이학석사학위논문

MALDI-TOF MS Identification of Peptide Citrullination Site Using Br Signature

MALDI-TOF 질량분석에서

브롬 동위원소 패턴을 이용한

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최 미 경

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Abstract

Enzymatic conversion of arginine to citrulline by peptidyl arginine deiminase (PAD) is associated with peptide presentation and development of autoimmunity in rheumatoid arthritis. In order to facilitate identification of the citrullination site, citrulline residue was modified using 4-bromophenyl glyoxal (BPG), and 194 Da mass increase and incorporation of the Br signature were confirmed by MALDI-TOF MS. Using this approach, we identified four and five citrullination sites of bovine serum albumin (BSA) and bovine fibrinogen, respectively. MALDI-TOF/TOF MS was used to unambiguously identify two citrullination sites from bovine fibrinogen.

Keywords : citrullination, deimination, citrulline, 4-bromophenyl glyoxal, MALDI-TOF MS

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1. Introduction

Citrullination is a post-translational deimination of arginine to citrulline catalyzed by peptidyl arginine deiminase (PAD). This enzymatic reaction is known to be associated with autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis.[1,2] Citrullinated proteins were increased in sera and synovial fluid of RA patients compared with those of osteoarthritis (OA) patients. These citrullinated proteins or peptides are suggested to be autoantigens which initiate immune reaction and induce systemic inflammation.[3,4] Anti-citrullinated protein antibodies have been widely used for the diagnosis of RA with 90-98% specificity.[5]

Western blot and 2D SDS-PAGE have been used to verify citrullination with high sensitivity;[6] however, they cannot be used for identification of citrullination site. Citrullination occurs only at specific arginine residues, and it becomes necessary to identify the citrullination site using mass spectrometry. Citrullination is accompanied by only 1 Da increase in mass (Fig. 1).[7] Therefore, citullinated peptide peaks and the isotope peaks from the control peptide overlap on mass spectrum making direct observation of citrullination by MALDI-TOF MS difficult. Moreover, peptides containing asparagine or glutamine could undergo deamidation

with 1 Da mass increase confounding the investigation of citrullination.

Several different approaches have been used for investigation of citrullination. Isotopic labeling using $H_2{}^{18}O$ was used for analysis of citrullination sites in human fibrinogen by LC-MS.[8] LC-MS/MS was used for analysis of citrullination sites in nucleophosmin.[9] Glyoxal derivatives have been known to react with arginine under basic conditions and with citrulline under acidic conditions. So, glyoxal derivatives such as phenylglyoxal and 2,3-butanedione were used for detection of citrulline. Specific modification of citrulline has been achieved using a combination of 2,3-butanedione and antipyrine or using 2,3-butanedione alone.[10-12] Specific binding of citrulline to 4-hydroxyphenylglyoxal bonded to beads has been utilized for enrichment of citrulline-containing peptides from myelin basic protein for subsequent analysis of citrullination sites by MALDI-TOF MS.[13]

We attempted to use 4-bromophenylglyoxal (BPG) for determination of the citrullination site taking advantage of the unique isotopic distribution of Br (^{79}Br, 50.69%; ^{81}Br, 49.31%) as demonstrated in earlier mass spectrometric studies.[14,15] BPG will react specifically with citrulline at low pH and the Br signature with increased mass will facilitate mass spectrometric identification of the citrullination site.

Trypsin hydrolyzes peptide bond after arginine and lysine, but not after citrulline.[16] Thus, comparison of the tryptic peptides from citrullinated protein with those from control protein can be used to obtain information about the citrullination site. A more definitive identification could be derived from BPG modification of tryptic peptides from citrullinated protein.

2. Experimental section

2.1 Materials

Bovine fibrinogen, bovine serum albumin (BSA), rabbit skeletal muscle peptidyl arginine deiminase 2 (PAD2), trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (CHCA), dithiothreitol (DTT), acetonitrile (ACN), glycerol, tris(hydroxymethyl)aminomethane (Tris), Lys-C and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-bromophenylglyoxal hydrate (BPG) was from Ochem Inc (Des Plaines, IL, USA). 2-mercaptoethanol was from Bio-Rad (Hercules, CA, USA). Zip-tip was from Millipore (Billerica, MA, USA).

2.2 BPG Modification of citrulline

Model peptides with or without citrulline (IAEFPSRGK-NH_2, IAEFPScitGK-NH_2, VGLGARGHRPYDK-NH_2) were synthesized by the solid-phase method of Merrifield. 1 mg model peptide was dissolved in 1 ml deionized water. 30 μl TFA and 10 μl 50 mM BPG solution were added to 10 μl of the peptide solution, and the mixture was incubated at 37℃ for 3 h. After removing the supernatant using speed-vac, the residue was dissolved in 10 μl deionized water and desalted with zip-tip.

Proteins (BSA and bovine fibrinogen) were dissolved at 1 mg/ml concentration in deimination buffer (80 mM tris-HCl, 8 mM $CaCl_2$, 4 mM DTT, 10% glycerol, pH 7.6). PAD (6.5 µl, 2U) was added to 500 µl of the protein solution in one tube, and 6.5 µl of the blank solution (20 mM tris-HCl, pH 7.4, 10 mM 2-mercaptoethanol, 1 mM EDTA) was added to another tube. The PAD reaction was allowed to proceed for 2 h at 37℃. A 10 µl aliquot of 0.1 mg/ml trypsin or Lys-C solution in 25 mM ammonium bicarbonate was added to both citrullinated and uncitrullinated protein and digestion proceeded overnight at 37℃. Peptides in the protein digest were treated with BPG as above for model peptides. A portion of the peptides from the control proteins was treated with PAD and BPG.

2.3 MALDI-TOF MS

Matrix solution was made by dissolving 10 mg CHCA in 1 ml of 50% acetonitrile in water containing 0.1% TFA. 1 µl sample was mixed with 1 µl of the matrix solution, and 1 µl of the mixture was loaded on a sample plate and dried at room temperature. MALDI-TOF and TOF/TOF mass spectra were obtained using Autoflex II MALDI time-of-flight mass spectrometer (Bruker Daltonics, Bremen, Germany). MALDI-TOF/TOF

spectra were also obtained using ABI 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA).

3. Results and discussion

3.1 BPG Tagging of Citrulline.

Citrullination of arginine residue to citrulline catalyzed by PAD and subsequent tagging with BPG is schematically shown in Fig. 1. When an arginine-containing model peptide (IAEFPSRGK-NH_2, 1002.56 Da, Fig. 2a) was treated with BPG, no change in the mass spectrum was observed by MALDI MS (Fig. 2b). When a similar peptide containing citrulline instead of arginine (IAEFPScitGK-NH_2, 1003.54 Da, Fig. 2c) was used, a complete modification of the peptide with a 194 Da shift accompanied by the Br isotopic pattern was observed after reaction with BPG for 3 h (Fig. 2d). Without the Br atom, the first isotopic peak is the strongest reflecting low abundance of C-13. However, with Br, the third isotopic peak becomes stronger than the first.

The molecular ion generated from BPG-modified IAEFPScitGK-NH_2, observed at *m/z* 1198.55 (Fig. 2d) was subjected to MS/MS analysis. Complete b- and y-series fragment ions were observed in the MS/MS spectrum (Fig. 3). The mass difference between y2 and y3 ions was consistent with BPG-citrulline. Moreover, the Br signature was observed from y3-y8 ions as well as the parent ion, but not from y1 and y2 ions.

These results clearly pinpoint the third residue from the C-terminus as the citrullination site. The mass difference between b6 and b7 ions was also consistent with the BPG-modified citrulline residue. The Br signature was observed from b7 and b8 ions, but not from b1-b6 ions.

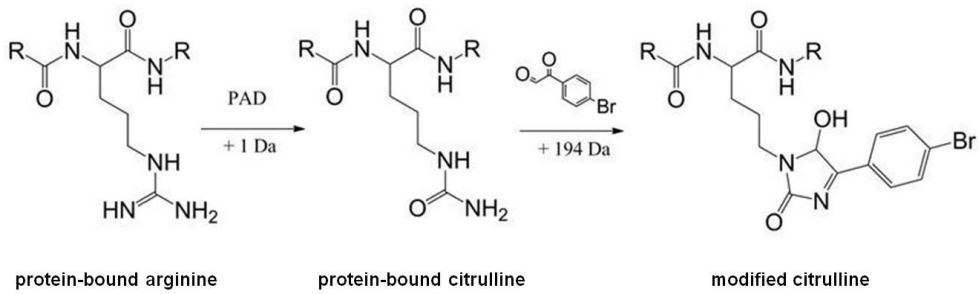

Figure 1. Citrullination by PAD with a mass shift of +1 Da followed by modification with BPG with additional mass shift of 194 Da.

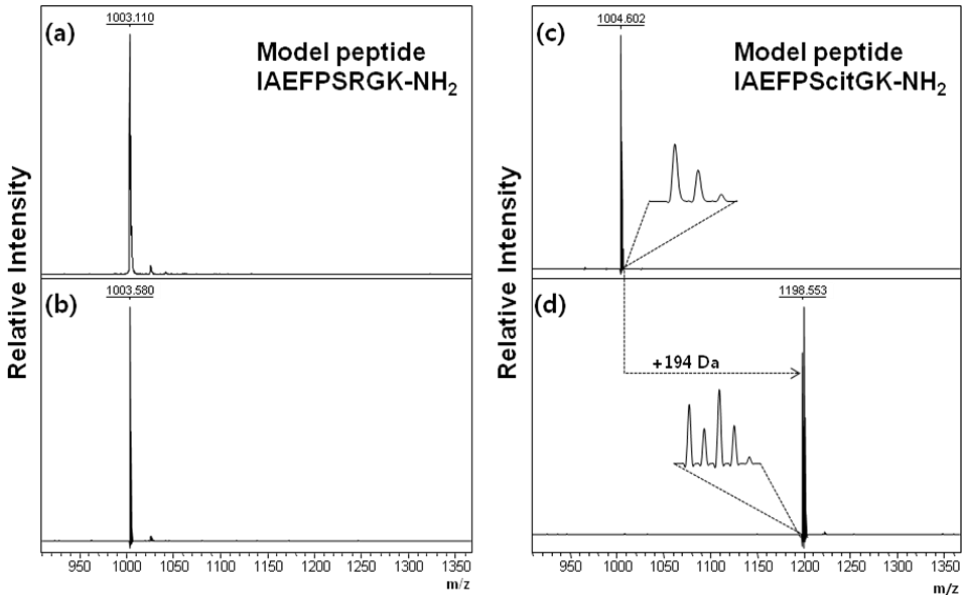

Figure 2. MALDI mass spectra showing an arginine-containing model peptide without citrulline before (a) and after (b) reaction with BPG; a model peptide with citrulline before (c) and after (d) reaction with BPG

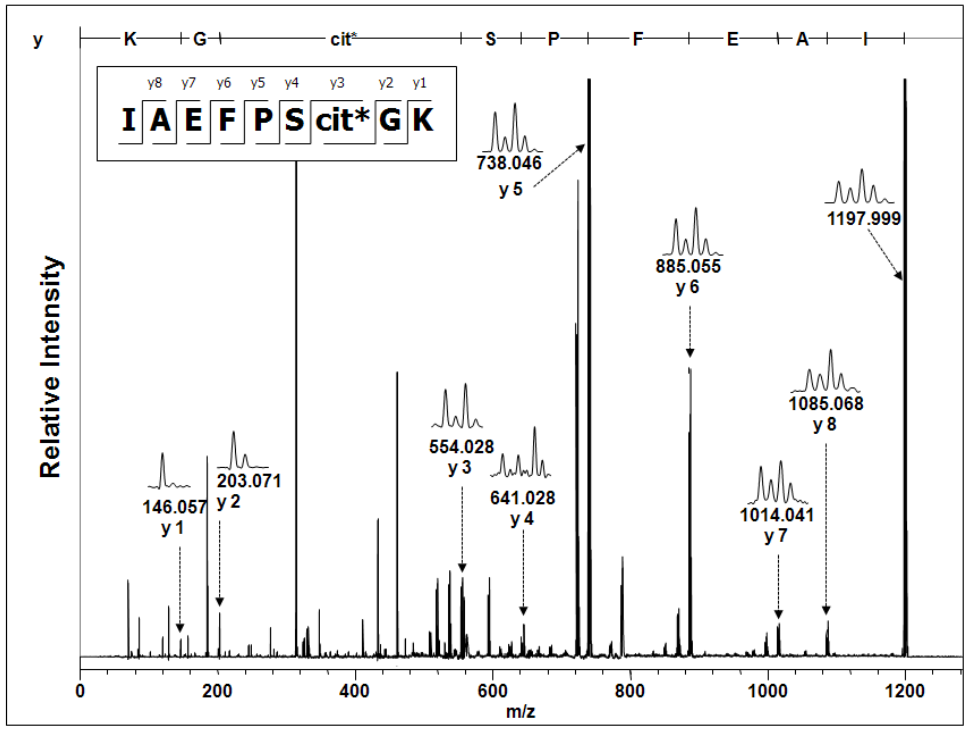

Figure 3. MALDI TOF/TOF spectrum obtained from the *m/z* 1198.55 peak in Fig. 2d

3.2 Citrullination sites of BSA

Since our goal was to identify the PAD-catalyzed citrullination sites in proteins utilizing the BPG tag, we next tested the efficacy of the citrullination reaction by PAD using BSA. BSA was treated with PAD in the presence of Ca^{2+}, which is known to facilitate the citrullination reaction.[17] When the tryptic digest of citrullinated BSA was treated with BPG, mass spectrum in Fig. 4b was obtained showing four peptide peaks with the Br signature. The original citrullinated peptide without the BPG tag could be identified from the mass spectrum in Fig. 4a by subtracting 194 Da from the peaks with the Br signature, and the amino acid sequence of the peptide could be determined from the BSA database. For example, the BSA peptide giving rise to the 1015.52 peak in Fig. 4b was identified as FGERALK. Similarly, the 1278.66, 1634.80, and 1663.76 peaks were identified as YLYEIARR, RHPEYAVSVLLR, and DTHKSEIAHRFK, respectively.

Both FGERALK and DTHKSEIAHRFK contain one arginine residue in between the N- and the C-terminus, which indicates that the R residues are citrullinated and thus the peptide bond between R and the adjacent residue is not subject to tryptic hydrolysis. Two peptides, YLYEIARR and RHPEYAVSVLLR, contain two R residues one of which

being at the C-terminus. If the C-terminal R were citrullinated, tryptic hydrolysis would have been blocked and such peptides would not have been detected. This result implies that citrullination of R is specific and depends on the chemical environment around the R residue. When trypsin treatment preceded the PAD and BPG reactions, the peptides ending with C-terminal R were not observed. For example, FGER which is a tryptic fragment of FGERALK was not citrullinated and subsequently detected with BPG tag. The same was the case with YLYEIAR and DTHKSEIAHR. Thus it appears that several amino acid residues after R are needed for the PAD reaction.

The mass spectrum obtained when BSA was first hydrolyzed with Lys-C, which works at the C-terminal side of lysine but not of arginine, and treated with PAD and BPG, was identical with the one obtained when BSA was first treated with PAD and then hydrolyzed with Lys-C and tagged with BPG (result not shown). This implies that citrullination by PAD does not depend on the three dimensional structure of a protein, but rather on specific residues around the arginine residue. BSA contains 23 arginine residues. Peptides observed by MALDI MS (Fig. 4a) covered 55% of the entire sequence and represented 18 arginine residues. Of these, only 4

residues were identified as citrullinated showing about 20% specificity. Citrullinated BSA peptides are summarized in Table 1.

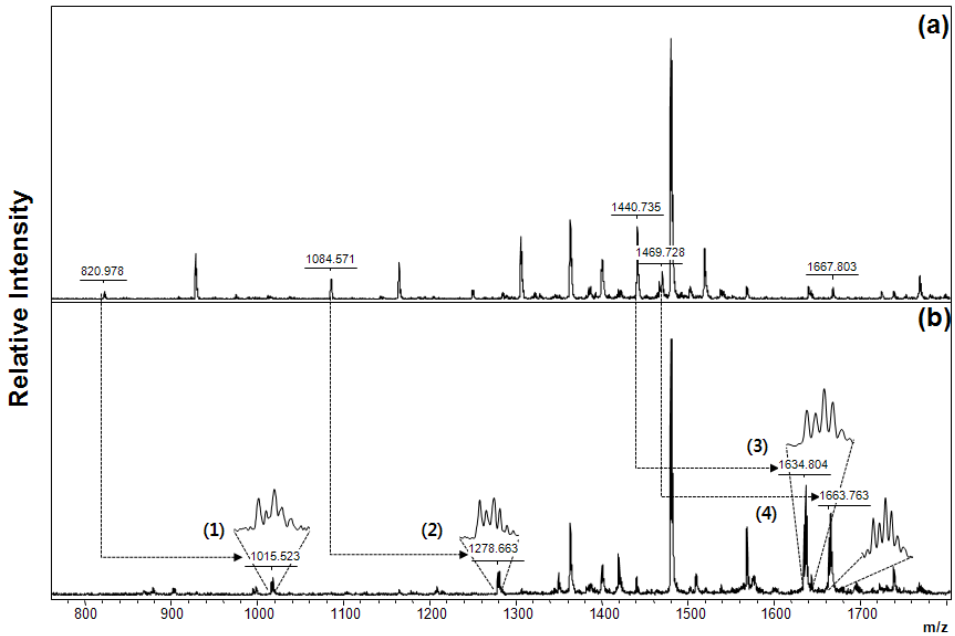

Figure 4. MALDI mass spectra obtained from tryptic digest of citrullinated BSA before (a) and after (b) reaction with BPG

No	Observed *m/z*	Sequence	Residue
(1)	1015.52	FGE**R**ALK	205-211
(2)	1278.66	YLYEIA**R**R	137-144
(3)	1634.80	**R**HPEYAVSVLLR	336-347
(4)	1663.76	DTHKSEIAH**R**FK	1-12

Table 1. Tryptic BSA peptides detected via citrullination and BPG tagging. Citrullinated arginine residues are in bold

3.3 Citrullination sites of fibrinogen

Five peaks showing the BPG tag were observed by MALDI MS (Fig. 5) from bovine fibrinogen citrullinated using PAD, treated with Lys-C and derivatized with BPG. Lys-C hydrolyzes after lysine, but not after arginine. Therefore, analysis of the results is more straightforward using Lys-C than trypsin, because trypsin hydrolyzes after the arginine residue from the control protein but not after citrullination by PAD. The four peptides containing the five citrullinated arginine residues were identified as summarized in Table 2. Two peptides were from the alpha chain and contained one R residue. A third peptide, VGLGARGHRPYDK, was from the beta chain and contained two R residues.

The three peptides giving rise to the four peaks, labeled 1 through 4, could be readily identified from the fibrinogen sequence from the observed *m/z* values taking citrullination and BPG tagging into account. However, the 1988.64 peak carrying the Br tag did not match any Lys-C digested peptide sequence from fibrinogen. MS/MS analysis was performed on this ion. A series of fragment ions with the Br signature was obtained as shown in Fig. 6. From the mass differences, the PTDYDEGQDD could be deduced definitively, which matched residues 3-12 of the N-terminal peptide from the bovine fibrinogen beta chain.

Therefore, it appeared that the 1988.64 peak represented the QFPTDYDEGQDDRPK sequence (residues 1-15). The ions, y1 and y2, corresponding to K and P, respectively, did not carry the Br signature. However, y3 and subsequent ions carried the signature clearly identifying the R, residue 13, as the citrullination site. The molecular weight of QFPTDYDEGQDDRPK is 1809.78 Da and after modification a signal was expected at 2004.78 instead of 1988.64. This difference of 16 Da suggests some modification of the N-terminal peptide. The nature of the modification is unknown.

It was hoped that the MS/MS spectrum derived from VGLGARGHRPYDK with one R citrullinated and derivatized with BPG could show which R is citrullinated first. The 1620.70 peak in Fig. 5 was not strong enough to yield the desired MS/MS spectrum. Therefore, VGLGARGHRPYDK-NH_2 was synthesized and citrullinated using PAD. After 1 h reaction with PAD followed by BPG tagging, a major peak with the Br signature was observed at 1618.99 followed by a smaller peak with 2 BPG tags. Figure 7 shows the MS/MS spectrum obtained from the 1618.99 peak. All b-ions up to b-8 did not show the Br signature. The b-9, b-10, and the parent ion carried the signature. These results indicate that the R which is the 9^{th} residue from the N-terminus is citrullinated first, while the 6^{th}

residue R remains uncitrullinated. It appears that the PAD reaction rate is influenced by the chemical environment around the R residue.

It should be noted that as the molecular weight of the peptide increases the first isotopic peak decreases making the symmetrical Br signature less evident. It was observed from peaks with the Br signature at *m/z* greater than 1400 Da in Fig. 4-6. The distinctly strong third isotopic peak still can be used to select the peak carrying the BPG tag.

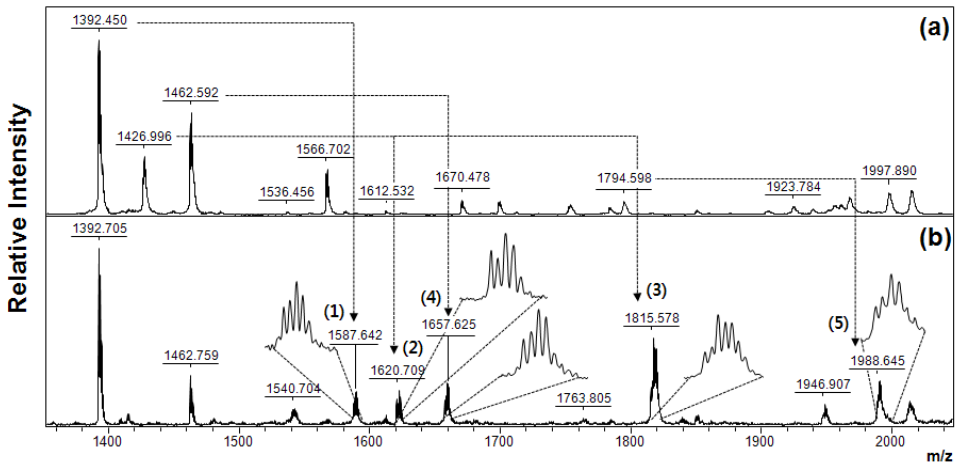

Figure 5. MALDI mass spectra obtained from peptides of citrullinated bovine fibrinogen before (a) and after (b) reaction with BPG

No	Observed *m/z*	Sequence	Residue	
(1)	1587.64	NIVELM**R**GDFAK	alpha	111-122
(2)	1620.70	VGLGA**R**GH**R**PYDK	beta	16-28
(3)	1815.57	VGLGA**R**GH**R**PYDK	beta	16-28
(4)	1657.62	MSTITGPVP**R**EFK	alpha	229-241
(5)	1988.64	QFPTDYDEGQDD**R**PK	beta	1-15

Table 2. Peptides from citrullinated bovine fibrinogen giving rise to the observed peaks showing the Br signature. Citrullinated arginine residues are in bold

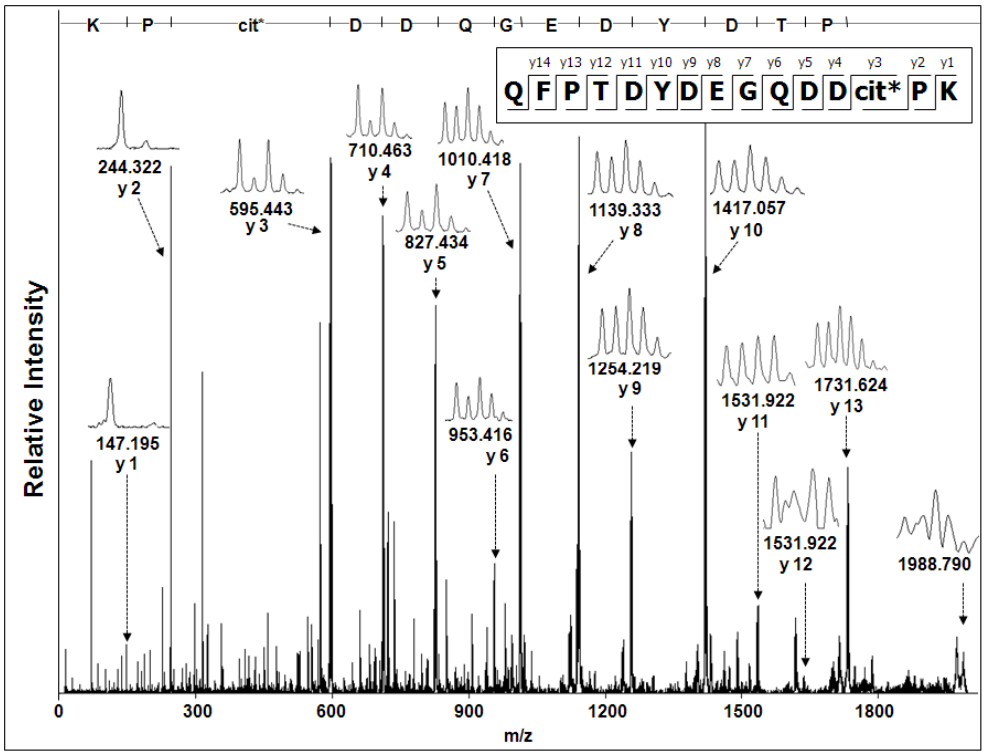

Figure 6. MALDI MS/MS spectrum obtained from the *m/z* 1988.64 peak in Fig. 5

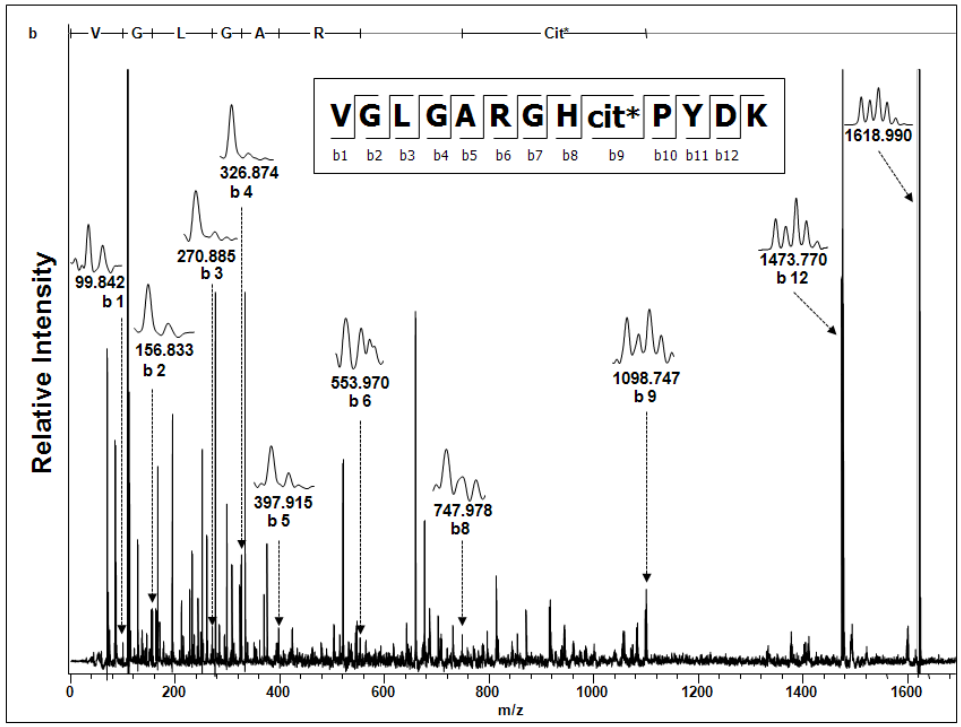

Figure 7. MALDI MS/MS spectrum derived from singly citrullinated VGLGARGHRPYDK-NH_2

4. Conclusion

Incorporation of Br signature via 4-bromophenyl glyoxal facilitates identification, by MALDI-TOF MS, of the peptide containing arginine residue citrullinated by peptidyl arginine deiminase. Unambiguous identification of the citrullination site through partial sequencing around the citrullination site is possible by MALDI-TOF/TOF MS.

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국문 초록

알지닌이 시트룰린으로 변형되는 시트룰린화는 peptidyl arginine deiminase (PAD)에 의한 효소화 반응으로 류마티스 관절염과 같은 자가면역질환과 관련되어 있다. 시르툴린화 위치를 확인하기 위해서, 시트룰린 잔기를 4-bromophenyl glyoxal (BPG)를 이용하여 변형하였고, 반응 뒤 194 Da 질량 증가와 브롬 동위원소 패턴을 MALDI-TOF 질량분석에서 확인하였다. 이 방법으로 bovine serum albumin (BSA) 에서 4 군데, bovine fibrinogen 에서 5 군데의 시트룰린화 위치를 각각 찾아내었다. 또한, MALDI-TOF/TOF 질량분석을 이용하여 bovine fibrinogen 의 시트룰린화 위치 두 곳을 명확하게 확인하였다.

주요 단어 : 시트룰린화, 시트룰린, deimination, 4-bromophenyl glyoxal, 매트릭스-지원 레이저 탈착 이온화 질량분석기 (MALDI-TOF MS)

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