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Purification of extracellular alkaline phosphatase released by *Escherichia coli* excretory mutants

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Summary. Alkaline phosphatase (APase) is the major protein released into the extracellular medium by strain 706, a periplasmic-excretory (*lky*) mutant of *Escherichia coli* K12. We developed a rapid three step procedure for APase purification from culture supernatants of *lky* mutants. Two ultrafiltration stages and an heat treatment were sufficient to obtain a 99% pure enzyme preparation. Batch culture conditions of strain 706 in a 15 l fermentor leading to an extracellular APase yield of 1250 U/ml were determined.

Introduction

Alkaline phosphatase (EC 3.1.3.1; APase) of *Escherichia coli* hydrolyses organic phosphate esters (Torriani 1967). Active enzyme is a dimer of 90,000 dalton molecular weight, localized in the periplasmic space between the inner and outer membranes of the bacterial cell envelope. The periplasmic space contains about one hundred proteins: degradative enzymes (APase, Ribonuclease I, Endonuclease I) and binding proteins involved in active transport systems (Malamy and Horecker 1961; Beacham 1979). APase is encoded by gene *pho*A; enzyme synthesis is induced in response to a low level of inorganic phosphate in the culture medium (Torriani 1960). *pho*A gene expression is under the control of the phosphate regulon (*pho* regulon); mutations in the *pho*S, *pstA* (*phoT*), *pstB, pstC, phoU, phoV,* or *phoR* genes result in the constitutive synthesis of APase (Tommassen and Lugtenberg 1982; Nakata et al. 1984; Levitz et al. 1984; Rao et al. 1986). Outer membrane PhoE as well as periplasmic proteins P2 (sn-glycerol-3-phosphate binding protein) and P4 (phosphate binding protein) are coregulated with APase (P1) (Argast and Boos 1980).

For many years, bacterial and calf intestinal APases were available commercially, and used for clinical analysis and biological research work; high quality APase preparations are required for molecular biology experiments, including DNA and RNA sequence analysis or gene cloning (removal of 5′-terminal phosphates before ^{32}P labeling) (Maxam and Gilbert 1977; Maniatis et al. 1982).

E. coli APase is currently purified according to a seven step procedure described by Torriani (1967). The first step consists of a specific release of periplasmic fluid: cells are converted into spheroplasts by EDTA and lysozyme treatment (Malamy and Horecker 1964), or alternatively bacterial outer membrane is destabilized by EDTA action and a cold water wash, the "osmotic shock" (Neu and Heppel 1965).

This prerequisite extraction is no more necessary with the availability of periplasmic-leaky mutants (*lky*) which spontaneously release into the culture medium up to 80% of total APase activity synthesized during growth (Lazzaroni and Portalier 1981, 1985). Extracellular APase yield has been improved by optimizing growth physiological conditions and by using *lky* strains carrying a constitutive APase mutation (*phoS*) allowing APase synthesis even during growth in a high phosphate rich medium (Atlan and Portalier 1984). In this paper, we report a new and easy method for facilitated APase purification from culture supernatant of a suitable excretory mutant which constitutively synthetized this enzyme, and we prove that APase extracellular production may

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Abbreviation: APase, *E. coli* alkaline phosphatase

be scaled up by growing *lky* mutants in fermentors.

Materials and methods

Strains. The *E. coli* K12 strain 706 used in this work was derived from the polyauxotrophic F^- PA 601 (Atlan and Portalier 1984). The relevant genotype of strain 706 was *thr, leuC, proA, lkyB207, his, rpsL phoS, argH.*

Media and culture conditions. Cells were grown in a modified Luria Broth medium (LB 8.3) as previously described (Atlan and Portalier 1984). Routinely, 5 ml cultures in 20 mm diameter test tubes or 25 ml cultures in 250 ml Erlenmeyer flasks were inoculated with 8 h subcultures developed in the same medium, and incubated overnight at 37° C under mechanical shaking for oxygenation. When necessary, 1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG; a gratuitous *β*-galactosidase inducer) sterilized by filtration on 0.22 μ Millipore membrane, or antifoam agents autoclaved at 120° C for 20 min were added to sterile medium culture.

For fermentor cultivation, we used PPL 8.0 medium containing the following ingredients (per liter): yeast extract (Difco) 5 g, proteose-peptone (Difco) 10 g, NaCl 4 g; pH was adjusted to 8.0 with 10 N NaOH before autoclaving at 120° C for 20 min.

Fermentor cultivation. Batch cultivation was carried out in a 15 l Biolafitte fermentor (INSA, Toulouse), steam sterilized, filled with 10 l of PPL 8.0 broth containing antifoam agent (Silicone Rhodorsil H26, 0.6 g/l) and inoculated with 550 ml of 9 h subculture obtained by shaken flask cultivation at 37° C. The agitation rate was 300 rpm (±1%) and the temperature was thermostatically controlled at 37±0.2° C. The automatic addition of 2.4 N HCl, controlled by an indicating-regulating Tacussel pH-meter (Villeurbanne, France), allowed to maintain pH at 8.0±0.1. The aeration rate was 0.8 VVM, and dissolved oxygen level was detected by an Ingold oxygen electrode and monitored with a type PO2-NUM Tacussel oxymeter.

Biomass. Biomass was estimated by measuring the turbidity of cell suspensions in a Lambda 3 Perkin Elmer spectrophotometer: 1.0 unit of absorbance at 600 nm corresponds to 0.4 mg of bacterial dry weight/ml.

Protein determination. The method of Schacterle and Pollack (1973) was used to estimate protein concentration with bovine serum albumin as standard.

Extracellular and intracellular extracts. 3 ml aliquots of culture were centrifuged (6,000 g, 10 min, 4° C); supernatants (extracellular extracts) were harvested and bacterial pellets (intracellular extracts), resuspended in 1 ml of 1 M Tris-HCl buffer pH 8.0, were treated with toluene as described by Miller (1972). 100% of enzyme activity corresponded to the total intra- plus extracellular activities.

Enzyme assays. APase and *β*-galactosidase activities were measured according to the procedures described by Torriani (1967) and Miller (1972), respectively. One unit (U) of APase or *β*-galactosidase activity was defined as the amount of enzyme that hydrolysed 1 nmol of substrate per minute. Total specific production (TSP) corresponded to the increase of total enzyme activity for an increase of 1 mg of bacterial dry weight.

Heat treatment. Samples were added to pre-warmed Eppendorf test tubes immersed in a water bath with temperature regulated at 84±0.1° C; then, aliquots were taken and immediately chilled in ice for 15 min. Denatured proteins precipitated and were removed by centrifugation (40,000 g, 20 min, 4° C). APase activity was determined in the supernatants.

Ultrafiltration. 25 ml of culture supernatants were ultrafiltered at room temperature in a model 52 Diaflo cell (Amicon) with a rate of approximately 1 ml/min. We used XM50 or XM100A membrane (Amicon) under a pressure of 2.0 kg/cm^2 or 0.4 kg/cm^2, respectively. Enzyme solutions were concentrated on a XM50 membrane, then diluted twice in TM buffer (0.01 M Tris-HCl pH 7.4 containing 0.01 M $MgCl_2$) and concentrated again to get rid of low molecular weight compounds.

Electrophoretic analysis. Before electrophoresis, purified protein extracts were concentrated by freeze-drying (after dialysis against distilled water for 48 h at 4° C in membrane tubings with a molecular weight cutoff of 12,000—14,000 daltons). Feeze-dried powders were resuspended in electrophoresis cracking buffer (Laemmli 1970). Samples were boiled for 3 min under reducing conditions in the presence of 0.1 M *β*-mercaptoethanol.

Sodium-dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as well as gel staining, were performed as described previously (Atlan and Portalier 1984).

Results and discussion

Preliminary experiments

Electrophoretic analysis of the protein composition of crude culture supernatants (Fig. 1A) showed that excretory mutant 706 released several minor polypeptides and four major periplasmic proteins: a protein with a molecular weight of 58,000 daltons (58 K) and three phosphate-regulated polypeptides, the APase monomer, P1, the sn-glycerol-3-phosphate binding protein, P2, and a peak called P4, corresponding to two comigrating compounds, the phosphate binding protein with a molecular weight of 32,000 daltons and an unidentified protein product. APase contributed for 30 to 35% of the total extracellular protein content (Table 1). Cell lysis, as estimated by assaying *β*-galactosidase activity in culture supernatants, was lower than 1%.

Proteins 58 K and P2, as well as several minor contaminants with apparent molecular weights higher than 45,000 daltons (data not shown), were for the most part removed after a 10 min treatment of extracellular fluids at 84° C (Table 1). Apase activity was not altered and accounted for 48% of the protein content of heat-treated extracts (Table 1). Increasing temperature of the heat treatment up to 90° C did not damage enzyme activity, but did not improve either APase purification (data not shown).

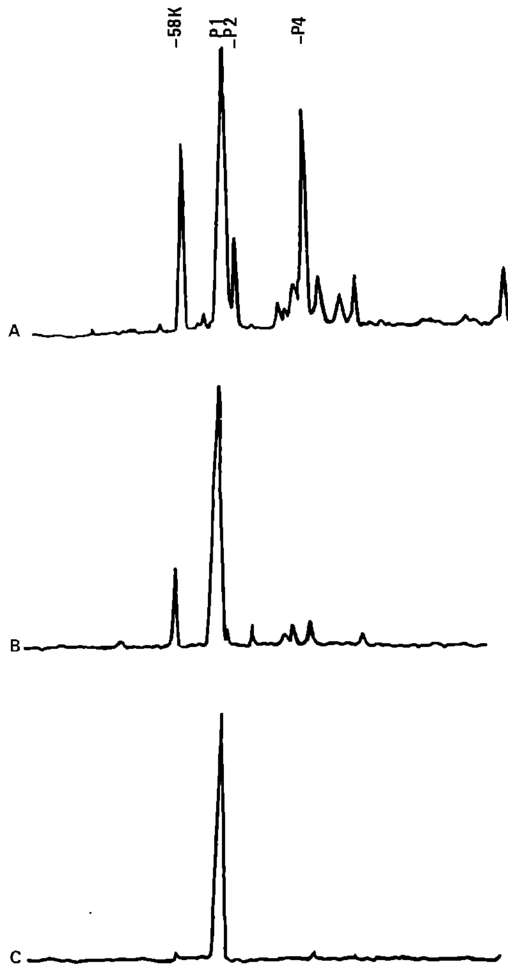

Fig. 1. Electropherograms of proteins from different extracts of APase purification steps: extracellular fluid from excretory mutant 706 (**A**); step 2 extract (**B**); step 3 extract (**C**)

Table 1. Relative contents of major periplasmic proteins contained in the extracellular fluid from *lky* mutant 706 and several purified extracts

APase preparation	Relative contribution of major periplasmic proteins (%)			
	58 K	P1	P2	P4
— Crude extract (extracellular fluid)	15	34	5	21
— Heat shock of crude extract (84° C 10 min)	1	48	2	25
— Concentrate of crude extract on XM50 membrane	1	75	0	10
— *Three step purification procedure*				
Step 2 extract	11	84	1	0
Step 3 extract	1	99	0	0

100% corresponded to the total protein content (undefined units) detected by scanning 12% polyacrylamide gels as described in *Materials and methods*

Protein P4 was not removed by heat treatment (Table 1); it was partially retained by ultrafiltration on XM50 membrane (Table 1) or filtration on G75 Sephadex gel (data not shown). These results suggested that the true molecular weight of protein P4 was higher than the one estimated on polyacrylamide gels. An additional purification step was then necessary to get rid of major contaminant P4.

In the final purification procedure (see below), we applied the heat treatment on small volume extracts obtained after concentration on XM50 membrane.

Purification procedure of extracellular APase

Purification steps are summarized in Table 2.

Step 1: ultrafiltration. Extracellular fluid from stationary cultures of strain 706 was used as crude extract. It was filtered on XM100A membrane to get rid of high molecular weight proteins including aggregates of protein P4. 81% of APase activity were recovered in the ultrafiltrate.

Step 2: concentration. The XM100A ultrafiltrate was concentrated 25 fold on XM50 membrane. At this stage, the main contaminant was protein 58 K (Figure 1B; Table 2). APase activity was distributed as follows: 10% escaped through the membrane, 22% adsorbed on it (and could be partially recovered by membrane washing with TM buffer) and 68% were concentrated. Enzyme solution was already 84% pure (Table 1).

Step 3: heat shock. Enzyme preparation from step 2 was heated for 10 minutes at 84° C and treated as described in "Materials and methods". Heat shock induced a 11% increase in APase activity, suggesting that protein inhibitors were removed by this treatment (Table 2 and Fig. 1C).

Moreover, high temperature destroyed the activities of periplasmic nucleases that could not be detected by protein staining techniques, but were released by excretory mutants (Lazzaroni and Portalier 1981).

Final APase preparation was 99% electrophoretically pure and enzyme activity was satisfactorily preserved by storage at −20° C.

Influence of antifoam agents on APase synthesis and excretion

As a prerequisite for studying the properties of excretory mutants in fermentor, we investigated

Table 2. Purification of APase from the extracellular fluid of excretory mutant 706

Purification step	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg protein)	Enzyme recovery (%)	Purification fold
Crude extract (extracellular fluid)	25.0	33553	160.6	209	100	1
Step 1: Ultrafiltration through XM100A membrane	23.0	27186	150.0	181	81	—
Step 2: Concentration on XM50 membrane	0.9	18382	1.3	14140	55	68
Step 3: Heat shock	0.9	20481	1.3	15755	61	75

Table 3. Effects of different antifoams on APase activity and growth of *lky* strain 706

Antifoam agent	Apase activity			Extracellular β-galactosidase activity	Biomass
	TSP	Extracellular activity			
(1 g/l)	(U/mg)	(U/ml)	(%)	(%)	(g/l)
No product	1699	1092	51	1.2	1.25
Structol	1778	1226	59	1.5	1.16
Super Montaline SLT65 (SEPPIC)	215	25	89	—	0.13
Octarox (SEPPIC)	2577	1268	75	3.7	0.66
Monarox 575 (SEPPIC)	2409	1295	72	7.2	0.75
Lutensol LF 711 (BASF)	3028	1226	86	5.1	0.47
Pluriol RPE 6100 (BASF)	2309	978	76	7.1	0.56
Pluriol RPE 3110 (BASF)	1511	1240	62	1.6	1.42
Silicone Rhodorsil H11 (PROLABO)	4482	1047	53	0.5	0.44
Silicone Rhodorsil H26 (PROLABO)	1519	1268	62	0.8	1.34
J4578 (SOPHOS)	1504	1281	66	3.6	1.30
J4137 (SOPHOS)	1573	1268	62	2.5	1.30
J4291 (SOPHOS)	2012	1516	67	3.4	1.13
E4019 (SOPHOS)	1303	579	48	1.3	0.92

Cells were grown for 16 h at 37° C in test tubes aerated by mechanical shaking

the effect of 13 antifoam products on cell lysis and APase synthesis and excretion during growth of mutant 706 in rich medium. Results reported in Table 3 showed that many antifoams increased cell lysis (Lutensol LF711, Monarox 575, Pluriol RPE 6100), inhibited cell development (Super Montaline SLT65, Octarox, Monarox 575, Lutensol LF711, Pluriol RPE 6100, Silicone Rhodorsil 411) or APase synthesis (Super Montaline SLT65). Three antifoam compounds (Structol, Pluriol RPE 3110 and Silicone Rhodorsil H26) did not exert any injurious effect and were used for fermentor cultivation.

Batch cultivation

Preliminary experiments carried out with a 5 l working volume fermentor SETRIC showed that medium pH values higher than 8.3 inhibited growth of excretory strain 706 (data not shown); so medium pH was regulated at a value of 8.0 ± 0.1 unit during batch fermentation.

During growth of strain 706 in a 15 l Biolafitte fermentor (Fig. 2) kinetics of APase production and excretion were biphasic. APase excretion rate (177 U/ml/h) and biosynthesis (201 U/ml/h) were associated with an 8 h increasing biomass phase; then, APase periplasmic content remained constant after growth ceased, reflecting the good stability of bacterial cells. Biomass (1.2 g/l), total specific APase production (1,400 U/mg of bacterial dry weight) and APase extracellular yield (1,250 U/ml) were similar to those observed in shaken Erlenmeyer cultures.

In conclusion, *E. coli lky* mutants are adapted to large scale growth conditions and could be used in the purpose of industrial APase production. Excretion across outer membrane corresponds to an efficient purification step, so extracellular fluids of excretory mutants are per se APase enriched extracts. From this high quality

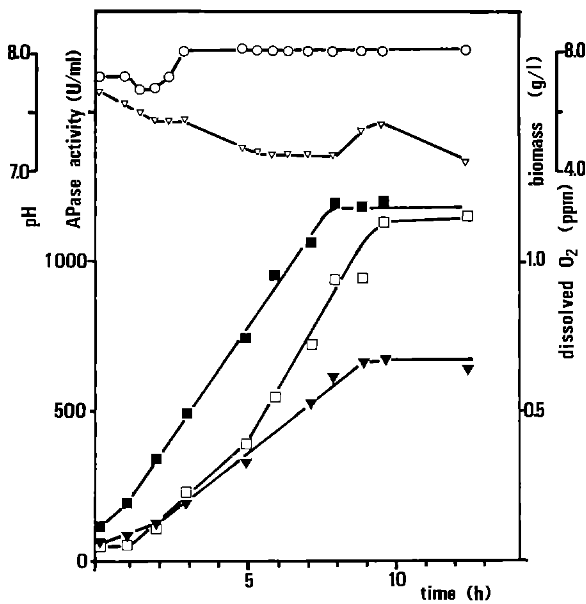

Fig. 2. Kinetics of growth (■—■), intracellular (▼—▼) and extracellular (□—□) APase activity of mutant 706 with monitoring of pH (O—O) and dissolved O_2 (▽—▽)

crude extract, we developed a rapid and low cost method of APase purification.

The yield in extracellular APase from *lky* mutants had been improved two times after introducing two copies of the $phoA^+$ gene on the bacterial chromosome (Atlan et al. 1986). On the other hand, higher yields of APase activity should be obtained through an increase in biomass: Gleiser and Bauer (1981) have reported high cell concentrations of *E. coli* W (55 g dry cell per medium liter).

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