A novel strategy for production of a highly expressed recombinant protein in an active form

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Under standard growth conditions, *E. coli* transformed with the high-level expression vector pMON5525 produces recombinant DMAPP/AMP transferase in inactive, insoluble complexes. We have produced large amounts of active, soluble protein by growing and inducing the cells under osmotic stress in the presence of sorbitol and glycyl betaine. This caused an increase of up to 427-fold in the active yield, and the disappearance of the protein from the pelletable fraction of cell extracts. This treatment may have wide applicability.

Recombinant protein; Activity; DMAPP/AMP transferase; *E. coli*

1. INTRODUCTION

The production of biologically active recombinant proteins is often hampered by the formation of insoluble inclusion bodies [1]. In these complexes the proteins co-precipitate in a denatured state with ribosomes, nucleic acids and other cytoplasmic proteins [2]. The denaturation of the protein and the heterogeneous nature of the inclusion bodies makes recovery of active proteins a formidable task. General techniques for achieving this have not been developed, although some successes have been reported [3,4]. A method is reported here for the production, in an active soluble form, of a highly expressed recombinant protein which normally accumulates as inclusion complexes in *Escherichia coli*. The method involves the use of osmotic stress to facilitate the uptake of the 'compatible solute', glycyl betaine [5]. Although developed for the production of the cytokinin biosynthetic enzyme dimethylallyl pyrophosphate:5′-AMP transferase (DMAPP:AMP transferase) from *Agrobacterium* [6,7], the technique may be applicable to the production of other recombinant proteins.

2. MATERIALS AND METHODS

E. coli transformed with pMON5525 [8] were grown on LB agar plates containing 200 μg/ml ampicillin. Cells to be grown with betaine and sorbitol (+B/S) (Sigma Chemical Co.) were pre-adapted by adding the appropriate concentration of sorbitol and 2.5 mM betaine. Colonies were inoculated into M9 salts medium [9] supplemented with

1% casamino acids. 2% sucrose and 5 m·gl^{-1} thiamine, 1 mM MgSO$_4$ and 0.1 mM CaCl$_2$, with and without B/S. Overnight cultures were used to inoculate 42 ml or 3.5 liter batches of the same media. The cultures were incubated at 37°C with shaking until the O.D.$_{660}$ was 0.5–0.6. One of each pair of duplicates was then cooled to 25°C. Expression of the transferase gene was initiated by adding nalidixic acid to 200 μg·ml^{-1}. Cultures were incubated for a further 3 h at 25°C or 37°C. Inclusion body content was estimated by light microscopy at 1000× magnification. Cells from the 42 ml cultures were extracted and the DMAPP/AMP transferase activities were assayed using the methods of Akiyoshi et al. [10]; incorporation into N^6-dimethylallyl purines being measured by radiocounting of HPLC separated compounds. This was repeated three times. Pellets from 1.5 liter of the 3.5 liter cultures were resuspended in 20 ml of sonication buffer (1 M betaine, 25 mM triethanolamine, 75 mM KCl, 5 mM MgCl, 5 mM mercaptoethanol, 0.1 mM PMSF·HCl to pH 7.8 plus growth medium to 30 ml final volume. 1.82 g of sorbitol was added to the buffers used to resuspend the −B/S pellets, so the sorbitol content of all samples was similar. The samples were sonicated and centrifuged at 200 000 ×g for 1 h, then rapidly assayed as follows. 8 × 100 μl portions of the supernatants were mixed with 500 μl of assay buffer (sonication buffer plus 5 mM KF, 1 mg/ml BSA, 3.5 mM DMAPP and 350 μM of tritiated AMP, adjusted to pH 7.5). The mixtures were incubated at 26°C for 15 min, after which 2.5 U of alkaline phosphatase (Boehringer Mannheim Cat No. 1097 075) in 50 μl of the assay buffer (minus DMAPP and AMP) was added to each sample. The incubation was continued for 10 min at 37°C. The cytokinins were extracted into 650 μl of ethyl acetate. 475 μl of the organic phase was rinsed with 475 μl of distilled H$_2$O and radioactivity was then counted in 325 μl of the ethyl acetate. Pellets and supernatants were boiled in appropriate amounts of the loading buffer of Laemmli [11] and were analysed by SDS-PAGE on a Bio-Rad mini-Protean system (5% stacking gel, 12.5% running gel, electrophoresis conditions as prescribed by the manufacturers).

Colonies were also inoculated into 42 ml of rich LB broth [9] supplemented with 0, 330, 660 or 1000 mM sorbitol plus 2.5 mM betaine and into LB broth with only 10% of the standard NaCl content. Cultures were grown at 37°C and induced at 25°C as above. Control cultures plus and minus 660 mM sorbitol and betaine were also induced at 37°C. Resultant transferase activities were assayed using the rapid assay (above) and the post-sonication 200 000 × g pellets and supernatants were analysed by SDS-PAGE as above. This was repeated.

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3. RESULTS

Microscopically visible inclusion bodies were formed in >90% of cells grown in the absence of sorbitol and betaine, and in <1% of cells grown in the presence of 660 mM or more sorbitol plus betaine. In initial experiments performed with cells grown in M9-based media, 30–180 times more enzymic activity was recovered in the soluble fraction of cells induced at 25°C with 1 M sorbitol than in extracts of control cells (those grown on –B/S medium and induced at 37°C) (Table I). Activity recovered from the control cells is almost too low to be detectable, introducing large standard errors in the control data. SDS-PAGE analysis of lysed cells grown on the M9 media revealed a marked reduction in the amount of the transferase in the pellets of cells grown +B/S and induced at 25°C compared to controls but not a clear corresponding increase in the supernatants, despite the large increase in active yield (Fig. 1). All assays used substrate concentrations previously shown to be saturating.

Since a higher yield of active protein could be achieved with cells grown on LB medium this was used for subsequent experiments. From Table I it can be seen that both the presence of betaine and sorbitol and the lower induction temperature increase active yield, that these increases are synergistic and that the yield from cells grown on LB +B/S and induced at 25°C was 427-fold higher than from the M9 grown control cells. PAGE gels confirmed the presence of a 27 kDa protein in the supernatant of the high-yield cells which was absent in untreated cells (Fig. 1).

4. DISCUSSION

Manipulation of growth conditions to favour production of active rather than denatured protein is an obvious approach in attempting to recover active protein from transformed organisms which form inclu-
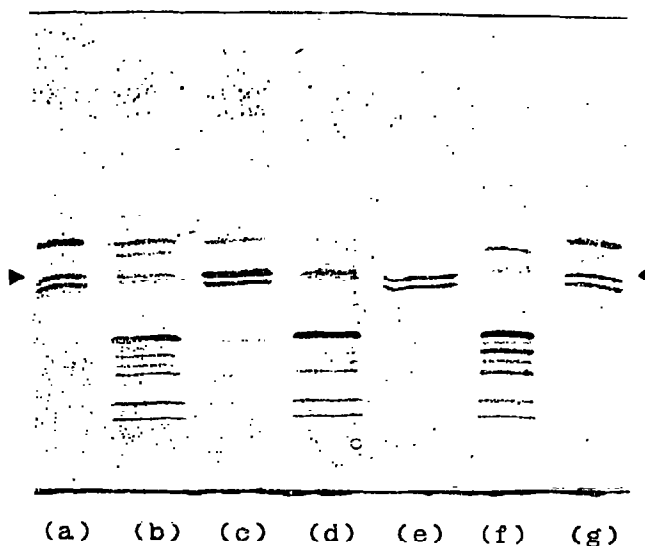

(a)   (b)   (c)   (d)   (e)   (f)   (g)

Fig. 1. SDS-PAGE analysis of sonicates of cells after 200 000 × g centrifugation. (a) and (g) Pellets from cells grown on M9 medium –B/S induced at 37°C, (b) supernatant from these cells, (c) and (d) pellet and supernatant respectively from cells grown on M9 medium +B/S and induced at 25°C, (e) and (f) pellet and supernatant respectively from cells grown on LB medium + 660 mM sorbitol induced at 25°C. The arrows mark the 27 kDa (recombinant transferase) band.

sion complexes under standard conditions. In some cases, active proteins have been produced by cells induced at 25–30°C rather than at the growth optimum of 37°C [12]. This suggested that other changes in the cellular environment which either encourage the adoption of the active conformation and/or increase the stability of proteins may also increase the yield of active protein. One such change could be higher internal concentrations of 'compatible osmolytes' such as glycyl betaine. which are believed to be excluded from the immediate domains of proteins, causing thermodynamically unfavourable 'preferential hydration' and, thus, minimisation of solvent–protein contact and stabilisation of protein structure [13]. This hypothesis was tested as follows. It is well known that *E. coli* is capable of growth in fairly low water potentials (in up to 800 mM NaCl, for instance) if betaine is present in the medium, betaine being taken up to such concentrations that it generates the major proportion of the osmotic balance with the medium [14]. We therefore grew *E. coli* with and without sorbitol and 2.5 mM betaine, in both 42 ml and 3.5 liter cultures and induced transferase production either at 37°C or 25°C and in M9 and richer LB-based media. The results clearly showed that the sorbitol/betaine treatment massively enhances production of soluble, active protein. It seems probable that it could significantly increase the yield of other recombinant proteins. The technique may, therefore, have wide and significant biotechnological application. The large difference in active yield obtained between LB and M9 CAS medium mentioned above is also of interest. This

Table I

DMAPP/AMP activities of extracts per ml of culture per unit O.D.$_{660}$ from cells grown with and without betaine/sorbitol (B/S). Means and SD are shown (n=8).

Basal medium	Induction temperature	Sorbitol concentration	Dpm (/1000) in cytokinin	Relative activity
M9 CAS	37°C	0 mM	1.2 ± 1.3	1
	25°C	1000 mM	51.2 ± 9.2	44
LB	37°C	0 mM	31.2 ± 0.6	26
	37°C	660 mM	74.4 ± 3.0	62
	25°C	0*mM	31.3 ± 0.5	26
	25°C	0 mM	80.0 ± 1.1	66
	25°C	330 mM	208 ± 4.5	173
	25°C	660 mM	378 ± 7.0	315
	25°C	1000 mM	512 ± 12.6	427

*LB medium with 10% normal NaCl.

may be at least partly due to LB having a considerably greater osmotic pressure than the M9 medium since it contains 170 mM NaCl and significant concentrations of amino acids and miscellaneous yeast extract solutes. The reduction of yield caused by reduction of the NaCl content of the LB broth supports this interpretation. No attempt was made to quantify the final yield of active protein as a proportion of the total production of recombinant protein in control cells generating the inactive inclusion bodies. We feel that the important pragmatic consideration is production of active protein in sufficient quantity to facilitate relatively easy purification, which was impossible from cells grown under the original conditions. It seems likely that further improvement in yield should be possible through further variation of media, induction conditions and length of induction. This is currently being tested.

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