A DIPEPTIDOCARBOXYPEPTIDASE FROM E. COLI*

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A sequence-ordered, α,N-substituted copolymer, α,DNP-poly-(Pro-Gly-Pro) was used as a specific substrate to follow, during purification, a proteolytic activity detected in E. Coli B extracts. The enzyme which releases glycyl-proline from the C-terminal end of the molecule is a dipeptidocarboxypeptidase (DCP). It was purified to homogeneity (purification factor of 1200) and shown to split C-terminal dipeptides from α,N-blocked tripeptides as well as from tetrapeptides and peptides of higher molecular weight. The enzyme has a pH optimum at 8.2, and is not deactivated by dialysis against EDTA (10^{-4}M). It is strongly activated by Co^{++}, slightly by Mn^{++} and Mg^{++} and deactivated by Zn^{++}, Cd^{++} and Ni^{++}. NaCl is not required and has little effect on enzyme activity. With tetrapeptide substrates Lineweaver-Burk plots were linear and gave $\overline{K}_m$ values in the range of 165-10,000 M^{-1} and k_{cat} of 30-220 sec^{-1}.

Isolation. The enzyme was isolated from Escherichia coli strain B. The cells were mechanically ruptured and the crude extract was subjected to the purification steps summarized in Table I (which also describes the assay of enzyme activity). The crude extract was treated with DNase and cell debris was centrifuged off. The supernatant was heated at 50° for 15 min causing precipitation of non-active proteins and also inactivating a dipeptidase which interferes with the assay. More protein was precipitated by adding $(NH_4)_2SO_4$ to 0.4 saturation and the activity was precipitated from the supernatant by increasing the saturation to 0.6. The precipitate obtained was dissolved in 0.05 M sodium acetate, pH 5.6 and acetone precipitation was performed

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TABLE I. Purification of Dipeptidocarboxypeptidase from E. coli-B[a]

Purification step	Volume (ml)	Activity[b] (units/ml)	Total units	Protein[c] (mg/ml)	Specific[d] Activity (units/mg)	Activity Yield (%)	Purification factor
Crude extract	40,000	0.87[e]	35,000[e]	31[e]	0.028[e]	-	-
Heating	40,000	0.96	38,500	10	0.096	100	1
Ammonium sulfate 0.4-0.6 saturation	7,000	1.81	12,600	15.1	0.12	32.7	1.24
Acetone fractionation 44-58%	1,000	6.03	6,030	7.83	0.77	15.7	8.0
DEAE-cellulose (ultrafiltration)	20	210	4,200	27.6	7.6	10.9	79
Sephadex Fr. I+II	67	34.2	2,305	1.72	19.7	6.0	204
Fr. III	40.3	23.4	945	1.90	12.3	2.45	127
Hydroxylapatite (ultrafiltration) Fr. I+II of step 5	30.4	42.5	1,290	0.67	63.5	3.3	660
Electrophoresis (ultrafiltration)	88.5	13.4	1,190	0.115	116	3.1	1,200

(a) Figures are given for 10 kg wet cells. (b) The activity was determined with α,N-DNP-poly-(Pro-Gly-Pro) as the substrate, using the ninhydrine colorimetric method (2) to measure the amount of Gly-Pro formed. The substrate in water (10 mg/ml), 7.2×10^{-3} M, 0.05 ml) was mixed with 0.05 M veronal buffer pH 8.15 (0.95 ml), placed into a 40°C water bath and the enzyme solution, (5-20 µl suitably diluted to produce 5-20 µg Gly-Pro in 15 min) added. After 15 min the reaction was stopped by adding the ninhydrine reagent. The ninhydrine color yields were determined with a Klett Summerson photoelectric colorimeter using filter No. 56. The amount of Gly-Pro formed was calculated from a calibration curve constructed with known amounts of Gly-Pro. One unit of activity is defined as the amount of enzyme which produces 1 mg Gly-Pro per hour under the assay conditions. (c) Protein concentrations were determined by the method of Lowry et al. (3). Bovine serum albumin was used as standard. (d) The specific activity is expressed in units per milligram of protein. (e) The figures obtained with the crude extract are approximate values, since interfering activities prevented accurate measurements.

at -3°. The precipitate obtained between 44% and 58% acetone was dissolved in 0.05 M acetate pH 5.6 and dialyzed against 0.005 M phosphate pH 6.0. This solution was fractionated on a DEAE-cellulose column (11.5x28.5 cm) with a linear gradient between the above buffer (18 liter) and 0.24 M KCl in the same buffer (18 liter). The active fractions were concentrated by ultrafiltration and applied to a Sephadex G-150 column. The active fractions were pooled, applied to a hydroxylapatite column (1.3x92 cm) and eluted with a linear gradient from 0.01 M phosphate pH 6.0 (1 liter) to 0.08 M phosphate pH 6.0 (1 liter).

The active fractions were concentrated by ultrafiltration and subjected to preparative acrylamide gel electrophoresis, using the Shandon apparatus with a locally modified collecting chamber. The individual steps were monitored by acrylamide disc electrophoresis (see Fig. 1).

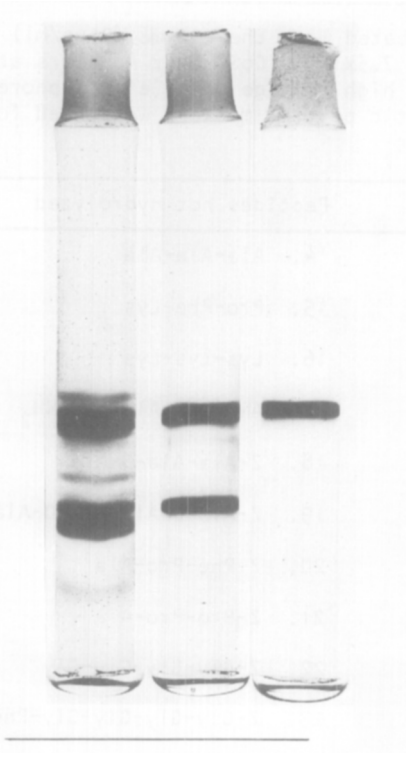

Fig. 1. Polyacrylamide gel electrophoresis (8) of dipeptidocarboxypeptidase preparations at various stages of purification. After gel filtration (left); after hydroxylapatite (center); after electrophoresis (right).

The preparation obtained in the last step was shown to be homogeneous by the above electrophoresis as well as by immunoelectrophoresis and immunodiffusion.

The enzyme does not require the addition of a metal for its catalytic action

Exhaustive dialysis against 10^{-4}M EDTA does not affect the specific activity. Co^{++} increases the specific activity about 5 to 8-fold, with quite a sharp optimum at $5x10^{-5}$M; slight activation was observed with Mg^{++} and Mn^{++} at 10^{-3}M; and partial deactivation with Zn^{++}, Cd^{++} and Ni^{++}. The pH of highest activity was 8.2.

Substrate specificity. The pure DCP from E. coli hydrolyzes the penultimate peptide bond of α,N-blocked tripeptides as well as tetrapeptides and higher peptides: $---R_{n-2}\overset{\downarrow}{-}R_{n-1}---R_n$ (see Table II). One peptide bond is hydrolyzed in tetra- (#2-7) and pentapeptides (#8-10) two peptide bonds are hydrolyzed successively in hexapeptide #11 and in the nonapeptide bradykinin (#12). The requirements of a free carboxyl group and stereospecificity are demonstrated

TABLE II. Hydrolysis of peptides by dipeptidocarboxypeptidase from E. coli

The peptides ($5x10^{-3}$M) tested were incubated with the enzyme (2 μg/ml) in 0.05 M borate buffer pH 8.15 containing $7.5x10^{-5}$M $CoSO_4$ for 48 hours at 40°C. The incubation solution was analyzed by high voltage paper electrophoresis (pH 1.4, 33 v/cm, 2.5-3 hours). Authentic peptide markers were used for comparison.

	Peptides Hydrolyzed		Peptides not Hydrolyzed
1.	Z-Ala↓Ala-Ala	14.	Ala-Ala-Ala
2.	Ala-Ala↓Ala-Ala	15.	Pro-Phe-Lys
3.	Lys-Lys↓Lys-Lys	16.	Lys-Lys-Lys
4.	Pro-Phe↓Gly-Lys	17.	Ala-Ala-Ala-Ala-NH_2
5.	Ala-Gly↓Phe-Ala	18.	Z-Ala-Ala-D-Ala
6.	Gly-Gly↓Phe-Ala	19.	Z-Ala-D-Ala-Ala-D-Ala
7.	Ala-Ala↓Phe-Ala	20.	Z-Phe-Pro-Ala
8.	Ala-Ala-Ala↓Lys-Ala	21.	Z-Pro-Pro-Ala
9.	Lys-Ala-Ala↓Ala-Ala	22.	Z-Gly-Gly-Gly-Gly
10.	Ala-Ala-Ala↓Lys-Phe	23.	Z-Gly-Gly-Gly-Gly-Phe
11.	Lys-Ala↓Ala-Lys↓Ala-Ala	24	poly-Pro
12.	Arg-Pro-Pro-Gly-Phe↓Ser-Pro↓Phe-Arg		
13.	$(Pro-Gly-Pro)_{n-1}$-Pro↓Gly-Pro ($\bar{n}$ = 4.6)		

by resistance to hydrolysis of peptides (#17-19). Peptide bonds in which the nitrogen is that of a proline residue were not hydrolyzed (# 20,21,24), neither were peptide bonds between two glycine residues (# 22,23).

Kinetic parameters. The rate of hydrolysis of several substrates was measured spectrophotometrically by the change of absorption at 225 nm. The initial rates increased linearly with enzyme concentration in the range tested ($1.0 - 6.0 \times 10^{-9}$M). Lineweaver-Burk plots were linear in the range 1.0×10^{-4} to

TABLE III. Kinetic parameters for the hydrolysis of peptides

The composition of the reaction solution was: substrate ($1.0 \times 10^{-4} - 7.5 \times 10^{-3}$M), enzyme ($1.0 - 6 \times 10^{-9}$M), 0.05 M borate buffer pH 8.15, (no metal added) at 40°. The difference in molar extinction coefficients, $\Delta\varepsilon_{225}$ accompanying the hydrolysis was determined with authentic mixtures of the product peptides and the substrates.

Substrate	$\overline{K}_m$ (M^{-1})	k_{cat} [a] (sec^{-1})	$C = \overline{K}_m k_{cat}$ ($M^{-1} sec^{-1}$)
Boc-Ala_3	1,400	34	47,800
Ala_4	2,280	139	318,000
Z-Ala_4	2,430	131	318,000
Ala-Ala-Phe-Ala	785	225	177,000
Gly-Ala-Phe-Ala	720	194	140,000
Ala-Gly-Phe-Ala	1,640	156	256,000
Gly-Gly-Phe-Ala	165	116	19,220
Lys_4	~10,000 [b]	~30 [b]	~300,000

[a] Assumed MW 100,000

[b] Substrate inhibition.

7.5×10^{-3}M substrate concentration. The values of $\overline{K}_m$ ($=1/K_m$), k_{cat} and C are given in Table III.

DISCUSSION

An enzymic activity compatible with the mode of action of DCP has been repeatedly reported to be present in mammalian tissue and studied in connection with conversion of the biologically inactive decapeptide angiotensin I to the active octapeptide angiotensin II with the liberation of the C-terminal His-Leu This "converting enzyme" (4) was partially purified from hog plasma (5,6) and shown to split C-terminal dipeptides (6) not only from angiotensin I but also from other peptides including bradykinin.

An activity with a specificity similar to our DCP has been shown to be present in Corynebacterium equi (7). It seems to differ in its capability to hydrolyze unprotected tripeptides . Since the presence of tripeptidases in the partially purified preparation cannot be excluded , only further purification of the enzyme will show if this is an intrinsic property of C. equi DCP.

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