Analytical Biochemistry 548 (2018) 78–81

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Establishment of potent and specific synthetic substrate for dipeptidyl-peptidase 7

Takayuki K. Nemoto*, Toshio Ono, Yuko Ohara-Nemoto

*Department of Oral Molecular Biology, Course of Medical and Dental Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki 852-8588, Japan*

ARTICLE INFO

*Keywords:*
Dipeptidyl-peptidase 5
Dipeptidyl-peptidase 7
Periodontopathic bacteria
*Porphyromonas gingivalis*
Substrate specificity

ABSTRACT

Bacterial dipeptidyl-peptidase (DPP) 7 liberates a dipeptide with a preference for aliphatic and aromatic penultimate residues from the N-terminus. Although synthetic substrates are useful for activity measurements, those currently used are problematic, because they are more efficiently degraded by DPP5. We here aimed to develop a potent and specific substrate and found that the $k_{cat}/K_m$ value for Phe-Met-methylcoumaryl-7-amide (MCA) ($41.40 \pm 0.83\ \mu M^{-1}\ s^{-1}$) was highest compared to Met-Leu-, Leu-Leu-, and Phe-Leu-MCA ($1.06–3.77\ \mu M^{-1}\ s^{-1}$). Its hydrolyzing activity was abrogated in a *Porphyromonas gingivalis dpp7*-knockout strain. Conclusively, we propose Phe-Met-MCA as an ideal synthetic substrate for DPP7.

## Introduction

Dipeptidyl-peptidases (DPPs) are exopeptidases that liberate a dipeptide from a polypeptide with an unmodified N-terminus. Based on the substrate specificities and enzymatic properties, 11 members of DPPs have been identified [1]. Among them, DPP4 [2,3], DPP5 [4,5], bacterial [6] and mammalian [7,8] DPP7, and DPP11 [9] are serine peptidases, and DPP 8–10 are allocated for subtypes of DPP4. DPP7 was allocated for two distinct DPPs, bacterial DPP7 [6] and mammalian one [7]. Bacterial DPP7 was first discovered in *Porphyromonas gingivalis* and is distributed solely in bacterial species [6]. On the other hand, human DPP7 is identical to DPPII and QPP/quiescent cell proline dipeptidase [8]. Although DPPs generally share distinct substrate specificities at the penultimate position from the N-terminus (P1 position), the P1-position specificity of DPP5 partially overlaps with that of bacterial DPP7, as DPP5 prefers Ala and aliphatic residues [4,5], and bacterial DPP7 prefers aliphatic and aromatic ones [8,9]. Nevertheless, there is no amino acid sequence similarity between DPP5 and bacterial DPP7 (11.7%), and the topologies of the three essential amino acid residues of serine peptidases are completely distinct; $Ser^{542}$, $Asp^{627}$, and $His^{659}$ in DPP5 [5], and $His^{89}$, $Asp^{225}$, and $Ser^{648}$ in DPP7 [11].

*Porphyromonas gingivalis*, an asaccharolytic periodontopathic bacterium, expresses all these serine peptidase family DPPs in periplasmic space, where they manage efficient production of nutritious dipeptides being incorporated into the organism [12]. In addition to their necessity for bacterial metabolism, DPPs may be associated with physiological events through degradation of bioactive peptides in humans. For example, it is well known that mammalian DPP4 is a key enzyme involved in regulation of blood glucose levels via degradation of incretin peptides, glucagon-like peptide 1 (GLP-1), and gastric inhibitory polypeptide (GIP) [13,14]. As for the host-parasite relationship, we recently demonstrated that periodontopathic bacterial DPP4 hydrolyzes GLP-1 and GIP as does mammalian DPP4, resulting in enhancement and prolongation of postprandial hyperglycemia [15]. In addition, DPP7 in a canine oral bacterium, *Capnocytophaga canimorsus*, degrades Factor X and retards blood coagulation [16]. Thus, it is of particular interest to elucidate whether DPP7 in human indigenous bacteria exhibits similar physiological effects. To examine these issues, development of a synthetic substrate for DPP7 to separately quantitate DPP7 and DPP5 activities in bacteria as well as clinical specimens is eagerly anticipated.

We previously found that *P. gingivalis* DPP7 prefers hydrophobic residues at the P2 as well as P1 position [10], while DPP5 no apparent specificity at the P2 position [5]. Hence, DPP7 more potently hydrolyzes Met-Leu-methylcoumaryl-7-amide (MCA) than Lys-Ala-MCA, despite the fact that Met-Leu-MCA is more efficiently hydrolyzed by DPP5 than DPP7. From the viewpoint of maximum utilization of the P2-position hydrophobic preference of DPP7, the combination of a hydrophobic P2 residue and non-hydrophobic P1 residue, such as Leu-Arg-, Leu-Gln-, and Leu-Glu-MCA, resulted in highly DPP7-specific hydrolysis [5]. However, the hydrolyzing efficiency of DPP7 for these substrates was substantially lower as compared to that for Met-Leu-MCA. To address this, the present study aimed to develop a synthetic dipeptidyl fluorescent substrate that is efficiently and specifically hydrolyzed by DPP7.

* Corresponding author. Department of Oral Molecular Biology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki 852-8588, Japan.
*E-mail address:* tnemoto@nagasaki-u.ac.jp (T.K. Nemoto).

https://doi.org/10.1016/j.ab.2018.02.008
Received 21 November 2017; Received in revised form 7 February 2018; Accepted 8 February 2018
Available online 10 February 2018
0003-2697/ 

## Materials and methods

### Materials

Lys-Phe-, Lys-Leu-, Lys-Val-, Lys-Met-, Phe-Leu-, Leu-Leu-, Phe-Met-MCA and dipeptides, Phe-Met, Leu-Asp, and Arg-Asp, were synthesized by Scrum (Tokyo, Japan). Other peptidyl MCA were purchased from the Peptide Institute (Osaka, Japan) and Bachem (Bubendorf, Switzerland). α-Chymotrypsin from bovine pancreas (type II), Gly-Gln, and Gly-Glu were purchased from Merck (NJ, USA).

### Methods

#### Bacterial culture

*P. gingivalis* strain ATCC 33277, and its derivatives NDP300 (*dpp5*::[*ermF ermAM*]) and NDP400 (*dpp7*::*tetQ*) [5] were grown under an anaerobic condition (80% $N_2$, 10% $CO_2$, 10% $H_2$) in Anaerobic Bacterial Culture Medium broth (Eiken Chemical, Tokyo, Japan), supplemented with 0.5 μg/ml of menadione with or without appropriate antibiotics (10 μg/ml of erythromycin or 0.7 μg/ml of tetracycline). Bacterial cells were harvested and washed with phosphate-buffered saline (PBS) at pH 7.4. The resulting cell pellets were re-suspended in PBS with absorbance adjusted at 600 nm to 2, then subjected to a DPP assay.

#### Expression and purification of recombinant proteins

The method for construction of plasmids, as well as the expressions of *P. gingivalis* DPP5 and DPP7 have been reported [5,9]. Recombinant forms of *P. gingivalis* DPP3 [5], DPP4 [17], DPP11 [9], and acylpeptide oligopeptidase (AOP) [18] were purified as previously described.

#### Measurement of peptidase activity

Assays of peptidase activity were started by adding recombinant proteins (1 ng–1 μg) or 5 μl of cell suspension to a reaction mixture (200 μl) composed of 50 mM sodium phosphate buffer (pH 7.0), 5 mM EDTA, and 20 μM dipeptidyl MCA. After 30 min at 37 °C, fluorescence intensity was measured with excitation at 380 nm and emission at 460 nm. Activity of DPP5 was measured in the presence of 0.1 M NaCl, and enzymatic parameters were calculated as described previously [5]. Experiments were conducted in triplicate and at least three times separately.

## Results

#### P1- and P2-position substrate specificities of P. gingivalis DPP5 and DPP7

First, to examine effects of the P1-position residue based on hydrophobicity, enzymatic activities were measured with five dipeptidyl MCA substrates with Lys at the P2 position (Fig. 1). DPP5 most efficiently hydrolyzed Lys-Ala-MCA, followed by Lys-Leu- and Lys-Phe-MCA, indicating an Ala preference at the P1 position. On the other hand, the order of hydrolysis efficiency was altered in DPP7, in which Lys-Met-MCA was most efficiently hydrolyzed by DPP7. Moreover, specific activities of DPP7 for these substrates were approximately one-tenth of those of DPP5, presumably because of the presence of the unwilling P2 residue. These results demonstrate that P1 Met [hydrophobicity index (H.I.) = 74] [19] was most preferred by DPP7 beyond its lower H.I. value compared to Phe (H.I. = 100), Leu (H.I. = 97), and Val (H.I. = 76).

Next, we examined the effects of the P2-position residues with six dipeptidyl MCA substrates carrying Phe (H.I. = 100) or Leu (H.I. = 97) as a hydrophobic residue at the P1 position. There was no apparent tendency shown by DPP5 between hydrophobicity of the P2 residue and efficiency. In contrast, the efficiency of hydrolysis by DPP7 was strictly dependent on P2-position hydrophobicity, because it more efficiently hydrolyzed Gly-Phe-MCA than Lys-Phe-MCA. Furthermore, among the four dipeptidyl MCA substrates with P1 Leu, DPP7 most efficiently hydrolyzed Phe-Leu-MCA, followed by Leu-Leu- and Met-Leu-MCA. Accordingly, DPP7 activity was strictly dependent on the H.I. of the P2-position residues. These findings suggest that Met was the best at the P1 position beyond its moderate H.I. of 74, while the most hydrophobic residue Phe (H.I = 100) was best at the P2 position.

#### Potency of Phe-Met-MCA as DPP7 substrate

Based on the data of Fig. 1, we newly synthesized Phe-Met-MCA and evaluated its potentials. Hydrolysis of Phe-Met-MCA by DPP7 was markedly higher as compared to hydrolyses of other substrates and all five substrates by DPP5 (Fig. 2). We determined the $k_{cat}$ (22,056 ± 1529 $s^{-1}$) and $K_m$ (51.1 ± 4.5 μM) of DPP7 for Phe-Met-MCA, and found that the resulting $k_{cat}/K_m$ value (41.40 ± 0.83 $\mu M^{-1} s^{-1}$) was substantially higher than those of Met-Leu-MCA (1.06 ± 0.25 $\mu M^{-1} s^{-1}$), Leu-Leu- (3.74 ± 0.11 $\mu M^{-1} s^{-1}$) and Phe-Leu-MCA (1.53 ± 0.06 $\mu M^{-1} s^{-1}$). The reason for these differences was mainly due to the highest $k_{cat}$ value for Phe-Met-MCA as compared to Met-Leu- (39.4 ± 7.9 $s^{-1}$), Leu-Leu- (75.4 ± 0.2 $s^{-1}$), and Phe-Leu-MCA (204.4 ± 25.4 $s^{-1}$).

We examined whether Phe-Met-MCA is hydrolyzed by other peptidases. When recombinant forms of *P. gingivalis* DPP3, DPP4, DPP5, DPP7, DPP11, and AOP were incubated with this substrate, DPP5 showed faint activity and no hydrolysis was observed with the other exopeptidases (Fig. 2). In addition, we confirmed that α-chymotrypsin, an endopeptidase with a preference for aliphatic P1 residues, did not exhibit activity towards Phe-Met-MCA (data not shown). Finally, we investigated the hydrolysis of Lys-Ala-, Phe-Met-, and Leu-Asp-MCA, *i.e.*, specific substrates for DPP5, DPP7, and DPP11, respectively, by *P. gingivalis* wild-type, and NDP300 (Δ*dpp5*) and NDP400 (Δ*dpp7*) cells. The hydrolyses of Phe-Met-MCA did not change in NDP300, while significantly reduced to 15.8% in NDP400 as compared to the wild type.

#### Inhibition of DPP7 activity by Phe-Met

We previously reported that the dipeptides Leu-Asp, Arg-Asp, and Arg-Glu inhibited DPP11 activity in this order [20]. Hence, we examined whether the dipeptide Phe-Met inhibits DPP7 activity in comparison with Leu-Asp, Arg-Asp, Gly-Gln, and Gly-Glu. When measured at 5 μM of Phe-Met-MCA. The activity was decreased to 83.5% by addition of 10-fold molar excess of Phe-Met, but not by others, which indicated that Phe-Met competitively inhibits the peptidase activity.

## Discussion

The present study demonstrates that Phe-Met-MCA (or Phe-Met-*p*-nitroaniline for a chromogenic assay) is an ideal synthetic dipeptidyl substrate with a fluorescent group for measurement of DPP7 activity. Authentic dipeptidyl substrates for DPP3 (Arg-Arg-MCA), DPP4 (Gly-Pro-MCA), DPP5 (Lys-Ala-MCA), and DPP11 (Leu-Asp/Glu-MCA) have already been established, thus establishment of a DPP7 substrate as well should greatly facilitate studies of DPPs in periodontopathic oral as well as enteric bacteria, which express these DPPs.

In addition to the role of DPP7 in bacterial metabolism, we consider it important to search for physiological substrates. We recently reported that DPP4 in human periodontopathic bacteria, *i.e.*, *P. gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia*, hydrolyzed the incretin peptides GLP-1 and GIP, resulting in decreased plasma insulin concentrations, while it inversely raised the level of postprandial hyperglycemia and retarded the recovery of blood glucose levels in a mouse model [15]. Furthermore, Hack et al. [16] reported that DPP7 from *C. canimorsus* degraded coagulation Factor X, thereby inducing bleeding tendency. Since identification of *in vivo* targets of DPPs is the basis for elucidation of novel physiological and pathological roles in