anisms may not be identical in these two microorganisms.

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Isolation of DD Carboxypeptidase from *Streptomyces albus* G Culture Filtrates*

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ABSTRACT: *Streptomyces albus* G secretes a soluble DD carboxypeptidase whose catalytic activities are similar to those of the particulate DD carboxypeptidase from *Escherichia coli*. Both enzymes hydrolyze the C-terminal D-alanyl-D-alanine linkage of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine and the enzyme–peptide interactions have identical Michaelis constants. Like the *E. coli* enzyme, the *Streptomyces* DD carboxypeptidase exhibits endopeptidase activities. The *Streptomyces* enzyme is lytic for those walls in which the peptidoglycan interpeptide bonds are mediated through C-terminal D-alanyl-D linkages. There is no strict requirement for a specific structure of the C-terminal D-amino acid residue. The tripeptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine is an excellent substrate for the *Streptomyces* DD carboxypeptidase.

The recent recognition of the presence in *Escherichia coli* of a particulate DD carboxypeptidase has excited considerable interest. This enzyme exhibits two important properties. It hydrolyzes the C-terminal D-alanyl-D-alanine sequence of the wall nucleotide precursor uridine-5′-pyrophosphoryl-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine (Araki *et al.*, 1966; Izaki and Strominger, 1968). It also exerts an endopeptidase action upon the peptide dimer of the *E. coli* wall peptidoglycan (Bogdanovsky *et al.*, 1969) by hydrolyzing the C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide bond (van Heijenoort *et al.*, 1969). Such an enzyme might thus be involved in the regulation of the size of the peptide moiety of the *E. coli* peptidoglycan either by limiting the number of wall peptide precursors or by hydrolyzing interpeptide bonds in the completed wall. More recently, particulate DD carboxypeptidase activities were also shown to occur in *Bacillus subtilis* (Strominger *et al.*, 1969; Matsuhashi *et al.*, 1969) and in the blue-green alga *Anabaena variabilis* (Matsuhashi *et al.*, 1969).

The wall peptidoglycan of *Streptomyces* sp. (Leyh-Bouille *et al.*, 1970a) belongs to a chemotype entirely different from that of *E. coli* (van Heijenoort *et al.*, 1969), but it presents structural features that indicate the active presence of a DD carboxypeptidase. The extent of peptide cross-linking is low and the C termini of the peptides are never D-alanyl-D-alanine. A program was therefore initiated whose aim was the study of the DD carboxypeptidase in strains of *Streptomyces*. The purpose of the present paper is to report the isolation of a soluble bacteriolytic DD carboxypeptidase which is secreted by *Streptomyces albus* G.

Materials and Methods

Analytical Techniques. Amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazinolysis

* From the Service de Bactériologie, Liège, Belgium. *Received December 8, 1969.* This research has been supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contracts 515 and 1000).

† On leave for absence from the Service de Chimie Biologique, Faculté de Pharmacie de Nancy. Supported by a NATO Fellowship.

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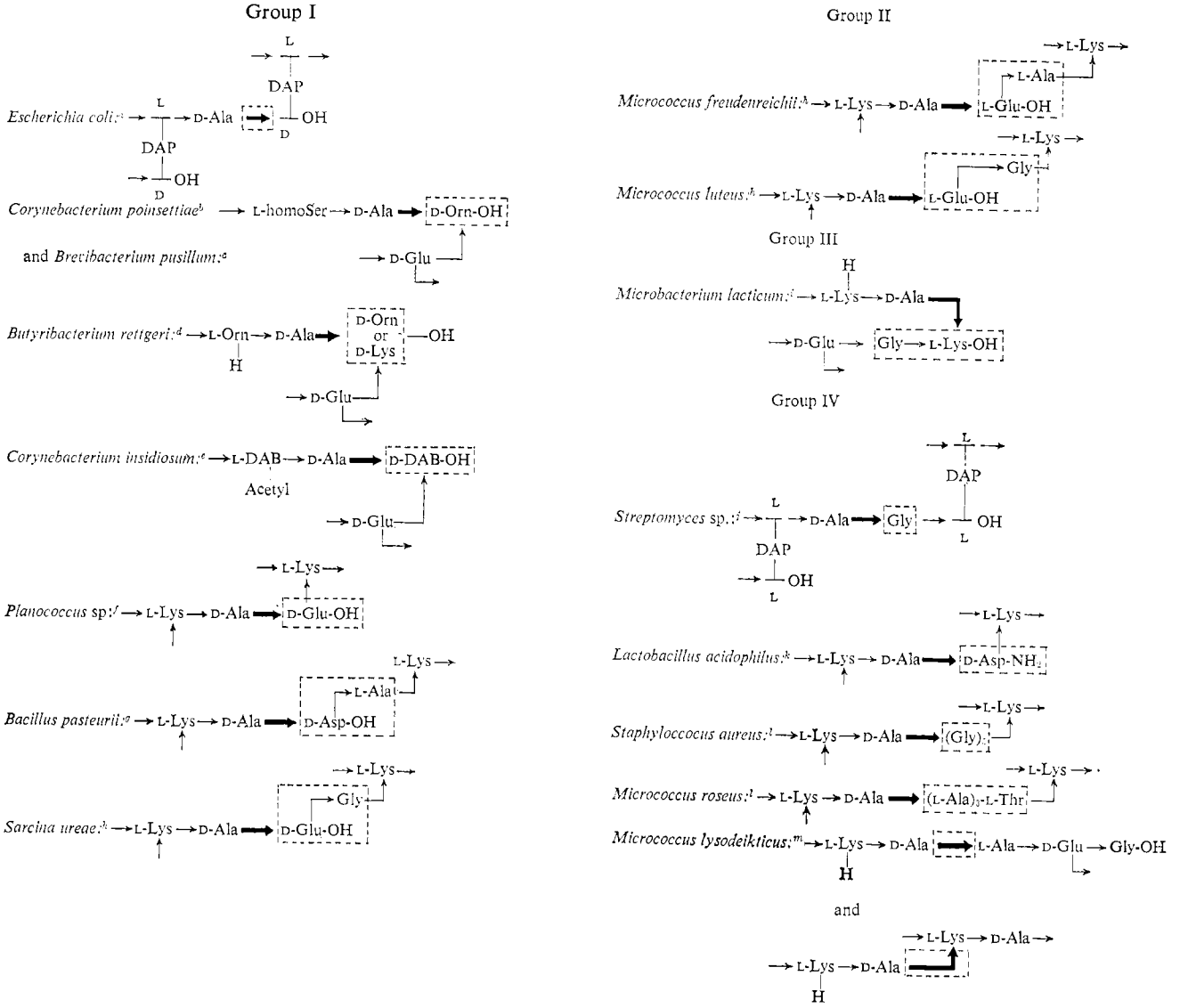
CHART I: The D-Alanyl Linkages in the Interpeptide Bridges in Wall Peptidoglycans.



[a] DAP, diaminopimelic acid; van Heijenoort *et al.*, 1969. [b] Perkins, 1967. [c] DAB, diaminobutyric acid; B. Ciharz, H. K. Schleifer, and O. Kandler, unpublished data. [d] Guinand *et al.*, 1969. [e] Perkins, 1968, and unpublished data. [f] K. H. Schleifer and O. Kandler, manuscript in preparation, 1970. [g] H. Ranftl, K. H. Schleifer, and O. Kandler, manuscript in preparation, 1970; [h] Niebler *et al.*, 1969. [i] Schleifer *et al.*, 1968a,b. [j] Leyh-Bouille *et al.*, 1970a. [k] Coyette and Ghuysen, 1970a; [l] Muñoz *et al.*, 1966. [m] Ghuysen *et al.*, 1968.

techniques, respectively) were measured as previously described (Ghuysen *et al.*, 1966, 1968). Proteins were measured using Lowry's procedure (Lowry *et al.*, 1951).

Walls were prepared from the bacteria listed in Chart I. These walls fall into four groups depending upon the nature of the D-alanyl interpeptide bond: N^{α}, C-terminal DD linkage (group I); N^{α}, C-terminal DL linkage (group II); N^{ω}, C-terminal DL linkage (group III); and DD, DL, or D-glycyl linkages in endo position (group IV). Moreover, depending upon the peptides, the residues preceding the interpeptide D-alanyl linkages were N^{α},N^{ϵ}-bisubstituted L-lysine, N^{α},N^{γ}-bisubstituted diaminobutyric acid, N^{α}-monosubstituted L-lysine, N^{α}-monosubstituted L-ornithine, LL-diaminopimelic acid, *meso*-diaminopimelic acid, or L-homoserine.

Peptides. (1) UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine was a gift of Dr. A. J. Garrett (Medical Research Council, London). It was isolated from *Bacillus subtilis* W 23 after inducing a high intracellular level of the nucleotide by Mg^{2+} deprivation (Garrett, 1969). (2) The *E. coli* peptide dimer (*i.e.*, two L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine tetrapeptides interlinked through a C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkage) was prepared by amidase treatment of the bisdisaccharide peptide dimer. The isolation of this latter compound from *E. coli* has been described (van Heijenoort *et al.*, 1969). (3) N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine was synthesized by conventional methods using both carbodiimide and *N*-hydroxysuccinimide ester coupling procedures (details will be published elsewhere; M. Nieto and H. R. Perkins, in preparation).

DD *Carboxypeptidase Unit.* By definition, one unit of enzyme catalyzed the hydrolysis of 1 mμmole of N^{α},N^{ϵ}-

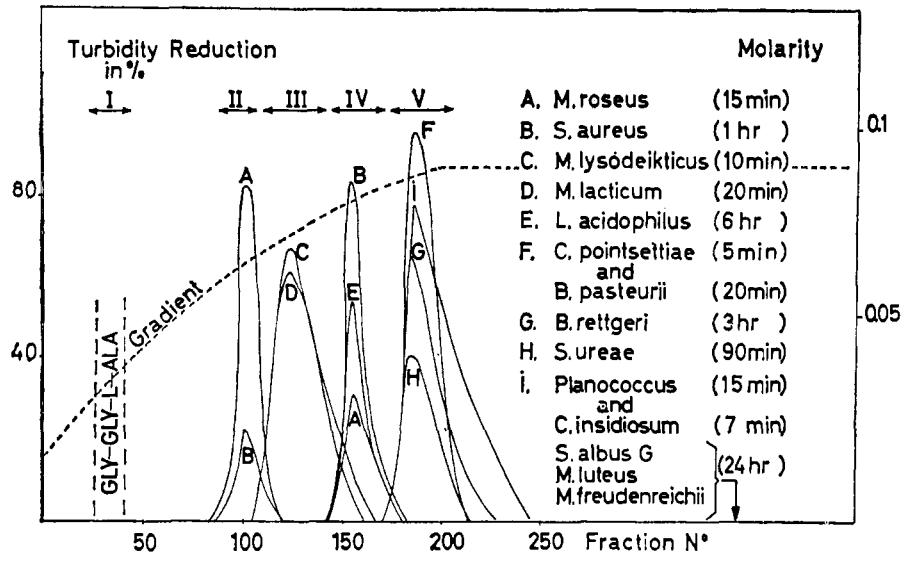


FIGURE 1: Separation on CM-cellulose of aminopeptidase (substrate: glycylglycyl-L-alanine), of MR endopeptidase (substrates: *M. roseus* and *S. aureus* walls), of ML endopeptidase (substrates: *M. lysodeikticus* and *M. lacticum* walls), of SA endopeptidase (substrates: *S. aureus* and *M. roseus* walls, and heat-inactivated *L. acidophilus* walls), and of DD carboxypeptidase (substrates: N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine; walls of *B. rettgeri*, *S. ureae*, *B. pasteurii*, *Planococcus* sp., *C. insidiosum*, and *C. poinsettiae*). No solubilization of walls of *M. freudenreichii*, *M. luteus*, and of *S. albus* G was observed. The curves show the activity of the eluent fractions on the designated substrates. Conditions of chromatography: after adsorption of the crude peptidases, the column was washed with 200 ml of the 0.02 M Tris buffer, pH 8.6, and then eluted with an increasing gradient of Tris buffer at pH 8.6 (mixing chamber at constant volume: 2 l. of 0.02 M Tris buffer; adding solution, 0.2 M Tris buffer). Walls (500 μg) were incubated at 37° with 75 μl of each fraction (8 ml), in a final volume of 500 μl. Time of incubation is shown in parentheses for each designated substrate. The tripeptide Gly-Gly-L-Ala (for aminopeptidase activity) was incubated under the conditions given in Ghuysen *et al.* (1969) (Figure 5). The peptide N^{α},N^{ϵ}-bisacetyl-L-Lys-D-Ala-D-Ala (20 mμmoles) (for DD carboxypeptidase activity) was incubated for 30 min with 6 μl of each fraction in a final volume of 30 μl. Free D-alanine was measured as its dinitrophenyl derivative. Activity was confined to peak V, but liberation of D-alanine is not represented in the figure. Fractions 1–175 were inactive. Fractions 180–190 gave rise to a maximal release of D-alanine (20 mμmoles). Fractions 195–230 performed decreasing activities. (I) Aminopeptidase; (II) MR endopeptidase; (III) ML endopeptidase; (IV) SA endopeptidase; (V) DD carboxypeptidase (formerly = KM endopeptidase in Ghuysen *et al.*, 1969).

bisacetyl-L-lysyl-D-alanyl-D-alanine per hr, at 37°, when 150 mμmoles of peptide was incubated with the enzyme preparation in 30 μl (final volume) of 0.02 M Tris-HCl–0.002 M $MgCl_2$ buffer, pH 7.5 (substrate concentration = 10 × K_m).

Experimental Section

Excretion of the DD *Carboxypeptidase. S. albus* G was grown at 27°, with shaking, in 1-l. flasks containing 500 ml of the following medium: 1% peptone Oxoid, 0.1% K_2HPO_4, 0.1% $MgSO_4 \cdot 7H_2O$, 0.2% $NaNO_3$, and 0.05% KCl. The extracellular DD carboxypeptidase activity was estimated by incubating aliquots of culture filtrates (usually 10 to 25 μl) with 15 mμmoles of N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine in a final volume of 30 μl and by measuring the amount of D-alanine liberated. Under these conditions, maximal activity was usually observed after 50 to 70 hr of culture and under the above conditions 1 ml of filtrate hydrolyzed 90–100 mμequiv of substrate/hr. It was repeatedly observed that the increase in enzyme activity of the filtrates paralleled the increase in mycelium mass produced by the cultures.

Isolation and Purification of the DD *Carboxypeptidase* was carried out using, with only minor modifications, the procedure previously described for the purification of the KM endopeptidase (Ghuysen *et al.*, 1969). The procedure essentially involves 5 steps (for more details, see Ghuysen *et al.*, 1969). Step 1: adsorption of the culture filtrate (50 l.) on Amberlite CG 50 or XE 64 (500 g) at pH 5. Elution of the enzymatic complex with K_2HPO_4. Concentration of the eluate up to 150 ml by dialysis against Carbowax. Step 2: filtration on Sephadex G-50 (25-ml aliquots of the concentrated extract were filtered separately in water on 75 × 4 cm columns). Adsorption of the salt-free filtrate on an 800-ml carboxymethylcellulose column, at pH 8.6, in water. Elution with 0.1 M phosphate. Concentration of the eluate almost until dryness by dialysis against Carbowax. Step 3 (not included in the original procedure): dialysis against a 0.06 M Tris-HCl buffer pH 8.6, at 4°, for 6 hr. Filtration, at 4°, through a 350-ml column of carboxymethylcellulose previously equilibrated against the same buffer. Concentration of the filtrate by dialysis against Carbowax. Step 4: dialysis against a 0.01 M Tris buffer pH 8.6. Adsorption on a 200-ml carboxymethylcellulose column equilibrated against a 0.02 M Tris buffer, pH 8.6. Elution with an increasing gradient of Tris buffer, pH 8.6 (Figure 1). Concentration of the carboxypeptidase fraction (V) by dialysis against Carbowax. Step 5: dialysis against a 0.06 M buffer, pH 8.6. Adsorption on a 180-ml carboxymethylcellulose column equilibrated against the same buffer. Elution with an increasing gradient of Tris buffer, pH 8.6 (mixing flask, at constant volume; 2 l. of 0.06 M Tris buffer; solution added: 0.2 M Tris buffer). The carboxypeptidase was eluted as a single peak and concentrated by dialysis against Carbowax. Filtration in water on Sephadex G-50 yielded the purified enzyme.

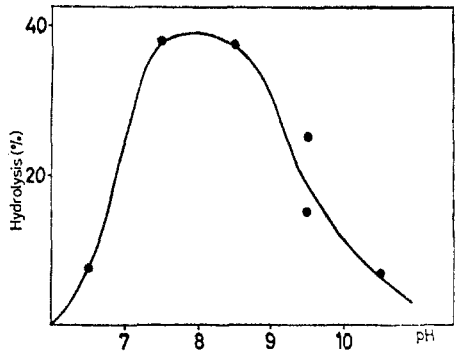


FIGURE 2: pH optimum of DD carboxypeptidase. N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (16 mμmoles) was incubated at 37°, for 20 min, in the presence of 0.7 μg of enzyme (specific activity = 100,000 units/mg of protein), in a final volume of 35 μl. Buffers, 0.02 M (final concentration), were: Na Hmaleate–NaOH (pH 6.5); Tris-HCl (pH 7.5; 8.5 and 9.5); Veronal-HCl (pH 8.5 and 9.5); glycine–NaOH (pH 9.5 and 10.5). Results are expressed in per cent of hydrolysis.

Table I gives the actual recoveries and improvement in specific activities, as determined with the help of the peptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine. Step 3 provided a twofold increase of the specific activity with an excellent yield. Moreover, it ensured a better subsequent separation of the peptidases (step 4) (Figure 1, to be compared with Figures 5 and 6 in Ghuysen *et al.*, 1969). The final enzyme preparation contained 100,000 DD carboxypeptidase units (see Materials and Methods) per mg of protein.

Bacteriolytic Action of the DD *Carboxypeptidase.* Walls of *B. rettgeri*, *C. poinsettiae*, *C. insidiosum*, *B. pusillum*, *S. ureae*, *B. pasteurii*, and *Planococcus* sp. in which the interpeptide bonds are mediated through C-terminal N^{α}-(D-alanyl)-D- linkages (Chart I), were also used for monitoring the fractionation procedure. The lytic activities upon these walls always remained associated with the activity upon N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine throughout the isolation and purification of the DD carboxypeptidase (Figure 1). On the contrary, those walls in which the interpeptide bonds are not mediated through C-terminal N^{α}-(D-alanyl)-D-

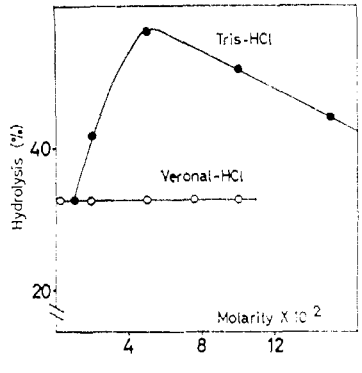


FIGURE 3: Effect of concentration of Tris (pH 7.5) and Veronal (pH 8.5) buffers on DD carboxypeptidase activity. N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (16 mμmoles) was incubated at 37°, for 20 min, in the presence of 0.7 μg of enzyme (specific activity = 100,000 units/mg of protein), in a final volume of 35 μl. Results are expressed in per cent of hydrolysis.

TABLE I: Isolation and Purification of the DD Carboxypeptidase

Steps[a]	Specific[b] Activity	Total Activity	Recovery[c] (%)
Culture filtrate		4,500,000	100
1	250	4,500,000	100
2	960	3,600,000	80
3	1,925	3,800,000	81
4	4,900	1,190,000	25
5	24,000	800,000	17

[a] See text. [b] N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (15 mμmoles) was incubated with the enzyme preparation, at 37°, in 35 μl (final volume) of 0.01 M Veronal buffer, pH 9, and the D-alanine liberated after increasing times of incubation was estimated. The activity of the preparation was estimated under the conditions required to liberate half the amount of D-alanine that is liberated at completion of the reaction. Specific activity is expressed in mμequiv of D-alanyl-D-alanine linkage hydrolyzed per hr, per mg of protein. Under the above conditions, the peptide concentration (450 μM) was almost equivalent to the K_m value (*vide infra*). With the exception of the culture filtrates, the amounts of enzymatic preparations used in the tests were usually so small that they did not modify the pH and molarity of the incubation mixtures. [c] In per cent of the total activity of the original culture filtrate.

linkages (see Chart I) were resistant to the DD carboxypeptidase action. These tests demonstrated that the carboxypeptidase preparation was lytic upon certain isolated walls, that is it behaved as an endopeptidase. Table II summarizes the lytic activity of the purified carboxypeptidase upon sensitive bacterial walls. Assuming that the nonpeptidoglycan components of the walls do not influence the activity of the enzyme upon the peptidoglycan layer, the fact that the walls of *C. poinsettiae*, *B. pusillum*, and *C. insidiosum* were much more sensitive than those of *B. rettgeri* was a puzzling observation. It will be further explained (Leyh-Bouille *et al.*, 1970b).

The rate of hydrolysis of the C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkage in the peptide dimer isolated from *E. coli* (see Chart I and Materials and Methods) was also estimated by measuring the amount of terminal amino group of diaminopimelic acid exposed. By incubating at 37°, 15 mμmoles of peptide dimer in 35 μl (final volume) of 0.01 M Veronal buffer, pH 9, in the presence of the purified DD carboxypeptidase, it was observed that 1 mg of the enzyme hydrolyzed 20,000 mμequiv of interpeptide linkages per hr. By comparison and under the same conditions, 1 mg of the enzyme hydrolyzed, per hr, 24,000 mμequiv of D-alanyl-D-alanine linkages in the peptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine.

pH and Salts Requirements of the Enzyme. pH optimum of the reaction in the presence of Veronal or Tris buffer was about 7.5 to 9 (Figure 2). Optimum concentration was about 0.05 M for the Tris buffer but the enzyme activity

TABLE II: Lytic Action of DD Carboxypeptidase on Walls Containing C-Terminal N^{α}-(D-Alanyl)-D Linkages.[a]

Walls	Content in Peptidoglycan (mμequiv/mg)	Time Required for Complete Lysis (in min)		Terminal Amino Groups (mμequiv/mg)	
				After Muramidase	After Carboxypeptidase
C. poinsettiae	400	2	N^{α}-Orn	130	400
B. pusillum	320	5	N^{α}-Orn	110	320
C. insidiosum	335	15	N^{α}-DAB	95	265
B. rettgeri	320	1080	N^{α}-Orn	57	115
			N^{α}-Lys	83	200
Planococcus sp.	480	15	*N*-Glu	126	400
B. pasteurii	400	20	*N*-Asp	110	300
S. ureae	550	120	*N*-Glu	115	390

[a] Walls (1 mg) were incubated at 37° in 1 ml of 0.01 M Veronal buffer, pH 9, in the presence of 80 μg of DD carboxypeptidase. Controls consisted of walls degraded by *Chalaropsis* endo-*N*-acetylmuramidase (in 0.01 M acetate buffer, pH 4.5). For more details on the structure of the interpeptide bridges, see Chart I. The other walls listed in Chart I were not sensitive to the DD carboxypeptidase (see also Figure 1). Specificity activity = 100,000 units/mg of protein (see Materials and Methods).

was not sensitive to Veronal buffer concentration between 0.005 and 0.1 M (Figure 3). Mg^{2+} and Ca^{2+} (0.002 to 0.01 M), when added to Tris buffer, pH 7.5 (0.01 or 0.02 M), activated the reaction (Figure 4). The involvement of cations in the reaction is also shown by the depressing effect exerted by cation-complexing buffers such as citrate, phthalate, or phosphate (Figure 5). Similarly, sodium ethylenediamine tetraacetate (0.002 M) completely abolished the activity of the enzyme when added to Veronal buffer, pH 8.5 (Figure 5).

Michaelis constant and maximal velocity were determined at 37° in 0.02 M Tris–0.002 M $MgCl_2$ buffer pH 7.5 on the basis of initial velocity measurements. The K_m for UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine was 400 × 10^{-6} M and the V_{max} was 10 × 10^{-6} mole of linkage hydrolyzed per mg of protein per hr. The K_m for N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine was 330 × 10^{-6} M and the V_{max} was 100 × 10^{-6} mole per mg per hr.

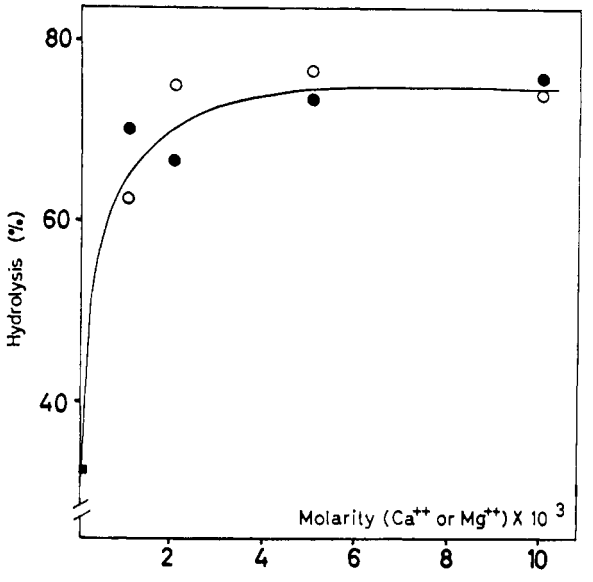


FIGURE 4: Activation of DD carboxypeptidase by Mg^{2+} and Ca^{2+} ions. N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (16 mμmoles) was incubated at 37°, for 20 min, in the presence of 0.7 μg of enzyme (specific activity = 100,000 units/mg of protein), in a final volume of 35 μl. Tris-HCl buffer (0.01 M, final concentration) was supplemented with $MgCl_2$ (●) or $CaCl_2$ (○) as indicated in the figure. The same maximal activity was observed when Mg^{2+} (0.002 M) was added to a 0.02 M Tris-HCl buffer. Results are expressed in per cent of hydrolysis.

Discussion

From the scanty information so far available (Araki *et al.*, 1966; Izaki and Strominger, 1968; Bogdanovsky

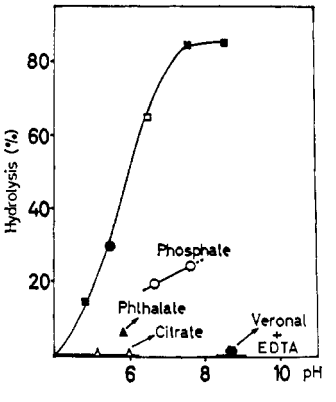


FIGURE 5: Effect of cation-complexing buffers on DD carboxypeptidase activity. N^{α},N^{ϵ}-Bisacetyl-L-lysyl-D-alanyl-D-alanine (16 mμmoles) was incubated at 37°, for 50 min, in the presence of 0.7 μg of enzyme (specific activity = 100,000 units/mg of protein), in a final volume of 35 μl. Buffers (0.01 M) were: acetate (pH 4.8 and 5.5); maleate (pH 6.5); Tris or Veronal (pH 7.5 and 8.5) and (see arrows) citrate, phthalate, phosphate, and Veronal + EDTA (0.002 M). Results are expressed in per cent of hydrolysis.

et al., 1969) and despite the fact that the *E. coli* enzyme preparation has apparently low specific activity,[1] it seemf that both the *Streptomyces* and the *E. coli* DD carboxypeptidases have identical catalytic activities. Indeed their Michaelis constants for UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine are virtually identical (K_m = 400 and 600 μM, respectively), and, furthermore, both enzymes hydrolyze the *E. coli* peptide dimer into monomers. The study of the endopeptidase action of the *Streptomyces* DD carboxypeptidase (formerly called KM endopeptidase, Bricas, 1968; Ghuysen *et al.*, 1969) has been extended to other types of bacterial peptidoglycans. The *Streptomyces* enzyme is able to hydrolyze not only those C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid linkages that occur in the *E. coli* peptidoglycan, but also many other types of C-terminal D-alanyl-D linkages. This property, of course, explains how this carboxypeptidase possesses a broad bacteriolytic spectrum. It also implies that the enzyme has no strict requirement for a specific structure of the C-terminal D-amino acid residue. The *E. coli* DD carboxypeptidase is a particulate, probably membrane-bound, enzyme (Izaki and Strominger, 1968). In contrast to it, the DD carboxypeptidase from *Streptomyces albus* G is excreted in the external medium, at least under the growth conditions that were used. It was observed that the increase in DD carboxypeptidase activity of the filtrates paralleled the *Streptomyces* growth curve, *i.e.*, the increase in mass of mycelium. The ability to secrete a DD carboxypeptidase activity might be restricted to *Streptomyces* sp. It offers an obvious advantage with regard to obtaining an enzyme preparation with a high specific activity. The observation that the synthetic tripeptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine was, by comparison with UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine, an excellent substrate for the *Streptomyces* DD carboxypeptidase (see K_m and V_{max} values), opened a new approach for the study of the specificity profile of this enzyme. The results of this study are reported in the ensuing paper of this series (Leyh-Bouille *et al.*, 1970b).

At this stage, the physiological significance of the bacterial DD carboxypeptidases is obscure. In *E. coli* (and in other bacteria; see Chart I, group I) the interpeptide bonds that are synthesized by the transpeptidase (*i.e.*, the enzyme which catalyzes the cross-linking between peptide units during the last step of the wall peptidoglycan biosynthesis, Tipper and Strominger, 1965; Wise and Park, 1965) are susceptible to hydrolysis by the DD carboxypeptidase. Clearly, a well-balanced growth requires a strict coordination of those synthetic and hydrolytic activities. If the carboxypeptidases are actually involved in the regulation of the size of the peptide moiety of the wall peptidoglycans (Izaki and Strominger, 1968), two types of mechanisms can be envisaged. The enzymes either hydrolyze interpeptide bonds within the completed wall or they hydrolyze the C-terminal D-alanyl-D-alanine sequences of the peptide units before they undergo transpeptidation. In *Streptomyces* sp. and in many other bacteria (see Chart I, groups II to IV), the interpeptide bonds synthesized by the transpeptidase are not sensitive to the carboxypeptidase, but, the active presence of this enzyme is demonstrated by the fact that D-alanyl-D-alanine sequences are never found at the C termini of the walls. This would suggest that the regulation of the size of the peptide moiety would be mediated through the control of the number of the peptide units that are allowed to undergo transpeptidation and, consequently, that the endopeptidase activity exerted by the carboxypeptidase in *E. coli*, would be, essentially, a means for autolysis (Pelzer, 1963). However, in walls of *L. acidophilus* there is a low extent of peptide cross-linking (Coyette and Ghuysen, 1970) but the interpeptide bonds are not sensitive to the enzyme and all the peptides have D-alanyl-D-alanine sequences at their C termini. Thus, at least in some bacteria, the size of the wall peptide is regulated by a mechanism other than carboxypeptidase action (Leyh-Bouille *et al.*, 1970b).

[1] In Tris-Mg^{2+} buffers and using UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine as substrate, the absolute activity (in mμequiv of hydrolyzed linkage per ml of protein per hr) of the *E. coli* enzyme is 330 (Izaki and Strominger, 1968). In these assays, however, the nucleotide concentration (10 μM) was very small, being equivalent to about K_m/60.

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Substrate Requirements of the *Streptomyces albus* G DD Carboxypeptidase*

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ABSTRACT: *Streptomyces albus* G secretes a carboxypeptidase which hydrolyzes C-terminal D-alanylglycine, C-terminal N^{α}-(D-alanyl)-D, and with a much lower efficiency, C-terminal glycyl-D-alanine linkages. The side chain of the C-terminal D residue may be a long peptide sequence. The K_m values for the binding of peptides ending in L → D-alanyl → D sequences, to the carboxypeptidase are essentially controlled by the structure of the L-amino acid that precedes the C-terminal D-alanyl-D linkages.

The substrate requirements of the soluble DD carboxypeptidase and of the membrane-bound transpeptidase involved in wall synthesis present striking similarities. It appears that the peptides which act as carboxyl donors in the transpeptidation reaction are those which are recognized by the DD carboxypeptidase. It is proposed that the soluble DD carboxypeptidase (or a very closely related enzyme) acts as a transpeptidase when it is integrated in the cell membrane.

Two types of enzymes, transpeptidases and carboxypeptidases, which characteristically recognize peptides ending in C-terminal acyl-D-alanyl-D-alanine sequences are known to occur in prokaryotic cells. Membrane-bound transpeptidases catalyze the last step of the bacterial wall peptidoglycan biosynthesis (Tipper and Strominger, 1965; Wise and Park, 1965). The mechanism of the reaction is such that the carbonyl group which is transferred from one peptide to the amino group of a second peptide always belongs to the penultimate C-terminal D-alanine residue of the peptide donor. As a result, interpeptide bonds are formed and D-alanine residues are released in equivalent amounts (Izaki *et al.*, 1968). DD Carboxypeptidases catalyze the simple hydrolysis of C-terminal D-alanyl-D-alanine linkages, *i.e.*, a reaction which is not coupled with peptide-bond formation. DD Carboxypeptidases have been detected in and partially purified from disrupted cells of *Escherichia coli* (Izaki and Strominger, 1968), *Bacillus subtilis* (Strominger *et al.*, 1969; Matsuhashi *et al.*, 1969), and the blue-green alga *Anabaena variabilis* (Matsuhashi *et al.*, 1969). More recently, a DD carboxypeptidase excreted by *Streptomyces albus* G was also isolated and purified (Ghuysen *et al.*, 1970). The purpose of the present paper is to describe the substrate requirements of this latter enzyme.

Materials and Methods

Analytical Techniques. Reducing groups (Park–Johnson procedure), acetamido sugars (Morgan–Elson reaction), amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazinolysis techniques, respectively) were measured as previously described (Ghuysen *et al.*, 1966, 1968).

Electrophoresis was carried out on Whatman No. 3MM paper using an Electrorheophor apparatus Pleuger at pH 6 (acetic acid–pyridine–water 0.33:4:1000 v/v).

Chromatography. The following solvents were used: (I) isobutyric acid–1 N NH_4OH (5:3 v/v); (II) 1-butanol–acetic acid–water (4:1:5 v/v upper phase); (III) chloroform–methanol–acetic acid (88:10:2 v/v); (IV) chloroform–methanol–acetic acid–water (65:25:13:8 v/v). Chromatography was performed on Whatman No. 1 paper, on thin-layer plates of cellulose MN 300 HR (Macherey, Nagel and Co., Düren), and on thin-layer plates of Stahl's silica gel G (Merck). Dinitrophenylamino acids were separated on silica gel plates using solvent III except N^{α}-dinitrophenylornithine and N^{δ}-dinitrophenylornithine which were separated using solvent IV.

* From the Service de Bactériologie, Liège, Belgium. *Received April 2, 1970.* This research has been supported in part by the Fonds de la Recherche Fondamentale Collective, Brussels, Belgium (Contracts 515 and 1000).

† On leave for absence from the Service de Chimie Biologique, Faculté de Pharmacie de Nancy. Supported by a NATO Fellowship.

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