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PURIFICATION OF PENICILLIN-INSENSITIVE DD-ENDOPEPTIDASE, A NEW CELL WALL PEPTIDOGLYCAN-HYDROLYZING ENZYME IN ESCHERICHIA COLI, AND ITS INHIBITION BY DEOXYRIBONUCLEIC ACIDS

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SUMMARY: Activity of a penicillin-insensitive DD-endopeptidase that splits the D-alanyl-meso-2,6-diaminopimelyl linkage in peptidoglycan was demonstrated in a sonic extract of Escherichia coli. The protein with this activity was partially purified. The activity was inhibited by 3 μg per ml of deoxyribonucleic acid, suggesting that this cell wall hydrolytic enzyme is regulated by deoxyribonucleic acid or its fragments.

Two enzymatic activities of DD-endopeptidase, which splits the D-alanyl-meso-2,6-diaminopimelyl(m-A_2pm) linkage in cell wall peptidoglycan, have been found in E. coli(1,2). One of these activities is very sensitive to penicillin G or ampicillin and is identical with D-alanine carboxypeptidase IB(1,3,4); its function is unknown(1,5,6). The other DD-endopeptidase activity(briefly described in ref. 1 and 2, but not elsewhere) is insensitive to penicillin. Recently it has become recognized that peptidoglycan-hydrolyzing enzymes are important, not only in cell-autolysis, but also in normal cell growth and cell division. Elongation and septation of peptidoglycan sacculus must be performed by a series of lytic (nicking) and synthetic (polymerizing and crosslinking) enzymes. Penicillin-insensitive peptidoglycan-hydrolyzing enzymes may also be important in cell-lysis induced by penicillin, an inhibitor of crosslinking enzymes. For studies on the character of the newly discovered DD-endopeptidase, this enzyme must be purified, and for studies on its function a mutant lacking the enzyme must be isolated or specific inhibitors of the enzyme must be found.

Abbreviations used are: m-A_2pm, meso-2,6-diaminopimelic acid; MurNAc, N-acetylmuramic acid; rRNA, ribosomal RNA; tRNA, transfer RNA.

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This report describes the partial purification and several properties of this new DD-endopeptidase in E. coli, showing that the enzyme is strongly inhibited by deoxyribonucleic acids.

MATERIALS AND METHODS

Culture of cells and preparation of a crude extract: E. coli strain JST752, derived from E. coli strain K-12 JE1011, was used as an enzyme source. The isolation and properties of this strain will be reported elsewhere. Strain JST752 has a defect in N-acetylmuramyl(MurNAc)-L-alanyl-amidase. The absence of amidase activity greatly facilitated DD-endopeptidase assay, because this amidase degrades both the substrate and product of the DD-endopeptidase reaction Cells grown in Difco Penassay broth (double strength) at 30°C were harvested in the late logarithmic growth-phase and washed once with 0.05 M Tris-HCl buffer, pH 7.6 (Buffer A). Cells from 1 liter of culture (3.5 g wet weight) were frozen at -20°C and thawed, and then suspended in 28 ml of Buffer A and sonicated at 10 KHz (Toyo-Riko Sonicator, Tokyo, Japan) for 10 min under cooling with ice cold water. Cell debris were removed by low speed centrifugation and the supernatant of high speed centrifugation at 100,000 x g for 60 min was used as the crude cell extract.

Purification of the enzyme: The crude cell extract (30 ml containing 330 mg protein) was fractionated with ammonium sulfate and the material precipitating between 0.35 and 0.60 saturation was dissolved in Buffer A and dialyzed overnight against 100 volumes of the same buffer with one change of the buffer. The enzyme solution at this step could be stored at -20°C for several weeks without appreciable loss of activity. The dialyzed enzyme solution (6.7 ml containing 102 mg protein) was applied to a column (1.8 cm x 11.5 cm) of DEAE-cellulose (Whatman DE-52) equilibrated with Buffer A. Most of the penicillin-insensitive DD-endopeptidase activity was recovered in the flow-through fraction with a little in the eluate obtained by washing the column with the same buffer solution containing 0.2 M NaCl. The portion of the flow-through fraction containing most of the enzyme activity was concentrated to about 2 mg protein per ml using 20 %(wt/vol) polyethylene glycol 6000 in 0.05 M Tris-acetate buffer, pH 6.2 (Buffer B), and then promptly dialyzed against Buffer B. The enzyme solution (1.22 ml containing 2.74 mg protein) was kept at 0°C for use in further experiments.

Enzyme assay: For enzyme assay, a solution in a final volume of 30 µl containing 0.05 M Tris-acetate buffer, pH 6.0, 2.2 nmol of labeled GlcNAc-MurNAc-L-Ala-D-Glu-m-[^{14}C]A2pm-D-Ala GlcNAc-MurNAc-L-Ala-D-Glu-m-[^{14}C]A2pm-D-Ala (called bis[disaccharide-tetrapeptide], 4.0 Ci/mol) and 1 mg per ml of potassium penicillin G, was preincubated for 2 min at 37°C and the reaction was started by adding 1 µl of enzyme solution containing 0.07-0.15 units of activity. After incubation for 5 to 10 min at 37°C, the reaction was stopped by adding EDTA to a final concentration of 20 mM and heating the mixture for 1 min at 100°C. Then the mixture was subjected to paper chromatography in isobutyric acid - 1 M ammonia (5:3, vol/vol) and the radioactivities in the positions of bis(disaccharide tetrapeptide) and disaccharide-tetrapeptide were located in a spark chamber (Birchover, England) and counted in a scintillation spectrometer using a toluene-PPO-POPOP (1 liter : 4 g : 100 mg) system (67 % counting efficiency). One enzyme unit was defined as the activity releasing 2 nmol of disaccharide-tetrapeptide per min at 37°C under these assay conditions.

Reagents: Crosslinked ^{14}C-labeled bis(disaccharide-tetrapeptide) was prepared from E. coli peptidoglycan labeled with m-[^{14}C]A2pm (New England Nuclear, 50 Ci/mol) as previously described(1-3). Penicillin G, potassium salt (Takeda Pharmaceutical Co., Osaka, Japan) and calf thymus DNA (PL-Biochemicals Inc.)

were commercial products. A purified preparation of Bacillus subtilis phage ϕ1E2Δ1 DNA was obtained from Dr. F. Kawamura, E. coli phage λ DNA was from Dr. K. Okada, E. coli ribosomal RNAs, E. coli tRNAs and E. coli phage MS2 RNA were from Dr. T. Ohta, B. subtilis lipoteichoic acid was from Dr. D. Ayusawa and Salmonella minnesota Re-lipopolysaccharide was from Dr. O. LÜderits.

RESULTS AND DISCUSSION

The specific enzyme activity was increased 380-fold with 390 % yield by the procedures described in the MATERIALS AND METHODS (Table 1). The crude cell extract seems to contain some strong inhibitor(s), because the total enzyme activity was increased considerably by ammonium sulfate fractionation and then dialysis. The activity of the partially purified enzyme after DEAE-cellulose fractionation and dialysis showed a normal dependence on time of incubation and amount of enzyme added (Fig. 1A). The optimal pH for the reaction was 6.0 at 37°C. The apparent Km value for bis(disaccharide-tetrapeptide) at pH 6.2 was 1×10^{-4} M, estimated from a Lineweaver-Burk plot of activity under the assay conditions described in the MATERIALS AND METHODS with substrate concentrations of less than 3.3×10^{-4} M.

The enzyme reaction reached completion when sufficient enzyme and 5 mM $CoCl_2$ were added to the reaction mixture. The stoichiometry of the reaction obtained is shown in Fig. 1B. The product was confirmed to be disaccharide-tetrapeptide by paper chromatography and paper electrophoresis. Moreover the appearance of free ε-amino groups of m-[^{14}C]A_2pm was demonstrated: for this the reaction product was eluted from the paper chromatogram, subjected to dinitrophenylation with dinitrofluorobenzene, hydrolyzed in 6 M HCl for 8 hr at 99°C and then subjected to thin layer chromatography on Silica Gel G (Merck) in isobutyric acid - 1 M ammonia (5:3, vol/vol). The ratio of mono-DNP-[^{14}C]A_2pm to free [^{14}C]A_2pm found in the reaction product was 1 : 0.08 (theoretically 1 : 0), while that in the substrate bis(disaccharide-tetrapeptide) was 1 : 1.4 (theoretically 1 : 1).

The enzyme required divalent cations for maximal activity, Co^{++} being the most effective of the divalent cations tested (Table 2, exp. 1). Mg^{++} and Ca^{++}

TABLE 1

Enzyme Purification [a]

Step	Specific activity (units/mg)	Purification	Total activity (units)	Yield (%)
Crude cell extract [b]	0.20	1	53.1	100
Ammonium sulfate(0.35-0.6) and dialysis	4.29	21.3	434	822
DEAE-cellulose column(flow-through) and dialysis [c]	76.3	380	209	394

[a] The experimental conditions and definition of enzyme activity are described in the MATERIALS AND METHODS. Purifications from other strains gave similar results.

[b] Cells from 1 liter of culture.

[c] At this stage 20 % of the total DD-endopeptidase activity was penicillin-sensitive, but the preparation obtained from a dacB (D-alanine carboxypeptidase IB defective) mutant(1) did not have this activity.

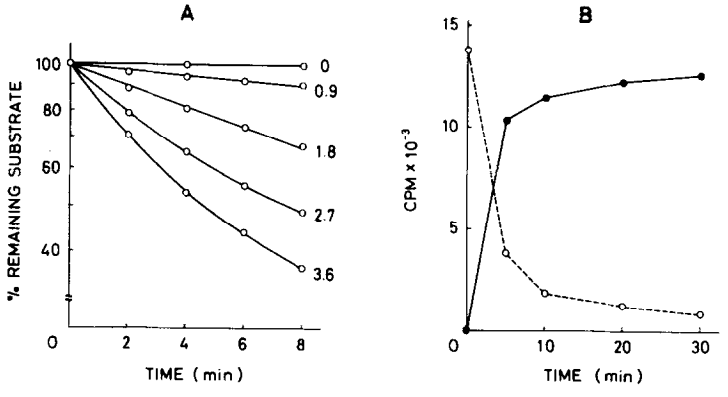

Figure 1. Enzyme kinetics. (A) Dependency of penicillin-insensitive DD-endopeptidase reaction on time of incubation and amount of enzyme added. Partially purified enzyme from the DEAE-cellulose step was used. For assay conditions see MATERIALS AND METHODS. Ordinate: percentage remaining bis(disaccharide-tetrapeptide); abscissa: time of incubation. Amounts of enzyme (in μg protein) are indicated in the figure. (B) Enzyme kinetics showing the stoichiometry of the reaction. The reaction mixture contained 0.52 units of enzyme, 2.2 nmol of bis(disaccharide-tetrapeptide) labeled with m-[^{14}C]A_2pm and 5 mM $CoCl_2$. Ordinate: cpm bis(disaccharide-tetrapeptide) (o) or disaccharide-tetrapeptide (•); abscissa: time of incubation.

TABLE 2

Compounds influencing the activity of penicillin-insensitive DD-endopeptidase in E. coli

Exp.	Addition	Activity (%)
1	None	100 [a]
	$CoCl_2$ 5 mM	240
	$CaCl_2$ 5 mM	137
	$MgCl_2$ 5 mM	133
	$MnCl_2$ 5 mM	115
	EDTA 1 mM	29
	Na citrate 10 mM	12
	Na phosphate (pH 6.0) 50 mM	19
2	None	100 [b]
	Calf thymus DNA 4.0 µg/ml	47
	" " 10.0 µg/ml	14
	" " 11.1 µg/ml, DNase treated	88
	Phage λ DNA 2.1 µg/ml	58
	" " 15.8 µg/ml	6
	" " 15.8 µg/ml, DNase treated	60
	Phage φ1E2Δ1 DNA 2.3 µg/ml	47
	" " 9.3 µg/ml	10
	$MgCl_2$ 5 mM	120
	$MgCl_2$ 5 mM plus calf thymus DNA 5.0 µg/ml	55
3	None	100 [c]
	E. coli rRNAs 15.5 µg/ml	99
	" " 77.7 µg/ml	62
	" tRNAs 23.6 µg/ml	81
	Phage MS2 RNA 17.8 µg/ml	67
4	None	100 [d]
	B. subtilis lipoteichoic acid 0.016 nM	59
	" " 0.16 nM	10
	" " 0.16 nM plus 5 mM $MgCl_2$	52
	S. minnesota lipopolysaccharide 5.0 µg/ml	46
	" " 50 µg/ml	3
	" " 100 µg/ml plus 5 mM $MgCl_2$	50
5	None	100 [e]
	Crude cell extract 1 µl	2
	" " 1 µl, boiled	1
	" " 1 µl, boiled & pronase treated	5
	" " 1 µl, boiled & DNase treated	48

The experimental conditions were as described in the MATERIALS AND METHODS, except that 1.1 nmol substrate was used in experiments 2 to 4. Concentration of lipoteichoic acid was calculated from the phosphate content assuming that 40 phosphate residues are involved in the molecule of lipoteichoic acid. Enzyme activity used was: a 0.10 units, b 0.14 units, c 0.12 units, d 0.07 units, and e 0.15 units. The reaction was carried out for 5 min.

were also stimulatory, but Ni^{++}, Zn^{++} and Cu^{++} were inhibitory (data not shown). Chelating agents such as EDTA, citrate and phosphate were also inhibitory. None of the substrate analogues so far tested, such as L-alanine, D-alanine, DL-alanyl-DL-alanine and glycyl-glycine (each 10 mM) were inhibitory (data not shown).

The most remarkable feature of this enzyme reaction is its inhibition by low concentrations of deoxyribonucleic acids (Table 2, exp. 2). Deoxyribonucleic acids of various origins, i.e., calf thymus, B. subtilis phage ϕ1E2Δ1, and E. coli phage λ, were all inhibitory, their 50 % inhibitory concentrations being 2.5 - 3.5 μg per ml. When these DNAs were treated with pancreatic DNase before incubation with the enzyme, their inhibitory effects were lost. Deoxyribonucleic acids were also inhibitory in the presence of 5 mM $MgCl_2$ or $CoCl_2$ (data not shown), their 50 % inhibitory concentrations being 2 - 5 μg per ml in these conditions. Ribonucleic acids, i.e., E. coli ribosomal RNAs, E. coli tRNAs and phage MS2 RNA, had no appreciable inhibitory effects (Table 2, exp. 3). Lipoteichoic acid from B. subtilis and Re (deep rough) lipopolysaccharide from Salmonella minnesota were also inhibitory and they did not lose their inhibitory effects when digested with DNase (Table 2, exp. 4). Lipoteichoic acid and lipopolysaccharide were also inhibitory in the presence of 5 mM $MgCl_2$, but their 50 % inhibitory concentrations increased 10 - 20 times in the presence of 5 mM $MgCl_2$. It is possible, that some contaminants in the preparations rather than lipoteichoic acid and lipopolysaccharide themselves are actually inhibitory. In any case, however, the results suggest that some compounds or factors other than deoxyribonucleic acids are also strong inhibitors of this enzyme.

Strong enzyme inhibition was observed in the crude cell extract (Table 2, exp. 5). The inhibitory factor(s) was stable to heat or pronase treatment but was inactivated by exhaustive digestion with pancreatic DNase. Although some other unknown inhibitors could be present, it seems probable that DNA fragments formed during sonic disintegration of the cells are the main inhibitors of this endopeptidase in the crude cell extract of E. coli. We are now attempting to determine the chain lengths and base sequences of the polydeoxyribonucleotides in the crude extract that are most inhibitory.

Penicillin-insensitive DD-endopeptidase may correspond to at least one of the three unidentified peaks of E. coli DD-endopeptidase activity separated by DEAE-sephadex column chromatography as described by Pelzer(7). The discovery

that the enzyme is specifically inhibited by DNA suggests a new view of the function of this enzyme: very probably the enzyme is important in cell-lysis, growth or septation of cells and its activity is regulated by DNA or polydeoxyribonucleotides. Deoxyribonucleotide-mediated enzyme regulation has been observed in "SOS functions" of E. coli(8), and the relation of this phenomenon with the present DD-endopeptidase should be investigated.

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