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Formation of polyamine-modified peptides during protein digestion

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Abstract

The effect of polyamines on the digestion of proteins by serine proteases was examined. Based on the mechanism of action of serine proteases, it was anticipated that nucleophilic functionalities such as amino groups in polyamine, rather than hydroxyl ions, would react with peptide bonds during the hydrolysis process. If this were the case, polyamine might be covalently linked to the C-terminus of the product peptides during protein digestion. In order to test this hypothesis, hemoglobin was used as a model protein and was digested with trypsin in the presence of polyamine. The product peptides were separated, collected by HPLC, and analyzed by MALDI-TOF MS using post-source decay. The results showed that some peptides were indeed modified with polyamine at the C-terminus.

Keywords: Spermine; Spermidine; Polyamine; Alkylamine; Serine protease; Modification; C-terminus

Spermidine (Spd) and spermine (Spm) are natural polyamines widely distributed in biological systems. Polyamines are essential in cellular proliferation and differentiation, and are generally present in cells as low molecular weight alkylamines. These polyamines are present in significant levels in many foods, with the daily adult polyamine intake (including putrescine, a polyamine precursor) estimated at 350–550 μmol [1,2]. Given these levels, polyamines could affect intestinal functions, food absorption and food digestion. Spd or Spm administered orally to suckling rats causes structural and biochemical changes in the intestinal mucosa, comparable to those observed at weaning [2]. In addition, exogenous polyamines have been shown to effectively substitute for endogenously synthesized polyamines in duodenal mucosal repair processes, and to increase the normal healing rate [2]. We previously reported that Spm significantly increased the absorption of macromolecules in the jejunum [3], but studies on the effect of polyamines on food digestion have been limited.

As the absorption of food proteins and polypeptides is generally poor, their digestion by proteases (pepsin, trypsin, chymotrypsin, elastase, carboxypeptidase A, B and intestinal brush-border membrane peptidase) is necessary for the efficient utilization of dietary proteins. Serine proteases are the primary enzymes involved in the digestion of food proteins. The mechanism of action of serine proteases, which involves a serine residue in the active site, is shown in Fig. 1 [4]. Proteins (or peptides) are hydrolyzed by the protease in two steps. The first step is the acylation of the serine residue, resulting in the liberation of the C-terminal peptide. The second step is hydrolysis of the acyl-enzyme intermediate, resulting in elimination of the N-terminal peptide. Removal of the C-terminal peptide produces sufficient space in the vicinity of the acyl-enzyme intermediate to allow a hydroxyl ion with an unshared electron pair to attack the acyl-enzyme intermediate. Instead of a hydroxyl ion, an alkylamine such as polyamine could potentially react with the acyl-enzyme intermediate. If this were the case, peptides would become C-terminally modified with polyamine during protein digestion in the

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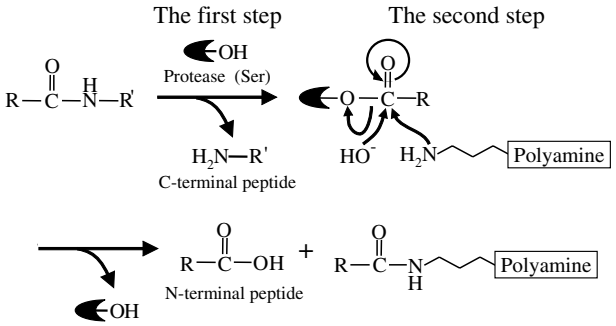

Fig. 1. Serine protease-catalyzed amide hydrolysis and aminolysis by polyamine. Proteins (or peptides) are hydrolyzed by trypsin in two steps. The first step is the acylation of the serine residue in the active site, resulting in liberation of the C-terminal peptide. The second step is hydrolysis of the acyl-enzyme intermediate for eliminating the N-terminal peptide. Polyamines rather than hydroxyl ions might react with the acyl-enzyme intermediate.

presence of polyamine (see step two in Fig. 1). The aim of the present study was to evaluate the possibility of polyamine-modified peptide production *in vitro*.

Materials and methods

Materials. Bovine hemoglobin (HB) and HPLC grade trifluoroacetic acid were obtained from Wako Pure Chemical Industries (Osaka, Japan), modified trypsin (sequencing grade) from Promega (Madison, WI), Spd trihydrochloride, Spm tetrahydrochloride, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid, angiotensin I, bovine insulin, bovine insulin β chain, and α-cyano-4-hydroxycinnamic acid were obtained from Sigma (St. Louis, MO, USA), and HPLC grade acetonitrile was purchased from KANTO KAGAKU (Tokyo, Japan).

Enzyme digestion. HB digestion with trypsin was performed at a concentration of 1.0 mg/ml in 100 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid buffer, pH 7.5, in the presence or absence of Spd (1 or 50 mM) or Spm (1 or 50 mM). The mass ratio of HB to trypsin was 1:20. The reaction mixture was incubated at 37 °C for 3 h.

Fractionation of enzyme digests. The protease digests were fractionated by high performance liquid chromatography (HPLC) on a packed C_{18} column (TOSOH ODS 120T, 4.6 × 250 mm). Solvents for HPLC were: A, 5% acetonitrile containing 0.1% trifluoroacetic acid; B, 80% acetonitrile containing 0.1% trifluoroacetic acid. The column was equilibrated with 100% A. The sample injection volume was 10 μl. The HPLC protocol consisted of 100% A for 10 min, followed by a gradient of 0–100% B over 90 min, all at a flow rate of 1.0 ml/min and a column temperature of 35 °C. Protein digest fractions were collected every 30 s. Each fraction was lyophilized, then dissolved in 20 μl 2:3 acetonitrile/0.1% trifluoroacetic acid.

Mass spectrometry. All mass spectra were obtained in reflectron mode using an AXIMA-CFR MALDI-TOF (Shimadzu/Kratos, Manchester, UK) equipped with a 337 nm pulsed nitrogen laser. External mass calibration was performed with a mixture of peptide standards (angiotensin I, *m/z* 1297.5; insulin bovine β chain, *m/z* 3496.9). The matrix, α-cyano-4-hydroxycinnamic acid, was prepared at a concentration of 10 mg/ml in 2:3 acetonitrile/0.1% trifluoroacetic acid. The sample (1.0 μl) was spotted onto the sample plate, then 1.0 μl of matrix was added and the spot was allowed to dry at room temperature. Peptide sequences were determined by post-source decay (PSD).

Results and discussion

In order to examine the modification of peptides with polyamine during protease digestion, HB and trypsin were used as a model protein substrate and serine protease, respectively. HB was treated with trypsin in the presence of 50 mM Spd or Spm, the peptide products were separated by HPLC, and fractions were collected every 30 s. Fig. 2 shows 90 fractions collected from 15 to 60 min. Digested peptides eluted between 25 and 60 min. Similar chromatograms were observed for HB tryptic digests in the presence of Spd (A) and Spm (B), and in the absence of polyamines (C).

Each digest fraction analyzed by MALDI-TOF MS contained peptides with masses corresponding to the calculated masses of polyamine-modified peptides (Table 1). Although not detected by HPLC as distinct peaks, five Spd-modified peptides and four Spm-modified peptides were detected by mass analysis. Polyamine-modified peptides were detected in digests conducted in the presence of 1 mM Spd or Spm. Spd- and Spm-modified peptides were not detected in control sample fractions (data not shown).

To confirm this C-terminal modification, all mass peaks having molecular weights corresponding to the calculated mass of a polyamine-modified peptide were analyzed by MALDI-TOF MS using PSD. The PSD spectra of peptides (eluting between 35.0 and 35.5 min) from samples digested in the presence of Spd or Spm are shown in Fig. 3A and B, respectively. The HB α chain Spd-modified peptide spanning residues 17–31 (VGGHAAEYGAEALER), has a mass of 1657 Da. When used as a parent ion for PSD, sequential fragment b ions from 3 to 15 and y ions from 2 to 15 were observed. Furthermore, the y-ion peaks had satellite peaks at −17 Da due to loss of ammonia from arginine [5]. The peaks marked '1*', '2*' and '3*' in Fig. 3A are also assigned as Spd fragment ions. Based on the parent ion and the fragment ions, the sequence of the 1657 Da peptide was identified as V-G-G-H-A-A-E-Y-G-

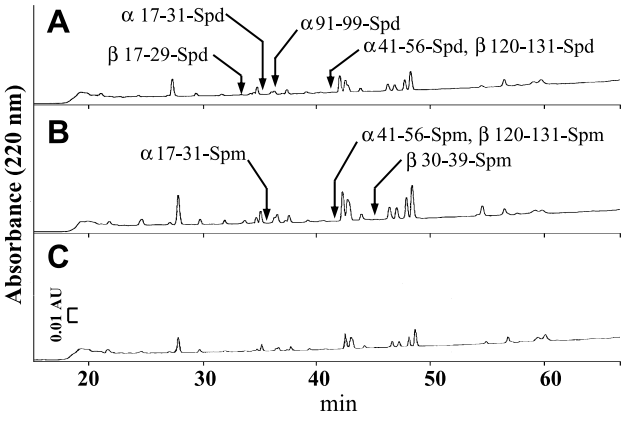

Fig. 2. HPLC chromatograms of tryptic digests of HB. (A) Tryptic digests of HB in the presence of 50 mM spermidine. (B) Tryptic digests of HB in the presence of 50 mM spermine. (C) Tryptic digests of HB in the absence of spermidine and spermine. The protease digests were fractionated by HPLC on a packed C18 column (TOSOH ODS 120T, 4.6 × 250 mm). UV absorption was monitored at 220 nm. Eluate was collected every 30 s. Arrows indicate retention time of polyamine-modified peptides identified by mass analysis.

Table 1
MALDI-TOF MS detection of peptide fragments with molecular weights corresponding to the calculated mass of polyamine-modified peptides

Polyamine	Fraction (A–B min)[a]	Region of HB	Monoisotopic MH^+ at *m/z* (calculated monoisotopic mass)
50 mM Spermidine	32.5–33.0	β 17–29	1455.94 (1454.86)
	35.0–35.5	α 17–31	1657.96 (1656.88)
	36.0–36.5	α 91–99	1214.85 (1214.77)
	41.0–41.5	α 41–56	1961.12 (1961.03)
	41.0–41.5	β 120–131	1549.95 (1549.87)
50 mM Spermine	35.0–35.5	α 17–31	1714.05 (1713.94)
	41.0–41.5	α 41–56	2018.20 (2018.09)
	41.0–41.5	β 120–131	1607.04 (1606.93)
	45.0–45.5	β 30–39	1459.04 (1458.93)
1 mM Spermidine	35.0–35.5	α 17–31	1657.9 (1656.88)
1 mM Spermine	35.0–35.5	α 17–31	1713.8 (1713.94)

HB digestion with trypsin was performed in the presence of spermidine (1 or 50 mM) or spermine (1 or 50 mM). Those protease digests were separated and fractionated every 0.5 min by HPLC, and every fraction was analyzed by MALDI-TOF MS.

[a] Fraction eluted between A min and B min.

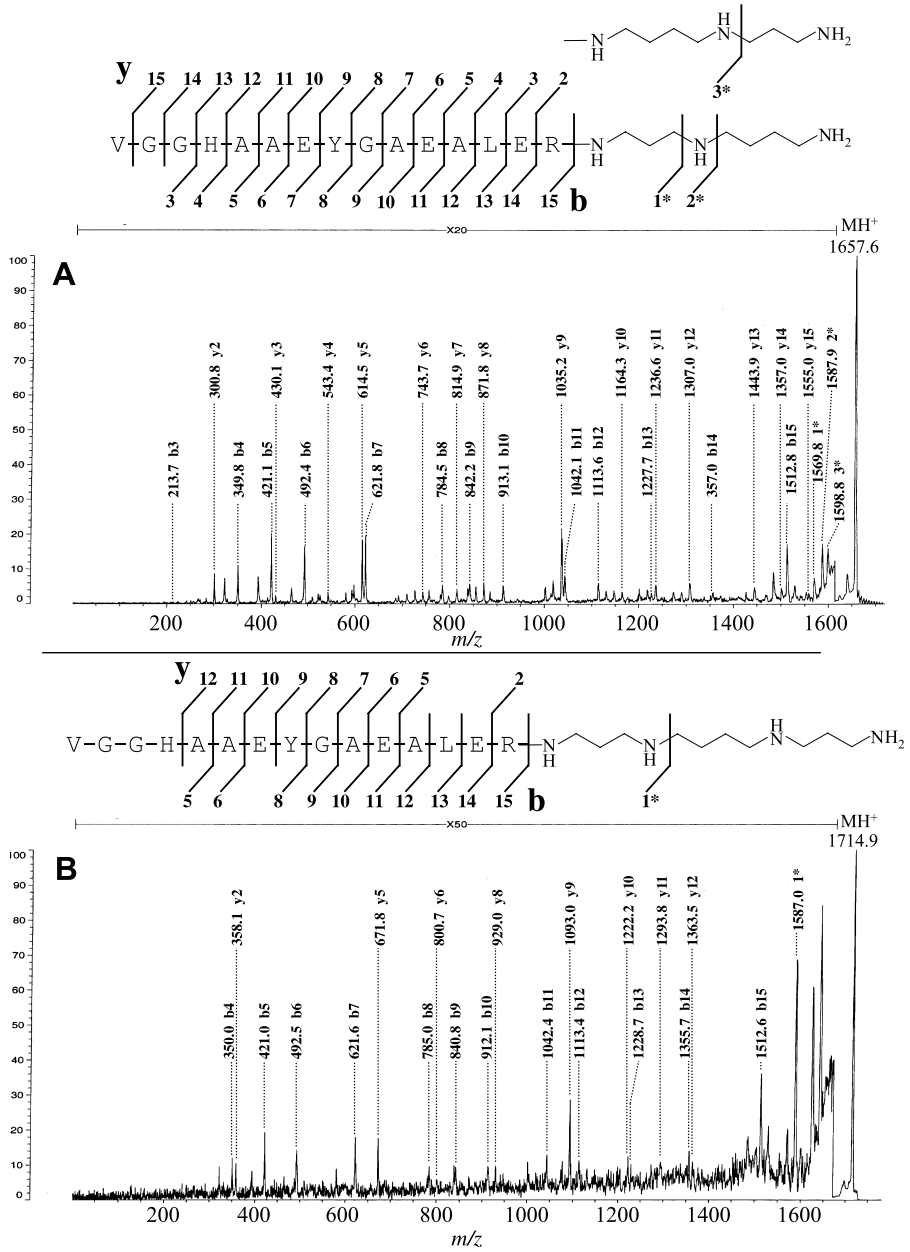

Fig. 3. PSD spectra of protonated C-terminus spermidine-modified 1657.6 Da peptide (A) and C-terminus spermine-modified 1714.9 Da peptide (B).

A-E-A-L-E-R-Spd, which corresponds to a portion of the HB sequence (Swiss-Prot Accession No. P01966 and P02070). Additionally, the PSD spectrum of the Spm-modified peptide corresponding to residues 17–31 of the HB α chain is shown in Fig. 3B. Peaks marked '1*' are also assigned as Spm fragment ions. Based on the parent ion and the fragment ions, the sequence of this peptide was identified as V-G-G-H-A-A-E-Y-G-A-E-A-L-E-R-Spm. All polyamine-modified peptides were modified at the C-terminus (data not shown).

Fragmentation of the polyamine moiety of polyamine-modified peptides using PSD analysis was attempted. Fragment peaks of the Spd moiety of Spd-modified peptides are indicated as 1^* and 2^* in Fig. 3A, and the Spm moiety of Spm-modified peptides are indicated as 1^* in Fig. 3B. The specificity of the polyamine fragmentation pattern may be useful for identifying polyamine-modified peptides.

Our data provide evidence that peptides are modified at the C-terminus with polyamine when proteins are digested by trypsin in the presence of 1 or 50 mM polyamine. Shortly after a meal, polyamine concentrations in the duodenal and jejunal lumen reach almost millimolar levels [6]. Therefore, it is possible that C-terminal-polyamine-modified peptides are produced in the intestinal lumen following a meal.

It has been shown that polycationic materials such as poly-L-arginine, poly-L-lysine, protamine, chitosan and *N*-trimethyl chitosan have the potential to promote transmucosal delivery of macromolecules without producing severe epithelial toxicity [7,8], and that cationic-labeled proteins have increased membrane permeability compared with non-labeled proteins [9]. Thus, it is feasible that polyamine-modified peptides may have increased membrane permeability compared with non-modified peptides.

It was recently shown that amino acids attached to polyamine via amide linkages have novel actions. For example, N^1-spermine-L-lysinyl amide is a polyamine transport inhibitor [10], and N^1-spermidine-L-arginyl amide has been shown to be neuroprotective in *in vitro* models of neurodegeneration and in *in vivo* ischaemia, yet it does not suppress synaptic transmissions [11]. Thus, if C-terminal polyamine-modified peptides are absorbed from the gastrointestinal tract, it is possible that the polyamine-modified peptides would have several unexpected effects on the body. Effects of polyamine-modified peptides remain to be resolved.

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References

[1] S. Bardócz, G. Grant, D.S. Brown, A. Ralph, A. Pusztai, Polyamines in food-implications for growth and health, J. Nutr. Biochem. 4 (1993) 66–71.
[2] D. Patricia, P. Olivier, D. Guy, Dietary polyamines and non-neoplastic growth and disease, Eur. J. Gastroenterol. Hepatol. 13 (2001) 1027–1032.
[3] Y. Sugita, K. Takao, Y. Toyama, A. Shirahata, Enhancement of intestinal absorption of macromolecules by spermine in rats, Amino Acides (2007), in press.
[4] J.B. Tobin, S.A. Whitt, C.S. Cassidy, P.A. Frey, Low-barrier hydrogen bonding in molecular complexes analogous to histidine and aspartate in the catalytic triad of serine proteases, Biochemistry 34 (1995) 6919–6924.
[5] P. Chaurand, F. Luetzenkirchen, Peptide and protein identification by matrix-assisted laser desorption ionization (MALDI) and MALDI-post-source decay time-of-flight mass spectrometry, J. Am. Soc. Mass Spectrom. 10 (1999) 91–103.
[6] E.R. Seidel, J.L. Scemama, Gastrointestinal polyamines and regulation of mucosal growth and function, J. Nutr. Biochem. 8 (1997) 104–111.
[7] K. Ohtake, T. Maeno, H. Ueda, M. Ogihara, H. Natsume, Y. Morimoto, Poly-L-arginine enhances paracellular permeability via serine/threonine phosphorylation of ZO-1 and tyrosine dephosphorylation of occludin in rabbit nasal epithelium, Pharm. Res. 20 (2003) 1838–1845.
[8] A. Yamamoto, Improvement of transmucosal absorption of biologically active peptide drugs, Yakugaku Zassi 121 (2001) 929–948. Article in Japanese.
[9] J. Futami, M. Kitazoe, T. Maeda, E. Nukui, M. Sakaguchi, J. Kosaka, M. Miyazaki, M. Kosaka, H. Tada, M. Seno, J. Sasaki, N.H. Huh, M. Namba, H. Yamada, Intracellular delivery of proteins into mammalian living cells by polyethylenimine-cationization, J. Biosci. Bioeng. 99 (2005) 95–103.
[10] M.R. Burns, C.L. Carlson, S.M. Vanderwerf, J.R. Ziemer, R.S. Weeks, F. Cai, H.K. Webb, G.F. Graminski, Amino acid/spermine conjugates: polyamine amides as potent spermidine uptake inhibitors, J. Med. Chem. 44 (2001) 3632–3644.
[11] B. Morrison III, A.K. Pringle, T. McManus, J. Ellard, M. Bradley, F. Signorelli, F. Iannotti, L.E. Sundstrom, L-Arginyl-3,4-spermidine is neuroprotective in several in vitro models of neurodegeneration and in vivo ischaemia without suppressing synaptic transmission, Br. J. Pharmacol. 137 (2002) 1255–1268.