Crystallization and preliminary X-ray crystallographic studies of dipeptidyl peptidase 11 from *Porphyromonas gingivalis*

Yasumitsu Sakamoto,^a Yoshiyuki Suzuki,^b Ippei Iizuka,^a Chika Tateoka,^a Saori Roppongi,^a Mayu Fujimoto,^a Hiroaki Gouda,^c Takamasa Nonaka,^a Wataru Ogasawara^b and Nobutada Tanaka^{c*}

Received 14 September 2014

Accepted 9 January 2015

Keywords: dipeptidyl peptidase; DPP11; *Porphyromonas gingivalis*.

Supporting information: this article has supporting information at journals.iucr.org/f

^aSchool of Pharmacy, Iwate Medical University, 2-1-1 Nishitokuta, Yahaba, Iwate 028-3694, Japan, ^bDepartment of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Nigata 940-2188, Japan, and ^cSchool of Pharmacy, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan. *Correspondence e-mail: ntanaka@pharm.showa-u.ac.jp

Dipeptidyl peptidase 11 from *Porphyromonas gingivalis* (PgDPP11) preferentially cleaves substrate peptides with Asp and Glu at the P1 position [NH₂-P2-P1(Asp/Glu)-P1'-P2'...]. For crystallographic studies, PgDPP11 was over-produced in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. X-ray diffraction data to 1.82 Å resolution were collected from an orthorhombic crystal form belonging to space group C222₁, with unit-cell parameters $a = 99.33$, $b = 103.60$, $c = 177.33$ Å. Structural analysis by the multi-wavelength anomalous diffraction method is in progress.

1. Introduction

Periodontitis is a bacterially induced inflammatory process that results in destruction of the connective tissue and bone that support the teeth and is widely regarded as the second most common disease worldwide (Peterson & Ogawa, 2005). Chronic periodontitis affects about 750 million people as of 2010 (Vos *et al.*, 2012). *Porphyromonas gingivalis*, an oral anaerobic bacterium, is implicated as a major pathogen associated with periodontitis. *P. gingivalis* is an asaccharolytic bacterium and its growth is dependent on an external peptide source. *P. gingivalis* secretes various proteases/peptidases that are capable of digesting external proteins into peptides. It is well known that *P. gingivalis* produces two cysteine proteases, Arg-gingipain and Lys-gingipain, which show specificity for arginine and lysine, respectively, and the inhibition of these proteases suppresses the virulence of *P. gingivalis* (Kadowaki *et al.*, 2004). Cell-associated peptidases, such as dipeptidyl peptidase 4 and prolyl tripeptidyl peptidase A, as well as Arg-gingipain and Lys-gingipain, are responsible for the energy metabolism of *P. gingivalis* because the organism mainly incorporates nutritional amino acids in the form of dipeptides and tripeptides, not as single amino acids. Interestingly, a relative of *P. gingivalis*, *P. endodontalis*, does not have proteolytic activities equivalent to those of the gingipains (Könönen *et al.*, 2011; van Winkelhoff *et al.*, 1987). Two types of novel dipeptidyl peptidases (DPPs), DPP7 and DPP11, have been characterized from *P. gingivalis* and *P. endodontalis* (Banbula *et al.*, 2001; Ohara-Nemoto *et al.*, 2011). DPP7s

liberate dipeptides from oligopeptides and have a broad specificity for both aliphatic and aromatic residues in the P1 position [$\text{NH}_2\text{-P2-P1}(\text{aliphatic/aromatic})\text{-P1'-P2'...}$]. In contrast, DPP11s specifically remove dipeptides from oligopeptides with a penultimate N-terminal Asp and Glu [$\text{NH}_2\text{-P2-P1}(\text{Asp/Glu})\text{-P1'-P2'...}$]. The most important role of these peptidases appears to be the digestion of proteinaceous substrates as carbon and energy sources rather than the virulence of *Porphyromonas* species (Sheets *et al.*, 2008). DPP11 from *P. gingivalis* (PgDPP11) plays a critical role in metabolism in *P. gingivalis* by degrading polypeptides carrying Asp and Glu, as Asx (Asp and Asn) and Glx (Glu and Gln) are the most abundantly utilized amino acids in the bacterium (Takahashi *et al.*, 2000; Takahashi & Sato, 2001). The predicted amino-acid sequences of DPP7s and DPP11s from *Porphyromonas* species revealed that these enzymes are ~80 kDa serine proteases and are classified into clan PA family S46 in the MEROPS database (Rawlings *et al.*, 2012). The S46 enzymes are widely distributed in anaerobic Gram-negative species in the genera *Bacteroides*, *Parabacteroides* and *Porphyromonas*, but are not found in mammals (Ohara-Nemoto *et al.*, 2011; Suzuki *et al.*, 2014). In order to further characterize the properties of the S46 enzymes, we initiated structural studies of PgDPP11.

To date, several crystal structure analysis of DPPs have been reported, for example those of mammalian DPP4s belonging to clan SC family S9 exhibiting a catalytic α/β domain and a regulatory β -propeller domain (Engel *et al.*, 2003; Hiramatsu *et al.*, 2003; Rasmussen *et al.*, 2003; Thoma *et al.*, 2003) and that of a mammalian DPP7 belonging to clan SC family S28 exhibiting a catalytic α/β domain and a regulatory α -helical cap (Bezerra *et al.*, 2012). The mammalian DPP4 plays a major role in the degradation of incretins such as GLP-1 and is a target for the treatment of type 2 diabetes. The mammalian DPP7 is ubiquitously expressed and has been reported to participate in several biological functions. Note that the mammalian DPP7s belonging to the S28 family are not related to the bacterial DPP7s belonging to the S46 family described above. Recently, we have determined the first crystal structure of a clan PA family S46 peptidase, dipeptidyl aminopeptidase BII (DAP BII) from *Pseudoxanthomonas mexicana* WO24 (Sakamoto, Suzuki, Iizuka, Tateoka, Roppongi, Fujimoto *et al.*, 2014; Sakamoto, Suzuki, Iizuka, Tateoka, Roppongi, Okada *et al.*, 2014). Our study revealed that DAP BII contains a peptidase domain including a typical double β -barrel fold, which is characteristic of clan PA peptidases such as chymotrypsin, and a previously unreported large α -helical domain. Although the overall structure of the S46 peptidase has been revealed by crystal structure analysis of DAP BII, exhibiting a broad specificity for the substrate P1 position, the determinants of the strict P1 specificity of the other S46 peptidases (*e.g.* DPP11s) remain to be fully understood. Thus, the three-dimensional structure of PgDPP11 is essential to clarify the molecular basis for the strict substrate specificity of the Asp/Glu-specific DPP DPP11. Here, we report the expression, crystallization and preliminary X-ray crystallographic analysis of PgDPP11.

2. Materials and methods

2.1. Overproduction and purification

A synthetic gene coding for full-length PgDPP11 (residues 1–720), codon-optimized for *Escherichia coli* expression, was purchased from GenScript. A variety of parameters that are critical to transcription (*e.g.* GC content) and translation (*e.g.* codon-usage bias and mRNA secondary structure) were optimized using the proprietary algorithm of GenScript (Supplementary Fig. S1). NdeI (CATATG) and BamHI (GGATCC) cloning sites were added at the 5'-end and 3'-end, respectively, of the synthetic gene. The N-terminal region (residues 1–21) was a signal peptide. The mature PgDPP11, residues 22–720, was composed of 699 amino acids with a theoretical molecular weight of 79 618.27 and an isoelectric point of 5.87. An *E. coli* BL21 Gold(DE3) (Agilent Technologies) transformant harbouring the full-length PgDPP11 sequence inserted into the pET-22b (Novagen) expression plasmid was used for the production of recombinant PgDPP11. The cells were grown in 2×YT medium at 298 K. Overproduction of PgDPP11 was performed using the Overnight Express Autoinduction System 2 (Merck). After this period, the cells were harvested by centrifugation at 8000g. The cells were disrupted using BugBuster Protein Extraction Reagent (Novagen). The cell extract was obtained by centrifugation at 27 000g for 30 min. PgDPP11 was precipitated by 70% saturated ammonium sulfate and the precipitate was dissolved in 30% saturated ammonium sulfate solution in 50 mM Tris-HCl pH 9.0 (buffer A). The enzyme solution was filtrated with a 0.22 μm pore filter and applied onto a 20 ml HiPrep 16/10 Butyl column (GE Healthcare) equilibrated with 30% saturated ammonium sulfate in buffer A. The column was washed with two column volumes of wash buffer (30% saturated ammonium sulfate in buffer A). After washing, PgDPP11 was eluted with five column volumes of elution buffer (a 30–0% gradient of ammonium sulfate in buffer A). The fractions containing PgDPP11 were pooled and applied onto a 50 ml HiPrep 26/10 Desalting column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 9.0 (buffer B). The PgDPP11 was then applied onto a 1 ml MonoQ 5/50 GL anion-exchange chromatography column (GE Healthcare) equilibrated with buffer B. The column was washed with five column volumes of buffer B. After washing, PgDPP11 was eluted with 30 column volumes of elution buffer (a 0–0.3 M gradient of sodium chloride in buffer B). The fractions containing PgDPP11 were pooled, buffer-exchanged to 80 mM Tris-HCl pH 8.5 and concentrated to 10 mg ml⁻¹ using a Vivaspin 20 concentrator (GE Healthcare). The protein concentration was determined by the Bradford assay using a bovine serum albumin as a standard (Bio-Rad Laboratories).

2.2. Crystallization

Initial sparse-matrix crystal screening (Jancarik & Kim, 1991) was manually conducted using Crystal Screen, Crystal Screen 2, Crystal Screen Cryo, PEG/Ion, PEG/Ion 2 and Index from Hampton Research, Wizard I, II and III, Ozma 1K, 4K, 8K and 10K and Cryo I and II from Emerald Bio and The

Table 1
Overproduction of PgDPP11.

In the synthetic DNA, the ATG sequence of the NdeI site corresponds to Met1 of PgDPP11. NdeI (CATATG) and BamHI (GGATCC) cloning sites were designed at the 5'-end and the 3'-end, respectively, of the synthetic gene.

Source organism	<i>P. gingivalis</i>
DNA source	Synthetic (codon-optimized DNA for <i>E. coli</i> expression)
Forward primer	None
Reverse primer	None
Cloning vector	pUCS7
Expression vector	pET-22b
Expression host	<i>E. coli</i> BL21 Gold(DE3)
Complete amino-acid sequence of the construct produced	MKKRLLPLFAALCLSQIAHADEGMWLMQQLGRK- YAQMKEGRLKMEYDLYNPNGTSLKDAVVLFD- GGCTGEVVSRLGLVLTNNHCGYDMLQAHSTLE- HNYLENGFWAMREADELPNKDISVVFIDKIED- VTDYVKKELKAIKDPNSMDYLSPKYLQKLADK- KAGKNFSAKNPGLSVEIKAFYGGNLYLMFTKK- TYTDVRLVGAPPSSIGKFGADTDNWIWPRHTG- DFSIFRIYADKNGNPAPYSEDNVLPKPRFFN- ISLGGVQENDYAMIMGFPGTTHRYFTASEVDE- WKSIDNDIRIMRDIRQGVMLREMLADPQIKI- MYSAKYAASQNAIKRAIGANWAIKTRGLRQNK- QAMQDRLLIAGAKQGTTRYEEAVHEIDATVAK- RADLRRRYWMIEEIGIRGIEFARSPITDETE- KALQGNDSARKEAIDKIRTRYSKFANKDYSA- EVDKKVAVAMLTLEYLKEIPYENLPLHLRLVKD- RFAGDVQAYVDDIFARSVFGSEAFDAFAAVP- SVEKLAEDPMVLFASSVFDEYRKLYNELRPYD- DPILRAQRTYIAGLLEMDGDQDQFPDANLTLR- FTYGVQVGYSPRDNVYGHQTTLDGVMKEDEP- DNWEFVDPKLKAVYERKDFGRYADRSGRMPV- AFCATHTTGGNSGSPVMNANGELIGLNFDRN- WEGVGGDIQYLADYQRSIIIVDIRYVLLVIDKV- GGCQRLLEDMNIVP

PACT and JCSG+ Suites from Qiagen. Crystallization was carried out by the hanging-drop method, in which 1 μ l protein solution (10 mg ml⁻¹) was mixed with the same volume of crystallization buffer and incubated at 293 K. The drops were suspended over 200 μ l reservoir solution in 48-well plates.

2.3. Data collection and crystallographic analyses

Data collection was performed by the rotation method at 95 K using an ADSC Q270 CCD detector with synchrotron radiation ($\lambda = 1.000$ Å) on beamline NE3A of the Photon

Figure 1
An orthorhombic crystal of PgDPP11.

Table 2
Crystallization of PgDPP11.

Method	Hanging-drop vapour diffusion
Plate type	Hampton Research 48-well plates
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	0.08 M Tris-HCl buffer pH 8.5
Composition of reservoir solution	16%(v/v) PEG 8000, 0.16 M tripotassium citrate, 20%(v/v) glycerol
Volume and ratio of drop	1 μ l protein + 1 μ l reservoir
Volume of reservoir (μ l)	500

Factory Advanced Ring (PF-AR), Tsukuba, Japan. The Laue group and unit-cell parameters were determined using *XDS* (Kabsch, 2010). Self-rotation and native Patterson functions were calculated using *POLARRFN* and *FFT* from the *CCP4* suite (Winn *et al.*, 2011).

3. Results and discussion

3.1. Overproduction, purification and crystallization

PgDPP11 was successfully overproduced (Table 1) and purified to homogeneity with an approximate yield of 8 mg protein per litre of bacterial culture at the end of the purification process. SDS-PAGE of the purified enzyme revealed a single 76 kDa protein band by Coomassie Brilliant Blue staining. Initial crystal screening produced several microcrystals within one week. The crystals grew from condition Nos. 30 [20%(m/v) PEG 8000, 0.2 M potassium phosphate] and 47 [20%(m/v) PEG 8000, 0.2 M tripotassium citrate] of Ozma 8K (Emerald Bio) and Nos. 40 [25%(v/v) *tert*-butanol in 0.1 M Tris pH 8.5], 44 [20%(v/v) ethanol in 0.1 M Tris pH 8.5] and 46 [20%(v/v) PEG MME 550 and 0.1 M sodium chloride in 0.1 M Bicine pH 9.0] of Crystal Screen 2 (Hampton Research). To obtain crystals suitable for X-ray analysis, a droplet was prepared by mixing equal volumes (1 μ l + 1 μ l) of the protein solution (10 mg ml⁻¹) described above and reservoir solution [20% glycerol, 16%(m/v) PEG 8000, 0.16 M tripotassium citrate] and suspended over 200 μ l reservoir solution in 48-well plates (Table 2). Bipyramidal-shaped crystals with typical dimensions of approximately 0.29 $\times$ 0.28 $\times$ 0.15 mm grew within one week (Fig. 1).

3.2. X-ray analysis

Since the crystallization conditions of PgDPP11 described above contained 20%(v/v) glycerol in the reservoir solution, X-ray data collection could be performed under cryogenic conditions without further addition of cryoprotectant. Crystals from the hanging drop were directly mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 95 K just prior to data collection. The Laue group of the PgDPP11 crystals was found to be *mmm* and the unit-cell parameters were $a = 99.33$, $b = 103.60$, $c = 177.33$ Å. Only reflections with $h + k = 2n$ and $l = 2n$ were observed for *hkl* reflections and along the (00 l) axes, respectively, indicating the orthorhombic space group *C22₁*. The current best diffraction data from a PgDPP11 crystal were collected to 1.82 Å resolution (Fig. 2).

Table 3
Data-collection statistics for PgDPP11.

Values in parentheses are for the outer shell.

Diffraction source	NE3A, PF-AR
Wavelength (Å)	1.000
Temperature (K)	95
Detector	ADSC Q270
Crystal-to-detector distance (mm)	244.0
Rotation range per image (°)	0.2
Total rotation range (°)	190
Exposure time per image (s)	1.5
Space group	C222 ₁
Unit-cell parameters (Å, °)	$a = 99.33$, $b = 103.60$, $c = 177.33$, $\alpha = \beta = \gamma = 90$
Resolution range (Å)	40.00–1.82 (1.92–1.82)
Total No. of reflections	455735 (22222)
No. of unique reflections	79248 (10000)
Completeness (%)	96.8 (84.9)
Multiplicity	5.8 (2.2)
$\langle I/\sigma(I) \rangle$	14.3 (2.4)
$R_{\text{meas}}^{\dagger}$	0.075 (0.311)
Mosaicity (°)	0.37
Overall B factor from Wilson plot (Å ²)	19.8

$\dagger R_{\text{meas}} = \sum_{hkl} \{N(hkl)/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th intensity measurement of reflection hkl , $\langle I(hkl) \rangle$ is the average intensity of multiple observations and $N(hkl)$ is the data multiplicity.

Data-collection statistics are summarized in Table 3. Assuming the presence of one subunit per asymmetric unit led to an empirically acceptable V_M value of $2.79 \text{ Å}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 55.9% (Matthews, 1968). Since another S46 peptidase, bacterial DAP BII (Ogasawara *et al.*, 1996; Sakamoto, Suzuki, Iizuka, Tateoka, Roppongi, Fujimoto *et al.*, 2014; Sakamoto, Suzuki, Iizuka, Tateoka, Roppongi, Okada *et al.*, 2014), was observed to be a dimer, we

assume that PgDPP11 also forms a dimer. In that case, the molecular twofold axis of the dimeric PgDPP11 molecule must coincide with a crystallographic twofold axis of the C222₁ crystal of PgDPP11. Initially, we tried to solve the structure of PgDPP11 by molecular-replacement techniques. Structures of DAP BII complexed with or without a peptide (Sakamoto, Suzuki, Iizuka, Tateoka, Roppongi, Okada *et al.*, 2014), belonging to the S46 peptidase family (Suzuki *et al.*, 2014) and having 29% sequence identity with PgDPP11, were used as search models. Since these attempts failed, a search for heavy-atom derivatives intended for phasing by the multiple/single isomorphous replacement method and the preparation of selenomethionine-substituted PgDPP11 using LeMaster medium (Hendrickson *et al.*, 1990) and *E. coli* B834(DE3) cells for phasing by Se-MAD/SAD methods are under way.

Acknowledgements

We thank Drs Y. Yamada and N. Matsugaki of the Photon Factory for their help with data collection at the synchrotron facilities. This work was supported in part by the Program for the Strategic Research Foundation at Private Universities (to NT and YSa), the Platform for Drug Discovery, Informatics and Structural Life Science (to NT and YSa) and a Grant-in-Aid for Scientific Research (C) 25462872 (to YSa) from the MEXT of Japan.

References

- Banbula, A., Yen, J., Oleksy, A., Mak, P., Bugno, M., Travis, J. & Potempa, J. (2001). *J. Biol. Chem.* **276**, 6299–6305.
- Bezerra, G. A. *et al.* (2012). *PLoS One*, **7**, e43019.
- Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H.-U. & Brandstetter, H. (2003). *Proc. Natl Acad. Sci. USA*, **100**, 5063–5068.
- Hendrickson, W. A., Horton, J. R. & LeMaster, D. M. (1990). *EMBO J.* **9**, 1665–1672.
- Hiramatsu, H., Kyono, K., Higashiyama, Y., Fukushima, C., Shima, H., Sugiyama, S., Inaka, K., Yamamoto, A. & Shimizu, R. (2003). *Biochem. Biophys. Res. Commun.* **302**, 849–854.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Kabsch, W. (2010). *Acta Cryst. D* **66**, 125–132.
- Kadowaki, T., Baba, A., Abe, N., Takii, R., Hashimoto, M., Tsukuba, T., Okazaki, S., Suda, Y., Asao, T. & Yamamoto, K. (2004). *Mol. Pharmacol.* **66**, 1599–1606.
- Könönen, E., Wade, G. & Citron, M. (2011). *Manual of Clinical Microbiology*, 10th ed., edited by J. Versalovic, K. C. Carroll, G. Funke, J. H. Jorgensen, M. L. Landry & D. W. Warnock, pp. 858–880. Washington: ASM Press. doi:10.1128/9781555816728.ch51.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Ogasawara, W., Kobayashi, G., Okada, H. & Morikawa, Y. (1996). *J. Bacteriol.* **178**, 6288–6295.
- Ohara-Nemoto, Y., Shimoyama, Y., Kimura, S., Kon, A., Haraga, H., Ono, T. & Nemoto, T. K. (2011). *J. Biol. Chem.* **286**, 38115–38127.
- Petersen, P. E. & Ogawa, H. (2005). *J. Periodontol.* **76**, 2187–2193.
- Rasmussen, H. B., Branner, S., Wiberg, F. C. & Wagtmann, N. (2003). *Nature Struct. Biol.* **10**, 19–25.
- Rawlings, N. D., Barrett, A. J. & Bateman, A. (2012). *Nucleic Acids Res.* **40**, D343–D350.
- Sakamoto, Y., Suzuki, Y., Iizuka, I., Tateoka, C., Roppongi, S., Fujimoto, M. *et al.* (2014). *Sci. Rep.* **4**, 4977.

Figure 2
X-ray diffraction image from a PgDPP11 crystal. The circles indicate resolutions of 7.00, 3.00 and 1.82 Å.

- Sakamoto, Y., Suzuki, Y., Iizuka, I., Tateoka, C., Roppongi, S., Okada, H., Nonaka, T., Morikawa, Y., Nakamura, K. T., Ogasawara, W. & Tanaka, N. (2014). *Acta Cryst.* **F70**, 221–224.
- Sheets, S., Robles-Price, A., McKenzie, R., Casiano, C. & Fletcher, H. (2008). *Front. Biosci.* **13**, 3215–3238.
- Suzuki, Y., Sakamoto, Y., Tanaka, N., Okada, H., Morikawa, Y. & Ogasawara, W. (2014). *Sci. Rep.* **4**, 4292.
- Takahashi, N. & Sato, T. (2001). *J. Dent. Res.* **80**, 1425–1429.
- Takahashi, N., Sato, T. & Yamada, T. (2000). *J. Bacteriol.* **182**, 4704–4710.
- Thoma, R., Löffler, B., Stihle, M., Huber, W., Ruf, A. & Hennig, M. (2003). *Structure*, **11**, 947–959.
- Van Winkelhoff, A. J., Kippuw, N. & De Graaff, J. (1987). *J. Dent. Res.* **66**, 1663–1667.
- Vos, T. *et al.* (2012). *Lancet*, **380**, 2163–2196.
- Winn, M. D. *et al.* (1994). *Acta Cryst.* **D67**, 235–242.