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Expression and purification of plasmid-encoded *Thermoplasma acidophilum* citrate synthase from *Escherichia coli*

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The citrate synthase gene from the thermophilic archaebacterium *Thermoplasma acidophilum* was expressed in *Escherichia coli*, yielding an active product of the expected molecular weight. Manipulation of the citrate synthase gene in a series of pUC19 constructs showed that the presumed *Thermoplasma* ribosome binding site is recognized by the *E. coli* ribosome. A rapid purification of the expression product to homogeneity was achieved, based on the thermostability of *Thermoplasma* citrate synthase.

Archaebacterium; Citrate synthase; Gene expression, thermophilicity; *Thermoplasma acidophilum*

1. INTRODUCTION

Thermoplasma acidophilum is a thermophilic archaebacterium, growing at pH 1–2 and 55°C. As part of investigations into the comparative enzymology of the central metabolic pathways of the archaebacteria, eubacteria and eukaryotes [1,2], we have previously reported the cloning and sequencing of the gene encoding the *Tp. acidophilum* citrate synthase [3]. In the present paper, we report a protocol for the purification and characterisation of the expressed protein from the *Escherichia coli* host.

Heat stability studies on *Tp. acidophilum* citrate synthase demonstrated that the enzyme is stable to 70°C [4]. This thermostability suggested the use of a heat step as the basis for purification of the archaebacterial enzyme from *E. coli*.

2. MATERIAL AND METHODS

2.1. *Bacterial strains*

The *E. coli* strain TG1 (*supE hsdΔ5 thiΔ(lac-proAB)* F′[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) was used throughout.

2.2. *Plasmids, enzymes and chemicals*

A plasmid construct (pTaCS19), carrying the *Tp. acidophilum* gene encoding citrate synthase, has been previously described [3]. pUC vectors and restriction endonucleases were purchased from Northumbria Biologicals Ltd. (Cramlington, UK). [α-^{35}S]dATP was obtained from Amersham International (Amersham, UK). Sequenase kits were purchased from United States Biochemical Corporation, (Cambridge Bioscience, Cambridge, UK). CoA and oxaloacetate were from Boehringer (Mannheim, Germany). 5,5′-dithiobis-(2-nitrobenzoic acid) was from Sigma Chemical Co. (Poole, UK). Gradipore (12%) SDS-polyacrylamide gels were from Flowgen (Sittingbourne, UK). Protein standards, purchased from Pharmacia LKB (Uppsala, Sweden), consisted of rabbit muscle phosphorylase b (M_r = 94 000), bovine serum albumin (M_r = 66 000), chicken ovalbumin (M_r = 45 000), bovine carbonic anhydrase (M_r = 30 000) and soybean trypsin inhibitor (M_r = 20 100). Polybuffer 96 was also from Pharmacia LKB.

2.3. *Genetic manipulation of pTaCS19*

A 1 kb region of *Tp. acidophilum* DNA, upstream of the citrate synthase gene in pTaCS19, was deleted (as shown in Fig. 1) to yield the plasmid pCSEH19. Further manipulation of pCSEH19 resulted in the removal of a 4 base segment, generating clone pCSSS. Sequencing by the method of Sanger et al. [5], using the synthetic oligonucleotide [5′-GTTTTCCCAGTCACGAC-3′] as a primer, confirmed that these manipulations had been carried out successfully.

2.4. *Assay of citrate synthase*

Citrate synthase was assayed spectrophotometrically at 412 nm and 55°C by the method of Srere et al. [6].

2.5. *Production and purification of plasmid-encoded* Tp. acidophilum *citrate synthase in* E. coli

Cells of *E. coli* carrying the recombinant vector pCSEH19 were grown for 16 h at 37°C in 250 ml of nutrient broth with 100 μg ampicillin/ml. The cells were harvested by centrifugation (10 min at 10 000 × *g*) and resuspended in 5 ml of 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. After lysis by sonication, the cell debris was removed by centrifugation (10 min at 8 000 × *g*) and the cell lysate was incubated at 65°C for 10 min. Denatured protein was removed by centrifugation (10 min at 8 000 × *g*). The supernatant was dialysed against 25 mM diethanolamine-HCl (pH 9.5) and then loaded onto a Pharmacia LKB FPLC mono P chromatofocussing column pre-equilibrated with the same buffer. Citrate synthase was eluted with 10% (v/v) Polybuffer 96 (pH 6.0)

3. RESULTS AND DISCUSSION

3.1. *Expression of* Tp. acidophilum *citrate synthase in* E. coli

The construct pCSEH19 was found to give enhanced

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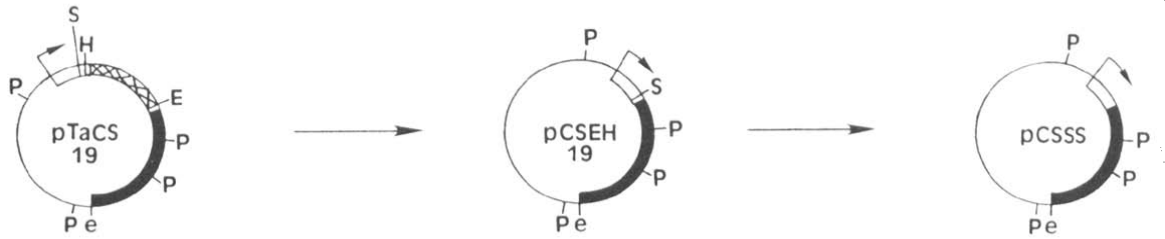

Fig. 1. Construction of plasmid pCSEH19. The construction of plasmid pTaCS19, carrying the *Tp. acidophilum* gene encoding citrate synthase, has been described previously [3]. The *lac* control region and the 5' end of the *lacZ* gene, carried on the pUC19 vector, are unshaded. An arrow indicates the direction of transcription for *lacZ*. *Tp. acidophilum* DNA, prior to the start of the citrate synthase gene (shaded), is represented by the hatched region. pTaCS19 was digested with *Hinc* II and *Eco* RV, and then religated to generate the clone pCSEH19. Digestion of pCSEH19 with *Sph* I, followed by S1 nuclease treatment and religation gave the clone pCSSS. e, *Eco* RI; E, *Eco* RV; H, *Hinc* II; P, *Pvu* II; S, *Sph* I.

levels of expression of the *Tp. acidophilum* citrate synthase compared to those obtained from the original construct pTaCS19. The transcription of the citrate synthase gene from pCSEH19 appears to be initiated from the *lac* promoter, since no expression was obtained from a similar construct in which the citrate synthase gene is in reverse orientation. However, the inclusion of isopropyl-β-D-thiogalactoside at a concentration of up to 5 mM had no effect on the level of expression of citrate synthase from pCSEH19, presumably due to the high plasmid copy number.

In the pCSEH19 construct, the *Tp. acidophilum* citrate synthase coding sequence is out of frame with the preceding segment of the *lac-Z* gene. Expression of this construct in *E. coli* produced a protein of M_r = 43 000, as predicted from the gene sequence. Deletion of a 4 base segment at the *Sph* I site in pCSEH19 generated a construct (pCSSS) with the citrate synthase gene in the *lac-Z* reading frame. Expression of pCSSS in *E. coli* produced proteins of M_r = 43 000 and 47 000, corresponding to *Tp. acidophilum* citrate synthase and a fusion protein which includes an additional N-terminal 19 residues from the *lac-Z* gene. The expression of *Tp. acidophilum* citrate synthase of M_r = 43 000 from both constructs shows that the *Tp. acidophilum* ribosome binding site, which is retained in both pCSEH19 and pCSSS, is efficiently recognized by the *E. coli* ribosome [7].

The *Tp. acidophilum* citrate synthase was expressed in *E. coli* at a specific activity of up to 5 units/mg protein, that is at a level up to 20 times higher than that found in native *Tp. acidophilum* cells [8,9]. Production amounted to maximally 10% of total *E. coli* cell protein.

3.2. *Purification of* Tp. acidophilum *citrate synthase from* E. coli

The thermostability of the *Tp. acidophilum* citrate synthase allowed a simple heat treatment to be used as an initial step in purification of the enzyme from heat-labile *E. coli* proteins. Subsequent passage through the

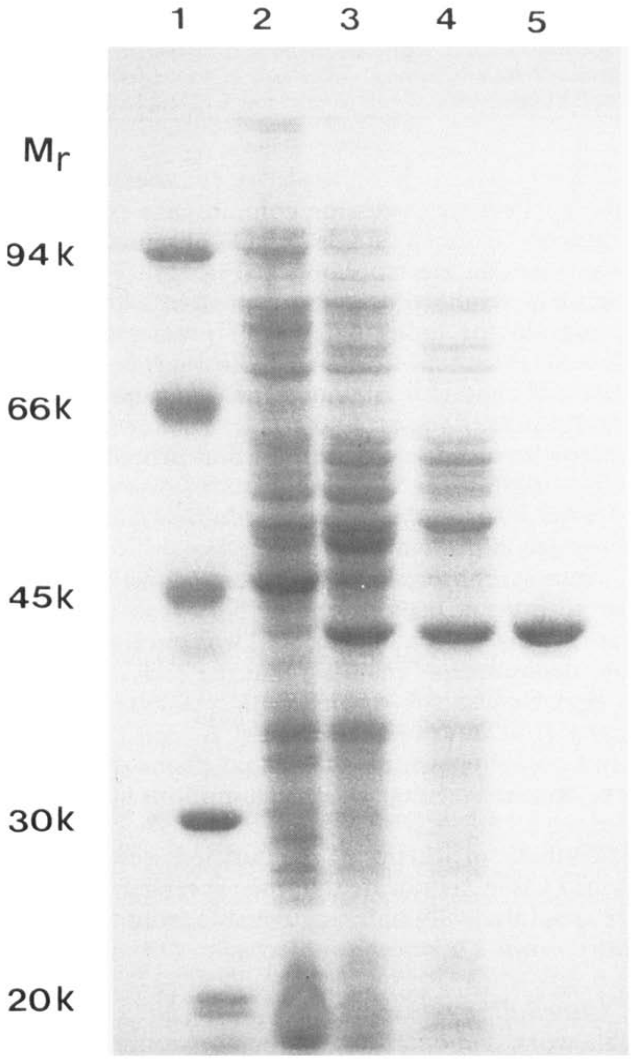

Fig. 2. SDS-PAGE of *Tp. acidophilum* citrate synthase. Lanes: (1) Standard proteins (see text); (2) cell-free extract of *E. coli* TG1; (3) cell-free extract of *E. coli* TG1 containing plasmid pCSEH19; (4) cell-free extract from (3) after heat treatment; (5) supernatant from (4) after FPLC Mono P chromatofocussing. The gel was stained with Coomassie Blue R. The relative molecular masses of the standard proteins are shown.

Table I

Purification of *Tp. acidophilum* citrate synthase from *E. coli* TG1 containing plasmid pCSEH19

Purification step	Volume (ml)	Total enzyme (units)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Overall purification (-fold)
Cell extract	4.0	320	60	5.3	100	-
Heat treatment (65°C)	2.8	241	10	24	75	4.8
Chromatofocussing (Mono P)	3.0	148	3.5	42	46	8.4

One unit of enzyme activity is defined as 1 μmol product produced per min, under standard assay conditions (see text).

Table II

Properties of the plasmid-encoded and native *Tp. acidophilum* citrate synthases

Property	*Tp. acidophilum*		*E. coli*
	Plasmid	Native	
Specificity activity (Units per mg protein)	42	41	>80
Polypeptide M_r	43 000	43 000	48 000
K_m-oxaloacetate (μM)	3.9 (±0.8)	4.5 (±0.5)	400 ($S_{0.5}$)
K_m-acetyl-CoA (μM)	7.7 (±1.0)	6.3 (±0.6)	55 ($S_{0.5}$)

Mono P chromatofocussing column gave pure protein as judged from a single band obtained by SDS polyacrylamide electrophoresis (Fig. 2). The subunit molecular weight of the pure protein (43 000) corresponds to the molecular weight predicted from the amino acid sequence derived from the gene sequence (42 942) [3] and that obtained for the enzyme purified from *Tp. acidophilum* (43 000) [9]. Table I summarises the steps involved in the purification procedure.

3.3. *Characterisation of the plasmid-encoded* Tp. acidophilum *citrate synthase*

Citrate synthase assays were carried out at 55°C in order to prevent background activity from the *E. coli* citrate synthase. In addition, KCl was omitted from the assay medium, thus ensuring that the activity of the *E. coli* enzyme was sub-optimal [10]. Assays of cell-free extracts from untransformed host *E. coli* cells, which did not contain plasmid, showed no citrate synthase activity, confirming that these assumptions were indeed the case.

The kinetic properties of the purified archaebacterial enzyme (Table 2) indicate that the expressed citrate synthase is catalytically indistinguishable from the enzyme isolated from *Thermoplasma* cells.

3.4. *Concluding remarks*

This work demonstrates that expression in *E. coli* of the citrate synthase gene from the thermophilic archaebacterium *Tp. acidophilum* is accompanied by folding of the protein into a native, active conformation. The thermostability of the *Tp. acidophilum* citrate synthase has enabled the enzyme to be purified after expression of the cloned gene in *E. coli*. A similar strategy has been employed for glyceraldehyde dehydrogenase from the thermophilic archaebacteria *Methanothermus fervidus* [11] and *Pyrococcus woesei* [12]. We find that approximately 10 mg of purified citrate synthase can be obtained per litre of *E. coli* culture, enabling the undertaking of detailed structural studies on this protein and the investigation of the basis of its thermostability.

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