Large Scale Preparation of Purified Exocellular DD-Carboxypeptidase–Transpeptidase of *Streptomyces* Strain R61

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Summary

The exocellular DD-carboxypeptidase–transpeptidase that *Streptomyces* R61 excretes during growth has been produced in large fermentation units of 15 m^3 total capacity. The yield from 15,000 liter culture filtrate was 1.080 g purified enzyme (92% purity) with a total recovery of 29% and at least a 2000-fold increased specific activity.

INTRODUCTION

Peptide crosslinking during synthesis of the bacterial wall peptidoglycan is catalyzed by a cell-bound DD-carboxypeptidase–transpeptidase enzyme system which is the lethal target of penicillins and cephalosporins.[1,2] The functioning of the enzyme system and the mechanism of its inhibition by the β-lactam antibiotics are complex phenomena. A well-defined model was devised using the exocellular DD-carboxypeptidase–transpeptidase that is excreted by *Streptomyces* strain R61 during growth. This soluble enzyme (abbreviated as the R61 enzyme) has been purified to protein homogeneity. It consists of one single polypeptide chain with a molecular weight of 38,000. Many of its physicochemical and enzymatic properties are described in recent papers.[1–3] By combining industrial tools and laboratory techniques, large quantities of purified R61 enzyme were prepared. The goal of the present paper is to describe the procedure used.

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MATERIALS AND METHODS

Maintenance of the Strain

Streptomyces R61, a soil isolate, was grown on slants of agar-APG,[3] containing per liter of final volume: 20 g agar; 0.5 g asparagine; 0.5 g peptone oxoid; 10 g glucose; and 0.5 g K_2HPO_4. Abundant sporulation occurred after four to five days of growth at 28°C. The slants were maintained at 4°C for one month.

Culture Medium

The same glycerol–casein medium[3] was used for the preculture, the inoculum, and the main culture. The glycerol–casein medium contains per liter of final volume: 40 ml 10% casein solution (w/v); 20 g glycerol; 0.25 g NaCl; 0.05 g $CaCO_3$; 0.05 g $MgSO_4 \cdot 7H_2O$; 0.05 g $FeSO_4 \cdot 7H_2O$; 0.5 g K_2HPO_4. The casein was dissolved at 70°C with the addition of KOH (0.04 g/g casein). The solution was then cooled and neutralized to pH 7.3 by careful addition of HCl, in order to avoid precipitation. Sterilization of the media was carried out at 120°C for 45 min. After cooling, the pH was adjusted to 7.15 by addition of a 50% H_3PO_4 solution.

Culture Devices

The precultures were carried out in 6 liter conical, unbaffled flasks, each containing 3 liter culture medium, on a rotating shaker

TABLE I
Dimensional and Mechanical Characteristics of the Two Fermentors Used to Produce DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R61

	Inoculum fermentor	Main fermentor
Total capacity (liter)	1,100	14,900
Charge (liter)	500	7,500
Total height (m)	1.5	4.2
Diameter (m)	0.9	2.0
Drive power (hp)	3	30
Agitation turbines	6 flat blades (6 × 10 cm)	6 ovoid blades (obliquity: 57°; total diam: 0.68 m)
Agitation rate (rpm)	200	165
Aeration rate (v/v/m)	0.25	0.25
Head pressure above atmospheric pressure (atm)	1	1

at 150 rpm. The inocula and the main cultures were carried out in fermentation tanks of 1.1 and 14.9 m^3, total capacity, respectively. Table I gives the technical characteristics of these fermentation units.

Large-Scale Filtration Devices

1) **Materials.** The following materials and devices were used: Clarcel DIF, Clarcel DCB, and Primisil 511 (diatomaceous earths; CECA, Paris, France); Actisil TSL (a mixture of metal silicates; Duchausel-Hebert, Reims, France); Fibraflow (a mixture of asbestos and perlite; Johns Manville, 92505 Rueil Malmaison, France); EKSA pads (Cofram Seitz, 91420 Morangis, France). Ultrafiltration was carried out with a DDS apparatus (De Danske Sukkerfabrikker, DK 1011 Copenhagen, Denmark).

2) **Elimination of mycelium.** The main cultures (7,500 liter), cooled to 18°C, were filtered on a precoated rotary filter of 3 m^2 total area. The 6 cm thick precoat consisted of a mixture of Clarcel DIF (four parts) and Clarcel DCB (one part). The rate of rotation of the filter (120 cm diam) was 0.45 rpm and the rate of advance of the knife was 9 mm/hr. Washing was carried out with 3,000 liter water.

3) **Sterilization of culture filtrate.** The culture filtrate and the washings were pooled and the resulting solution (10,500 liter) was supplemented with 13.5 kg Primisil 511 and 13.5 kg Actisil TSL. The suspension was then submitted to a second filtration using a plate-and-frame device. The first section of this device had a total area of 12.5 m^2 and was equipped with asbestos pads (K7 porosity) coated with 0.5 kg/m^2 Fibraflow; it yielded a clear filtrate. The second section had a total area of 10 m^2 and was equipped with sterilizing EKS pads.

4) **Concentration by ultrafiltration.** This operation was carried out at 15°C with a DDS apparatus of 22 m^2 total area, equipped with No. 600 membranes.

Growth Parameters during Main Culture

The pH of the culture was not readjusted during growth. The temperature (29°C) and addition of antifoam (a $^1/_{10}$ dilution of Rhodorsil 426R) were under automatic control. Culture samples were removed after increasing times and the mycelium was collected by filtration on sintered glass filters. The biomass (in dry weight of mycelium per volume unit) was determined after washing with water and drying at 105°C for 24 hr. The culture filtrates were

used for pH measurements and for the estimation of total soluble nitrogen (using the technique of Kjeldahl), glycerol (by measuring with chromotropic acid[4] the amount of formaldehyde produced after periodic oxidation), and enzyme activity.

Enzyme Activity

One unit of DD-carboxypeptidase[3] catalyzes the hydrolysis of 1 μmol Ac_2-L-Lys-D-Ala-D-Ala into D-Ala and Ac_2-L-Lys-D-Ala per min, at 37°C and under conditions of enzyme saturation by the substrate (K_m = 12mM). The tests were carried out in 5mM sodium phosphate, pH 7.0, and the D-Ala liberated was measured enzymatically with D-amino acid oxidase (for more details, see ref. 3).

Criteria of Purity

The purity of the enzyme preparations at various stages of the purification was estimated on the basis that the pure R61 enzyme:

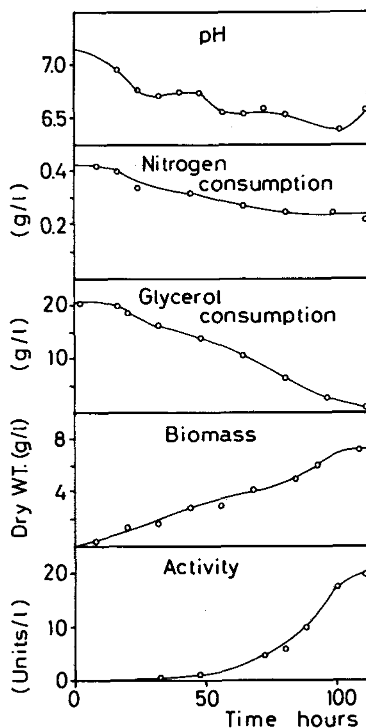

Fig. 1. Growth parameters during culture of *Streptomyces* R61 in a 14.9 m^3 fermentor. For conditions see text.

1) has on Ac_2-L-Lys-D-Ala-D-Ala a specific activity of 86 units/mg protein and a molar activity of 300 min^{-1}; 2) gives rise by polyacrylamide gel electrophoresis in the presence of SDS, with or without 2-mercaptoethanol, to one single band with a mobility corresponding to a molecular weight of 38,000; and 3) formed a stoichiometric complex with benzylpenicillin with the result that the end-point of the titration of the enzyme with the antibiotic, based on the disappearance of enzyme activity, occurs at a molar ratio of benzylpenicillin to enzyme of 1:1 (for more details, see ref. 3).

Protein Estimation

Protein concentrations at the various stages of the enzyme purification were determined either by using Kalckar's[5] equation:

$$[\text{proteins}](\text{mg/ml}) = 1.54\ A_{280} - 0.76\ A_{260}$$

or by measuring the amount of total amino groups available to fluorodinitrobenzene after 6*M* HCl hydrolysis (100°C; 20 hr; standard: bovine serum albumin).[3]

RESULTS

Preparation of Crude Enzyme

Step 1: Main culture. A 12 liter preculture (made from a suspension of conidiospores and after 30 hr of growth at 30°C under the conditions described in Materials and Methods) was used to inoculate the 1.1 m^3 fermentation tank containing 500 liter culture medium. After 29 hr of growth at 29°C, during which time the pH shifted from 7.15 to 6.74, this culture was in turn used to inoculate the 14.9 m^3 fermentation tank containing 7,500 liter culture medium. The growth was maintained for 112 hr at 29°C (for other conditions, see Materials and Methods). Figure 1 shows how the various growth parameters developed with time. The enzyme titles are approximate values as they depended upon the sizes of the samples used for the estimation; the larger the sample the lower the apparent enzyme activity, suggesting the presence of inhibitor(s) in the culture. After 112 hr of growth, the enzyme activity started to level off and over 90% of the glycerol initially present was consumed. At this stage, the culture was heavily brown pigmented and the mycelium represented 7% of the total volume.

Step 2: Filtrations. The filtration on precoat (Materials and

Methods) was a 7 hr process and the sterile filtration was a 12 hr process. The ultrafiltration took more time than expected because of the clogging of the filter by pigmented materials. The flow rate which initially was 800 liter hr^{-1} progressively decreased to 200 liter hr^{-1}. The whole operation took 32 hr resulting in a 34-fold concentration of the initial culture filtrate (final volume: 310 liter). After use, the DDS 600 membranes were discarded because all attempts to restore their original filtration properties failed.

Step 3: Ammonium sulfate precipitation. Solid $(NH_4)_2SO_4$ (pure grade) was added to the concentrated culture filtrate to 50% saturation at 15°C. Part of the insoluble material thus formed floated at the surface of the preparation and was eliminated by filtration on a fine stainless-steel screen. The other part of the insoluble material precipitated and was eliminated by filtration on a 0.5 m^2 total area Buchner, equipped with a nylon cloth covered with 1 kg Primisil 511. The filtrate thus obtained was supplemented with solid $(NH_4)_2SO_4$ to 95% saturation at 15°C. Filtration with the help of Primisil 511 as above gave rise to a paste (5 kg) that consisted of a mixture of proteins, filtration adjuvant, and pigments, and contained more than 90% of the initial enzyme activity.

Preparation of Purified Enzyme

The following laboratory scale procedure, which is an adaptation of the technique of Frère et al.,[3] was applied to a crude enzyme preparation (as obtained after step 3) originating from two main culture, i.e., from 15,000 liter filtrate containing about 300,000 total enzyme units. All the steps described below were carried out at 4°C. Table II gives the total recoveries and enrichments in specific activity after each step of the purification procedure.

Step 4: Solubilization of crude enzyme. The wet paste containing the active enzyme was homogenized and divided into five identical samples. Each sample was suspended in 4 liter water at 4°C and after stirring the suspension was filtered on a sintered glass filter. The extraction was repeated three times, the 16 liter resulting solution concentrated to 500 ml with the aid of a hollow fiber H1D P10 Amicon apparatus, and the concentrated solution was dialyzed four times against 10 liter 10m*M* Tris-HCl buffer, pH 8.0. The average yield in step 4 was 89%.

Step 5: Chromatography on DEAE-cellulose (MN 2100 DEAE, Machery-Nagel and Co). Each of the five concentrated and dialyzed enzyme solutions obtained after step 4 were separately

TABLE II
Purification of the DD-Carboxypeptidase–Transpeptidase from *Streptomyces* R61

Step	Total volume at the end of the relevant step (liter)	Number of samples treated	Total protein (g)	Activity (total units) ($\times 10^{-3}$)	Specific activity (units/mg protein)	Yield (%)	Purity (%)
Main culture	15,000		11,000	300	0.03	100	
4	2.5	5	492	250	0.50	83	0.6
5	0.5	5	62	193	3.1	64	3.6
6	2.4	20	11.2	147	13.1	49	15
7	0.1	5	3.23	111	34.5	37	40
8	0.05	4	1.08	85.5	79	29	92

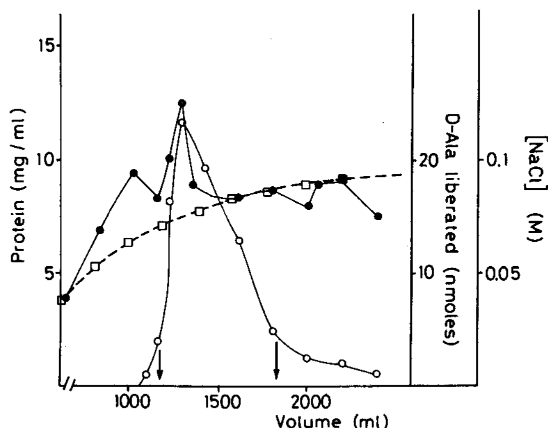

Fig. 2. Enzyme purification. Chromatography on DEAE cellulose (step 5). (●—●) Protein concentration (mg/ml); (○—○) enzyme activity in nmol D-Ala liberated after incubation of 0.1 μl each fraction with 50 nmol Ac_2-L-Lys-D-Ala-D-Ala in 30 μl, final volume of 5m*M* sodium phosphate, pH 7.0, for 15 min at 37°C; (—□—□—) NaCl gradient. Pooled fractions used for step 6 of the purification are those located between the arrows. For conditions of chromatography, see text.

supplemented with 700 g wet DEAE cellulose previously equilibrated against 10m*M* Tris-HCl, pH 8.0. The exchange resin was added progressively under stirring. The resin with the enzyme and virtually all the pigments adsorbed on it was introduced into a 6.5 × 20 cm column previously packed with a 4 cm thick layer of clean DEAE cellulose. After washing with 4 liter 10m*M* Tris-HCl, pH 8, the column was treated with an increasing convex gradient of NaCl (mixing flask at constant volume: 1 liter 10m*M* Tris-HCl, pH 8; solution added: same buffer supplemented with 0.1*M* NaCl). The collected active fractions, between 1,100 and 1,700 ml effluent (Fig. 2), were pooled and concentrated to 100 ml by ultrafiltration. The average yield of step 5 was 77%. At this stage, the total volume of enzyme solution originating from the 15,000 liter culture filtrate was 500 ml.

Step 6: Gel filtration on Sephadex G-100 (Pharmacia). Twenty 25 ml samples of the enzyme solution after step 5 were filtered independently on 800 ml columns of Sephadex G-100 previously equilibrated against 10m*M* sodium cacodylate–HCl, pH 6.0. The collected active fractions, between 350 and 470 ml effluent (Fig. 3), were pooled, giving rise to a total enzyme solution of about 2,400 ml. The average yield of step 6 was 76%.

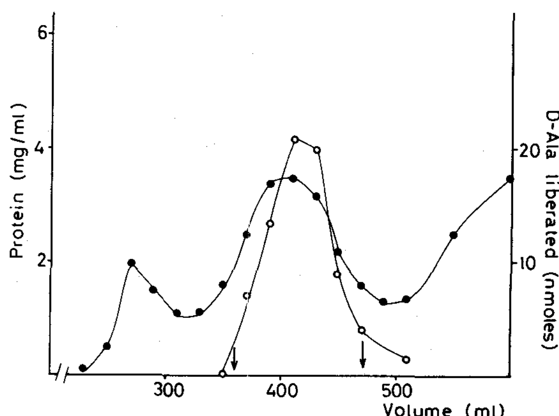

Fig. 3. Enzyme purification. Gel filtration on Sephadex G-100 (step 6). (●—●) Protein concentration (mg/ml); (○—○) enzyme activity in nmol D-Ala liberated under the same incubation conditions as those of Fig. 2. Pooled fractions used for step 7 of the purification are those located between the arrows. For conditions of gel filtration, see text.

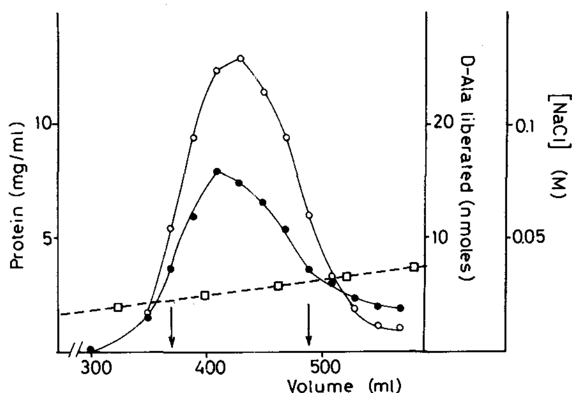

Fig. 4. Enzyme purification. Chromatography on DEAE Sephadex (step 7). (●—●) Protein concentration (mg/ml); (○—○) enzyme activity in nmol D-Ala liberated under the same incubation conditions as those of Fig. 2 except that 0.05 μl each fraction were incubated with the substrate for 10 min; (—□—□—) NaCl gradient. Pooled fractions used for step 8 of the purification are those located between the arrows. For conditions of chromatography, see text.

Step 7: Chromatography on DEAE-Sephadex A-50 (Pharmacia). Five 480 ml samples of the enzyme solution after step 6 were adsorbed independently on 3 × 15 cm columns of DEAE-Sephadex A-50 previously equilibrated against 10m*M* cacodylate–HCl, pH 6.0. The column was treated with a linear gradient of NaCl (800 ml

10mM sodium cacodylate–HCl, pH 6.0 plus 800 ml the same buffer supplemented with 0.1M NaCl). Most of the pigments remained fixed on the top of the columns. The collected active fractions, between 370 and 490 ml effluent (Fig. 4), were slightly colored. They were pooled, concentrated to 20 ml by ultrafiltration, and dialyzed against 10mM sodium phosphate, pH 6.8, containing 5mM NaCl. The average yield of step 7 was 75%. At this stage the total volume of enzyme solution was about 100 ml.

Step 8: Equilibrium chromatography on DEAE cellulose (MN 2100 DEAE, Machery-Nagel and Co). Four 25 ml samples of the enzyme solution after step 7 were filtered independently on 3 × 30 cm columns of DEAE cellulose previously equilibrated against 10mM sodium phosphate, pH 6.8, containing 5mM NaCl. The columns were then treated with the same phosphate–NaCl buffer with the result that the pigments remained fixed at the top of the columns, whereas the colorless enzyme eluted between 190 and 270 ml effluent at a constant specific activity (Fig. 5 and insert). The active fractions from the four equilibrium chromatographies were pooled, concentrated to 50 ml by ultrafiltration, and dialyzed

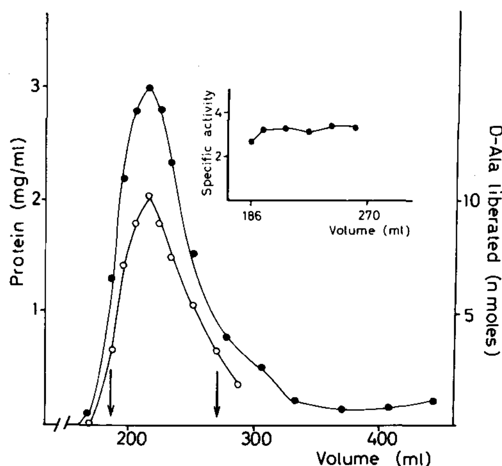

Fig. 5. Enzyme purification. Equilibrium chromatography on DEAE cellulose (step 8). (●—●) Protein concentration (mg/ml); (○—○) enzyme activity in nmol D-Ala liberated under the same incubation conditions as those of Fig. 2 except that 0.02 μl each fraction were incubated with the substrate for 10 min; insert: specific activity (i.e., enzyme activity/protein concentration). Fractions located between the arrows yielded the final enzyme preparation (92% purity). For conditions of chromatography, see text.

against 10m*M* sodium phosphate buffer, pH 7.0, giving rise to a 92% pure enzyme preparation. The yield of step 8 was 78%.

CONCLUSION

The above procedure combines: 1) industrial procedures which through one series of three steps yielded a crude enzyme preparation in the form of an insoluble material consisting of a mixture of proteins, pigments, and filtration adjuvant; 2) laboratory-scale procedures which through several series of five steps yielded a 92% pure enzyme preparation. The overall yield of the procedure was 1.080 g enzyme from 15,000 liter culture filtrate. A 95–99% pure enzyme can be easily obtained by means of one additional equilibrium chromatography as described in step 8. Steps 4–8, as they are described, were perfectly reproducible but attempts to scale them up, at least with the laboratory equipments available, resulted in a decreased effectiveness of the purification procedure. The final enzyme preparation (2% (w/v) in 10m*M* phosphate buffer, pH 7.0) can be stored frozen at −20°C for months without any loss of activity. An extensive description of the chemical and physicochemical properties of the R61 enzyme including the effects of pH, salts, heat, and denaturing agents, can be found in ref 1.

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