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SHORT COMMUNICATION

Determination of meaty peptide in enzymatic hydrolyzate of beef protein by HPLC-MS

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Abstract−The purpose of this study is to detect beefy meaty peptide (BMP) in beef hydrolyzate. The synthesized BMP is used as a standard sample in the study. High performance liquid chromatography (HPLC)/ion trap electrospray ionization mass spectrometry (ESI-MS) with UV detection was applied in qualitative analysis of the peptides. Six beef protein enzymatic hydrolyzate samples were separated on a Surveyor HPLC system through a SUPELCO Discovery® C_{18} analytical column (5 μm, 15 cm×2.1 mm i.d.). The column was eluted at a flow rate of 0.2 mL/min in a linear gradient elution mode of acetronitrile-water solution with 0.1% trifluoroacetic acid. The concentration of acetronitrile was increased from 5% to 50% in 40 minutes. A Finnigan LCQ Advantage MAX instrument was used as detector to analyze with ESI-MS and ESI-MS/MS in positive mode. Among the six samples of beef protein enzymatic hydrolysate, the BMP is detected and confirmed in sample No.4 with a higher intensity of characteristic peak and is further investigated by ESI-MS/MS. As a result, BMP exists in sample No.4. The study proves that HPLC-ESI-MS/MS is a simple, rapid, sensitive method to analyze target peptides from complex polypeptides.

Key words: Beef, Enzymatic Hydrolysis, Beefy Meaty Peptide (BMP), HPLC-MS

INTRODUCTION

Beefy meaty peptide (BMP) is an octapeptide isolated from the beef that initially digested by papain. The primary structure of this peptide was proposed as H-Lys-Gly-Asp-Glu-Glu-Ser-leu-Ala-OH (K-G-D-E-E-S-L-A) [1]. There are synergisms of BMP with salt and MSG (monosodium glutamate). BMP is a heat-stable flavor peptide, which makes it suitable for heat treatment as required in the food industry [2,3]. Therefore, it is possible that BMP can be used as a new natural flavor enhancer and has a broad market potential.

With the process of hydrolysis, beef protein was digested to a mass of complex polypeptides with different molecular weight, and small peptides were the main component. For routine peptide analysis, bioactive peptide identification from complex hydrolyzate is comprised of several purification steps, facilitated by a combination of different chromatographic techniques. Each separation step involves solvent evaporation and evaluation of the biological activity [4]. Under these conditions, it is very difficult and labor-intensive to isolate and identify the peptide in the hydrolyzate.

Usually, high performance liquid chromatography (HPLC or LC) is used in separation, purification, collection of the single component [5], and the retention time of the corresponding component is provided. Liquid chromatography-mass spectrometry (LC-MS) is a commonly used tool in biological analysis, forming an effective basis for identifying and quantifying proteins and peptides [6]. LC-MS is normally used to provide initial identification according to molecular weight, and confirmation is obtained by structural specific fragmentation analysis by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The combination of LC-MS and LC-MS/MS analysis enables detailed and accurate identification of peptides in complex sample matrices [6].

In this study, using synthesized BMP as a standard, we identified the peptide sequences of BMP in beef protein hydrolyzate through the technique of HPLC-MS/MS. Consequently, a new method was developed for qualitative analysis of target peptides of other food commodities in food technology.

MATERIALS AND METHODS

1. Materials

1-1. Raw Materials

Beef used in the analysis was purchased from a local market of agricultural products in Tianjin, China.

1-2. Reagents and Chemicals

Acetonitrile (ACN): HPLC grade (Merck, Germany). All other chemicals used were of analytical grade. Double distilled water was filtered through a 0.45 μm filter membrane prior to use. BMP (95% purity) was commercially synthesized in Shanghai Sangon Biological Engineering Technology & Services CO., Ltd. (Shanghai, China) and used as a standard. Six different proteolytic enzymes were provided by Tianjin Chunfa Food Ingredient Limited Company (Tianjin, China). They are designated as No.1, No.2, No.3, No.4, No.5, and No.6.

1-3. Instruments

The LC-ESI-MS/MS experiment was carried out with a Surveyor-LCQ Advantage MAX (Thermo Finnigan Company, USA).

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The analytical column was a SUPELCO Discovery® C_{18} (5 μm, 150 mm×2.1 mm. i.d.) (SUPELCO, USA).

2. Methods

2-1. Enzymatic Hydrolysis

10 g of minced beef was mixed with 50 mL water. The mixture was preheated for 30 min in water bath with continuous agitation. Before addition of the proteolytic enzyme, the optimal pH and temperature conditions were adjusted. Then 1 g enzyme preparation was added to initiate hydrolyzation. The sample was taken from the hydrolyzates after 0.5 h, 1 h, 1.5 h, and 2 h hydrolyzation. The optimal pH was maintained with the 1 M HCl and 1 M NaOH during the hydrolyzation. The enzyme reaction was stopped by raising the temperature to 95±1 °C for 10 min. The hydrolyzate was centrifuged at 12,000 rpm for 20 min. The supernatant was filtered through a 0.45 μm filter membrane. The filtrates were concentrated *in vacuo* and then stored in the refrigerator at −20 °C for HPLC-MS analysis later. Six same samples were treated with six different proteolytic enzymes in the same way as above.

2-2. Preparation of BMP Standard Sample

We dissolved the BMP standard sample in DI water to 10 μg/mL and filtered through 0.45 μm filter membrane; the filtrate was stored in a refrigerator at 4 °C prior to use.

2-3. Analysis Conditions

2-3-1. HPLC Conditions

Samples were analyzed by a Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) through a SUPELCO Discovery® C_{18} analytical column (5 μm, 15 cm×2.1 mm i.d.) (SUPELCO, USA). A 10 μL sample was injected and the UV detection wavelength was set at 203 nm. The column was eluted at a flow rate of 0.2 mL/min in a gradient mode by using the mixture of mobile phase A (H_2O+0.1% trifluoroacetic acid) and mobile phase B (acetonitrile +0.1% trifluoroacetic acid). The mobile phase B was increased from 5% to 50% in 40 min period. All HPLC separations were performed at ambient temperature.

2-3-2. Mass Spectrometry Conditions

Electrospray ionization mass spectrometry (ESI/MS) analysis was performed with a Finnigan LCQ Advantage MAX instrument (Thermo Electron Corporation, San Jose, CA, USA). Sample was automatically infused by the HPLC system. The ESI-MS/MS condition was as follows: nitrogen was used as both the sheath gas (35 arbitrary units) and the auxiliary gas (5 arbitrary units); Helium was used as the damping and collision gas; capillary heating temperature was set at 300 °C; spray voltage 4.5 kV; collision energy 100 V; and detection of negative or positive ions 300-2,000u in a full scan mode. The data processing software used was Xcalibur 1.3.

RESULTS AND DISCUSSION

1. Confirmation of HPLC and ESI-MS of BMP Standard Sample

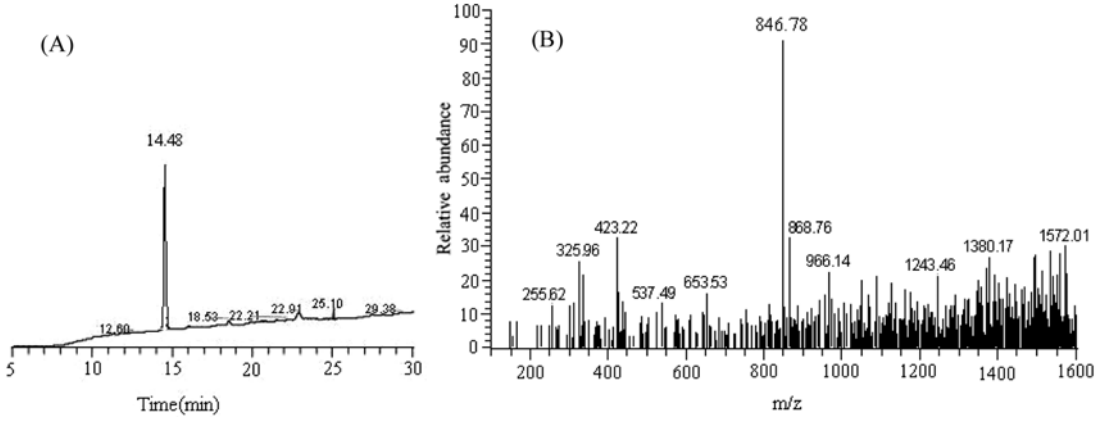

Fig. 1. HPLC (A) and ESI-MS (B) of BMP standard sample.

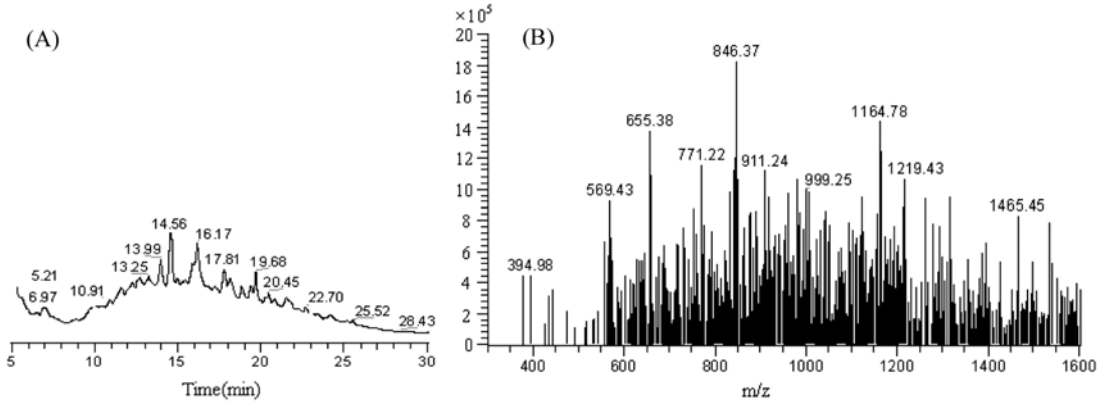

Fig. 2. HPLC (A) and ESI-MS (B) of sample No. 4.

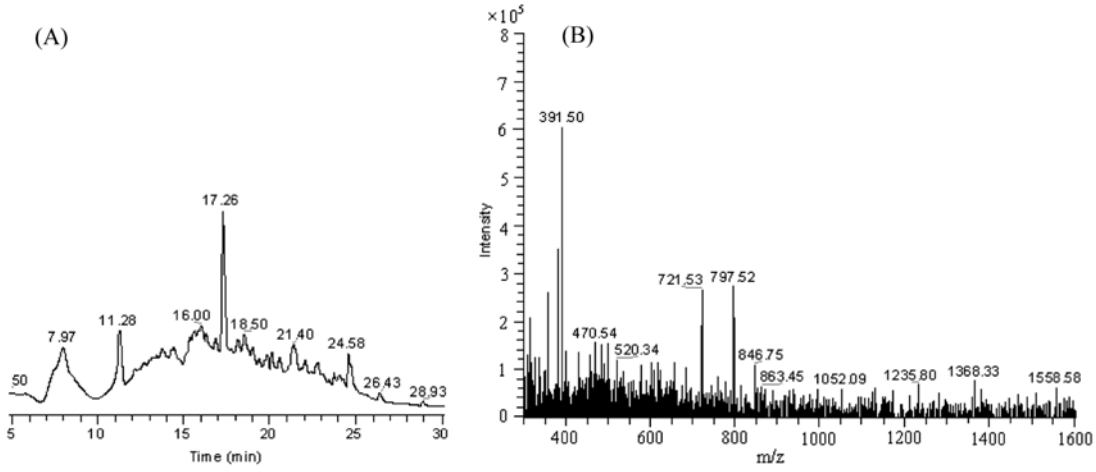

Fig. 3. HPLC (A) and ESI-MS (B) of sample No. 6.

Under the established LC-MS condition, a major peak of the BMP standard sample was obtained. Its retention time is 14.48 min (Fig. 1A). In negative ionization mode, a characteristic fragment (m/z 846.78) of the BMP standard sample was produced corresponding to $[M-H]^-$ ion at m/z 847 (Fig. 1B).

2. Confirmation of HPLC and ESI-MS of Beef Enzymatic Hydrolysis

In light of the method of determining the BMP standard sample, six beef protein hydrolyzates were tested through LC-MS. A characteristic peak was displayed if fragments were similar to those of the BMP standard sample. Out of the six samples, the test result indicated the possible presence of BMP in samples No.4 and No.6 of 2 h hydrolyzed samples. Comparison between the spectrum of sample No.4 and No.6 revealed that sample No.4 had a significantly higher intensity of characteristic peaks (as shown in Fig. 2 and 3). Therefore sample No.4 was selected for further examination by ESI-MS/MS.

3. ESI-MS/MS of BMP Standard Sample

ESI-MS/MS was operated in positive ion mode. The precursor ions spectra were recorded in a range of m/z 848.1-848.9 for the scan analysis. All data was processed by a Finnigan Xcalibur™ core data system Rev. 1.3 (Thermo Quest Corporation, San Jose, CA, USA).

Based on the experiment observation, as peptides undergo collision-induced dissociation (CID), the main types of ions produced are as follows: If the charge is retained on the N-terminus, the fragment ion is classified as a, b, or c, and if the charge is carried on the C-terminus, the fragment ion is classified as x, y, or z [7]. The nomenclature for fragmentation ions is shown in Fig. 4.

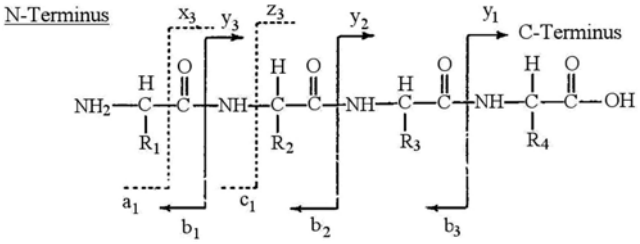

Fig. 4. Sketch map of fragment peptide backbone and nomenclature.

The relative abundance of fragments is highly associated with the applied collision energy [8-10]. The ESI-MS/MS experiments were conducted by varying collision energy to obtain multiple characteristic peaks. A series of fragment ions were obtained from in-source CID. Amino acid sequences of BMP could then be deduced from the fragment ions such as b-ions and y-ions. In this study, collision energies were set at 42.5% and 40% for the ESI-MS/MS scans, respectively. The ESI-MS/MS spectra of the BMP standard sample for collision energies of 42.5% (A) and 40% (B) are illustrated in Fig. 5.

Fig. 5A shows the ESI-MS/MS fragment spectrum of the BMP

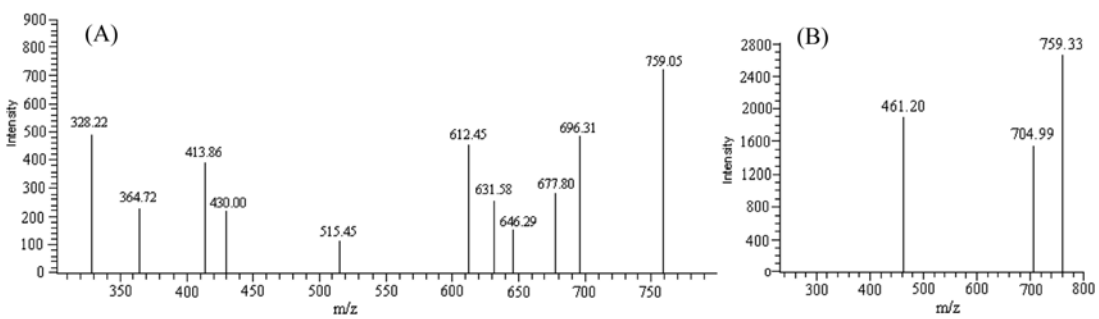

Fig. 5. ESI-MS/MS of BMP standard sample under collision energy of 42.5% (A) and 40% (B).

Table 1. Fragment ions of BMP standard sample determined by mass spectrometry under collision energy of 42.5% and 40%

Fragment ion	Sequence	Expected mass (m/z)	Measured mass (m/z)
b1	K	129	------
b2	KG	186	------
b3	KGD	301	------
b4	KGDE	430	430.00
b5	KGDEE	559	------
b6	KGDEES	646	646.29
b7	KGDEESL	759	759.05
$[b5(-CO_2)]^+$	KGDEE	515	515.45
$[x6(-CH_2-CO_2)]^+$	GDEESL	631	631.58
$[y7(-CH-(CH_3)2)]^+$	GDEESLA	678	677.80
$[b7(-CO_2-H_2O)]^+$	KGDEESL	697	696.31
b7	KGDEESL	759	759.33

standard sample under collision energy of 42.5%. Fragment ions of b4, b6, and b7 displayed signals at m/z 430.00, 646.29, and 759.05, respectively. Whereas $[b5\text{-}CO_2]^+$, $[x6\text{-}CH_2\text{-}CO_2]^+$, $[y7\text{-}CH\text{-}(CH_3)_2]^+$ and $[b7\text{-}CO_2\text{-}H_2O]^+$ showed signals at m/z 515.45, 631.58, 677.80 and 696.31, respectively. In order to obtain more fragment ions to verify the sequence of the BMP, the collision energy was adjusted to 40%. Fig. 5B shows signal of b7 at m/z 759.33. The analysis results are listed in Table 1.

According to the ESI-MS/MS analysis conducted in this study, the sequence of the BMP standard sample was correctly identified as follows: Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (K-G-D-E-E-S-L-A). It clearly indicates that the method of determination is highly practicable.

4. ESI-MS/MS of BMP in Beef Enzymatic Hydrolysis

The method for determining BMP in No.4 beef protein hydrolyzate by ESI-MS/MS was performed under the same experimental conditions as those for the BMP standard sample. The ESI-MS/MS spectra of No.4 beef protein hydrolyzate at collision energies of 42.5% (A) and 40% (B) are presented in Fig. 6.

Fig. 6A shows the ESI-MS/MS fragment spectrum of No.4 beef protein hydrolyzate under a collision energy of 42.5%. Signals for $[x5\text{-}H_2O]^+$, y7, $[b7\text{-}H_2O]^+$ and $[M\text{-}H_2O]^+$ were detected at m/z 556.97, 720.29, 740.56 and 830.27, respectively. In order to obtain more

Table 2. Fragment ions of sample No.4 determined by mass spectrometry under collision energy of 42.5% and 40%

Fragment ion	Sequence	Expected mass (m/z)	Measured mass (m/z)
x5	DEESL	556	556.97
y7	GDEESLA	720	720.29
$[b7\text{-}H_2O]^+$	KGDEESL	741	740.56
$[M\text{-}H_2O]^+$	KGDEESLA	830	830.27
b5	KGDEE	559	559.02
b7	KGDEESL	759	759.27

fragment ions to verify the first order of the BMP in sample No.4 of the beef protein hydrolyzate, the collision energy was adjusted to 40%. Fig. 6B shows the signals of b5 and b7 occurring at m/z 559.02 and 759.27. The results of analysis show that the peptide sequence of sample No.4 of beef protein hydrolyzate could be deduced from a series of fragment ions shown in Fig. 6 and Table 2. The same peptide sequence Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala (K-G-D-E-E-S-L-A) was identified in the synthesized BMP and in sample No. 4 of beef protein hydrolyzate. These results prove that BMP is present in the beef protein hydrolyzate of sample No. 4 (after 2 hours of hydrolyzation).

CONCLUSION

The study confirmed that BMP was existing in the beef protein hydrolyzate through the HPLC-ESI-MS/MS technology.

The BMP in beef protein hydrolyzate could easily be marked by the use of an HPLC system coupled with electrospray mass spectrometry (ESI/MS). This technique allowed us to identify the target peptide BMP in the beef protein hydrolyzate. This report presents an alternative procedure in the search for biologically active peptides derived from food proteins. Our results demonstrate that HPLC-ESI-MS/MS is a simple, rapid, sensitive method to analyze target peptides from complex polypeptides.

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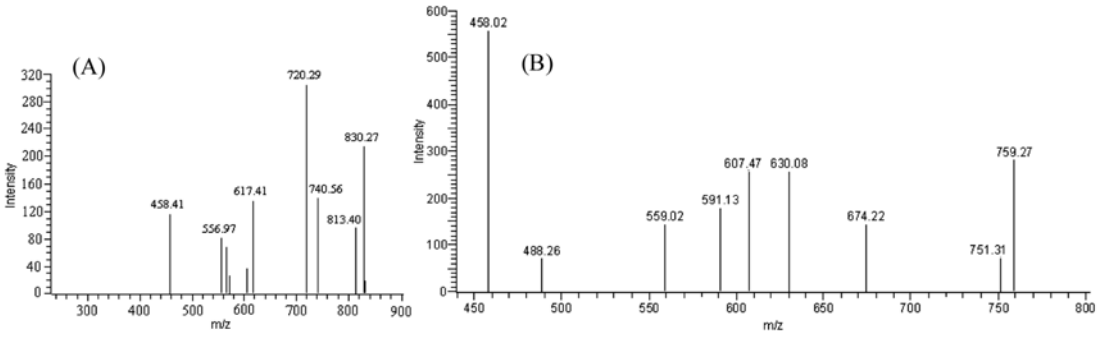

Fig. 6. ESI-MS/MS of sample No.4 under collision energy of 42.5% (A) and 40% (B).

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