Short Communication

Post-proline Dipeptidyl Amino-peptidase from *Flavobacterium*

Tadashi YOSHIMOTO and Daisuke TSURU

Faculty of Pharmaceutical Sciences,
Nagasaki University,
Nagasaki, Nagasaki 852, Japan

Received March 10, 1980

During the past fifteen years, several endo- and exo-peptidases specific for peptide bonds involving the amino or carboxyl group of prolyl residue were discovered and characterized.^{1~10)} But most of them were from mammalian tissues.^{1~7)} Recently, we have found that certain strains of *Flavobacterium meningosepticum* produce a proline specific endopeptidase,^{8,9)} whose substrate specificity is quite similar to that of post-proline cleaving enzyme (EC 3.4.21.26) from lamb kidney.^{2,11,12)} The bacteria also showed deamidase activities on Gly-Pro- β -naphthylamide (Gly-Pro-2-NNap), Ala-2-NNap, Leu-2-NNap, Cys-di-2-NNap together with proline specific endopeptidase activity.⁹⁾ The present paper describes the purification and substrate specificity of Gly-Pro-2-NNap deamidase from the bacteria.

The enzyme activity was assayed as described previously^{6,13)} and one unit of the activity was defined as the amount of enzyme which released 1 μ mol of β -naphthylamine per min at 30°C under the condition. *Flavobacterium meningosepticum* IFO 12535 was aerobically cultivated at 30°C using a Microferm Fermentor (New Brunswick Co., U.S.A.) in bouillon medium at pH 7.2. After 18 hr, the cells were harvested and suspended in about 100 ml of 50 mM Tris-HCl buffer, pH

7.0, and then disrupted with a Sonicator (Ultrasonics, INC. N.Y.) for 5 min in the presence of 1% sodium-cholate. The disrupted mixture was centrifuged, and the Gly-Pro-2-NNap deamidating enzyme in the supernatant was purified by ammonium sulfate fractionation (50~80% saturation), chromatographies on columns of DEAE-Sephadex, Sephadex G-150 and hydroxyapatite. By the procedures, the specific activity toward Gly-Pro-2-NNap was increased about 250-fold while the enzyme preparation was free from amidase activity on Z-Gly-Pro-2-NNap, Leu-2-NNap and Ala-2-NNap. The enzyme activity toward Gly-Pro-2-NNap was completely inhibited by 1 mM diisopropyl phosphorofluoridate but not by phenylmethane sulfonyl-fluoride, *p*-chloromercuribenzoate, iodoacetic acid, ethylenediaminetetraacetic acid and *o*-phenanthroline. Naturally occurring proteinase inhibitors such as trasylol, trypsin

FIG. 1. Time Course of Hydrolysis of Substance P by Post-proline Dipeptidyl Amino-peptidase-like Enzyme from *Flavobacterium meningosepticum*.

Substance P, 0.55 mg, in 200 μ l of 10 mM Tris-HCl buffer, pH 7.0, was digested by 0.07 units of the enzyme at 37°C. Twenty μ l aliquots of the reaction mixture were periodically withdrawn and spotted on the paper. The numbers indicate the time in hr after incubation with the enzyme. Spots A and B were composed of Arg, Pro, and Lys, Pro, respectively. Spot C contained Glu₂, Phe₂, Gly₁, Leu₁, and Met₁.

The constituent amino acids of substrates used here are of L-configuration, unless otherwise specified.

Abbreviations: 2-NNap, β -naphthylamide; MCA, 4-methylcoumarinamide; TLC, thin-layer chromatography; *p*-NA, *p*-nitroaniline.

TABLE I. SUBSTRATE SPECIFICITY OF POST-PROLINE DIPEPTIDYL AMINO PEPTIDASE-LIKE ENZYME FROM *Flavobacterium meningosepticum*

Linkages hydrolyzed readily

↓
Gly-Pro-2-NNap ($K_m=0.095$ mM)↓
Gly-Pro-MCA↓
Gly-Pro-Leu-Gly↓
Gly-Pro-Ala↓
Gly-Pro-*p*-NA ($K_m=0.10$ mM)↓
Ala-Pro-2-NNap↓
Gly-Pro-Leu-Ala↓
Gly-Pro-Phe

Linkages hydrolyzed sparingly

↓
Ala-Ala-2-NNap,↓
Ala-Ala-Ala

The followings were not hydrolyzed: Z-Gly-Pro-2-NNap, Z-Ala-Gly-Pro-2-NNap, Z-Pro-2-NNap, Pro-2-NNap, Cys-di-2-NNap, Leu-2-NNap, Ala-2-NNap, Gly-Pro-D-Ala, Benz-Arg-*p*-NA, Benz-Tyr-*p*-NA, Thr-Lys-Pro-Arg, Pro-Leu-Gly-NH₂, Gly-Leu-Tyr, Leu-Tyr, Leu-Gly-Phe, Z-Gly-Pro-Leu-Gly, Z-Gly-Pro-Leu-Gly-Pro, Z-Gly-Pro-Ala.

inhibitors from egg-white and soybean were all inert toward the enzyme.

Substrate specificity of the enzyme is summarized in Table I. Equal volumes of substrates (5 mM in 50 mM Tris-HCl buffer, pH 7.0) and enzyme (0.1 unit/ml) were incubated at 30°C for 1 hr. Aliquots of the reaction mixtures were removed and spotted on a TLC cellulose plate (Merck) and developed using butanol-acetic acid-water (4:1:2, by vol). The hydrolysis products were identified by comparing the *R_f* values with those of authentic markers. The enzyme action on various peptides and amides were also checked by liberations of β -naphthylamine, *p*-nitroaniline and 4-methyl-7-aminocoumarin and the ninhydrin reaction, as described previously.¹³⁾ The enzyme hydrolyzed peptides and amides at the carboxyl side of prolyl residue locating at the penultimate positions of unprotected amino termini. The enzyme also split peptide bonds involving carboxyl group of alanyl residue, though the activity was quite low, as was the case of mammalian post-proline dipeptidyl aminopeptidases.^{6,11)}

The time course of hydrolysis of substance P* by the enzyme was followed by high voltage

paper electrophoresis (2,000 volt, pH 3.2, 1 hr) and the digestion products were analyzed for amino acid composition after acid hydrolysis. As shown in Fig. 1, substance P was cleft to produce amino terminal Arg-Pro and the subsequent peptide fragment, Lys-Pro by the enzyme. These substrate specificities shown in Table I and Fig. 1 are very close to post-proline dipeptidyl aminopeptidase of mammalian source so far reported.^{4,6,7,11)} This may be the first report concerning the post-proline dipeptidyl aminopeptidase of microbial origin. The enzymatic and physicochemical properties of this microbial enzyme are now under investigation and will be reported elsewhere.

REFERENCES

- 1) M. Koida and R. Walter, *J. Biol. Chem.*, **251**, 7593 (1976).
- 2) T. Yoshimoto, R. C. Orlowski and R. Walter, *Biochemistry*, **16**, 2942 (1977).
- 3) A. Nordwig and H. Meyer, *Hoppe-Seyler's Z. Phys. Chem.*, **354**, 380 (1973).
- 4) V. K. Hopsu-Havu and S. R. Sarimo, *ibid.*, **348**, 1540 (1967).
- 5) P. Deam and A. Nordwig, *Eur. J. Biochem.*, **17**, 364, (1970).
- 6) T. Yoshimoto and R. Walter, *Biochim. Biophys. Acta*, **485**, 391 (1977).

* A putative peptide neurotransmitter in brain.¹⁴⁾

- 7) H. Oya, I. Nagatsu and T. Nagatsu, *ibid.*, **258**, 591 (1972).
 - 8) T. Yoshimoto, R. Walter and D. Tsuru, *J. Biol. Chem.*, **255**, 4786 (1980).
 - 9) T. Yoshimoto and D. Tsuru, *Agric. Biol. Chem.*, **42**, 2417 (1978).
 - 10) A. Yaron and D. Mlynar, *Biochem. Biophys. Res. Commun.*, **32**, 658 (1968).
 - 11) T. Yoshimoto, M. Fischl, R. C. Orlowski and R. Walter, *J. Biol. Chem.*, **253**, 3708 (1978).
 - 12) R. Walter and T. Yoshimoto, *Biochemistry*, **17**, 4139 (1978).
 - 13) T. Yoshimoto, K. Ogita, R. Walter, M. Koida and D. Tsuru, *Biachim. Biophys. Acta*, **569**, 184 (1979).
 - 14) T. Takahashi, S. Konishi, D. Powell, S. E. Leeman and M. Otsuka, *Brain Res.*, **73**, 59 (1974).
-