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PURIFICATION AND PROPERTIES OF THE D-ALANYL-D-ALANINE CARBOXYPEPTIDASE OF *BACILLUS COAGULANS* NCIB 9365

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Summary

After solubilisation with urea and the non-ionic detergent Genapol X-100, the membrane-bound DD-carboxypeptidase (UDP-*N*-acetylmuramoyl-tetrapeptidyl-D-alanine alanine-hydrolase, EC 3.4.12.6) of *Bacillus coagulans* NCIB 9365 was purified to homogeneity, as verified by sodium dodecyl sulphate gel electrophoresis, by chromatography with an ampicillin-agarose affinity resin and DEAE-cellulose. The properties of the purified DD-carboxypeptidase were similar to those of the membrane-bound enzyme; these include enhancement of activity by divalent cations, Pb^{2+} and Cd^{2+} being the most effective. The enzyme also catalysed a simple unnatural model transpeptidation reaction between UDP-*N*-acetylmuramoyl pentapeptide (donor) and D-alanine or glycine (acceptors).

The enzyme consisted of a single polypeptide chain with a molecular weight (M_r 29 000), considerably lower than values obtained previously for most other DD-carboxypeptidases. However, its molecular weight and its degree of relatedness, as assessed by amino acid composition, were similar to several β-lactamases.

Introduction

Penicillin interacts with several enzymes present in the cytoplasmic membrane of bacteria [1—6], some of which are associated with the multiple penicillin-binding proteins present in the membranes of all bacteria so far

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examined, which bind [^{14}C]benzyl penicillin covalently [1,5—10].

Of these enzymes, the DD-carboxypeptidase (UDP-*N*-acetylmuramoyl-tetrapeptidyl-D-alanine alanine-hydrolase, EC 3.4.12.6) has proved the most suitable for investigation and purification. Although the DD-carboxypeptidases from several microorganisms have been purified partially [11,12] or to homogeneity [8,9,13—15] using conventional techniques of protein purification, the phenomenon of the interaction of the enzyme with β-lactam antibiotics has been exploited by the purification of this enzyme and other penicillin-binding proteins by affinity chromatography [1,5—7,10,16—23]. By employing this technique, sometimes in conjunction with conventional methods, the purification of the DD-carboxypeptidase to total or virtual homogeneity has been achieved in several instances [5—7,10,19].

The properties of the Pb^{2+}-enhanced membrane-bound DD-carboxypeptidase of *Bacillus coagulans* NCIB 9365, and its solubilisation, have been described previously [24,25]. In this paper we therefore report its purification to homogeneity, and the properties of the purified enzyme.

Materials and Methods

Enzyme preparation and assay. DD-Carboxypeptidase was solubilised from protoplast membranes with urea and Genapol X-100 (3.25 M and 2% final concentrations, respectively) as described previously [25]. Solubilisation was, however, carried out in 0.05 M sodium cacodylate-HCl, pH 7.4 instead of 0.05 M Tris-HCl, pH 7.5.

DD-Carboxypeptidase and unnatural model transpeptidase were assayed as before [24,25]. In addition the DD-carboxypeptidase activity of column fractions was assessed by the colourimetric procedure [9].

Affinity chromatography. Succinylaminodipropylamino agarose was prepared from Sepharose 4B [26]; it had a binding capacity of 12—18 μmol · ml^{-1} of packed resin (for L-[^{14}C]alanine). To a stirred suspension of succinylaminodipropylamino agarose at 4°C in 0.1 M sodium cacodylate-HCl (pH 5.0) was added (per ml of resin), 6 mg sodium ampicillin and 20 mg 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, dissolved in the above buffer; the final volume of the gel-antibiotic-coupler suspension was equivalent to 1.5 vols of the original resin. The pH was readjusted to 5.0, and the mixture stirred gently at 4°C for 16 h. The affinity resin was then recovered by filtration and washed successively with 50 vols. of ice-cold 0.05 M sodium cacodylate-HCl (pH 7.4) containing 1 M NaCl, and the same buffer in the absence of NaCl. No L-[^{14}C]-alanine could be coupled to the ampicillin-agarose.

For purification of the solubilised DD-carboxypeptidase, the resin was equilibrated in 0.05 M sodium cacodylate-HCl, 0.1% Genapol X-100 (pH 7.4) and dried briefly under suction. The soluble enzyme was added to the gel (at an excess of approximately 5 : 1, v/v), the resulting slurry stirred at room temperature for 30 min and then poured into a 5 × 3 cm column. Unadsorbed material was washed from the column at 4°C with the equilibrating buffer, and the column then washed with 0.05 M sodium cacodylate-HCl, 0.1% Genapol X-100, 1 M NaCl (pH 7.4), in each instance until no 280 nm-absorbing material was detected in the eluate. The DD-carboxypeptidase was then eluted at 20°C

with 1.5 bed vols. of 0.05 M sodium cacodylate-HCl, 0.1% Genapol X-100, 0.8 M neutral hydroxyammonium-HCl (pH 7.4).

Polyacrylamide gel electrophoresis. Discontinuous electrophoresis in 10% polyacrylamide slab gels containing SDS was carried out by the method of Laemmli [27] as modified by Anderson et al. [28], at a constant current of 40 mA; gels were stained with Coomassie brilliant blue [28]. The molecular weight of the DD-carboxypeptidase was estimated by comparing its mobility with those of bovine serum albumin (M_r 68 000), aldolase (subunit M_r 40 000), glyceraldehyde-3-phosphate dehydrogenase (subunit M_r 36 000), trypsin (M_r 23 800) and lysozyme (M_r 14 300). A linear plot for log (molecular weight) against electrophoretic mobility was obtained.

S-Carboxy[^{14}C]methylation. Purified enzyme (approx. 1 mg) was dialysed extensively against glass-distilled water at 4°C to lower the concentration of detergent and the protein freeze-dried. The DD-carboxypeptidase was *S*-carboxy[^{14}C]methylated with iodo[2-^{14}C]acetate (1.5 Ci/mol) according to the method of Gibbons and Perham [29].

Amino acid analysis. Samples containing 50 μg of *S*-carboxy[^{14}C]methylated DD-carboxypeptidase were hydrolysed and quantitated as described by Baldwin et al. [30].

N-terminal analysis. *S*-Carboxy[^{14}C]methylated DD-carboxypeptidase was examined for free N-terminal residues by the dansyl procedure. Dansyl-amino acids were separated and identified by chromatography on polyamide thin-layer sheets [31].

Protein estimation. Protein was determined by the method of Lowry et al. [32] modified for assay in the presence of detergents [7], using bovine serum albumin as a standard.

High-voltage electrophoresis. Products were separated by electrophoresis at pH 6.5 (pyridine/acetic acid/H_2O, 800 : 24 : 7200, by vol.) on Whatman 3 MM paper at 60 V · cm^{-1} for 60 min.

Materials. Genapol X-100 was kindly provided by Farbwerke Hoechst AG (Frankfurt/Main-Hoechst, F.R.G.). DEAE-cellulose (DE 52) was obtained from Whatman (Maidstone, U.K.); Sepharose 4B from Pharmacia (Uppsala, Sweden); polyamide sheets from BDH (Poole, U.K.) and sodium ampicillin from Beecham Research Labs. (Brockham Park, U.K.). D-[U-^{14}C]alanine, L-[U-^{14}C]-alanine and [U-^{14}C]glycine were purchased from the Radiochemical Centre (Amersham, U.K.) and 2,6-diamino[1,7-^{14}C]pimelic acid from New England Nuclear (Dreieich, F.R.G.).

Results

Purification of solubilised DD-carboxypeptidase

Affinity chromatography. Penicillin affinity resins show a low degree of stability in Tris-HCl [6,19], the buffer initially used for the solubilisation of the membrane-bound enzyme; hence alternative systems which buffer around the optimal pH for the solubilisation of the DD-carboxypeptidase (pH 7.5) [25] were investigated. Enzyme activity was inhibited considerably by buffers containing phosphate; this was most pronounced with citric acid/Na_2HPO_4 in which the activity was inhibited by 70% relative to Tris-HCl. However, the

enzyme activity and the efficiency of solubilisation of the DD-carboxypeptidase were equivalent in sodium cacodylate-HCl (pH 7.4) and Tris-HCl. Ampicillin was very stable in the former buffer (as assessed by the starch/iodine assay [33]) and hence sodium cacodylate was employed.

The data from a typical purification experiment are shown in Table I. As observed previously [25], enhancement of DD-carboxypeptidase activity occurred on treatment of protoplast membranes with urea-Genapol X-100. Virtually all the enzyme activity was bound to the column, less than 0.5% of the soluble enzyme being unadsorbed. Binding of the enzyme to the affinity resin required intact ampicillin. Thus treatment of the ampicillin with β-lactamase prior to its coupling to the succinylaminodipropylamino agarose, or of the ampicillin-agarose with 0.8 M neutral hydroxylamine prevented subsequent adsorption of the DD-carboxypeptidase.

The enzyme showed the 'covalent' association with the affinity resin characteristic of DD-carboxypeptidases and most other penicillin-binding proteins from Gram-positive microorganisms [5—7,10,20,21]; thus the enzyme was not eluted by high salt concentrations which have proved effective with *Escherichia coli* [18] and *Proteus mirabilis* [19], and the β-lactamases from *Bacillus licheniformis* and *Bacillus cereus* [22,23]. Between 75 and 90% of the DD-carboxypeptidase applied to the column was eluted by 1.5 bed vols. of buffer containing 0.8 M neutral hydroxylamine. No further activity could be released by increasing the volume of the hydroxylamine wash. Polyacrylamide gel electrophoresis of the dialysed hydroxylamine eluate (Fig. 1a) showed a major protein band with a molecular weight of about 29 000, although small amounts of higher molecular weight proteins were also present.

Stability of the partially purified enzyme. The DD-carboxypeptidase activity of the dialysed hydroxylamine eluate was lost rapidly on storage at −20°C; however, virtually no inactivation of the enzyme occurred for up to three weeks on the addition of either 1 mM EDTA or 50% glycerol (w/v) to the eluate. The stability of the purified DD-carboxypeptidase of *E. coli* [17] has also been enhanced by 0.1 mM EDTA. Although this chelating agent has been

TABLE I

PURIFICATION OF THE DD-CARBOXYPEPTIDASE

Treated membrane values were obtained from a dialysed sample after treatment with urea and Genapol X-100 but prior to centrifugation [25].

Fraction	Total protein (mg)	Total activity ($mU \times 10^{-3}$)	Specific activity ($mU \cdot mg^{-1}$)	Yield (%)	Purification (-fold)
Protoplast membranes	438	3.04	6.94	42	—
Treated membranes	438	7.23	16.51	100	—
Soluble enzyme	355	6.15	17.37	85	—
Unadsorbed activity from affinity column	263	0.02	0.08	0.3	—
Salt wash	81	0	—	—	—
Hydroxylamine from affinity column	5	5.18	1036	72	63
DEAE-cellulose	1.3	3.52	2708	49	164

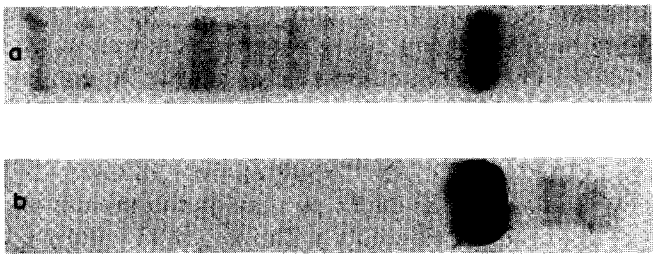

Fig. 1. SDS gel electrophoresis. SDS-polyacrylamide gels of hydroxylamine (a) and DEAE-cellulose fractions (b), see Table I.

used frequently to inhibit the activity of metal proteases, its role in these systems is unclear. In order to increase the stability of the DD-carboxypeptidase of *B. coagulans*, EDTA was incorporated into all buffers employed for dialysis or further fractionation at a final concentration of 1 mM.

DEAE-cellulose chromatography. The hydroxylamine eluate from the affinity column was dialysed exhaustively against 0.05 M sodium cacodylate-HCl, 0.1% Genapol X-100, 1 mM EDTA (pH 7.4) and applied to a DEAE-cellulose column (5 × 2 cm), equilibrated previously in the above buffer. The adsorbed enzyme was then eluted with a 50 ml gradient of 0—0.3 M NaCl; all enzyme activity was released between 0.11 and 0.15 M NaCl. The active fractions were pooled and concentrated by ultrafiltration on Amicon PM 10 membranes.

Molecular weight. Polyacrylamide gel electrophoresis of the DEAE-cellulose eluate (Fig. 1b) showed a single band of protein with a molecular weight of 29 000 ± 500, as determined from ten separate measurements. The molecular weight was the same in the presence or absence of dithiothreitol; hence the enzyme does not consist of subunits connected by sulphydryl linkages. Attempts were made to confirm the molecular weight of the DD-carboxypeptidase by gel filtration on Sephadex, however, no enzyme activity could be recovered from such resins. The reason for this is unclear, but similar results have been obtained with the DD-carboxypeptidase of *Bacillus stearothermophilus* (Mackenzie, C.R. and Reynolds, P.E., unpublished data). Table I shows a typical result for the recoveries and enrichments in specific activity during the purification procedure.

Properties of the purified enzyme

The homogeneous enzyme preparation was dialysed extensively against 0.02 M sodium acetate/acetic acid, 0.1% Genapol X-100, 1 mM EDTA (pH 4.9). divided into 0.25 ml aliquots and stored at −20°C until required. No reduction in enzyme activity was detected for at least three months.

pH optimum and thermal stability. In an analogous manner to the membrane-bound enzyme, the purified DD-carboxypeptidase had a pH optimum of 4.9 in the presence or absence of metal ions, and showed a similar degree of thermal stability. Thus the enzyme was stable up to the maximum growth temperature of the bacterium (56°C), although once this figure had been exceeded the thermal stability was sharply reduced.

Metal ions. As had been observed with the particulate enzyme [24], divalent cations enhanced activity considerably; again the greatest enhancement was found with Pb^{2+} and Cd^{2+} (970% and 841%, respectively, relative to enzyme lacking ions = 100%). This was greater than that obtained for the membrane-bound enzyme (Pb^{2+}, 703%; Cd^{2+}, 481%). Although Zn^{2+} (613% relative to 348%) also followed this trend, Mn^{2+} (350%), Ni^{2+} (307%), Co^{2+} (322%), Ca^{2+} (248%) and Mg^{2+} (204%) gave similar enhancement to that of the membrane-bound enzyme, while this was reduced considerably with Cu^{2+} (191% purified; 470% membrane bound). The optimal cation concentration in each instance reached a plateau at 10 mM, which was slightly less than that found previously for the membrane-bound enzyme (15 mM) [24].

Removal of DL-*carboxypeptidase.* Although DL-carboxypeptidase activity was present in the urea-Genapol X-100-solubilised material, the purified DD-carboxypeptidase was devoid of this activity. Thus the liberation of D-[^{14}C]alanine from substrates labelled in either the terminal or both D-alanine residues proceeded at precisely the same rate until the release of virtually 1 mol of D-[^{14}C]alanine/mol of substrate had occurred.

Kinetic parameters. K_m values for pentapeptide and its UDP-*N*-acetylmuramoyl derivative were 5 mM ± 0.5 and 2.3 mM ± 0.2, respectively, while V values were 1.25 U · mg^{-1} ± 0.2 and 2.40 U · mg^{-1} ± 0.3; these were mean values from six preparations. The effect of cations on the kinetic parameters for UDP-*N*-acetylmuramoyl pentapeptide is shown in Table II. Cation enhancement manifested itself on the V of the enzyme as the K_m remained virtually constant; the degree of enhancement being similar to that found above. Lineweaver-Burk plots characteristic of substrate inhibition were obtained at high substrate concentrations (Fig. 2a) under standard assay conditions (15 mM Mn^{2+}). Similar results were obtained in the presence of other divalent cations at this concentration, although no profiles indicative of substrate inhibition were found in the absence of cations nor when the Mn^{2+} concentration of assay mixtures was raised to 30 mM (Fig. 2b). This would imply that a substrate-metal ion complex may be the preferred or actual substrate for the enzyme [13,34].

Sulfhydryl reagents. The DD-carboxypeptidase was inhibited by sulfhydryl reagents, implying the presence of a thiol group at or near the active site of the

TABLE II

THE EFFECT OF VARIOUS CATIONS ON THE KINETIC PARAMETERS OF DD-CARBOXYPEPTIDASE

Cation	K_m (mM)	V (U · mg^{-1})
0	2.5	0.74
Mg^{2+}	2.3	1.67
Mn^{2+}	2.5	2.52
Co^{2+}	2.5	2.50
Zn^{2+}	2.1	4.55
Cd^{2+}	2.4	6.29
Pb^{2+}	2.4	7.69

K_m and V values for DD-carboxypeptidase activity were calculated in the presence of various cations (10 mM final concentration). Axial intercepts were obtained by extrapolation of the linear portion of the curve, and were verified by linear regression.

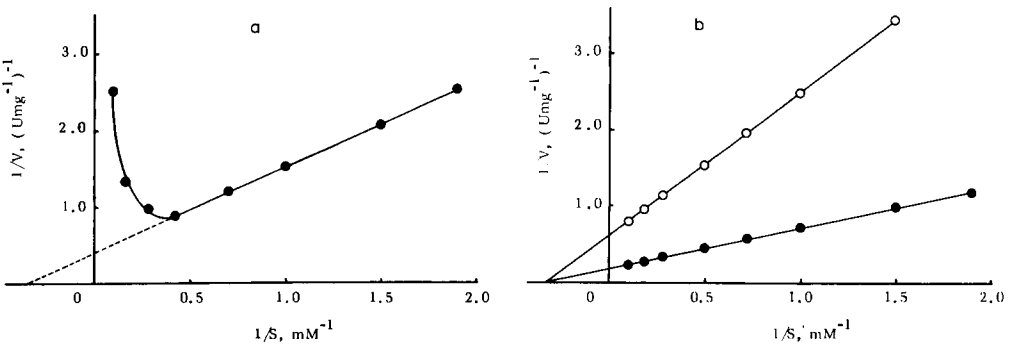

Fig. 2. DD-Carboxypeptidase kinetics. Lineweaver-Burk plots were constructed for UDP-*N*-acetylmuramoyl-L-Ala-D-Glu-*meso*-A_2pm-D-[^{14}C]Ala-D-[^{14}C]Ala, under standard assay conditions (15 mM $MnCl_2$) (a), in the absence of $MnCl_2$ (b; ○), or with $MnCl_2$ at a final concentration of 30 mM (b; ●).

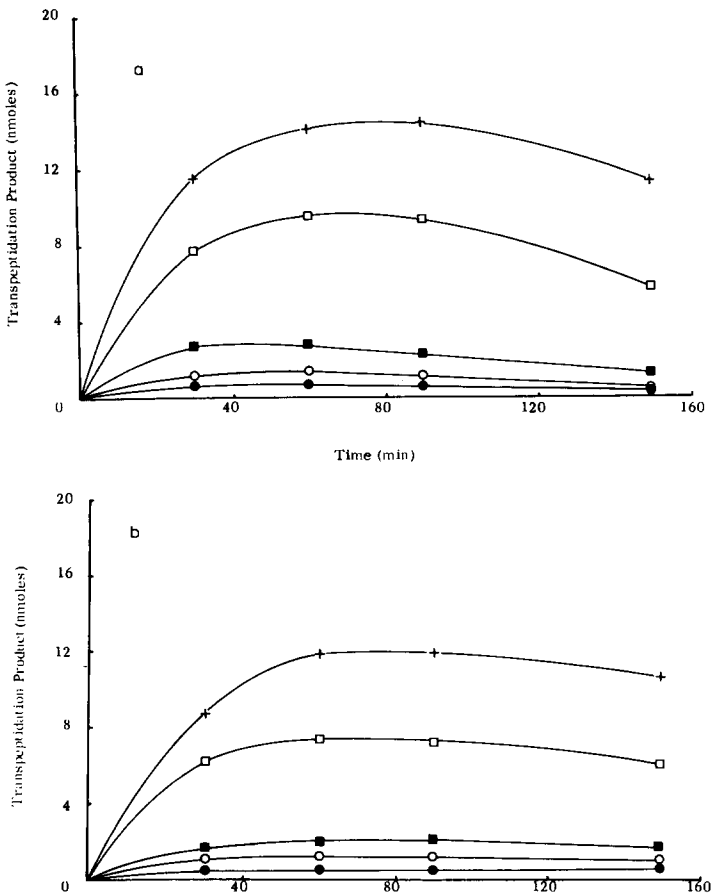

Fig. 3. Transpeptidase activity. Assay mixtures contained: 0.025 M sodium acetate/acetic acid (pH 4.9); 0.015 M $MnCl_2$; 0.1% Genapol X-100; UDP-*N*-acetylmuramoyl-L-Ala-D-Glu-*meso*-A_2pm-D-Ala-D-Ala (donor), 20 nmol; purified DD-carboxypeptidase (0.5 μg protein) and acceptor (a, D-[^{14}C]alanine; b, [^{14}C]glycine) as indicated, in a total volume of 32 μl. Incubation was at 37°C for the appropriate time. Acceptor:donor ratios; +, 100 : 1; □, 50 : 1; ■, 10 : 1; ○, 6 : 1; ●, 3 : 1.

enzyme [35]. The concentration required for 50% inhibition of enzyme activity was 150 mM, 20 mM, 2 mM and 0.05 mM, for *N*-ethylmaleimide, iodoacetate, 5,5′-dithiobis(2-nitrobenzoic acid) and *p*-hydroxymercuribenzoate, respectively. The serine protease inhibitor phenylmethylsulfonyl fluoride had no effect up to concentrations of at least 20 mM.

Transpeptidation. Although the formation of dimers from L-Ala-D-Glu-*meso*-A_2pm-D-[^{14}C]Ala-D-[^{14}C]Ala was not found, the purified DD-carboxypeptidase possessed an unnatural model transpeptidase activity. This simple transfer reaction had also been found in membrane preparations [24]. [^{14}C]-Glycine could additionally function as an acceptor although [^{14}C]diaminopimelic acid (a mixture of LL-, *meso*- and DD-isomers) and L-[^{14}C]alanine were totally ineffective. Time courses of transpeptidation for D-[^{14}C]alanine and [^{14}C]glycine at various acceptor concentrations are shown in Fig. 3a and b.

The amount of UDP-*N*-acetylmuramoyl [^{14}C]pentapeptide increased initially as a result of transpeptidase activity; however, as the incubation became prolonged, degradation of this radioactive product by the DD-carboxypeptidase occurred. The maximum amount of product formed was 14.6 nmol and 12.0 nmol, at acceptor to donor ratios of 100 : 1, for D-[^{14}C]alanine and [^{14}C]-glycine, respectively; this corresponded to the reaction of 72% and 60% of the donor present at the commencement of the assay. Although a greater degree of transpeptidation was obtained with D-[^{14}C]alanine at equivalent acceptor concentrations, the generated UDP-*N*-acetylmuramoyl pentapeptide was also

TABLE III

AMINO ACID COMPOSITION OF THE DD-CARBOXYPEPTIDASE

Cysteine was quantified as *S*-carboxymethylcysteine. The molecular weight was taken as 29 000. Percent polar (○) or hydrophobic (●) residues were calculated from Ref. 47 and 48, respectively. Amino acids recovered correspond to at least 92% of the nominal applied protein.

Amino acid	Average	Integral	Mol%	Total mass
○, Asx	27.3	27	10.1	3110
○, Thr	15.1	15	5.6	1518
○, Ser	17.4	17	6.4	1481
○, Glx	27.3	27	10.1	2195
●, Pro	10.4	10	3.8	971
Gly	23.9	24	9.0	1370
●, Ala	26.0	26	9.7	1849
●, Val	18.4	18	6.7	1786
Cys	1.2	1	0.4	102
●, Met	7.3	7	2.6	918
●, Ileu	15.3	15	5.6	1698
●, Leu	22.0	22	8.2	2490
Tyr	8.8	9	3.4	1469
●, Phe	9.8	10	1.5	1472
○, His	3.8	4	1.5	549
●, Trp	4.6	5	1.9	931
○, Lys	18.6	19	7.1	2436
○, Arg	11.3	11	4.1	1218
		267	100.0	28 981
			% hydrophobic residues 42.3	
			% polar residues 44.9	

hydrolysed more rapidly by the DD-carboxypeptidase than the molecule with the terminal [^{14}C]glycine.

As had been found previously with *B. stearothermophilus* [7], the release of D-[^{14}C]alanine from the donor was independent of the concentration or nature of the acceptor, indicating that donor and acceptor molecules have different binding sites.

Amino acid and N-terminal analyses. The results for the amino acid analysis of the DD-carboxypeptidase are shown in Table III. Analysis of nominally 150 μg of protein confirmed the presence of 1 mol carboxymethylcysteine/mol of enzyme. Reaction of the enzyme with iodo[^{14}C]acetate gave radioactivity corresponding to 0.87 mol of [^{14}C]carboxymethylated derivatives/mol of protein. Electrophoresis (pH 6.5) of acid-hydrolysed material showed that all the radioactivity was associated with a derivative with a similar mobility to authentic *S*-carboxymethylcysteine [36]. Thus the DD-carboxypeptidase contains a single cysteine residue.

No N-terminal amino acid could be detected by the dansyl technique, although large amounts of dns-*O*-tyrosine and dns-ε-lysine were found. Thus it would appear that the amino terminus of the enzyme is blocked.

Discussion

An investigation of the general properties of the purified DD-carboxypeptidase of *B. coagulans* has shown few differences in the values obtained for the membrane-bound enzyme [24], indicating that solubilisation and purification do not alter its properties significantly. Thus the enzymes had identical pH optima and similar values for thermal stability. Maximal activity was obtained in the presence of divalent cations; with the exception of Cu^{2+}, the pattern of enhancement was similar to that obtained previously, with Pb^{2+} and Cd^{2+} being the most effective. Although certain differences were observed in the kinetics, the alteration in K_m obtained on solubilisation [25] was maintained during purification.

With the exception of *Streptomyces albus* G [14], all DD-carboxypeptidases hitherto purified catalyse additionally the unnatural model transpeptidase reaction. However, none of the Gram-positive DD-carboxypeptidase tested can utilise diaminopimelic acid in this reaction, nor accomplish the natural model transpeptidase activity which more closely resembles the in vivo cross-linkage of peptidoglycan [5,7,10,13], although in each instance the latter reaction has been catalysed by the DD-carboxypeptidases of Gram-negative bacteria [8,9,19].

The molecular weight of the DD-carboxypeptidase of *B. coagulans* (M_r 29 000) is considerably lower than that found in other microorganisms which are all between 38 000 and 50 000 (5—10,14,19] (with the exception of *Strep. albus* G, M_r 18 500 [15]); however it is comparable to the molecular weight of several β-lactamases [37—40]. The degree of relatedness among proteins may be assessed from their amino acid compositions by calculating their $S\Delta Q$ values [41], and these are shown in Table IV for several DD-carboxypeptidases and β-lactamases; 98% of unrelated proteins differ by more than 100 $S\Delta Q$ units. The DD-carboxypeptidase of *B. coagulans* does not bear much

TABLE IV

ESTIMATION OF RELATEDNESS AMONGST DD-CARBOXYPEPTIDASES AND β-LACTAMASES

SΔQ values, to determine the degree of relatedness among various DD-carboxypeptidases and β-lactamases were calculated from amino acid composition according to the method of Marchalonis and Weltman [41]. Data in brackets are the reference numbers.

		1	2	3	4	5	6	7	8	9	10
	M_r:	29 000	50 000 [13]	18 500 [15]	38 000 [51]	53 300 [51]	31 000 [37]	28 000 [38]	22 000 [38]	28 823 [39]	28 900 [40]
DD-Carboxypeptidases											
B. coagulans	1	0	43	150	77	121	49	20	90	175	48
B. subtilis	2	43	0	280	139	252	74	61	82	125	112
Strep. albus G	3	150	280	0	154	97	245	200	334	512	193
Strep. R61	4	77	139	154	0	89	133	124	138	335	81
Strep. R39	5	121	252	97	89	0	206	180	240	493	133
β-Lactamases											
B. licheniformis	6	49	74	245	133	206	0	32	107	140	73
B. cereus (type I)	7	20	61	200	124	180	32	0	92	128	81
B. cereus (type II)	8	90	82	334	138	240	107	92	0	147	134
S. aureus	9	175	125	512	335	493	140	128	147	0	298
E. coli	10	48	112	193	81	133	73	81	134	298	0

similarity to the enzymes of the streptomycetes, but considerable correlation was found with the DD-carboxypeptidase from *B. subtilis*. However, the *B. coagulans* enzyme showed a close similarity to several of the β-lactamases tested, where the $S\Delta Q$ values were of the same order as those obtained between different β-lactamases; this was most pronounced with the β-lactamase I of *B. cereus*. Less correlation was obtained with the DD-carboxypeptidase of *B. subtilis* while the streptomyces enzymes showed virtually no relatedness.

The possible evolution of 'native' β-lactamases from DD-carboxypeptidases has been postulated [42—44]. The penicillinase activity of purified DD-carboxypeptidase is well established [3—5,8,45] and in two instances the degradation product is penicilloic acid [5,8], the product of 'native' β-lactamases. A penicillinase activity is also found with the purified DD-carboxypeptidase of *B. coagulans*, and a reduction in molecular weight of the membrane-bound enzyme is obtained under some conditions of solubilisation, or on treatment with small amounts of trypsin (unpublished data), in an analogous manner to the β-lactamase of *B. licheniformis* [37,46]. Indeed the absence of either a low number of polar amino acids or a high percentage of hydrophobic amino acid residues in the DD-carboxypeptidase of *B. coagulans*, which are often indicative of a membrane-bound enzyme [47,48], are at variance with the considerable resistance of the enzyme to solubilisation [25]. This may indicate the loss of a hydrophobic portion of the enzyme which aids its binding to the membrane, found with the β-lactamase of *B. licheniformis* [37,46].

In order to investigate this possible correlation further, it is necessary to determine the amino acid sequence of the DD-carboxypeptidase and to obtain X-ray crystallographic data, as the β-lactamases of *B. cereus* (type I), *B. licheniformis*, *Staphylococcus aureus* and *E. coli* show a considerable degree of structural similarity at the primary [39,40,43,49] and also, apparently, at the secondary levels [44,50]. The use of the purification technique described in this communication permits readily the preparation of large amounts of pure DD-carboxypeptidase, thus enabling such investigations to be carried out.

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