Purification of D-Alanine Carboxypeptidase from *Escherichia coli* B on a Penicillin–Sephadex Column

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1. A soluble D-alanine carboxypeptidase from *Escherichia coli* strain B was purified on a *p*-aminobenzylpenicillin–Sephadex column. This one-step chromatography followed by an $(\text{NH}_4)_2\text{SO}_4$ precipitation yielded an enzyme purified 1200-fold and some of its properties are reported. 2. The pure D-alanine carboxypeptidase was devoid of D-alanine carboxypeptidase II activity and migrated as a single protein band on analytical disc gel electrophoresis. 3. Triton X-100 in the purification procedure is an absolute requirement for obtaining a stable enzyme. 4. The enzymic activity of D-alanine carboxypeptidase was greatly affected in solution of high salt concentrations and varied somewhat with the nature of the cation tested.

D-Alanine carboxypeptidase hydrolyses the terminal D-alanyl-D-alanine peptide bond present in the nucleotide pentapeptide, UDP-MurNAc*-L-Ala-D-Glu-meso-A₂pm-D-Ala-D-Ala, which is utilized for the biosynthesis of bacterial cell wall peptidoglycan (Izaki *et al.*, 1968; Blumberg & Strominger, 1974). The enzyme from various species is inhibited either reversibly or irreversibly by extremely low concentrations of penicillin, therefore many studies on the mechanism and mode of penicillin-inhibition action were directed towards this enzyme.

The isolation of D-alanine carboxypeptidase from a number of organisms has been reported. In *Escherichia coli* B the enzyme was found in both the soluble and particulate fractions (Izaki & Strominger, 1968). Membrane-bound carboxypeptidases were found in *Bacillus subtilis* (Lawrence & Strominger, 1970), *Bacillus megaterium* KM (Wickus & Strominger, 1972) and *Bacillus stearothermophilus* (Barnett, 1973). *Streptomyces* strains contain a soluble D-alanine carboxypeptidase which is excreted into the culture medium (Ghuysen *et al.*, 1970; Leyh-Bouille *et al.*, 1970a,b, 1971, 1972). Only the *Streptomyces* enzymes were obtained in pure form. However, studies of the molecular properties of these enzymes and their peculiar sensitivity to penicillin demand an easy and reproducible method for purification.

The technique of affinity chromatography permits fast and convenient purification of an enzyme by its selective adsorption on a suitable matrix to which a

specific inhibitor has been covalently attached (Cuatrecasas *et al.*, 1968; Cuatrecasas & Anfinsen, 1971). Penicillin–Sephadex was used successfully in isolation of penicillin-binding proteins from membranes of *B. subtilis* by covalent affinity chromatography (Blumberg & Strominger, 1972). The proteins covalently bound to the immobilized penicillin were released with NH_4OH . The use of cephalosporin–Sephadex permitted partial purification of penicillinase from *Bacillus licheniformis* (Crane *et al.*, 1973). Use of insoluble penicillins and cephalosporins can therefore be a promising tool in the studies of proteins that are vulnerable to β -lactam antibiotics.

The present paper describes purification of D-alanine carboxypeptidase from the soluble fraction of *E. coli* B by affinity chromatography on a *p*-aminobenzylpenicillin–Sephadex column.

Materials and methods

Sephadex 4B was purchased from Pharmacia, Uppsala, Sweden; CNBr was from Fluka A.G., Buchs SG, Switzerland; D-[U-¹⁴C]alanine (30 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; vancomycin was from Eli Lilly Co., Indianapolis, Ind., U.S.A.; and cycloserine was from Sigma, St. Louis, Mo., U.S.A. *p*-Aminobenzylpenicillin was a gift from Beecham Research Laboratories, Betchworth, Surrey, U.K. Benzyl-oxy-carbonyl-D-alanine *N*-hydroxysuccinimide ester was prepared by Miss G. Saltoun in our laboratory. *E. coli*, strain B, was grown in a medium containing 1% (w/v) yeast extract (Difco Laboratories Inc., Detroit, Mich., U.S.A.), 2.18% (w/v) K_2HPO_4 , 1.7%

* Abbreviations: MurNAc, N-acetylmuramic acid, A₂pm, α -mesodiaminopimelic acid; DNAase, deoxyribonuclease; RNAase, ribonuclease.

(w/v) KH_2PO_4 and 1% (w/v) glucose. The cells were harvested during their exponential-growth phase. Wet cells (1500g) were suspended in 3 litres of 0.05M-Tris-HCl, pH 8.2, and freeze-dried.

Synthesis of D-[^{14}C]Ala-D-Ala

A sample of D-[^{14}C]alanine (40 μCi) was dried over phosphorous pentoxide. Dioxan saturated with phosgene (2ml) was added and the mixture was stirred for 16h at 24°C. The solvent was evaporated by a stream of dry N_2 and the residue was redissolved in 0.5ml of acetonitrile and cooled to -15°C. A solution of D-alanine (10mg in 0.5ml of 0.1M- NaHCO_3) and 0.5ml of acetonitrile were added and the reaction mixture was stirred for 1h at -10°C. The D-[^{14}C]Ala-D-Ala was purified by high-voltage electrophoresis on Whatman no. 3 paper (pH 3.5, 50V/cm, 45min). The radioactive dipeptide was located by radioautography and eluted with water. The yield was 92%.

Synthesis of D-Ala-D-[^{14}C]Ala

Benzoyloxycarbonyl-D-alanine *N*-hydroxysuccinimide ester (6.4mg) was dissolved in dioxan (0.6ml) and added to 100 μCi of D-[U- ^{14}C]alanine in 0.5ml of 1M-sodium phosphate buffer (pH 7.5). The reaction mixture was stirred at room temperature for 16h after which the solvent was evaporated to dryness, and 1ml of HBr in acetic acid was added. After 1h, the acid was evaporated in a desiccator over NaOH under reduced pressure. The residue was redissolved in water, applied to a 50cm column of a Beckman 120C amino acid analyser and eluted with 0.2M-sodium citrate buffer (pH 4.25). Radioactivity in the column effluent was determined with a Packard Tri-Carb flow analyser scintillation spectrometer 3022, with anthracene as scintillant. The appropriate fractions were pooled and desalted on a column (1cm $\times$ 15cm) of Dowex 1X4 (OH^- form). The column was first washed with 50ml of water and then the dipeptide was eluted with 1M-acetic acid and freeze-dried. The yield was 95%.

Preparation of UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm-D-Ala-D-Ala

This nucleotide pentapeptide was isolated from *Bacillus cereus* by inhibiting growth with vancomycin. The bacteria were grown at 37°C in a medium containing 0.5% bacto-peptone, 0.5% yeast extract and 0.3% K_2HPO_4 to a middle exponential phase; then vancomycin (20mg/l) was added and the incubation continued for 60min. The bacteria were harvested, washed twice with 0.9% NaCl and the cells (50g wet packed cells) were suspended in 125ml of 0.9% NaCl. The cooled cells were homogenized in trichloroacetic

acid (20%, 125ml) with 150g of glass beads in an Omnimixer for 30min. The cellular debris was pelleted by centrifugation and the supernatant saved. The pellet was resuspended in 60ml of trichloroacetic acid (20%) and homogenization was repeated for an additional 30min. The debris was removed by centrifugation and the combined supernatants were extracted three times with ether. The traces of ether were removed by evaporation. The solution was adjusted to pH 7.0 with 1M- NH_3 , and freeze-dried. The residue was dissolved in 10ml of water and applied to a column (4cm $\times$ 100cm) of Sephadex G-25, equilibrated with 1M-HCl. The column was developed with the same solvent. The nucleotide pentapeptide which emerged in the first u.v. peak was localized by modified Morgan-Elson assay for *N*-acetylhexosamine (Reissig *et al.*, 1955; Sharon *et al.*, 1966). The corresponding fractions were pooled and freeze-dried. The residue was redissolved in 10ml of water and applied to a column (1.5cm $\times$ 30cm) of Dowex 1 (formate form). The column was eluted with 3M-ammonium formate. The tubes containing the hexosamine pentapeptide were pooled, desalted by ultrafiltration in an Amicon apparatus (UM-05 Diaflo membrane) and then freeze-dried. The compound, located by ninhydrin stain and u.v. absorption was found to be homogeneous by paper chromatography in isobutyric acid-1M- NH_3 (5:3, v/v). Amino acid analysis of the hydrolysed nucleotide pentapeptide showed the molar ratios of alanine/glutamic acid/ α -mesodiaminopimelic acid/muramic acid to be 2.7:1.0:0.9:0.9.

The yield was 100 μmol of nucleotide pentapeptide.

Preparation of UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm

This compound was isolated from *B. cereus*, treated with cycloserine (Izaki *et al.*, 1968). The procedure for isolation and purification of UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm was the same as described earlier for the UDP-MurNAc-pentapeptide. Amino acid analysis of a hydrolysed sample revealed the molar ratios of alanine/glutamic acid/ α -mesodiaminopimelic acid/muramic acid to be 0.92:1.0:0.95:0.9.

Preparation of UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm-D-Ala-D-[^{14}C]Ala and UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm-D-[^{14}C]Ala-D-Ala

These peptides were prepared by enzymic addition of D-Ala-D-[^{14}C]Ala or D-[^{14}C]Ala-D-Ala to UDP-MurNAc-L-Ala-D-Glu-meso-A₂pm. The adding enzyme was prepared from *E. coli*, strain B. The procedure of Comb (1962) was followed up to the first protamine sulphate precipitation step. The

supernatant thus obtained was dialysed and concentrated by ultrafiltration through a Diaflo UM-05 membrane (Amicon apparatus).

The enzymic addition was performed in a mixture containing 20 μ Ci of D-Ala-D-Ala, 10 μ mol of UDP-MurNAc-tripeptide, 50 μ mol of $MgCl_2$, 25 μ mol of ATP, 200 μ mol of Tris-HCl (pH 8.6) and 1 ml of a crude solution of the adding enzyme (obtained from 5 g of wet bacteria) in a total volume of 5 ml. After incubation for 3 h at 37°C the reaction mixture was spotted on a Whatman no. 3MM paper and subjected to high-voltage electrophoresis (pH 3.5; 50 V/cm; 45 min). The radioactive pentapeptide, located by radioautography as determined by co-migration with unlabelled UDP-Mur-Nac-pentapeptide, was eluted with water and freeze-dried. The pure compound was obtained by repeated paper chromatography on Whatman no. 3MM paper in isobutyric acid-1 M-NH₃ (5:3, v/v) for 48 h.

Preparation of penicillin-Sephadex columns

Sephadex 4B was activated with CNBr by the method of Cuatrecasas *et al.* (1968). The washed activated Sephadex (50 ml) was suspended in 50 ml of ice-cold 0.1 M-NaHCO₃, containing 0.5 g of *p*-aminobenzylpenicillin. The mixture was stirred gently at 4°C for 16 h, then sequentially washed with water, 1 M-NaCl and an appropriate buffer. Under these conditions 1–2 μ mol of penicillin was coupled per ml of gel as determined by sulphur analysis. Penicillin G does not bind to the CNBr-activated Sephadex. Therefore it can be assumed that the nature of attachment of *p*-aminobenzyl-penicillin is via free *para*-amino group.

Gel electrophoresis

Analytical polyacrylamide-disc-gel electrophoresis was carried out by the method of Davis (1964) (7.5%, w/v, gels, pH 8.9). The protein bands were stained with Coomassie Brilliant Blue G250 by the improved method of Diezel *et al.* (1972).

Electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate was performed by the procedure of Weber & Osborn (1969).

Determination of protein

Total protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard.

Activity assay

The enzymic activity of D-alanine carboxypeptidase was assayed in a mixture containing 5 μ l of enzyme (1 μ g), 15 μ l of 0.05 M-Tris-HCl (pH 8.2)–0.1 mM-

EDTA and 5 μ l of UDP-MurNAc-D-[¹⁴C]Ala-pentapeptide solution (12000 c.p.m.) diluted with unlabelled UDP-MurNAc-pentapeptide to a final concentration of 3 mM, in a volume of 25 μ l. The mixture was incubated at 30°C, for 30 min and 200 μ l of 0.2 M-sodium citrate buffer, pH 2.2, was added. The reaction mixture was centrifuged at 8000g for 2 min (Eppendorf microcentrifuge) and the supernatant removed for analysis of [¹⁴C]alanine. Analysis was performed on the 20 cm column of the amino acid analyser (Beckman 120C) and elution was carried out with 0.2 M-sodium citrate buffer (pH 4.25). The effluent from the column was directly connected to a Packard Tri-Carb flow analyser scintillation spectrometer. In this system UDP-MurNAc-D-[¹⁴C]Ala-pentapeptide is eluted in the void volume and [¹⁴C]alanine emerges 15 min later. The enzymic activity was expressed as nmol of substrate hydrolysed/min per mg of protein.

Results

Purification of D-alanine carboxypeptidase

Step I: crude enzyme. Freeze-dried *E. coli* cells (100 g) were suspended in 4 litre of water and stirred for 10 min. The suspension was centrifuged at 10000g in a Sharpless centrifuge. The supernatant (3.5 litres) was incubated for 3 h at 23°C with 2 mg of DNAase and 2 mg of RNAase. The clear solution was chilled to 4°C and 1400 g of solid (NH₄)₂SO₄ was added. The precipitate was collected by centrifugation using a Sharpless centrifuge, redissolved in 500 ml of 0.05 M-Tris-HCl (pH 8.2) and dialysed overnight at 4°C against the same buffer. The insoluble material was removed by centrifugation and discarded and the supernatant was freeze-dried. The dried crude enzyme is stable on storage at –20°C for several months.

Step II: affinity chromatography. Dried crude enzyme (1 g) was dissolved in 100 ml of 0.05 M-Tris-HCl (pH 8.2) at 22°C containing 1 mM-EDTA and 1% Triton X-100. This solution was applied to a *p*-aminobenzylpenicillin-Sephadex column (1.4 cm $\times$ 10 cm) equilibrated with 0.05 M-Tris-HCl–0.1 mM-EDTA (pH 8.2). All of the enzymic activity was adsorbed by the column and unbound protein was removed by washing the column with the equilibrating buffer. D-Alanine carboxypeptidase was then eluted with 0.5 M-NaCl (Fig. 1a).

Step III: ammonium sulphate precipitation. The fractions that contained enzymic activity from Step II were pooled and solid (NH₄)₂SO₄ was added to 60% saturation at 4°C. The precipitate was collected by centrifugation at 15000g for 10 min, redissolved in 1 ml of 0.05 M-Tris-HCl–0.1 mM-EDTA–0.025% Triton X-100 (pH 8.2), and dialysed against the same buffer lacking Triton X-100 for 3 h at 4°C.

By this procedure the enzyme solution was concentrated and could be stored for several weeks at

Fig. 1. Affinity chromatography of D-alanine carboxypeptidase on *p*-aminobenzylpenicillin-Sepharose (Step II) in the presence (a) and absence (b) of Triton X-100

The elution of the pure enzyme was achieved with 0.5M-NaCl (arrow A). While the affinity chromatography was performed without detergent, the affinity column was washed with 0.1M-NaCl containing buffer (arrow B) before elution of the enzyme. For the conditions see the Results section, E_{280} ; ○, enzyme activity.

Table 1. Purification of D-alanine carboxypeptidase

The purification was carried out as described in the text. One activity unit is defined as the hydrolysis of 1 nmol of substrate/min.

Step	Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Total activity* (units)	Purification	Yield (%)
1	Extract	100	800	425	1250	1	—
2	Affinity chromatography	20	0.9	1112	1125	800	90
3	(NH ₄) ₂ SO ₄ fractionation	1.5	0.55	1000	1025	1200	82

* The activity of the enzyme preparation was measured in 0.1M-NaCl.

−20°C without any detectable loss of activity. Dilute solutions of the enzyme in 0.025% Triton X-100 retained all their enzymic activity when kept at 4°C for 2–3 weeks. Pure enzyme can be dried by freeze-drying and stored as dry powder for several months.

Table 1 summarizes the extent of purification and yields of isolation of D-alanine carboxypeptidase.

Analytical disc gel electrophoresis of D-alanine carboxypeptidase (from Step III) revealed one protein band and a slowly migrating broad band which is due to traces of Triton X-100. The hydrophobic detergent was probably adsorbed with the enzyme on the affinity column and emerged together with the enzyme by elution with 0.5M-NaCl.

Pure enzyme, devoid of Triton X-100, was obtained by a modified affinity-chromatography procedure; the crude material from Step I was applied to the affinity column in the absence of Triton X-100, washed with 0.05M-Tris-HCl (pH 8.2) containing

1mM-EDTA and 0.1M-NaCl to remove non-specifically bound proteins which otherwise emerge together with D-alanine carboxypeptidase (Fig. 1b). The enzyme was eluted with 0.5M-NaCl and concentrated as described in Step III. This preparation loses activity when stored for several days at −20°C. Gel electrophoresis of this D-alanine carboxypeptidase showed one main band migrating identically with the enzyme prepared in the presence of Triton X-100. All preparations aggregate during electrophoresis as is demonstrated by the appearance of a protein band on the top of the gel. Analytical gel electrophoresis performed with 0.1% sodium dodecyl sulphate showed that the enzyme migrates as a single band corresponding to a protein with a molecular weight of approx. 43000.

The specific activity of D-alanine carboxypeptidase purified in the absence of Triton X-100 is doubled by the addition of the detergent, although the stability of

the enzyme is not restored. Therefore an affinity-chromatography procedure in which the buffer contained Triton X-100 was adopted for routine preparations of purified D-alanine carboxypeptidase. Partial stabilization of the enzyme can be also achieved by the addition of 0.1% bovine serum albumin or 10% (v/v) glycerol.

D-Alanine carboxypeptidase II

The D-alanine carboxypeptidase preparations were assayed for the presence of D-alanine carboxypeptidase II (Izaki & Strominger, 1968), which removes the terminal D-alanine from UDP-MurNAC-L-Ala-D-Glu-A₂pm-D-Ala. The assay was performed with Mur-NAC-pentapeptide labelled in the penultimate D-alanine residue exactly as described for D-alanine carboxypeptidase. Radioactive alanine could be released only by a combined action of D-alanine carboxypeptidase which removed the terminal unlabelled D-alanine, followed by the D-alanine carboxypeptidase II which hydrolyses the remaining UDP-MurNAC-tetrapeptide, releasing its radioactive ultimate D-alanine residue.

D-Alanine carboxypeptidase II activity was found in the crude extract (Step I). However, it was about 50 times lower than the activity of D-alanine carboxypeptidase present in the same preparation. Purified enzyme obtained by affinity chromatography was completely free from D-alanine carboxypeptidase II activity.

Variability of the penicillin-Sephadex conjugates

p-Aminobenzylpenicillin coupled to Sephadex by the CNBr method is a very stable conjugate which retains its ability to adsorb specifically D-alanine

carboxypeptidase even after storage in water at 4°C in the presence of 0.02% sodium azide, for several months. During these studies about 60% of all preparations of substituted gels were suitable for immediate use in affinity chromatography of the enzyme. However, some of the conjugates adsorb the enzyme irreversibly and no enzymic activity could be eluted by high salt concentrations. Exhaustive saturation of the gel by considerable amounts of crude enzyme (10 times as much enzyme as used for a normal purification procedure) converted the penicillin-Sephadex column into a reversible adsorbent of D-alanine carboxypeptidase, suitable for affinity chromatography, as described.

The above saturation effect was highly specific, since no other inert protein, e.g. bovine serum albumin, could replace D-alanine carboxypeptidase in the above treatment. We have also noticed that prolonged storage at 4°C of some of these highly adsorbing gels converted them into gels suitable for normal affinity chromatography. This change may be attributed to a decrease in the number of highly active 'adsorbing sites' on the surface of the gels, so that the enzyme can be bound reversibly to the gel conjugate. Hydrolysis of the β -lactam ring in the penicillin nucleus is not responsible for the change in the properties of the gels since prolonged treatment of the conjugate with a neutral solution of NH₂OH completely destroys their specific adsorbing properties.

Properties of purified D-alanine carboxypeptidase

The Michaelis constant K_m of D-alanine carboxypeptidase for the substrate UDP-MurNAC-pentapeptide at 30°C was 0.4×10^{-3} M. A similar value was reported by Izaki & Strominger (1968).

Fig. 2. Effect of cations on D-alanine carboxypeptidase activity

(a) Effect of cations (with Cl⁻ as anion) on pure D-alanine carboxypeptidase. (b) Effect of NaCl on pure D-alanine carboxypeptidase (—) and on the crude extract (----). The activities of crude and purified enzyme without any added NaCl were chosen as 100%.

Fig. 3. Reversal of the penicillin inhibition of D-alanine carboxypeptidase by penicillinase

D-Alanine carboxypeptidase (Step III) (5 μ l) was incubated with 2 μ l of penicillin G (25 μ g/ml) and 5 μ l of 0.1M-Tris-HCl buffer, pH 8.2, for 5 min at 4°C. Then 3 μ l of penicillinase (5mg/ml) was added and the reaction mixture was incubated at 37°C. At various time-intervals the residual D-alanine carboxypeptidase activity was assayed (○). In the control experiment, the activity of D-alanine carboxypeptidase in the presence of penicillinase was measured (Δ).

Metal-ion requirements. D-Alanine carboxypeptidase showed no absolute requirements for added cations, since EDTA did not inactivate the enzyme even at 0.1M, but rather stabilized it at low concentration. This led us to use buffer solutions containing 0.1mM-EDTA for all treatments of the enzyme. However, an increase in activity was caused by addition of Na^+ , Mg^{2+} or Mn^{2+} if tested in a limited concentration range (Fig. 2). Mg^{2+} and Mn^{2+} stimulate the enzyme activity about 2- and 3-fold respectively at a concentration of 5mM.

NaCl at concentrations higher than 0.15M inhibits pure D-alanine carboxypeptidase whereas activity of the crude extract is stimulated by 0.2–0.3M-NaCl and its inactivation is observed at much higher salt concentrations (0.6–0.8M) (Fig. 2).

Inhibition by penicillin. Pure D-alanine carboxypeptidase is inhibited strongly by extremely low concentrations of penicillins. K_i for penicillin G was 10^{-8} M. The affinity between D-alanine carboxypeptidase and penicillin G is not affected by Triton X-100 when measured in 1% solutions of the detergent. The inhibition caused by penicillin can be completely reversed by digestion with penicillinase (Fig. 3).

Discussion

A Sepharose column to which *p*-aminobenzyl-penicillin has been covalently bound was used as a

powerful adsorbent for purification of D-alanine carboxypeptidase from *E. coli* B. Affinity chromatography of a crude extract yielded a homogeneous protein with an excellent total activity recovery of 80%. As concentration of crude enzyme by $(\text{NH}_4)_2\text{SO}_4$ precipitation did not cause any significant loss in the activity of D-alanine carboxypeptidase, the scaling up of the purification procedure for larger quantities of the enzyme can be easily achieved. Moreover, this affinity-chromatography method may be adopted for purification of similar penicillin-sensitive D-alanine carboxypeptidases from various sources.

Partial purification of D-alanine carboxypeptidase by ion-exchange chromatography (120-fold) reported by Izaki & Strominger (1968), resulted in a low yield of about 1.5%. In our hands, such preparations could not be further purified because of their low stability. Similarly, an enzyme purified by affinity chromatography in the absence of Triton X-100 was unstable. The presence of the detergent before affinity chromatography stabilized the enzyme, and partially prevented its aggregation. Triton X-100 increases the enzymic activity of prepared enzyme, but does not stabilize it.

The penicillin-Sepharose column may be used for repeated and reproducible affinity chromatography for practically unlimited time. The immobilized penicillin is very stable and only on prolonged treatment with neutral hydroxylamine is it destroyed.

Penicillin, even a 10mM solution does not desorb D-alanine carboxypeptidase from the penicillin-Sepharose conjugate. It seems reasonable that because of steric hinderance by the polysaccharide matrix, the soluble inhibitor is impeded and cannot compete with the insoluble one. However, the affinity of the enzyme for the insoluble ligand is altered by the high salt concentrations, and its elution from the affinity column by NaCl ensues.

A similar situation was observed in purification of thymidine kinase by affinity chromatography on a 5'-amino-5'-deoxythymidine-Sepharose column. The enzyme was firmly bound to the column and could not be eluted with a solution of the substrate, but was readily released from the column by adding NaCl to the eluent (Rohde & Lezius, 1971).

The response of D-alanine carboxypeptidase to salt concentration depends on the purity of the enzyme preparation (Fig. 3). Although the activity of the crude enzyme is enhanced by increasing the ionic strength up to 0.2M and declines at higher concentrations, the purified enzyme is inhibited at lower salt concentrations. Moreover, if the recovery of the enzyme purified by affinity chromatography was calculated with respect to the amount of enzyme in the crude extract (assayed without adding Na^+ or Mg^{2+} ions) a peculiar increase in the total enzyme activity was noticed (Table 1).

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