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Molecular Weight and Amino Acid Composition of the Exocellular DD-Carboxypeptidase–Transpeptidase of *Streptomyces* R61

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A procedure allowing the purification of milligram amounts of the exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 to protein homogeneity (95% purity) is described. The isolated protein has a molecular weight of about 38000 and consists of one polypeptide chain. Its amino acid composition is presented.

Previous papers have described the isolation, substrate specificity and other enzymic properties of the exocellular DD-carboxypeptidase–transpeptidase produced by *Streptomyces* strain R61 (Leyh-Bouille *et al.*, 1971; Pollock *et al.*, 1972; Nieto *et al.*, 1973*a*; Perkins *et al.*, 1973). This enzyme is believed to be a soluble form of the membrane-bound transpeptidase which catalyses the peptide cross-linking of the nascent cell-wall peptidoglycan (Dusart *et al.*, 1973). In the course of these studies, only small amounts of the enzyme were isolated. The present paper describes the purification of milligram amounts of enzyme, and some physicochemical properties of the isolated protein.

Materials and Methods

Enzyme activity

One unit of enzyme catalyses the hydrolysis of 1 μequiv. of D-alanyl-D-alanine linkage/min at 37°C when the enzyme is exposed to Ac_2†-L-Lys-D-Ala-D-Ala at concentrations of 10 times the K_m value (12mM) in 5mM-sodium phosphate buffer, pH 7.5 (Leyh-Bouille *et al.*, 1971). In previous papers, one unit referred to the conversion of 1nmol of substrate/h.

Physicochemical methods

Diffusion coefficient ($D_{20,w}$). The enzyme solution (3mg of protein/ml) was dialysed against 0.01M-Tris–HCl buffer, pH8.0, containing 0.09M-NaCl and analysed at 20°C in a Spinco model E ultracentrifuge, equipped with a 'Rotor internal temperature control unit' and a schlieren optical system. Experiments were carried out in the epon–aluminium double-sector capillary-type synthetic-boundary cell at a speed of 13410 rev./min. The rate of diffusion was measured by plotting $A^2/(H^2F^2)$ versus time (A, area; H, maximum height of the peak; F, total enlargement used, i.e. 15).

Equilibrium sedimentation and molecular-weight determination. The equilibrium sedimentations were performed in 0.01M-Tris–HCl buffer, pH8.0, containing 0.09M-NaCl at 12590rev./min for 22h at an initial protein concentration of 3mg/ml, by using cells with a 12mm filled-epon double-sector centrepiece and sapphire windows. The solution columns were about 2mm long. Initial solute concentrations were determined by a complementary run with the double-sector capillary-type synthetic-boundary cell. The apparent molecular weight ($M_{app.}$),

$$M_{app.} = \frac{RT}{(1-\bar{v}\rho)\omega^2} \frac{1}{Cx} \frac{dC}{dx},$$

at any point x of the column, where $\bar{v}$ is the partial specific volume of the protein, ρ is the density of the solvent and C the concentration at point x was calculated according to O'Donnell & Woods (1962). The $\bar{v}$ value was not experimentally measured and a value of 0.75cm$^3 \cdot$g^{-1} was used in the calculations. The molecular weight was determined by plotting $1/M_{app.}$ versus concentration at 0.1mm intervals of the column.

Polyacrylamide-gel electrophoresis. This was done at pH8.4 as previously described (Leyh-Bouille *et al.*, 1972).

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† Abbreviations: Ac, acetyl; A_2pm, diaminopimelic acid.

Polyacrylamide-gel electrophoreses were also carried out at pH7 in the presence of sodium dodecyl sulphate, according to the technique of Weber & Osborn (1969) and after pretreatment with 1% (final vol.) of mercaptoethanol. Gels (85mm × 6mm) contained 10.5% acrylamide, 0.27% *NN'*-methylenebisacrylamide and 0.1% sodium dodecyl sulphate. They were pre-run overnight at 2mA/tube before application of the samples.

Gel electrofocusing. This was performed as described by Wrigley (1969). Gels (6cm × 0.5cm) contained 2.5% carrier ampholytes (pH3–6), 7% acrylamide, 0.18% *NN'*-methylenebisacrylamide and 0.08% ammonium persulphate. The pH gradient was formed by passing the current (2mA/tube, up to a maximum of 240V) for 30min. The sample, in 10% sucrose, was layered on the top of the gel and protected from direct contact with the cathode solution by a layer of 2.5% ampholytes in 5% sucrose. Current was passed for 75min, the gels were removed from the tubes, the ampholytes eliminated by diffusion in 2.5% trichloroacetic acid for several days and the precipitated proteins were stained with Coomassie Brilliant Blue G 250, 0.25% in methanol–acetic acid–water (10:1:9, by vol.) for 2h at 0°C. Destaining was carried out at room temp. for 2 days by diffusion in methanol–acetic acid–water (1:1:8, by vol.).

Performic acid oxidation. This was carried out according to the technique of Hirs (1956). Samples of the R61 enzyme (5–10μg of protein) were evaporated to dryness and redissolved in 30μl of formic acid–methanol (5:1, v/v) previously cooled at −5°C. Performic acid (50μl) was then added and the mixture kept at −5°C. After 150min, 0.75ml of water was added and the mixture was evaporated to dryness.

Separation of radioactive peptides by high-voltage electrophoresis. Peptides were separated from each other by electrophoresis on Whatman 3MM paper at pH6.5 (collidine–acetic acid–water; 7:2.5:1000, by vol.) for 210min, a Gilson High Voltage (10000V) Electrophorator model DW (60V/cm) being used. The radioactive peptides were located on the strips by using a Packard Radiochromatogram Scanner. The radioactivity was determined by cutting strips into sections of 10mm, which were counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Amino acid composition of radioactive peptides. The radioactive peptides, purified by paper electrophoresis, were hydrolysed in 6M-HCl at 100°C for 16h. After being freeze-dried, the residues were dissolved in 1.5ml of 0.2M-sodium citrate buffer, pH2.2. The solutions were injected in a Bio-Cal BC200 amino acid analyser programmed for single-column runs. When diaminopimelic acid was not present in the hydrolysates, the temperature was kept constant at 50°C and the following buffers were used: (1) 0.2M-sodium citrate, pH3.25, in 20% ethanol (for elution of glycine and alanine); (2) 1.2M-sodium citrate, pH6.5 (for elution of lysine). When diaminopimelic acid was present in the hydrolysates, the conditions used were as follows: (1) 0.2M-sodium citrate, pH3.25, in 20% ethanol at 30°C (for elution of glycine and alanine); (2) 0.2M-sodium citrate, pH3.50, at 60°C (for elution of diaminopimelic acid; (3) 1.2M-sodium citrate, pH6.5, at 60°C (for elution of lysine).

Amino acid composition of the R61 protein. This was determined on a sample hydrolysed for 24h at 110°C in constant-boiling HCl (made from AnalaR HCl by addition of water and redistillation) after removal of air in a vacuum. The instrument was a Bio-Cal automatic analyser.

Chemicals and reagents

Carrier ampholytes were obtained from LKB (ampholine 8142). Myoglobin, chymotrypsinogen, carbonic anhydrase, aldolase and ovalbumin were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Sodium dodecyl sulphate was recrystallized twice from ethanol before use.

Results

Purification of the enzyme

Step 1. *Streptomyces* R61 (150 litres) was grown, the enzyme adsorbed from the culture supernatant on Amberlite CG 50 at pH4.0 and desorbed from the resin by 0.1M-K_2HPO_4 at pH8.0, as previously described by Leyh-Bouille *et al.* (1971). The adsorption of the enzyme on the resin and its elution as well as all subsequent steps were performed at 4°C. Solid $(NH_4)_2SO_4$ was added to the eluted enzyme solution and the precipitate obtained at 40% saturation was discarded. Protein precipitated when the $(NH_4)_2SO_4$ concentration was raised to 65% saturation was redissolved in a minimum of 0.01M-Tris–HCl buffer, pH8.0 (1 litre), and dialysed against 50 litres of the same buffer. The dialysed solution was divided into four 250ml portions, which were separately adsorbed on four 800ml columns of DEAE-cellulose previously equilibrated against 0.01M-Tris–HCl buffer, pH8.0. The enzyme was eluted from the DEAE-cellulose with an increasing NaCl gradient as described by Leyh-Bouille *et al.* (1971). The active fractions from the four columns were pooled, concentrated by ultrafiltration through a UM-10 membrane in an Amicon cell, dialysed and rechromatographed on one 200ml column of DEAE-cellulose under the same conditions as above. The active fractions were pooled, concentrated to 25ml by ultrafiltration and dialysed against water.

Step 2. After step 1, the enzyme preparation was still heavily contaminated by a dark-brown pigment. It was divided into three lots, which were separately

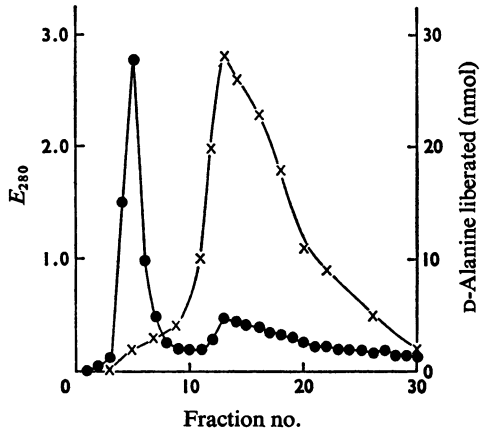


Fig. 1. *Elution pattern of preparative polyacrylamide-gel electrophoresis of R61* DD-*carboxypeptidase–transpeptidase (step 2)*

Fractions (7.5ml) were collected with a flow rate of 1ml/min. DD-Carboxypeptidase activity was estimated by incubating 2μl of each fraction with 50nmol of Ac_2-L-Lys-D-Ala-D-Ala for 15min at 37°C in a total volume of 30μl. ×, Enzyme activity; ●, E_{280}.

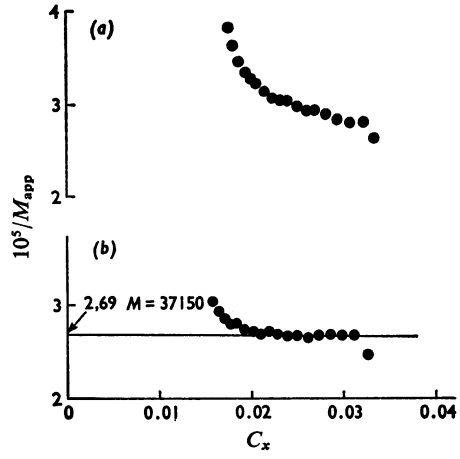


Fig. 2. *Reciprocal of the apparent molecular weight of R61* DD-*carboxypeptidase–transpeptidase in* 0.01M-*Tris–HCl buffer (pH8.0, ionic strength adjusted to 0.1 with NaCl) at initial concentration of 3mg of protein/ml, as a function of the concentration in the column (C_x) during sedimentation equilibrium*

For conditions, see the text. (*a*) Enzyme preparation after step 3; (*b*) enzyme preparation after step 4.

submitted to preparative polyacrylamide disc-electrophoresis in a Shandon apparatus (height of the gel: 5cm; current intensity: 40mA), by using the same gel/buffers as those described for the analytical procedure (Leyh-Bouille *et al.*, 1972). The brown pigment was eluted from the gel with the tracking dye (Bromophenol Blue) and the enzyme was eluted 60min later. The active fractions were pooled, concentrated to 8ml by ultrafiltration, dialysed against water and submitted to an additional electrophoresis under the same conditions as above. Fractions 11–22 (Fig. 1) were pooled and concentrated to 20ml by ultrafiltration.

Step 3. After step 2, the concentrated solution was faintly yellow. It was filtered through a 400ml column of Sephadex G-75 in 0.01M-Tris–HCl buffer, pH8.0. The active fractions were pooled and applied to a 100ml column of DEAE-cellulose equilibrated against 0.01M-Tris–HCl buffer, pH8.0. The enzyme and all the material absorbing at 280nm was fixed on DEAE-cellulose. The resin was treated with an increasing linear gradient of NaCl (500ml of Tris buffer+500ml of 0.2M-NaCl in Tris buffer). The elution profile of the enzyme activity closely followed that of the absorbancy at 280nm. The active fractions were concentrated by ultrafiltration. Although the preparation was colourless, analysis by equilibrium sedimentation (Fig. 2*a*) and polyacrylamide-gel electrophoresis at pH8.4 indicated the presence of contaminating materials of low molecular weight.

Step 4. The enzyme solution was dialysed against 0.015M-HCl–piperazine buffer, pH6.0, and applied to a short column (10cm×1cm) of DEAE-Sephadex A-50 previously equilibrated against the same HCl–piperazine buffer. The enzyme was eluted from the column with an increasing linear gradient of NaCl (200ml of HCl–piperazine buffer+200ml of 0.15M-NaCl in the HCl–piperazine buffer). The enzyme activities of the fractions were exactly proportional to their protein contents. The active fractions were pooled and concentrated to 0.5ml by ultrafiltration. Analysis by equilibrium sedimentation (Fig. 2*b*), polyacrylamide-gel electrophoresis at pH8.4 and electrofocusing (see below) indicated that not more than 5% of contaminating material was present in the final preparation. The specific activity was equal to 86 units/mg of protein, i.e. a value five times higher than that of the preparation previously obtained (Leyh-Bouille *et al.*, 1971). Table 1 gives the total recoveries and enrichments in specific activity after each step of the purification procedure.

Transpeptidase activity of the purified enzyme. During its purification, the enzyme was monitored by following its DD-carboxypeptidase activity upon Ac_2-L-Lys-D-Ala-D-Ala. The enzyme preparations of lower specific activity, which had been obtained earlier (17 units/mg of protein; Leyh-Bouille *et al.*, 1972), catalysed transpeptidation reactions when a

Table 1. *Purification of R61* DD-*carboxypeptidase–transpeptidase*

Step	Total protein* (mg)	Activity (total units)	Yield (%)	Specific activity (units/mg of protein)	Enrichment
Culture supernatant	134×10^3	1670	100	0.012	1
1	550	1250	75	2.3	186
2	30.5	800	48	26	2050
3	8.4	600	37	72	6000
4	3.0	260	15	86	6940

* The protein concentration was determined either by measuring extinction at 280 and 260nm and using the formula C (mg/ml) $= 1.54E_{280}-0.76E_{260}$, or by measuring the amount of total amino groups available to fluorodinitrobenzene after 6M-HCl hydrolysis (100°C, 20h; standard: bovine serum albumin).

Table 2. *Amino acid composition of R61* DD-*carboxypeptidase–transpeptidase*

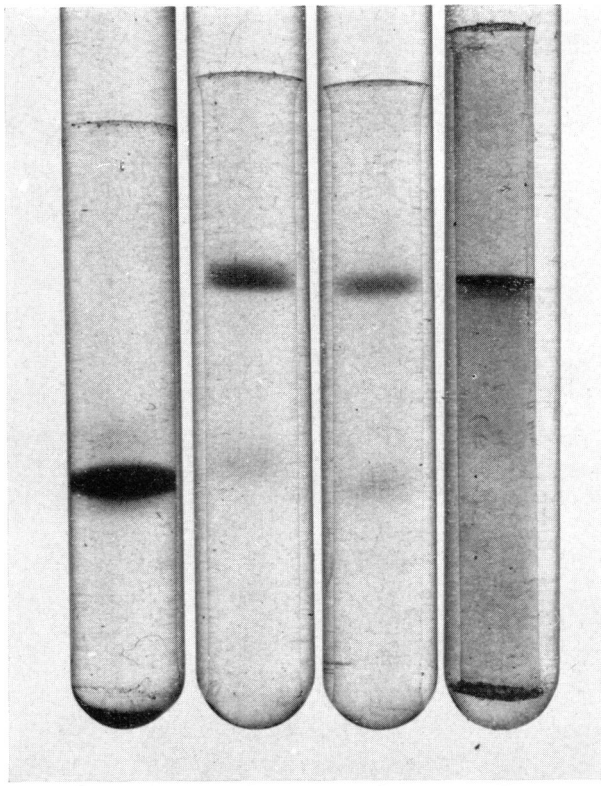
The amount of enzyme hydrolysed was 31.5μg

	Amount in sample (nmol)				
	Uncorrected	After correction		Wt. (μg)	Residues per enzyme molecule (M = 38000)
Asp	30.2			3.48	38
Thr	28.2	29.7		3.00	38
Ser	20.5	22.8		1.98	29
Glu	22.4			2.89	28
Pro	8.5			0.82	11
Gly	25.2			1.44	32
Ala	27.2			1.93	34
Cys (half)	2.4			0.25	3†
Val	23.8			2.36	30
Met	5.0			0.66	6
Ile	7.1			0.80	9
Leu	26.4			2.98	33
Tyr	10.1			1.65	13
Phe	9.3			1.37	12
Lys	6.0			0.77	8
His	6.5			0.89	8
Arg	11.2			1.75	14
Trp	3.0*			0.56	4
			Total	29.58	350

* Calculated from the u.v. spectrum in alkali (Nieto *et al.*, 1973*b*).

† Cystine alone was found; no cysteine occurred. Evidently the protein must contain one, or at most two, disulphide bridges.

suitable acceptor was added to the incubation mixture (Pollock *et al.*, 1972; Perkins *et al.*, 1973; Nieto *et al.*, 1973*a*). The demonstration that the highly purified preparation was also able to catalyse such transpeptidation reactions was made by using ^{14}C-labelled Ac_2-L-Lys-D-Ala-D-Ala as donor and either *meso*-diaminopimelic acid or glycyl-L-alanine as acceptors. A detailed study of the kinetics of these reactions is presented elsewhere (Frère *et al.*, 1973). After reaction, the excess of radioactive tripeptide donor, the hydrolysis product ^{14}C-labelled Ac_2-L-Lys-D-Ala and the transpeptidation products [either ^{14}C-labelled Ac_2-L-Lys-D-Ala-(D)-*meso*-A_2pm or ^{14}C-labelled Ac_2-L-Lys-D-Ala-Gly-L-Ala] were separated from each other by paper electrophoresis at pH6.5 (see the Materials and Methods section). The electrophoretic migrations were: 53cm for the tripeptide donor, 60cm for the hydrolysis product, 46cm for the transpeptidation product with *meso*-diaminopimelic acid and 48cm for the transpeptidation product with



EXPLANATION OF PLATE 1

Polyacrylamide gels stained with Coomassie Blue

From left to right: A, electrophoresis at pH8.4 of 20μg of R61 DD-carboxypeptidase–transpeptidase; B, electrophoresis of 7μg of R61 DD-carboxypeptidase–transpeptidase at pH7.1 in the presence of 0.1% sodium dodecyl sulphate (the fast moving band is myoglobin, used as an internal standard); C, electrophoresis of 7μg of performic acid-oxidized R61 DD-carboxypeptidase–transpeptidase at pH7.1 and in the presence of 0.1% sodium dodecyl sulphate (the fast-moving band is myoglobin); D, electrofocusing of 4μg of R61 DD-carboxypeptidase–transpeptidase. For conditions see the text.

glycyl-L-alanine. The radioactive compounds were eluted from the paper strips, purified by filtration on Sephadex G-15 in water and characterized by determining their amino acid composition after HCl hydrolysis (see the Materials and Methods section).

Molecular weight and diffusion coefficient of the purified R61 DD-carboxypeptidase–transpeptidase. Fig. 2(*a*) and Fig. 2(*b*) compare the plots of $1/M_{app.}$ versus concentration for the preparation obtained after step 3 (Fig. 2*a*) and the final enzyme preparation after step 4 (Fig. 2*b*). From this last experiment, a molecular weight of 37150 was obtained by extrapolation. The R61 enzyme exhibited a $D_{20,w}$ value (see the Materials and Methods section) of 8.45×10^{-7} cm$^2\cdot$s^{-1}. Since a spherical, non-hydrated protein of molecular weight 37150 has a diffusion constant D_0 of 9.50×10^{-7} cm$^2\cdot$s^{-1}, it follows that the purified enzyme has a frictional ratio ($f/f_0 = D_0/D_{20,w}$) of 1.12.

Electrophoresis

Polyacrylamide-gel electrophoresis of the purified R61 DD-carboxypeptidase–transpeptidase. After electrophoresis of 20μg of enzyme at pH8.4 and coloration with Coomassie Blue (Plate 1A), two very faint bands were visible in addition to the main protein band. As previously observed (Leyh-Bouille *et al.*, 1971), the R61 enzyme is a very anionic protein at pH8.4. Scanning of the stained gel indicated that altogether the two contaminating compounds represented less than 5% of the total protein content of the enzyme preparation.

Gel electrophoresis in the presence of sodium dodecyl sulphate. Chymotrypsinogen, carbonic anhydrase, aldolase and ovalbumin were used as molecular-weight standards. The mobility of the R61 enzyme and that of each protein were expressed as the ratio of their migration to the migration of myoglobin used as an internal standard. The mercaptoethanol-treated R61 enzyme (7μg) migrated as a single band. Its mobility, when compared with that of the likewise-treated protein standards, indicated a molecular weight of 39000$\pm$1600 (4 determinations) (Plate 1B).

Gel electrophoresis in the presence of sodium dodecyl sulphate after performic acid oxidation of the purified DD-carboxypeptidase–transpeptidase. If the enzyme were composed of two or several polypeptide chains held together through disulphide bridges, oxidation with performic acid (see the Materials and Methods section) would give rise to compounds of lower molecular weight. The dry residue obtained after performic oxidation of 7μg of enzyme was dissolved in 0.12ml of 0.1M-phosphate buffer, pH7.1, containing 1% mercaptoethanol and 1% sodium dodecyl sulphate, and submitted to polyacrylamide-gel electrophoresis under the same conditions as described above. A single band was detected, which had the same mobility as that exhibited by the non-oxidized enzyme (Plate 1C), thus demonstrating that the protein was composed of a single polypeptide chain.

Isoelectric point of the purified R61 DD-carboxypeptidase–transpeptidase

The R61 enzyme (4μg) was submitted to gel isoelectrofocusing (see the Materials and Methods section). Another gel, to which no protein had been applied, was also submitted to isoelectrofocusing under the same conditions but was not stained by Coomassie Blue. This second gel was sliced into sections, 3mm thick, which were extracted separately with 1ml of water. After 2h, the pH of each eluate was measured. The isoelectric point of the R61 enzyme was determined from the position of the protein band on the stained gel. It was found to be 4.8$\pm$0.14 (4 determinations). As shown in Plate 1D, a very faint band was seen about 1–2mm below the main band. Scanning of the stained gel indicated that this contaminating compound represented less than 5% of the total protein content of the enzyme preparation.

Amino acid composition of R61 enzyme

Amino acids, except tryptophan, were estimated after HCl hydrolysis. Tryptophan content was estimated from the u.v. spectrum given by the enzyme in alkali (Nieto *et al.*, 1973*b*). Results are given in Table 2. The total mass of amino acid residues, 29.58μg, compared fairly well with the nominal amount of enzyme analysed, namely 31.5μg.

Discussion

The exocellular DD-carboxypeptidase–transpeptidase excreted by *Streptomyces* R61 has been purified to near homogeneity (at least 95% purity), and the purified protein has been shown to perform both enzymic activities (see also Frère *et al.*, 1973). A molecular weight of 38000$\pm$1000 was assigned to the protein either by equilibrium sedimentation or by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate of the mercaptoethanol-treated protein. The turnover number was 3300mol of D-alanine liberated from Ac_2-L-Lys-D-Ala-D-Ala per mol of enzyme per min. Treatment of the protein with performic acid did not affect its molecular weight. Hence at ionic strength 0.1, the enzyme molecule is one single polypeptide chain. The enzyme molecule contains about three half-cystine residues. The acidic residues (20%) largely outnumber the basic ones (8%). This property explains the negatively charged character of the protein and its low isoelectric point (4.8). About 50% of the residues are non-polar ones, suggesting

the existence of two areas within the molecule. One of them might be strongly polar and the other one highly hydrophobic. From the experiments reported here and by Nieto *et al.* (1973*b*), the fluorophoric tryptophan and at least some of the tyrosine residues might be somehow buried in the latter hydrophobic area.

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