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RETENTION OF ENZYMATIC ACTIVITY BY N-TERMINAL DOMAIN (1-78) T4-LYSOZYME: EXPRESSION OF SYNTHETIC DNA IN ESCHERICHIA COLI

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DNA of 235 b.p. coding for N-terminal domain (1-78) T4-lysozyme was synthesized and cloned by ligating twelve synthetic fragments with a linearized plasmid pUCE8 followed by transformation. On expression in *E. coli* strain JM103 cells, colonies containing the synthetic DNA were found to be lytic. On purification, clone ptly.23-5 was found to contain polypeptide (M.W. 10,500), corresponding to N-terminal domain, its dimeric and aggregate form. It was identified by amino acid sequence analysis of the dimeric form. © 1987 Academic Press, Inc.

T4-lysozyme devoid of disulfide bridge, is a two-domain protein: the N-terminal domain (1-71) is form type ($\alpha+\beta$) and C-terminal domain (72-164) is "all-α" (1). The two domains are linked by a helical bridge (60-79). Attempts to obtain a large fragment corresponding to domains have failed so far (2). In this communication, we wish to report the chemical synthesis, cloning and stable expression of DNA coding for N-terminal domain (1-78) T4-lysozyme maintaining its enzymatic activity.

MATERIALS AND METHODS

(a) Deoxyribonucleotides, enzymes and general techniques

Deoxyoligonucleotides were synthesized by using a model 380A DNA synthesizer (Applied Biosystem, Foster City, CA).

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The unprotected oligomers were purified by electrophoresis in 12% polyacrylamide gel containing 7M urea. Transformations and plasmid DNA purification were performed according to standard procedures (3). Sequencing of DNA was carried out by the dideoxy method (4). T4-lysozyme activity was measured by a modification of the turbidity assay described by Tsugita et al. (5).

(b) Chemical synthesis and cloning of DNA coding for N-terminal domain (1-78) T4-lysozyme

The DNA of 235 b.p. was synthesized and cloned in the one step ligation procedure as outlined in Figure 1B.

Each of the twelve synthetic fragments (ℓ-1, ℓ-2, ℓ-3, ℓ-4, ℓ-5, ℓ-6, ℓ-7, ℓ-8, ℓ-9, ℓ-10, ℓ-11 and ℓ-12) (5 pmol) were phosphorylated individually in 5 µl kinase buffer (50 mM Tris.HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 5 mM DTT and 0.1 mM ATP) containing 0.5 u of T_4 polynucleotide kinase. After incubation at 37°C for 2 hr, samples were heated to 90°C for 5 min, chilled and centrifuged. Then, each fragment (1.5 µl) was mixed and annealed slowly in a water-bath to room temperature in a 3-hr time period. Plasmid pUCE8 (Narang et al., manuscript in preparation) was cut with EcoRI + BamHI, treated with calf intestinal alkaline phosphatase and added to the annealed fragments along with 3.5 µl of 10× ligase buffer, 3.5 µl mM ATP, 15.0 µl H_2O and 7 u of T_4 polynucleotide ligase. The reaction mixture was incubated at 12.5°C overnight and used for transforming competent E. coli strain HB101 cells. Transformant colonies were picked at random and analyzed by colony hybridization with two ^{32}P-labelled oligomers (ℓ-2 and ℓ-5) and confirmed by DNA sequence analysis.

(c) Expression

Expression was carried out in Escherichia coli strain JM103 [Δ(lac-pro), thi, strA, supE, endA, sbcB, hsd, R, ftraD36, proAB, lacI9, lacZΔM15].

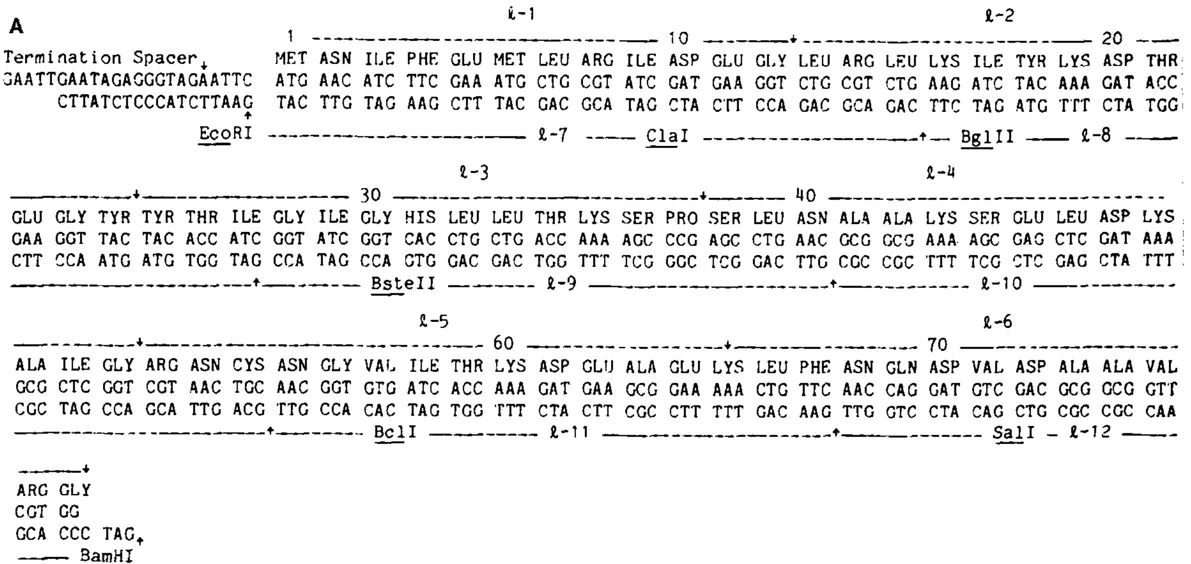
A batch of bacterium culture (9 l) was inoculated with a log phase inoculum (20 ml/l) and shaken in an incubator at 32° overnight. The culture was supplemented with ampicillin chloride (50 µg/ml) followed by further supplementation (50 µg/ml) next day. The incubation temperature was increased to 35-37°C for 4 h. Cells were harvested by centrifugation and frozen at -20° for 2 days at least prior to extraction.

The polypeptide containing T4-lysozyme activity was purified according to the procedure of Perry and Wetzel (5) with the following modification. Extraction buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 M PMSF, pH 8.0) 20 ml/g of wet cells was used. Cells were sonicated 5×, 15-sec strokes at 45-sec intervals, followed by the addition of 0.7 ml of 5% PEI-HCl pH 7.5 with stirring for 20 min. After 10 min, 0.5 g/100 ml of CDR (Waters) was added followed by stirring for another 10 min. Spinning at 10,000 rpm for 10 min provided a clear extract. The extract was then loaded onto tandem columns of DE-52 and CM-52 (6 ml each) and equilibrated with a buffer (50 mM Tris-HCl, 3 mM mercaptoethanol, 1 mM EDTA, pH 8.0). The enzyme was eluted from CM-52 column by eluting with a linear gradient of 0-1 M NaCl in equilibrating buffer. Fractions (2.3 ml) were collected and assayed for T4-lysozyme activity, protein level and mobility on

PAGE. Fractions containing the enzymatic activity were pooled, further purified and analysed by electrophoresis on SDS gel with the appropriate marker (Figure 2).

RESULTS AND DISCUSSION

DNA of 235 b.p. coding for N-terminal domain (1-78) T4-lysozyme (Figure 1A) was chemically synthesized by one-step ligation of twelve deoxyribooligonucleotide fragments with a linearized plasmid pUCE8 at EcoRI-BamHI sites followed by transformation of *E. coli* strain HB101 competent cells. Selection was done by colony hybridization with ^{32}P-labelled fragments (ℓ-2 and ℓ-5) and confirmed by DNA sequence analysis. For expression, *E. coli* strain JM103 cells were used. At 37°, the cells were lytic, indicating the appearance of enzymatic activity corresponding to T4-lysozyme. For isolation purposes, the cell growth conditions were modified to an incubation temperature at 32°C with half the desired dose of ampicillin. Under these conditions, the cell grew normally (i.e. without lysis). On purification from clone ptly.23.5, the polypeptide fractions containing T4-lysozyme activity were analysed by



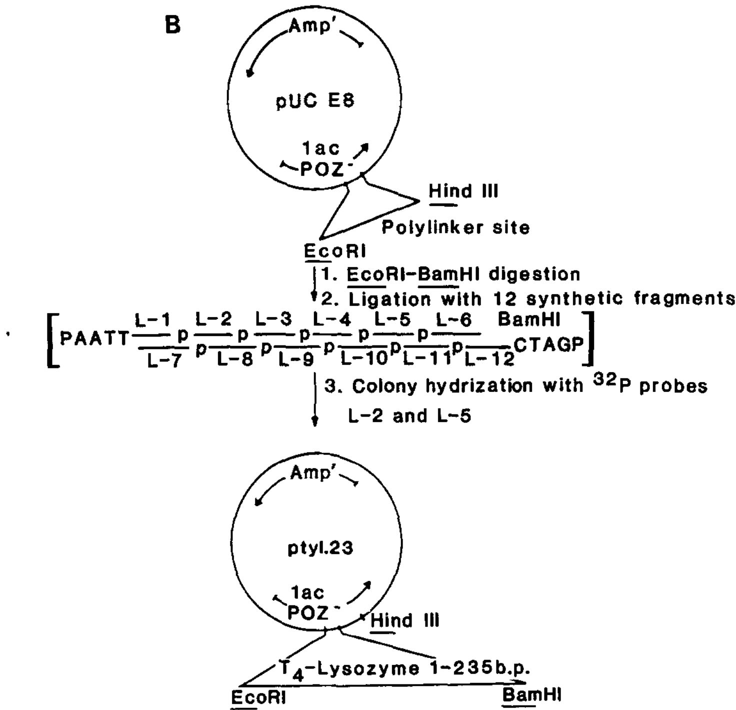


Figure 1A. Nucleotide sequence of synthetic DNA coding for N-terminal domain (1-78) T4-lysozyme DNA. This sequence begins with EcoRI restriction sites and ends with BamHI restriction sites. Synthetic DNA of 235 b.p. was assembled from 12 synthetic fragments, ℓ-1 (41-mer), ℓ-2 (42-mer), ℓ-3 (33-mer), ℓ-4 (42-mer), ℓ-5 (41-mer), ℓ-6 (36-mer), ℓ-7 (46-mer), ℓ-8 (41-mer), ℓ-9 (34-mer), ℓ-10 (42-mer), ℓ-11 (39-mer), ℓ-12 (33-mer). Vertical arrows indicate the junction of synthetic fragments.

Figure 1B. Assembly and cloning of synthetic DNA coding for N-terminal domain (1-78).

electrophoresis on SDS of a gel with appropriate markers (Figure 2). Two protein bands corresponding to monomer* (M.W.

*Extra nineteen amino acids were fused at the c-terminal of T4-lysozyme 1-78 polypeptide due to DNA construction in plasmid ptly.23.5.

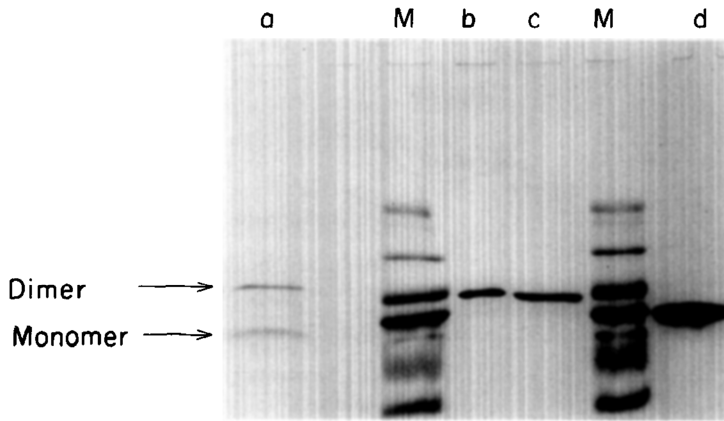


Figure 2. Electrophoresis on Na Dod SO_4/15% polyacrylamide gel and stained with Coomassie brilliant blue. Lane a: contains polypeptide pooled from fraction 6-11 from CM-52 column; Lane b: polypeptide in fraction 4 from CM-52 column; Lane c: native full-length T4-lysozyme; Lane d: HEW lysozyme; and Lane M: protein standard; from top to bottom: 43,700, 25,700, 18,400, 14,300, 12,300, 6,200 and 300. Arrow at left indicates bands for dimeric and monomeric form of T_4 lysozyme, half molecule under consideration.

10,500) and dimer (M.W. 21,000) were found in pooled fractions 6-11 whereas fraction 4 mainly contained a dimeric form. The dimeric polypeptide was purified and analysed for amino terminal sequence. Its 15 amino acid sequence corresponded to the expected data. On examination, cells containing plasmid ptly.23.5 under microscope showed inclusion bodies indicating the formation of aggregate (data not shown) which has also been observed in expressing eukaryotic proteins such as proinsulin (6). The formation of aggregate might be due to intramolecular interaction (7) of the left half of the T4-lysozyme portion. This aspect is under further investigation.

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