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Characterization of dipeptidyl peptidase–IV of *Porphyromonas gingivalis*

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Summary

Dipeptidyl peptidase IV (DPP–IV) was purified to homogeneity from the crude extract of *Porphyromonas gingivalis*. Isoelectric point and molecular weight of DPP–IV were 6.1 and 66 kDa, respectively. Km value for the appropriate substrate (H–Gly–Pro–pNA) was 0.25 mM, and Vmax/Km was 0.52. Optimum pH for the activity was found at pH 7.5. DPP–IV was considered a serine enzyme.

P. gingivalis has been implicated as a putative etiological agent of chronic periodontitis[1–4]. Since this organism lacks saccharolytic activity, it utilizes peptides as the energy source[5–8] which may be derived from proteins surrounding the organisms mainly by endogeneous proteinases. Suido et al.[9] evaluated formation of peptidases by many species of oral bacteria, and detected in *P. gingivalis* some hydrolyzing activities of C–termini of dipeptides such as Arg–Arg, Gly–Arg, Gly–Phe, Gly–Pro, Leu–Gly, Lys–Ala and Lys–Pro using *β*–naphthylamide conjugated–dipeptides. After that, DPP–IV of *P. gingivalis* was isolated from the sonicated crude extract and determined its molecular weight as 78 kDa and Km value for Gly–Pro–pNA as 0.28 mM[10]. Banbula et al.[11] isolated also DPP–IV of *P. gingivalis* from the Triton X–100 extract of cells. It was a serine enzyme with a molecular weight of 69 kDa. The discrepancy of molecular sizes (78 kDa and 69 kDa) between the two reports seems to be significant, thus we attempted to estimate it and characterize the enzymatic properties

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Abbreviations: dipeptidyl peptidase, DPP; *p–nitroanilide*, pNA; 50 mM Tris–HCl buffer, pH 8.2, Tris–buffer

of DPP–IV of this species.

Strains of *P. gingivalis* (ATCC 33277, W50, 381 and W50) maintained on blood agar plates were grown in a liquid medium consisting of trypticase peptone (17 g/l), yeast extract (3 g/l), NaCl (5 g/l), K_2HPO_4 (2.5 g/l), hemin (5 mg/l) and menadione (0.5 mg/l)[12]. Cultivation was carried out at 37℃ for 3 days anaerobically in a glove box filled with a mixture of gasses containing $N_2:H_2:CO_2=85:10:5$ at 37℃ for 3 days.

Whole culture (400 ml) was centrifuged at 10,000 xg for 15 min to separate culture fluid (supernatant) and cells (pellet). Cells (2.5 g) suspended in Tris–buffer were disrupted by ultrasonic treatment at 150W for 15 min. After removal of cell debris and unbroken cells from the sonicate by centrifugation at 6,000 xg for 15 min, the supernatant was further centrifuged by 100,000 xg for 60 min. The resultant supernatant and precipitate were designated the crude extract and the envelope, respectively. To solubilize the envelope, CHAPS（3–[(3–cholamidopropyl)dimethylammonio]–1–propanesulfonate) was added to the envelope suspension at a concentration of 1% and stirred gently at room temperature for 45 min, followed by centrifugation at 100,000 xg for 60 min. The resultant supernatant was referred to as the solubilized envelope. The rate of solubilization of the envelope by this procedure was 91.3%.

DPP–IV activity was determined photometrically, using H–Gly–Pro–pNA as a substrate by the methods described earlier[13]. Briefly, reaction mixtures containing 50 μl of enzyme source, 700 μl of 1 mM substrate in 50 mM Tris–maleate buffer (pH 7.5) and 150 μl of 50 mM Tris–maleate buffer (pH 7.5) were incubated at 37℃ for 30 min. Reaction was stopped by addition of 100 μl of 7.5 M acetic acid, and released *p*–nitroaniline was assayed by absorbance at 410 nm. One unit of the enzyme activity was defined as the liberation of 1 μmol of *p*–nitroaniline per min.

Distributions of DPP–IV in the crude extract, solubilized envelope and culture fluid are summarized in Table 1. It is quite obvious that DPP–IV was an intracellular enzyme. Simultaneously, the same experiments were performed to assess whether this enzyme is found in the crude extracts of other strains including 381, W50 and W83, as well as strain in ATCC 33277. Since cell yields were varied among strains, each activity was expressed after conversion of cell weights into 2.5 g. DPP–IV activities in the crude extract of the three strains were 3.32 U, 4.06 U and 3.85 U, respectively. These findings indicate that DPP–IV production may be ubiquitous among the *P. gingivalis* strains.

The crude extract was applied to a column (2.5×15 cm) of Q–Sepharose, which had been previously equilibrated with Tris–buffer, and the column was rinsed with the same buffer thoroughly. Then the column contents were eluted with a linear concentration gradient of NaCl from 0 to 700 mM, which was generated by mixing 200 ml of Tris–buffer containing 700 mM NaCl into an equal volume of Tris–buffer. The flow rate was 60 ml/h, and 5 ml fractions were collected. DPP–IV eluted around 250 mM of NaCl. The active fractions were collected, concentrated using an evaporator in vacuo, and dialyzed against Tris–buffer containing 200 mM NaCl. The concentrated and dialyzed material was subjected to gel filtration on Sephacryl S–300 column (2.6×100 cm) equilibrated to Tris buffer containing 200 mM NaCl. The column was eluted with the same buffer saline at a flow rate of 30 ml/h and eluates were collected 5 ml each in fraction tubes (Fig. 1). The active fractions

Table 1：Cellular locations of DPP–IV of *P. gingivalis*

Fraction	DPP–IV (U)	Rate (%)
Crude extract	5.83	100.0
Solubilized envelope	0.21	3.6
Culture fluid	0.01	0.2

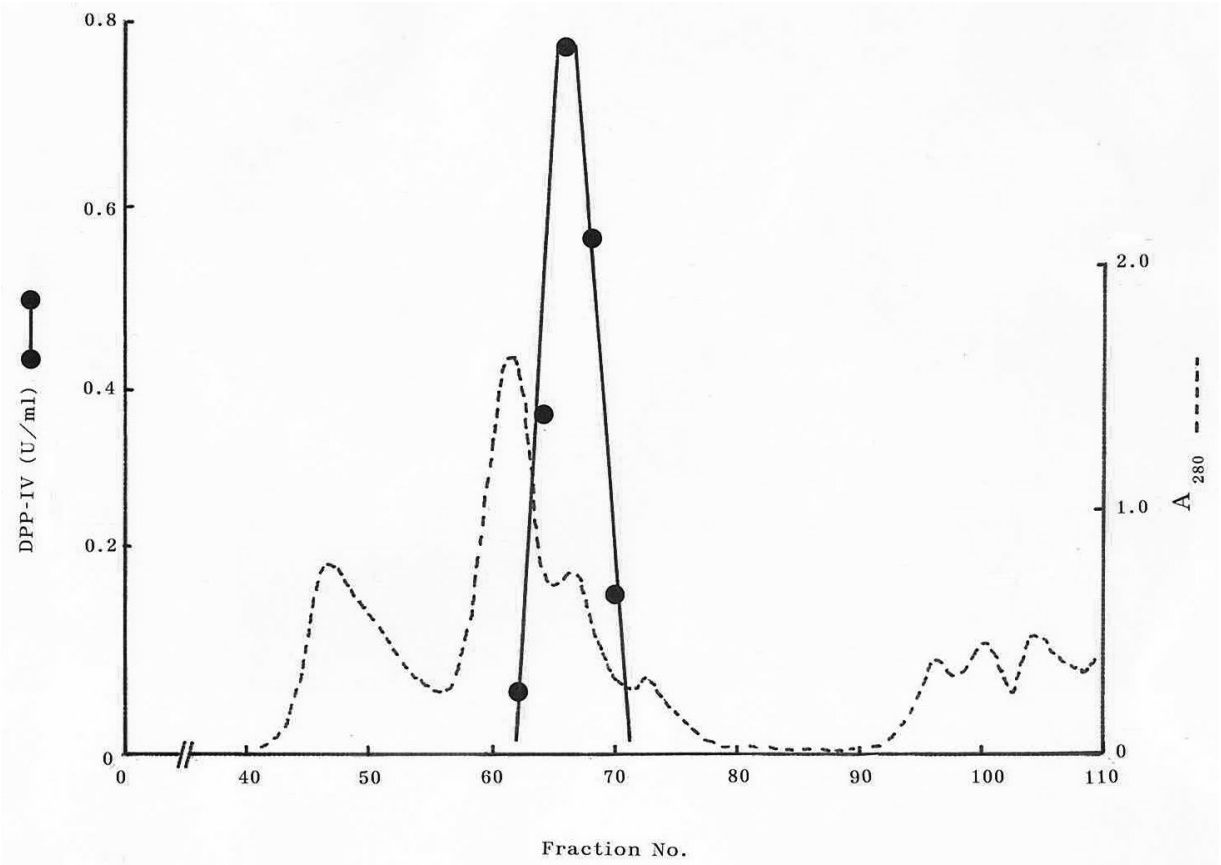

Fig. 1 : Sephacryl S–300 gel filtration. Experimental conditions are described in the text.

were dialyzed against Tris–buffer containing 700 mM ammonium sulfate and hydrophobic interaction chromatography using Phenyl Sepharose CL–4B column (0.9×17 cm), equilibrated with this dialysis solution. The column was eluted linearly descending gradient of ammonium sulfate concentration. In this chromatography, two protein peaks emerged, at concentrations about 300 mM and 50 mM of ammonium sulfate. DPP–IV activity was detected in the latter fraction. Finally, this fraction was dialyzed against water and electrophoresed on isoelectric focusing (110 ml capacity column) using ampholine (pH 3.5–10.0). Electrophoresis was carried out at 300 V for 20 h. DPP–IV activity was detected at a position of pH 6.1.

As shown in Fig. 2, a single Coomassie brilliant blue stained band was found. The molecular weight was calculated as 66 kDa by comparison of the migration rates with reference proteins.

Km value was estimated as 0.25 mM from the profile of Lineweaver–Burk plots of substrate (H–Gly–Pro–pNA) concentration and reaction velocity (Fig. 3). Since Vmax was calculated as 0.13, Vmax/Km was 0.52. This Km value was quite close to that of Kumagai et al., 0.28 mM for the same substrate[10].

The maximum enzyme activity was found in the pH 7.5 buffer. DPP–IV had no activity in buffers with pH below 6.0. Since in buffers with pH over 8.0, *p*–nitroaniline seemed to be released automatically from H–Gly–Pro–pNA, the activity could not be assayed under the alkaline condition (data are not shown).

DPP–IV was completely inhibited by Pefabloc SC, 3,4–diisocoumarin and diisopropylfluorophosphate, indicating this enzyme is a serine peptidase (Table 2). No inhibition by the other group specific reagents was confirmed.

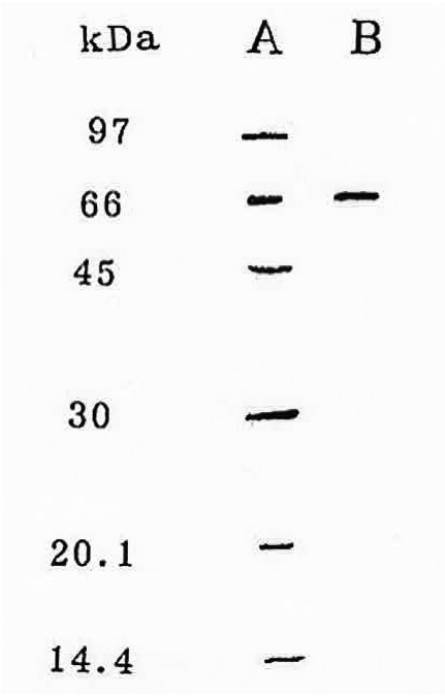

Fig. 2：SDS–PAGE. Gel (7.5%) was stained by Coomassie brilliant blue.
Lane A, Marker proteins　　Lane B, purified DPP–IV

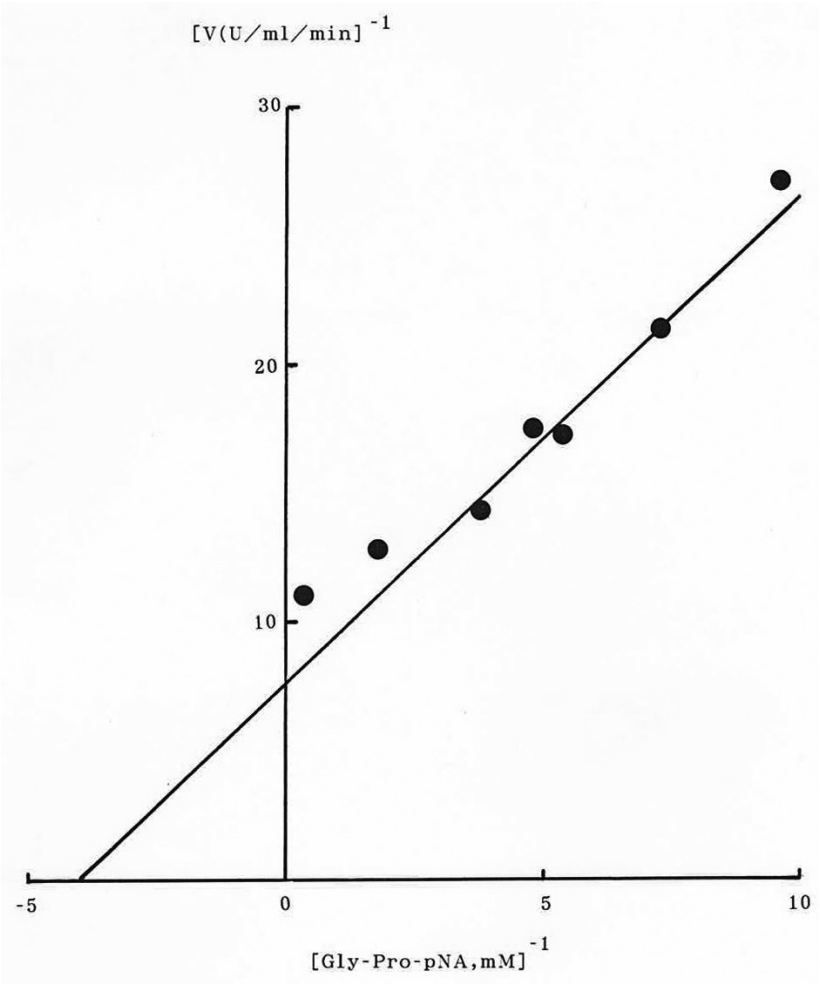

Fig. 3：Lineweaber–Burk plots

Table 2 : Effects of group specific reagents on DPP–IV

Reagents	Concentration	Activity (%)
Control	—	100
Leupeptin	2 mM	95
Antipain	10 mM	89
Bestatin	2 mM	102
E64[1)]	2 mM	105
Pefabloc SC[2)]	2 mM	6
Diisopropyl fluorophosphate	20 mM	59
	40 mM	5
3,4–Dichloroisocoumarin	2 mM	6
EDTA	10 mM	90
EGTA	10 mM	96
1,10–phenanthroline	2 mM	98

1): L–*trans*–epoxy–succinyl leucylamido–(4–guanidino)butane
2): 4–(2–aminoethyl)–benzenesulfonyl–fluoride hydrochloride

Residual activities after heating at various temperatures for 10 min were assayed to assess the thermostability of DPP–IV. Heating at 50℃ and 60℃ resulted in 30% and 90% loss of the activities, respectively.

DPP–IV described in this report may be the same enzyme characterized by Banbula et al.[11)] despite a small difference in molecular weights between the two reports. It should be considered that Triton–X100 extracted material of bacterial cells does not necessarily represent only intracellular soluble substances, since materials solubilized by the detergent possibly contain the cell surface components such as the envelopes and cell membranes.

In *P. gingivalis*, enzyme other than DPP–IV, an intracellular Xaa–Ala specific peptidase preferentially hydrolyzed Lys–Ala also was characterized[14)]. It was a serine enzyme with molecular weight of 64 kDa and isoelectric point of 5.7. However, further functional and enzymatic investigations of the other peptidases described by Suido et al.[9)] should be performed in future for a better understanding the pathogenic properties of the periodontopathogen.

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抄録：*Porphyromonas gingivalis* のジペプチジル ペプチダーゼ –IV の性状

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Porphyromonas gingivalis は有力な慢性歯周炎の原因菌と考えられるが，ATCC 33277株で調べたところ，そのジペプチジル ペプチダーゼ（DPP）–IV のほとんどが粗抽出液に認められ，エンベロープや培養上清中の活性は僅かであった．従って，DPP–IV は菌体内（細胞質内）に偏在する酵素であることが分かった．また，他の菌株，W50，381および W83でも，DPP–IV 活性は認められ，ATCC 33277同様，そのほとんどが，粗抽出液中に検出された．

ATTCC 33277株を培養し，その粗抽出液から，DPP–IVをイオン交換クロマトグラフィー，ゲル濾過，疎水性クロマトグラフィー，等電点電気泳動にて分離精製した．この DPP–IV はセリン酵素であり，分子量は66 kDa で，等電点は6.1であった．H–Gly–Pro–pNA に対する Km 値は0.25 mM で，Vmax/Km 値は0.44であった．反応の至適 pH は7.5であった．熱安定性テストでは，50℃，10分加熱で30％の失活，60℃，10分加熱では90％失活することが分かった．