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Chaperone Assisted Overexpression of D-carbamoylase Independent of the Redox State of Host Cytoplasm

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ABSTRACT

The N-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase) gene (dcb) from *Agrobacterium tumefaciens* AM 10 has been successfully cloned and expressed in *Escherichia coli*. Expression of D-carbamoylase gene under the T7 promoter in different host strains showed that the optimal expression was achieved in *E. coli* JM109 (DE3) with a 9-fold increase in enzyme production compared to the wild-type strain. The co-expression of the GroEL/ES protein with D-carbamoylase protein caused an *in vivo* solubilization of D-carbamoylase in an active form. The synergistic effect of GroEL/ES at 28°C led to 60 % solubilization of the total expressed target protein with a 6.2-fold increase in enzyme activity in comparison to that expressed without GroEL/ES and 43-fold increase in enzyme activity compared to *A. tumefaciens* AM 10. Attempts to express D-carbamoylase in an altered redox cytoplasmic milieu did not improve the enzyme production in an active form. The Histidyl-tagged D-carbamoylase was purified in a single step by Nickel-affinity chromatography and was found to have a specific activity of 9.5 U/mg protein.

INTRODUCTION

N-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase) catalyzes the hydrolysis of N-carbamoyl-D-amino acids to optically pure D-amino acids, which are valuable intermediates in the production of pharmaceutical chemicals including β-lactam antibiotics, small peptide hormones and pesticides (1). In cooperation with D-specific hydantoinase, which catalyzes the cleavage of the 5-monosubstituted hydantoin into a D-N-carbamoyl derivative, the hydantoinase-carbamoylase reaction process (2,3) is now a primary method for industrial production of D-amino acids owing to its lower reaction temperature, higher yield, rapidity and much lower amount of waste.

D-carbamoylase activity has been found in a number of microorganisms belonging to genus *Agrobacterium* (3,4), *Arthrobacter* (5), *Pseudomonas* (6), *Comamonas* (7) and *Blastobacter* (8). D-carbamoylase gene has been cloned and identified from *Agrobacterium* sp. (4,9) and *Pseudomonas* sp. (6). A recombinant strain producing large amounts of D-carbamoylase is important in decreasing the production cost of D-amino acids for the pharmaceutical industry. Therefore, the conditions for the overexpression of D-carbamoylase gene have been studied by several groups (10,11). As in case of other recombinant proteins, the bottleneck for the overproduction of D-carbamoylase in *Escherichia coli* has been the formation of biologically inactive aggregates, commonly referred to as inclusion bodies (9,10). While the mechanism behind inclusion body formation is not well understood, some novel approaches have been explored to overcome this problem *in vivo* (12-14). It has been shown that coproduced molecular chaperones prevent heterologous proteins, produced by *E. coli* recombinant cells,

from forming inclusion bodies (15,16). Another approach has been to select a lower growth temperature, which renders unstable folding intermediates to be less trapped towards off-pathway aggregation (15).

We have already reported the purification and characterization of D-carbamoylase from *A.tumefaciens* AM 10 (18) and herein, report the cloning of D-carbamoylase gene and its successful overproduction in an active form in *E.coli*. We report here, for the first time, the synergistic effect of coexpression of GroEL/ES and low induction temperature on the production of soluble recombinant D-carbamoylase in *E. coli* from *A. tumefaciens* AM 10. Furthermore, the effect of the host strain Origami (DE3) lacking thioredoxin (trx B) and glutathione reductase (gor) was investigated on the overexpression of disulfide containing D-carbamoylase (19), as the cytoplasmic redox state is known to influence the folding of such proteins.

MATERIAL AND METHODS

Bacterial culture and plasmids

A. tumefaciens AM 10 was isolated from soil samples, and cultivated at 30°C in a medium having the following composition (in g/l): glucose 10.0, yeast extract 10.0, $MnCl_2.2H_2O$ 0.02, KH_2PO_4 1.0, NaCl 0.5, pH 7.2. The plasmids and host strains used in this study are listed in Table 1. Plasmid pET-28a was used as the cloning and expression vector. The plasmid pKY206 (20) was derived from pACYC 184 and can coexist in the same cell which carry pET-DCB. Restriction enzymes, T4 DNA ligase and λ/HinD III DNA marker were obtained from New England Biolabs (Beverly, Mass.). Protein molecular weight markers and electrophoresis reagents were purchased from Sigma (St. Louis, MO). Polymerase chain reaction (PCR) was carried out with Vent DNA polymerase obtained from New England Biolabs. Oligonucleotide primers were purchased from IDT Inc. (Coralville, IA). DNA sequencing was carried

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant characteristics	Source
A..tumefaciens AM 10		Lab collection
E. coli		
DH5α	*deo*R *endA*1 *gyrA*96 *hsdR*17($r_k^-m_k^+$) *supE*44 *thi*-1 Δ(*lacZYA-argF169*) *recA*1 ϕ80 *lacZM*15	Lab collection
BL21 (DE3)	*ompT hsd* SB (r_Bm_B) *gal dem* λ (DE3)	Novagen
JM109 (DE3)	*endA*1 *recA*1 *gyrA*96 *thi hsdR*17(r_k^-,m_k^+) *relA*1 *supE*44 Δ(*lac-pro*AB) [F' *traD36 pro*AB *lacI*^{q}ZΔ*M*15] λ(DE3)	Promega
Origami (DE3)	Δ*ara-leu* 7697 Δ*lacX*74 ΔphoA Pvull *phoR araD*139 *galE* galK rspL F'[lac$^+$(lacI^q)pro] gor 522::Tn10(Tc^R) *trxB*::*kan* (DE3)	Novagen
Plasmid		
pET-28a	ColE1 origin Kan ^{r}, controllable T7promoter	Novagen
pKY206	P15A origin Tet ^{r}, GroEL/ES expressed constituitively	Mizobata
pET-DCB	pET-28a containing D-carbamoylase (*dcb*) gene under T7 promoter	*et al* (20) This study

out by using the BigDye™ Terminator Cycle Sequencing Kit in an automatic DNA sequencer model 310 (Applied Biosystems).

DNA manipulation and plasmid construction

Genomic DNA isolation from *A. tumefaciens* AM 10 and plasmid isolation was done according to the protocol described in Sambrook *et al* (21). The coding region of D-carbamoylase from *A. tumefaciens* AM 10 was engineered by PCR to add a Nhe I site upstream of the first ATG and a Xho I site downstream of the termination codon. The primers DCB-1 (5'-AGCGCTAGCATGACACGTC-3') and CBM-3 (5'-CTCTCGAGTCAGAATTCCGCGATCAG-3'), derived from the 5' and 3' coding region of D-carbamoylase were used for amplification. The PCR product was cloned into NheI/XhoI site of pET-28a carrying an inframe N-terminal Histidyl-tag and a thrombin cleavage site. The sequence engineered by PCR was verified by DNA sequencing.

Expression of D-carbamoylase

E. coli strain DH5α was used for routine cloning work, while BL21 (DE3)/JM109 (DE3) was employed for expression of the dcb gene. *E.coli* JM109 (DE3) was transformed with pET-DCB and pKY206. Transformants were grown in LB broth containing kanamycin (50 μg/ml) or kanamycin plus tetracycline (12.5 μg/ml). The target protein was induced by the addition of 5 μM IPTG (final conc.), when O.D. $_{600}$ reached 0.6 and the culture was then incubated at different temperatures on a rotary shaker set at 200 rpm. The cells were harvested by centrifugation and resuspended in 50 mM phosphate buffer, pH 7.0 containing 5 mM DTT and 0.5 mM PMSF. The cell suspension was sonicated using Heat Systems sonicator (Farmingdale, NY). The cell debris was removed by centrifugation at 28,000 g for 30 min. at 4°C, and the supernatant was used as the soluble fraction. The resultant pellet was resuspended in 50 mM phosphate buffer pH 7.0, 5 mM DTT and this was used as the insoluble fraction. The proteins in both the soluble and insoluble fractions were analyzed by 12.5% SDS-PAGE (22) followed by Coomassie staining. The stained gels were scanned by LKB Bromma 2202 Ultroscan Laser Densitometer coupled to Shimadzu Chromatopac C-R6A for integration.

Assay of D-carbamoylase activity

An appropriately diluted enzyme was incubated with 25 mM N-carbamoyl-D-phenylglycine in 50 mM phosphate buffer, pH 7.5 for 10 min. at 50 in a final volume of 100 μl. The reaction was terminated and deproteinized by the addition of 10 μl of 50% TCA. The samples were centrifuged at 13,000 g for 5 min. Activity was measured by the estimation of ammonia (stoichiometrically produced along with D-phenylglycine) by glutamate dehydrogenase (GDH) enzyme coupled assay (18). One unit of enzyme activity was defined as one ol of ammonium ions formed per minute under the assay conditions.

Protein concentration was measured by the method of Bradford (23); BSA was used as the standard.

Purification of D-carbamoylase

The soluble fraction of Histidyl-tagged D-carbamoylase was loaded onto Nickel-nitriloacetate (Ni-NTA)-agarose column (QIAGEN) equilibrated with buffer A (50 mM phosphate buffer pH 8.0 containing 300 mM NaCl, 2 mM DTT). The resin was washed with buffer A in 8 column volumes. The protein was then eluted with buffer A containing 250 mM imidazole. The eluted protein was dialyzed against 50 mM phosphate buffer pH 7.5 containing 2 mM DTT.

RESULTS AND DISCUSSION

Molecular cloning of D-carbamoylase

The D-carbamoylase gene of *A. tumefaciens* AM 10 was successfully cloned by use of PCR. The resulting ~1.0 kb NheI/XhoI fragment of D-carbamoylase gene (dcb) was ligated into plasmid pET28a which was transformed into *E. coli* DH5α. To confirm the cloned DNA, the sequencing of dcb gene was carried out, and the resulting nucleotide sequence of 915 bp was found to be identical to that listed in GenBank.

Expression of D-carbamoylase in *E.coli*

The expression plasmid was transformed into *E. coli* cells BL21 (DE3) and JM109 (DE3) in order to express the dcb gene placed under the control of T7 promoter on induction with IPTG. The clones were then screened for D-carbamoylase activity. However, no D-carbamoylase activity was detected in the soluble as well as the insoluble fraction of over 20 clones of BL21 (DE3). The expression status of D-carbamoylase varied in different host cells as shown in Fig. 1. The translated product of dcb gene in *E. coli* BL21 (DE3) cells, when analyzed on SDS/PAGE, was found to be of ~25 kDa. The protein subunit was ~10 kDa shorter as compared to the expected 38 kDa molecular weight of D-carbamoylase enzyme of *A. tumefaciens* AM 10. After isolation of the plasmid pET-DCB from BL21 (DE3) cells and restriction digestion by NheI/XhoI enzymes, both the insert and the pET28a vector was found to be truncated when compared with that isolated from *E. coli* DH5α

When dcb gene was expressed using *E. coli* JM109 (DE3) as the host strain, D-carbamoylase activity was detected in the uninduced as well as in the cells induced by IPTG, as described in Materials and Methods. The D-carbamoylase activity was found to be twice as much in the induced cells as compared to the uninduced cells. This suggested that the gene was not being tightly controlled by T7 promoter resulting in a basal level expression of D-carbamoylase protein, even in the uninduced state.

Effect of low induction temperature

pET vector is known as one of the most powerful expression systems. An extremely high level of expression of D-carbamoylase was achieved in *E. coli* JM109 (DE3) cells (Fig. 2), after induction by as

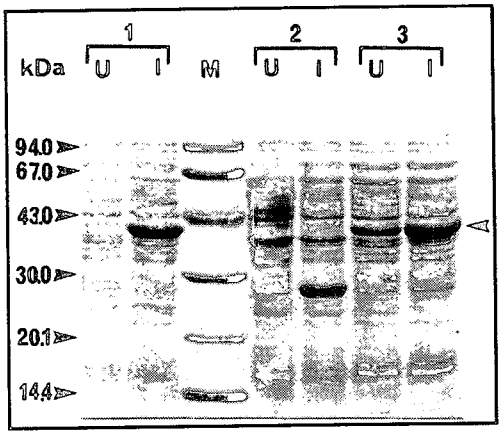

Fig. 1. Effect of host strains on the expression of D-carbamoylase.
Lane 1- Origami (DE3), lane 2- BL21 (DE3), lane 3- JM109 (DE3) where U stands for uninduced and I for induced fraction.

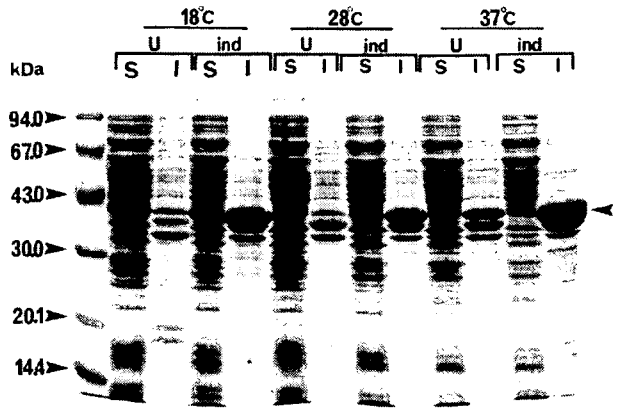

Fig. 2. Effect of temperature on induction by IPTG.
The culture was induced with 5 μM of IPTG at 18°C, 28°C and 37°C. The D-carbamoylase activity was estimated in the soluble fraction by GDH assay as described in Materials and Methods. S is soluble fraction and I stands for insoluble fraction, U stands for uninduced fraction and ind. for induced fraction.

little as 0.005 mM of IPTG. However, almost all of the enzyme was found in the insoluble fraction as an inclusion body, after induction for 3 h at 37°C, as has been found in earlier reports of overexpression of D-carbamoylase (9,10). Effect of high temperature has been shown to promote the formation of inclusion bodies (15). It has been suggested that a certain folding intermediate of the target protein is unstable at a high temperature *in vivo*, and hence the native conformation is not formed. Thus, it was likely that lower temperature might result in higher yields of the properly folded product (24). The activity was found to further increase 1.5-fold, when grown at 18°C overnight, as compared to cells grown at 37°C (Table 2). Densitometric analysis of total protein from overnight grown cells at 18°C revealed that the overproduced D-carbamoylase corresponded to more than 50 % of the total cellular proteins, out of which only 22% was present in the soluble fraction and the rest of it formed inclusion bodies and hence was detected in the insoluble fraction. Under these conditions, a 9-fold increase in enzyme production (expressed as μmol/min./O.D.$_{600}$) was achieved in recombinant *E. coli* cells when compared with the wild type strain *A. tumefaciens* AM 10.

Expression level of D-carbamoylase in *E. coli* Origami™ (DE3)

It has been suggested that *E. coli* cytoplasm is too reducing for many disulfide bonds to form (25). In fact, when many exported proteins that ordinarily form disulfide bonds are expressed in the cytoplasm, they do not form these bonds (26,27) and the presence of oxidized cytoplasmic environment of *E. coli* mutants missing thioredoxin reductase allows proper disulfide bond formation (28). Since, D-

Table 2. Effect of induction temperature and GroEL/ES on D-carbamoylase production by *E. coli* JM109 (DE3)

	D-carbamoylase activity (μmol/min./O.D.$_{600}$)		
-GroEL/ES	18 °C	28 °C	37 °C
Uninduced	0.032	0.023	0.025
Induced	0.040	0.030	0.026
+GroEL/ES			
Uninduced	0.101	0.062	0.008
Induced	0.173	0.186	0.009

carbamoylase is known to possess 2 disulfide bonds (19), it was highly likely that the inclusion body formation was the result of lack of proper disulfide bond formation in the overexpressed protein and hence the active conformation was not being attained. Therefore, it would be interesting to see the expression level of D-carbamoylase using Origami (DE3) as the host strain (25) which has a mutation in the components of two reducing pathways i.e. thioredoxin (trx B) and glutathione pathway (gor). For this, *E. coli* Origami (DE3) strain was electrotransformed by pET vector carrying dcb gene. The recombinant strains were grown and induced by 0.005 mM IPTG at 37°C for recombinant protein production. However, the results showed a further decrease in the active protein production as judged by measuring the enzyme activities (data not shown). Effect of induction temperature was further checked for the possible increase in enzyme activity. However, unlike the results with JM109 (DE3) cells, D-carbamoylase still tended to aggregate in the insoluble fraction on expression in Origami (DE3) cells, regardless of the temperature used for its induction (Fig. 3).

On the contrary, Chao et al (17) coproduced increased level of thioredoxin alongwith the recombinant D-carbamoylase in an attempt to make the redox state of the *E. coli* cytoplasm more reducing. They expected that the reducing environment would favour the proper folding of the recombinant protein, as it would prevent the aberrant formation of intramolecular disulfide bonds. However, the results showed no improvement in the active protein production. The fusion of D-carbamoylase with thioredoxin further resulted in 22-fold less activity than the unfused enzyme. The observations of this group and our study on Origami (DE3) strain shows that the formation of inclusion bodies by the overexpressed D-carbamoylase is not attributed to disulfide bond formation.

Coexpression of D-carbamoylase with GroEL/ES

Protein folding is the process by which the linear information contained in the amino-acid sequence of a polypeptide gives rise to the well-defined three-dimensional conformation of the functional protein. How this is accomplished constitutes a central problem in biology. Because unfolded proteins can reach their active state spontaneously *in vitro* (29), it was assumed that the folding (acquisition of tertiary

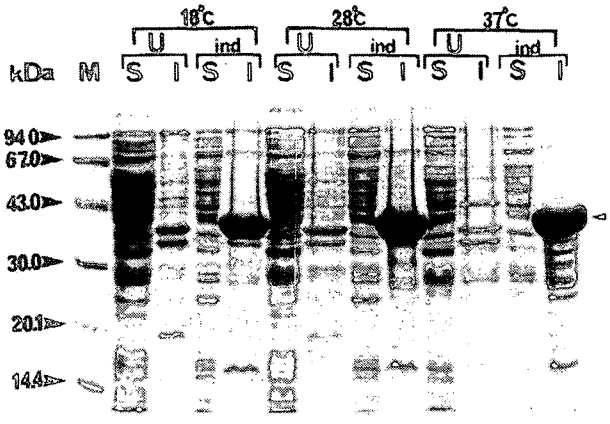

Fig. 3. Effect of postinduction temperature on the solubility of D-carbamoylase in uninduced and induced *E. coli* strain Origami (DE3).

The culture was induced with 5 μM of IPTG at 18°C, 28°C and 37°C. The D-carbamoylase activity was estimated in the soluble fraction by GDH assay as described in Material and Methods. S is soluble fraction and I stands for insoluble fraction, U stands for uninduced fraction and ind. for induced fraction.

structure) and assembly (formation of protein oligomers) of newly synthesized polypeptides *in vivo* does occur essentially uncatalyzed and without the input of metabolic energy. This long-held view has been revised in recent years owing to the discovery that in the cell, the correct folding of many proteins depends on the function of a pre-existing protein machinery-the molecular chaperones (30-32). One of the best characterized molecular chaperones is GroEL from *E. coli*, that binds the unfolded proteins tightly and releases them in a folded form, after hydrolysis of ATP and interaction with GroES (33). It has been demonstrated that GroEL is able to facilitate the correct folding of some recombinant proteins by preventing the formation of inactive aggregates (15-17). In light of earlier reports, we investigated co-expression of D-carbamoylase with the *E. coli* chaperonin GroEL/ES. For this, plasmid pKY206 carrying GroEL/ES gene, was cotransformed with pET-DCB into *E. coli* strain JM109 (DE3). The GroEL/ES was produced constituitively, while the production of D-carbamoylase was induced by the addition of 0.005 mM IPTG (Fig. 4). Interestingly, it was found that the coexpression of GroEL/ES sharply increased the solubility of D-carbamoylase, only when the cells were induced at a lower

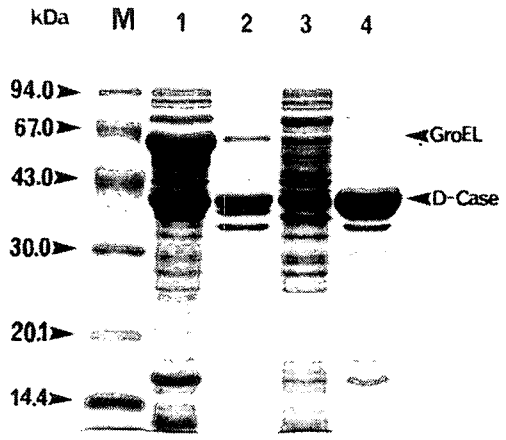

Fig. 4. Effect of molecular chaperones on the solubility of recombinant D-carbamoylase.
The enzyme was induced with 5 μM IPTG at 18°C for overnight, where lane 1- soluble fraction of cells carrying both pKY206 and pET-DCB, lane 2 - insoluble fraction of the same, lane 3 - soluble fraction of the cells carrying only pET-DCB, lane 4 - insoluble fraction of the same.

Table 3. Ratio of D-carbamoylase overproduced in soluble* and insoluble* fractions

Induction temperature (°C)	Soluble (%)	Insoluble (%)
- GroES/EL		
37	2	98
28	10	90
18	22	78
+ GroES/EL		
37	5	95
28	60	40
18	80	20

* Fractions were prepared as described under Materials and Methods. Each fraction was electrophoresed on 12.5% SDS-PAGE followed by Coomassie staining and was scanned by the densitometer. Total amount of D-carbamoylase produced under the specified conditions was taken as 100%.

temperature. Densitometric analysis revealed that the overexpressed D-carbamoylase accounted for 40% of the total cellular proteins.

Synergistic effect of low temperature and coexpression of GroEL/ES

The presence of overexpressed GroEL/ES hardly made any difference in the solubility of D-carbamoylase, when the cells were induced with IPTG at 37°C. But, on induction at 28°C or 18°C, the ratio of the soluble form of D-carbamoylase exceeded the insoluble form (Fig. 5). More than 75 % of the D-carbamoylase was expressed as the soluble form, on induction at 18°C (Table 3) and the total activity increased 4.5-fold when compared without coexpression of GroEL/ES (Table 2). The overall increase in productivity in this system was 43-fold in comparison to the wild-type strain *A. tumefaciens* AM 10. The growth rate was comparatively slow at 18°C and the final growth yield also remained at a lower level, than the cells cultured at 28°C. The level of expression of the target protein also decreased with decrease in growth temperature and this lower target protein concentration might have helped in preventing the formation of the insoluble aggregates. Although, the cells cultured at 28°C resulted in 60% solubilization of the total expressed target protein, but the enzyme activity was found to increase 6.2-fold than the cells grown under the same conditions without coexpression of GroEL/ES. This system led to 43-fold increase in productivity of D-carbamoylase than the wild type strain *A. tumefaciens* AM 10. Chao et al (17) obtained a four-fold increase in enzyme activity of the recombinant D-carbamoylase from *Agrobacterium radiobacter* NRRL B11291 by the coproduction of GroEL/ES.

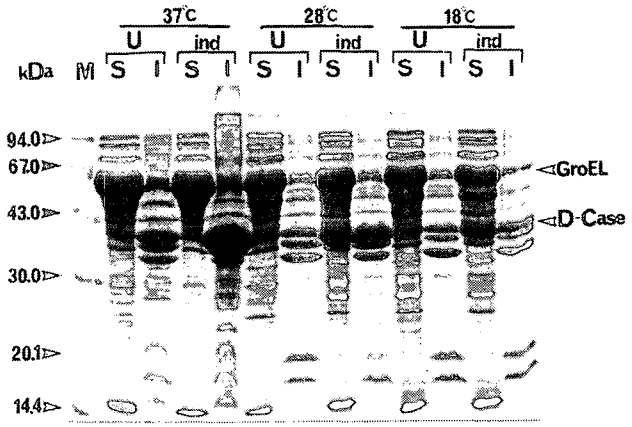

Fig. 5. Synergistic effect of post-induction temperature and co-expression of GroEL/ES on the solubility of D-carbamoylase in uninduced and induced *E. coli* strain JM109 (DE3).

The culture was induced with 5 μM of IPTG at 18°C, 28°C and 37°C. The D-carbamoylase activity was estimated in the soluble fraction by GDH assay as described in Materials and Methods. S is soluble fraction and I stands for insoluble fraction, U stands for uninduced fraction and ind. for induced fraction.

Table 4. Purification of D-carbamoylase from *E. coli* JM109 (DE3) with coexpressed GroEL/ES chaperonin

Purification stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
Cell free extract	3.8	4.35	1.14	100
Immobilized metal affinity chromatography	0.34	3.23	9.5	74

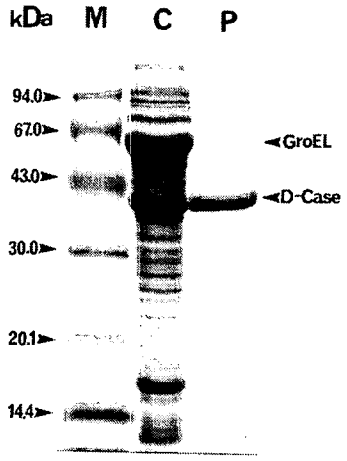

Fig. 6. Purification of D-carbamoylase by single step Ni-affinity chromatography.
Lane C shows total soluble proteins prepared from *E. coli* JM109 (DE3) carrying both pET- DCB and pKY206, lane P- purified D-carbamoylase. The standard proteins used were phosphorylase b (94 kDa), albumin (bovine, 67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

However, the enzyme still tended to aggregate as inclusion bodies regardless of the temperature used to grow *E. coli* cells.

Purification of D-carbamoylase

The Histidyl-tagged D-carbamoylase was purified by immobilized metal affinity chromatography on Ni-NTA resin in a single step from the culture induced at 28°C (Fig. 6). The enzyme was found to have a specific activity of 9.5 U/mg protein (Table 4). This was in accordance with the specific activity of the enzyme (7.88 U/mg protein) purified from *A. tumefaciens* AM 10 utilizing two steps (18). This shows that tagging of six consecutive histidyl residues and a thrombin cleavage site to the amino terminus did not affect D-carbamoylase activity, so the cleavage of the tag by thrombin was not required. A production yield of ~45 mg protein was obtained per liter of the bacterial culture.

The present study thus shows that the overproduction of GroEL/ES protein and low induction temperature resulted in a decrease in the amount of inclusion bodies with a significant increase in that of the active enzyme and was independent of the redox state of the cell.

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