D-ALANINE CARBOXYPEPTIDASE FROM *Streptococcus faecalis*

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SUMMARY

A particulate D-alanine carboxypeptidase that can cleave the terminal residue of D-alanine from UDPMurNAc-L-ala-D-isoglu-L-lys-D-ala-D-ala was isolated from *Streptococcus faecalis*. The enzyme was inhibited by penicillin G non-competitively with a K_i of 0.8 μM.

The carboxypeptidase was solubilized with Triton X-100 without loss of catalytic activity. In this form it could also be inhibited by penicillin G.

INTRODUCTION

Bacterial carboxypeptidases that cleave the terminal D-alanine residue from a DAP[2] containing nucleotide-sugar-peptide have been described (1-5). These enzymes are strongly inhibited by penicillins (1,5), as are the transpeptidases that catalyse the terminal cross-linking reaction of the cell wall peptidoglycan (6,7). In this study, we report the existence and some properties of a particulate D-alanine carboxypeptidase in *S. faecalis*. The preferential activity of this enzyme on a lysine-containing UDPMurNAc-pentapeptide distinguishes it from other reported bacterial carboxypeptidases.

MATERIALS AND METHODS

Uniformly labeled ^{14}C-D-alanine was obtained from Radiochemical Centre (Amersham) and D-cycloserine from the Sigma Chemical Co. UDPMurNAc-L-ala-D-isoglu-L-lys was isolated from *Staphylococcus aureus* cells that had been treated with D-cycloserine (7).

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[2]Abbreviations: DAP, diaminopimelic acid; tBoc, tert-butyloxycarbonyl-$(CH_3)_3C-O-\overset{O}{\overset{\|}{C}}-$; Osu, succinimide ester.

Preparation of D-ala-^{14}C-D-ala: Quantitative yields of D-ala-^{14}C-D-ala were obtained as follows: 50 mg of tBoc-D-ala-Osu[2], 3 mg of ^{14}C-D-alanine (1 mCi, 30mCi/mmole), 100 µl of 1M $NaHCO_3$ and 100 µl of dioxane were stirred overnight at room temperature. The mixture was then diluted with water to 3 ml, centrifuged, and the supernate lyophilized. The tBoc group was removed by addition of 2 ml of 4N HCl in dioxane followed by stirring the mixture for 90 min at room temperature. The solution was dried in vacuo; the residue was then dissolved in water and applied on Whatman paper 3 MM. Separation was achieved by electrophoresis at pH 3.5, 60V/cm for 40 min, and the labeled dipeptide located by autoradiography.

Preparation of tBoc-^{14}C-D-ala-Osu: To 25 mg of ^{14}C-D-alanine (1 mCi), dissolved in 100 µl of water, were added 200 µl of dioxane and 150 µl of 2N NaOH. Then 180 mg of tBoc-azide was added and the mixture stirred at room temperature for 48 hrs. The pH was maintained above 10 by addition of small volumes of 2N NaOH. Excess tBoc-azide was extracted into ether, and the pH of the aqueous phase lowered to 3 by gradual addition of 10% citric acid. The tBoc-^{14}C-D-alanine was then extracted into ethyl acetate, and the citric acid removed by extraction into water. The ethyl acetate solution was dried with anhydrous Na_2SO_4, and the supernate then dried under vacuum. A 94% yield of tBoc-^{14}C-D-alanine was obtained.

tBoc-^{14}C-D-alanine was dissolved in ethyl acetate and treated with molar equivalents of N-hydroxysuccinimide and dicyclohexylcarbodiimide. The mixture was stirred in an ice bath for 30 min and then centrifuged to remove insoluble by-products; the supernate containing the tBoc-^{14}C-D-ala-Osu was dried under vacuum.

Preparation of ^{14}C-D-ala-D-ala: tBoc-^{14}C-D-ala-Osu was coupled with a four-fold excess of unlabeled D-alanine to give the protected dipeptide. The protecting group was removed as described above for D-ala-^{14}C-D-ala. Starting with the tBoc-D-ala-Osu, a quantitative yield of alanine dipeptide was obtained.

Preparation of the radioactive UDPMurNAc-pentapeptides: UDPMurNAc-L-ala-

D-isoglu-L-lys-D-ala^{14}C-D-ala was prepared by enzymatic conjugation of chemically synthesized D-ala-^{14}C-D-ala with biologically prepared UDPMurNAc-L-ala-D-isoglu-L-lys; for this purpose the "adding enzyme" (EC 6.3.2.10) was prepared and used as previously described (7). This enzyme was also used for the synthesis of UDPMurNAc-L-ala-D-isoglu-L-lys-^{14}C-D-ala-D-ala starting with UDPMurNAc-tripeptide and ^{14}C-D-ala-D-ala. The radioactive nucleotide-pentapeptides were purified and analyzed as previously described (7). UDPMurNAc-L-ala-D-glu-DAP-D-ala-^{14}C-D-ala was a gift from Dr. M. Gorecki.

Growth of bacteria, preparation of the particulate fraction and solubilization of enzyme: A strain of S. faecalis derived at the Hadassah Medical School, Jerusalem, was used. The bacteria were grown in liquid media containing 1% Bactopeptone, 1% yeast extract, 0.5% KH_2PO_4 and 0.5% glucose. The cells were harvested at early logarithmic phase of growth, and from them a particulate fraction was prepared as described previously (7). The particulate fraction (27.6 mg protein/ml) was suspended in 1 ml of 50mM Tris-HCl at pH 7.5 containing 100mM KCl, 1mM 2-mercaptoethanol and 0.1mM $MgCl_2$, and the suspension mixed with an equal volume of 2% Triton X-100 in the same buffer. The mixture was incubated for 45 min at 25° C and centrifuged at 105,000 xg for 2 hrs. The specific activities of both the pellet and the supernate were determined.

Kinetic measurements with D-alanine carboxypeptidase: D-alanine carboxypeptidase activity was assayed in a reaction mixture (150 µl) containing: UDPMurNAc-L-ala-D-isoglu-L-lys-D-ala-^{14}C-D-ala, 48 nmoles (8.7 x 10^6 cpm/µmole) Tris-HCl at pH 7.6, 10 µmoles; $MgCl_2$, 5 µmoles; 2-mercaptoethanol, 2 µmoles; and KCl, 10 µmoles. Reaction was initiated by addition of the particulate enzyme preparation (0.5 mg of protein). The mixture was incubated at 37° C for 15 min, and the reaction terminated by immersing the assay tube in boiling water for 5 min. After the tube was cooled the denatured protein was centrifuged and washed with an equal volume of water. The combined supernate and water wash was passed through an Amberlite IR-120 column (0.9 x

TABLE I

Distribution of D-alanine carboxypeptidase in cell-free preparations of S. faecalis [a)]

	Supernatant fraction	Particulate fraction
Total activity (units)	1,800	4,700
Protein concentration (mg/ml)	5.0	27.6
Specific activity (units/mg protein)	1.8	17.1

a) A suspension of disrupted S. faecalis cells (representing 15 gm wet weight of cells was centrifuged for 2 hrs at 105,000 xg. The washed pellet was suspended in the original buffer and assayed. The supernatant fraction was dialyzed against the same buffer and its enzymatic activity determined.

58 cm) at 58° C and eluted with 0.2 M citrate buffer, pH-4.25 at a flow rate of 60 ml per hour. Under these conditions, UDPMurNAc-pentapeptide elutes after 10 min and the released ^{14}C-alanine after 30 min. A Tri-Carb flow analyzer was used to monitor radioactivity. Under the specified conditions of assay, the rate of ^{14}C-alanine release was linear for 30 min with amounts of enzyme protein as large as 1 mg. A unit of activity is defined as the amount of enzyme catalyzing release of 1 nmole of terminal D-alanine from the lysine-containing UDPMurNAc-pentapeptide in 15 min at 37° C.

RESULTS AND DISCUSSION

Over 70% of the total activity of D-alanine carboxypeptidase was found in the particulate fraction obtained after centrifugation of the cell-free preparation at 105,000 x g for 2 hrs (Table I).

The enzyme preferentially cleaves the lysine-containing UDPMurNAc-pentapep-

TABLE II

Substrate specificity of particulate D-alanine carboxypeptidase from S. faecalis and its inhibition by penicillin G [a]

Substrate	Inhibitor (μg/ml)		^{14}C-alanine released	
	Penicillin G	D-Cycloserine	nmoles	% inhibition
UDPMurNAc-L-ala-D-glu-L-lys-D-ala-^{14}C-D-ala	--	--	16.4	--
17.7 x 10^6 cpm/μmole, 36 nmoles	0.1	--	13.1	20
	1.0	--	3.8	73
	10	--	0.4	97
	100	--	0.1	99
	--	300	16.5	0
UDPMurNAc-L-ala-D-glu-L-lys-^{14}C-D-ala-D-ala				
2 x 10^6 cpm/μmole, 64 nmoles	--	--	0.5	--
UDPMurNAc-L-ala-D-glu-DAP-D-ala-^{14}C-D-ala	--	--	--	--
3 x 10^5 cpm/μmoles, 53 nmoles	100	--	0	100

[a] The reaction was carried out for 30 min at 37° C with 30 μl of particulate fraction containing 790 μg protein. Other details are described in Methods.

tide, although significant release of the terminal D-alanine from the DAP-containing nucleotide-pentapeptide also occurs (Table II). This enzyme, therefore, has specificity different from that of the particulate D-alanine carboxypeptidase from B. subtilis or of the soluble D-alanine carboxypeptidase from E. coli. The last two enzymes are specific for the DAP-containing UDPMurNAc-pentapeptide. DAP is a constituent of the cell wall peptidoglycans (8) of these organisms. On the other hand these last two enzymes do not release terminal D-alanine from UDPMurNAc-L-ala-D-isoglu-L-lys-D-ala-D-ala (1,5). In view of the relationship between the composition of the cell wall peptidoglycan and the specificity of the D-alanine carboxypeptidases in B. subtilis and E. coli, we examined the peptidoglycan from the strain of S. faecalis used for isolation of the enzyme described here. As had been reported for other strains of S. faecalis (8), the peptidoglycan was found to contain lysine but not DAP.

Crude soluble enzyme preparations from E. coli and particulate enzyme preparations from B. subtilis eventually release both ultimate and penultimate D-alanine residues from the DAP-containing nucleotide-pentapeptide (5). The release of penultimate alanine is caused by a second enzyme, D-alanine carboxypeptidase II (1,5). With the S. faecalis preparations as studied here, however, using the lysine-containing nucleotide-pentapeptide labeled specifically in its penultimate D-alanine as substrate, D-alanine carboxypeptidase II activity was extremely low in the particulate fraction (Table II) and in the soluble fraction.

The use of the nucleotide-pentapeptide labeled exclusively in the terminal D-alanine position already has been described in studies of particulate transpeptidase activity in S. aureus (7,9). It has an obvious advantage over the substrate labeled in both the terminal and penultimate D-alanine, since accurate measurements can then be made of carboxypeptidase I activity in enzyme preparations that contain both carboxypeptidase I and II activities. The nucleotide-pentapeptide labeled in the penultimate D-

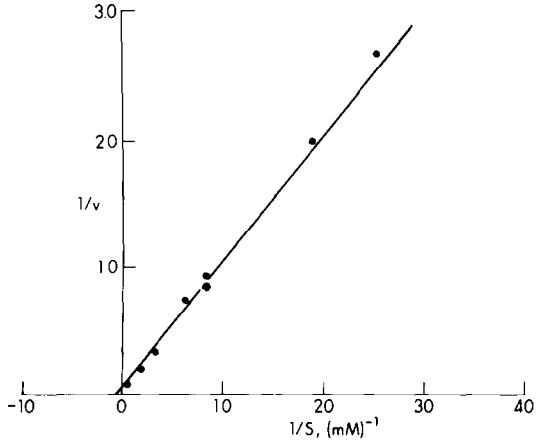

Fig. 1. Determination of the Km for UDMurNAc-L-ala-D-isoglu-L-lys-D-ala-D-ala. The range of substrate concentrations used was 0.01 mM-1.66 mM. The enzyme preparation contained 1 mg protein per tube. Other details are described in Methods.

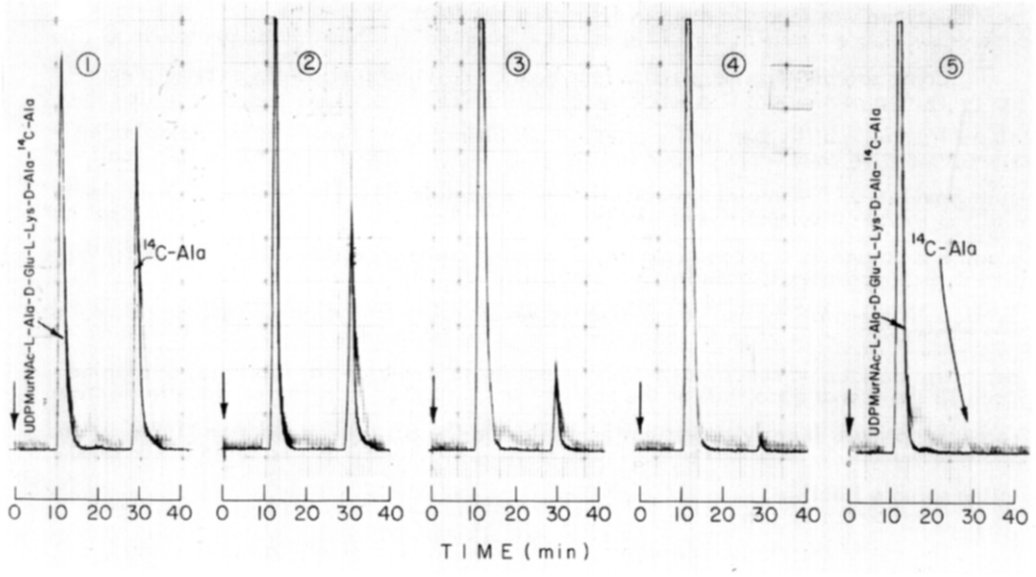

Fig. 2. Release of ^{14}C-alanine from UDPMurNAc-L-ala-D- isoglu -L-lys-D-ala-^{14}C-D-ala and inhibition of the release by penicillin G. The reaction conditions are as described in Methods, (1) - complete system; (2-5) in the presence of penicillin G (μg/ml): (2), 0.1; (3), 1.0; (4), 10 and (5), 100.

alanine, introduced here, can serve for detection of carboxypeptidase II in cell-free extracts containing both carboxypeptidase I and II.

Kinetic parameters of D-alanine carboxypeptidase from S. faecalis: The Km of this enzyme with UDPMurNAc-L-ala-D-isoglu-L-lys-D-ala-D-ala was found to be 2mM

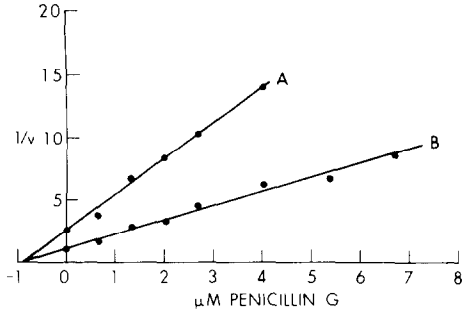

Fig. 3. Inhibition of D-alanine carboxypeptidase by penicillin G. A, 0.22 mM and B, 0.53 mM of UDPMurNAc-L-ala-D-isoglu-L-lys-D-ala-D-ala. One mg of enzyme protein was added in each tube.

(Fig. 1), a value not too different from Km values found for other bacterial carboxypeptidases I (1,5). The particulate enzyme from S. faecalis, like that from B. subtilis (1), is strongly inhibited by penicillin G (Fig. 2, Table II). However, the soluble D-alanine carboxypeptidase I from E. coli is much more sensitive to inhibition by penicillin G (5). Moreover, the particulate D-alanine carboxypeptidase from S. faecalis is inhibited in a non-competitive manner [$K_I = 8 \times 10^{-7}$ M (Fig. 3)], in contrast to that from E. coli which is inhibited competitively [$K_I = 1.6 \times 10^{-8}$ M (Ref. 5)]. D-cycloserine does not inhibit carboxypeptidase activity of S. faecalis (Table II).

Solubilization of particulate D-alanine carboxypeptidase by Triton X-100: Table III shows that almost all of the activity was extracted from the particulate fraction by 1% Triton X-100 with a two-fold increase in specific activity and retention of sensitivity to inhibition by penicillin G. Triton itself did not affect the carboxypeptidase activity in assay.

While this manuscript was in preparation, Umbreit and Strominger (10) reported solubilization (by various non-ionic detergents) of particulate D-alanine carboxypeptidase from B. subtilis, and Coyette et al (11) described penicillin-sensitive DD-carboxypeptidase and transpeptidase activities in membranes of S. faecalis.

Additional studies on the D-alanine carboxypeptidase with a unique preferential

TABLE III

Solubilization of particulate D-alanine carboxypeptidase with Triton X-100 and its inhibition by penicillin G

	particulate fraction	fraction solubilized by Triton X-100	insoluble residue after Triton treatment
Activity (nmoles/mg protein) [a]	17.1	36.1	2.6
Total units of enzyme	942	903	38
Recovery (%)	100	96	4
% inhibition by penicillin G (1μg/ml)	76	69	

[a] The assay conditions as well as solubilization with Triton X-100 are described in Methods. The amount of protein was 0.68-0.72 mg per tube, and of UDPMurNAc-pentapeptide (1.4×10^6 cpm/μmole), 75 nmoles.

activity on the lysine-containing nucleotide-pentapeptide of S. faecalis may contribute to a better understanding of the mode of action of penicillin.

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