Dihydroorotase from *Escherichia coli*

CLONING THE prc GENE AND PRODUCTION OF TRYPTIC PEPTIDE MAPS*

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We have inserted a 1.7-kilobase pair *Escherichia coli* DNA fragment containing the 1-kilobase pair *prc* gene into the high copy number plasmid pKC16. Dihydroorotase expressed by the *prc* plasmid in *E. coli* constituted 6.3% of the soluble protein in frozen cell paste. Pure dihydroorotase derived from this frozen cell paste was compared with pure enzyme derived from an *E. coli* strain lacking the *prc* plasmid: tryptic peptide maps from the two dihydroorotase preparations, produced using reverse-phase high performance liquid chromatography, were indistinguishable. We conclude that the entire *prc* gene is present on the hybrid plasmid and that the dihydroorotase produced from this plasmid is identical to the wild type.

Dihydroorotase (L-5,6-dihydroorotate amidohydrolase; EC 3.5.2.3) has been purified previously from a strain of *Escherichia coli* (ATCC 29477) that contains the chromosomally derived enzyme as 0.23% of its soluble protein, a 5- to 8-fold enrichment over wild type (Washabaugh and Collins, 1984). That purification procedure required approximately 1 month to produce 10 mg of enzyme, beginning with frozen *E. coli* (ATCC 29477) cell paste. We present here procedures for inserting the 1-kilobase pair *E. coli prc* gene into a high copy number plasmid to produce a strain of *E. coli* containing elevated levels of dihydroorotase. This strain will allow the convenient preparation of gram quantities of dihydroorotase. We also compare peptide maps from purified *prc* plasmid-derived and chromosomally derived enzyme to evaluate the effects of our cloning procedures on the structure of the enzyme.

After our cloning was completed, Jensen *et al.* (1984) reported the insertion of the *E. coli prc* gene on a 3-kilobase pair DNA fragment from the same Clarke-Carbon collection plasmid (Clarke and Carbon, 1976) into pBR322. This recombinant plasmid, which produced dihydroorotase as approximately 0.5% of the total soluble protein, was used for studies on the control of gene expression. The work we report here, which uses a smaller *E. coli* DNA insert, is focused on maximizing the yield of dihydroorotase and establishing that the overproduced enzyme is unaltered.

EXPERIMENTAL PROCEDURES, RESULTS, AND DISCUSSION

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*Experimental Procedures," *Results," *Table 1, Figs. 1–4, "Discussion," and "Acknowledgments" are presented in miniprint at the end of this paper. The abbreviations used are: TPC, L-1-tosylamido-2-phenylchloromethyl ketone; HPLC, high performance liquid chromatography; kb, kilobase pair. Miniprint is easily read with the aid of standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9560 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-3381, cite the authors, and include a check or money order for $7.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press."
Cloning E. coli pyrC

**EXPERIMENTAL PROCEDURES**

**MATERIALS:**

Deionized water was used in the experiments. The E. coli strain used in this study was a derivative of the wild-type E. coli strain K-12. The strain was grown in Luria Broth (LB) media. The plasmid used in this study was pBR322, which contains the pyrC gene. The plasmid was isolated from E. coli K-12 using the alkaline lysis method.

**METHODS:**

**Cloning E. coli pyrC**

The pyrC gene was isolated from a genomic library of E. coli K-12. The library was screened using a probe corresponding to the pyrC gene. A positive clone was identified and the plasmid was isolated. The plasmid was then sequenced and the sequence was verified.

**GROWTH CONDITIONS**

The E. coli strain was grown in LB media supplemented with 10 µg/mL of trimethoprim (TMP) and 50 µg/mL of streptomycin (STR). The growth was monitored by measuring the optical density at 600 nm (OD600).

**SEROLOGICAL AND PROTEIN ANALYSIS**

Antisera for enzyme assay were prepared according to Watson and Gouin (1985). Antibody specificity was determined by Western blot analysis. The antibodies were affinity-purified using the pyrC gene fusion protein embedded in polyethylene glycol (PEG). The antibodies were then used to detect the expression of the pyrC gene in vivo.

**GROWTH OF CELLS WITH HIGH LEVELS OF DIPHOTOPHOSPHATE**

Bacterial cultures were grown at 30°C for 6-8 h and shifted to 42°C for production of diphosphoglycerate (DPG) with the presence of 50 mM glucose. The growth was monitored by measuring the optical density at 600 nm (OD600) and the formation of DPG was analyzed by thin-layer chromatography (TLC). The cultures were harvested and the cells were disrupted using a French press. The resulting crude extract was subjected to enzyme assay.

**PREPARATION OF CELL FREE SYSTEMS**

Cell-free extracts were prepared using E. coli strain K-12 transformed with the plasmid pBR322 containing the pyrC gene. The cells were grown in LB media supplemented with 10 µg/mL of trimethoprim (TMP) and 50 µg/mL of streptomycin (STR) at 30°C. The cells were harvested by centrifugation and the cell pellets were suspended in 50 mM potassium phosphate (pH 7.5) and 10 mM magnesium chloride. The cell suspension was then disrupted using a French press. The resulting crude extract was subjected to enzyme assay.
Cloning E. coli pyrC

Our failure to produce high levels of dihydroorotase using the E. coli promoter in pEE led us to clone the 89-22 segment into the high-copy-number plasmid pHC (Figure 3). Figure 2 shows that when E. coli strain ET/19a (pET) containing pHC was grown in modified M56 medium for 4 h at 30°C, and then shifted to 42°C for 6 h, dihydroorotase was found at high levels in the time interval 12-24 h.

The highest specific activity was consistently attained when E. coli strain MM13 containing pHC was incubated in modified M56 plus 1 g/liter tryptone in a volume of 0.05 ml, adjusted to pH 7.0, and incubated at 30°C for 5-6 h. The highest specific activity was then shifted to 42°C for growth, and harvested on an agar plate to increase the specific activity (Figure 5).

A 1-liter culture of E. coli strain MM13 containing the cloned pHC was grown in a fermentor under those conditions, yielding 350 g of cell paste. The specific activity of dihydroorotase in the sample taken from the fermenter during growth was 150 units per mg of protein when assayed at 30°C at pH 7.0 at a final substrate concentration of 0.03 M. The specific activity of dihydroorotase in the sample taken from the fermenter during growth was 23 units per mg of protein when assayed at 30°C at pH 7.0 at a final substrate concentration of 0.03 M. The specific activity of dihydroorotase in the sample taken from the fermenter during growth was 23 units per mg of protein when assayed at 30°C at pH 7.0 at a final substrate concentration of 0.03 M. The specific activity of dihydroorotase in the sample taken from the fermenter during growth was 23 units per mg of protein when assayed at 30°C at pH 7.0 at a final substrate concentration of 0.03 M.

The presence of the pyrC gene was confirmed by hybridization to the cloned DNA. The specific activity of dihydroorotase increased at least ten-fold when grown dihydroorotase concentrations less than about 100 mg/ml were used in the assay mixture (also see Hashbash & Collins, 1981).

Cloning plasmid pHC16 from E. coli strain ET/19a provided the representative tryptic peptide maps illustrated in Figure 7.

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