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Microbial degradation of linear peptides by strain B-9 of *Sphingosinicella* and its application in peptide quantification using liquid chromatography-mass spectrometry

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The bacterial strain *Sphingosinicella* sp. B-9 was originally discovered to have the ability to degrade cyanobacterial cyclic peptides (microcystins), and has three hydrolytic enzymes (MlrA, MlrB, and MlrC). The purpose of this study was to examine in detail the degradation of glucagon/vasoactive intestinal polypeptide (VIP) family peptides by B-9, and to investigate the substrate specificity of B-9 proteases and the possibility of using a B-9 protease as a novel protease for peptide quantification by using a surrogate peptide and mass spectrometry (MS). The effective use of inhibitors revealed the following hydrolytic capability of B-9: One of the B-9 proteases (presumably MlrB) that was not inhibited by ethylenediaminetetraacetic acid (EDTA) cleaved bioactive peptides into medium-sized peptides with broad selectivity, similar to neutral endopeptidase, and another protease that was not inhibited by phenylmethylsulfonyl fluoride (PMSF) corresponded to MlrC and cleaved the resulting medium-sized peptides to smaller peptides or amino acids. The former property was desirable to obtain a suitable surrogate peptide, which was used successfully to quantify peptide using liquid chromatography (LC)-MS. Thus, the present study verified that one of the B-9 proteases has broad cleavage selectivity and cleavage sites, not seen in commercially available proteases, and is applicable to protein and peptide quantification using LC-MS.

[**Key words:** B-9 strain; Glucagon/vasoactive intestinal polypeptide family peptide; Inhibitor; Protease; Surrogate peptide]

Owing to its high specificity, sensitivity, and relatively short method-development time, there has been an increasing interest in using liquid chromatography mass spectrometry (LC-MS) for the quantification of peptides from complex biological matrices (1–3). However, larger intact peptides such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptides (GIP), as well as proteins, are more difficult to analyze by electrospray ionization mass spectrometry (ESI/MS) because of the occurrence of many multiple charge states (4) and occasional adsorption problems during sample preparation (3,5,6). To overcome undesirable properties, Siskos et al. proposed the use of a surrogate peptide (4). We have also successfully developed a quantification method for GIPs using LC-MS via their surrogate peptides, in which we selected the endoproteinase Asp-N instead of trypsin because the surrogate peptide $GIP_{1\text{-}8}$ obtained with Asp-N was excellent in terms of oxidation resistance, compared to $GIP_{1\text{-}16}$ obtained with trypsin (7). This strongly indicates the importance of careful selection of both the targeted peptide and the protease for the quantification of peptides. Since few commercial proteases with specific cleavage sites are available at present (8), it would be worthy to find novel proteases for the quantification study of proteins or peptides using LC-MS.

The strain B-9 was isolated from Lake Tsukui, Japan, as a bacterium exhibiting degradation activities against microcystins (MCs) (9). Bacterial degradation of MCs was first confirmed by an isolated *Sphingomonas* sp. laboratory strain ACM-3962 (10). The cloning and molecular characterization of four genes from the strain ACM-3962 revealed the presence of three hydrolytic enzymes (MlrA, MlrB, and MlrC) together with a putative oligopeptide transporter (MlrD) (11,12). The three hydrolytic enzymes have been putatively characterized as metalloproteases (MlrA and MlrC) and a serine protease (MlrB) (12). It has been reported that strain B-9 hydrolyzes not only cyclic peptides but also linear peptides (13–16).

The purpose of this study is to develop an LC-MS based method for determining the levels of peptides using surrogate peptide obtained by B-9 proteases. For this purpose, the digestion conditions were optimized for B-9 protease and substrate specificity of the protease of B-9 was investigated. Glucagon/vasoactive intestinal polypeptide (VIP) family peptides (Table 1) were selected as the substrates, which have a narrow range of molecular weight (3200–5000 Da) and have been researched well with regard to their physiological activities and the biodegradation behavior. In the present study, we observed the degradation behavior of the glucagon/VIP family peptides by B-9 in the absence or presence of two protease inhibitors, ethylenediaminetetraacetic acid (EDTA)

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TABLE 1. Tested glucagon/vasoactive intestinal polypeptide family peptides.

Group	Peptide	Number of amino-acid residues	Molecular weight
Glucagon/ VIP family	GLP-1 (human, 7–36 amide)	30	3297.6
	GLP-2 (human)	33	3766.1
	GIP (human)	42	4983.5
	Glucagon (human)	29	3482.7
	VIP	28	3325.8
	Exendin-4	39	4186.6
Others	Fragment peptides no. 1 from GLP-1 (FTSDVSSYLEGQAAKE)	16	1731.8
	Fragment peptides no. 2 from GLP-1 (YLEGQAAKEFIA)	12	1339.5
	NERP-2 (human)	38	4064.5

and phenylmethylsulfonyl fluoride (PMSF). Finally, we pursued the possibility of B-9 protease as a new tool for determining the levels of GLP-1.

MATERIALS AND METHODS

Peptides used for experiments GLP-1 (7–36 amide, purity: >99.0%), GLP-2 (purity: >99.0%), glucagon (purity: >99.0%), GIP (purity: >99.0%), VIP (purity: >99.0%), and neuroendocrine regulatory peptide-2 (NERP-2, purity: >99.0%) were purchased from Peptide Institute, Inc. (Osaka, Japan). Exendin-4 (purity: 98.1%) was purchased from PolyPeptide Laboratories (San Diego, CA, USA). The fragment peptides from GLP-1 (GLP-1_{12-27}; FTSDVSSYLEGQAAKE (purity: 97.54%), GLP-1_{19-30}; YLEGQAAKEFIA (purity: 97.66%), GLP-1_{7-23}; HAEGTFTSDVSSYLEGQ (purity: 97.77%)) were purchased from Life Technologies Japan Ltd. (Tokyo, Japan). GIP_{1-8} (purity: >95%) was purchased from BEX Co, Ltd. (Tokyo, Japan). These peptides were dissolved in distilled water or 1% acetic acid and are listed in Table 1, with the exception of GLP-1_{7-23} and GIP_{1-8}.

Chemicals Tris–HCl buffer (pH 7.6) and Charcoal (charcoal activated A, powder) were purchased from Nacalai Tesque (Kyoto, Japan). EDTA-2Na (purity: >99.5%) and PMSF (purity: >98.5%) as protease inhibitors were purchased from Dojindo Laboratories (Kumamoto, Japan) and Sigma–Aldrich Japan (Tokyo, Japan), respectively. Acetonitrile (ACN, LC/MS grade, purity: 99.8%), methanol (MeOH, LC/MS grade, purity: 99.7%), ethanol (EtOH, special grade, purity: 99.5%), formic acid (FA, LC/MS grade, purity: 99.5%), acetic acid (AcOH, LC/MS grade, purity: 99.5%), trifluoroacetic acid (TFA, special grade, purity: 98.0%), ammonium carbonate (special grade), and 28% ammonia solution (NH_4OH, special grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water used for the preparation of all solutions was purified through a Milli-Q apparatus (Millipore, Billerica, MA, USA). Sitagliptin (purity: >99.9%), an inhibitor of DPP-4, was synthesized in Sanwa Kagaku Kenkyusho Co., Ltd. (Mie, Japan). OASIS MAX 96-well plate (30-μm particle size, 30 mg) was purchased from Waters (Milford, MA, USA).

MC-degrading bacterium The bacterial strain B-9, isolated from the surface water of Lake Tsukui, Kanagawa, Japan, has been previously reported to degrade various MCs and nodularin (13,14). This bacterium was inoculated into a flask containing 100 mL of Sakurai medium composed of peptone, yeast extract, and glucose and incubated at 27°C and 200 rpm for 3 days.

Enzyme inhibition Enzyme inhibitors were prepared as follows: EDTA was prepared as a 200 mM stock solution in water and was used at an assay concentration of 10 mM. PMSF was prepared as a 200 mM stock solution in EtOH and was used at an assay concentration of 10 mM. The cell broth and required inhibitor were preincubated at 27°C for 30 min.

Degradation of glucagon/VIP family peptides Fifty microliters of biologically active peptides at 0.1 mM concentration were added to 500 μL of the preincubated cell broth of B-9 (containing approximately 3×10^6 colony forming units (CFU) mL^{-1}) and incubated at 27°C for 5, 15, 30, 60, and 120 min. After incubation, 50 μL of each of these mixtures were added to 50 μL of MeOH containing 0.2% FA and centrifuged at 15,000 ×*g* for 5 min at 4°C to stop the degradation and to eliminate proteins. Each supernatant was then analyzed using LC/ion trap mass spectrometry (ITMS) equipped with an ESI interface.

LC/MS conditions for the degradation analysis The LC separation was performed using an Alliance 2690 HPLC system (Waters Corporation, Milford, MA, USA). Ten microliters of the sample were loaded onto a Cadenza CD-C18 column (3 μm, 1.0 mm ID × 150 mm, Imtakt, Kyoto, Japan) at 40°C. Mobile phase A was 0.1% FA/2% ACN and mobile phase B was 0.1% FA/95% ACN. The flow rate was 50 μL/min. The analytical gradient profile was as follows (min/% B): 0/0, 20/60, 25/60, 30/0. LC-MS and LC-MS/MS analysis were performed with an ITMS (LCQ Deca XP plus, Thermofisher Scientific, San Jose, CA, USA) equipped with an ESI interface, and operated in a mode that alternated single MS scans (*m/z* 400–1500) with MS/MS scans (data-dependent scan mode in which the most intense ion peak in the previous MS scan was isolated and subjected to collision-induced dissociation). The ESI conditions in the positive ion mode were as follows: capillary temperature, 225°C; sheath gas flow rate, 10 (arbitrary unit); ESI source voltage, 5 kV.

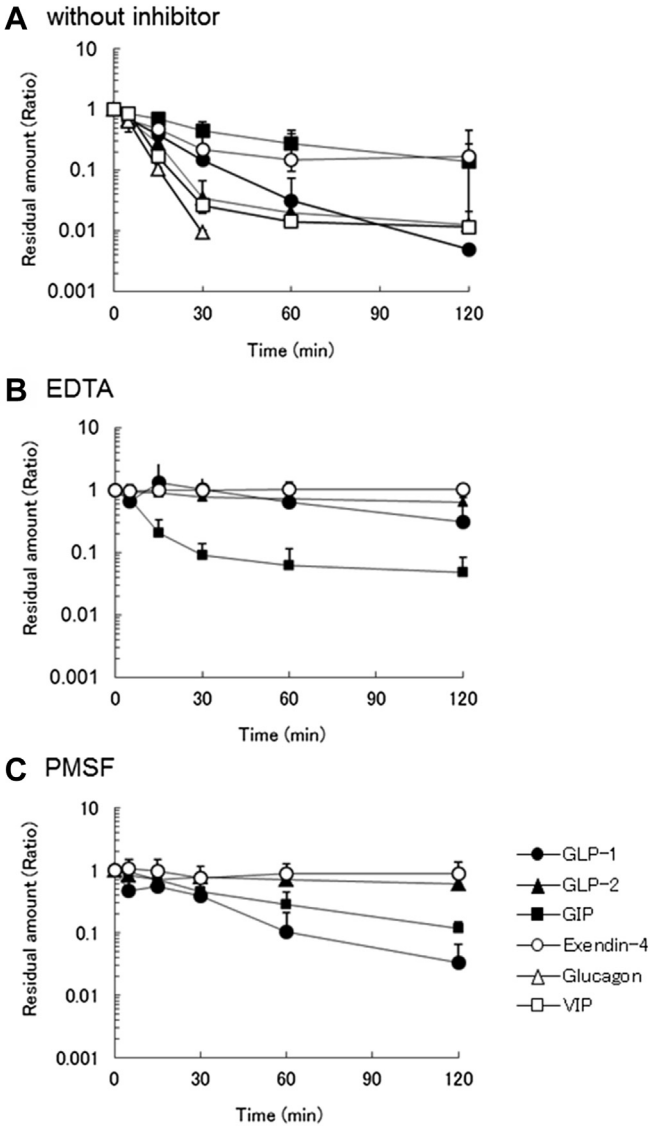

FIG. 1. Degradation behavior of GLP-1 and related peptides by B-9 without inhibitor (A), with EDTA (B), and with PMSF (C). Each point represents the mean ± S.D. ($n = 3$).

Preparation of plasma without GLP-1 (GLP-1-free plasma) Two grams of charcoal were added to every 10.0 mL of plasma, and this mixture was agitated for 12 h at 4°C. After centrifuging for 1 h at 4°C and 105,000 ×*g*, the supernatant was filtered using a 0.22-μm filter (7).

Protein precipitation for quantification of human GLP-1 (7–36 amide) In order to prepare a standard curve and quality control (QC) samples, a charcoal-stripped plasma sample aliquot, 100 μL in volume, and 5 μL of a solution containing 0.2 μg/μL sitagliptin were added to a 2-mL tube. Then, aliquots of GLP-1 standard solution were added in order to prepare a standard curve ranging in concentration from 1 to 500 nM, and QC samples for GLP-1. The calibration curve for GLP-1 consisted of six calibrators, as follows: 1, 2, 10, 50, 200, and 500 nM. QC samples consisted of 5 replicates of samples spiked with lower limit, low, middle, and high levels of GLP-1: 1, 2, 50, and 500 nM. Subsequently, 100 μL of 180 mM ammonium carbonate solution and 600 μL of ice-cold EtOH were added and the solution was stored for 20 min on ice. After centrifuging for 10 min at 4°C and 20,400 ×*g*, the supernatant was evaporated to dryness for 1 h under a stream of nitrogen at 40°C.

Digestion of human GLP-1 (7–36 amide) by B-9 culture broth One hundred 20 μL of B-9 culture broth pre-incubated with 10 mM EDTA was added to the concentrated extracts obtained by protein precipitation. Then, samples were incubated at 27°C for 15 h. After incubation, 10 μL of GIP_{1-8} solution was added as an internal standard to all samples, resulting in a final plasma concentration of 500 nM for GIP_{1-8}.