16-18h. After centrifugation, the cells were washed and resuspended in 50ml of 0.9% (w/v) saline. Flasks (250ml) containing 50ml of medium were inoculated immediately with 0.01 vol. of the resuspended starter cells.

*Enzyme Assays*  Unless otherwise stated, enzyme concentrations for the wild type and Ala190 proteases were measured as follows. $V_{max}$ values were determined for the enzymes in cell-free culture supernatants using succinyl-AlaProAla-$p$-nitroanilide and succinyl-AlaAlaProPhe-$p$-nitroanilide as substrates, respectively. The enzyme concentrations in the supernatants were then calculated using published $k_{cat}$ values for the two proteases (Silen *et al.*, 1989; Bone *et al.*, 1989). Thereafter, initial rates were measured at one of the substrate concentrations used previously, and the levels of enzyme protein were estimated as a proportion of that determined originally.

**RESULTS**

*Induction*  The enzyme levels and cell densities obtained in cultures grown using Terrific Broth containing 0.4% (v/v) glycerol are shown in Table 1. Full induction was achieved by adding IPTG to a final concentration of 1mM. Uninduced cultures were also grown to serve as controls. Surprisingly, we found that the levels of wild-type enzyme in the uninduced cultures were 12-fold greater than the highest levels obtained in the induced cultures, and also that levels of the Ala190 enzyme were 46-fold higher in the absence of inducer. Induced cultures grew more slowly than uninduced ones. Where induction with IPTG was performed, we observed higher levels of enzyme in cultures induced later in the exponential growth phase compared with those induced soon after inoculation.

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