# A NOVEL AMINOPEPTIDASE FROM CLOSTRIDIUM HISTOLYTICUM

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Received November 27,1972

An aminopeptidase was found in the culture filtrate of Cl. histolyticum and purified to homogeneity (130 times) in a two-step procedure. All types of N--terminal amino acids, including proline and hydroxyproline are cleaved by the enzyme from small peptides and from polypeptides. A low rate of hydrolysis was observed for β-naphthylamides and for alanine amide; p-nitroanilides were not hydrolyzed. Kinetic parameters ($K_m$ and $V_{max}$) for several tripeptides and the tetrapeptide Pro-Gly-Pro-Pro were determined. The enzyme has a pH optimum at 8.6. The presence of either $Mn^{++}$ or $Co^{++}$ is essential for its activity. Only slight activation was observed with $Ni^{++}$ and $Cd^{++}$, while $Zn^{++}$ and $Cu^{++}$ were inhibitory. The molecular weight of the native enzyme is about 340,000, and a molecular weight of about 60,000 was determined for the reduced and denatured enzyme by gel electrophoresis in sodium dodecyl sulfate (SDS).

The culture filtrate of Cl. histolyticum has been shown to contain various proteolytic enzymes, in addition to collagenase[1-5]. In a search for enzymes acting on proline-rich peptides, we tested the crude filtrate with $(Pro\text{-}Gly\text{-}Pro)_n$, $(Pro\text{-}Gly\text{-}Pro)_n$-OMe, α,DNP-$(Pro\text{-}Gly\text{-}Pro)_n$ and poly-L-proline as substrates. Proline was formed only from $(Pro\text{-}Gly\text{-}Pro)_n$ and its methyl ester. This showed the presence in Cl. histolyticum filtrate of an aminopeptidase which cleaves N-terminal proline from polypeptides but not from polyproline. The purification and some of the properties of this clostridial aminopeptidase (CAP) are described in this communication.

Isolation. Cl. histolyticum cells were grown according to Warren et al.[6]. The cells were removed by centrifugation and 1M EDTA was added to the clear supernatant to a final concentration of $10^{-3}$M. This solution was stored at 4°C and retained full activity for at least 1 week.

The purification process described below is summarized in Table 1. (The routine assay is described in the legend.)

TABLE 1. Purification of CAP

| | Volume (ml) | Activity[a] units/ml | Total Activity (units) | Recovery of Activity (%) | Protein[b] (mg/ml) | Specific activity units/mg protein | Purification factor |
|---|---|---|---|---|---|---|---|
| Crude Culture Filtrate | 51,500 | 0.115[c] | 5922 | 100 | 0.047[c] | 2.45 | 1 |
| DEAE - Cellulose | 1,235 | 1.310 | 1618 | 28 | 0.090 | 14.50 | 6 |
| Sephadex G-150 | 34.2 | 32.00 | 1094 | 18 | 0.101 | 317.00 | 130 |

[a] Activity was determined by measuring the rate of release of the N-terminal proline of the tripeptide Pro-Gly-Pro as follows: The enzyme solution (5-50 µl) containing 0.01-0.1 units was added to a reaction solution which was prepared by mixing 0.05M veronal buffer pH 8.6 (0.7 ml), $10^{-2}$M Pro-Gly-Pro in water (0.05 ml, final concentration $5 \times 10^{-4}$M) and 0.2 ml of a fresh Mn-citrate solution. The latter was prepared by mixing 0.4 M sodium citrate, 0.1 M $MnCl_2$, 0.1 M NaOH, and 0.05 M veronal pH 8.6 in the ratios 1:1:1:5, respectively.

The mixture was incubated at 40°C for 30 minutes. The reaction was stopped by adding the ninhydrin reagent (2.5 ml) and free proline was determined by the method of Troll and Lindsley[16], as modified by Sarid et al.[13]. The unit of activity is defined as the amount of enzyme which produces 1 µmole proline under the assay conditions.

[b] Protein concentration was determined by the method of Lowry et al.[17], using bovine serum albumin as standard.

[c] These values were determined following concentration by ultrafiltration and dialysis against 0.05 M phosphate pH 7.4, $10^{-3}$M in EDTA.

The culture filtrate (51.5 liters) was diluted five times with $10^{-3}$M EDTA and applied to a DEAE-cellulose column (12x20 cm). A linear gradient between 0.05M K-phosphate pH 7.4, $10^{-3}$M in EDTA (18 liters) and 0.33M NaCl in the same buffer (18 liters) was applied. After concentration by ultrafiltration (Diaflo, PM-30 membrane) the active fractions were applied to a Sephadex G-150 column (2.8x245 cm), and eluted with 0.05M phosphate pH 7.4, $10^{-3}$M in EDTA. The preparation obtained in the last step was shown to be homogeneous by polyacrylamide gel electrophoresis at pH 8.9,[7] by immunodiffusion and by immunoelectrophoresis (Fig. 1).

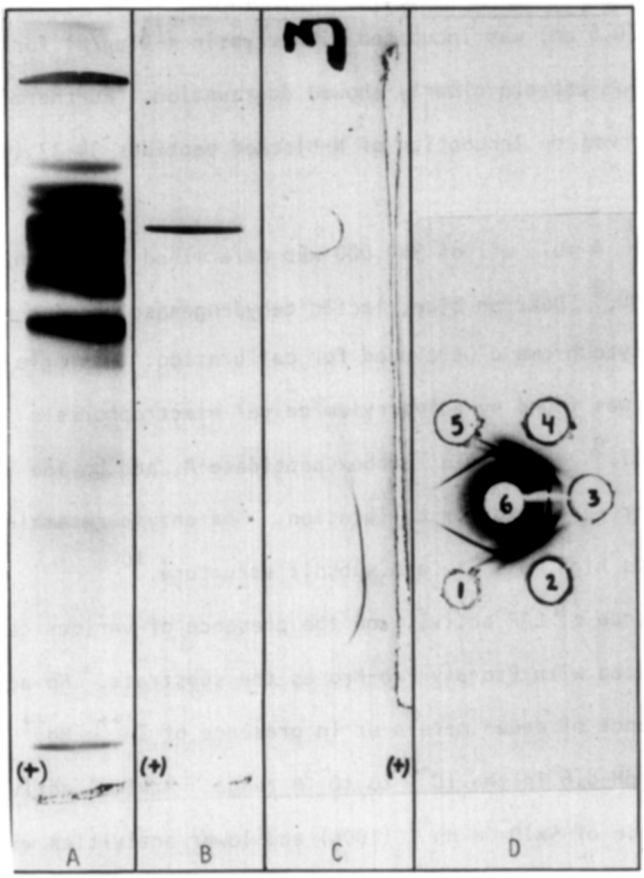


Fig. 1. A, Polyacrylamide gel electrophoresis of the crude filtrate; B, Polyacrylamide gel electrophoresis of purified CAP; C, Immunoelectrophoresis of purified CAP; D, Immunodiffusion: Well 1,3 and 5, purified CAP; well 2 and 4, partially purified CAP obtained after DEAE-cellulose chromatography; well 6, antiserum. The antiserum for C and D was obtained by injecting rabbits with a crude filtrate of C. histolyticum in complete Freund's adjuvant.

The absence of endopeptidase activity in the purified CAP preparation was demonstrated as follows: Performic acid oxidized lysozyme (POL) (0.3 mM) was incubated with CAP (25 μg/ml) at 40°C for 18 hours under the assay conditions. The incubation solution was applied to a Sephadex G-25 column (1.2x93 cm) and eluted with 0.05M ammonium bicarbonate. A single component, migrating identically with that of POL incubated in absence of CAP, was observed. (Because of the high contents of ionized amino acid residues in the N-terminal part of lysozyme, only very limited digestion by CAP can be expected.) On the other

hand, when POL (0.6 mM) was incubated with trypsin (40 μg/ml for 2.5 hours at 40°C), the elution pattern clearly showed degradation. Furthermore, no degradation was observed on incubation of N-blocked peptides 34-37 (Table 2) with CAP.

Properties. A mol. wt. of 340,000 was determined by gel chromatography on Sephadex G-200.[8] Dextran blue, lactic dehydrogenase, bovine serum albumin, ovalbumin, and cytochrome c were used for calibration. A single component of mol. wt. 63,000 was found by polyacrylamide gel electrophoresis in SDS and 2-mercaptoethanol.[9] Hemoglobin, carboxypeptidase-A, and bovine serum albumin monomer and dimer were used for calibration. The enzyme resembles other aminopeptidases in its high mol. wt. and subunit structure.[10]

The dependence of CAP activity on the presence of various cations and on pH was investigated with Pro-Gly-Pro-Pro as the substrate. No activity was observed in absence of added metals or in presence of $Zn^{++}$, $Mg^{++}$, $Ba^{++}$, $Ca^{++}$, $Sr^{++}$ or $Cu^{++}$ at pH 8.6 in the $10^{-6}$ to $10^{-3}$M range. Maximal activation was obtained in presence of $5x10^{-5}$M $Mn^{++}$ (100%) and lower activities with $Co^{++}$ (73%), $Cd^{++}$ (15%), and $Ni^{++}$ (12%) at their optimal concentrations. In presence of $5x10^{-5}$M $MnCl_2$, complete inhibition was observed with $10^{-5}$M $Zn^{++}$ or with $10^{-3}$M $Cu^{++}$. A similar $Zn^{++}$ effect was observed with aminopeptidase I,[11] but not with leucine aminopeptidase (LAP), which has been shown to be a $Zn^{++}$ enzyme.[12] The pH optimum in presence of $5x10^{-5}$M $Mn^{++}$ in 0.05 M veronal was at 8.6. The effects of pH, $Mn^{++}$, $Zn^{++}$ and $Cu^{++}$ were also examined with the tripeptide Pro-Gly-Pro, essentially with the same results.

The effect of p-mercuribenzoate (PMB) and diisopropyl phosphorofluoridate (DFP) on CAP activity was investigated with Pro-Gly-Pro and Pro-Gly-Pro-Pro. With PMB, 30-100% inactivation was observed at $1.2x10^{-3}$M concentration after preincubation at room temperature for 30 minutes in 0.01 M K-phosphate pH 7.4, $10^{-3}$M in EDTA, at an enzyme concentration of $6x10^{-8}$M (mol. wt. 340,000). DFP was not inhibitory at $10^{-6}$M.

The peptides listed in Table 2 were tested as substrates for CAP (for con-

TABLE 2. Hydrolysis of various peptides and amino-acid amides by CAP

| | | |
|---|---|---|
| 1. Met⇓-Met | 16. Ala⇓-Gly-Gly | 30. Ala⇓-Ala⇓-Ala↓-Ala |
| 2. Pro⇓-Phe | 17. Met⇓-Gly-Gly | 31. Pro↓-L-Ala-D-Ala-L-Ala |
| 3. Pro⇓-Leu | 18 Val⇓-Gly-Gly | 32. Pro⇓-Gly-Pro-(Pro-Gly-Pro)$_{\overline{36}}$ |
| 4. Ala⇓-Lys | 19. Ser⇓-Gly-Gly | 33. Pro-Pro$_{\overline{60}}$ (= poly-L-proline) |
| 5. Gly↓-Phe | 20. Glu↓-Ala-Ala | 34. Z-Ala-Ala-Ala |
| 6. Pro↓-Gly | 21. Lys↓-Ala-Ala | 35. Z-Ala-Ala-Phe-Ala |
| 7. Phe↓-Ala | 22. Ala⇓-Ala↓-Ala | 36. Acetyl-Gly-Phe-Ala |
| 8. Ala↓-Ala | 23. Gly⇓-Phe↓-Ala | 37. α,DNP-Pro-Gly-Pro-(Pro-Gly-Pro)$_{\overline{36}}$ |
| 9. Gly-Gly | 24. Gly↓-Gly↓-Phe | 38. Pro⇓-Gly-Pro-(Pro-Gly-Pro)$_{\overline{36}}$ - OMe |
| 10. Gly-Pro | 25. Pro-Pro-Ala | 39. Ala⇓-Ala⇓-Ala⇓-Ala↓-$NH_2$ |
| 11. Val-Pro | 26. Gly-Pro-HyPro | 40. Leu↓-β-naphthylamide |
| 12. HyPro⇓-Ala↓-$NH_2$ | 27. Pro⇓-Gly-Pro-Pro | 41. Pro↓-β-naphthylamide (traces)* |
| 13. Pro⇓-Gly-Gly | 28. Ala⇓-Ala⇓-Phe↓-Ala | 42. Pro-p-nitroanilide |
| 14. Phe⇓-Gly-Gly | 29. Gly⇓-Gly⇓-Phe↓-Ala | 43. Ala-p-nitroanilide |
| 15. Leu⇓-Gly-Gly | | 44. Ala↓-$NH_2$ |

Peptides were incubated with the enzyme (0.5 µg/ml) for 3 hours under the assay conditions (legend to Table 1) at concentrations: $4\times10^{-3}$M for 1-29, 31, 34, 36, 43, 44 and $4\times10^{-4}$M for 27, 30, 32, 33, 35, 37-42. Compounds which hydrolyzed slowly or were resistant to CAP, were incubated also for 16 hours. The products were analyzed chromatographically and by high voltage paper electrophoresis. The extent of hydrolysis is indicated by arrows: ⇓ 95-100%; ⇓ >20%; ↓ <20%. The absence of arrows indicates bonds resistant to hydrolysis.

* By fluorescence.

ditions see legend to the Table). The enzyme acts as a typical aminopeptidase, splitting various amino acid residues from the N-terminal end of peptides. The free α-amino group is essential, since α-N blocked peptides are resistant to CAP (34-37). Peptide bonds between an N-terminal amino acid residue and proline, which are known to be hydrolyzed by specific aminopeptidases,[13-15] are resistant to hydrolysis by CAP (10, 11, 25, 26, 32 (2nd bond), 33). The ex-

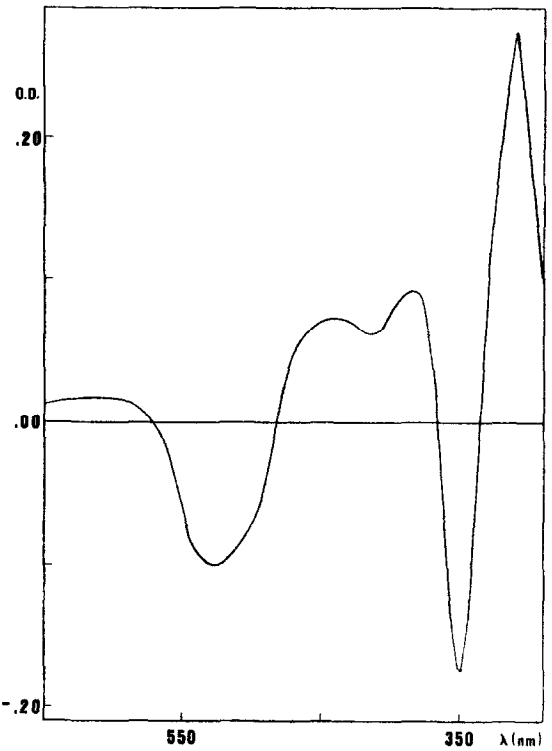


Fig. 1 Spectrum obtained in the reaction of $e^-_{aq}$ and $10^{-5}$M argon saturated solution of $B_{12}$ containing $10^{-1}$M tert-butanol (pH-6.1), taken 40 μsec after the pulse.

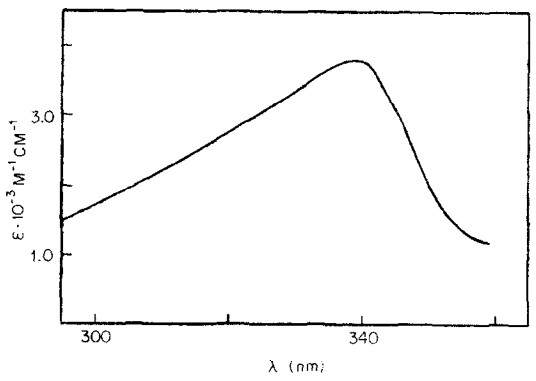


Fig. 2 Spectrum of transient obtained from the reaction $e^-_{aq}$ + 5,6 - dimethylbenzimidazole in argon saturated solution.

TABLE 3. Kinetic parameters for the hydrolysis of various peptides.

| No. | Peptide | $\overline{K}_m$, ($M^{-1}$) | $V_{max}$ ($\mu$moles.$sec^{-1}$.$mg^{-1}$) | $V_{max} \cdot \overline{K}_m$ |
|---|---|---|---|---|
| 1. | Leu-Gly-Gly | 155 | 16.5 | 2,560 |
| 2. | Phe-Gly-Gly | 119 | 12.0 | 1,430 |
| 3. | Val-Gly-Gly | 54 | 4.9 | 264 |
| 4. | Ala-Gly-Gly | 12.5 | 32.8 | 410 |
| 5. | Pro-Gly-Gly | 25 | 24.2 | 605 |
| 6. | Pro-Gly-Pro | 50 | 10.0 | 500 |
| 7. | Pro-Gly-Pro-Pro | 225 | 6.0 | 1,350 |

The peptides ($5 \times 10^{-4}$M to $10^{-2}$M) were incubated with enzyme (0.44 - 0.88 µg/ml) under the assay conditions (legend to Table 1). Samples were removed at different time intervals and the reaction was stopped by acidification. The progress of reaction was followed by the increase of ninhydrin color according to Matheson et al.[18]. The acid ninhydrin method[13] was used with peptides 5-7. Initial rates were linear with enzyme concentration up to 1.5 µg/ml.

activity of the other known aminopeptidases with the exception of proline iminopeptidase.[10,13] 3) The rate of hydrolysis seems to be dependent on the substrate chain length. The tetrapeptide Pro-Gly-Pro-Pro is a better substrate than the tripeptide Pro-Gly-Pro (increased $V_{max} \cdot \overline{K}_m$ and $\overline{K}_m$ values). Furthermore, dipeptides are not as good substrates as the higher peptides (Table 2).

CAP may prove useful because of its broad substrate specificity (including the hydrolysis of prolyl-peptides), and the simple purification procedure, which yields the enzyme in a pure form. Scaling up of the isolation procedures is now being attempted in order to make CAP available for further studies of its properties and its use in structural studies on proteins

Acknowledgment: This investigation was supported by Agreement 06-003-1 with the National Institutes of Health.

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